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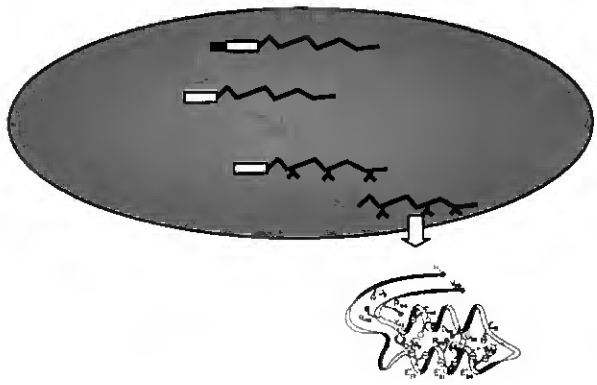

Cloning of the Bone Gla Protein gene from the teleost fish *Sparus aurata* (gilthead seabream). Molecular organization, developmental appearance and evolutionary implications

Idílio Jorge Matias Pereira Pinto

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2808T.

The content of this dissertation is of the exclusive responsibility of the author

(Idílio Jorge Matias Pereira Pinto)

Aos meus pais

AGRADECIMENTOS

No já longínquo ano de 1980, frequentava eu o 7° ano de escolaridade, uma professora de ciências, hoje anónima, porque esqueci o seu nome, cativou-me. Ao revelar-me a magia e o mistério das ciências, e da Biologia em particular, desvendou-me o meu destino. A Biologia, numa ou noutra vertente, nunca mais me iria deixar e a essa senhora, à professora dos bichos-da-seda, devo um sincero e tardio obrigado.

Numa universidade pequena, como era a Universidade do Algarve em 1987, quando iniciei a licenciatura em Biologia Marinha e Pescas, encontrei um conjunto de professores empenhados em fazer evoluir uma universidade periférica, em condições muito adversas. Àqueles que, talvez sem o saberem, mais me deram, em particular aos professores Karim Erzini, Margarida Castro, Fátima Rosado, Delminda Moura, Lucília Coelho e Helena Galvão, o meu agradecimento.

Num dia, que não sei já localizar, dirigi-me ao gabinete da professora Leonor Cancela e disse-lhe que gostaria de fazer um doutoramento em Biologia Molecular e de passar algum tempo nos Estados Unidos. Alguns dias passados e eu estava já certo de que encontrara a pessoa que me iria orientar. Ao longo destes anos fizemos juntos esta viagem e, no seu decorrer, a professora Leonor ensinou-me, não só a conduzir o barco, mas, acima de tudo, onde ele nos pode levar. E mostrou-me também que não é fácil chegar ao porto, e que não chegamos como partimos. À professora Leonor, por tudo o que me ensinou, e pela amizade que nos une, um enorme e sentido obrigado.

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Em termos mais pessoais, o meu primeiro e maior agradecimento vai para aqueles a quem dedico esta tese. Para os meus pais, que não só me educaram e incentivaram para que fizesse o que mais gostava, como me apoiaram, nos sucessos e fracassos, mostrando-me que os terei sempre ali, alegrando-se ou sofrendo, como se deles se tratasse.

Para agradecer aos meus amigos Teresa Modesto e Luís Faísca não bastaria uma página inteira, tanto é o que lhes devo e tanto o carinho que lhes tenho. Não posso ocupar uma página, mas posso dizer-lhes que este obrigado que aqui deixo é muito mais do que isso. Obrigado.

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Estava eu no segundo ano do curso de Biologia Marinha e Pescas e, a propósito de um trabalho sobre radiolários, dirigi-me ao Professor Sadat Muzavor em busca de orientação. Encontrei a orientação, mas encontrei também um amigo a quem muito estimo e a quem agradeço tudo o que me tem dado.

Houve bons e maus momentos nesta viagem. Tempos de acalmia em que o vento soprava a favor e era apenas uma questão de tempo chegar a bom porto. E tempos de tormenta, em que as vagas cavavam abismos de onde parecia não haver engenho nem arte que nos pudessem salvar. Nos bons e nos maus momentos tive quase sempre alguém a meu lado, para me dar razão, mesmo quando a não tinha. À minha amiga Natércia, a minha parceira da "dupla invencível", obrigado por tudo.

E por fim, só porque foi o último a chegar, para o Marco, que trouxe o que faltava e fez de mim uma pessoa melhor, um derradeiro e enorme obrigado.

ABBREVIATIONS AND ACRONYMS

In addition to standard abbreviations for metric measurements (e.g., ml) and chemical symbols (e.g., HCl), the abbreviations and acronyms below are used throughout this work.

~:	approximately
$1,25(OH)_2D_3$:	1,25-dihydroxyvitamin D_3
aa:	amino acid
AP:	Alkaline Phosphatase
bp:	base pair
BGP:	Bone Gla Protein
BMP:	Bone Morphogenetic Protein
DNA:	Deoxyribonucleic acid
ddH_2O:	Bidestilled water
dph:	Days post-hatching
dsDNA:	double-stranded DNA
EDTA:	Ethylenediaminotetracetate
Gla:	γ-carboxyglutamic acid
GRE:	Glucocorticoid Responsive Element
KO:	Knock Out, Depleted
MGP:	Matrix Gla Protein
nt:	nucleotides
O/N:	Overnight
OP:	Osteopontin
ORG:	Osteocalcin-Related Gene
PCR:	Polymerase Chain Reaction
RNA:	Ribonucleic Acid
rpm:	rotations per minute
R/T:	Room Temperature
RXR:	Retinoid X Receptor
S.L.	Standard Length (length measured from the jaw to the base of the caudal fin)
spBGP:	*Sparus aurata* BGP
Tris-HCl:	Tris(hydroxymethyl)aminomethane adjusted to the referred pH with HCl
U:	Enzyme units (the amount of restriction enzyme necessary to digest one microgram of DNA under established conditions)
VDR	Vitamin D Receptor
VDRE:	Vitamin D Responsive Element
vol.:	volume

RESUMO

A proteína Bone Gla (BGP, osteocalcina) é uma pequena proteína dependente-da.vitamina K que apresenta resíduos de ácido glutâmico γ-carboxilados. A presença destes aminoácidos modificados permite à proteína ligar-se a iões Ca^{2+} e interagir com os cristais de hidroxiapatite dos tecidos mineralizados.

Embora a BGP tenha sido isolada pela primeira vez em 1976, só recentemente se demonstrou a sua função de modo convincente, permanecendo o mecanismo de acção ao nível molecular essencialmente desconhecido.

O alinhamento das sequências de aminoácidos de todas as BGPs previamente conhecidas revela uma notável conservação de certas regiões, as quais se acredita serem essenciais para o funcionamento correcto da proteína, ao longo de vários milhões de anos de evolução. O facto de o gene da BGP ter sido clonado apenas em mamíferos (humano e roedores) e de apenas se encontrar disponível a sequência parcial de aminoácidos da BGP de um vertebrado inferior (um peixe teleósteo) impedia a realização de estudos evolutivos desta proteína, assim como a avaliação do seu papel no aparecimento e evolução do osso.

O presente relatório descreve a clonagem do gene da BGP de um vertebrado inferior, o peixe teleósteo *Sparus aurata* (Dourada), a sua expressão ao longo do desenvolvimento e correspondente distribuição tecidular d do RNAm. A organização molecular do gene da BGP de *Sparus* (spBGP) é semelhante à dos genes das BGPs de mamíferos, diferenciando-se apenas por um Intrão II mais longo e pela localização dos sítios de inserção dos três intrões. Tal como nos mamíferos, a expressão do gene da BGP em *Sparus* está restricta aos tecidos ósseos e o início da sua expressão ao longo do desenvolvimento segue-se ao início da calcificação do esqueleto. Estes resultados foram obtidos independentemente por diferentes técnicas de detecção.

Desenvolveu-se uma cultura celular de células derivadas de osso de *Sparus* a fim de testar a funcionalidade de uma construção promotor spBGP/vector pβGal, demonstrando-se a capacidade deste promotor em induzir a transcrição deste vector de expressão nestas células.

Com base na sequência do gene de spBGP e em outras sequências parciais de RNAm de peixe e amfíbio igualmente obtidas pelo nosso grupo, efectuou-se uma análise filogenética e avançaram-se hipóteses relacionando as BGPs com outra proteína Gla (proteína Matrix Gla), em particular, e com a família das proteínas Gla, em geral. Os nossos dados suportam a hipóteses de que todas as BGPs terão tido a mesma origem, partilhando um ancestral comum com a proteína Matrix Gla. Juntamente com as semelhanças observadas ao nível da distribuição tecidular e do

aparecimento no decorrer do desenvolvimento da mensagem da BGP em todos os modelos estudados, estes resultados reforçam a hipótese de que a BGP terá atravessado mais de 200 milhões de anos sem alterações significativas, desempenhando provavelmente o mesmo papel desde a alvorada dos vertebrados.

ABSTRACT

The Bone Gla Protein (BGP, osteocalcin) is a small vitamin K-dependent protein which presents three γ-carboxylated glutamic acid residues. The presence of these modified amino acids enables the protein to bind to Ca^{2+} ions and to interact with hydroxyapatite crystals of mineralized tissues.

Although BGP was first isolated in 1976, only recently has its function been convincingly demonstrated, the mechanism of action at the molecular level remaining essentially unknown.

Amino acid sequence alignment of all previously known BGPs show a remarkable conservation of certain regions, which are believed to be essential for the correct functioning of the protein, throughout several million years of evolution. The fact that the BGP gene had only been cloned in mammalian systems (human and rodents) and that partial BGP amino acid sequences were available only from one lower vertebrate (the teleost bluegill) prevented evolutionary studies of this protein, as well as the assessment of its role in the appearance and evolution of bone.

This report describes the molecular cloning of the BGP gene from a lower vertebrate, the teleost fish *Sparus aurata* (gilthead seabream), its developmental expression and corresponding mRNA tissue distribution. The molecular organization of the *Sparus* BGP (spBGP) gene is similar to that of mammalian BGP genes, the only differences being a longer intron II and the sites of insertion of the three introns. As in the mammalian models, BGP gene expression in *Sparus* is restricted to bony tissues and its expression throughout development follows the onset of skeletal calcification. These results were independently obtained by different detection techniques.

Different *Sparus* bone-derived cell strains were obtained and used to test the functionality of an spBGP promoter/pβGal vector construction, proving the ability of this promoter to drive the transcription of this expression vector in these cells.

Based on the spBGP gene sequence and on other partial fish and amphibian BGP mRNA sequences also obtained by our group, a phylogenetic analysis was performed and evolutionary assumptions were advanced relating BGPs with another Gla protein (Matrix Gla Protein), in particular, and with the Gla family of proteins, in general. Our data support the hypothesis that all BGPs have a single origin and share a common ancestor with Matrix Gla protein. Together with the similarities observed in

the tissue distribution and timing of appearance of the BGP message in all studied models, these findings strengthen the hypothesis that BGP has crossed more than 200 million years with only minor changes, probably playing the same role since the dawn of the vertebrates.

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CHAPTER I: INTRODUCTION

The first chapter of this thesis is meant to give an overview on the current knowledge of Gla proteins, in general, and on Bone Gla protein, in particular. Easily noticeable, the vast majority of information arises from studies using mammals, mostly, and avian, more scarcely, as models. Also, the kind of information available is influenced by the "use" this protein has been given throughout most of the 23 years that spanned since its discovery, as an indicator of bone remodelling in clinical studies. As described, only more recently has a shift occurred in the approach chosen to study this protein and new information has been obtained concerning such aspects as its function and regulation in mammalian models. In this report we describe the first BGP cDNA and gene structure from a lower vertebrate, and give the first results on its tissue distribution in fish and appearance during development. We also report the development of bone-derived primary cell cultures, suitable to further analyse BGP gene expression and regulation *in vitro*. With this work we introduce a lower vertebrate, the teleost fish *Sparus aurata*, as a new model for the study of the bone-related proteins.

1. BONE GLA PROTEIN

1.1. DISCOVERY AND PURIFICATION FROM BONE

Bone Gla protein (BGP, osteocalcin) is a small protein originally isolated from the mineralized phase of bovine bone (420, 421). It is the most abundant non-collagenous protein of bone in all species analysed (90, 207, 209, 212, 216, 288, 298), with a molecular weight of up to 6500 (chicken; 209). BGP is a very acidic protein, with an isoelectric point of 4.0 and a net negative charge of 9 at pH 8.0 (414, 434). The name osteocalcin (Gr. *osteo*, bone + Lat. *calc*, lime salts + *in*, protein) derives from (i) its Ca^{2+} affinity (209, 214, 422) and (ii) its abundance in bone tissue [1-20% of noncollagenous proteins, depending on species, age and site (90, 207, 209, 212, 216, 288, 436)].

Chicken (70, 207, 209) and cow (420) bone were the first tissues from which BGP was isolated. More recently, the protein has been purified and sequenced from bones of human (415), monkey (215), pig, goat, sheep and wallaby (232), cat (486), rat (387, 400), mouse (Hauschka and Gundberg, unpublished observations), rabbit (Hauschka and Triffitt, unpublished observations), emu (234), toad (64), swordfish (422) and seabream (64), and has been inclusively detected at low levels in fossil bones, possibly protected from degradation through its binding affinity for hydroxyapatite (233, 358).

1.2. THE BGP GLA RESIDUES: LOCATION, ORIGIN AND FUNCTION

Location and origin

One of the most striking features of BGP is the presence of three residues of the vitamin K-dependent aminoacid γ-carboxyglutamic acid (Gla) [located at positions 17, 21 and 24 in human, bovine and rat, and at equivalent positions in all other species], from swordfish to mammals (this study, 117, 261, 371, 527), which are thought to be positioned on the same face of one helix, spaced at intervals of about 5.4 Å (214). Gla residues result from a post-translation modification (γ-carboxylation) in which specific glutamic acid residues are modified in a reaction catalysed by a vitamin K-dependent carboxylase (113, 143, 207, 308, 327, 420, 438, 443, 506), a process that requires the intervention of vitamin K in its reduced form, as a cofactor (385, 517, 544). The enzymatic reaction generates γ-carboxyglutamate and vitamin K 2,3,-epoxide, which is then recycled back to the hydroquinone form by a reductase enzyme (173).

Function

Since its discovery in the early 70s, the presence of Gla residues has been implicated in the ability of vitamin K-dependent proteins to bind to divalent cations, hidroxyapatite, and acid phospholipids (301, 370, 501, 505, 541, 545), although the strength of this association is relatively weak (439). Scatchard plot analysis of Ca^{2+} binding to bovine BGP revealed the presence of three Ca^{2+} binding sites with an average dissociation constant of 2-3 mM (214,

373, 422). Since there are three Glas in bovine BGP, it seemed resonable to postulate that the three Ca^{2+} binding sites were provided by the side chain of Gla. This interpretation is supported by the observation that decarboxylation of Gla to Glu abolishes Ca^{2+} binding to BGP (414) and by the prediction that the organization of the Gla residues on the BGP structure is similar to the spacing of Ca^{2+} in the hydroxyapatite crystal (214). Such coordination of Ca^{2+} by Gla residues in BGP would leave two Ca^{2+} coordination sites unoccupied by protein ligands and therefore free to function in binding interactions with bone mineral (439). Inhibition of the γ-carboxylation by the vitamin K antagonist warfarin, by selective proteolysis of the Gla domain, or by chelation of the calcium ions results in loss of binding of Gla-containing proteins to metal ions, hydroxyapatite or membranes (375, 428, 430, 435).

Do Gla residues play a role in BGP secretion?

It is unclear whether Gla residues may play an intracellular role in modulating protein secretion. From the comparatively low Ca^{2+} affinity of Gla proteins (K_d=0.2-3 mM, far lesser than the affinity for hydroxyapatite) and the paucity of intracellular ionic Ca^{2+} ($<10^{-6}$M), it might be expected that functionally important Gla- and Ca^{2+}-dependent conformational changes will occur only as these proteins leave the cell (218). However, carboxylation does appear to play a role in the intracellular fate of vitamin K-dependent proteins (218). *In vitro* studies performed on cells and tissues (286, 375, 400) demonstrate inhibited secretion of BGP and accumulation of intracellular precursor in the absence of correct γ-carboxylation, suggesting that processing occurs intracellularly and that the presence of Gla residues in the protein may be required for processing into the mature secreted peptide. Although the cleavage enzyme [which may be related to the furin/PACE specific endopeptidase known to cleave the propeptide of profactor IX (54)] may prefer the presence of Gla in its substrate, the presence of Gla residues is not absolutely required for production of the mature BGP species, which has been shown to circulate in the non-carboxylated form (for a revision on this subject see 218).

Calcium binding to Gla shapes the BGP protein

Ca^{2+} binding to fully carboxylated BGP alters the circular dichroism spectrum and the immunochemical properties of the protein (105, 214). The apparent α-helical content of bovine BGP increases from 1% in the absence of Ca^{2+} to 14% in its presence (105), the same phenomenon ocurring for chicken BGP (214). It is interesting to note that the α-helical content of carboxylated BGP in the presence of Ca^{2+} approaches that of uncarboxylated BGP much more than that of carboxylated BGP in the absence of Ca^{2+} (105). One interpretation for this phenomenon is that the Gla-containing region of BGP can only exist as an α-helix when the Gla residues bind Ca^{2+}. In the absence of this metal, Gla residues would destabilize the α-helix, perhaps because of the extra negative charge on the Gla side chains (439).

Combined chemical, immunochemical, spectral, and predictive investigations of BGP structure (214) have yielded the model shown in Figure I-1. The model consists of two antiparalell α-helical domains, the "Gla helix" (residues 16-25 in the chicken protein), and the "Asp-Glu helix" (residues 30-41) connected by a peptide segment containing a β-turn (residues 26-29) and stabilized by the Cys-23-Cys-29 disulfide bond. Other β-turns occur at positions 5-8 and 12-15, with β-sheet structure in the COOH-terminus from residues 42 to 48. Additional constraints derive from immunochemical studies that indicate contiguity of the NH_2- and COOH-termini of BGP (214, 216). From the few two-dimensional NMR spectra data available, it appears that the Ca^{2+} -induced helical conformation is extremely rigid, with hydrophobic stabilization of the helical domain by the COOH-terminal domain (214, 216). The adjacent carboxyl groups in the Gla residues are aligned in such a way that they project from the helix in a plane, potentially facilitating the protein adsorption to hydroxyapatite (214). Millimolar levels of Ca^{2+} cause normal BGP to change from a random-coil to an alpha-helical conformation. Apparently, these milimolar levels of Ca^{2+} or other specific cations are required to offset electrostatic repulsion if the highly anionic BGP molecule is to achieve its full potential of ~ 40% α-helix (200, 214, 240).

Not only must Gla residues be present, they must also be in helical register to fully achieve the adsorption specificity for hydroxyapatite (414). The binding of BGP to hydroxyapatite probably involves the bidentate chelation of Ca^{2+} atoms on the crystal

surface by the malonate side chain of Gla, which is supported by the fact that binding to hydroxyapatite completely prevents the thermal decarboxylation of Gla residues to Glu residues (439).

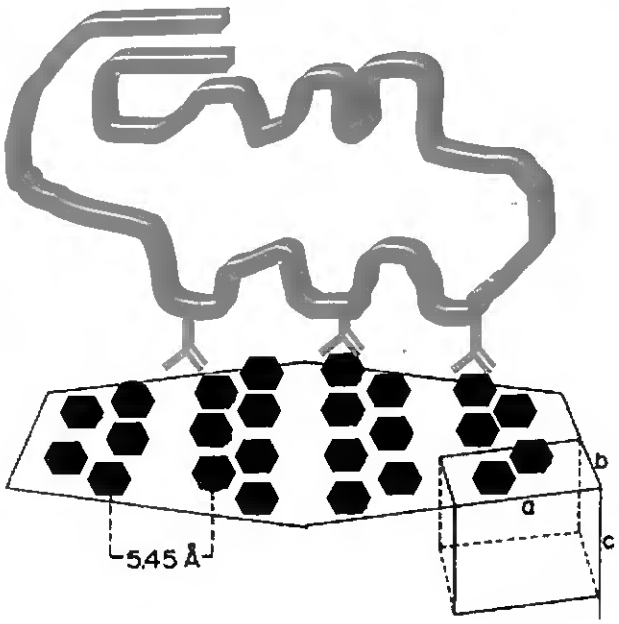


Figure I-1. Model for Ca^{2+}-induced structure of BGP, showing its hypothetical mode of interaction with Ca^{2+} in the hydroxyapatite crystal lattice. The polypeptide backbone of BGP is represented by a blue ribbon and the Gla residues by a red inverted Y. Hydroxyapatite crystals are schematic represented, including the three axes, a, b and c. Adapted from Hauschka *et al.* (218)

The affinity of metal-free BGP for hydroxyapatite is increased fivefold by the addition of 5mM Ca^{2+} (214, 563). Interestingly, Mg^{2+} is known to induce a somewhat aberrant α-helical conformation in BGP (214) and simultaneously inhibits the binding to hydroxyapatite (219, 560). Magnesium ions compete rather well for the Ca^{2+} binding sites, but Sr^{2+} and Ba^{2+} show little or no competition (200, 209). In contrast, the Gla-rich fragment-1 portion of prothrombin shows increased hydroxyapatite binding in the presence of either Ca^{2+} or Mg^{2+} (560). Other inhibitors of BGP adsorption to hydroxyapatite are described in Table I-I. Finally, it seems interesting to refer that although BGP is distinguished by its normal content of Gla residues, the human protein may contain only two fully γ-carboxylated Glas (415), a fact that may be related to the low concentration of BGP in human bone and plasma (~5% of most other species, including other mammals; 425, 427, 431).

Table I.I. Most important ions and molecules that inhibit BGP adsorption to hydroxyapatite, suggested mechanism of action and corresponding references.

Inhibitors of BGP adsorption to hydroxyapatite	Mode of action	References
Magnesium (Mg^{2+})	Induces aberrant α-helical conformation in BGP	219, 560
Diphosphonates	Competes with BGP for hydroxyapatite	219, 561
Dicoumarol	Prevents γ-carboxylation by inhibiting vitamin K production	211
Warfarin ($C_{19}H_{16}O_4$)	Prevents γ-carboxylation of Glu residues to Gla in BGP	82, 289, 375, 430, 437, 441
Decarboxylation	Reverts Gla residues to Glu, which are unable to bind hydroxyapatite	213, 219, 414, 560
Phenprocoumon $C_{18}H_{16}O_3$	With a structure very similar to warfarin, inhibits vitamin K epoxide reductase and, indirectly, the activity of vitamin K.	138, 226, 277, 410, 538

BGP in free solution binds between 2 and 3 mol Ca^{2+} /mol protein with a dissociation constant ranging from 0.8 to 3 mM (200, 209, 214, 422). Binding sites for Ca^{2+} are probably formed by carboxyl groups of Gla residues, as well as by opposing carboxyls of aspartic acid and glutamic acid in the two helical domains of BGP (218). The interaction of Gla with Ca^{2+} is such that only two of the six to nine likely coordination sites are occupied (62, 214). Thus the sequestered Ca^{2+} is available for other types of interaction. Candidate ligands for sharing in the interaction of Ca^{2+} bound to Gla residues of BGP include other Ca^{2+} -binding proteins, acidic phospholipid surfaces, and calcium phosphate mineral surfaces, such as hydroxyapatite. Although BGP apparently fails to bind phosphate anions in solution (422, 444), a potential arginine moiety for phosphate interaction is absolutely conserved (Arg-20). The Arg-20 is positioned optimally to provide for both i) internal neutralization of electrostatic charge in the Gla helix and ii) the complementary charge array in residues 17-24 (Gla-X-X-Arg-Gla-X-X-Gla), which appears to promote adsorption to the hydroxyapatite

surface lattice (Ca^{2+} . PO_4^{3-} . Ca^{2+}) (214, 217).

1.3. BIOCHEMICAL CHARACTERISTICS OF BONE GLA PROTEIN

The biosynthetic pathway of BGP

The most recent advances in defining the biosynthetic pathway for BGP include (i) isolation of the BGP gene and characterization of promoter regulatory elements, (ii) sequencing of cDNAs coding for preproBGP from several species, (iii) characterization of proBGP, (iv) description of the possible role of the propeptide as a vitamin K-dependent carboxylase recognition site, (v) *in vitro* synthesis of the Gla residues and delineation of their potential role in the processing and the physiological functioning of BGP, (vi) investigation of tissue specificity for BGP synthesis, and (vii) study of the modulation of BGP synthesis by the hormone $1,25(OH)_2D_3$ and other effectors of BGP activity (218).

The current knowledge of the mammalian BGP biosynthetic pathway is partially summarized in Figure I-2. A preproBGP, approximately twice the size of the mature peptide, is first synthesized, bearing a signal peptide (which directs the protein to the endoplasmic reticulum) and a propeptide, both cleaved off in separate reactions prior to secretion (73, 320, 374, 399, 400). After removal of the 23-residue hydrophobic leader sequence by the signal peptidase, concomitant or immediately after translation (399), it appears that the intracellular proBGP (374, 375, 400) undergoes posttranslation modifications, leading to synthesis of the three Gla residues, by a processus that requires vitamin K as a cofactor and is CO_2- and O_2-dependent (143, 286, 399, 400). This reaction is catalysed by the γ-carboxylase enzyme, which also contains Gla residues (34), event that seems to be coincident with membrane binding potential (182) and proceeds by a mechanism similar to that described for other vitamin K-dependent proteins (143, 542). The proregion contains a gamma-carboxylation recognition site homologous to corresponding regions in the vitamin K-dependent clotting factors. Arg at position -1 and Phe at -16 are strictly conserved and appear to be critical for the binding of the carboxylase enzyme to its substrates (246, 399). Another homologous structure possibly involved in substrate recognition by the carboxylase is the invariant

occurrence of the peptide sequence Glu-X-X-X-Glu-X-Cys (with X for variable amino acid) in all Gla-containing clotting factors, BGPs and Matrix Gla proteins (MGPs) sequenced (442). The two glutamic acid residues in this consensus sequence are normally carboxylated to Gla, along with other specific glutamic acid residues, depending on the protein. This potential recognition site is part of the Gla-helix domain in BGP (214), and thus substrate conformation may play an important role in determining carboxylation specificity (218). After carboxylation, the propeptide is removed and the protein is secreted (198).

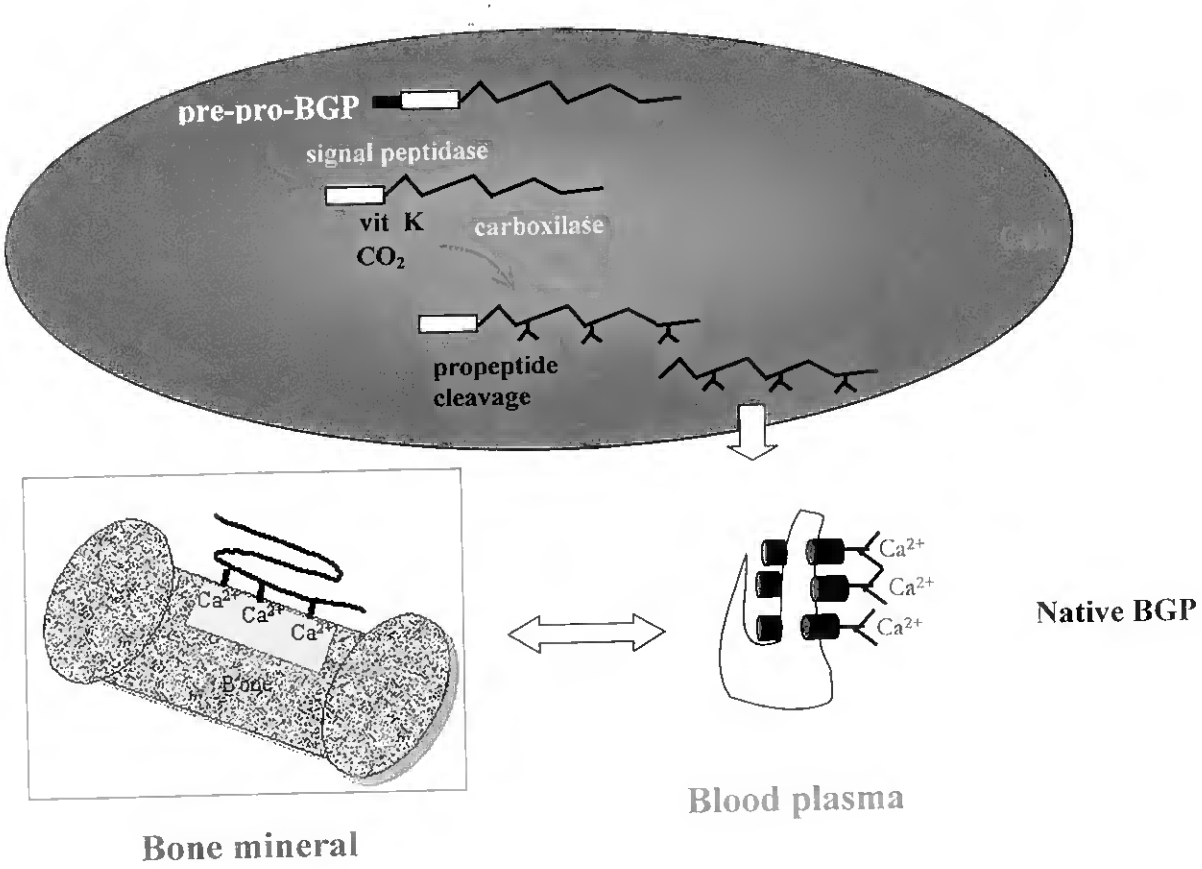


Figure I-2. General pathway of BGP biosynthesis and secretion by osteoblasts. Prepro BGP ($M_r \approx 10.000$) consists of a 23-26 residue prepeptide, a 22-28-residue propeptide, and a 45 to 50-residue BGP sequence (this study, 117, 261, 371, 527), the exact size of each fragment being dependent on the species analysed. After cleavage by signal peptidase resulting preBGP is presumably targeted for carboxylation by its propeptide (73, 246, 399, 442). Gla (symbolized by inverted and horizontal **Y**s) is normally formed at residues 17, 21 and 24 (218). Adapted from Hauschka *et al.* (218).

Two contiguous basic amino acids appear to the amino-terminal side of the propeptidase cleavage site in most of the vitamin K-dependent proteins. However, additional studies with vitamin K-dependent coagulation factors and protein C mutants (30, 160) suggest that the propeptidase substrate specificity is not restricted to the basic residues at –1 and –2, but also includes the carboxy-terminal four aminoacids of the propetide.

1.4. BIOSYNTHESIS, TISSUE DISTRIBUTION AND ABUNDANCE OF BGP

BGP in bone

BGP is synthesized in bone, where it represents 1-20% of all non-collagenous proteins (90, 207, 209, 212, 216, 288, 436), being associated with the calcified extracellular matrix (214, 236, 336, 337, 458). Several lines of evidence point to osteoblasts as the cells in bone which synthesize BGP (e.g., 73, 218, 295, 375, 503, 571) and the protein is also synthesized *in vitro* by bone-derived cells, such as primary cultures of osteoblastic cells (25, 33, 57, 273, 305, 368), and by clonal osteosarcoma cells which display features of the osteoblastic phenotype such as high PTH responsiveness and high alkaline phosphatase activity (5, 324, 374, 375).

BGP in teeth

Besides osteoblasts, BGP is also synthesized by odontoblasts in teeth (57, 123). In fact, rat incisor dentin has yielded a Gla protein identical to rat bone BGP (123, 303), in amounts comparable to those found in that tissue (302), an observation consistent with the fact that dentine has a calcified collagenous matrix generally similar to that of bone (304). Interestingly, BGP has never been detected in tooth enamel (420), a mineralized tissue that differs from dentine and bone by the average size of its hydroxyapatite crystals, which are far larger, and also by the absence of a collagenous matrix.

BGP in atherosclerotic lesions

BGP has been also identified in calcified atherosclerotic plaques (158, 485). Already in 1863 the mineral component in calcified arteries was described as "*an ossification, and not a mere calcification; the plates which pervade the inner wall of the vessel are real plates of bone...we see ossification declare itself in precisely the same manner as when an osteophyte forms on the surface of bone...following the same course of development*" (547). This bone associated with atherosclerosis inclusively possesses marrow (156). Not unexpectedly, we find in this atherosclerotic lesions, besides BGP, the same proteins present in normal bony tissues, namely MGP (485), Bone Morphogenetic protein-2a (BMP-2a (a potent osteogenic differentiation factor (50, 259))) and osteopontin (445). The cells responsible for the presumable active calcification observed in atherosclerosis are not known, but it is possible that vascular cells may be able to act in a manner similar to osteoblasts (445). In addition, at least some of these cells have morphological and immunocytochemical features of macrovascular pericytes (50; a type of mural cell).

BGP in plasma

BGP has been detected in the plasma of all vertebrates examined (e.g., 197, 425, 427, 433), suggesting that a significant amount of BGP synthesized by bone cells circulates in plasma. Studies have shown that the BGP found in the circulation derives from new protein synthesis and not from bone resorption or release of pre-existing BGP from the bone matrix (433). This fact, together with the evidences that serum BGP binds strongly to added hydroxyapatite [(433) which indicates that the plasma protein has a full complement of γ-carboxyglutamate residues] has raised the possibility that BGP found in bone represents serum BGP bound to bone hydroxyapatite (425). However, several evidences point against that conclusion (e.g., the fact that less than 7% of the ^{125}I-labelled BGP injected into rats actually accumulates in bone (433)), and an alternative model appeared suggesting that the high BGP concentrations adjacent to hydroxyapatite were due to its secretion near mineralizing sites (425). The BGP found in serum would then represent the fraction of newly synthesized BGP escaping from hydroxyapatite binding and diffusing away from the mineralizing site. This model also accounts for the increase in serum BGP upon warfarin

administration, which creates an abnormal BGP that cannot bind to hydroxyapatite, or upon ethylhydroxydiphosphate injection, which competitively blocks the binding of BGP to hydroxyapatite (432).

The molecular weight of plasma BGP is identical to that for BGP extracted from bone (425-427). The turnover of BGP in plasma is quite fast (433), as indicated by the complete shift in the ability of serum BGP to bind to hydroxyapatite within 3 hours of warfarin administration. Since warfarin inhibits the γ-carboxylation of BGP at the microsomal level, this result indicates that the interval between new BGP synthesis within the cell and complete turnover of BGP in serum is only 3 hours (439).

BGP in blood

Sensitive radioimmunoassays (RIAs) for BGP have shown that this protein circulates in blood at concentrations ranging from 2 to 15 ng/ml in normal adult humans (72, 103, 522, 524) to as much as 900 ng/ml in young rats (2, 406, 430). Comparable levels of BGP are found in both serum and plasma samples of humans and other species (197), at least in those checked until present by this method.

In contrast with the short half-life of the BGP protein in the circulation (around 5 minutes), being rapidly metabolized by the kidney (433), the lifetime of the BGP message in unstimulated or fully $1,25(OH)_2D_3$-stimulated cells appears to be quite long, which is indicated by the fact that 15 hours of exposure to transcriptional inhibitors affects neither basal nor fully stimulated BGP synthesis (398).

Various fragments of BGP are known to circulate in blood. The majority of circulating BGP is composed of the intact molecule and a large N-terminal mid-molecule fragment, which is thought to encompass residues 1-43 (199). Recent studies indicate that there are other smaller (< 30 residues) N-terminal immunoreactive species of BGP in the serum (80, 178, 194). The origin and significance of the N-terminal mid-molecule fragment is uncertain. Some authors state, based on animal studies, that the BGP found in the circulation is derived from new protein synthesis rather than from bone resorption or release of existing BGP from the bone matrix. The dynamics of plasma BGP have been clarified by studies on rats treated with various amounts of warfarin and vitamin K_1 (430, 433, 439). There are still those who

believe that the breakdown of BGP may also occur during osteoclastic dissolution of bone (467). Proteins like cathepsins (24) and plasmin (379) are capable of degrading BGP, producing fragments of diverse lenghts. Osteoblastic degradation of BGP could also serve as a mechanism to regulate BGP concentration (199). Whether the generation of BGP fragments during bone resorption is related or not to BGP biological function in regulating bone turnover is not clear.

1.5. DEVELOPMENTAL APPEARANCE OF BGP

Investigations into the developmental appearance of BGP in calcifying tissues have relied either on direct biochemical and immunochemical assays for BGP itself or on chemical analysis for γ-carboxyglutamic acid. These two approaches, using human and rat models, yield different results for the developmental appearance of BGP. Direct assays for BGP in demineralized extracts of calcifying tissues indicate that the protein itself does not appear in parallel with the accumulation of mineral, but rather 1-2 weeks later, at the approximate time when the initial mineral phase matures to hydroxyapatite (439, 525). Accordingly, the level of BGP in rat bone rises rapidly after birth, from 1.7% of the adult level in newborn rats to 65% of the adult level in 24-day-old rats (427). The same conclusion was reached by studies analysing the developmental expression of the mouse and rat BGP gene by *in situ* hybridization and Northern techniques, with accumulation of mRNA for BGP occurring at a late stage of osteoblast differentiation, suggesting that the protein is not necessary for initial mineralization of the bone matrix (79, 331, 362, 497). In contrast, chemical analysis of γ-carboxyglutamic acid in developing bone shows that the appearance of this amino acid parallels the accumulation of mineral, rather than subsequent mineral maturation (427). These results are in agreement with those obtained in a previous study (210), where the appearance of γ-carboxyglutamate in developing chicken bone was shown to correlate with the appearance of bone mineral, and with a later study (216) on the developmental appearance of BGP in bovine and chicken bone. However, this result is now attributable to the presence in bone matrix of at least one other Gla-containing protein, MGP, whose accumulation is known

to occur much earlier than that of BGP during mouse development (19, 309, 316, 388, 497).

Another interesting fact is that serum BGP in newborn rats is within the adult range, what makes clear that the 100-fold lower content of BGP in newborn rat bone is not due to an inhibition in expression of the BGP gene (427). The fact that a great number of cells stains strongly with anti-BGP antibodies in the bones of 2- and 3-day-old rodents (32) reinforces the conclusion that BGP is synthesized at a high rate by osteoblasts in the newborn rat bone, but mostly fails to accumulate in the mineral phase. The hypothesis that the initial bone mineral phase is deficient in a binding domain required for a strong interaction with BGP is strengthened by the finding that, *in vitro*, BGP does not bind to amorphous calcium phosphate (420), a less ordered mineral phase than hydroxyapatite.

From these results we can expect BGP not to be dispersed evenly throughout developing bones. Accordingly, a study using tibial bones of 2- and 4-week-old rats (431) has shown that BGP concentrates mostly in the midshafts, with the proximal and distal growth plates containing less that 5% of the midshaft levels of BGP, a result consistent with the fact that most new mineral is deposited at the growth plate. Two weeks later, however, in this model, the segments previously located near the growth plate have become part of the bone diaphysis and the BGP levels have risen to midshaft levels. Other studies (451) have reached the same conclusion, i.e., in rat, mineral accumulation precedes BGP appearance by about 2 weeks. The explanation for this delay lies surely in the still not evident mode of action of BGP, which will be discussed in the next section.

1.6. BGP FUNCTION

The function(s) of BGP

Because BGP is one of the most abundant noncollagenous proteins in bone, an important, but elusive, function has been inferred since the time of its discovery. However, and despite the concerted efforts of an important number of scientists, the precise function of BGP in the formation and metabolism of bone has remained, until recently, unclear. Because of its specific interaction with hydroxyapatite, BGP was thought to affect the growth or

maturation of Ca^{2+} -phosphate mineral phases. In agreement with this, BGP developmental appearance roughly parallels the onset of mineralization and the increase in synthesis of this protein is concomitant with hydroxyapatite deposition during skeletal growth (210, 288).

The adsorption affinity of BGP for hydroxyapatite may be an important factor in mineral dynamics of bone. The transition of brushite ($CaHPO_4.2H_2O$) to hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is inhibited by very low concentrations of BGP (205). BGP also inhibits precipitation of hydroxyapatite from supersaturated solutions (414, 420, 537) and from seeded hydroxyapatite systems (416, 462), but has no effect on Ca^{2+} -phospholipid-PO_4-dependent crystallization (48). The degree to which BGP retards cystallization, at least in supersaturated solutions of calcium phosphate, depends critically on the concentration of BGP, with a doubling of the time required for half-maximal crystal formation at a BGP concentration of 6 μM (414). Since the final amount of hydroxyapatite is not affected by BGP, the effect of BGP is exclusively on the kinetics of mineral formation, rather than on the thermodinamic end point (solubility product) of the mineral phase (439). Similar studies performed with supersaturated solutions of calcium phosphate seeded with a small amount of hydroxyapatite (416) led to the same conclusion: BGP is a potent inhibitor of mineralization, but only if it contains Gla residues and an intact disulfide bond. Its effect is kinetic, rather than thermodinamic. The protein binds poorly to amorphous calcium phosphate of unspecified surface area (420). BGP adsorption to fluorapatite [$Ca_{10}(PO_4)_6F_2$] exhibits a fivefold greater affinity constant than hydroxyapatite (219, 560), which may account for some of the known disparate effects of fluoride in bone mineral metabolism.

Studies addressing the developmental appearance of BGP (see previous section) also give insight into the possible function(s) of BGP. The finding that fetal rat bone is nearly devoid of BGP (0.09 mg/g of bone; 427) indicates that this protein is not required to be present at this stage of rat development but also that any structure or property already present in fetal rat bone can be excluded as a possible biological function for BGP. For example, BGP cannot be required for the formation of the first mineral phase of rat bone, since a 20-day old fetal rat with 0.1% of the adult BGP level has already 40% of the adult mineral levels (427). BGP also cannot be required for osteoclastic bone resorption in response to hormones such as parathyroid hormone, since fetal rat bone is resorbed in response to such hormones

(449, 450). The same information is given by the differences in BGP content observed in different bone regions, according to its age (see previous section). The fact that, in rats, BGP accumulates in calcifying tissues only 1-2 weeks after the accumulation of mineral may be related to the maturation of initially deposited mineral to hydroxyapatite.

When one tries to analyse changes in bone structure and physiology which occur between rat birth and the 24-day old-stage, when the BGP level rises to 65% of the adult level (427), we find that one change in bone structure which approximately parallels the appearance of BGP is the transition from the initially formed amorphous calcium phosphate mineral of fetal bone to the hydroxyapatite phase characteristic of adult bone (525). BGP may appear concurrently with this mineral transition because its function only requires it to be present at this stage. For example, its function could be to catalyze the transition to hydroxyapatite, to regulate the size or shape of the hydroxyapatite crystals formed, or to orient the crystals epitactically allong the collagen fiber (427). Alternatively, BGP could appear in bone paralleling the appearance of hydroxyapatite because only this phase of the mineral binds BGP (414, 420). The first of these two hypothesis was strenghtened by the studies described in the next paragraph.

Depletion studies carried out in rodents have shed some light on the contribution of BGP in bone formation. In one of these studies (135), gene targeting has produced a mouse that has had the BGP gene "knocked out" (KO). These mice are characterized by a progressive increase in bone mass, with an accelerated rate of bone formation without changes in osteoclast or osteoblast number. No changes in mineral content of the bones of BGP-depleted mice were detectable by von Kossa staining or histomorphometry. However, a more sensitive assay of mineralization, Fourier transform infrared microspectroscopy, revealed differences in the size and perfection of the crystallites (49). In wild-type animals the crystals were larger and more "perfect" in the cortical bone than in trabecular bone. In contrast, in the BGP KO animals, the crystal size and perfection were the same in both the trabecular and cortical bone. These findings are consistent with impaired mineral maturation in the BGP-deficient bone and imply the presence of newer (less remodeled) mineral. Also interesting is the fact that the expression of other non-collagenous proteins, such as MGP, osteopontin and bone sialoprotein was not significantly affected by the absence of BGP. Over

time, the mutant developed abnormalities of bone remodeling which became noticeable in 6-month-old animals (135). These findings have led to the intriguing conclusion that, despite its abundance in skeletal tissues and its ability to bind calcium and apatite, BGP may actually serve as an inhibitor of bone formation.

Chronic treatment of rats for 2 months with the vitamin K antagonist warfarin reduced BGP in bone matrix to less than 2% of the control levels, but did not affect bone formation or structure (430). A visible effect on bone structure was only detected when the same warfarin treatment was prolonged for 8 months (435), and resulted on excessive mineralization and closure of the growth plate, with cessation of all longitudinal growth. This phenotype resembles the "fetal warfarin syndrome", a defect characterized by radiological stippling of the growth plate in children born to mothers who have received warfarin during gestation (204). The hypothesis that defective synthesis of vitamin K-dependent coagulation factors, which results in sporadic bleeding, is responsible for these phenotypes seems to have been ruled out, since fetal warfarin syndrome is absent when other anticoagulants, like heparin, are used. One possible explanation for the absence of visible bone alterations may be the existence of backup systems that may compensate for a BGP deficiency in rodents. In any case, the elucidation of the BGP function in the rodent depletion model may require the imposition of an external stress to bone metabolism other than those tested to date.

In fulfilling its yet conceptual role, it is possible that BGP may act in combination with other hydroxyapatite binding proteins, such as osteopontin, which potentiates osteoclast adhesion to mineral surfaces and forms a complex with BGP *in vitro* (452, 456). It is possible that this and other BGP-protein complexes may function as a bone remodelling signal. Further studies in animals depleted of combinations of matrix proteins should provide clues to the function of BGP and other bone specific proteins.

Several *in vivo* and *in vitro* studies reinforce the above stated conclusion. First, disrupted collagen fibrillogenesis in the cloned mouse calvarial cell line MCT3T3-E1 results in increased turnover of the collagenous matrix, a decrease in alkaline phosphatase, but a five-fold increase in BGP biosynthesis (567). Second, the pattern of BGP distribution in human osteons changes with gender and age, and localized reductions of BGP in the extracellular matrix are associated with reduced cortical remodeling (237). Several early

studies have suggested that BGP is involved in recruitment and activation of bone resorbing cells. The protein is a chemoattractant for peripheral mononuclear cells and giant osteoclast-like cells from tumours (81, 291, 325).

Immunolocalization studies show that BGP is distributed throughout the mineralized regions of bone matrix, dentin and calcified cartilage (330, 338). However, a growing accumulation of evidence indicates that BGP is not related to events that allow mineral deposition to occur, but rather that it participates in regulation of mineralization or bone turnover. Alternatively, Thiede *et al.* (529) suggest that, since BGP can chelate calcium ions, it may act as a natural anticoagulant within bone.

It is interesting that the presently most credited hypothetical BGP function, inhibition of hydroxyapatite crystal growth, seems to be also performed by a non-γ carboxylated protein, osteonectin. This protein has a very acidic NH_2 terminus containing glutamic acid and aspartic acid residues which, if appropriately spaced, could interact with the hydroxyapatite crystal lattice (462). However, the inhibitory effect on crystal growth of a mixture of BGP and osteonectin seems to be additive (462), which indicates that not only osteonectin and BGP do not undergo any synergistic interaction nor can compete for binding to hydroxyapatite, but also that these proteins may play, even if slightly, different roles in preventing hydroxyapatite crystal growth and maturation.

Finally, it is interesting to note that the odontoblasts already engaged in synthesizing predentine but not yet in mineralizing it to form dentine can already be strongly stained with the anti-BGP antibody (57). This observation reveals a temporal dissociation between BGP synthesis and mineralization and suggests that the protein could act to delay the mineralization of predentine.

Bone pathologies and BGP

Although, as referred above, the first clear evidence concerning the exact biological function of BGP has been provided only recently (49, 135), the protein has been extensively studied since its first identification in bone, mostly as a clinically important diagnostic parameter of bone pathologies (e.g., as a marker of bone turnover; 58, 116, 218, 529, 557), role that is favoured by its short half-life in the circulation (around 5 minutes), being rapidly

metabolized by the kidney (433). Serum BGP concentrations are correlated with histomorphometric indices of bone formation (58, 59, 91, 106, 139, 177), leading most investigators to agree that assay of serum BGP is a measure of bone formation in particular and bone turnover in general. The serum concentration of BGP reflects that portion of the newly synthesized protein that does not bind to the mineral phase of the bone but is released directly into the circulation. It is estimated that >90% of the newly synthesized protein is deposited in bone in one-month-old rats, but as the animal mature a greater proportion of protein is released directly into the serum (340). In normal human adults approximately one-third of BGP synthesis ends up in the circulation (340). Deviations from normal concentrations of circulating BGP are a consequence of changes in the synthesis or degradation pathways of the protein. Such changes may result from physiological alterations in skeletal homeostasis that accompany normal development or may be associated with specific disease states. The rate of glomerular filtration or renal catabolism also influences circulating BGP levels. Finally, serum concentrations may reflect drug- or disease-induced alterations in the normal hydroxyapatite-protein interaction, resulting in an altered proportion of existing or newly synthesised protein that binds to bone (218).

Plasma BGP content is elevated dramatically in patients with metabolic bone diseases characterized by increased bone turnover (426). Consequently, this protein can be of great utility as a diagnostic tool for such important diseases as (i) osteoporosis (a metabolic bone disease characterized by a defect in bone remodelling and the loss of the normally mineralized bone), where it may serve as an early diagnostic criteria (103, 104, 142, 372, 426), (ii) Paget's disease, as a marker of response to treatment (107, 563), or (iii) metastatic bone cancer (423). The level of BGP carboxylation has also been proposed as an indicator of the nutritional state of bone with respect to vitamin K (279), which is supported by the results of studies showing that BGP can be up to 40% undercarboxylated in postmenopausal women when compared with premenopausal women (412).

Serum BGP is commonly measured by both in house methods and commercial kits with various assay formats (e.g., 419, 463). These include high performance liquid chromatography (HPLC), radioimmunoassay (RIA), immunoradiometric assay (IRMA), enzyme linked immunosorbent assay (ELISA), and luminescence immunoassay (LIA). Also,

BGP has been increasingly used as a highly specific osteoblastic marker produced during bone formation, more explicitly as a marker of late osteoblast differentiation (121, 333, 371, 524).

Is the role of BGP crucial to the same extent in all vertebrates?

There is a possibility that the role of BGP in bone development/formation may not have the same degree of importance in all vertebrates. The fact that normal BGP levels are dramatically low in man, when compared, for example, with rat and calf (see section 1.4) suggests that the protein may play a less crucial role in the metabolism of the human skeleton when compared to other vertebrates, a feature that can provide an important clue to its function. However, additional studies using different species are required to address this question.

1.7. THE BONE GLA PROTEIN GENE

To date, only mammalian BGP genes have been characterized. Therefore it is not possible to compare structures and patterns of regulation across other than mammalian species.

Chromosome location and gene organization

The transcribed regions of the BGP genes expressed in bone of human, rat and mouse contain three introns and four exons. The promoters of these mammalian BGP genes have a similar overall organization and contain comparable promoter regulatory elements (*H. sapiens*: 73; *M. musculus* (OG1): 117; *R. norvegicus*: 527). Thus these bone-specific BGP genes appear to be organized in a manner that supports analogous responsiveness to homeostatic physiological mediators and developmental expression in relation to bone cell differentiation.

The human BGP gene has been localized to the 1q distal region of chromosome 1

(446). A mouse gene has been mapped to chromosome 3, which contains genes homologous to those located in the distal region of the human chromosome 1q (244). From these and other studies, the BGP gene was initially described as a single copy gene. However, more recent analysis of several mouse and rat strains has indicated that, in these models, BGP is part of a gene cluster (116, 117, 448). In the mouse, three contiguous genes were identified in all strains examined, while in the rat, either one or multiple copies were detected dependent on the strain (448). Of the three genes in mouse, two have identical promoters, and one gene (ORG, for Osteocalcin-Related Gene) has a variant promoter that is developmentally expressed *in vivo* in several non-bony tissues, such as brain, lung and kidney (116, 117). The coding region of ORG has a similar intron/exon organization to the BGP gene expressed in bone but carries five amino acid substitutions, one at the propeptide cleavage site. ORG also contains an additional exon that is not translated, and a 3 Kb insertion separates the ORG coding sequence from its promoter. The inserted sequence has the structure of a typical retrovirus, an attribute that leads to the downregulation of transcription, possibly explaining the low levels of expression of ORG in non-osseous tissues (117).

The function of the nonosseous expressed gene remains to be established. According to some authors (192), consideration should be given to the possibilities that it encodes (i) Gla-containing nephrocalcin, a calcium oxalate crystal growth inhibitor found in kidney, although never cloned (363-366), (ii) BGP associated with platelets (529) and the hematopoietic system (311), and (iii) BGP associated with cartilage and other tissues (281, 307). Relatively to the first hypothesis, the primary sequence of this protein remains unknown, and human specific BGP antibodies do not cross-react with partially purified human nephrocalcin (F.L. Coe, Y. Nakagawa and C. Gundberg, unpublished observations).

In contrast to other bone-related genes (e.g., type I collagen and alkaline phosphatase) (29, 577), only a single mRNA transcript has been observed from the BGP gene, in all species analysed to date (mammals and chicken). It should be noted that although regulation of expression does not appear to be modulated by changes in the organization of the mRNA transcripts, this does not preclude the presence of sequences in the transcribed region of the BGP gene which contribute to regulation of transcription. A silencer element has been identified within the BGP gene (284) and is similar to silencer elements observed in several

prokaryotic (326) and eukaryotic genes (66, 175, 465, 560, 570). Interestingly, the BGP gene silencer was the first to be identified within the protein coding region of a gene in higher eukaryotes (504).

The bone GLA protein promoter

A model of the three dimensional organization of the BGP gene promoter is presented in Figure I-3, showing postulated interactions between distinct promoter elements to support transcriptional control within a three dimensional context of cell structure and regulatory requirements at the cell and tissue level.

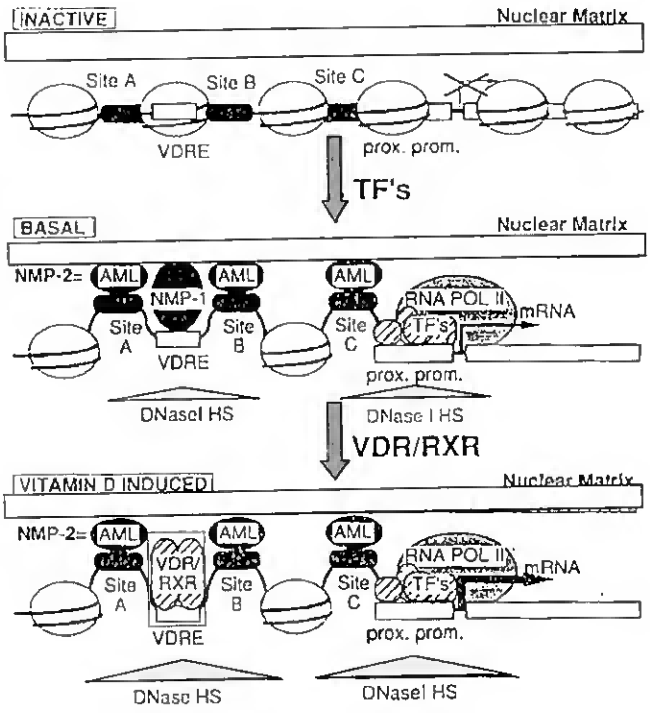


Figure I-3. General model of BGP gene regulation. Schematic representation of promoter organization and occupancy of regulatory elements by transcription factors to either i) suppress transcription in proliferating osteoblasts, ii) activate expression in differentiated osteoblasts, or iii) enhance transcription by vitamin D. Placement of nucleosomes is indicated, as well as remodelling of chromatin structure and nucleosome organization to support supression, basal expression and vitamin D-enhanced transcription of the BGP gene. Representation and magnitude of Dnase I hypersensitive sites (see 347) are shown by solid triangles. AML, AML-related nuclear matrix bound factor; NMP-1 and NMP-2, nuclear matrix protein-1 and 2; RNA POL II, RNA polymerase II; prox. prom., proximal promoter; TF's, transcription factors. Adapted from Stein *et al.* (504).

Regulatory sequences in the proximal BGP promoter

Transgenic studies indicate that sequences residing within the proximal 1,800 bp of

the rat BGP gene promoter support tissue-specific transcription (12). *In vitro* deletion-mutant experiments have shown that a 200 bp fragment of the rat promoter (521) and a 160 bp fragment of the mouse OG2 promoter (134) are necessary and sufficient to confer osteoblast-specific expression to a reporter gene. Frendo *et al.* (167) have shown that 647 bp of the mouse OG2 promoter contain all the regulatory elements necessary and sufficient to direct bone expression of a reporter gene, including the cis-acting elements required for time-specific expression of the BGP gene. However, this does not preclude the contributions of additional upstream sequences to BGP gene promoter activity (504). When transgenic animals were constructed with 3,900 bp of the human BGP promoter fused with a CAT reporter (258), expression was observed predominantly in bone but additionally at reduced levels in hypertrophic chondrocytes and kidney. Subtleties in regulatory sequences and/or nuclear proteins that account for these differences remain to be defined.

Typical sequences associated with most genes transcribed by RNA polymerase II are found in 5'-flanking regions of the rat BGP gene (e.g., TATA, CAAT, AP1 and AP2) (295, 527, 572). In addition, the BGP promoter also contains a small stretch of alternating purines and pyrimidines just 5' of the CCAAT sequence. This sequence has the potential of forming Z-DNA, a structure which may play a role in gene regulation (224). Table I.II summarizes all the factors known to regulate the expression of the BGP gene at the level of transcription and corresponding DNA binding elements.

Developmental and tissue specific-control

Combined activities of overlapping regulatory elements and associated transcription factors provide a mechanism for complex developmental control of BGP expression during osteoblast growth and differentiation (147, 367, 383, 392, 472, 504). One such example is Osteocalcin box, an highly conserved regulatory sequence required for basal expression of the rat (295) and mouse (134) BGP genes, which contains multiple regulatory elements, including AP-1 and homeodomain binding sites. The OC box contributes to both bone tissue-specific expression and species-specific regulation of the BGP gene (222, 223, 227, 228, 521, 530).

Table I.II. Main regulatory factors of BGP gene expression and corresponding DNA binding sequences. Also indicated are the proposed mechanisms of action of these regulatory factors and corresponding references.

REGULATION FACTOR	MODE OF ACTION	RESPONSIVE ELEMENT	REFERENCES
1,25 $(OH)_2D_3$	Direct and indirect transcriptional activation/ inhibition	VDRE	46, 393, 394
9-*cis*-retinoic acid	Mostly indirect activation/inhibition. Generally with no effect when alone (some exceptions; e.g. 384).	RARE/VDRE	47, 264, 319, 349, 376, 384, 408, 477, 480
Fos-jun proteins	Transcriptional regulation	AP-1 sites	296, 392, 503
Dexamethasone	Transcriptional inhibition/activation	GREs	9, 46, 222, 513
17β-Estradiol	Induction of estrogen receptor gene transcription	ERE	20
Id-HLH	Transcriptional regulation, conferring tissue-specificity	E box	357, 381, 487, 504, 521
AP1	Transcriptional inhibition; regulator of osteoblast-specific gene expression	VDRE; OSCARE-2	111, 191, 296, 392, 395, 396, 480
Cbfa1	Transcriptional activator	OSE2	18, 134, 136, 254, 283
Osf1	Transcriptional activator	OSE1	474
Parathyroid hormone	Transcriptional inhibitor	PTHRE	33, 276
Tumour Necrosis Factor (TNF- α)	Transcriptional inhibitor	TNFRE	283
TGF-β	Transcriptional inhibitor	TGRE	17, 299
Nuclear Matrix Protein-2 (NMP-2)	Regulator of osteoblast-specific gene expression	Nuclear matrix protein binding sites (AML-1 recognition motif)	39, 342, 346

Developmental and tissue-specific control

Contributions of multiple sequences appear to be operative in tissue-specific regulation (39, 341, 504), thereby providing opportunities for expression of the BGP gene in bone under diverse biological circumstances.

Osteocalcin *cis*-acting response element (OSCARE-1) was identified by Goldberg *et al.* (190) and consists of a short DNA sequence in BGP that contributes to basal promoter activity. It has two regulatory elements: a G/C-rich element and an adjacent reverse CCAAT element that binds to protein factors in a mutually exclusive manner. Homologous sequences have been characterized both as negative and positive basal regulatory elements in the promoter of the collagen type I (αI) gene (190), which suggests that OSCARE-1-like units may be a common regulatory feature of osteoblast-expressed genes.

A negative regulatory domain contributing to developmental expression of the BGP gene resides within the coding region, overlapping the first exon (168, 169). This domain, designated the osteocalcin silencer (504), contains a ACCCTCTCT motif, which is present in a series of tissue-specific genes (for example, collagen II, insulin and growth hormone; 85, 471). The BGP silencer is a multipartite element that contributes to transcriptional activity in a position- and orientation-dependent manner; the silencer is also operative in nonosseous cells, further participating in control of tissue-specific expression. In transient transfection assays, deletion of this sequence allows for a 50-fold higher level of transcription to occur in proliferating normal diploid osteoblasts, implicating the OC silencer in regulating developmental expression during differentiation and possibly tissue-specific control. This control might be mediated by variations in protein-DNA interactions (504).

In conclusion, it is important to reinforce the idea that information encoded within the modularly organized regulatory elements from the promoter of the BGP gene renders the gene competent to support expression during bone tissue development and remodelling, as well as when skeletal involvement is required to control calcium homeostasis. Subtleties in structure and organization of the BGP gene regulatory sequences must be related to the extent to which the gene is transcribed. The overlap of nuclear factor binding domains within BGP gene regulatory sequences reflects the possibility of using several regulatory pathways to expand the potential for responsiveness. The multipartite characteristics of factors binding to

each promoter element broaden the diversity of regulatory responsiveness. Apparent redundancy in transcription factor recognition for BGP gene promoter elements supports fine tuning of control and the generation of transcription factor heterogeneity, by selective splicing of primary gene transcripts for transactivation factors which mediate BGP gene promoter activity, adds possibilities for modulating expression (504).

1.8. BONE GLA PROTEIN GENE REGULATION

> On the field where this battle was fought I saw a very wonderful thing which the natives pointed out to me. The bones of the slain lie scattered upon the field in two lots, those of the Persians in one place by themselves, as the bodies lay at the first - those of the Egyptians in another place apart from them: if, then, you strike the Persian skulls, even with a pebble, they are so weak, that you break a hole in them; but the Egyptian skulls are so strong, that you may smite them with a stone and you will scarcely break them in. They gave me the following reason for this difference, which seemed to me likely enough: The Egyptians (they said) from early childhood have the head shaved, and so by the action of the sun the skull becomes thick and hard. The same cause prevents baldness in Egypt, where you see fewer bald men than in any other land. Such, then, is the reason why the skulls of the Egyptians are so strong. The Persians, on the other hand, have feeble skulls, because they keep themselves shaded from the first, wearing turbans upon their heads.
>
> Herodotus, *The Persian Wars*, Book III

Studies on regulation of BGP expression have been conducted exclusively in higher vertebrates, mainly in mammals and chicken. Those studies have shown that BGP gene expression is tightly regulated at multiple levels, which may account for variations in the activity and inducibility of the gene during the osteoblast developmental pathway. Although there are species-specific differences in the regulation of BGP, common patterns can be identified. The expression of the BGP gene is transcriptionally regulated by a broad spectrum of hormones and other physiological mediators, as established by activities of the rat as well as the human BGP gene promoters, *in vivo* and *in vitro* (e.g., 146, 196; reviewed in 298 and 504).

BGP regulation by Vitamin D and analogs

Evidence for transcriptional regulation - effects on bone metabolism and calcium homeostasis. The first suggestion that vitamin D modulated BGP synthesis derived from the observation of decreased bone and serum BGP concentrations in vitamin D-deficient chicks (287) and rats (292, 436, 439). Furthermore, when vitamin D-deficient rats were maintained on high-calcium diets that normalize serum calcium, decreased BGP levels were still observed in the vitamin D-deficient animal (218). Further studies confirmed the hypothesis that the active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] plays a key role in the transcriptional regulation of BGP gene expression in osteoblasts, both *in vivo* and *in vitro* [(109, 295, 398, 429, 572); for reviews on the molecular actions of vitamin D see, for example, 60, 245, 510, 559].

Under basal conditions, the production of BGP by cultured osteoblasts is usually low but may be enhanced several fold following stimulation with the hormonally active form of vitamin D_3, $1,25(OH)_2D_3$. This has been demonstrated *in vivo* (287, 292, 436), as well as in a number of rat and human osteoblast-like cell culture systems (32, 77, 248, 420). In the ROS 17/2 cell line, administration of $1,25(OH)_2D_3$ increases intracellular BGP levels up to six fold above basal levels within 12 hours (424), with a corresponding six-fold increase in the rate of BGP secretion observed by 15 hours. In malignant osteoblast cell lines BGP synthesis is modulated by, rather than dependent upon, vitamin D, since there is often substantial BGP synthesis in the absence of $1,25(OH)_2D_3$ (252). In contrast, the synthesis of BGP by normal human bone-derived osteoblast-like cells is always dependent upon the addition of $1,25(OH)_2D_3$ (33, 490).

It is interesting to note that during development of the osteoblast phenotype BGP gene expression can be regulated by transcriptional and post-transcriptional mechanisms. In fact, as previously stated, $1,25(OH)_2D_3$ can control the rate of transcription during the transition from the proliferative to the postproliferative period. Later in the mineralization phase, however, accumulated basal mRNA levels are stabilized and transcriptional control of the hormone is minimal within 24 hours (484).

$1,25(OH)_2D_3$ has multiple actions but its major role is related to bone metabolism and

mineral homeostasis. The genomic action of $1,25(OH)_2D_3$ is mediated by the nuclear vitamin D receptor (VDR), a phosphoprotein member of the nuclear transacting receptor superfamily that binds its ligand with high affinity (108, 271). The family which includes the VDR and the nuclear receptors for all-*trans* retinoic acid (RA), thyroid hormone (3,5,3' - triiodothyronine [T_3]), and peroxisome proliferators is classified as Class II, according to the response element recognized (257, 404, 575). Following the binding of $1,25(OH)_2D_3$, the VDR undergoes covalent modifications resulting in rapid protein phosphorylation (411). This phosphorylated receptor form heterodimerizes with the retinoid X receptor (RXR) and then associates specifically with vitamin D reponsive elements (VDREs) in target genes (92, 265, 345). The regulatory role of vitamin D on BGP gene expression occurs, therefore, at the level of transcription (398, 401, 424, 429), which is confirmed by the complete blocking of the BGP response by the transcription inhibitors α-amanitin and actinomycin D (398).

Characterization of the VDRE. The VDRE of the rat (110, 332, 526, 572) and human (257, 349) BGP genes has been identified and characterized structurally and functionally by several approaches. The BGP VDRE was the first of the VDREs to be identified (257, 349) and bears similarities with the members of the superfamily of related steroid response-elements, that includes the estrogen responsive element (ERE), the thyroid hormone responsive element (TRE), the glucocorticoid responsive element (GRE), and the retinoic acid responsive elements (RAREs) (40, 98, 297, 312, 396, 533, 535, 574). The minimal VDRE is characterized by two half steroid motifs (either perfect or imperfect direct repeats) separated by 3 nt. (54). The variable that determines receptor specificity seems to be the number of nucleotides separating the half-sites. The RXR has been shown to have a preference for two half-sites separated by a single nucleotide, termed a DR-1 response element, whereas the VDR binds to a DR-3 element, the TR to a DR-4 element, and the RAR to a DR-5 element (535). Interestingly, almost all of the naturally occurring VDRE isolated from genes upregulated by the VDR have fallen into the DR-3 category (378, 429, 580). The genes negatively regulated by VDR, such as PTH and IL-2, were thought not to follow this rule. Initial work with the PTH gene indicated that the vitamin D responsive element in this case consisted of only a single half-site. However, more recent work indicates that the PTH VDRE

may in fact consist of two half-sites (99), and this leaves only a few exceptions, such as the IL-2 gene, to this rule (3). Table I.III summarizes most of the information concerning vitamin D-regulated genes and their respective VDREs, when known.

Confirmation of protein-DNA interactions and related functionality has been provided by *in vitro* protein-DNA binding studies (332) and mutational analysis (111). Characterization of the VDRE in other vitamin D-regulated genes, osteopontin (378), calbindin D9K (97), calbindin D28K (187), parathyroid hormone (112), and the hydroxylase enzyme (382, 580) revealed similar features.

The VDRE of the rat and human BGP genes functions as an enhancer (257, 495). $1,25(OH)_2D_3$ does not induce transcription but requires basal expression (504). This integration of promoter regulatory activities supports the well documented accomodation of vitamin D-responsive transcriptional control to several physiological mediators simultaneously. Additionally, the VDRE is sensitive to growth and to the physiological status of the cell (504). As an example of the two previous statements, in imature osteoblasts dexamethasone antagonizes the marked vitamin D-mediated upregulation of BGP transcription, as it does in ROS 17/2.8 cells and human osteoblasts, whereas in mature osteoblasts within a mineralized matrix, dexamethasone further increases vitamin D stimulation of BGP by mRNA stabilization (504).

In contrast to the rat and human model, $1,25(OH)_2D_3$ inhibits mouse BGP expression (e.g., 481), at least partially by abolishing the binding of osteoblast nuclear extracts to OSE2, a critical osteoblast-specific cis-acting element present in the BGP promoter (579). Hypothetically, the $1,25(OH)_2D_3$ treatment would inhibit the transcription of the gene coding for Cbfa1, an osteoblast-specific activator of transcription (see section 1.9.9). Taken together, these relationships provide potential explanations for positive or negative activity of a single regulatory factor under different biological conditions.

Table I.III. Genes known to be regulated by Vitamin D and accepted putative function of their corresponding proteins. VDRE sequence and type (when available with a high degree of confidence) present in their respective promoters, the effect of the hormone in the transcription of the target gene and significant references are also indicated.

Target Gene	Protein function	VDRE sequence[1] in gene promoter	VDRE type*	Transcriptional effect of vitamin D	References
Human BGP	Bone crystal maturation? Inhibitor of mineralization?	GGGTGAacgGGGGCA	DR3	Induction	257, 349, 395
Rat BGP	Idem	GGGTGAatgAGGACA	DR3	Induction	109, 295, 424, 572
Rat BGP	Idem	TGCACTgggtgaatgAGGAC A	IP-9	Induction	477
Mouse BGP	Idem	GGGCAAatgAGGACA	DR3	Inhibition	86, 579
Chicken BGP	Idem	Unknown	Unknown	Inhibition	56
Rat collagen type I	Among other functions, forms fibrils that are a matrix for calcium salts and hydroxyapatite deposition.	GGGGGCAGAAGAACT	-	Induction/Inhibition	42, 162, 247, 272, 392, 393, 550
Rat bone sialoprotein	Regulation of hydroxyapatite crystal nucleation and growth? Promotes attachment and spreading of cells	AGGGTTtatAGGTCA	DR3	Inhibition	383
Mouse osteopontin	Bone morphogenesis and remodeling? Kidney morphogenesis?	GGTTCAcgaGGTTCA	DR3	Induction	114, 115, 378, 461
Chicken osteopontin	Idem	Unknown	Unknown	Inhibition	56

[1] Nuclear protein binding sites are indicated in capital and spacers in low case letters.

Target Gene	Protein function	VDRE sequence	VDRE type*	Transcriptional effect of vitamin D	References
Chicken carbonic anhydrase II	Encodes an enzyme in bone-resorbing cells derived from the fusion of monocytic progenitors.	AGGGCAtggAGTTCG	DR3	Induction	447
Human PTH	Stimulates osteoblast proliferation; stimulates or supresses osteoblast differentiation; inhibits collagen synthesis; activates osteoclasts.	GGTTCAaagCAGACA	DR3	Inhibition	112, 253, 488
Bovine PTH	Idem	Unknown	Unknown	Inhibition	221, 253
Rat PTH	Idem	Unknown	Unknown	Inhibition	488
Avian PTH	Idem	GGGTCAggaGGGTGT	DR3	Inhibition	269, 306
Human calbindin D_{9k}	Transcellular flux of calcium?	TGCCCTtccttatggGGTTCA	IP-9	Induction	477, 552
Mouse calbindin D_{28k}	Idem?	GGGGGAtgtgAGGAGA	DR4	Induction	187, 552
Avian calbindin D_{28k}	Idem?	Unknown	Unknown	Induction	552
Avian β3 integrin	Transmembrane glycoproteins that serve as receptors for a wide variety of ligands	GAGGCAgaaGGGAGA	DR3	Induction	67, 498, 564
Rat 24-Ohase-Distal	Metabolism and regulation of vitamin D3 function	GGTTCAgcgGGTGCG	DR3	Induction	580
Rat 24-Ohase-Proximal	Metabolism and regulation of vitamin D3 function	AGGTGAgtgAGGGCG	DR3	Induction	248, 378, 382

*According to Umesono *et al.* (535).

The specificity of induced BGP synthesis by only the $1,25(OH)_2D_3$ metabolite of vitamin D_3 has been confirmed in several studies (33, 77, 556). The $24,25(OH)_2D_3$ metabolite that has been implicated in mineralization and calcification of cartilage does not strongly influence BGP synthesis (33, 78, 556). However, synthetic analogues of the $1,25(OH)_2D_3$ metabolites, for example, 1,25-dihydroxy-hexadeuterocholecalciferol and 1,25-dihydroxy-26-trifluorocholecalciferol in fetal rat calvariae cultures and human bone osteoblast cultures (294, 490), and 1,25-dihydroxyvitamin D-2 and 24-epi-1,25-dihydroxyvitamin D-2 in the ROS 17/2.8 osteosarcoma cell line (5) do stimulate BGP synthesis. It is important to note, however, that, in several model systems, decreased or induced levels of $1,25(OH)_2D_3$ are accompanied by paralleled changes in serum BGP values but are not necessarily related to bone BGP content (218).

In vitro (121) and *in vivo* (287, 436) studies show that synthesis of BGP occurs in the absence of vitamin D at relatively high levels (~60% of normal in vitamin D-deficient chickens and mice), suggesting a possible role for BGP in bone metabolism independently of vitamin D. Furthermore, this suggests that the physiological role of the $1,25(OH)_2D_3$ regulation of BGP synthesis is to accelerate a normal BGP action in bone, in order to adjust bone metabolism to stresses such as dietery Ca^{2+} deficiency. As indicated in section 1.6, one possible physiological role for BGP as a mineralization inhibitor would be to reduce the flux of calcium and phosphate to bone. The increased rate of BGP synthesis and secretion induced by $1,25(OH)_2D_3$ would, by inhibiting mineralization, reserve for serum the fraction of Ca^{2+} which would otherwise be incorporated into bone. Serum calcium homeostasis would be, then, the "reason" why BGP is regulated by vitamin D, while in the non-stimulated phase BGP production in bone and dentine probably plays a role in matrix formation rather than in regulating serum Ca^{2+} concentration (439 and references therein).

Regulation by other steroids. Steroid and steroid-like hormones are potent modulators of transcription through binding to nuclear receptors. The DNA binding domain of a typical steroid hormone receptor consists of a structure in which two zinc atoms are coordinated in two finger-like domains (166). The N-terminal finger confers specificity to the binding, whereas the second finger stabilizes the complex (195). Steroid hormone receptors form homodimers (152) and some of them heterodimerize with other members of the family (159,

478) or with other nuclear proteins (96, 275, 356).

Among the steroids, retinoic acid (RA), a vitamin A metabolite, is an important signaling molecule involved in the regulation of growth during embryonic development and cell differentiation, and plays an important role in the normal development of bone (e.g., 119). RA has been demonstrated to increase the number of 1,25-dihydroxyvitamin D_3 receptors in the ROS 17/2 cell (408). Pretreatment with RA also increases BGP secretion in 1,25-dihydroxyvitamin D_3 -treated cultures compared to controls (376).

Effects of glucocorticoids. Glucocorticoids (GR) have significant effects on bone and mineral metabolism (183). *In vivo*, the skeletal effects of glucocorticoids, which include increased bone resorption (102) and decreased bone formation (183), are associated with diminished osteoblastic activity, a decline in serum levels of BGP (140, 314) and a reduction of BGP gene expression (46, 349). The initially characterized glucocorticoid responsive-element (GRE) of the human BGP gene is associated with the TATA domain (513). In rat, the presence of GR binding sites in close proximity to the basal TATA box and CCAAT motifs (504) suggests that interference of GR with the positive transcription factors, such as TFIID, could be responsible for the negative regulation of BGP by glucocorticoids documented in ROS 17/2.8 cells (8, 255, 413) and in human osteoblasts (482, 514, 566). From studies on the rat BGP promoter, it is now known that several GRE domains are involved in modulating glucocorticoid effects on BGP gene transcriptional activity (222), all these GREs appearing to be functionally active (504).

Interaction of other transcription factors with the proximal GREs, that include NF-IL6, has been reported (531), further expanding the potential of the BGP gene to be transcriptionally regulated by glucocorticoids. It is reasonable to consider that the GREs in the BGP gene may be selectively used in a developmentally and/or physiologically responsive manner, and the possibility of functional interactions with transcription factors other than glucocorticoid receptors under certain conditions should not be dismissed (504).

Effects of dexamethasone. Dexamethasone, a synthetic glucocorticoid, affects rat osteoblast-like cells via its effect on 1,25-dihydroxyvitamin D_3 receptors (76). Dexamethasone alone suppresses BGP gene transcription (222, 349, 473, 513). However, the combined effect of

$1,25(OH)_2D_3$ and dexamethasone is a synergistic increase in the transcriptional activity of the BGP gene (47, 376, 480). Differences in the influences of vitamin D and dexamethasone on BGP gene transcription occur and appear to be dependent on the developmental stage of normal diploid osteoblasts and on maintenance of the normal diploid phenotype (26, 393, 480, 566). In human osteosarcoma cells, rather than a synergism between $1,25(OH)_2D_3$ and dexamethasone, a glucocorticoid-dependent abrogation of vitamin D-related transcriptional enhancement is observed when these cells are simultaneously treated with the two steroid hormones. It remains to be determined whether the same promoter regulatory sequences are involved in the positive and negative responses associated with independent, compared with combined, activities of vitamin D and dexamethasone in normal and in tumour cells. However, the possibility can be considered that variations may reflect transformation-related modifications in cellular signaling pathways that determine the targeting and/or activity of vitamin D and dexamethasone to promoter regulatory elements. Influences on both receptors and other components of the multipartite transcription factor complexes that contribute to transcriptional control by vitamin D and dexamethasone are viable considerations within this context (504). Indeed, the presence of multiple glucocorticoid-responsive elements in the rat BGP gene promoter (8, 9, 222) with positive and negative glucocorticoid-responsive motifs and activities lends credibility to these explanations.

Regulation by growth factors. Growth factors are small peptides, originally thought only to act on tranformed cells, but now known to be key players in regulating cell growth and embryonic development (93 and references therein).

BGP is downregulated upon treatment with TGF-β1 (53, 189, 206). Mutational analysis, coupled with results of *in vitro* gel shift and competition assays, led to the conclusion that the response of the rat BGP gene to TGF-β1 is mediated through at least one cis-acting DNA element with similarity to the AP-1 binding consensus sequence and that Fra-2 and Jun-B proteins are involved in the interactions at this site (17).

Regulation by the nuclear matrix. Involvement of the nuclear matrix in control of the BGP gene transcription is provided by several lines of evidence. One of the most compelling is the association of a bone-specific nuclear matrix protein designated Nuclear Matrix Protein-2

(NMP-2) with sequences flanking the VDRE of the rat BGP gene promoter (39). Several results (39, 341) implicate the nuclear matrix in regulatory events that mediate structural properties of the BGP VDRE domain and may also contribute to link BGP gene expression with modulation of pattern formation required for skeletal tissue organization during bone formation and remodeling.

Regulation by Parathyroid- / Thyroid-produced hormones. Parathyroid hormone (PTH) is one of the major regulators of bone, exerting various effects on bone cells, namely stimulating osteoblast cell proliferation (318), stimulating or suppressing osteoblastic differentiation (27, 571) or inhibiting collagen synthesis in osteoblasts (272). These effects seem to be mainly mediated by classical PTH/parathyroid hormone-related protein (PTHrP) receptor, although novel receptors, such as PTH2 receptor and C-PTH receptor (238, 536) have been reported.

The precise relationship between parathyroid gland activity and serum BGP concentrations has not been clearly established. However, a study by Tsuji *et al.* (534) showed a decrease in serum BGP concentration upon admnistration of PTH to normal and hypophosphatemic (HYP) mice. Other studies have reached the same conclusion, demonstrating not only that PTH directly impairs BGP synthesis by osteoblasts *in vitro* (33, 276), but also that in humans, infusion of PTH to healthy subjects (161) and HYP patients (88) leads to significant decreases in serum BGP. The acute effect of PTH on serum BGP is in contrast to its long-term effect, which is associated with elevated levels of serum BGP (455).

Regulation by *runt*-like nuclear factors (CBFA, AML, MSX).

The CBFA family. The nomenclature of the first gene identified from this family (Cbfa1) has changed several times since it was first isolated. It was originally called Pebp2a1, then Aml3 (279), and in 1995 the gene nomenclature committee decided to use the Cbfa nomenclature for all the genes of this family (500).

The Cbfa (Core binding factor a) /AML family of transcriptional activators, which are mammalian homologs of the *Drosophila* segmentation gene *runt*, are critical factors for the development of hematopoietic and skeletal tissues. Each of the three known genes, Cbfa1 (human AML-3/mouse Pebp2a [hAML-3/mPebp2a]), Cbfa2 (hAML-1/mPebp2b), and Cbfa3

(hAML-2/mPebp2c), encodes several mRNA splice variants (1, 279, 500). Several isoforms of Cbfa1 have been described (for a review on Cbfa1 see 264); one is expressed in hematopoietic tissues (578), and another is highly expressed in osteoblast lineage cells of bone (18, 568) and in hypertrophic chondrocytes (267). Ablation of the Cbfa1 gene in mice reveals the importance of this factor in development of the skeleton, resulting in a phenotype in which the skeleton is composed of cartilage and fibrous tissue, with complete lack of ossification (267, 354, 389). Osteoblasts of the mutant mice expressed low levels of alkaline phosphatase but barely detectable levels of osteopontin and BGP (267, 389). In addition, Ducy *et al.* (137) have recently shown, through a time-specific, cell-specific, and stage-specific inhibition-of-function experiment that Cbfa1 is required for bone formation by differentiated osteoblasts after birth. By controlling its own expression positively, Cbfa1 is at the top of a gene regulatory cascade, regulating bone extracellular matrix deposition. Inhibition of this autoregulatory loop in differentiated osteoblasts results in an osteopenic phenotype caused by the near abolition of expression of extracellular matrix-related genes, including type I collagen genes, without any overt effect on osteoblast differentiation. These results uncover a transcriptional pathway governing bone formation by differentiated osteoblasts and identify Cbfa1 as the first transcriptional activator of this process.

Cbfa proteins were also shown to regulate tissue-specific expression of the BGP promoter (16, 134, 136, 167). Although overexpression of the Cbfa1/AML-3, Cbfa2/AML-1, or Cbfa3/AML-2 factors in non-osseous cells can confer expression of the BGP gene (16), the DNA binding activity present in mature osteoblasts consists primarily on the Cbfa1 gene product (18, 136).

The rat BGP promoter contains three recognition sites for Cbfa interactions [sites A, B, and C (342)]. Notably, all three motifs bind a similar osteoblast-specific DNA binding complex, first designated NMP-2 (39, 342). While only one Cbfa site fused to a minimal BGP promoter is sufficient to confer enhancer activity in osseous and nonosseous cells (16), the presence and positioning of multiple Cbfa sites suggest that spatial organization of the native BGP promoter may be important for interaction of Cbfa proteins with other BGP promoter regulatory factors. For example, as described in 1.9.1. of this chapter, transcription of the rat and human BGP genes is strongly influenced by $1,25(OH)_2D_3$. Cbfa sites A and B flank the vitamin D response element which mediates a 3- to 10-fold enhanced activity of the

rat and human BGP promoters. A third Cbfa site, C [also designated OSE2 in mice (134)], is located in the proximal promoter. A nucleosome is positioned between Cbfa sites B and C in the rat BGP promoter (348). Because Cbfa factors associate with the nuclear matrix and can recruit other factors to form complexes (7, 239, 280, 397, 500), Cbfa binding sites may impose structural constraints on the BGP promoter to facilitate interaction between proximal and distal regulatory elements (e.g., the vitamin D response element [VDRE] and TATA domains). Cbfa1 is, therefore, an indispensable regulator, not only of BGP, but also of osteoblast differentiation (e.g., 268), that fullfills a function dominant to and nonredundant with the function of any other gene product.

The MSX family. Another class of transcription factors that belong to the family of runt-homologous proteins bind to BGP promoter sequences, more specifically to homeodomain protein binding sites within the OC box (222, 530). Competition and mutational analysis, as well as binding of purified homeodomain peptides and Msx-1 and Msx-2 proteins in gel shift assays provide evidence for interaction of this type of factors with the CCAATT motif (227, 530). Msx-1 gene encodes an ubiquitous transcription factor expressed in most cells and in osteoblasts at a constitutive level throughout differentiation, while Msx-2 appears to be tissue restricted and expressed developmentally during the osteoblast growth and differentiation periods (528 and references therein). The $1,25(OH)_2D_3$-dependent upregulation of Msx-2 expression and its binding to homeodomain sequences within the OC box are consistent with a functional relationship between the degree of Msx-2 binding within the OC box and the level of BGP gene transcription.

The AML family. The transcription factor Acute Myelogenous Leukemia-1 (AML-1) has a DNA binding domain that shares 69% identity to runt homology domain proteins and is somewhat tissue restricted, found predominantly in T-limphocytes (504). The AML-related transcripts are found in osteoblasts by Northern analysis but specific bone-related proteins have not been characterized. Two confirmed AML binding sites flank the rat BGP VDRE, one within the proximal promoter adjacent to the TGF-β responsive element, which is essential for transcription.

2. OTHER VITAMIN K-DEPENDENT PROTEINS

The vitamin K-dependent proteins comprise a group of macromolecules that contain the aminoacid γ-carboxyglutamic acid (Gla), a protein family that is important in a variety of tissues and cellular functions. This family comprises the proteins involved in the blood coagulation cascade, and a few others, functionally not related to the first group, including some just recently discovered. The initial discovery of Gla in prothrombin (507) and the existence of vitamin K-dependent protein carboxylation in liver microsomes (143) were milestones in unraveling the complexity of the blood coagulation pathway. The substrates for carboxylation were found to include prothrombin (factor II) and coagulation factors VII, IX and X (143, 509, 517, 519), and the post-translationally acquired content of Gla was understood to confer on these proteins a new and functionally important potential for interaction with Ca^{2+} (214, 414, 507) and acidic phospholipid surfaces (369, 509, 541). Subsequently, protein carboxylation, resulting in the vitamin K-dependent formation of Gla residues, was observed in a wide variety of other tissues, including bone (286), kidney (208, 543), urine (155), renal calculi (285), placenta (171), skin (100), and spleen, lung and testis (61, 499, 543). Gla containing proteins occur in cartilage (165, 202, 211, 388), dentin (123, 188, 303), cementum (188), antler and bone of all vertebrate classes, including Amphibia and Reptilia (240).

Table I.IV. synthetises some of the information concerning all the presently known vitamin K-dependent proteins.

Matrix GLA protein (MGP). Matrix Gla Protein (MGP) deserves here a special emphasis, due to its affinities with BGP, some of which will be addressed in the following paragraphs (see also chapter IV, Section 7, on the phylogenetic analysis of spBGP).

Matrix Gla protein (named that way because of its firm association with the organic phase of bone and cartilage) belongs to the family of vitamin K-dependent proteins (Table I.IV). It was the second vitamin K-dependent protein discovered in bone, being isolated from bovine bone in 1983 (438). Upon amino acid sequence, bovine MGP was shown to have 5

Gla residues and be 79 residues in length. Subsequently, in each species where the initial MGP translation product was determined (e.g., 260, 442) it was observed that it comprises a transmembrane signal peptide and the mature protein. MGP was the first example of a vitamin K-dependent protein that lacks a propeptide (442), demonstrating that the γ-carboxylation and secretion of vitamin K-dependent proteins need not be linked to the presence of a propeptide or to its proteolytic removal.

During bone development, MGP appears before the onset of mineralization, consistent with its presence in cartilage (388). The final concentration of MGP attained in mature rat bone (388, 430) [0.4 mg/g] is ~10% of the BGP level on a molar basis. Approximately 10-20% of the total protein-bound Gla in adult bone is accounted for by MGP, and BGP contributes the remainder (218).

While BGP biosynthesis is restricted to bone and dentin, MGP is synthesised in a variety of tissues and cell types, its mRNA being present in all soft tissues examined, mainly in lung, heart and kidney (165, 167), but also in bone and cartilage (69 for bone; 202 and 388 for cartilage). The accumulation of the MGP protein in bone and cartilage greatly exceeds that of the soft tissues, which fail to accumulate it (165). A wide variety of cartilage types, including those that do not normally calcify, contain MGP (202).

The detection of MGP mRNA in a wide variety of tissues (165) raises new issues about the biological function of this protein. Although its function, as with BGP, is not fully understood, MGP is thought to have a role in clearing excess calcium from tissues into the circulation, thus protecting against calcification (165, 309). Results showing the protein to be a potent inhibitor of hydroxyapatite crystal formation *in vitro*, via γ-carboxylated Gla residues (420, 462) support this hypothesis. Recent genetic studies have shown that mice lacking a functional MGP gene are viable, but exhibit increased calcification of growth plate cartilage, short stature, osteopenia, and fractures (317). The MGP-deficient mice die between one and two months of age as a result of excessive abnormal calcification of their arteries leading to blood vessel rupture.

Table I.IV. Summary of information on vitamin K-dependent proteins (excepting BGP). Information on the function, site of production of protein, number of GLAs, molecular weight, site of protein localization and relevant references is displayed. Intervenients on the blood coagulation cascade are signaled by an asterisk (*).

Protein	Function(s)	Produced by	Number of Glas	M_r (Da)	Tissues of presence	References
Prothrombin*	Blood Clotting; feed back activation of factor XI; converts fibrinogen into fibrin.	Liver	10	72,000	Blood plasma	132 and reviews therein; 174, 313, 359
Coagulation factor VII*	Forms a complex with Tissue-Factor (TF) that activates factor IX.	Liver	10	50,000	Blood plasma	174, 278
Coagulation factor IX*	Blood clotting; cofactor of factor VIIIa; converts prothrombin in thrombin; activator of factor X.	Liver	12	56,000	Blood plasma	55, 126, 174, 278, 479
Coagulation factor X*	Converts Prothrombin in Thrombin, in the presence of Ca^{2+} and factor Va.	Liver	11	56,000	Blood plasma	174, 479
Gas 6[2]	Anticoagulant factor ?; growth regulation?; cell survival factor?; ligand for the receptor tyrosine kinases.	Liver	11-12	75,000	Heart, lung, stomach, kidney, cartilage and human chondrocytes.	327, 360, 361, 475, 512, 540
Protein S*	Anticoagulant factor; cofactor for protein C in the inactivation of coagulation factors Va and VIIIa and enhancement of fibrinolysis. Provides a link between osteoblast and osteoclast? Bone turnover	Mainly in liver; also in endothelial cells, megakaryocytes, brain, spleen,	10	69,000	Blood, bone and cartilage	31, 101, 125, 180, 323, 409

[2] First discovered in 1988

Protein	Function(s)	Produced by	Number of Glas	M_r (Da)	Tissues of presence	References
	and metabolism? Mitogen for smooth muscle cells? Some function in the brain?	osteoblasts and cells of the nervous system.				
Nephrocalcin	Inhibition of the growth of calcium oxalate monohydrate crystals; calcium elimination through kidney ?	Kidney	2-3	14,000	Kidney; urine	363, 364
Protein C*	Anticoagulant factor; co-responsible for the inactivation of factors Va and VIIIa by cleavage of polypeptide chain; also with fibrinolytic activity.	Liver	11	62,000	Blood	144, 508, 539
Protein Z*	Unknown	-	-	-	Plasma	199
MGP	Inhibition of ectopic calcification?	Vascular smooth muscle tissue and chondrocytes	5	10,000	Lung, heart, kidney, bone and cartilage	69, 165, 167, 202, 388
PRGP1[3]	Unknown	Spinal chord	-	23,000	Spinal chord	274
PRGP2[4]	Unknown	Thyroid	-	17,000	Thyroid and kidney, among others.	274

[3] First discovered in 1997.

[4] First discovered in 1997.

More recent results (355, 569) confirm that MGP is also a powerful and developmentally regulated inhibitor of cartilage mineralization, controls mineral quantity but not type, and appears to have a previously unsuspected role in regulating chondrocyte maturation and ossification processes.

3. *SPARUS AURATA*: THE GILTHEAD SEABREAM

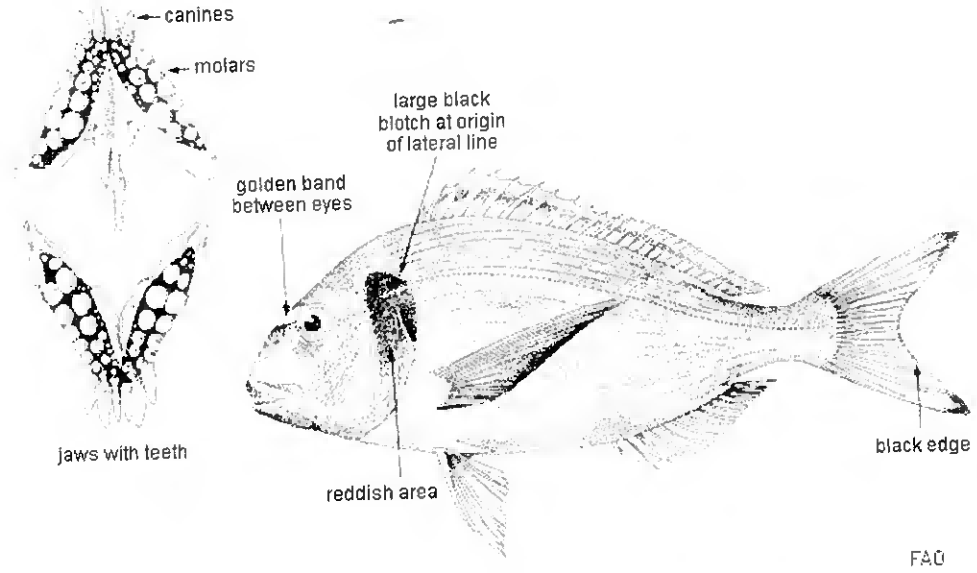


Figure I-4. Drawing showing an adult *Sparus aurata* specimen, with particular relevance to the characteristic head and tail markings. Also displayed are the jaws and teeth, showing canines and molars (in 476).

3.1. TAXONOMICAL CLASSIFICATION OF Sparus aurata

Kingdom: ANIMALIA
Type: CHORDATA
Subphylum CRANIATA
Infraphylum: VERTEBRATA
Superclass: PISCES
Class: OSTEICHTHYES
Subclass: ACTINOPTERYGII
Division: TELEOSTEI
Subdivision: EUTELEOSTEI
Series: PERCOMORPHA
Order: PERCIFORMES
Suborder: PERCOIDEI
Family: SPARIDAE
Genus: *Sparus*
Species: *Sparus aurata* (Linnaeus, 1758)

[according to Long (310)]

3.2. GENERAL Sparus aurata BIOLOGICAL FEATURES

Sparus aurata, the gilthead seabream, is a marine, teleost fish, which is common in all the Mediterranean Sea and in the east Atlantic Ocean (from the British islands to the Canaries and Cape Vert), being rare in the Black Sea (157, 558). In the Mediterranean, the juveniles can be found during Spring in the salt marches, returning to the marine environment by the end of Autumn. It is a litoral species, the juveniles being found until 30 m of depth and the adults until 150 m (157), either isolated or in small groups, mainly near sandy bottoms surrounded by rocks, or in the vicinity of muddy bottoms (22, 23). As is typical of litoral-dwelling species, *S. aurata* is an eurihaline and eurithermic fish, withstanding salinities ranging between 4 and 70 ‰ (157), and temperatures between 5 and 32°C (87).

S. aurata is a carnivorous species, although sporadically herbivorous (157, 558). Its feeding regime is formed mainly by bivalves, crustaceans, worms, other fishes and, sometimes, by algae (6).

The reproductive physiology of *S. aurata* makes it an interesting species, since it is a protandrous hermaphrodite species, its first sexual maturation being masculine, and with a sexual inversion that occurs at a given period of its development (157). In fact, in captivity and during the first year of life, all specimens are males, the same occurring, when specific conditions are established, throughout the second year. From this date on a percentage of males suffers a sexual inversion (386, 582, 583) that seems to be socially controled concerning the proportion of males experiencing it (205). The presence of a high number of young fish from May to September, period when social and hormonal factors influence sex inversion, triggers an increase in the number of older fishes suffering sexual inversion; also, the presence of a high number of old females can inhibit sexual inversion in younger specimens.

The laying season of *S. aurata* extends for several months, with multiple layings (71), due to an asynchronous ovocitary development. In the Mediterranean sea the reproduction season spans from November until the end of January (6), although in warmer waters, like near Israel, it can be extended up to May (584). Laying is bentonic (between 5 and 35 m) and eggs and larvae are pelagic. The laying of eggs and the extension of the embrionary

development are greatly conditioned by external factors, such as salinity (583), photoperiod (75) and water temperature (243).

At the time of eclosion the *S. aurata* larvae measures about 2 mm and is rather transparent. Already formed are the primordial fin, the notochord and the miomers (262). The vitelin sac, localised in the antero-ventral region of the body, encompasses nearly half of the larvae length and represents its source of nourishment for the first 3-4 days of life after eclosion (546, 576). During this period the pectoral fins start to develop (262) and the eyes become fully functional (576). In these first days of exogenous life, the *S. aurata* brain is completely differentiated and the branchial arches are developing (250).

After the vitellin sac has been reabsorbed the mouth and the remaining parts of the digestive tract become functional and the primordial pectoral fins are completely formed (546). With transition to exogenous feeding the larvae experience a critical period, with very high mortalities (315, 584).

The larval stage ends when the specimens develop scales and fins are differentiated, which corresponds to an age around 40-45 dph. Calcified structures begin to appear at around 30 dph (or 70-80 mm in lenght), although a high variability has been observed, depending on a series of extrinsic and intrinsic factors which regulate individual development. Around 90 dph almost all calcified structures have developed, only the structures necessary for growing of the fish remaining in the cartilaginous state (6 and references therein; 150, 151).

Its highly appreciated meat makes *S. aurata* a commercially important species and justifies the long-lasting habit of intensive and extensive culture, which dates back to the Acient Greece (21). Since the late seventies a growing interest has been focused on the semi-intensive and intensive culture of this species and presently it is possible to control all stages of its life cycle (e.g., 94, 127, 148).

Sparus aurata has been the subject of various studies, focusing mainly on larval development and nutritional requirements of this species (e.g., 249, 523, 546). However, information on cartilage and bone development exist largely for related species (e.g.: 13, 14, 172, 230) and only recently some studies focusing on the developmental ossification of the main *Sparus aurata* skeletal structures appeared: 4 and 402: malformations of the axial skeleton; 262 and 469: developing of the notochord; 150 and 151: development of osseous structures and fins.

4. APPEARANCE, STRUCTURE AND IMPORTANCE OF THE SKELETON IN VERTEBRATES IN GENERAL AND IN FISHES IN PARTICULAR

This section will i) give an overview on the appearance and importance of skeleton for vertebrates and ii) focus on the available information concerning cartilage, bone and other calcified structures on *S. aurata*, in particular, and on the fish class, in general.

The parts of the vertebrate body which mineralize during life, comprising at least 60% of its dry weight, constitutes the skeleton. Besides its obvious mechanical function as the body's scaffolding, anchoring muscles and providing levers and articulations for locomotion, food acquisition and food processing, as well as holding and protecting fragile organs and soft tissues, the skeleton performs equally important (if less obvious) physiological functions during the life of the animal, from which can be mentioned its involvement in the storage and release of calcium and phosphorous salts, as part of the control of body homeostasis. These varied and apparently conflicting functional demands are exemplified by the process of skeletal growth, which accomodates simultaneous changes in skeletal size, shape and proportions with the continuous fulfillment of all its mechanical and physiological functions.

4.1. THE ORIGIN OF THE SKELETON

It is usually accepted that true bone is an acquisition of the vertebrates, and that its appearance has possibly occurred about 500 million years ago, in the United States Ordovicean (L1). However, the discovery of conodonts (the enigmatic, microscopic phosphatic remains of a group of primitive chordates; they are mainly tooth-like in shape and functioned as a food-gathering apparatus) raised a strong doubt on the accuracy of that timespan. Conodonts are found over a very wide stratigraphic range, spanning from long before the previously accepted earliest vertebrates are recorded (at least 30 MY prior to this), to the period where all major vertebrate groups (except for the mammals) had already diverged (*circa* 350 MY ago), i.e., from the Cambrian to the Triassic (163 and 494, and references therein).

According to Moss (350, 352, 353), the earliest ossified vertebrates had the intrinsic capacity to produce the entire spectrum of vertebrate skeletal tissues. However, there is still

some controversy concerning the relative timing of appearance and the relationship between the several primordial hard structures. Whether dermal bone, dentine or bone evolved first is contentious (493). According to Moss (352), dentin did not evolve from bone, based on two assumptions claiming that (i) teeth and dermal denticles are homologous, constrained by common developmental patterns, and (ii) dermal denticles in the oral cavity gave rise to teeth during the evolution of jawed vertebrates (352 and references therein).

It is fairly consensual that the first hard structures were exoneous, i.e., served primarily the function of protection, and that they could have extended from head to tail in those early vertebrates possessing an extensive bony armor of separate or fused denticles, i.e., in the majority of early vertebrates (494). This mineralized dermoskeleton, formed of dental tissues and bone, was well developed in the first jawless fishes (Heterostraci) as early as in the Late Cambrian. However, endoskeletal calcified cartilages are already well known in at least one Ordovician and many Silurian jawless vertebrates, e.g. in the Heterostraci and Osteostraci as well as in early jaw-bearing fishes (acanthodians, placoderms, chondrichtyans, etc.), in the Devonian. Thus, dermal and periosteal (perichondral) ossification seems to have evolved before true endochondral ossification (for more information on ossification mechanisms see section 5.1 of this chapter), but the early history of this process is poorly known (163).

Previous work on conodont histology had looked for a resemblance to acellular bone (aspidin) because this was the tissue characteristic of heterostracans, the primitive agnathan group. This phylogeny predicts that aspidin would be present in a more primitive outgroup (acellular bone assumed to precede cellular bone). The new interpretations that cellular bone is present in conodonts and primitive vertebrates have major implications for the evolution of vertebrate skeletal tissues, namely, that cellular bone is more primitive than acellular bone. This would suggest that this type of vertebrate tissue, with only the cell processes in spaces without the inclusion of the cell body, is a later evolutionary development. Suggestions that cellular bone is more primitive than acellular were discussed by Smith (1991, in 494) with a new evaluation of the earliest vertebrates with well-preserved histology. In one early vertebrate with good preservation of histology this author described enamel, cellular dentine and cellular bone.

The evolutionary interpretation of the difference in distribution of potential skeletogenic and odontogenic developmental mechanisms can be either that "odontodes

only", is the primitive condition for vertebrates, accepting conodonts without an exoskeleton but with tooth elements as a stem-group vertebrate, because heterostracans represent the basal verebrate group with an extensive bony armor. Most authors recognize the great antiquity and overall evolutionary stability of vertebrate skeletal tissues, and thus of their underlying cytological and molecular mechanisms. Indeed, skeletal structures of the third and fourth orders, as defined by Petersen (1930, in 163), appear to have been established with the earliest known vertebrate fossils. Perhaps since the Late Cambrian and certainly during the Ordovician, mineralized vertebrate tissues show the main characteristics of living forms, and some components seem to be already specialized in ways almost identical to modern vertebrates. It thus seems clear that both the fundamental genetical-molecular mechanisms and the epigenetical constraints which produce the various specialized lineages of scleroblasts and their specific cell-products appeared very early during vertebrate phylogeny and have later experienced little, if any, change (203, 454). Collagen fibrils, stellate scleroblasts, bone-trapped blood vessels, etc., have not changed their basic structure since the time of their initial appearance. The history of bone and other vertebrate skeletal tissues can hardly be interpreted as demonstrating an increasingly complex and irreversible evolutionary trend. Instead, this history shows ever-changing rearrangements of stable, basic components which evolved early in the history of the group (141).

4.2. SOME IMPORTANT NOTIONS

Regarding the deposition of mineral as a biologic event, it seems usefull to clearly distinguish between some often confused terminology.

By *mineralization* we consider the biologically mediated or controlled deposition of crystalline or amorphous solid mineral in or on a pre-existing matrix. Most biological mineralization requires specialized cells (scleroblasts).

Calcification is the specific process of mineralization which deals with the deposition of calcium carbonate (e.g. in otholits) or calcium phosphate (e.g. in enamel), generally in or on a pre-existing organic matrix (42, 256). No generally accepted hypothesis yet exists that explains the events occurring during calcium phosphate deposition on bone matrix (247). It is known that calcification begins by the deposition of calcium salts on collagen fibrils, a process induced by proteoglycans and high affinity calcium-binding glycoproteins. The

deposition of calcium salts is probably accelerated by the ability of osteoblasts to concentrate them in intracytoplasmic vesicles, which also contain annexin V and alkaline phosphatase (263) and to release, when necessary, their contents to the extracellular medium (247). Calcification is aided, in some unknown way, by alkaline phosphatase produced by osteoblasts and present at ossification sites (247).

Ossification, a process peculiar to vertebrates, is the formation of hydroxyapatite on a collagenous matrix in the histologically defined bony tissues (247); in other words, is the calcification of specific organic matrices made of collagen, non-collagenous proteins and complex glucides, by mineral deposits consisting primarily of crystaline hydroxyapatite, a calcium phosphate.

All hard tissues in the vertebrate skeleton are mineralized by apatites (although not all ossify) and most have a mesodermal origin (e.g., bone, cartilage and dentine), irrespective of their location in the body and of the pattern of mineralization (163).

4.3. THE FISH SKELETON

The origin of ossified tissues in fish is complex but can be subdivided into two main groups, intramembranous (achondral) which occurs in the absence of a cartilage matrix and gives rise to dermal bone (247), or cartilage replacement bone (which includes parachondral, perichondral and endochondral), in which a matrix of cartilage is progressively substituted by bone. This and other aspects of the fish skeleton will be dealt with in the following paragraphs, divided according to the location of the skeletal part.

4.3.1. The exo and the dermoskeleton

The exoskeleton includes skeletal elements which differentiate from the external surface of the skin epidermis (163). It includes denticles, scales, armor (dorsal and ventral plates, branchial plates, etc.), fin lepidotrichia, and teeth, both in agnathan and in gnathostome fishes.

The greatest diversity of skeletal tissues is apparent among the exoskeletons of lower vertebrates. Variations occur in tissue type (enamel, enameloid; mesodentine, semidentine, orthodentine); pulp (open, closed, or infilled with dentine or bone); bone (cellular or acellular); arrangement of bone tissues (spongy or compact); bone composition (from surface parallel lamellae, or lamellae concentric with vessels) (38).

The dermoskeleton includes bones originally formed in the skin dermis, the teeth, tooth-like organs and tissues or their derivatives among the lower vertebrates, and the bony scutes of terrestrial vertebrates (163). The scales of fishes form a more or less continuous dermal skeleton on the body, which modulates locally into the specialized dermal elements of fins (fin rays or dermotrichia *sensu lato*) on one hand, and of the mouth and pharynx (teeth) on the other (163). Scales are mineralized elements which form in the upper part of the dermis, generally close to the epidermis. They differ from dermal bones which form in the deeper part of the dermis. Scales can regenerate after removal (35).

Contrary to what happens in Osteichthyes (rhomboid) scales, it is generally believed that in Teleost (elasmoid) scales dentine and enamel disappeared, except in *Latimeria*, where obvious odontodes are inserted on the posterior area of the scale. Elasmoid scales are transparent, thin, lamellar, imbricated osseous plates. They consist of two main layers: a thick, lamellar, partially mineralized basal plate with a plywood-like structure (= isopedin), covered by a thin, ornamented superficial layer (the external layer, or osseous layer). In the posterior region, the scale is covered by the epidermis and an outer limiting layer is deposited on the surface of the external layer. These scales are localized in a pocket and they are obliquely inserted into the dermis (35).

4.3.2. The endoskeleton

Teleosts have a well-calcified but nonetheless lightly built skeleton (44). The term endoskeleton refers to the internal position in the body of the skeletal elements involved, and to their ontogenic development, i.e. preformed by a cartilaginous matrix, hence the name "substitution bones". However, not all endoskeletal material is formed by the substitution of cartilage with bone, since a great part is formed by periostal or endosteal deposition (163).

Derived from the sclerotomic mesenchyme of the embryonic mesoderm, the material of the endoskeleton differentiates deep within the body, below the striated musculature. It is first formed as cartilage, but it is later associated with periosteal (perichondral) and endochondral ossification. In its final, mature form the endoskeleton consists of bones of both periosteal and endochondral origin associated with permanent cartilages in the joints, and with tendons and ligaments which connect bones to muscles and to each other, respectively. The tendons and ligaments may themselves become mineralized and thus integrated within the mineralized part of the skeleton system (163).

In the next paragraphs, the main elements of the vertebrate endoskeleton will be concisely characterized, with the main emphasis, when possible, in the fish endoskeleton.

The skull. The relatively straightforward axial skeleton of elasmobranchiomorphs contrasts with the more complex skull and vertebral column of bony fishes. Although the skull begins in development as a cartilaginous neurocranium similar to that of sharks, it is soon altered by endochondral bones ossifying in this cartilaginous framework, and by the addition of many dermal (membrane) bones, supposedly derived from a scale layer in the skin. The end result is that the bony fish skull is a composite and complicated structure (44).

In fishes, specially in the most complex forms, the skull is a very complex bony structure, having several functional units (310). There is a general similarity among vertebrates in the embryonic development of the skull, although various groups show characteristic differences. The neurocranium forms at the anterior end of the notochord, beginning with the formation of two paracordal cartilages from somatic mesoderm. These cartilages, one on each side of the notochord, enlarge and form a structure called the basal plate by fusing around the notochord. The basal plate enlarges and fuses with paired occipital arch cartilages that develop over the hindbrain, thus forming the backwall of the neurocranium. Also uniting with the basal plate and occipital arch cartilages are paired otic capsules that form around the inner ears. The synotic tectum, a small cartilage that forms over the posterior part of the brain, joins with the occipital arch cartilages. Anteriorly, two precordal cartilages, called trabeculae, form from the neural crest. As the trabeculae grow, they fuse at their anterior ends to form the ethmoid plate, and unite with the developing basal plate posteriorly. Nasal capsules form anteriorly and orbital cartilages extend forward from

the otic capsules. Enlargement and dorsal growth of the parts results in the cartilaginous cranium, which will be partially covered or replaced by bone in the bony fishes (43, 118).

Fishes such as the salmonids still retain much cartilage in the cranium, but this is mostly replaced by bone in the majority of fishes (118). Ossifications forming around and replacing cartilage are called cartilage bones, perichondral and endochondral, whereas bones that are not preceeded by cartilage but are formed in the dermis are called membrane or dermal bones (see 1.2.1. of this Chapter). In teleosts, the endochondral bones are especially prominent in the posterior region of the neurocranium. To these, bones originating in the dermis are added dorsally and ventrally. The latter, called dermal bones, membrane bones, or investing bones, may include plates originating from scales, plates formed from coalescing tooth bases, and bones that form directly from membranes. For a detailed description of the origin and composition of the teleost skull see (43).

The vertebrae. The vertebrae of most teleosts are typical endoskeletal bones which sometimes involve little, if any, endochondral ossification (163). In fact, this last kind of ossification intervenes only in the vertebral arches. For this type of bones Patterson (405) proposed to restrict the term "membrane bone", i.e., endoskeletal elements which differentiate directly during ontogeny without a transitory cartilaginous stage. The vertebrae of the typical teleost have ossified biconcave (amphicelous) centra, with the notochord filling the concavities. Basapophyses are present, as well as neural arches and spines, and the caudal vertebrae have haemal arches and spines. In every case, arches and centra ossify independently, only secondarily becoming attached. Ventral ribs (pleural ribs) usually attach to the basapophyses. Intermuscular bones that extend into the horizontal skeletogenous septum are often called dorsal ribs. Other intermuscular bones may take their names from the structure that bear them. The numerous intermuscular bones of bony fishes directly ossify within myosepta, the sheets of connective tissues lining each successive myotome (37, 163). There is much modification in the vertebral column in the region of the caudal fin. In higher teleosts the vertebral column ends in an urostyle, which is a raised portion of the last vertebral centrum. The hypurals of higher teleosts are usually fused into larger plates, sometimes with one supporting the upper lobe of the caudal fin and one supporting the lower (37).

The fin rays. Fin rays (dermotrichia) are dermoskeletal elements supporting fish fins, which differ in structure and origin in the various groups of fishes. The dermal fin rays of teleosts are generally called lepidotrichia because of their presumed origin from rows of scales (36, Goodrich, 1904 in 163).

In teleosts each ray of the median fins is tipically supported by two ossified and one cartilaginous pteryogophores. Proximal pteryogophores are elongate tapered bones set deeply into the median skeletogenous septum, usually between the neural or haemal spines. The middle pteryogophores are ossified and jointed flexibly to the proximal elements on one end and to the distal pteryogophores, if present, on the outer end (36).

In the pectoral girdle of typical teleosts the scapula and caracoid are ossified as endochondral bones and part of their outer edges form the articular surface for the radials (actinosts) of the pectoral fin (36).

The pelvic fin skeleton in teleosts is made up of plate-like basipterygia, one for each fin. These bones usually are joined to each other posteriorly and may meet anteriorly (36).

5. THE BONE TISSUE: FORMATION AND MAJOR CHARACTERISTICS

5.1. MECHANISMS UNDERLYING BONE FORMATION AND MAIN CHARACTERISTICS

Bone is a dynamic connective tissue, composed of an exquisite assembly of functionally distinct cell populations that are required to support both the structural, biochemical, and mechanical integrity of this mineralized tissue and its central role in mineral homeostasis (300).

Bone always forms from a pre-existing connective tissue (42, 247, 511). Its formation can take place by two distinct ways: (i) direct mineralization from the matrix secreted by osteoblasts (intramembranous ossification), or (ii) deposition of an osseous matrix in a pre-existing cartilaginous matrix (endochondral ossification). Ossification includes all processes resulting in bone formation.

According to the mechanisms underlying bone formation, there are several kinds of

ossification:

Intramembranous ossification: is the source of most of the flat bones. The process begins when groups of cells differentiate into osteoblasts. New bone matrix is formed and calcification follows, resulting in the encapsulation of some osteoblasts. These islands of developing bone are known as spicules. The connective tissue that remains among the bone spicules is penetrated by growing blood vessels and additional indifferentiated mesenchymal cells, giving rise to the bone marrow cells. Cells of the mesenchymal tissue condensation divide, giving rise to more osteoblasts, which are responsible for the continued growth of the ossification center. The portion of the connective tissue layer that does not undergo ossification gives rise to the endosteum and the periosteum of intramembranous bone (42, 247, 511).

Parachondral ossification: bone forms directly within the connective tissue in the vicinity of a cartilage. The cartilaginous element appears to induce the shape of the bone, and the two elements are always separated by at least a layer of perichondrium and/or periosteum.

Perichondral ossification: bone appears in contact with an already formed cartilaginous element. The perichondrium stops making cartilage and it changes into periosteum. The cartilage then assumes the role of a mold upon which the osseous substance is deposited and thickens. This process is especially conspicuous in the branchial arches of teleosts (41). This term connotes a topographical relationship rather than an histological process, which is also true for parachondral ossification.

Periosteal ossification: this results from the activity of the periosteum at the outer surface of a bone, and it is characterized by a centrifugal mode of deposition [Gross, 1934 in (163)].

Endosteal ossification: results from the activity of the endosteum on the inner surfaces of the cavities of a bone. This term is generally applied to ossification occuring after the resorption of a pre-existing bony tissue, and this endosteal secondary bone is separated from the primary bone by a cementing line. It is characterized by a centripetal mode of deposition [Flourens, 1845 in (62)].

Endochondral (Gr. *endon*, within + *chondros*, cartilage) **ossification:** substitution of a preformed cartilaginous element with bony tissue, with the concomitant destruction of the cartilage model: blood vessels invade and the cartilage is broken up into strands. Erosion of the cartilage is only partially complete so that many cartilaginous regions may persist within the osseous trabeculae, behind the front of erosion, at least in young bone. When the bone is fully grown the areas of ossification unite and the cartilage plates disappear (42, 247, 511). This process characterizes the endochondral bones, which constitute the deep endoskeleton of vertebrates (short and long bones) (247). This process is also met in fishes, where it occurs primarily in the vertebrae and skull (41).

Metaplastic ossification. Two types can be defined in this case:

I. In pre-existing connective tissue: involves the progressive mineralization of the matrix and inclusion of the cells without cell multiplication and cell hypertrophy (201). Where mechanical requirements preclude the presence of a periosteum, a new bone is formed by "metaplasia", i.e., transformation of a pre-existing fibrous matrix of connective tissue direcly into bone. This mode of metaplastic ossification can occur in the formation of osteoders in reptiles.

II. In cartilage: Chondro-osseous metaplasia is a direct transformation of cartilage into bone without concomitant destruction of the cartilage. The only non-pathological example of this kind might be the "tissu mixte" of Stephan [1900 in (163)].

Centrifugal and centripetal ossification: centrifugal ossification is bone accretion occuring at the free external surface of a bone and proceeds outwards. Centripetal ossification proceeds inwards from the periphery of cavities within the bone. Deposition seems to be much slower in centrifugal than in centripetal bone tissues.

Inorganic matter represents about 50% of the dry weight of the bone matrix. Calcium and phosphorous are specially abundant (calcium is the fifth most abundant inorganic element in the body and phosphorous is the sixth; (457) and references therein), but bicarbonate, citrate, magnesium, potassium, fluoride and sodium are also found (163, 247,

511). The skeleton contains the highest percentage of the total calcium of the animal body and acts as a calcium reservoir. Calcium and phosphorous form hydroxyapatite crystals with the composition $Ca_{10}(PO_4)_6(OH)_2$ [Posner, 1973 in (510)]. Significant quantities of amorphous (noncrystalline) calcium phosphate are also present. The organic matter is type I collagen and amorphous ground substance, which contains proteoglycan aggregates. Also, several glycoproteins have been isolated from bone. The association of hydroxyapatite with collagen fibers is responsible for the hardness and resistance that are characteristic of bone (42, 247).

Bone modeling is the acquisition and transformation of overall bone form and shapes by differential variation of local growth rates. During morphogenesis, the bone may homothetically maintain its initial shape (isometric growth) or change its proportions and form (allometric growth). Beginning with an initial shape such as a cartilaginous model, conservation or transformation of shape results from the interplay of local osteogenetic rates in various directions (42, 247).

In order to acquire an adequate form during normal ontogenic growth and following fractures, bone needs to be resorbed at specific spatial and temporal intervals. Bone resorption may involve different processes: a) *osteoclasia*, the erosion of bone surfaces by osteoclasts, which produces bone surfaces named Howship's lacunae; and b) *periosteocytic osteolysis*, bone resorption by osteocytes at their own periphery. Osteoclastic resorption and periosteocytic osteolysis deal with the total removal of bone tissue, both organic matrix and mineral. The third process, which involves the removal of mineral without destruction of the organic matrix, was named halastatic demineralization by Rutishauser *et al.* (466). "Halastasis" is a word of greek origin which means "unstable salts". In bony fishes, remodeling is generally weak, except among some forms with a very active metabolism (tunas) (163).

Concerning the presence or absence of living cells entraped in the matrix, bone is classified as follows:

Cellular bone, which is any kind of bone tissue containing bone cells (osteocytes) enclosed in (periosteocytic) bone lacunae. Tipically, bone cells remain alive and physiologically active, communicating with each other by means of fine cytoplasmic extensions housed in minute bone canaliculi. Osteocytes are "mature bone cells" resulting from the entrapment of former osteoblasts within the mineralizing bone matrix. However,

some osteocytes do not derive from typical osteobasts of the periosteum or endosteum. This is the case for bone cells within the densely fibrous mineralizing tissues forming the insertion sites of ligaments and tendons.

Acellular bone, which is a tissue devoid of osteocytes. More precisely, acellular bone never contains complete osteocytes in typical periosteum lacunae, but, at most, fine cytoplasmic extensions of cells housed in long minute canaliculi. Although acellular bone occurs in various groups of vertebrates, it is by far most common among advanced teleosts (highly evolved bony fishes), as first recognized by Kölliker [1859, in (163)]. Hence, at least among bony fishes, acellular bone represents an apomorphic (advanced or specialized) rather than a plesiomorphic (primitive or generalized) condition (343,403). Alternatively, acellular bone may entrap young bone cells (osteoblasts) within the bone matrix, but these ultimately shrink and die before mineralization takes place (351).

5.2. THE BONE CELLS

The contribution of the cellular elements to the bone total mass is small (445). In actively growing bones four different cell types can be distinguished: osteoprogenitor cells, osteoblasts (*osteon* + Gr. *blastos*, germ), osteocytes (Gr. *osteon*, bone + *kytos*, cell) and osteoclasts (*osteon* + Gr. *klastos*, broken). Excepting osteoclasts, this division represents four different functional states of the same cell type. The reversible changes that this cells experience represent examples of celular modulation, a process which is dintinct from differentiation, this last term applicable to the, apparently irreversible, progressive specialization of the structure and function (42).

Osteoblasts: Osteoblasts synthesise the organic components (type I collagen, proteoglicans and glicoproteins) of the matrix (247, 445) and deposition of the inorganic components of bone is also dependent on their presence (247). They are exclusively located at the surfaces of bone tissue, side by side (247). Secretion of matrix components occurs at the cell surface, which is in contact with older bone matrix, producing a layer of new (but yet uncalcified) matrix, called osteoid, between the osteoblast layer and the previously formed

bone. This process, bone apposition, is completed by subsequent deposition of calcium salts into the newly formed matrix (247).

More detailed, the temporal sequence of gene expression defines four principal developmental periods in osteoblast development (1- proliferation; 2- maturation; 3- mineralization; 4- remodelling). The more interesting, in this case, are: the first, when proliferation supports expansion of the osteoblast cell population and biosynthesis of the type I collagen bone extracellular matrix; and the third (mineralization period), which involves gene expression related to the ordered deposition of hydroxyapatite. Osteopontin and BGP exhibit maximal expression at this time, when maturation of bone-tissue like organization is ongoing. Once surrounded by the matrix which has synthesised, the osteoblast becomes an osteocyte (247, 445, 504).

Osteocytes: Osteocytes, which derive from osteoblasts, are buried within the mineralized bone matrix. Newly formed osteocytes are very similar to osteoblasts, but when more mature, they become flatter, loose some of their abundant cytoplasm and experience a regression of some organelles, such as the Golgi apparatus and the endoplasmic reticulum. Though these cells seem less active in what refers to protein synthesis, they are not metabolically inert (42). Osteocytes vary widely in size and shape (390) and they are connected to one another and to osteoblasts on the bone surface by extensive projections of canaliculi (42, 247, 511). These cells are actively involved in the maintenance of the bony matrix, and their death is followed by resorption of this matrix. Osteoblasts and osteocytes do not divide after having been formed from the osteoprogenitor cell (247).

Osteoclasts: Bone is the only organ that contains a cell type, the osteoclast, whose only function is to constantly destroy the organ hosting it. Osteoclasts are derived from the fusion of blood derived monocytes and thus belong to the mononuclear phagocyte system (42, 247, 515). Osteoclasts are a heterogenous group of multinucleate cells (2 to 100 nuclei) with several properties in common. They are generally larger than other bone cells, ranging in diameter from 20 to 100 μm; secrete acid, collagenase, and other proteolytic enzymes, such as acid phosphatase, and are found where bone is being resorbed. Chenu *et al.* (81) demonstrated a chemotactic activity of osteocalcin for osteoclast-like cells, suggesting that this protein may represent a component of bone matrix involved in the mechanism for

attraction of the osteoclasts to the bone surface. In areas of bone undergoing resorption, osteoclasts are found to lye within enzymatically etched depressions in the matrix known as Howship's lacunae (42, 247, 511) and, when in contact with the bone surface, their membranes form many processes which appear to penetrate the bone surface. They attack the bone matrix, liberate the calcified ground substance, and are actively engaged in elimination of debris formed during bone resorption (247). It is suggested that one osteoclast is able to remove as much bone as is laid down by 100-1000 osteoblasts, a conclusion that takes into account their relative numbers (511).

6. GENERAL INTRODUCTION AND OBJECTIVES

From what was presented in the previous sections it can be inferred that Bone Gla protein plays an important role in the correct development of the vertebrate bone. Its high abundance, stringent regulation and diversified interactions are sufficient "molecular reasons" to assume the importance of this protein.

Bone deficiencies associated with a large array of pathologies have been, still are and, with the expected increase in human longevity, will increasingly be, a serious health problem, justifying the large amount of research done in this area (e.g., 80, 122, 128, 131, 176, 242, 581). Other important health problems, like atherosclerosis, have also led to studies involving BGP (e.g., 242, 553), without, however, elucidating in a decisive manner the mechanism of action of this small protein. In fact, as it is stressed in the "Bone Gla protein" section of this chapter, although the first isolation and sequencing of this protein dates back to 1976 (420, 421), only recently has the search for its biological function produced convincing results (49, 135), although its mechanism of action at the molecular level remains largely unknown. With this in mind, and taking into account the fact that the majority of research involving this protein has been done using mammalian models (human, mouse and rat), the use of a different, non-mammalian, system might give a new insight into the function and regulation of BGP, in particular in lower vertebrates.

The major objective of this work was, therefore, to determine the molecular organization of the fish BGP gene and study its tissue distribution and appearance during development, thus providing the necessary molecular tools and information to initiate studies on its function in lower vertebrates. Comparison between results obtained in lower and higher vertebrates may prove usefull to further understand BGP function throughout evolution.

The teleost fish *Sparus aurata* was chosen as model organism for several reasons:

1. It is a commonly raised and abundant fish in aquaculture facilities, due to important economical issues, being already implemented as model organism at the Universidade do Algarve. Indeed, gilthead seabream aquaculture is presently in expansion, clearly leaving behind the natural catches for this fish, and representing an increasingly important alternative source of proteins. Concomitantly, the amount of money and effort involved, at least in south-

European countries like Portugal and Spain, make *Sparus aurata* a comercially important resource, which justifies all efforts applied to avoid high juvenile morbidity and mortality.

2. It reproduces fairly easily, throughout the entire year upon stimulation.

3. It produces a fast-growing, abundant offspring, which constitutes a clear advantage over the mammalian models.

4. It has an external embryonic development, with transparent embryos and larvae, which makes this species an adequate model to bring additional insight into the function of BGP, in particular for those aspects related to appearance, regulation and basic role during early development.

5. When raised in closed systems frequently develops various skeletal abnormalities (151, 270, 402), mainly at the larval stages, thus being an adequate model to analyse a possible relationship between skeletal deformities and changes in BGP expression.

Finally, it was of interest to analyse the evolutionary relationships of BGP with other vitamin K-dependent proteins. In fact, as was already described in previous sections of this chapter, BGP shares some features with other proteins, namely Matrix Gla protein (218) and the vitamin K-dependent coagulation factors (174). It is likely that at a given time in the evolutionary history, a polypeptide precursor originated BGP and MGP. Since fishes evolved as the first creatures to have a skeleton, making them the ancestors of all vertebrates (310), *S. aurata* BGP constitutes a good candidate to further investigate these questions.

CHAPTER II: MATERIAL AND METHODS

1. RNA EXTRACTION

Total RNA from *Sparus* tissues and whole larvae/juveniles at different developmental stages was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (83). Briefly, the specimens were reduced to powder in liquid nitrogen and 1 ml of solution D [Guanidinium Isothiocyanate Solution (Appendix II) : 2-Mercaptoethanol, 100:0.72] was added, per 100 mg of tissue. After adding 0.1 vol. 2M $NaC_2H_3O_2$, 1 vol. Acidic phenol (pH 5-6) and 0.2 vol. Chloroform : Isoamyl alcohol (49:1), the mixture was homogenized for 10 seconds, incubated on ice for 15 minutes and centrifuged at 10.000g for 15 minutes at 4°C. One volume of Isopropanol was added to the aqueous phase, followed by incubation at -30°C for one hour. The mixture was centrifuged at 10.000g for 15 minutes, at 4°C, and the pellet ressuspended in 500 µl of solution D. One volume of isopropanol was added to the mixture, followed by incubation at -30°C for one hour and centrifugation at top speed for 15 minutes, at 4°C. The pellet was washed with 75% ethanol, air-dried and ressuspended in 100 µl of DEPC-treated water.

2. AMPLIFICATION OF A PARTIAL spBGP CLONE BY RT-PCR

Reverse transcription (RT) of 1 µg of total RNA, extracted from a *Sparus* juvenile with a mineralized skeleton, was performed with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) (Promega), using an oligo (dT) linked to an universal adapter, under conditions suggested by the supplier (see Protocol 2, Appendix I). Half (10 µl) of the RT reaction was used as a template for amplification by the Polymerase Chain Reaction (PCR), using a 5' degenerated primer (SBG2F: 5'-TGC/TGAA/GCAC/TATGATGGA-C/TACA/C/G/TGA-3'; see Table II.I for localization) designed according to the spBGP protein sequence previously obtained (64) and a reverse universal adapter (5'-ACGCGTCGACCTCGAGATCGATG -3'). PCR reactions were conducted for 35 cycles

(one cycle corresponds to 40 seconds denaturation at 95°C, 30 seconds annealing at 52°C and 45 seconds elongation at 72°C; an initial denaturation of 3 minutes at 95°C and final elongation of 7 minutes at 72°C were used), according to the following protocol: 5 mM $MgCl_2$, 1x Mg-free buffer (Perkin Elmer), 4 µl dNTPs (2.5 mM each), 1 µM forward and reverse primers, 0.5 µl Ampli *Taq* DNA polymerase and ddH_20 to complete 50 µl.

3. CLONING AND SEQUENCING OF DNA FRAGMENTS RESULTING FROM PCR AMPLIFICATION

PCR products of the expected size were identified by agarose (Promega) gel electrophoresis, excised from the gel, and eluted from the agarose using the Qiaex II Gel Extraction Kit (Qiagen), following the manufacturer's instructions. The DNA fragments thus obtained were inserted into the pGEM-T plasmid vector (Promega) and this chimeric DNA used to transform *Escherichia coli* DH5α strain (GibcoBRL) (Appendix II). Briefly, 2 µl of the ligation reaction were added to 100 µl of a suspension of competent DH5α *E. coli* cells, incubated on ice for 30 minutes and heat-shocked at 42°C for 45 seconds. The cells were then placed on ice and 500 µl of SOC medium (LB-Broth (GibcoBRL) / 1% 2M Glucose) were added. Following incubation at 37°C with gentle agitation for 45 minutes, 5 µl of a 1 M isopropilthio-β-D-galactoside (IPTG; Sigma) solution (in H_2O) were added to the bacterial cells, which were then plated in LB-Agar (Sigma) supplemented with 50 µg/ml of ampicilin and 0.8 mg/plate of a 5-bromo-4-cloro-3-indolil-β-galactopiroside (X-Gal; Sigma) solution (in N-dimethylformamide; Sigma). The plates were incubated overnight (O/N) at 37°C and the positive clones (white colonies) were obtained based on colour selection. Each positive clone was transferred to liquid LB medium (Appendix II), supplemented with 50 µg/ml ampicilin and allowed to grow O/N with agitation, at 37°C.

Isolation of the plasmid DNA containing the inserts of interest was performed by the "boiling technique" (229; Protocol 1, Appendix I). In each case, the cDNA was excised from the plasmid by digestion with *EcoR*I restriction enzyme (Pharmacia), electrophoresed in a 1.4% ethidium bromide-containing agarose gel, and positive clones (those containing a DNA insert with the expected size) were sequenced. DNA sequence analysis was performed with the ^{T7}Sequencing Kit (Pharmacia) and the SP6 and T7 vector-specific primers, following established procedures (see Appendix I, Protocol 3). The labelled DNA thus obtained was

loaded on a 19:1 (acrylamide:bisacrilamide) 6% sequencing gel and electrophoresed at 60 W. After drying the gel, a Kodak X-Omat AR film was exposed O/N.

4. AMPLIFICATION AND CLONING OF THE 5'- END OF spBGP cDNA by 5' RACE PCR

Sparus poly(A^{+}) RNA was prepared from total RNA (extracted from whole juveniles with a calcified skeleton) using the QuickPrep Micro mRNA Purification kit (Pharmacia), following the suppliers instructions (Appendix I, Protocol 4). The *Sparus* mRNA thus obtained was reverse-transcribed and amplified using the Marathon™ cDNA Amplification Kit, according to the supplier's indications. The first step consisted on the synthesis of a *Sparus* cDNA library. In order to do that 4 µl of poly(A^{+}) RNA (approximately 2 µg) were added to 1 µl of 10 µM cDNA synthesis primer (5'-TTCTAGAATTCAGCGGCCGC$(T)_{30}$N$_{-1}$N-3', where N_{-1}= G, A or C; N= G, A, C or T), incubated for 2 minutes at 70°C and then at 4°C for 2 minutes. After this annealing step, 2 µl of 5x first-strand buffer (Promega), 1 µl of 10 mM dNTP mix (Promega), 1 µl of DEPC-treated ddH_2O and 1 µl of M-MLV Reverse Transcriptase (100 U/ µl; Promega) were added. Incubation was performed at 42°C for 1 hour, after what the second-strand cDNA synthesis was performed, following manufacturer's instructions (Appendix I, Protocol 5). Two microliters of the resulting double-stranded cDNA were analysed by electrophoresis in 1.2% agarose gel (Promega), to check its integrity.

The next step in the cloning of the 5'-end of the BGP cDNA was the Adapter Ligation, for which in a 0.5 ml tube were added 5 µl of double-stranded cDNA, 2 µl of 10 µM Marathon cDNA Adaptor (Clontech), 2 µl of 5x DNA ligation buffer (Clontech) and 1 µl of T4 DNA ligase (1U/ µl) (Clontech).

The reaction mixture was incubated O/N at 16°C, and then heated to 70°C for 5 minutes to inactivate the enzyme. From the 10 µl ligation reaction, 1 µl was dissolved in 83 µl of Tricine-EDTA buffer (10 mM Tricine-KOH (pH 8.5), 0.1 mM EDTA), heat denatured at 94°C for 2 minutes, and immediatelly cooled on ice for 2 minutes.

The next step consisted in the 5'-Rapid Amplification of the cDNA End (5'-RACE), in which the Advantage KlenTaq Polymerase Mix (Clontech) was used. Into a 250 µl sterile PCR tube were added 36 µl of sterile ddH_2O, 5 µl of 10x Klen *Taq* PCR Rx Buffer (Clontech), 1 µl of 10 mM dNTPs (Clontech) and 1 µl of 50X Advantage Klen *Taq* Polymerase Mix (Clontech), which includes TaqStart Antibody (Clontech) for automatic hot

start PCR. The mixture was completed with 5 µl of diluted adaptor-ligated cDNA (previous paragraphs), 1 µl of 10 µM AP1 primer (Clontech) (5'-CCATCCTAATACGACTC-ACTATAGGGC-3') and 1 µl of 10 µM Gene-specific primer (SBG4R; Table II.I). After mixing and centrifuging briefly, the reaction was incubated in a Perkin-Elmer GeneAmp PCR System 2400 with the following program: 94°C, 1 minute; 94°C, 10 seconds and 72°C, 4 minutes (5 cycles); 94°C, 10 seconds and 70°C, 4 minutes (5 cycles); 94°C, 10 seconds and 68°C, 4 minutes (25 cycles).

Five microliters of the resulting PCR products were analysed by electrophoresis in a 3% FMC Seakem GTG agarose gel. The DNA fragments of interest were excized from the gel with a clean scalpel and eluted from the agarose with the Qiaex II Gel Extraction Kit, following the manufacturer's instructions. These eluted DNA fragments were cloned into the pGEM-T plasmid vector (Promega), and inserted in *E. coli* DH5α strain, as described in Appendix I and section 3 of this chapter. The plates were incubated O/N at 37°C and the positive clones were colour-selected and grown O/N on LB medium (Sigma) supplemented with 50 µg/ml ampicilin. Plasmid DNA was prepared by the "alkaline lysis" method, as described in Protocol 7 (Appendix I), according to Sambrook *et al.* (468).

Eight microliters of the purified plasmid DNA were digested with *EcoR*I restriction enzyme, and the presence of inserts was analysed by electrophoresis in a 1.4 % agarose gel. These DNA inserts were sequenced using the Universal and SP6 primers, and the Pharmacia's ^{T7}Sequencing kit (Protocol 3, Appendix I).

In order to get both strands of the cDNAs sequenced, an extra set of primers was designed: SBG8F and SBG9R (Table II.I).

5. EXTRACTION OF GENOMIC DNA

Genomic DNA was extracted from 0.3 g of muscular tissue of a freshly sacrified adult *Sparus* specimen, using a method adapted from Sambrook *et al.* (468). Tissue was sliced into small fragments with a sterile scalpel and added to 3 ml of Digestion Buffer [100 mM NaCl, 10 mM Tris.Cl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5 % SDS and 0.1 mg/ml Proteinase K). The mixture was incubated O/N at 55°C, with agitation, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform. The aqueous phase

was transferred to a sterile 1.5 ml centrifuge tube and 0.1 volumes of 3M $NaC_2H_3O_2$ (pH 4.8) were added, followed by 2 volumes of 100% R/T ethanol. The precipitated DNA was transferred (with a plastic pipete tip) to a sterile 1.5 ml centrifuge tube, washed with ice-cold 80% ethanol, dried at R/T for ~15 minutes and dissolved in 300 µl of TE buffer (see composition in Appendix II). Genomic DNA integrity was analysed by electrophoresis in a 0.8 % agarose gel (Promega).

6. AMPLIFICATION OF THE spBGP GENE

PCR amplification using different pairs of primers (SBG5F+SBG4R, SBG8F+SBG4R, SBG13F+SBG15R, SBG8F+SBG10R and SBG8F+SBG11R; Table II.I) was performed in a GeneAmp PCR System 2400 (Perkin Elmer), under the following conditions: 1.5 mM $MgCl_2$, 5 µl 10x PCR buffer (GibcoBRL), 0.05 mM of each nucleotide, 0.4 µM of each primer, 1 unit of *Taq* DNA Polymerase (GibcoBRL); denaturation was performed at 95°C for 2 minutes, annealing at 60°C for 1 minute and elongation at 68°C for 2 minutes, with 35 cycles; an initial denaturation step of 5 minutes and a final elongation step of 12 minutes were performed.

DNA fragments obtained were cloned in pGEM-T Easy, as described in II-3, and sequenced (Protocol 3, Appendix I). All exons and exon/intron borders were sequenced on both strands.

7. AMPLIFICATION OF THE 5'-FLANKING REGION OF THE spBGP GENE

The 5' flanking region of the spBGP gene was obtained with the Universal Genome Walker™ Kit (Clontech). In summary, 2.5 µg of *Sparus* genomic DNA were digested with 1 µl (20U) of *BamH*I (DLI) or 1,5 µl (18U) of *Pst*I (DLII) restriction enzymes (Pharmacia) for 18.5 hours at 37°C. Enzymes were inactivated by incubation of the reaction at 65°C for 10 minutes. The digested DNA was then treated with the Klenow (Boheringer) enzyme (4 U), after adding 2 µl of 2.5 µM dNTPs (Promega), in order to generate DNA fragments with blunt ends; incubation was accomplished for 15 minutes at 25°C. The enzyme was inactivated at 65°C for 15 minutes, the reactions dialysed for 20 minutes against water to

remove salts [using a 0.025 µm dialysis membrane (Millipore)], lyophilised, and the resulting pellets dissolved in 20 µl of TE buffer (pH 7.4). One microliter of each dissolved DNA was electrophoresed in a 0.8% agarose gel (Promega), in order to check its integrity.

An Adaptor (5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGG-CCCGGGCTGGT-3') was then ligated to each digested DNA fragment (4 µl of DNA + 1.9 µl 25 µM GenomeWalker Adaptor + 1.6 µl 5x Ligation Buffer + T4 DNA Ligase (1U/µl)) for 48 hours at 16°C. The enzyme was inactivated at 70°C for 5 minutes and 72 µl of TE were added.

The 5'-flanking DNA of the spBGP gene was amplified by nested PCR. Briefly, a primary PCR amplification was performed using a forward AP1 (Adaptor Primer 1; GTAATACGACTCACTATAGGGC) and a reverse SBG19R (see Table II.I) as Gene Specific Primer 1 (GSP1; Protocol 6, Appendix I). Five microliters of the PCR reaction thus obtained were electrophoresed in a 0.8% agarose gel. After analysis of the results of the primary PCR reaction, a second PCR reaction was performed, using Adaptor Primer 2 (AP2; 5'-ACTATAGGGCACGCGTGGT-3') as forward primer, and SBG19R as reverse primer, following the manufacturer's intructions (Protocol 6, Appendix I). Fifteen microliters of the secondary PCR reaction were electrophoresed in a 1.5% agarose gel (Promega). The resulting amplified DNA fragments were excised and extracted from the gel with the Qiaex II Gel Extraction Kit (Qiagen), cloned in pGem-T Easy (Promega) and sequenced as described (II-3).

8. GENOMIC SOUTHERN ANALYSIS

Aliquots (20 to 50 µg) of *Sparus* genomic DNA were incubated for 3-4 days with sterile ddH_2O and enzyme buffer at 4°C, according to a procedure described in Sambrook *et al.* (468), and then digested with ~ 50 U of selected restriction enzymes (*BamH* I, *Bgl* I, *Hind* III and *Pst* I and with *Bgl* I, *Bgl* II, *EcoR* I, *Hind* III and *Pst* I), at 37°C for 30 minutes. After this period, approximately 25 U of the respective restriction enzymes were added *de novo* and the reaction allowed to proceed for an extra 3 hours at 37°C. The digested DNA was then ethanol precipitated (0.1 vol. 3M $NaC_2H_3O_2$, 2 vol. 100% ethanol), re-suspended in sample loading buffer and size-fractionated by electrophoresis on a 0.8% agarose gel (Promega) for 9 hours at 35 Volts. DNA markers (lambda DNA-HindIII digested and 1.0 kb ladder from

GibcoBRL) were loaded in adjacent lanes in the gel. Following electrophoresis, DNA in the gel was transferred to an N^+-Nylon membrane (Schleicher & Schuell) by a capillary method (468) and pre-hybridized at 42°C for 3 hours in 6x SSPE (see Appendix II for composition), 10x Denhardt's solution, 0.5% SDS and 50 µg/ml calf thymus DNA. Hybridization was carried for 24 hours in a solution containing 6x SSPE, 0.5% SDS, 50% formamide, 50 µg/ml calf thymus DNA and either a 280bp (from nucleotides 323 to 603 of the cDNA) or the full length spBGP cDNA, labeled with α^{32}P-dCTP. Labelling was performed with the Prime-It® II Random Primer Labeling Kit (Stratagene) and the unincorporated nucleotides were separated from the labelled DNA with NACS 52 PREPAC® columns (GibcoBRL), according to manufacturer's instructions. The labeled probe thus purified was added to the hybridization solution and incubated with the membrane O/N, at 42°C. Blots were washed twice in 6x SSPE, 0.1% SDS at R/T for 15 min, twice in 1x SSPE, 0.1% SDS at 37°C for 15 minutes and once in 0.1x SSPE, 0.5% SDS at 65°C for 30 minutes. Autoradiography was performed with Kodak X-Omat AR film and two intensifying screens at –80°C for 8 days.

9. DETERMINATION OF THE TRANSCRIPTION START SITE OF THE spBGP GENE

The transcription start site of the spBGP gene was identified using a 5'-primer extension technique. Fifteen micrograms of total RNA, extracted from the calcified jaw of a juvenile *Sparus* by established methods (II-1), were annealed to a reverse primer (SBG14R; Table II.I) extending from +75 to +94 bp upstream from the initiation ATG codon, as follows: 10 µl RNA / 15 µl 1x Hybridization Buffer [0.15 M KCl, 10 mM Tris.Cl (pH 8.3), 1 mM EDTA] / 10 pmol SBG14R primer, for a final volume of 30 µl; the annealing was achieved by incubating the mixture at 65°C for 1.5 hours, after what the tube was allowed to cool at R/T.

The resulting RNA was recovered by precipitation with 0.1 vol. DEPC-treated 3M $NaC_2H_3O_2$ and 2.5 vol. ice-cold 100% Ethanol, incubated at -20°C for 10 minutes, centrifuged at 12000 g for 10 minutes, washed with ice-cold 70% ethanol and dried for 10 minutes at R/T.

The extension reaction was performed for 5 minutes, at 37°C, using the M-MLV

reverse transcriptase (GibcoBRL), 4 µl of 5X First-Strand Buffer (GibcoBRL), 2 µl of 0.1 M DTT, 1 µl of each 10mM dNTP, 4.5 µl of 1 mg/ml actinomycin D and DEPC-treated ddH_2O to complete 19 µl. An additional incubation of 60 minutes, at 37°C, was performed, after addition of 10 µCi (1 µl) [α^{32}P] dCTP. The resulting single strand cDNA was RNase-treated in the presence of non-specific DNA [100 µg/ml Calf Thymus DNA (GibcoBRL), 20 µg/ml Rnase A] for 15 minutes at 37°C and then phenol/chloroform-extracted and ethanol precipitated. Pellets were re-suspended in 5 µl of formamide-containing sequencing dye (Pharmacia's ^{T7}Sequencing kit Stop Solution), heat-denatured (5 min at 65°C) and loaded on a 9% acrylamide, 7M urea sequencing gel (468). Autoradiography was performed using Kodak X-Omat AR film with two intensifying screens at –30°C for 15 hours. The size of the amplified fragments was determined from a known DNA sequence reaction [corresponding to a partial spBGP intron II clone, sequenced with the SGB29F primer] loaded on adjacent lanes.

10. NORTHERN BLOT ANALYSIS

Total RNA, extracted from *Sparus* tissues (vertebra, jaw, heart and liver) and from whole larvae collected at different developmental stages (18, 75, 82, 86 and 130 dph), was size-fractionated by electrophoresis over a 1.4% formaldehyde-containing agarose gel, transferred onto N^+ Nylon membranes (Schleicher & Schuell) by a capillary method (468) and pre-hybridized at 42°C in 50% formamide, 5x Denhardt's solution, 5x SSPE and 50 µg/ml Calf Thymus DNA for 2-3 hours. A partial spBGP cDNA (spanning from nucleotide 325 to 602 of the spBGp mRNA) was labeled with [α-^{32}P] dCTP using the Prime-It® II Random Primer Labeling Kit (Stratagene) and separated from unincorporated nucleotides on a NACS 52 PREPAC® column (Gibco-BRL). The labeled probe thus purified was added to the pre-hybridization solution and incubated with the membrane O/N under the same conditions described for pre-hybridization. Blots were washed twice in 6x SSPE (Appendix II), 0.1% SDS at R/T for 15 minutes and twice in 1x SSPE, 0.1% SDS at 55°C for 30 minutes. Autoradiography was performed with Kodak X-Omat AR film with two intensifying screens at –30°C for up to one week.

11. RT-PCR SOUTHERN BLOT ANALYSIS

One microgram of total RNA extracted from various *Sparus* tissues (vertebra, jaw, heart, liver and muscle) and from whole specimens at various developmental stages (neurula, 2, 3, 18, 27, 37, 47, 61, 75, 82, 91 and 130 dph) was treated with Rnase-free DNAse I for 3 hours (37°C) and reverse transcribed using the conditions described in Protocol 2 (Appendix I). One twentieth of each reaction was amplified by PCR, using two specific oligonucleotide primers designed according to the spBGP cDNA sequence previously obtained (SBG8F and SBG11R; Table II.I), either for 20, 25 or 35 cycles (one cycle is 30 sec at 95°C, 40 sec at 60°C and 45 sec at 68°C) followed by a final extension period of 10 minutes at 68°C with *Taq* DNA polymerase (Promega). Positive and negative controls were made by amplifying respectively a clone of spBGP cDNA and a sample without DNA template with the same primers. Resulting PCR products obtained with 20 and 35 cycles were Southern transferred onto a N^+ Nylon membrane (Schleicher & Schuell) and prehybridization/hybridisation performed following the procedures described in Section 3 of this chapter. Autoradiography was performed with Kodak X-Omat AR film and two intensifying screens at –30°C. As an internal control for the relative amount of RNA used for each sample, *Sparus* β-actin was also amplified from an equal volume of RT reaction, using two specific primers designed according to the published (470) *Sparus* beta-actin cDNA sequence (forward primer: 5'-TTCCTCGGTATGGAGTCC-3'; reverse primer: 5'-GGACAGGGAGGCCAGGA-3').

12. DETECTION OF CARTILAGINOUS AND MINERALIZED STRUCTURES IN *S. AURATA*

The technique of whole mount double staining with Alcian Blue/Alizarin Red was used for the detection of cartilage and bone, based in established procedures (124, 266, 417, 554).

Sparus specimens with ages ranging from 15 to 150 dph were collected and fixed in 4% paraformaldehyde, at 4°C, from 24 hours to 7 days, according to size. Specimens were then hydrated by bathing in a 50% ethanol solution for 30 minutes (60 minutes for specimens older than 59 dph), in a 25% ethanol solution for 3x 30 minutes and, finally, in ddH_2O for

3x 30 minutes. For detection of cartilaginous tissue, specimens were incubated in a solution containing 10 mg Alcian Blue 8GX (Sigma; # A-5268), 70 ml 100% ethanol and 30 ml glacial acetic acid for 16 hours (for younger specimens) and 20 hours (specimens older than 59 days), at R/T. Specimens were then re-hydrated by bathing in a decreasing ethanol series: 100% ethanol (2x 30 minutes), 95%, 70%, 40% and 15% ethanol (30 minutes each) and ddH_2O (2x 30 minutes). For specimens older than 59 dph the 100% ethanol and the ddH_2O baths were applied for 2x 60 minutes. This procedure, followed by incubation O/N with 1% KOH, removed all the excess blue colour and prepared the specimens for the following step, the detection of calcified tissues. Calcification was detected by incubating specimens in a 1% Alizarin Red S solution (pH 12.8; Sigma; # A-5533, in 0.5% KOH) for 24 hours, followed by removing the excess dye with 2.5% KOH:glycerol (3:1; only for 89 and 150 dph specimens), 1% KOH:glycerol (3:1; to this solution, 40 µl/ml of 30% H_2O_2 were added, to remove colour from chromatophores) (3 days) and then with 1% KOH:glycerol (1:1) and 1% KOH:glycerol (1:3) for variable periods of time, according to specimens age. Finally, specimens were stored in 100% glycerol, supplemented with a few microliters of phenol, to prevent development of bacteria and fungi.

13. INCLUSION OF *S. AURATA* BONE IN METHYLMETACRYLATE

A juvenile *Sparus* specimen (~ 15 cm S.L.) was sacrified and the bones of the jaw and vertebra extracted, cleaned of adherent tissue and fixed in 100 ml of Fixation Solution (2 vol. 0.2M sodium cacodylate buffer, 1 vol. 40% formaldehyde (Sigma), 2 vol. methanol; pH 7.3) for 6 days at 4°C. After fixation, bones were cut into small pieces, transferred to 20 ml glass vials and incubated in acetone for 2x 12 hours, at 4°C. Acetone was then replaced by the Impregnation Solution[1] (5 ml Solution A + 30 µl Solution B) and incubation took place for 3 days at 20°C, with this solution replaced every 24 hours. Inclusion was made with the same Impregnation solution, in air-free vials, at R/T.

After inclusion, the blocks were moulded into a rounded shape (more suitable to be held by the cutting apparatus) and stored at R/T until used.

For sectioning, both the methylmetacrilate blocks and the blade used to section them were kept constantly wet with 35 % Ethanol. Several 5 µm sections were prepared, placed on

[1] For details on preparation of solutions see Appendix II

a histological slide covered with 35 % ethanol, and kept over a heating plate, to spread the section. A small plastic sheet was then placed over each section and all was covered by another glass blade. The assemblage was kept at R/T for 48 hours, held together by forceps.

14. HISTOLOGICAL DETECTION OF ALKALINE PHOSPHATASES IN OSSEOUS TISSUES OF *S. AURATA*

Alkaline phosphatases were detected in methylmetacrylate-included sections of juvenile *Sparus* bone according to an established procedure. Briefly, a detection solution was prepared [20 mg Naphtol ASBI Phosphate (Sigma; # N-2125); 1 ml N-N-dimethylformamide (Sigma; # D-8654); 10 ml TRIS, pH 8.5; 8 ml NaCl 9.5‰; and 6 mg Fast Red Violet LB Salt (Sigma; # F-3381)], and a few drops added to each section, followed by incubation O/N at 37°C. Sections were then washed with ddH_2O, fixed O/N with 10% formaldehyde, dehydrated in a crescent alcoholic series and mounted in DPX. Alkaline phosphatase activity was detected in a Zeiss Axiovert 25 inverted microscope, by the red colour staining resulting from the degradation of the substrate.

15. HISTOLOGICAL DETECTION OF ACID PHOSPHATASES IN OSSEOUS TISSUES OF *S. AURATA*

A Pararosanilin-HCL stock solution was prepared as follows: 1 g Pararosanilin (Sigma; # P-7632) was dissolved in 20 ml of distilled water and 5 ml of concentrated hydrochloric acid were added; the solution was heated gently, cooled, filtered and stored in the refrigerator. The solution was then added, drop by drop, to an equal volume of 40mg/ml sodium nitrite, with thorough shaking after each addition. A second solution was prepared by adding 0.5 ml Naphthol ASB1 phosphate stock (50 mg Naphthol ASB1 Phosphate, 5 ml Dimethyl formamide), 2.5 ml Veronal Acetate Buffer Stock (3.88 g Sodium Acetate ($3H_20$) and 5.88 g Sodium Barbitone, dissolved in 200 ml distilled water) and 6.5 ml distilled water. The pararosanilin/sodium nitrite solution was then added to this last solution and the pH adjusted to 4.7-5.0, followed by filtering.

Tissue sections were incubated in the solution described above for up to 60 minutes, at 37°C, washed well in distilled and then tap water and counterstained with 2% Methyl green for 15-30 seconds. Sections were then washed, dehydrated, cleared with xylol and mounted in DPX.

16. *IN SITU* HYBRIDIZATION ANALYSIS

Animals and tissue preparation:

S. aurata specimens collected at various developmental stages were fixed overnight at 4°C in freshly made 1% paraformaldehyde solution, then washed 3x10 min. in TBST buffer (50mM Tris, pH 7.4; 150mM NaCl; 0.1% Triton X-100) and stored in methanol at 4°C. Samples were dehydrated by passing them through an increasing alcohol series and embedded in paraffin. Tissues were cut into longitudinal 5µm thick sections and mounted on slides pre-coated with 10% poly-L Lysine (Sigma) or Vectabond® (Vector Laboratories), dried for 48 hours at 42°C and kept at R/T until use.

In situ hybridization:

Ten micrograms of a 326 bp fragment of spBGP cDNA [spanning from nucleotide 323 to the 3´end of the cDNA and cloned in pGem®-T vector (Promega)], was linearized with either alternatively with *Apa*I or *Pst*I restriction enzymes, phenol:chlorophorm:isoamyl alcohol (25:24:1) extracted, precipitated with 0.1 vol. $NaC_2H_3O_2$ / 1 vol. ice-cold 100% ethanol, washed briefly with ice-cold 75% ethanol, air-dried and the pellet dissolved in 25 µl DEPC-treated H_2O. One microliter of each digestion was electrophoresed in a 1.4% agarose gel to check for complete digestion and used to generate sense and anti-sense riboprobes.

Digoxigenin-11-UTP-labeled single strand RNA probes were prepared with a DIG RNA Labeling kit (Boehringer-Mannheim Biochemica), according to the manufacturer's instructions (Protocol 8, Appendix I). One tenth of the RNA obtained was analyzed by electrophoresis in a 1.4% formaldehyde-containing agarose gel in order to check for RNA size and integrity (results not shown).

The tissue sections obtained as described above were then treated as follows.

a) Deparafination: in a rocking platform, sections were treated for 2x 10 min. with xylol, 2x 8 min. with 100% Methanol, 1x 5 min. with 75% Methanol + 25% H_2O DEPC, 1x 5 min. with 50% Methanol + 50% H_2O DEPC, 1x 5 min. with 25% Methanol + 75% PTW (1x PTW: 1x PBS + 0.1% Tween 20) and 2x 10 min. with PTW.

b) Proteinase K digestion and fixation: a few drops of Proteinase K (40µg/ml in 1M TRIS, pH 7.4) were placed over each section and incubated at R/T for 15 minutes; sections were washed in PTW for 3 min. and re-fixed in 4% formaldehyde (in PTW) for 30 min., followed by a final washing with PTW, 2x 5 min..

c) Pre-hibridization: the Pre-Hybridization solution was prepared as follows: 2.5 ml deionized formamide (Sigma; # F-5786), 1.0 ml 20x SSC (Appendix II), 0.1 ml 50x Denhardt's (Sigma; # D-2532), 0.1 ml 50 µg/ml Yeast tRNA, 0.1 ml 2% CHAPS ((3-Cholamidopropyl) dimethylammonium-1-propanesulfonate, Sigma; # C-5070), 10 mg/ml Heparin (Sigma; # H-6279), and the final volume brought to 5 ml with DEPC-treated ddH_2O. A few drops of pre-hybridization solution were placed on top of each section, which was covered with parafilm and incubated at 55°C for 3 hours, in an atmosphere saturated with 20xSSC.

d) Hybridization: the spBGP probes, prepared as described above, were denatured by heating at 80°C for 5 min. and immediatelly placed on ice. Twenty microliters of each probe were added to 300µl of pre-hybridization solution and the mixture was immediatelly placed over each section, which was then covered with parafilm. Hybridization took place O/N, at up to 62°C, in an atmosphere saturated with 20x SSC.

e) RNase Digestion: sections were washed 3x 20 min. in RNase-free 2x SSC, at 55°C. Non-hybridized RNA was digested with a few drops of 10 µg/ml RNase A (GibcoBRL 10777-019), placed over each section, which was then covered with parafilm and incubated for 30 minutes at R/T.

f) Washing and Blocking: sections were washed as follows: 2x20 min., at 55°C, with 2x SSC/CHAPS (600µl 2% CHAPS + 1.5ml 2x SSC); 1x5 min. with PTW, at R/T; 1x10 min. with PTW/MAB (1:1), at R/T. Sections were then covered with ~ 200µl of blocking buffer [a 1% (w/v) blocking buffer (Boehringer) was prepared in 1x Maleic Acid Buffer (MAB; Appendix II)] and incubated for 1 hour at R/T.

g) Colour development: 0.5µl of Anti-digoxigenin-AP antibody (Boehringer) were added to 1ml of blocking buffer. Sections were covered with this solution, and then with parafilm, and incubated for 2 hours, at R/T, in an atmosphere saturated with 20x SSC. Sections were then washed with PTW for 3x 20 min., at R/T, and incubated for 10 minutes with Colour Buffer (50mM Tris, pH9.5; 50mM $MgCl_2$; 100mM NaCl; 0.1% Tween 20), at R/T. The solution was then replaced by fresh Colour Buffer, supplemented with NBT and BCIP (Boehringer; # 1383213 and 1383221, respectively) (337.5 µg/ml of nitroblue tetrazolium salt, 165µg/ml of 5-bromo-4-chloro-3-indolyl-phosphate in colour buffer). Incubation took place at R/T, protected from light, with constant agitation. Sections were visualized periodically in a reflecting light microscope to check for signal/noise development, and the reaction stopped when the signal to noise ratio began to decrease.

h) Dehydration and Mounting: sections were briefly washed with tap water, with ddH_2O, dehydrated in a crescent alcohol series (2x 70%, 1x 80%, 1x 90%, 2x 100%; 30 seconds each) and cleared in xylol for 1-2 minutes. A drop of DPX (BDH) was then placed over each section and topped with a lamella.

17. ESTABLISHMENT OF PRIMARY CELL CULTURES DERIVED FROM DIFFERENT *Sparus* TISSUES

17.1. FROM CALCIFIED TISSUES

Primary cell cultures of mixed phenotype were obtained from vertebra, jaw and cartilage of *Sparus* using an adaptation of previously established procedures (459, 565). The vertebra, jaw and branchial arches of juvenile *Sparus* were extracted immediately after sacrifice, briefly rinsed in 100% ethanol, placed on PBS supplemented with 1% penincillin / streptomycin (GibcoBRL 10378-032) + 1‰ Fungisone (Amphotericin B; GibcoBRL 15290-026) and cleaned of adherent soft tissue. The bones were then fragmented into pieces of 0.1-0.5 cm, transferred to sterile 50 ml polypropilene capped tubes (Falcon) and incubated with 5 ml of Digestion Solution [90% L15 medium (GibcoBRL 11415-049) + 8% sterile ddH_2O + 1% penincillin / streptomycin + 1‰ Fungisone + 0.125% Collagenase (Sigma C-0130)] for 30 minutes, at R/T, with rocking. The supernatant was discarded, replaced by 5 ml of fresh Digestion Solution, and the incubation prolonged for an extra 2 hours, with rocking, at R/T.

The tubes were then centrifuged at 2000 r.p.m. for 5 min., the supernatant discarded and the fragments spread on Tissue Culture (TC) ∅10 cm Petri dishes (Sarstedt). About 5 ml of Medium I (L15 medium supplemented with 10% Foetal Calf Serum (GibcoBRL 10270-106), 1% penincillin / streptomycin, 1‰ Fungisone) were added to the fragments and incubated on a humidified atmosphere at 22°C.

Several days after culture was initiated, cells that migrated from bone fragments filled most of the culture plate and were collected by centrifugation following treatment with Solution T (140mM NaCl, 2.7mM KCl, 200mM Na_2HPO_4, 1.5mM KH_2PO_4, 1mM EDTA and 2 ‰ Trypsin). Cells were then re-plated in two TC Petri dishes (Sarstedt) at a density of approximately $8x10^4$ cells/ml, and re-trypsinizated each 3-5 days, according to the division rate. Medium changes were performed each 4-5 days.

17.2. FROM SCALES

Following sacrifice, scales were extracted from *Sparus* specimens with a forceps, briefly rinsed in PBS supplemented with 1% penincillin / streptomycin (GibcoBRL 10378-032) + 1‰ Fungisone (Amphotericin B; GibcoBRL 15290-026) and immediatelly placed, with the interior side facing down, on TC Petri dishes (Sarstedt), to which a small volume of Medium I, just enough to cover the bottom surface, had been previously added. Incubation took place at 18-22°C, on a humidified atmosphere.

18. PRESERVATION OF *Sparus*-DERIVED CELL TYPES

Periodically (approximatelly each fifth trypsinization), cells obtained from *Sparus* tissues were frozen for later use. Cells were typsinizated as described above, re-suspended in culture medium and centrifuged at 2000 r.p.m. for 5 minutes. The pelleted cells were then resuspended in 10% serum-containing culture medium, supplemented with 10% Dimethyl sulfoxide (DMSO; Sigma, D-8779), transferred to 2 ml criovessels (Nunc), incubated for 15 minutes on ice and then O/N at -80°C, in a Cryo1C Freezing container (Nalgene, 5100-0001). The next day cells were transferred to liquid nitrogen and stored until needed.

To thaw frozen *Sparus* cells, tubes containing cells were removed from the liquid N_2,

quickly thawed and transferred to an appropriate volume of culture medium. After incubation at the suitable temperature for 2 to 3 hours, the culture medium was replaced by fresh medium, to eliminate the DMSO.

19. AMPLIFICATION OF THE BGP MESSAGE FROM PRIMARY CELL CULTURES OF *S. AURATA*

Total RNA was extracted from cells derived from vertebra, jaw and branchial arches of *Sparus*, following the guanidinium thiocyanate-phenol-chloroform method (83), as described in Section 1 of this chapter. One microgram of total RNA was then reverse transcribed with M-MLV-RT (Promega), using an oligo (dT) linked to an universal adapter (Protocol 2, Appendix I). Half (10 µl) of the RT reaction was used as a template for amplification by the Polymerase Chain Reaction (PCR), using SBG5F and SBG4R as primers (Table II.I), according to the protocol detailed in Section 11 of this chapter. The remaining RT volume was used to amplify spβactin, which served to assess the quality of the RNA used. Specific primers [designed according the published spβActin cDNA sequence (470)] were used in order to amplify a DNA fragment of 240 bp.

The resulting PCR products were electrophoresed on a 1.4% agarose gel and the bands corresponding to the expected size for spBGP excized with a clean scalpel and eluted from the agarose using the Qiaex II Gel Extraction Kit (Qiagen), cloned in pGEM-T Easy (Promega), inserted in *E. coli* DH5α strain (GibcoBRL) (see Section 3 of this chapter for description of methodology and Appendix II for information on vector and bacterial strain characteristics) and sequenced with the ^{T7}Sequencing Kit (Pharmacia) and the SP6 and T7 vector-specific primers (Protocol 3, Appendix I).

20. DETECTION OF ALKALINE PHOSPHATASE ACTIVITY IN VERTEBRA-DERIVED PRIMARY CELL CULTURES OF *S. AURATA*

Sparus vertebra-derived cells were trypsinizated (T4, i.e., fourth trypsinization after beginning of culture) and plated at approximately 40% confluency. After 24 hours incubation at 22ºC in Medium I (see 17.1 for composition), medium was removed and cells washed 3X with PBS (pH 7.0), followed by fixation in 70% ethanol (30 minutes, R/T) and five washes

with ddH_2O. Cells were covered by the detection reagent [prepared by adding 10 mg of Naphtol ASBI (Sigma, N-2125) to 0.5 ml of N,N-Dimethyl-Formamide (Sigma, D-8654); 5 ml of Tris buffer, pH 8.6; and 4 ml of 9.5 ‰ NaCl; the solution was mixed and 3 mg of Fast Red Violet LB salt (Sigma, F-3381) were added and mixed., incubated for 1 hour at 37°C and rinsed with ddH_2O]. Detection of sites of alkaline phosphatase activity was performed by visual examination of the cell layer under a Zeiss Axiovert 25 inverted microscope.

21. DETECTION OF MINERAL DEPOSITION IN THE EXTRACELLULAR MATRIX OF *S. AURATA* BONE-DERIVED CELLS

Cells derived from *Sparus* vertebrae were grown into confluency and then treated with a "mineralization solution" consisting of 10mM β-Glycerophosphate (Sigma, G-9891), 50 μg/ml Ascorbic acid (Sigma, A-4034), in Medium I, for 30 days, at 20°C, with medium replacements every 3-4 days. Negative controls consisted of cells with exactly the same origin incubated simultaneously in Medium I without the mineralizing supplements.

After one month of incubation with the "mineralization solution" the presence of mineral deposition was revealed by either the Von Kossa method, or the Alizarin red-S staining, according to the procedures described below.

Von Kossa: The mineralizing medium was discarded and cells were thoroughly washed (3 times) with PBS, pH 7.0. After fixation with 4% formaldehyde, for 15 minutes, at R/T, cells were thoroughly washed with ddH_2O (5 times), at R/T, and incubated with 1% silver nitrate ($AgNO_3$; aqueous solution) for 30-60 minutes, under ultraviolet light. Cells were then washed with ddH_2O (3 times), at R/T, and incubated with 2.5% sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$; aqueous solution) for 5 minutes. A final wash was performed with ddH_2O (once), at R/T, followed by air-drying.

Alizarin red staining: The mineralizing medium was discarded and cells were thoroughly washed (3 times) with PBS, pH 7.0. After fixation with 4% formaldehyde, for 15 minutes, at R/T, cells were thoroughly washed with ddH_2O (5 times), at R/T, and incubated with a saturated aqueous solution of Alizarin red-S, for ~5 minutes, at R/T. Cells were then washed with ddH_2O (3 times) and air-dried.

22. CLONING THE SPBGP 5'-FLANKING REGION IN THE PβGAL-BASIC EXPRESSION VECTOR

Preparation of the spBGP promoter

A DNA fragment containing 1.2 kb of the 5' flanking region of the spBGP gene and extending 37 bp into Exon 1 (from -1200 to +37), was obtained by PCR amplification of *S. aurata* genomic DNA, using two spBGP gene-specific oligonucleotide primers (SBG24F and SBG25R; Table II.I). Each oligonucleotide carried also the sequence for either *Xho*I or *Hin*dIII restriction enzymes. Briefly, 100 ng of DNA (3 µl) were mixed with 2 µl (50mM) of $MgCl_2$ (GibcoBRL), 5 µl of 10x Mg-free buffer (GibcoBRL), 1 µl of 50 µM SBG24F oligonucleotide (Pharmacia), 1 µl of 50 µM SBG25R oligonucleotide (Pharmacia), 4 µl (2.5 mM each) dNTPs (Promega), 0.3 µl (1.2 Units) *Taq* Polymerase (GibcoBRL), and the volume completed to 50 µl with ultrapure H_2O (Sigma).

Negative controls were made by adding only one or the other primer to the reaction mix, to test for non-specific amplifications, and with all reagents but no DNA, to test for possible contaminations. Amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400 for 35 cycles (one cycle was 95°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute), with an initial denaturion step of 3 minutes at 95°C, and a final elongation step of 10 minutes at 72°C.

Five microliters of the resulting PCR products were analysed by electrophoresis in a 1.4% ethidium bromide-containing agarose gel and visualized under ultraviolet light. The expected band was excized from the gel with a clean razor blade and eluted from the agarose with the Qiaex II Gel Extraction Kit (Qiagen), following the manufacturer's instructions. The DNA fragment obtained was cloned into the pGEM-T Easy plasmid vector (Promega), according to the manufacturer instructions, inserted in *E. coli* DH5α strain (GibcoBRL) (see Section 3 and Appendix II). The plates were incubated O/N at 37°C and the positive clones were colour selected, isolated and grown O/N on LB medium (Sigma) supplemented with 50 µg/ml ampicilin.

Plasmid DNA was prepared following the "alkaline lysis" method (Protocol 7, Appendix I). Six microliters of the purified DNA thus obtained were digested with *Eco*RI restriction enzyme, using the appropriate buffer, and the presence of positive clones (selected by the presence of the right size DNA insert) was confirmed by electrophoresis in a 1.4 %

agarose gel (Promega). One of the positive clones obtained was chosen and digested with *Hin*dIII and *Xho*I restriction enzymes, in order to create the protruding ends that would facilitate cloning into the pβgal-Basic vector. The digestion reaction was performed by mixing together 20 µl of DNA (~ 100 ng/ml), 6 µl of 10x OPA^+ buffer (Pharmacia), 0.5 µl of *Xho*I (7 Units; Pharmacia), 0.5 µl of *Hin*dIII (6 Units; Pharmacia), and 3 µl of H_2O, and incubating at 37°C for 1.5 hours. Enzymes were inactivated at 85°C for 30 minutes and cooled until R/T for 20 minutes.

Preparation of the plasmid DNA

Two microliters of the pβgal-Basic vector were digested with the *Hin*dIII and *Xho*I restriction enzymes, by mixing together 1.5 µl of pβgal-Basic, 6 µl of OPA^+ buffer (Pharmacia), 0.5 µl of *Xho*I (Pharmacia), 0.5 µl of *Hin*dIII (Pharmacia) and 21.5 µl of ddH_20, and incubating at 37°C for 1.5 hours. In order to avoid re-ligation of the digested plasmid DNA, dephosphorilation of the digested ends was performed by adding 1 µl (200 units) of Calf Alkaline Phosphatase (Appligene; # 120221), at 37°C, for 30 minutes, followed by a phenol:chloroform extraction (30 µl digested/dephosphorilated DNA, 400 µl phenol:chloroform (1:1); centrifugation for 5 minutes at 14000 rpm and removal of super-natant into a sterile 1.5 ml tube), and re-precipitation (400 µl plasmid DNA, 40 µl 3M $NaC_2H_3O_2$, 800 µl 100% ethanol; 15 minutes incubation at -80°C, centrifugation for 15 minutes at 14000 rpm, wash with 75% ethanol and air-dry for 15 min.). Pelleted plasmid DNA was then dissolved in 20 µl of ddH_2O and 4 µl were electrophoresed in a 1.4% ethidium-bromide stained agarose gel.

Cloning of the spBGP promoter into the pβgal-Basic vector

The spBGP gene putative promoter DNA was inserted into the digested pβgal-Basic vector, by adding 2 µl (100 ng) of plasmid DNA to 5.8 µl (290 ng) of spBGP promoter DNA, and incubating for 5 minutes at 45°C. One microliter of 10x ligase buffer (Stratagene), 1 µl of rATP (1mM final concentration) and 0.2 µl of T4 DNA Ligase (1 U; Stratagene; # 600011) were then added to the DNA mixture and incubated O/N at 4°C.

Three microliters of the ligation product were inserted in *E. coli* DH5α strain (Appendix II), as described in Section 3 of this chapter. Ten white colonies were picked with sterile toothpicks, transferred to LB supplemented with 50 µg/ml ampicilin and incubated

O/N, at 37°C. Plasmid DNA was extracted from the bacteria using the "alkaline lysis" method (Protocol 7, Appendix I). Six microliters of the DNA thus obtained were digested with the *Stu*I restriction enzyme (Pharmacia), using the appropppriate buffer, and the presence of positive clones (as assessed by the presence of a DNA insert with the expected size) was analysed by electrophoresis in a 1.4 % agarose gel (Promega). One of the presumable positive clones obtained was chosen and 44 microliters were used to verify the orientation and integrity of the promoter-vector construct, by double-strand DNA sequence analysis (Protocol 3, Appendix I). DNA was prepared in large scale using the Wizard Maxiprep DNA purification kit (Promega Corp., Madison, WI), following the manufacturer's recommended protocol.

23. TRANSIENT TRANSFECTION OF BONE-DERIVED PRIMARY CULTURES OF *S. AURATA*

Sparus vertebra-derived cells (obtained following the procedure described in Section 17 of this chapter) were cultured to confluency, trypsinized and plated at a density of approximately $8x10^4$ cells/ml. The next day cells were transiently transfected by lipofection, using the DOTAP Liposomal Transfection Reagent (Boehringer Mannheim, cat.# 1202375), with either spBGPprom/pßgal construct, the pßgal under the control of human cytomegalovirus (CMV) promoter (pCMVβgal), as positive control, or the pβgal-Basic (Clontech; #6044-1) vector, as negative control. Five micrograms of each DNA construct were diluted in Hepes buffer (Sigma, H-0763) to a final concentration of 0.1 µg/µl in 50 µl, in a sterile 1.5 ml centrifuge tube. Thirty microliters of DOTAP were diluted in Hepes buffer to a final volume of 100 µl, in a sterile 1.5 ml centrifuge tube, added to the DNA/Hepes solution and mixed by pipeting. The transfection mixture was incubated for 15 minutes, at R/T, and then 5 ml of cell culture medium (see composition in Section 17 of this chapter) were added to the transfection solution. The mixture was added to the cells and incubated at 22°C for 6 hours. After this period, the transfection solution was replaced by cell culture medium and incubation continued for an additional 48 hours, at 22°C.

Activation of the reporter gene was detected by exposing the fixed cells to an X-Gal containing solution (5mM $K_4Fe(CN)_6.3H_2O$, 5mM $K_3Fe(CN)_6$, 2mM $MgCl_2$, 1mg/ml Xgal) at 37°C for 24-48 hours.

24. CLONING OF A PARTIAL Halobatrachus didactylus (TOAD FISH) BGP cDNA

Total RNA was extracted from the vertebra of an adult specimen of *H. didactylus* according to the acid guanidinium thiocyanate-phenol-chloroform method (83), as detailed in Section 1 of this chapter. One microgram of this RNA was reverse transcribed, following the procedure described in Protocol 2 (Appendix I), and the resulting single strand cDNA amplified by PCR, using SBG5F and SBG4R as primers (Table II.I). The mixture was incubated in a Perkin Elmer GeneAmp PCR System 2400, programmed for an initial denaturation step of 9 minutes at 95°C (for enzyme activation), and 35 cycles of denaturation at 95 °C for 1 minute, annealing at 60°C for 30 seconds and extension at 65°C for 45 seconds, followed by a final extension of 10 minutes at 65°C.

Fifteen microliters of the resulting PCR product were electrophoresed on a 1.4% agarose gel and the observed DNA fragment cut from the gel with a clean razor blade. DNA was eluted with the Qiaex II Gel Extraction Kit (Qiagen), cloned in a pGEM-T plasmid vector (Promega), and inserted in *E. coli* DH-5α strain, as described (Section 3). Three positive clones (white colonies) were selected, transferred to liquid LB medium (see composition in Appendix II), supplemented with 50 μg/ml ampicilin, and allowed to grow O/N with agitation, at 37°C. Purification of the plasmid DNA was performed by the "alkaline lysis technique" (Protocol 7, Appendix I). DNA thus obtained was excised from the plasmid by digestion with *Apa*I and *Pst*I restriction enzymes (Pharmacia) and three of the positive clones selected for sequencing. DNA sequence analysis was performed with the Sequenase 2.0 kit (USB) and the SP6 and T7 vector-specific primers (Protocol 3, Appendix I).

25. PHYLOGENETIC ANALYSIS

Phylogenetic analysis was performed using all available BGP, MGP and mouse nephrocalcin amino acid sequences, and using as outgroup human Coagulation factor II and hagfish prothrombin amino acid sequences. Character state changes were all weighted equally. Polymorphisms were treated as uncertain and insertions/deletions were coded as missing data, in order to avoid including them as if they were many independent events, while still retaining the information about substitutions in other taxa in the indel regions. All

phylogenetic analyses were performed using the PAUP 4.0 b2a software. Heuristic searches with 50 repetitions using random stepwise additions were performed under maximum parsimony. This method allows the construction of trees that require the fewest nucleotide replacements to explain the descent of extant sequences (192, 193).

The level of confidence in each node of the maximum parsimony tree was assessed using non-parametric bootstrapping (153) based on 1000 replicates.

Table II.1
Oligonucleotides used for PCR amplification of *Sparus* BGP cDNA and gene

Primer	Sequence[a]	Localization in the gene[c]	Localization in the cDNA
SBG2F	TGYGARCAYATGATGGAYACNGA[b]	+ 2453	322
SBG3R	GGGGATCGGTCCGTAGTAGG	+ 2518	387
SBG4R	GTTAGGGGAAATGATCGAATCACAGTGGG	+ 2733	602
SBG5F	TGCGAGCACATGATGGACACTGAGGGAATC	+ 2453	322
SBG7R	GAACCAGGAAGGCCAGAGTC	+ 124	124
SBG8F	TTCGTGGAGAGGGACCAGGC	+ 2121	211
SBG9R	GGGGATCGGTCCGTAGTAGG	+ 2518	387
SBG11R	CCATCAGCTGTCGTAGTAAGGC	+ 2548	417
SBG12F	GAGCTGGAAGTCTCCGGTCCG	+ 33	33
SBG13F	CCAGCCTGCCAGTGACAACCC	+ 371	174
SBG14R	GGTTGTCACTGGCAGGCTGG	+ 390	193
SBG15R	CCGCTCTCTTCTGTCTCACC	+ 2169	259
SBG18F	CGGTAAGTTGCATCAAACGG	+ 295	-
SBG19R	CCACTGCGGAGGCCTGGTCCCTCTCC	+ 2126	241
SBG20R	GTGGAGGCATCTGAGGGAAAACATCTCG	+ 364	-
SBG21R	CGGACCGGAGACTTCCAGCTCTGTCAC	+ 53	53
SBG22F	GACAAGGCACCAGCATTGACC	-1119	-
SBG26R	GACGTTTCTATCGGCCAT	+ 1912	-
SBG27R	CCCGGGTTGTATGTGC	+1589	-
SBG28F	CGGAACACTGTTTGAAG	+ 573	-
SBG29F	CCCCAAACTACATAGTGC	+ 774	-
SBG30R	CAGAGTACAACTGAGCAC	+ 1385	-

[a] All sequences are described in the 5' to 3' direction.

[b] Y, pyrimidine; R, purine; N, G+a+T+c.

[c] Corresponds to localization of the first nucleotide (5' end of oligonucleotide) in the sequence of the spBGP gene.

CHAPTER III: **RESULTS**

1.MOLECULAR CLONING OF spBGP cDNA

1.1. CLONING OF A PARTIAL spBGP cDNA CONTAINING THE 3'-END

Total RNA extracted from a *Sparus* juvenile with a completely mineralized skeleton (as assessed by alizarin red staining) was used for the reverse transcription and amplification of spBGP mRNA with SBG2F (Table II.I) and Universal dT. After size fractionation of the resulting PCR products by agarose gel electrophoresis, three bands within the expected size were observed (Figure III-1). After cloning and sequencing, the 314 bp band (band 14) proved to be a partial cDNA for spBGP, encoding part of the aminoacid sequence already available (64). This DNA fragment spun from amino acid 23 of the mature protein to the stop codon, and extended an additional 239 bp to the site of insertion of the poly-A tail, 15 bp after a consensus polyadenylation signal (Figure III-3).

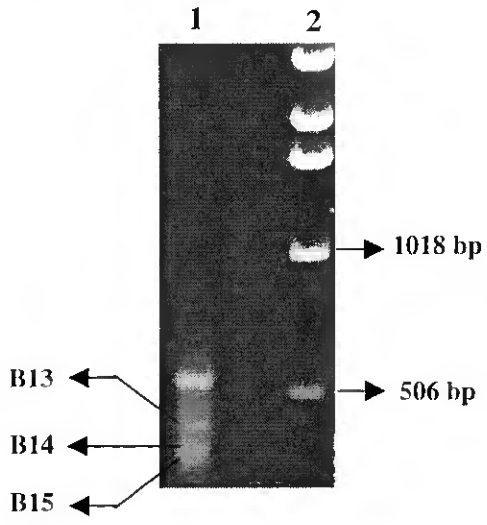


Figure III-1. RT-PCR amplification of *Sparus* bone RNA with SBG2F and Universal Adapter. Fifteen microliters of RT-PCR product were electrophoresed in a 1.4% ethidium-bromide containing-agarose gel (lane 1), together with a 1 Kb DNA ladder (GibcoBRL; lane 2). Bands 13, 14 and 15 (B13, B14, B15) were extracted from the gel for further analysis.

DNA from the two other bands observed in Figure III-1 (bands 13 and 15), as well as from another band obtained in a previous RT-PCR (not shown) were also cloned and sequenced, and, after searching the NCBI database, were shown to correspond to partial cDNAs for *Sparus* Pancreatic amylase (band 13), Calmodulin (band 15) and Trypsin. These

sequences were submitted to GenBank, being assigned the accession numbers AF316854, AF316853 and AF316852, respectively. The spTrypsin clone was later used for testing the *in situ* hybridization technique.

1.2. CLONING OF THE 5'- END OF spBGP cDNA

The 5' end of the spBGP cDNA was obtained by 5' RACE PCR, using the Marathon™ cDNA Amplification kit (Clontech), as described in Chapter II. Two major amplified fragments were obtained, named Band A and B (Figure III-2), which were excised from the gel and cloned in pGEM-T Easy, as described in the Methods chapter. Following cloning and sequence analysis of the DNA fragments purified from bands A and B, it was shown that they all corresponded to spBGP cDNA, although extending to different lenghts in their 5' ends. This was attributed to the existence of different sized single strand cDNAs, due to an early stop of the reverse transcriptase and, therefore, we chose to consider the clone presenting the longest 5'-extremity as the most representative of the *Sparus* cDNA.

The spBGP cDNA thus obtained spans 635 bp in length and comprises a 5'-untranslated region (UTR) of 87 bp, an open reading frame of 291 bp, coding for a polypeptide with 97 amino acid residues, and a 257 bp 3'- UTR, from the stop codon to the site of insertion of the poly-A tail (Figure III-3 and Table III.I).

As deduced from the comparison with the complete cDNAs for mammalian and chicken BGPs (Table III.I), the spBGP cDNA encodes a pre-peptide of 24 residues, a pro-peptide of 28 residues and a mature protein of 45 residues. Although the *Sparus* cDNA is longer than its mammalian and avian counterparts, this difference is due to longer 5´and 3´UTRs, while the coding region is of a size comparable to those from all known BGP cDNAs (Table III.I).

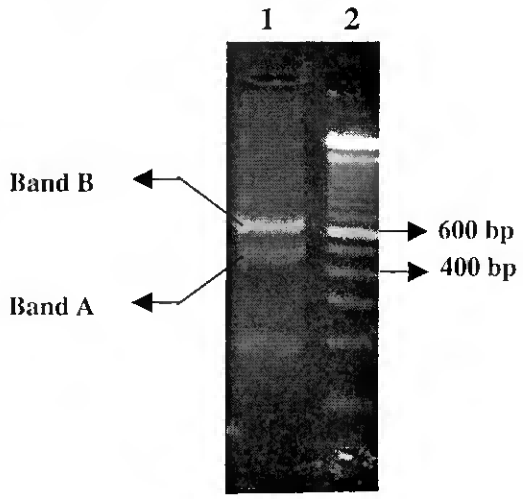


Figure III-2. Amplification of *Sparus* mRNA by 5' RACE PCR, with AP1 and SBG4R. Five microliters of the PCR reaction were electrophoresed onto a 3% FMC Seakem GTG agarose gel (lane 1), together with a 100 bp DNA ladder (GibcoBRL; lane 2), and bands A and B were extracted from the gel for further analysis.

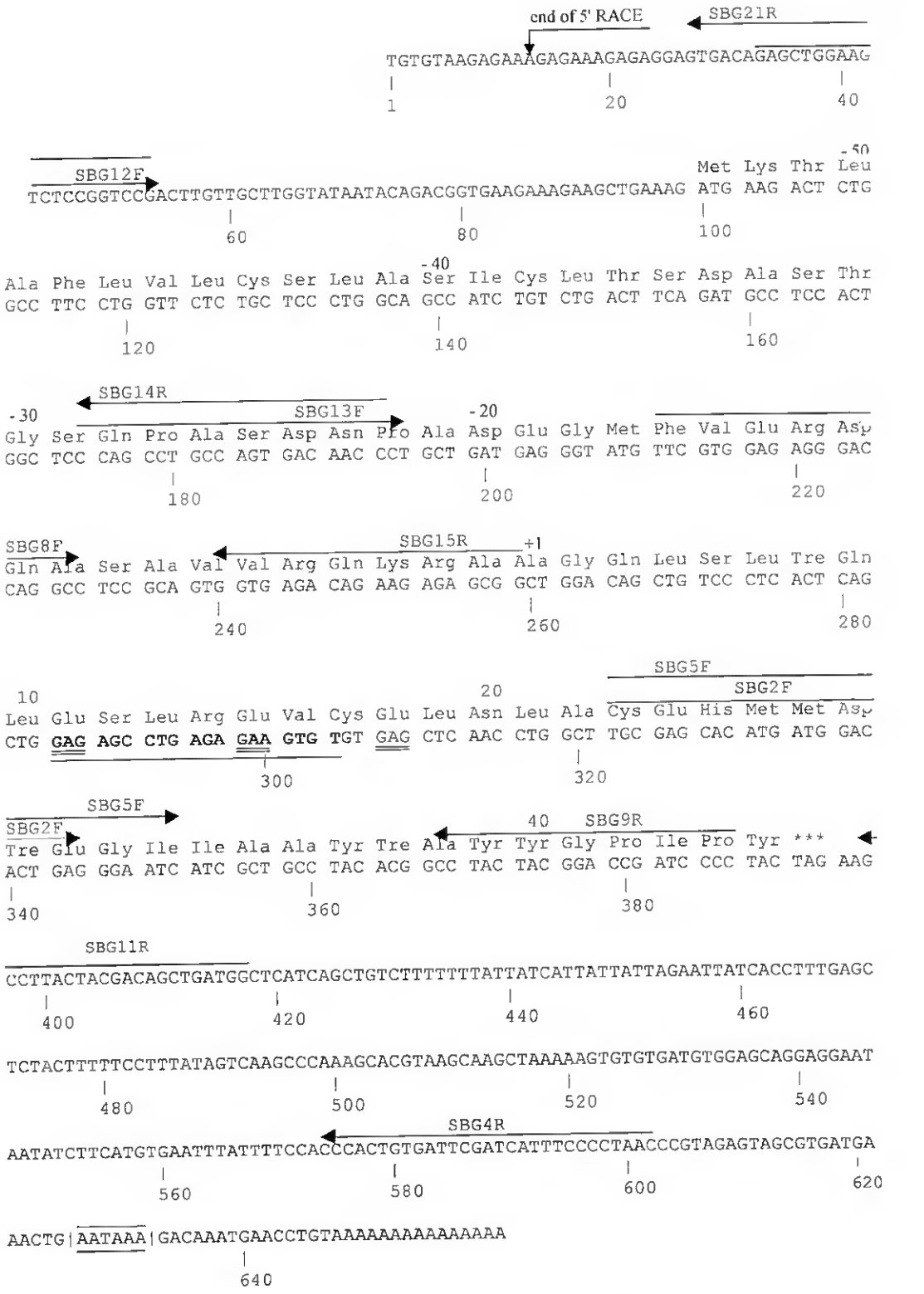


Figure III-3. Complete nucleotide sequence of *Sparus* BGP cDNA and deduced amino acid sequence of polypeptide. Numerical positions in the nucleotide and amino-acid sequences are notated below and above each line, respectively. Numbering of the nucleotides in the cDNA sequence begins in the cap site. The first amino-acid of the mature protein is identified as amino-acid +1. The stop codon following the protein coding region is indicated by three asterisks. A box indicates the polyadenylation signal and a bar is drawn under the putative γ-carboxylase recognition site, which sequence is in bold. The codons for γ-carboxyglutamate residues are underlined twice. Location of the oligonucleotide primers used to obtain the complete cDNA is indicated by arrows.

Table III-I. Characteristics of known BGP cDNAs and corresponding proteins. Sizes of all known BGP cDNAs including 5´and 3´untranslated regions (UTR) and coding regions are indicated. Comparison of sizes for pre, pro and mature forms of corresponding BGPs are also shown. Genbank accession numbers are provided in the right column.

Species	cDNA size (bp)*	5' UTR (bp)	Coding Region (bp)	3' UTR (bp)*	Protein Size (aa) Pre | Pro | Mat.	Accession numbers
Homo sapiens	451	18	300	133	23 | 28 | 49	X53698
Bos taurus	437	27	300	110	23 | 28 | 49	X53699
Mus musculus	458	48	285	125	23 | 26 | 46	X04142
Rattus norvegicus	474	36	297	141	23 | 26 | 50	X04141
Gallus gallus	421	18	291	112	24 | 24 | 50	U10578
Sparus aurata	647	99	291	257	24 | 28 | 45	AF048703

2. MOLECULAR CLONING AND ORGANIZATION OF THE spBGP GENE

The spBGP gene was cloned by a mixture of genomic amplification, using specific DNA primers designed according to the spBGP cDNA, and the gene walking technique.

The spBGP protein was alligned with the known mammalian BGPs for which the gene structure is known (Figure III-4) and sites of possible exon-intron borders deduced by comparison. Several primers were designed flanking the region of the *Sparus* BGP thought to contain the introns. The primers designed for amplification of the spBGP introns were SBG12F+SBG14R (intron 1), SBG12F+SBG15R (intron 2), and SBG8F+SBG9R (intron 3) (Table II.I). Also, other primers were constructed in other regions of the spBGP cDNA in order to check the possible existence of other introns, not identified by comparison with the published BGP gene sequences.

For each amplification of the genomic DNA with a specific pair of primers only one band was visible on agarose gel following electrophoresis of the resulting PCR product. Sequencing of the bands obtained with the three primer pair combinations showed that they corresponded to three introns in the BGP gene, starting and ending, in each case, within the spBGP cDNA sequence already available. Sequence analysis led us to conclude, therefore, that the spBGP gene (Figures III-8 and III-9) is organized into four exons and three introns

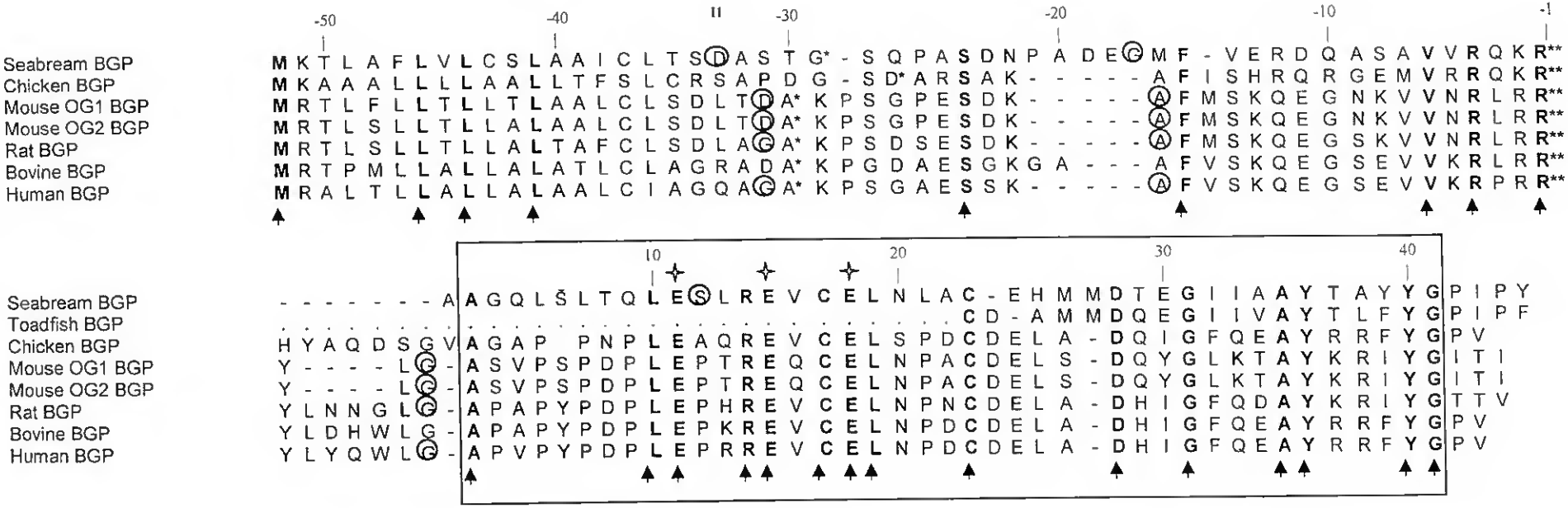


Figure III-4. Amino acid sequences of all known complete pre-proBGPs. Sequences were aligned and conserved residues marked by vertical arrows. A box encloses the most conserved region of the protein and a star is located above each gamma-carboxyglutamate residue. Dashes indicated gaps in the sequence introduced to increase homology; dots stand for unavailable data. A circle encloses aminoacids corresponding to sites of intron insertion in the corresponding genes. Residues are numbered according to residue 1 of mature spBGP protein. Sequence references are: this study for seabream and toadfish; 371 for chicken; 117 for mouse OG1 and OG2; 527 for rat; and 261 for human and bovine.

(the first two of phase I, while the third intron is of phase II, Table III.II), in agreement with the structure of all known mammalian BGP genes (Table III.III). It spans 2778 bp, from the major start site of transcription to the site of insertion of the poly(A) tail, and it is approximately twice as long as the mammalian genes, mostly due to the presence of a very large intron 2. The three introns account for nearly 78% of the total DNA of this gene and each exon encodes roughly the same protein domain in both fish and human genes (Table III.III).

Table III-II. Exon-intron splice junctions and phase of the introns in *Sparus aurata* BGP gene. 5' and 3' borders for each intron are indicated. The consensus motif (5'gt....ag 3'), as described by Breathnach *et al.* (52), is shown in **bold**. The phase of each intron is shown according to Patthy (407).

Intron Number	Intron borders 5' border.....3'border		Phase of Intron
Intron 1	..TCA.G **gtag**..	..tc**ag** AT.GCC.	I
Intron 2	..GAG.G **gtaa**	..gc**ag** GT.ATG.	I
Intron 3	.GAG.AG **gtat**	..tc**ag** C.CTG.	II

All exon-intron splice junctions in the spBGP gene (Table III.II) conform to the AG/GT rule (52). Although the overall organization of the gene has been maintained from fish to man, the sites of insertion of the three introns within the protein coding sequence differ in *Sparus* as compared to the mammalian genes (Figures III-4).

Table III-III. Characteristics of known BGP gene structures. Gene, exon and intron sizes (in base pairs, bp) are indicated for all known BGP genes. Phase of intron is defined according to Patthy (407). Genbank accession numbers for all gene sequences are indicated in the far right column. References for BGP gene sequences are as follows: *H. sapiens*: 73; *M. musculus* (OG1): 117; *R. norvegicus*: 527; *S. aurata*: this study.

Species	Gene size (bp)	Exon 1	Intron 1 (phase)	Exon 2	Intron 2 (phase)	Exon 3	Intron 3 (phase)	Exon 4	Accession N°
Sparus aurata	2778	99 (5' UTR)+58	197 (I)	47	1713 (I)	86	221 (II)	100 + 257 (3' UTR)	AF289506
Rattus norvegicus	1145	49 (5' UTR)+64	148 (I)	33	143 (I)	70	200 (II)	130 + 308 (3' UTR)	M25490
Mus musculus	950	48 (5' UTR)+64	144 (I)	33	142 (I)	58	206 (II)	130 + 125 (3' UTR)	L24429
Homo sapiens	1077	18 (5' UTR)+64	257 (I)	33	175 (I)	70	201 (II)	127 + 132 (3' UTR)	X04143

3. DETERMINATION OF THE START SITE OF TRANSCRIPTION OF THE spBGP GENE

Determination of the site of transcription initiation of the spBGP gene was performed by primer extension analysis, as described in the Methods chapter, revealing two possible sites of transcription initiation, 99 and 78 bp upstream from the first ATG (Figures III-3 and III-5). The site corresponding to the longest 5'UTR was found to be located, in the genomic DNA, 26 bp downstream from a consensus TATA box motif and was considered to correspond to the major start site of transcription of the spBGP gene (Figure III-9).

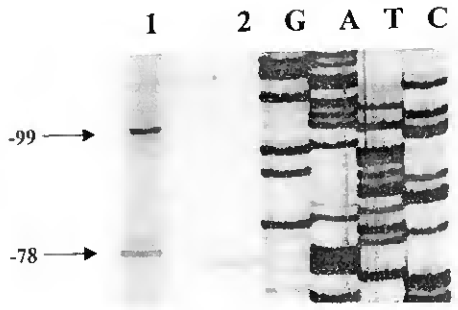


Figure III-5. Identification of transcription start site of the spBGP gene as determined by primer-extension. Poly $(A)^+$ RNA isolated from *Sparus* jaw was annealed to a spBGP reverse primer, reverse-trancribed and subjected to Rnase digestion, electrophoretic fractionation and autoradiography. The extension products are indicated in lane 1 and their sizes are shown on the left margin. Lane 2 shows a non-related fragment of known size. A sequencing ladder (lanes G, A, T and C) was fractionated in the same gel for assignment of transcription start sites at single nucleotide resolution

4. CLONING AND ANALYSIS OF THE 5' FLANKING DNA OF THE spBGP GENE

Cloning of the 5' flanking DNA

The 5' flanking DNA of the spBGP gene was cloned by a gene walking strategy, using the Gene Walker Kit from Clontech, as described in the Methods chapter. The results from the electrophoresis of the primary PCR using AP1 and SBG19R as primers are depicted in Figure III-6 and those from electrophoresis of the secondary PCR using AP2 and SBG20R as primers in Figure III-7. Only one proeminent band (lane 2A) was obtained with the *BamH*I digestion, while a major band (lane 2B) and two minor bands were obtained from *Pst*I digested DNA.

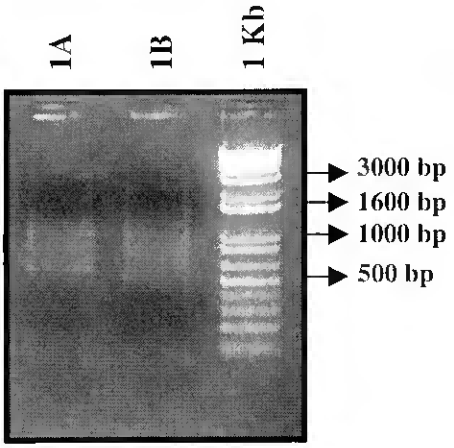


Figure III-6. Primary amplification of *Sparus* genomic DNA with Adaptor-specific primer 1 (AP1) and SBG19R. Resulting PCR products were electrophoresed in a 0.8% agarose gel (1A-*BamH*I; 1B-*Pst*I; 1Kb DNA ladder (GibcoBRL)).

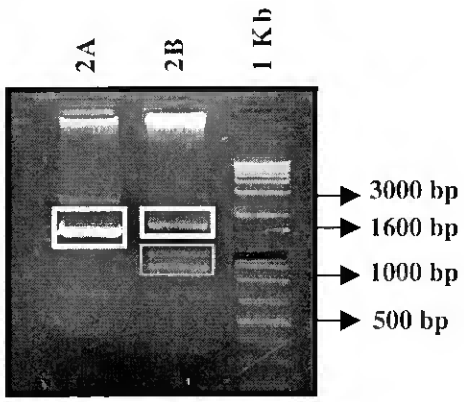


Figure III-7. Electrophoresis of the secondary amplification of primary PCR (Figure III-6) with Adaptor-specific primer 2 (AP2) and SBG20R (2A-*BamH*I; 2B-*Pst*I; 1Kb-DNA ladder (GibcoBRL)).

Sequencing of the bands 2A and 2B (Figure III-7) showed that they corresponded to overlapping DNA fragments containing the 5' upstream sequence from the spBGP gene. We thus identified a 5'-flanking DNA sequence with 1145 bp. These results also confirmed the position of the introns previously obtained. The sequence of the complete spBGP gene is shown in Figure III-8. The two minor bands seen in lane 2B of Figure III-7 (blue square) were also sequenced, revealing a sequence which we were unable to align with our previously obtained BGP or BGP 5'-flanking sequences.

Analysis of the spBGP promoter region

The spBGP 5' -flanking DNA sequence obtained was compared with the available databases for identification of specific regulatory sequences present, in particular, in other BGP genes, revealing that the spBGP gene promoter has a modular organization with sequence motifs typical of a gene transcribed by the RNA polymerase II (Figure III-8) such as the canonical TATA box (TATAAA), located from -31 to -26 bp, and putative CCAAT boxes found at positions -256 and -67. We have also identified a series of putative consensus sequences for steroid receptor binding sites and bone-specific transcription factors, known to be physiological mediators of BGP gene expression in higher organisms. Between positions -406 and -390, a sequence motif sharing homology with a steroid hormone response element (SRE) was identified. In addition, several OSE1 and OSE2-like motifs (134) and one AP1-like motif (391) in intron I were also identified. Interestingly, intron II was found to contain copies of many of the putative response elements found within the proximal promoter of the *Sparus* BGP gene, including a TATA consensus sequence and two CCAAT boxes.

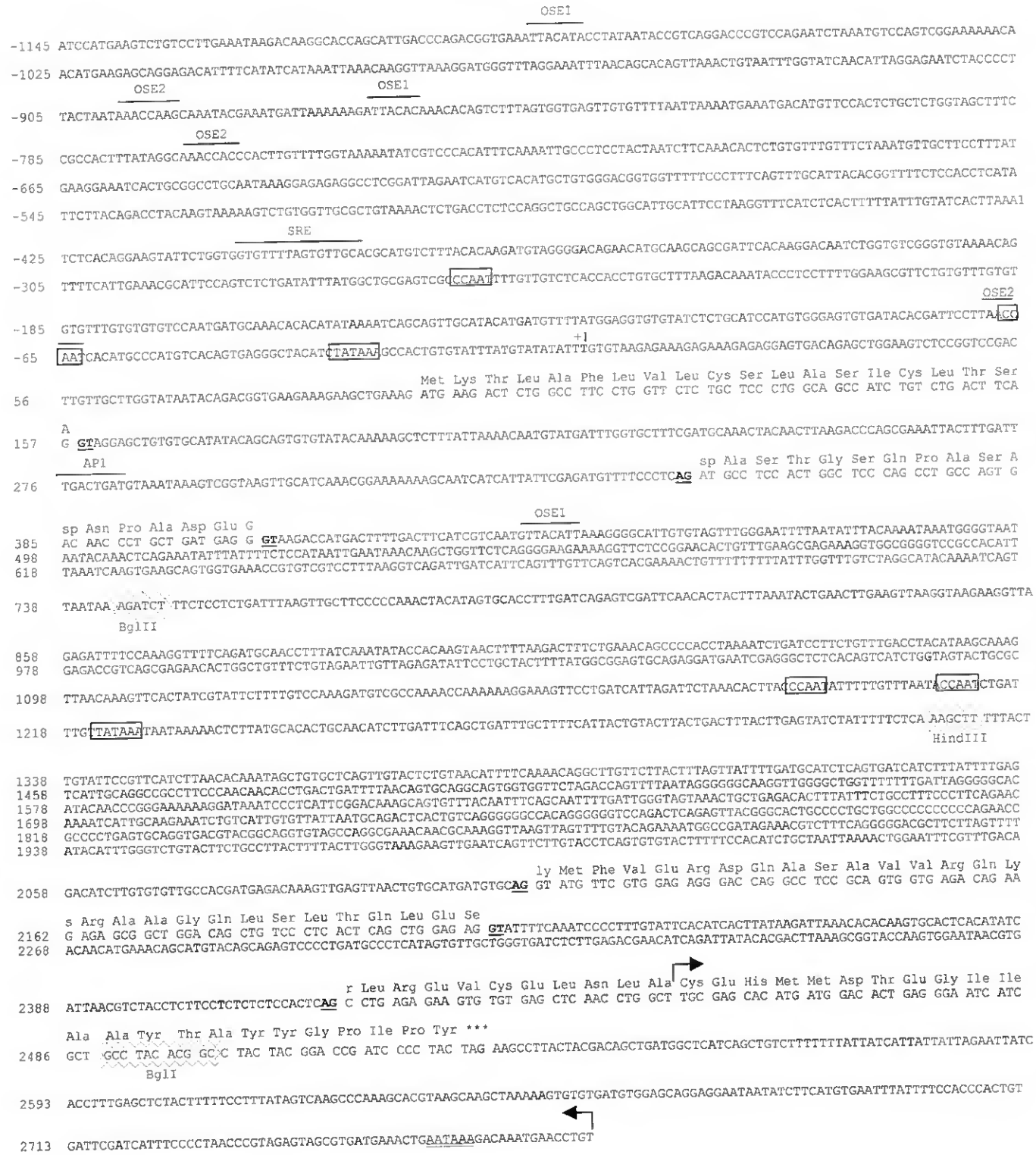


Figure III-8. Sequence of *Sparus* BGP gene and 5'-flanking region. The major site of transcription initiation is designated as +1 and the corresponding nucleotide is bold. Nucleotides are numbered in the left margin and the predicted amino acid sequence is shown above the coding sequence. The stop codon is indicated by asterisks and the polyadenylation signal is underlined twice. Consensus sequences at the intron borders are underlined and bold. Putative TATA and CCAAT motifs are boxed. The location of putative steroid-responsive elements (SRE) and transcription factor consensus sequences (OSE1, OSE2 and AP1) is indicated. Curved arrows mark the localization of the partial spBGP cDNA probe used in southern genomic hybridisation (Figure III-10B).

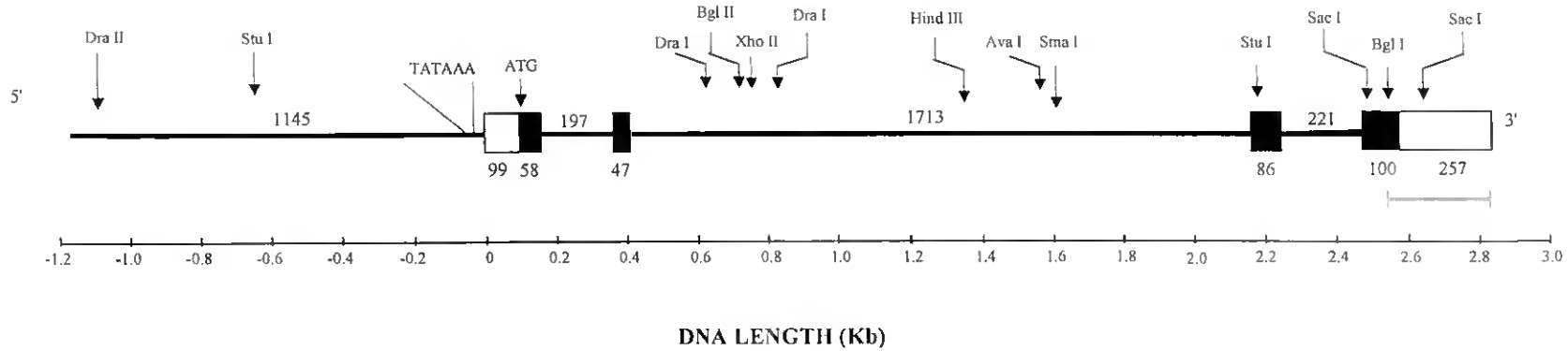


Figure III.9. Map of the *Sparus* BGP gene and 5'flanking region, showing the major restriction endonucleases sites and the position of exons (boxes) and introns (lines between exons). Sizes (in nucleotides) of coding (black boxes) and non-coding (white boxes) sequences are indicated underneath the corresponding exons. Sizes of the introns are also shown. Localization of the cDNA probe used for Genomic Southern B (Figure III-10B) is shown in grey. The gene begins with the Cap site of the first exon and ends at the site of insertion of the poly-A tail, 15 bp downstream from the polyadenylation site located in exon 4 (as deduced from the cDNA sequence).

5. HOW MANY BGP GENES EXIST IN *Sparus aurata*?

Search for the presence of more than one BGP gene was performed through genomic southern analysis and genomic PCR. In each case, we obtained evidences for the presence of only one BGP gene within the *Sparus* genome.

Genomic Southern approach

Samples of *Sparus* genomic DNA were digested with restriction enzymes which, according to our sequence, should cut within the *Sparus* BGP gene (*Bgl*I, *Bgl*II and *Hind*III; Figure III-9) or in its flanking DNA (*EcoR*I and *Pst*I), and analyzed by Southern hybridization using a specific probe spanning the full length spBGP cDNA. The positive signals observed for each restriction enzyme digestion (Figure III-10A) corresponded to the expected number of fragments based on the known restriction map of the spBGP gene (Figures III-8 and III-9).

A second genomic southern (using DNA digested with *Hind*III, *BamH*I, *Pst*I and *Bgl*I) was hybridized with a partial spBGP cDNA (spanning from nucleotide 322 to 602; see Figure III-8 for localization). This clone was expected, from the known restriction map of the *Sparus* BGP gene, to hybridize only with the genomic restriction fragments located at the 3'end of the spBGP gene. The results obtained showed only the one expected fragment for each enzyme digestion (Figure III-10B), with the exception of *Bgl*I. In this case one site was located within the genomic DNA covered by the probe used (spBGP cDNA spanning from bp 322 to the 3'end of the cDNA; see Figures III-3 and III-8), and therefore two genomic fragments should have hybridized to this probe. However, since this *Bgl*I site was located 43 bp from the 5' end of the probe used (Figure III-9), the second genomic fragment (the top *Bgl*I fragment seen in Figure III-10A) would give only a very weak positive signal and was not detected in the autoradiography.

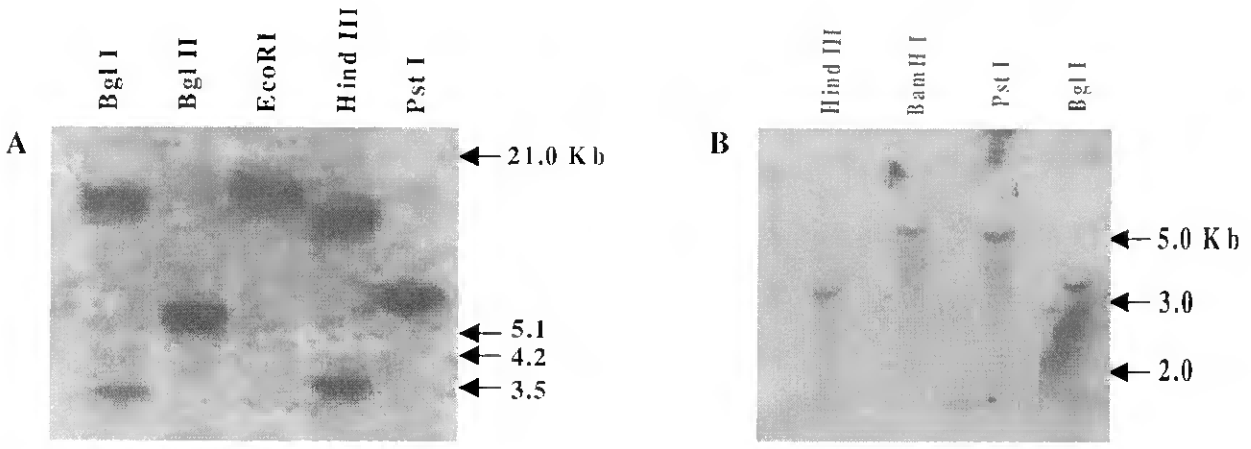


Figure III-10. Analysis of the *Sparus* BGP gene locus by Southern hybridization. Following restriction enzyme digestion with *Bgl*I, *Bgl*II, *EcoR*I, *Hind*III and *Pst*I (panel A) or with *Hind*III, *BamH*I, *Pst*I and *Bgl*I (panel B), genomic DNA samples were electrophoresed on a 0.8% agarose gel (Promega), transferred to a nylon membrane (Nytran+) and hybridized with either the complete (panel A) or a partial (panel B) spBGP cDNA, as referred in section 3.1 of Chapter II. Results from the autoradiography are shown. Sizes of the DNA markers are indicated on the right margin.

6. SEARCH FOR MORE THAN ONE spBGP mRNA

To analyse the possibility of the existence of more than one transcript of the spBGP gene in different tissues or phases of development, spBGP mRNA obtained from several adult *Sparus* tissues and from two stages of development (before and after calcification: 27 and 130 dph, respectively) was used to amplify the spBGP cDNA by RT-PCR, using two primer sets covering distinct intervals in the spBGP cDNA (SBG5F+SBG11R and SBG12F+SBG4R; Table II.I and Figure III-3). Following PCR reaction, DNA fragments were cloned and identified by sequence analysis, revealing the presence in two independent clones of a deletion in the 3' UTR region (Figure III-11A). Specific primers were constructed (see Figure III-11A for primer location) in order to determine if these clones represented a true cDNA entity or were the result of an artifact. RT-PCR amplification of spBGP mRNA from different tissues [brain, muscle, heart (not shown), kidney and vertebra (Figure III-12)]

and developmental stages [18, 37 and 130 dph (Figure III-12)] was performed according to the procedure detailed in section 2 of the Methods chapter. A positive control for the PCR reaction was performed by co-amplification of a spBGP genomic clone with the SBG8F+SBG11R primer pair (lane BGP in Figure III-12). The resulting products were size fractionated by electrophoresis over an ethidium bromide-containing 1.4% agarose gel (Promega) and observed in a U.V. transilluminator (Figure III-12). A positive signal was observed only when amplification was performed with SBG8F+SBG11R, i.e., with primers designed to amplify DNA only in the absence of the deletion (-d). No amplification was obtained with primers designed to amplify DNA in the presence of the deletion (+d), either in the several tissues or in the developmental stages considered. No DNA amplification was observed in the negative controls (not shown). These results showed that this deletion is most probably a PCR artifact, possibly resulting from the formation of an hairpin due to the existence of a perfect inverted repeat in this region (Figure III-11B).

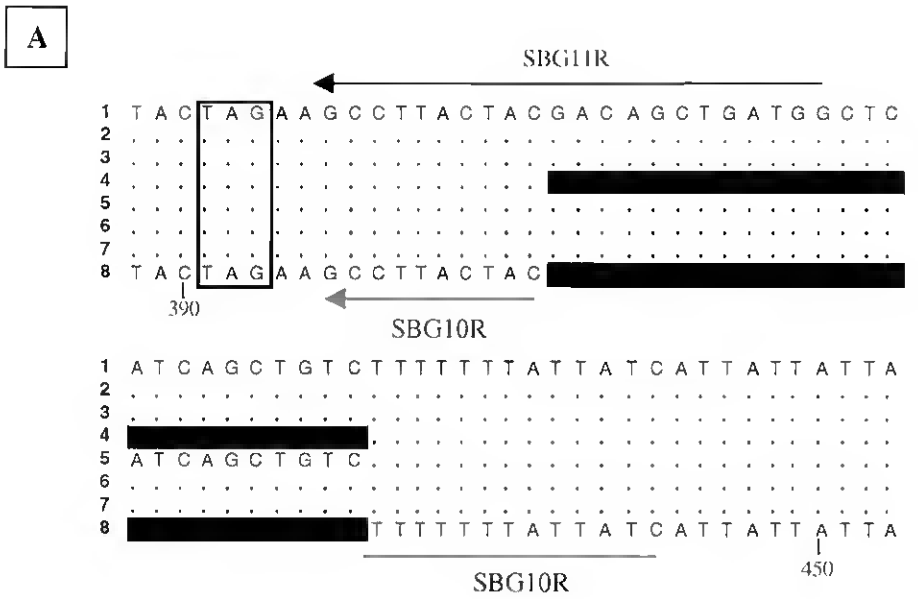


Figure III-11. Analysis of the deletion observed in clones obtained by RT-PCR. **A.** Partial nucleotide sequences of *Sparus* BGP cDNA from different clones obtained in independent experiments by RT-PCR (1 to 8). A box signals the stop codon TAG, dots stand for identical nucleotides in the different sequences and black bars signal a deletion in the sequence when compared to spBGP cDNA (Figure III-3). Reverse primers used in PCR amplification are signalled by arrows under and above the sequence. Numbering is shown under the sequence as in cDNA (Figure III-3). **B.** Representation of the putative loop structure formed within the site spanned by the deletion.

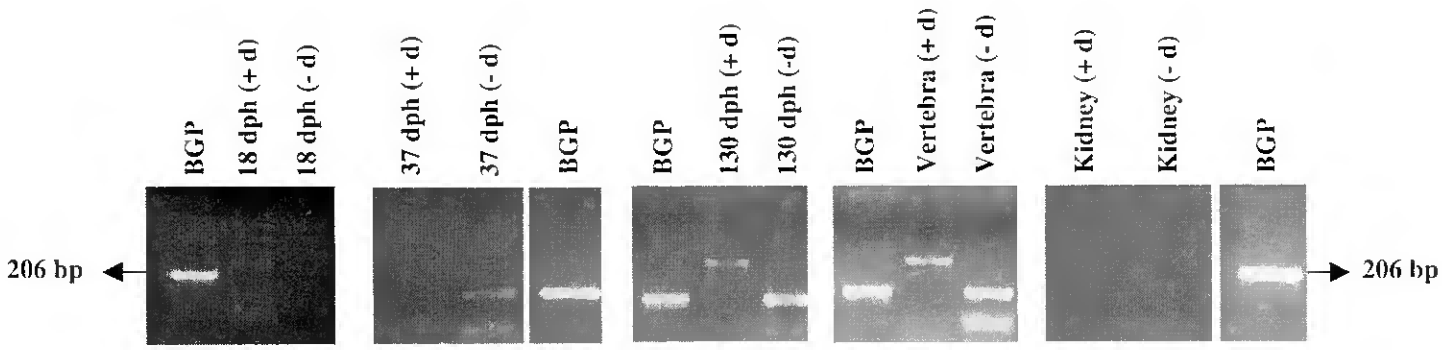


Figure III-12. RT-PCR amplification of RNA extracted from tissues (vertebra and kidney) and developmental stages (18, 37 and 130 dph) of *Sparus*, using primer pairs designed to test the existence of a deletion in the spBGP cDNA sequence (Figure III-11A). The primers used were SBG8F+SBG10R, for the presence of deletion (+d), and SBG8F+SBG11R for the absence of deletion (-d). A genomic spBGP clone (BGP lanes) was amplified with SBG8F+SBG11R for control of PCR reaction.

7. EXPRESSION OF THE spBGP GENE

7.1. TISSUE DISTRIBUTION

Northern Blot analysis

Figure III-13 depicts the result of the Northern blot analysis. Of the different tissues analyzed, only calcified tissues (vertebra and jaw) showed the presence of BGP mRNA, with the strongest signal observed in vertebra-derived RNA. No positive hybridization was seen in the other tissues analyzed (liver and heart, Figure III-13; kidney, muscle and brain, results not shown), even after a longer exposure of the membrane (up to two weeks).

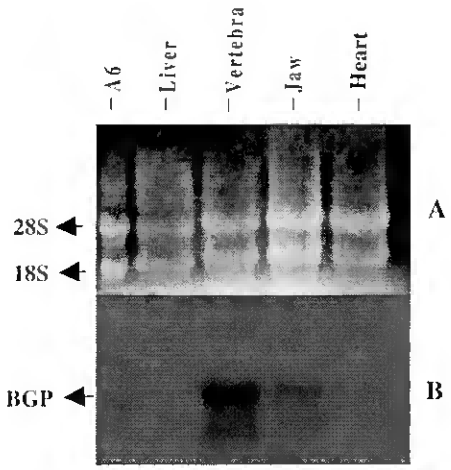


Figure III-13. Tissue distribution of spBGP mRNA by Northern analysis. Total RNA was extracted from several tissues (liver, vertebra, jaw and heart) of *Sparus*, size fractionated by denaturing agarose gel electrophoresis and transferred to a nylon membrane. RNA integrity was controlled by ethidium bromide staining of 28 and 18S ribosomal RNAs (panel **A**). Expression of spBGP was detected following hybridization with a ^{32}P-labelled spBGP cDNA (panel **B**). Total RNA from the *Xenopus laevis* cell line A6 was used as negative control.

RT-PCR Southern Blot analysis

RT-PCR amplification of spBGP from several tissues of *Sparus* produced diverse results according to the number of cycles used in the amplification (Figures III-14 and III-15). With 35 cycles (Figure III-14) BGP amplification was detected not only in mineralized tissues (vertebra and jaw), but also in heart, although with a lower intensity. When the number of cycles was reduced to 20 (Figure III-15), detection of spBGP message was

restricted to vertebra and jaw, no signal being present for muscle, heart or liver, even after a longer exposure (result not shown).

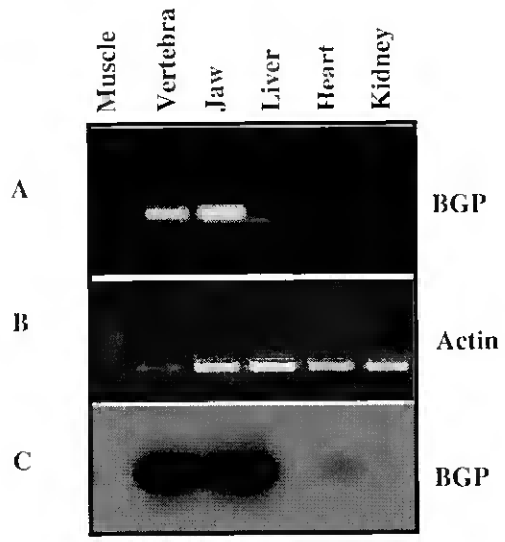


Figure III-14. Detection by RT-PCR (35 cycles) of BGP from several tissues of *Sparus*. Total RNA was extracted from tissues (Muscle, Vertebra, Jaw, Liver, Heart and Kidney) of *Sparus* and used to amplify spBGP (panel A) mRNA by RT-PCR with two specific primers (SBG8F and SBG4R); Figure III-3), followed by visualisation in agarose gel, as described. The resulting PCR products for spBGP were transferred to a nylon membrane and hybridized with a ^{32}P-labelled spBGP cDNA (panel C). The same RT reaction was used to amplify spActin as a positive control for the integrity and amount of mRNA (panel B)

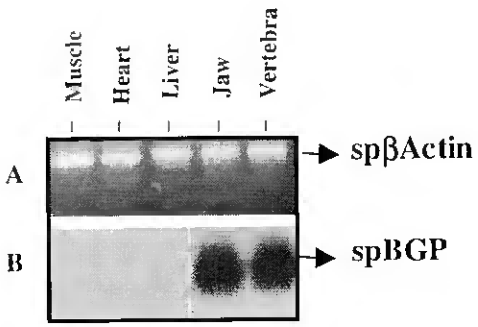


Figure III-15. Detection of BGP mRNA in *Sparus* tissues by RT-PCR (20 cycles), coupled with Southern hybridization. Total RNA was extracted from tissues (Muscle, Heart, Liver, Jaw and Vertebra) of *Sparus* and used to amplify spBGP mRNA by RT-PCR, using two specific primers (SBG8F and SBG4R; Figure III-3), as described. The resulting PCR products were size separated by agarose electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labelled spBGP cDNA (panel B). To check for RNA integrity, the same RT reactions were used to amplify spβActin mRNA, using two specific primers based on the published sequence (panel A).

7.2. DEVELOPMENTAL EXPRESSION

The expression of the BGP gene was analysed during the different stages of *Sparus* larvae and juvenile development by Northern analysis and RT-PCR. BGP gene expression was detected only in stages where calcification was apparent during *Sparus* development, as assessed by histological techniques (alizarin red staining).

Northern Blot analysis

Northern analysis (Figure III-16) showed that spBGP mRNA is abundant in fish larvae with 86 dph and older, which correspond to post-larval (juvenile) stages known to have a nearly fully mineralized skeleton (as detected by alcian blue/alizarin red staining). No BGP mRNA was detected at 27 dph with this method, a stage where calcification of the larvae skeleton is still restricted to some structures in the head region.

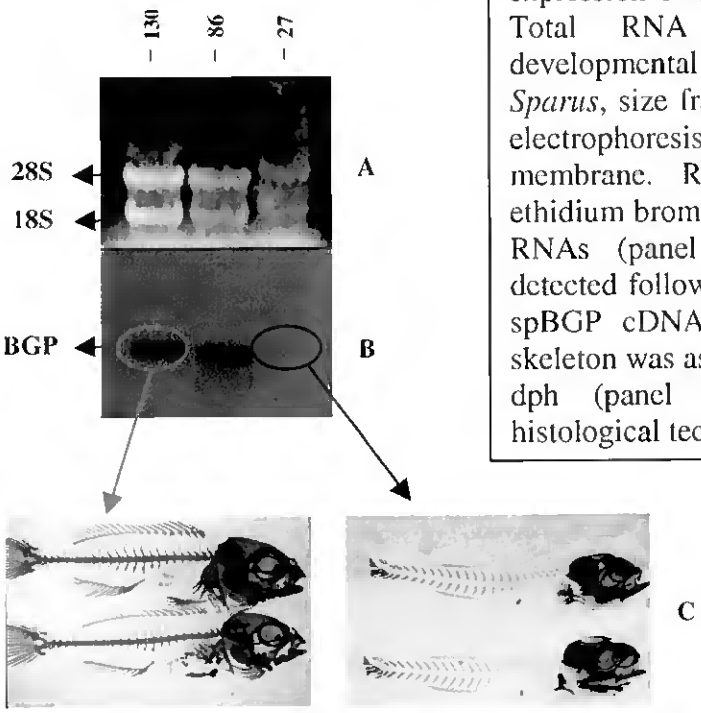


Figure III-16. Analysis of developmental expression of spBGP mRNA by Northern blotting. Total RNA was extracted from several developmental stages (27, 86 and 130 dph) of *Sparus*, size fractionated by denaturing agarose gel electrophoresis and transferred to a nylon membrane. RNA integrity was controlled by ethidium bromide staining of 28 and 18S ribosomal RNAs (panel A). Expression of spBGP was detected following hybridization with a ^{32}P-labelled spBGP cDNA (panel B). Mineralization of the skeleton was assessed in specimens with 27 and 130 dph (panel C) by alcian blue/alizarin red histological technique, as described.

RT-PCR Southern Blot analysis

RT-PCR amplification of spBGP from several developmental stages of *Sparus* produced, in general terms, the same results as those obtained by Northern blot analysis (Figure III-16). However, the lower limit of amplification was different when a different number of cycles was performed. In fact, with a 35 cycle amplification a signal for spBGP

can be observed since 18 dph (Figure III-17), whereas with 20 cycles onset of spBGP mRNA production was only observed at 37 dph (Figure III-18). The intensity of the amplified signal increased with age, with the strongest hybridization detected in the older (75 dph and above) specimens. No signal was observed, in either case, for stages younger than 18 dph, even after longer autoradiography exposure (not shown).

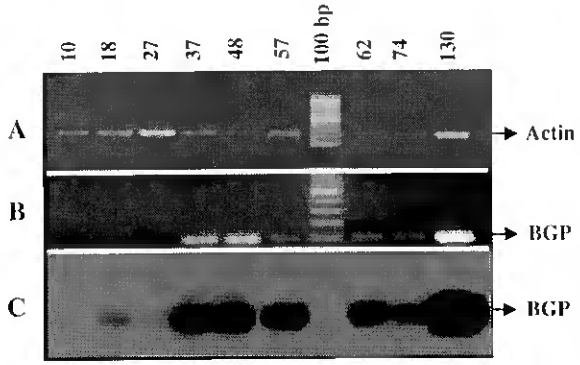


Figure III-17. Detection of BGP mRNA by RT-PCR (35 cycles) in *Sparus* developmental stages. Total RNA was extracted from whole Sparus specimens at different developmental stages (10 to 130 dph) and used to amplify spBGP mRNA by RT-PCR, as described in the Methods section. The resulting PCR products were size separated by ethidium bromide-stained agarose gel electrophoresis (panel B), or transferred to a nylon membrane and hybridized with a ^{32}P-labelled spBGP cDNA (panel C). To check for RNA integrity, the same RT reactions were used to amplify spβActin mRNA, using two specific primers based on the published sequence (panel A).

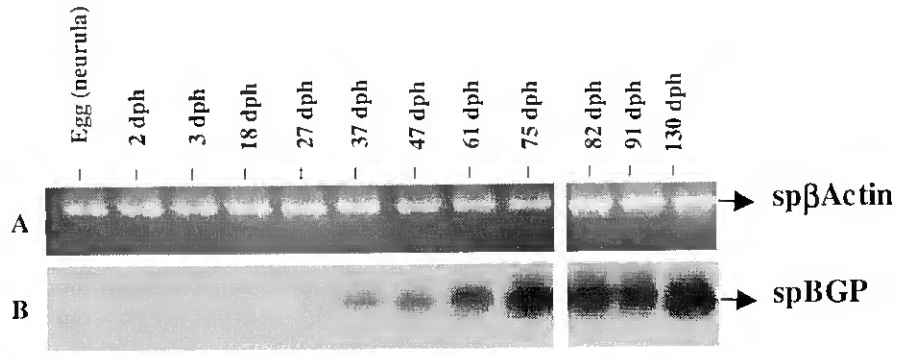


Figure III-18. Detection of BGP mRNA by RT-PCR (20 cycles) in developmental stages of *Sparus*. Total RNA was extracted from whole Sparus specimens at different developmental stages (neurula to 130 dph) and used to amplify spBGP mRNA by RT-PCR, using two specific primers (SBG8F and SBG4R; Figure III-3), as described in the Methods chapter. The resulting PCR products were size separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labelled spBGP cDNA (panel B). To check for RNA integrity, the same RT reactions were used to amplify spβActin mRNA using two specific primers based on the published sequence (panel A).

7.3. CORRELATION BETWEEN BGP APPEARANCE AND BONE DEVELOPMENT IN SPARUS

Detection of cartilaginous and calcified structures

Variables such as stock origin and culture conditions have a high impact on the early development of fish specimens (e.g., 179, 460). The objective of this part of the work was to obtain information about the cartilaginous/calcified status of the skeleton of the individuals used in this study. To accomplish that we have used two dyes: (a) alcian blue, specific for proteoglycans (∴ staining of cartilage) and alizarin red, specific for calcium (∴ staining of mineralized structures).

During embryonic development and immediately after birth *Sparus* specimens do not possess any calcified structures, and only some of the head bones present a cartilaginous nature (Figures III-19), as well as the branchial arches. The axial skeleton, in the absence of a rigid structure, is formed by the notochord (N in Figure III-19), which allows for a greater flexibility, although poorly efficient in terms of helping the locomotion abilities. The only other cartilaginous structure in the axial skeleton are the hypural 1 and a small ventral cartilaginous structure, the coracoid-scapula complex (CO-SCA). Although not visible in Figure III-19, the cleitrum, the first calcified structure, is already present at this stage.

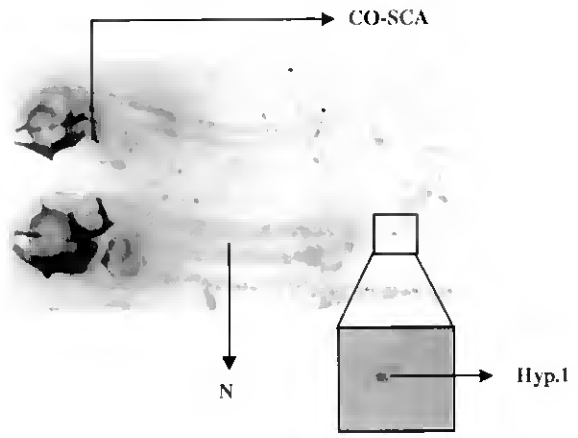


Figure III-19. Alcian blue/alizarin red staining of 15 days (4.1-5.1 mm) *Sparus* larvae (20x). Cartilaginous structures are stained in blue. **N** signals the notochord, the coracoid-scapular complex is denoted by **CO-SCA** and the box shows a magnification of the hypural 1 (Hyp.1).

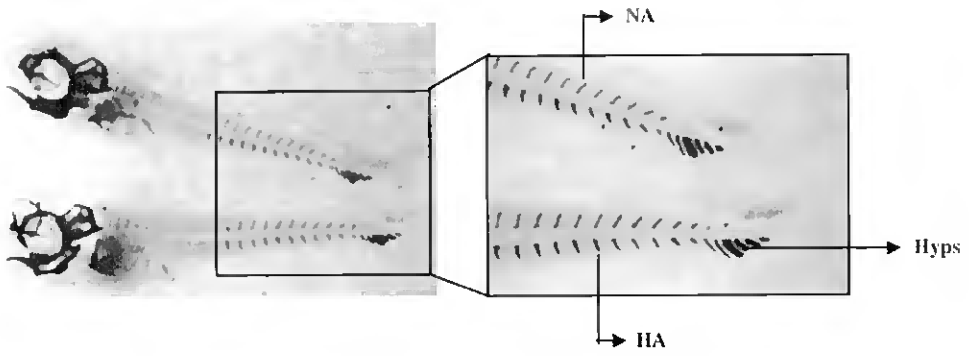


Figure III-20. Alcian blue/alizarin red staining of 20 dph (6.0 mm) *Sparus* specimens (16.5 x). Appearance of neural (**NA**) and hemal (**HA**) arches can be observed, as well as an increase in the number of hypurals (**Hyps**) and cartilaginous structures of the head (in blue). The box shows a magnification (31 x) of the posterior halfs of the two specimens.

At 20 dph (~6.0 mm) the *Sparus* larvae show an increase in the number of cartilaginous structures, relatively to the previous state (Figure III-20). We can see that most neural (NA) and hemal (HA) cartilaginous arches are formed, their appearance ocurring in the antero-posterior (neural) or postero-anterior (haemal) sense (150). Also, an increase in the number of hypurals (Hyps) and of cartilaginous structures of the head can be observed.

In the individuals with 27 dph (6.2-6.6 mm) we can notice the onset of calcification, demonstrated by the red-stained structures in Figure III-20. Calcification begins by the structures that are, presumably, more important for an efficient exogenous feeding, which are the jaw (J; efficient seizing of preys), the caudal soft rays (CR; rapid swimming) and the branchial arches (BA; increased respiratory activity → more active metabolism). No other structures are visibly calcified at this stage. It is noteworthy the appearance of the dorsal (DPT) and anal (APT) pteriogophores, structures that support, respectively, the dorsal and anal fins. As can be observed in Figure III-21, 12 dorsal pteriogophores exist between the 11^{th} and the 17^{th} neural spines, whereas 11 ventral pteriogophores are situated between the 2^{nd} and the 7^{th} haemal spines. Appearance of the remnant pteriogophores takes place in the anterior and posterior senses, starting from the primordial group [this study and (150)].

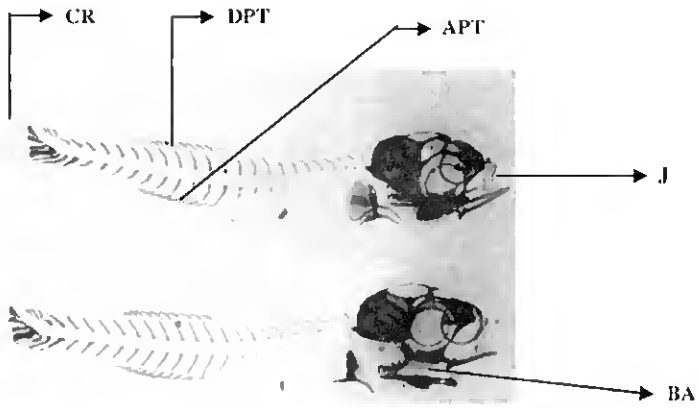


Figure III-21. Alcian blue/alizarin red staining of 27 dph (6.2 - 6.6 mm) *Sparus* larvae (16 x). Onset of ossification is noticeable by the red staining in the jaw (**J**), in the caudal rays (**CR**) and in the branchial arches (**BA**). Previously undetected cartilaginous dorsal pterigiophores (**DPT**) and anal pterigiophores (**APT**) are signalled.

At 35 dph (6.7-8.8 mm) more calcified structures are identifiable. Besides the previously observed, calcification begins to occur in the vertebral centra (Figure III-22). As observed with the appearance of cartilaginous neural arches, calcification of the centra occurs in an anterior-posterior sense, beginning in the 2nd centra and progressing in the posterior sense. The first centra only seems to ossify after ossification of the 6th/7th centra (326). Also conspicuous is the presence of organized pectoral fins and of the basipterigium (BT), although still in a cartilaginous state. Calcified soft rays are still absent but structures such as the four actinosts (ACT) and the distal radials (DR) are obvious.

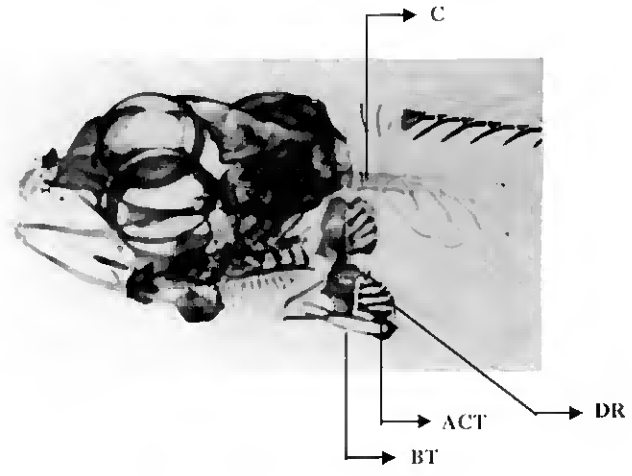


Figure III-22. Anterior half of an alcian blue/alizarin red-stained *Sparus* specimen with 35 dph (6.7-8.8 mm; 17x). Ossification is observable in the jaw and in the vertebral centra (**C**). Previously undetected cartilaginous actinous (ACT), basipterigium (BT) and pectoral distal rays (DR) are signalled.

At 72 dph (11-14 mm), ossification has progressed into most of the previously soft and cartilaginous squeletal tissues (Figure III-23). The urostyle (U; the 24th centrum) is already completely calcified as are most of the neural and haemal arches. As can be observed in Figure III-23C (arrow), this calcification proceeds in a proximal-distal sense, starting from an ossification centre (149). Also calcified are the dorsal and ventral soft rays, the caudal soft rays (CFSR) and the spines (not shown). The calcified soft rays of the pectoral fins (PFSR) are present, the same occurring with the soft rays of all other fins. Still in the pectoral fin, we can observe ossification in progress in the actinosts, which, according to Faustino (149) occurs in the dorso-ventral sense. The pelvic fin soft rays (PeFSR) are completely calcified and the onset of ossification is noticeable in the basipterigium. Some structures remain, however, in its former cartilaginous state, namely the epurals (EP), the acessory cartilages (AC), the proximal ends of the caudal soft rays, the distal rays of the pectoral fins and the dorsal and ventral pteriogophores, which are all already present.

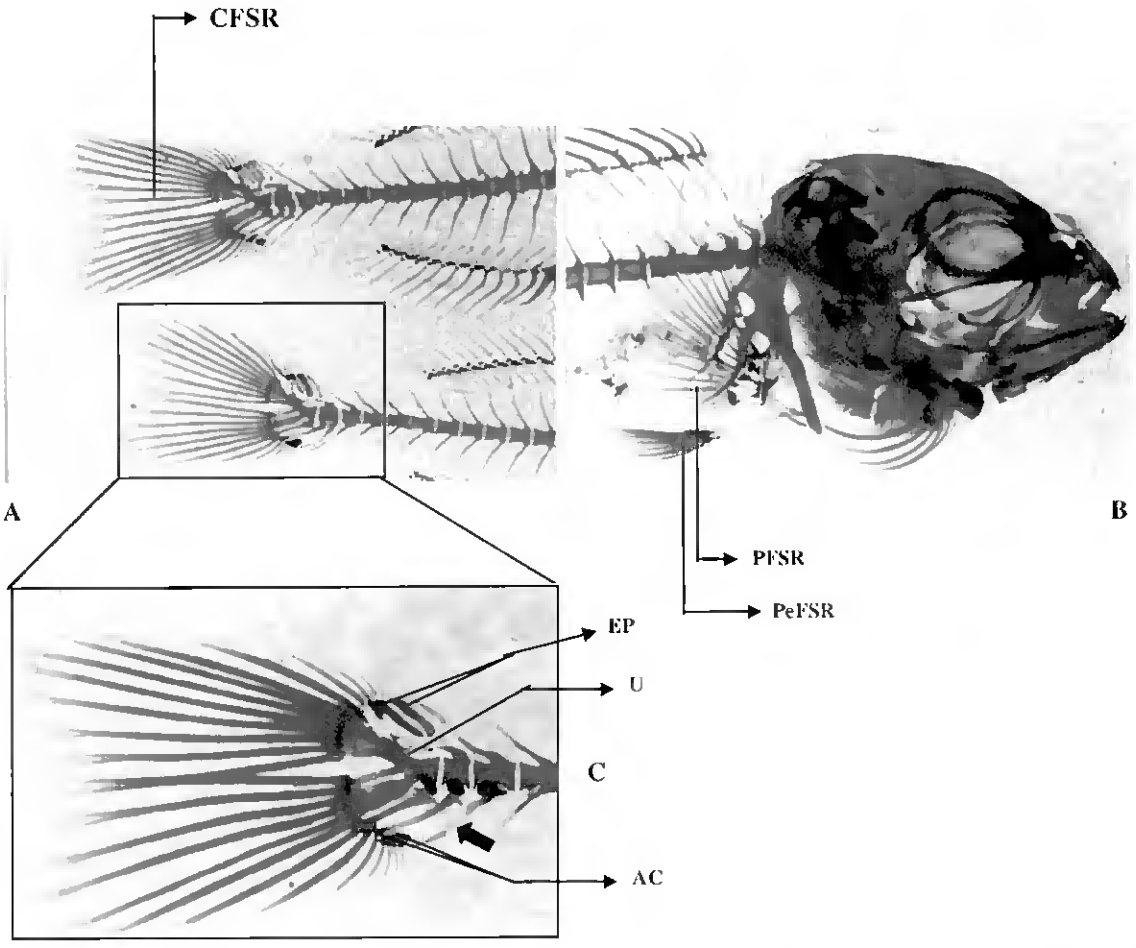


Figure III-23. Alcian blue/alizarin red staining of 72 dph (11-14 mm) *Sparus* juveniles. A- posterior half (6 x); B- Anterior half (14 x); C- magnification (22.5 x) of the caudal fin of one of the specimens displayed in A. Most skeletal structures show ossification (red staining), only a small number of structures remaining in the cartilaginous state. **PFSR**: pectoral fin soft ray; **PeFSR**: pelvic fin soft ray; **CFSR**: caudal fin soft ray; **EP**: epurals; **U**: urostyle; **AC**: acessory cartilages.

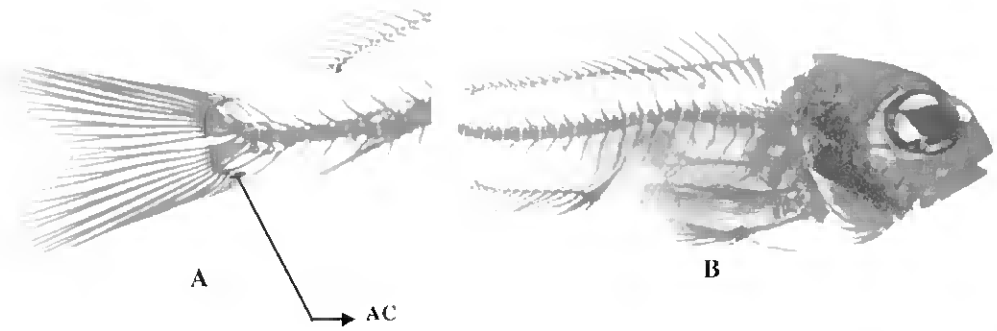


Figure III-24. Alcian blue / alizarin red staining of 110 dph (19-22 mm) *Sparus* juveniles. A- posterior half (9.5 x); B- anterior half (6 x). Almost all skeletal structures are ossified (red), only some cartilaginous structures (blue) remaining. **AC**: acessory cartilage.

At 110 dph (19-22 mm), ossification is nearly complete (Figure III-24). All the fin soft rays, the vertebra, almost every bone from the head and the jaw are ossified. The sole structures that remain cartilaginous are the acessory cartilage (AC), the pectoral distal radials, the proximal ends of the caudal soft rays and the last ventral and dorsal pteriogophores.

The oldest specimens analysed (150 dph; 24-27 mm) showed an almost complete ossification of the skeletal structures (Figure III-25), the only remaining skeletal structures unossified being the accessory cartilage, the pectoral distal radials and some of the head bones.

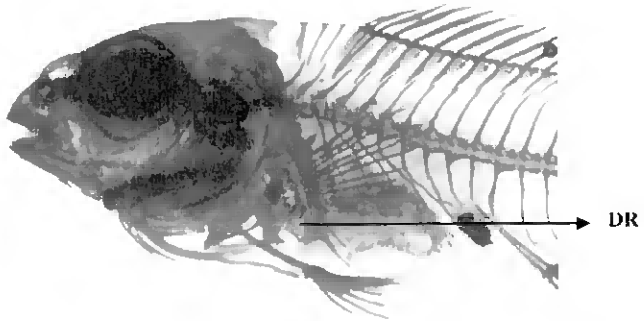


Figure III-25. Alcian blue / alizarin red staining of 150 dph (24-27 mm; 6 x) *Sparus* juvenile (anterior end). **DR**: pectoral fin distal rays.

Detection of BGP mRNA by *in situ* hybridization

Localization of specific sites of expression of the BGP gene was determined by *in situ* hybridization in *Sparus* sagital sections, using an antisense riboprobe specific to spBGP mRNA. *Sparus* BGP mRNA was detected (Figure III-26; arrows) in vertebrae (A1 and A2), jaw (not shown), sites of fin insertion (not shown) and dermis (A3). Hybridization of *Sparus* sections with a sense spBGP riboprobe, which functioned as negative control, produced no signal (A4).

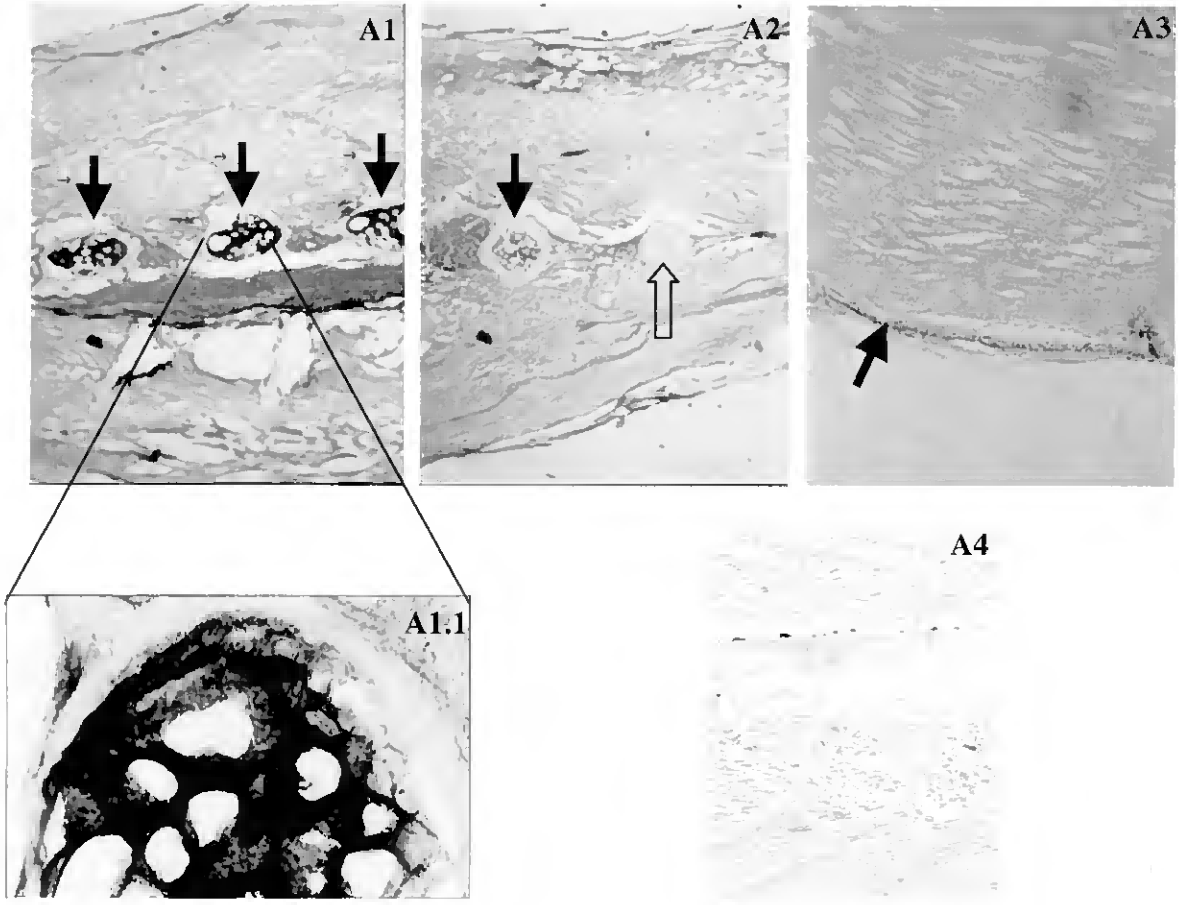


Figure III-26. Localization of spBGP mRNA in *Sparus* tissues by *in situ* hybridization. Longitudinal 5 µm sections of whole undecalcified 90 dph *Sparus* were hybridized with an antisense digoxigenin-labelled spBGP riboprobe and positive hybridization revealed as described in Chapter II. *Sparus* BGP mRNA was detected (black arrows) in vertebrae (A1 and A2) and dermis (A3), while distal vertebrae showed less or no hybridization (black and white arrows, respectively, in panel A2). Hybridization with sense digoxigenin-labelled spBGP probe produced no visible signal (A4).

8. STUDY OF THE FUNCTIONALITY OF THE spBGP PROMOTER IN *S. AURATA* BONE-DERIVED CELLS

8.1. OBTENTION OF PRIMARY CELL CULTURES DERIVED FROM Sparus TISSUES

Primary cell culture derived from vertebra, jaw and branchial arches

Significant migration of cells from jaw and branchial arches ocurred after 48-96 hours, whereas vertebra started to release cells usually more than one week after the beginning of incubation (Figure III-27).

The behaviour of cells in culture conditions was different according to their respective origin (vertebra-, jaw- or branchial arches-derived). The vertebra-derived cells seemed to adapt best to culture conditions, with a doubling time of 2-3 days, and without visible phenotype changes for the first 4-6 weeks (Figure III-28). After this period, differentiation (phenotype changing, division rate slowing/arresting) was observed. These cells were able to be preserved frozen in liquid nitrogen with a reasonable survival rate after thawing (> 60%). When these cells were maintained in the same dishes for approximately 30 days, with regular medium changes, some differentiation was observed, with development of some tissue-like structures (Figure III-29A, B).

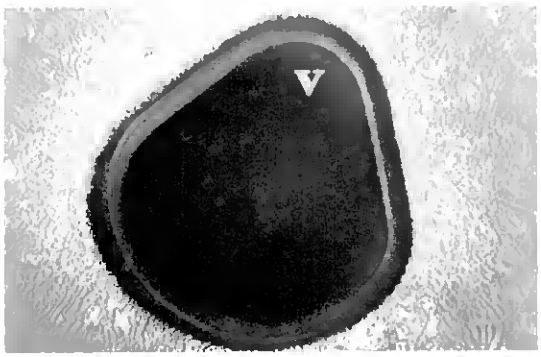


Figure III-27. Significant migration of cells from *Sparus* vertebrae (V) was observed only after one week incubation. Amplification 100x.

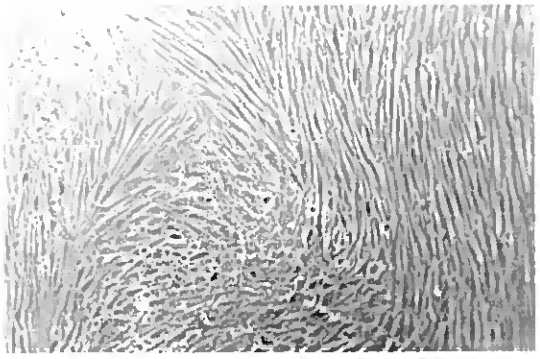


Figure III-28. Phenotype of *Sparus* vertebra-derived cells in active growing state. Amplification 200x.

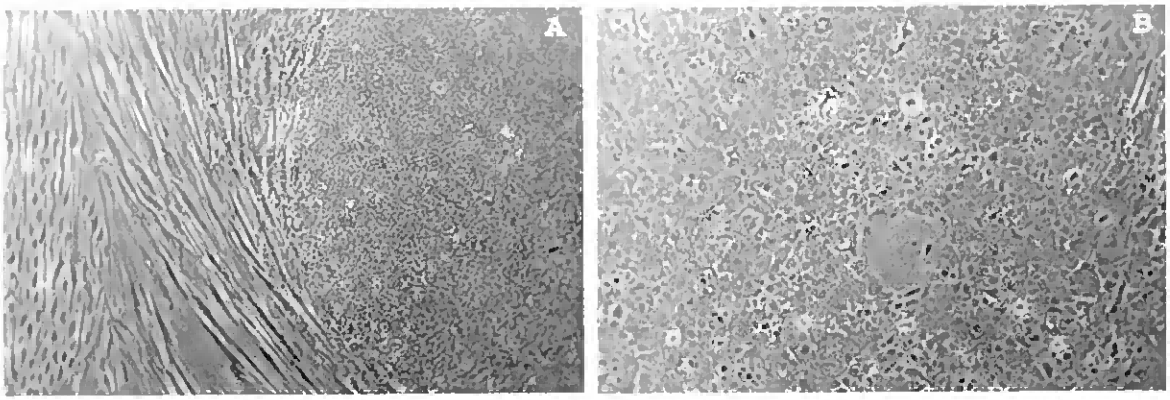


Figure III-29. *Sparus* vertebrae-derived cells kept without passage for approximately 30 days experienced differentiation, originating a tissue-like structure (A and B). Amplification 200x.

In comparison with vertebrae-derived cells, jaw- and branchial arches-derived cells have a very distinct behaviour. They migrate rapidly from the tissue fragments and withstand the freezing procedure, but do not endure as many tripsinizations as do vertebra-derived cells. After no more than 10 passages they substantially decrease their division rate, under these culture conditions.

Primary cell culture obtained from scales of *S. aurata*

Significant migration of cells from scales ocurred few hours after the beginning of incubation of *Sparus* scales in medium I (Figure III-30). However, they rapidly begin to change phenotype (Figure III-31), hindering any attempt to trypsinize them, since most of them do not survive the procedure, and those who do are unable to divide.

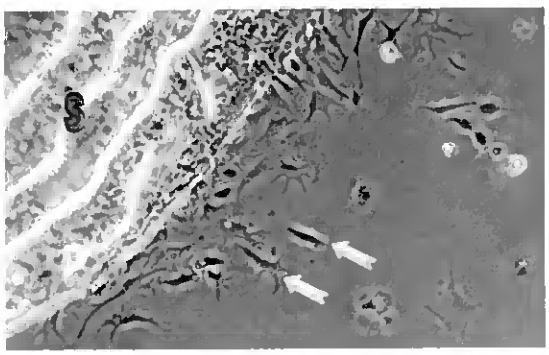


Figure III-30. Migration of cells (arrows) from *Sparus* scales (S) ocurres a few hours after beginning of incubation. Amplification 200x.

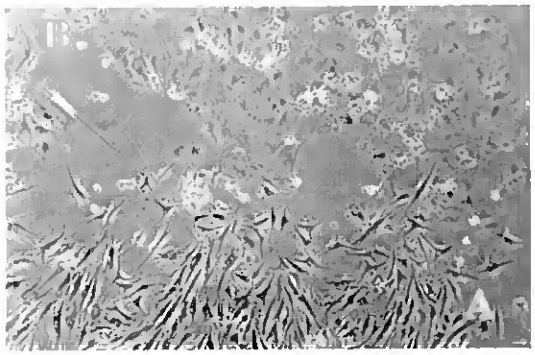


Figure III-31. A few days after migration from scales, cells begin to differentiate (A → B). Amplification 200x.

Sparus bone- and cartilage-derived primary cell cultures survive the freezing procedure

With the exception of cells derived from *Sparus* scales, which, with the present culture conditions, rapidly differentiate, do not divide and, perhaps for that reason, are unable to survive the freezing procedure, vertebra-, jaw- and branchial arches-derived cells remain viable after being frozen for several months in liquid N_2, with an high survival rate. According to our experience, they show an high sensitivity to DMSO, demanding a rapid change of media after thawing.

Sparus vertebra- and jaw-derived cells produce BGP mRNA

RT-PCR amplification of spBGP transcripts from total RNA extracted from primary cultures of cells obtained from vertebra and jaw produced a putative spBGP DNA fragment of the expected size. Sequence analysis revealed that these bands corresponded to spBGP. Therefore, both vertebra- and jaw-derived (i.e., bony tissue-derived) cells produce BGP mRNA, although the signal could only be detected by RT-PCR and was not strong enough to be seen in Northern hybridization (results not shown).

Sparus vertebra-derived cells produce alkaline phosphatase

Detection of alkaline phosphatase activity by the Naphtol/Fast red violet method showed that this protein was present in some of the cells derived from vertebra of *Sparus* (Figure III-33). Also, these red-staining cells showed a distinct phenotype, with a variable, irregular form, contrasting with the more regular, non-staining cells.

Figure III-32. Detection of alkaline phosphatase activity in *Sparus* vertebra-derived cells of *Sparus*. Red-staining cells (square) show alkaline phosphatase activity, a feature distinctive of osteoblasts. Asterisks signal non-staining cells. Amplification 200x.

Cells derived from *Sparus* vertebra produce mineralized structures

Sparus vertebra-derived cells maintained in confluent culture for 30 days and treated with ascorbic acid and β-glycerophosphate formed a distinct "hill and valley" morphology, with cell retraction from some areas and coalescence into multicellular foci, or nodules, in other areas (Figures III-34 to 36). Calcification was found to be associated with the nodules by von Kossa and Alizarin Red -S staining (Figures III-34 and III-35, respectively).

In addition to mineralized structures, vertebra-derived cells produced other structures (Figure III-36A), which do not stain with either of the two methods used to detect mineralization. These structures were also observed in jaw- and branchial arches-derived cells (Figure III-36B and C, respectively). None of these structures, either mineralized or non-mineralized, were observed in negative controls (Figure III-37).

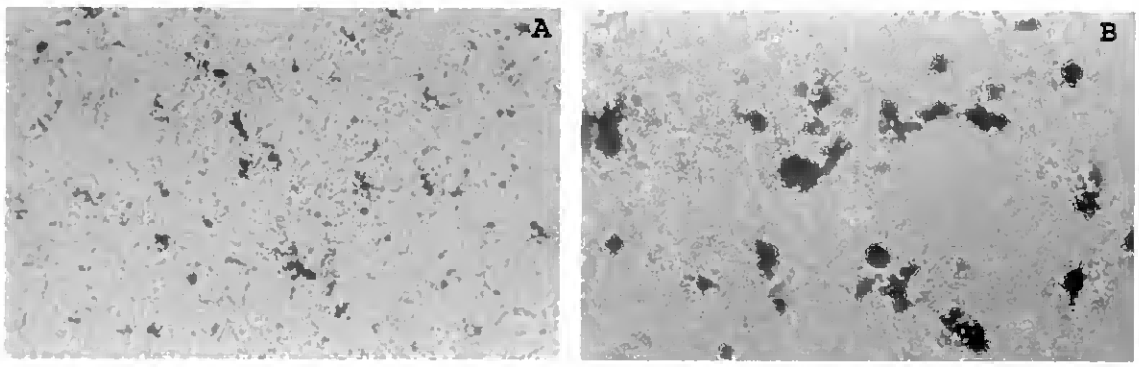


Figure III-33. Von Kossa staining of mineralized structures (in dark brown) in *Sparus* vertebra-derived cells incubated for 30 days in Medium I supplemented with 10mM β-Glycerophosphate and 50 µg/ml Ascorbic acid. Amplification 200x.

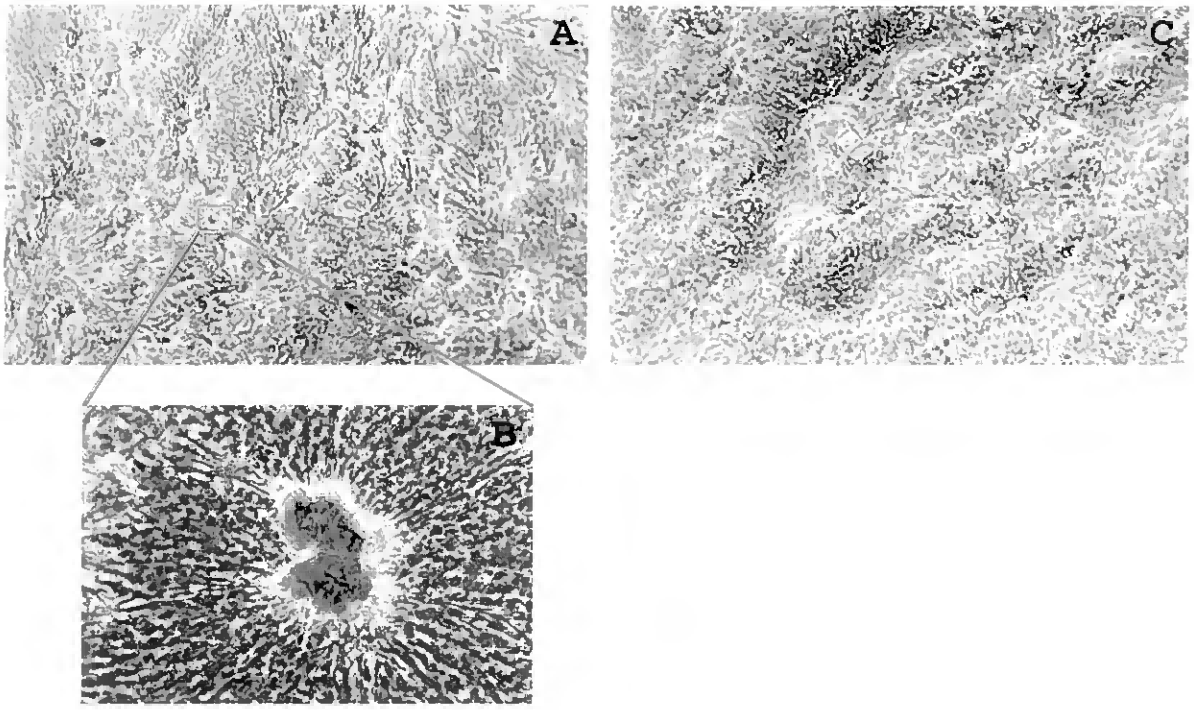


Figure III-34. Staining of mineralized nodules produced by *Sparus* vertebra-derived cells with alizarin red S. A) General aspect of cultures after 30 days incubation with "mineralization medium", with mineralized nodules (red) clearly visible; amplification 100x. B) Magnification of a stained nodule; amplification 500x. C) Some of the nodules were poorly mineralized, staining weakly in red; amplification 320x.

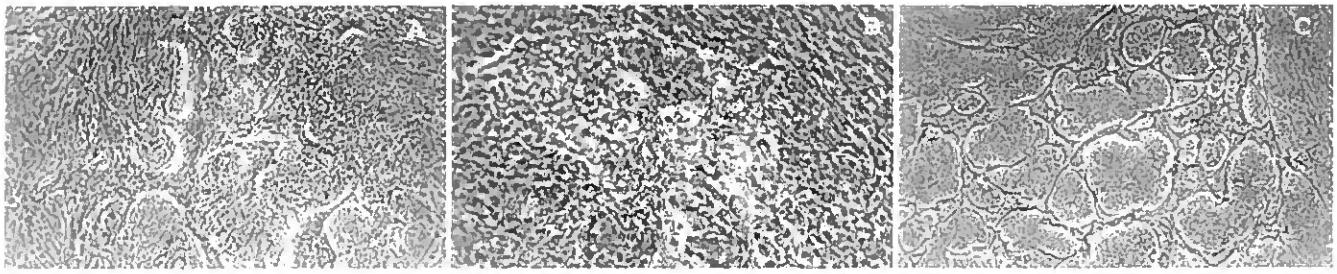


Figure III-35. Phenotype of *Sparus*-derived cells of different origins cultured in mineralising medium. Structures distinct from the mineralized nodules were observable after 15 days of incubation with L15 supplemented with ascorbic acid and β-glycerophosphate, spreading to the entire culture after 30 days, either in vertebra- (A), jaw- (B) or branchial arches-derived cells (C). Amplification 100x.

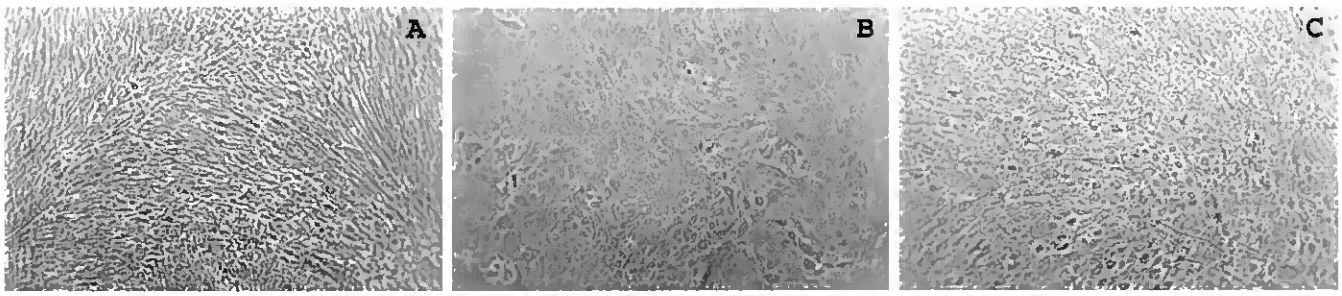


Figure III-36. Von Kossa staining of *Sparus* cells derived from vertebra (A), jaw (B) and branchial arches (C) detects no signs of mineralization when cells are incubated in Medium I, at 22°C, for 30 days. Amplification 100x.

8.2. IS THE spBGP 5´ FLANKING REGION ABLE TO FUNCTION AS A PROMOTER IN VITRO?

To determine if the 5' flanking DNA of the spBGP gene could function as a regular promoter and drive expression of a reporter gene in *Sparus* bone-derived cells we have cloned the entire 5' flanking sequence (-1145 to +37) of the spBGP gene (see Figure III-7) in the Multiple Cloning Site (MCS) of the pβgal-Basic expression vector, upstream from a *lacZ* reporter gene. The expression of this gene produces the β-galactosidase enzyme, capable of degrading the X-Gal substrate (in which we incubate our transfected cells), giving rise to a blue-greenish compound that accumulates inside the cells, which permits to assess the functionality of our putative promoter sequence and identify the transfected cells.

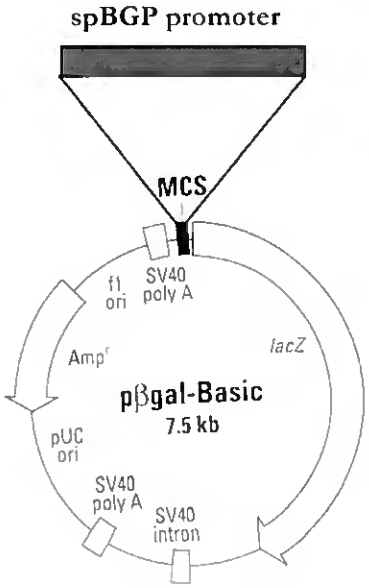


Figure III-37. Schematic representation of the cloning of the spBGP gene 5' flanking region in the multiple cloning site (MCS) of the pβgal-Basic reporter vector. Also signaled are the gene for ampicillin resistance (Amp^r), the *lacZ* gene, and the f1 and pUC sites for origin of replication; GenBank accession number U13184.

Visual assessment of transfection efficiency revealed that less than 1% of the cells were transfected. A small number of cells, however, was stained in blue (Figure III-39A and B), proving the ability of the putative spBGP promoter to induce transcription of reporter genes in these cells. Transfection with positive control (β-galactosidase reporter gene under the control of pCMV promoter) registered a higher efficiency. Endogenous β-galactosidase was controlled by transfection with β-galactosidade reporter gene alone, which did not produce any blue stained cells (not shown).

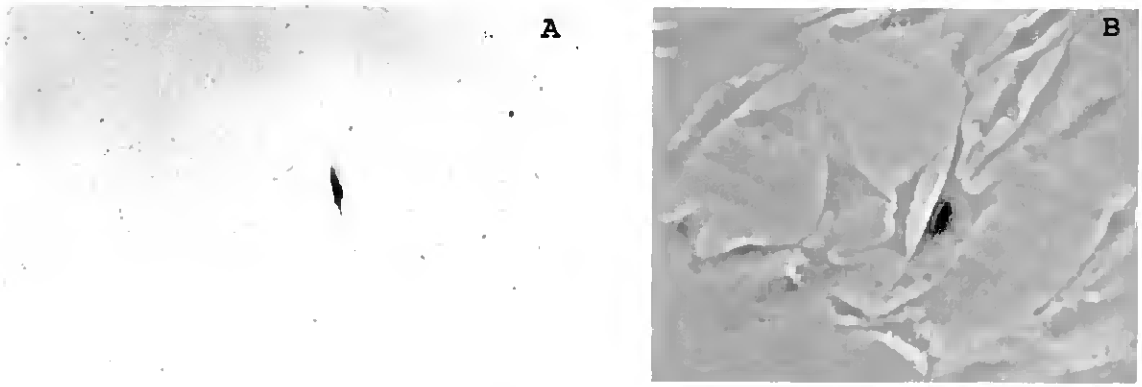


Figure III-38. Transient transfection of bone-derived primary cell cultures of *S. aurata* with spBGPpromoter/pßGal construct. Cells were transiently transfected by lipofection with either the spBGPprom/pßGal construct or the pßGal vector as negative control. Activation of the reporter gene, detected by exposure to an X-Gal containing solution, resulted in a number of blue stained cells. A-amplification 200x; B- amplification 320x.

9. *Sparus aurata* BGP AND OTHER VITAMIN K-DEPENDENT PROTEINS: AN EVOLUTIONARY PERSPECTIVE

9.1. CLONING OF A PARTIAL Halobatrachus didactylus (TOAD FISH) BGP cDNA

Amplification of a putative toad fish partial cDNA was performed by RT-PCR using total RNA from toad fish vertebra, as described in the Methods chapter. Electrophoresis of 15 µl of the RT-PCR product produced a major, clearly visible, band (lane A in Figure III-39). Several identical clones were obtained, following excision of this band from the gel and

cloning of the corresponding PCR product. Three of them (tf1, tf4 and tf5) were sequenced, revealing the same sequence of DNA which, after comparison with the NCBI database, showed to be a novel BGP sequence corresponding to a partial toad fish BGP (tfBGP) cDNA. This DNA fragment contained a putative coding sequence of 69 nucleotides, followed by a stop codon and an additional 216 bp to the site of insertion of the poly-A tail (Figure III-40). This sequence was submitted to GenBank, being assigned the acession number AF144707.

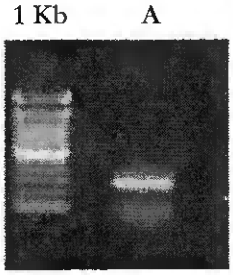


Figure III-39. RT-PCR amplification of a partial *Halobatrachus didactylus* BGP cDNA. Fifteen microliters of the RT-PCR product were electrophoresed in a 1.4% agarose gel and the major band (A) excised, cloned and sequenced. A 1 Kb DNA marker (GibcoBRL) was loaded in an adjacent well.

```
1                    SBG5F
                     ------------------>
Cys Asp Ala Met Met Asp Gln Glu Gly Ile Ile Val Ala Tyr Thr Leu
TGT GAC GCA ATG ATG GAT CAG GAG GGA ATC ATC GTC GCC TAC ACG CTT

49
Phe Tyr Gly Pro Ile Pro Phe ***
TTT TAC GGG CCG ATT CCC TTT TAGaccgcaaaaccgcaacaaatggttgaattgac


105
ctttcgtgacctttacctggtgctgttatcatttattgcatctcctttcctgcagtgaagggt

168
gtgtgcccgtaactgtcatgtgaaggaggcaaacaagcgaattaaattttctatctctgagac

231
tttactttccgttcattctgtagtgtagtgtgataaatgtcaataaaatgaatctgtc
```

Figure III-40. Partial *Halobatrachus didactylus* BGP cDNA and deduced amino acid sequence of corresponding polypeptide. Numerical positions in the nucleotide sequence are notated above each line. The stop codon following the protein coding region is indicated by three asterisks and a box indicates the polyadenylation signal. Sequence corresponding to the region of annealing of the SBG5F oligonucleotide is in italics and signaled with an arrow.

9.2. EVOLUTIONARY ANALYSIS OF spBGP

The phylogenetic analysis performed using all known amino acid BGP, MGP, murine nephrocalcin, human coagulation factor II and hagfish thrombin sequences yielded thirty Maximum Parsimony (MP) trees, which were summarized using Strict Consensus (i.e., retaining only the clades that are common to all thirty MP trees). This can be considered to be the most conservative estimate of the evolutionary patterns. The Strict Consensus tree (Figure III-41) shows two clearly distinct clades assembling, respectively, BGPs *versus* MGPs (with the exception of shark MGP). Inside the BGP clade is notorious the clear separation between fish BGP and other BGPs, the same occurring with birds and amphibian BGPs.

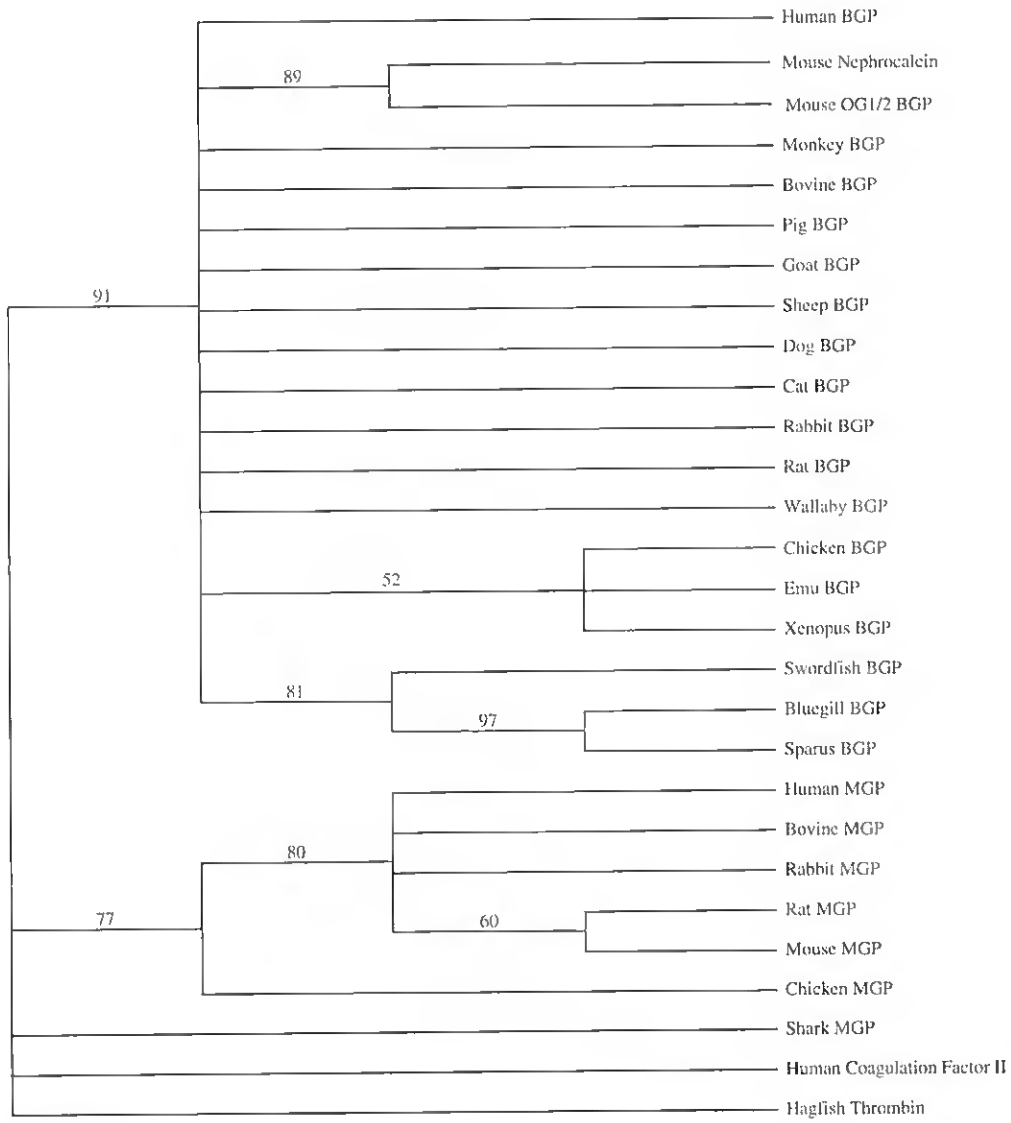


Figure III-41. Evolution of BGP in relation to other vitamin K dependent proteins. Strict consensus tree of the 30 Maximum Parcimony Trees generated with the available data on BGPs, MGPs, Human Coagulation Factor II and Pacific Hagfish Thrombin amino acid sequences. Numbers indicate confidence limits of individual clades, estimated by bootstrap analysis with 1000 replicates.

CHAPTER IV: **DISCUSSION**

1.THE *Sparus aurata* BGP cDNA

Molecular cloning of the *Sparus* BGP cDNA, by a mixture of RT-PCR amplification, RACE PCR and 5' primer extension, showed that the spBGP cDNA comprises 647 bp, numbered from the major cap site of the spBGP mRNA to the site of insertion of the poly(A) tail and encodes a polypeptide of 97 amino acid residues (Figure III-3). Based on its homology with the mammalian proteins, it contains a pre-region encoding the signal peptide and a pro-region whose sequence is homologous to the gamma carboxylase recognition site found in the pro-peptides of all other known BGPs (Table III-I and Figure III-5). The sequence of the mature form is in full agreement with the sequence of the mature BGP protein previously purified from *Sparus* bone (64). The proBGP sequence ends, as expected, with two basic residues, Lys-Arg, as in the chicken protein (371), and not Arg-Arg as in the mammalian BGPs (Figure III-3). Dibasic residues such as arginine are a common feature in pro-peptide sequences from proteins known to require proteolytic activation such as peptide hormones and clotting factors (e.g., 84). Both motifs are probably cleaved prior to secretion, as in higher vertebrates, to yield the mature spBGP.

2.THE *Sparus aurata* BGP GENE AND 5' FLANKING DNA

The spBGP gene encompasses 2778 bp, being approximately twice as long as the mammalian genes, mostly due to an unusually long intron II, ~10x larger than its mammalian counterpart (see Table III.III). Although the overall organization of the spBGP gene has been maintained from fish to man, the sites of insertion of the three introns within the protein coding sequence differ in *Sparus* as compared to the mammalian genes (Figures III-5 and III-9).

Identification of promoter regulatory elements that are responsive to basal and tissue-restricted transactivation factors, steroid hormones, and other physiological mediators, provides a basis for understanding regulatory mechanisms contributing to developmental expression of BGP, tissue specificity and biological activity. We have made an attempt to

identify several such motifs in the spBGP gene 5' flanking DNA sequence, by comparison with the published sequence motifs.

All known BGP gene promoters have a modular organization reflected by the presence of RNA polymerase II canonical sequences and of a series of consensus sequences for hormone receptor binding sites and nucleotide-responsive elements, which are physiological mediators of BGP gene expression (109, 295, 527, 572). A series of elements contributing to basal expression in mammalian BGP genes include a TATA sequence and osteocalcin box (OC box), a 24-nucleotide element with a CCAAT motif as a central core, both required for efficient transcription of the gene (227, 255, 530). Likewise, the spBGP gene promoter shows an organization with sequence motifs typical of a gene transcribed by the RNA polymerase II such as TATA and CCAAT boxes, but no clear OC box could be identified.

Sequences with similarities to the core OSE1 and OSE2 sequences (134) were identified, both in the 5'-flanking region and in intron II of the spBGP gene. In the mammalian system, the OSE1 sequence binds OSF1, an osteoblast-specific transcription factor present in nuclear extracts of osteoblastic cell lines and primary osteoblasts, thought to be required for the early steps of osteoblast differentiation (474). As for the OSE2, it binds Cbfa1/OSF2, a transcription factor which, in mammals, is specific to osteoblastic cells and is responsible for the tissue specificity of BGP through transcriptional control of expression (136, 137, 254). Both the mouse and rat genes contain, within their promoters, sites of interaction for these transcription factors (136, 241). Since the tissue distribution of BGP in *Sparus* (Figures III-13, III-15 and III-26) appears to be confined to mineralized tissues, it is likely that in fish, as in mammals, bone specific transcription factors could be involved in this tissue specificity. In this case, the putative OSE1 and OSE2 response elements found in the promoter of the spBGP gene could be functional.

Vitamin D, through its active metabolite 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] plays a key role in the transcriptional regulation of BGP gene expression in mammalian osteoblasts, both *in vivo* and *in vitro* (54, 86, 295, 398). Several VDREs have been characterized in the promoter region of vitamin D-regulated genes, including the rat (46, 110, 572) and human (145, 257, 395) BGP genes. Although there is considerable variation between natural VDREs, a consensus positive VDRE can be defined as a direct repeat (DR) of two six-base half elements of the sequence AGGTCA, separated by a spacer of three nucleotides (DR-3; Table I.III in Chapter I). This sequence directs the VDR-RXR

heterodimer to the promoter region of $1,25(OH)_2D_3$-regulated genes (68, 220, 322, 328). In contrast with its mammalian counterparts, the proximal promoter of the spBGP gene contains no obvious VDRE regulatory elements. Although fish are known to store vitamin D (520), and in some fish species vitamin D or its metabolites have been shown to affect plasma parameters such as calcium (516) and phosphorous (11) levels, no vitamin D responsive elements have been, as yet, identified in any fish gene. On the other hand, since even for relatively closely related species, such as human and rat, the conservation between regulatory elements can be low (395, 447), it may be the case that consensus sequences for regulatory elements are significantly different in fish gene promoters from those known in higher vertebrates.

Although vitamin D induces BGP expression in humans and rats *in vivo* and in rats and human-derived primary osteoblast cultures and osteoblast cell-lines *in vitro* (218, 293), a negative regulation of the BGP gene by vitamin D has been also described for mouse (86, 489, 573) and chicken (56), and preliminary results of *in vivo* treatments of *Sparus* with $1,25(OH)_2D_3$ seem to point in the same direction (Pinto, J.P. and Gavaia, P., unpublished). VDRE motifs involved in the vitamin D-induced repression of downstream genes seem to present a lower interspecific conservation in the core sequence, as can be concluded by examining Table I.III (Chapter I). Therefore, it is possible that failure to find a putative VDRE in the spBGP promoter sequence may be related with an inhibitory effect of vitamin D on the spBGP gene. Finally, the tripartite sequence found in intron II (Figure III-9) resembling the motifs identified in close proximity in the VDRE of the rat BGP gene (110, 332), leads us to hypothesize that not only a VDRE should be present in the spBGP gene, but also that intron II may have some regulatory role previously not advanced for other BGP genes. Work with deletion mutants from the spBGP gene promoter, gel mobility shift assays and DNase I footprinting analysis is important, in order to identify and characterize functional regulatory sequences within the fish BGP gene promoter, and is now being initiated.

3. *Sparus aurata* HAS ONE SINGLE BGP GENE?

Several lines of evidence resulting from our different approaches of the question of one/versus multiple spBGP genes, point in the direction of the existence of one single copy of this gene in *Sparus*. The results obtained with the hybridization of the genomic Southerns, either with a complete or a partial spBGP cDNA probe (Figures III-10 A and B, respectively), conform with the "single gene hypothesis", since we should expect different genes to have different sizes, most probably due to intronic differences, since these parts of the genes should be subjected to less evolutionary pressure than exons. We reached this same conclusion, i.e., that there seems to be no additional spBGP genes with different sized introns, with the RT-PCR approach I, since we were only able to amplify a single-size band corresponding to each of the three introns. All these three bands corresponded to the introns we already knew from previous sequencing.

An hypothesis we conceived was that if more than one spBGP message would exist it could be differentially tecidular and/or developmentally expressed. The strategy of amplification of an spBGP cDNA partial clone from RNAs extracted from several *Sparus* tissues and developmental stages (Figures III-12, III-14, III-15, III-17 and III-18) indicates that, at least for the region of the cDNA covered by the SBG5F+SBG4R and SBG8F+SBG11R primer pairs there are no alternative messages.

The appearance of a deletion in the spBGP cDNA sequence of two of the clones obtained when trying to obtain the 5'-end of the spBGP cDNA (Figure III-11A) could also result from the existence of an additional message for the spBGP. However, its presence could not be confirmed by RT-PCR amplification in any of the tissues or developmental stages analysed by us (Figure III-12). After sequence analysis of this deletion we found that it consists of an inverted repeat, a type of sequence that would favour the formation of a loop at this site (Figure III-11B), thus probably leading to a PCR artifact.

In conclusion, our attempts to find more than one gene coding for *Sparus* BGP were unsuccessful. Whilst we cannot eliminate the possibility of gene duplication, we consider the available data to favour the existence of just one BGP gene in this species. These results are in contrast with those obtained in rodents where analysis of several mouse and rat strains have indicated that BGP is part of a gene cluster. While in the rat either one or multiple copies were detected, depending on the strain (448), in the mouse two BGP and one BGP-related genes were identified side by side in the genome of all strains examined (117). Our

genomic southern approach followed the same conditions referred in the mouse study, but the output points to the existence of one single spBGP gene, instead of the cluster found in the mouse genome. Given the very high degree of identity between these various mouse genes, it is possible that the duplication of the BGP gene in rodents occurred quite recently, after the branching of bony fish nearly 200 million years ago.

4. EXPRESSION OF THE spBGP GENE

4.1. Tissue distribution: the spBGP gene is only expressed in mineralized tissues

The results reported in section 7.1 of Chapter III show that in *Sparus*, as previously reported for other species (117, 443, 458, 555), the expression of BGP is specific to bone tissues undergoing mineralization such as vertebra and jaw, as clearly seen by Northern blot and RT-PCR (Figures III-13 to III-15). This conclusion is supported by *in situ* hybridization results, where specific message was detected exclusively in vertebra, jaw bone, sites of fin insertion and dermis (Figure III-26), this last result being attributed to the production of BGP mRNA by the scale-forming cells. The identification, by histological methods, of osteoblasts and osteoclasts in the same vertebra and jaw sections (Figure IV-1) used for detection of BGP mRNA by *in situ* hybridization showed that not only the cells resposible for producing the spBGP transcripts are present but also that bone deposition and possibly some remodelling is actively occurring in the vertebra and jaw of these juvenile specimens.

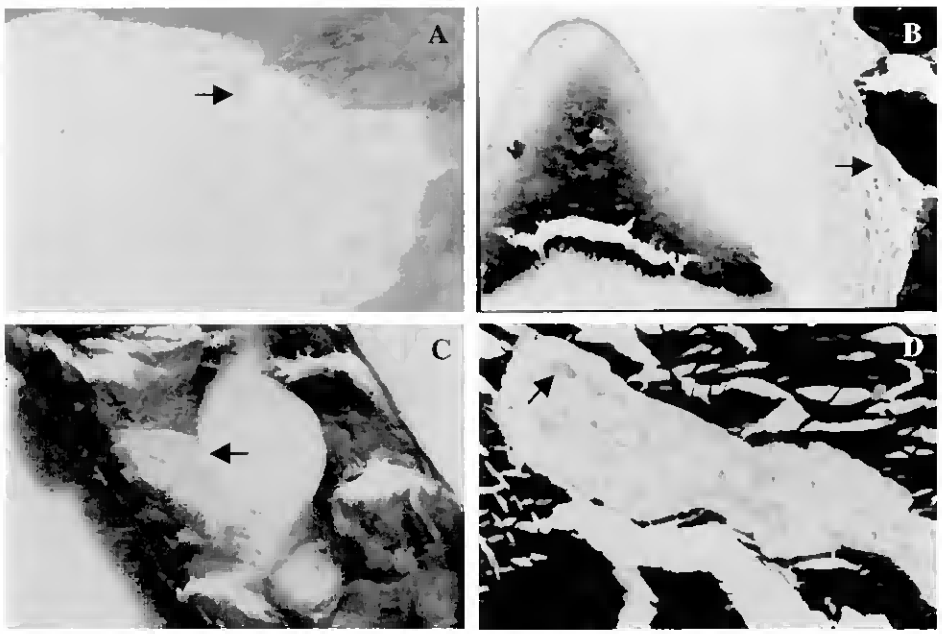


Figure IV-1. Identification of osteoblast-like and osteoclast-like cells in uncalcified sections of a *Sparus* juvenile (90 dph) by histological detection, respectively, of alkaline and acid phosphatases activity. **A-B**- Osteoblast-like staining (red stained cells; arrows) in vertebra (A) and jaw (B); **C-D**- Osteoclast-like staining (arrows) in vertebra (C) and jaw (D).

A faint signal for spBGP was also observed in *Sparus* brain (35 cycles RT-PCR and *in situ* hybridization) and heart (35 cycles PCR). These results, however, must be prudently considered, since there is the possibility that they correspond to background noise, i.e., detection of residual levels of spBGP mRNA, due to the very high sensitivity of both detection techniques used, with no physiological meaning. BGP mRNA presence in non-mineralized tissues has previously been reported, namely for tissues such as brain, intestine, and kidney (158), although it is not clear whether these low levels of BGP gene expression are derived from the BGP genes or from the BGP-related genes described in mice and rats (117, 448). This doubt is further strenghtened by the fact that during the past two decades, radioimmunoassay data have never convincingly demonstrated the presence of BGP protein in non-osseous cell cultures or tissues, except in ectopic calcifications (282), where mineral adsorption of circulating BGP is a likelihood. The most unassailable proof of the bone specificity of BGP derives from Northern blot analysis performed by several groups (73, 165, 295). No BGP mRNA was detected in preparations of rat liver, lung, intestine, heart, spleen,

spleen, kidney, brain, skin, or sternal cartilage with a rat BGP cDNA probe, whereas bone mRNA was strongly positive (165, 295). Fibroblasts do not synthesize BGP (10) or BGP mRNA (73), even when stimulated with 1,25-dihydroxyvitamin D_3. In various chondrocyte cultures of sternal and vertebral cartilage origin, it has been confirmed that BGP synthesis does not occur (185, 186). BGP synthesis has been demonstrated by radioimmunoassay in numerous osseous culture systems: 1) bone organ culture models, including chick embryonic bone (287), fetal rat calvariae (290), and bovine trabeculae (373) normal osteoblast-like cells grown out of human bone trabeculae (10, 33, 490) or isolated by serial enzymatic digestion of chick (184) or rat (77, 78) bone; and 2) osteosarcoma cell lines (164, 374, 424). BGP was also shown to be synthesized in a cell-free system directed by mRNA from rat bone (320). There are, therefore, no truly convincing reports of BGP synthesis in cells or tissues other than those originating from bone, cementum and dentin and so BGP may still be considered specific to bone cells, a conclusion strenghtened by our results.

4.2. Developmental expression: the onset of spBGP gene expression follows the appearance of bony structures

Developmental appearance of cartilaginous and mineralized structures

Detection of cartilaginous and mineralized structures in developmental stages of *Sparus* (Figures III-19-25) produced results that are in general agreement with those of Faustino (149), mainly in what refers to the relative timing of appearance of the cartilaginous and calcified structures. Immediatelly after birth *Sparus* specimens have already some cartilaginous structures, no ossification being detected at that stage. The first clear sign of ossification of the skeleton (as determined by alizarin red staining) is detected at 27 dph[1] (6.2-6.6 mm), localized on the structures that are presumably more important for food catching and seizing: the jaw, the caudal soft rays and the branchial arches. At this stage the number of skeletal cartilaginous structures has increased and its ossification will take place during the ontogenic development of the individual. At 35 dph (6.7-8.8 mm) the *centra* of the vertebral column begin to ossify and at 110 dph (19-22 mm) most of the skeleton shows ossification. At 150 dph (24-27 mm) ossification of *Sparus* skeleton is virtually complete and

[1] An improvement of the alcian blue/alizarin red double staining technique used in this work has recently been developed and published (181). Use of this technique allows detection of mineralized stuctures in *Sparus* earlier than 20 dph, a stage where BGP was never detected, either by Northern, PCR-Southern or *in situ* hybridization.

only some structures remain cartilaginous, presumably because of its importance for the maintenance of growth.

According to Faustino and Power (151) "*three principal phases of cartilaginous/osteological development can be identified in Sparus aurata, ≤3.1 mm L_N, 3.1 mm L_N-11.6 mm L_S; and > 11.6 mm L_S. The first phase corresponds to the yolk-sac stage (or free embryo) and the cartilaginous/osteological structures which develop are those that are necessary for exogenous feeding, the bones of the head which permit opening and closing of the mouth and the structures which will ultimately support the pectoral fins. The second phase is associated with the larval stage, when most of the fin structures form and ossify, leading naturally to the transition to the juvenile stage. In S. aurata the third distinct phase occurs before the rays segment*".

In comparison with other teleost species, developmental appearance of cartilaginous and osseous structures in *Sparus* resembles most with what was observed for *Pagrus major* (334), a closely related species (see Figure IV-2), in terms of timing and of relative order of appearance of the structures. Other species show considerable differences in these parameters (e.g., 14, 181, 230, 418).

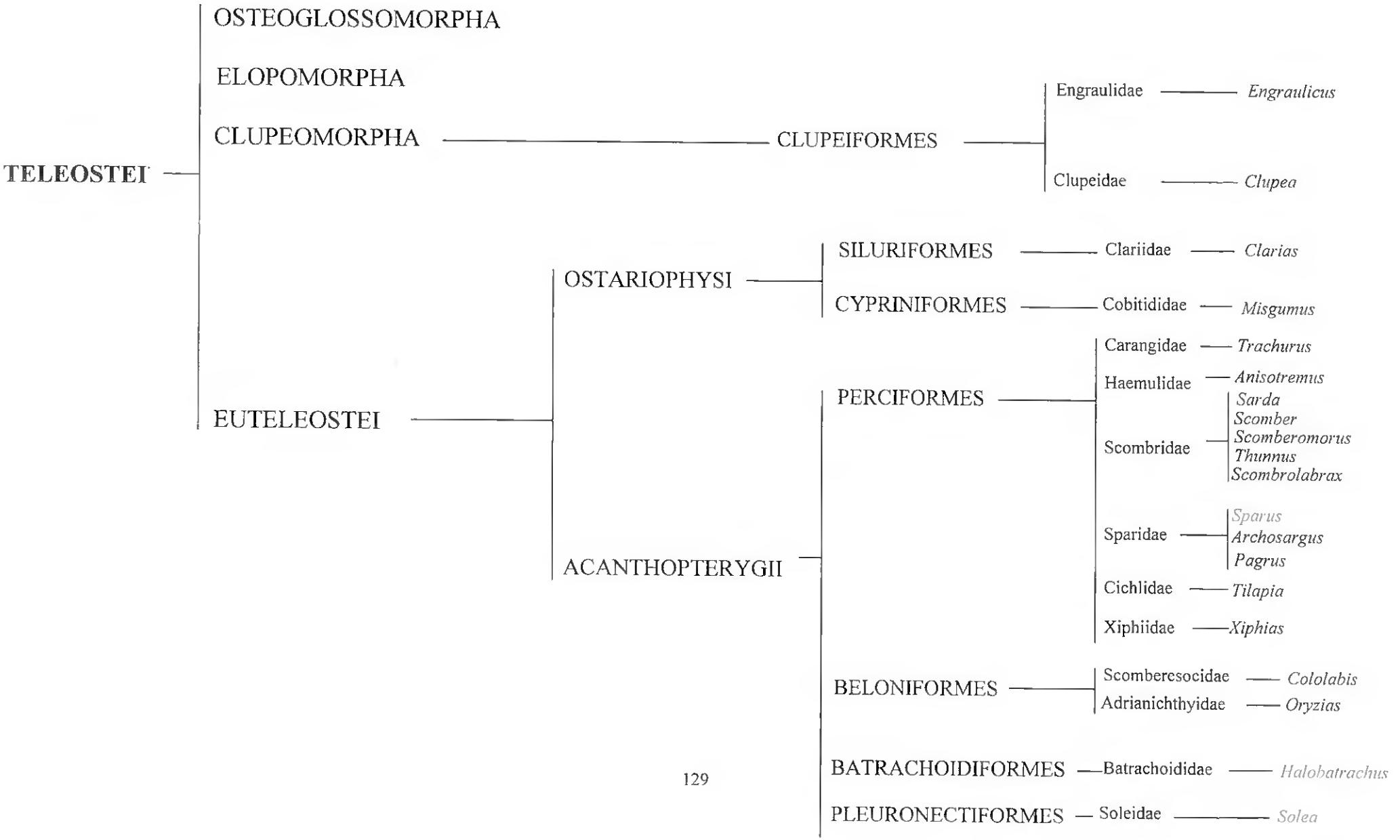
Developmental appearance of spBGP message

Results displayed in Figures III-16 to III-18 show a discrepancy relatively to the onset of BGP gene expression during *Sparus* development. The 35 cycle RT-PCR allows detection of message with a greater sensitivity than either the 20 cycle RT-PCR or the Northern analysis (Figures III-18 and III-16, respectively). We could, therefore, conclude that BGP mRNA is present very early during the *Sparus* development, at least at 18 dph., covering a period where ossification is not yet detected by histological techniques. Other studies where gene messages, including BGP, was detected by RT-PCR (e.g., 117, 137, 154, 273) have used a variable number of cycles (25-35), depending on the expected abundance of the message to be amplified. However, we believe that such an high number of PCR cycles as 35 might be amplifying a residual expression of the BGP gene, so that the positive signals observed for the younger *Sparus* stages in Figure III-17 might not reflect the presence of a physiologically significant level of spBGP mRNA (the hypothesis of genomic DNA contamination can, in principle, be ruled out, since all RNA samples were previously treated with DNase) and the corresponding protein. Accordingly, the 20 cycle RT-PCR amplification, together with the Northern blot analysis, might give the correct image of

the real situation, concerning the presence of BGP message in *Sparus* developmental stages. In that case, the spBGP mRNA is only present after 27 dph, exclusively, which signifies that, in *Sparus*, presence of BGP message is only unambiguously detected after the onset of ossification, as detected by alizarin red staining (previous section). This pattern of developmental expression of the BGP gene parallels what was previously observed for mammalian species (117, 443), with the onset of expression of the gene following the appearance of calcified structures and with its level of expression increasing as mineralization is extended to all the skeletal structures.

Debate on the question of whether initial mineralization occurs in the presence or absence of BGP has endured the last two decades. In embryonic chick tibiotarsus, the presence of BGP as measured by Gla content (210) and radioimmunoassay (216) appears coincident with the onset of mineralization at 8 days, judged by alizarin red staining and atomic absorption measurements of Ca^{2+} in ashed samples (216). *In vivo* studies have shown that BGP is expressed relatively late in the developmental sequence in long bones of embryonic chicks (210, 212, 216) and fetal rats (427, 431, 572), well after the cartilaginous anlagen have formed, and other studies also contend that BGP appears only after the onset of mineralization (329, 427, 431). *In vivo* transcription studies of the BGP gene show also age-correlated expression (483). Developmental control is reflected by the absence of BGP expression in proliferating osteoblast progenitor-like cells and expression in postproliferative committed cells, albeit at low levels. Expression is then upregulated in osteoblasts within a mineralizing extracellular matrix (391). This developmental expression is stringently regulated by steroid hormones and growth factors in normal diploid osteoblasts (393, 394, 480). However, transcription control is abrogated to a significant extent in osteosarcoma cells, where BGP expression and cell proliferation occur concomitantly (47, 503). The more recent hypothesis concerning the BGP function state that this protein might be involved in the maturation of the hydroxyapatite crystals, i.e., in the transition from the initially formed amorphous calcium phosphate mineral of fetal bone to the hydroxyapatite phase characteristic of adult bone (49, 135, 525). If this is the case, then we should expect to obtain a mineralization signal (by the alizarin red technique), as indeed was observed, earlier than the BGP mRNA signal, since this protein would only be necessary when the transition to the

Figure IV-2. Brief classification of the Teleostei Division [adapted from Smith and Heemstra (492)]. The genus for which BGP cDNA has been obtained are highlited in red.



hydroxyapatite phase takes place. Debate on this question will surely continue, with problems of detection method and sensitivity remaining a central issue.

The increase in the spBGP message with age, observed in our results (Figure III-18 and III-19), has also been observed in other studies, where BGP content was shown to increase with increasing bone density (e.g., 288). It is interesting to note, however, that after ~75 dph (when the majority of the skeletal structures are calcified; see Figure III-14) the amount of spBGP message does not seem to significantly increase with age, which, in functional terms, can be interpreted as meaning that BGP needs increase during formation of new bony structures, a stage where mineral deposition and maturation is stronger. Once the fish skeleton is mostly calcified the level of BGP mRNA stabilizes, at least in non-pathological conditions. A parallel study on the localization of BGP protein during the various stages of development of *Sparus* using a fish antibody is currently being initiated in our lab (Simes *et al.*, in prep.), which should bring additional information to further understand this question.

5. *Sparus aurata* BONE-DERIVED CELLS AND ASSESSMENT OF spBGP GENE PROMOTER ACTIVITY

Sparus bone-derived cells can be used as a system for *in vitro* studies

We have shown that bone-derived *Sparus* cell cultures have an heterogenous morphology and express several osteoblastic differentiation markers. After confluency, they aggregate into cellular condensations, which later mature into nodules where mineralization is localized (Figure III-34 to III-36). Similar-looking nodules were shown to be composed of calcified deposits similar to hydroxyapatite, the type of calcium phosphate found in bone (445). *Sparus* bone-derived cell cultures retain their phenotype and mineralization capability through several passages, and they exhibit osteoblastic markers such as alkaline phosphatase enzymatic activity and BGP mRNA.

From the four different mineralized tissues used as cell sources, vertebra was the one that produced better results, mainly in terms of cell resistance and simultaneous presence of alkaline phosphatase positive cells and synthesis of BGP mRNA. It is, however, possible that the use of different culture conditions, as we are testing presently, will allow the culturing of

cells derived from other mineralized tissues we have tested. In fact, the medium we have used in our cell culture experiments, Leibowitz Medium L-15, is commonly used in culture of fish cells (170), but others, such as Eagle's Minimum Essential Medium (MEM) or Medium 199, all supplemented with 10% fetal bovine serum, are also reported (170). For the culture of osteoblast-like cells, either primary or immortalized, most studies use Dulbecco's Modified Eagle´s Medium (DMEM; e.g., 28, 47, 74, 120, 335) or Modified Eagle´s Medium (MEM; e.g., 95, 502, 567) and, at least the first of these two culture media, in an atmosphere of 10% CO_2, seems to be a suitable medium for the cells derived from calcified tissues of fish, since not only they migrate and divide faster than when grown in L-15 but also they mineralize more eficiently than these (Laizé *et al.*, in prep.). Zebrafish cells have been reported to be very sensitive to conventional culture media containing high concentrations of mammalian serum, being grown in complex media containing insulin and trout embryo extract (51, 89). Other studies, however report success with fish primary and immortalized cell culture using DMEM (133, 532) and L-15 (524), supplemented with mammalian serum.

We have shown that although distinct cellular types migrate from the mineralized tissues, some cells display osteoblast-like properties, such as the ability to synthesize BGP and alkaline phosphatase and the capacity to mineralize nodules. This last capacity, however, was not always present since in some cases cells were able to form nodules but were unable to mineralize them (see Figure III-36). It is possible that the conditions we have used are sub-optimal, either for cell culture, or for mineralization, since this structures are not observable in our more recent experiments using a different culture medium. Indeed, preliminary results indicate that, among other factors, *Sparus* bone-derived cells require a higher calcium concentration in the culture medium than mammalian cells, in order to be able to mineralize nodules *in vitro* (Laizé *et al.*, in prep.).

The spBGP 5' flanking region is able to induce transcription of a downstream reporter gene

Obtaining a culture of *Sparus* bone-derived cells was critical in order to perform all kinds of *in vitro* studies, and thus approach the question of regulation of the spBGP gene expression at the level of transcription.

Assessment of transfection with the spBGPpromoter/pßGal construct revealed a low efficiency (less than 1%), which may reflect the low number of osteoblast-like cells present

in the mixed primary cultures. This low efficiency, however, did not prevent showing that the 1.2 Kb sequence located upstream from the spBGP gene was capable of acting as an active promoter *in vitro* and drive the expression of the reporter gene (lacZ). The low efficiency of the transfection procedure could have different causes. Among them, the presence of an heterogenous cell population may have been a critical factor. In fact, an higher number of cells may have been transfected, although not being able to support lacZ expression, due to their non-osteoblastic phenotype. Collagenase digestion of bony tissues releases all the phenotypic variants of the osteoblastic lineage, as well as other "contaminant" cell types. The first, which are variably represented in primary osteoblast cultures (565), are all capable of proliferation under the appropriate culture conditions (218). However, one of the properties of the BGP promoter, at least in higher vertebrate models, is the almost complete restriction of the BGP gene transcription to mineralized tissues and cells from certain osteoblast/odontoblast lineages (see Chapter I for a more complete approach to this subject). Additionally, normal rat (77, 78), chick (82, 184), and human (10, 32, 33) primary osteoblast cell cultures usually produce modest levels of BGP, a situation that shifts if osteoblasts are maintained until confluency in a rich medium for production of a dense collagenous matrix that can be induced to calcify with β-glycerophosphate, with increases in BGP synthesis up to 100-fold (184). Also, primary and transformed osteoblast cultures often require exposure to 1,25-dihydroxyvitamin D_3 for osteocalcin synthesis to be detected (376, 490). The *Sparus* cell cultures we have transfected with the spBGPprom/pßGal construct proved to contain BGP producing cells, albeit at very low levels. They also contained cells capable of synthesizing alkaline phosphatase, a protein also expressed in osteoblasts (e.g., 565). However, not only these osteoblast-like cells were far from being the majority, but also they were not either confluent or hormonally treated, in order to stimulate BGP production.

Another plausible reason for the low efficiency obtained might be that the culture medium we have used is not possibly the most suitable for bone-derived cells, an hypothesis that seems to be validated by our preliminary results with different culture media. We may have, in some way, prevented the bone-derived cells from displaying osteoblast-like properties and, concomitantly, inhibited the functionality of the spBGP promoter.

In conclusion, the development of primary fish bone-derived cell cultures is a first step towards obtaining *Sparus* bone-derived cell lines and their availability will broaden the usefulness of this vertebrate model system. Co-transfection assays in such cells will allow the convenient study of the influence of transcription factors and other regulatory proteins on

the expression levels of genes previously found to be important for the control of normal *Sparus* bone development. Transfection studies provide a powerful tool to efficiently study specific gene expression in fish. A *Sparus* cell line could also be used to test whether this species is susceptible to any known viral vectors. Retroviruses are an excellent tool for insertional mutagenesis in mice (133) and may prove to be of equal quality for *Sparus* mutagenesis. In addition, due to their ease of propagation and limited requirement for specialized incubation equipment, *Sparus* cells can be used for teaching the art of cell culture and the study of cell-virus interactions in fish.

6. *Halobatrachus didactylus* BGP CLONE SHOWS HIGH SIMILARITY WITH ITS SPARUS COUNTERPART

Comparison with spBGP cDNA (see Figure III-9) shows that *Sparus aurata* and *Halobatrachus didactylus* (toad fish) BGP cDNAs present, at least for the available tfBGP cDNA partial sequence (Figures III-5 and III-40), a high degree of conservation (~ 70%), whereas similarity between tfBGP and human BGP goes down to 43%. The remaining cDNA sequence is presently being obtained, which will allow a more complete comparison between these apparently very similar BGP sequences.

7. PHYLOGENETIC ANALYSIS OF spBGP

Evolution of the sp BGP gene

Our data support the hypothesis that all BGPs have a single origin, in agreement with a previous suggestion (64). Within the BGP clade (Figure III-34), the fish form a clearly distinct group that might represent the most ancestral forms of BGP. It is known that fishes evolved as the first creatures to have a skeleton, its evolution spanning 500 million years (from the Ordovician), of which the first 150 million years were a time span when the fish lived on the earth as the pinnacle of evolutionary achievement (310). The first to diverge were the jawless *Agnatha*, of which only two genera are extant: the lampreys and the hagfish. The placoderms followed, originating in the Silurian, some 420 MY ago, and extinguishing in the end of the Devonian, 65 MY after (310). They sit in the evolutionary tree somewhere between the sharks and the true bony fishes and are characterized by their armour made

of overlapping bony plates, forming a head and a trunk shield. The first teleosts seem to have arisen only in the Middle Triasic, some 220 million years ago (310), that is, contemporary to the first birds, possibly simultaneously with sharks, coevolving from separate ancestors (464).

Figure III-5 shows the existence of a high degree of homology between a region of all mature BGPs, even when we compare species whose origins are separated by more than 200 million years of evolution. The structural features common to these different proteins include the sequence positions of the three Gla residues and the associated disulfide bond. Indeed, in the region between residues +2 and +41, in *Sparus*, which contains the three Gla residues, only 23 amino acids have changed, in comparison with the human protein. The conservation of this region, which must be sufficiently important to have survived until our present time, and is known to be crucial for the protein to adopt the correct conformation and adsorb to hydroxyapatite (62, 182, 369, 399, 507), lead us to conclude that BGP is probably playing the same role since the dawn of the vertebrates, its invention probably explaining and allowing the invention of bone itself.

Bone Gla protein versus Matrix Gla protein

BGP is evolutionarily related to Matrix Gla protein, or MGP, a 10 KDa vitamin K-dependent protein which, although also found in bone matrix, has a more wide tissue distribution than BGP. MGP, the second vitamin K-dependent protein to be discovered in bone (438), has, in almost every species where it has been isolated, among other features, 5 Gla residues, a transmembrane signal peptide and a mature portion, being the first example of a vitamin K-dependent protein that lacks a propeptide (442). In contrast with BGP, MGP is synthesised in a variety of tissues and cell types: lung, heart and kidney (165), as well as in bone and cartilage (69 for bone; 202 and 388 for cartilage), the bone and cartilage concentrations greatly exceeding that of the soft tissues (165). Also differently from BGP, MGP appears before the onset of mineralization, which is consistent with its presence in cartilage (388). Its function, although not entirely understood, seems to be related with the clearing of excess calcium from tissues into the circulation, thus protecting against abnormal or ectopic calcification (165, 309, 420, 462, 569; for more details on MGP see 1.2.1 of chapter I and references therein).

Comparison of the amino acid sequence of BGP with the COOH-terminal 42 residues of MGP (see box in Figure IV-3) reveals a high degree of sequence identity (31%).

Although MGP is generally γ-carboxylated at five sites, two of the Gla residues are adjacent to a disulfide bond as in BGP, potentially facilitating the interaction of MGP with hydroxyapatite. Also, like BGP, MGP contains a 19-amino-acid N-terminal hydrophobic transmembrane secretion signal that is cleaved during translocation into the rough endoplasmic reticulum (62). These facts lead us, and others, to raise the hypothesis that BGP and the carboxy-terminal region of MGP could have arisen from a common ancestor by gene duplication, preceding the divergence of bony fish from other vertebrates (440). This last statement is corroborated by the fact that bovine MGP is more closely related to swordfish BGP than to bovine BGP (442), which can be explained if the putative gene duplication event that gave rise to BGP and MGP preceeded the divergence of swordfish from other vertebrates.

Another possibility raised by some authors (e.g., 453) is that MGP was the ancestral template for BGP, as sharks synthesize MGP, but, until now, no BGP was ever detected in these fishes. Indeed, one of the lower vertebrates known to synthesize MGP is the shark, namely the soupfin shark *Galeorhinus galeus*, from which the protein has been sequenced in 1994 (453). Sharks belong to a group that appeared about 400 million years ago, in the Silurian Period, and since then they have changed very little (310). The mistery remains as to how they first evolved. Did they evolve from an heavily scaled agnathan group, like the thelodonts, or from an as-yet-undiscovered ancestral fish group? The only clue we have to their distant origins is the similarity that exists between the scales of early sharks and those of the jawless thelodonts (310). Independently of their precise origin, sharks were among the first groups to benefict from the vertebrate condition, since, although they lack an internally ossified bony skeleton, they possess a special type of cartilage forming the braincase, jaws, gill arches, vertebrae and fin supports. The only hard bone tissues are developed in their defensive fin-spines, teeth and scales. This cartilaginous condition, although once regarded as primitive, as a precursor to the evolution of true bone, is now considered by an increasing number of authors as a highly specialized condition (15, 310).

Certainly due to its lack of bone, BGP was never sequenced in sharks. Nevertheless, given the previous suggestion (64, 453) that BGP and MGP arose from a common ancestor, one could hope to find extra similarities between shark MGP and fish BGP. However, our data (Figure III-34) show that fish BGP is more closely related with mammalian MGP than with shark MGP, an intriguing result that is strenghtened by the recent finding that teleost MGP is very different from both xenopus and shark MGP (Simes and Cancela, in prep.).

In fact, teleost MGP shares with shark MGP a C-terminal extension, and both share some homologies with the other known MGPs only in the regions where Glas are present and where phosphorylation occurs. Hence, lower vertebrate MGPs seems to be considerably different from all other known MGPs (see relative position in Figure III-41), while amphibian, birds and mammalian MGPs and BGPs seem to show a stronger relationship among themselves. This could mean (i) that teleost and shark MGPs, along its evolutionary path until our present time, have changed considerably in relation to its ancestor and/or higher vertebrates; or (ii) that lower vertebrate MGPs retain more of the primordial sequence, in relation to higher vertebrates. Since, for example, sharks have changed little since its first apearance, 400 million years ago, this last hypothesis seems to be the more plausible.

The doubts stated above may be softened by going even lower in the evolutionary scale. The search for BGP in shark teeth and of MGP, or related proteins, in invertebrates may give us some clues to solve the riddle of the BGP/MGP origin and evolution. However, we must always be aware that the ancestors of vertebrates are now extinct, and that attempts to trace the origin of these proteins within the invertebrates, in groups such as the tunicates, are speculative and assume that some living animals are close relatives of the vertebrate ancestor, which may or may not be true.

The Gla protein family

The study of the appearance of BGP cannot disregard the analysis of the appearance and evolution of the other Gla-containing proteins. In the previous section we analysed the possible relationships between BGP and the other known bone-related Gla protein, MGP. In the following paragraphs we will address what is presently known and hypothesized about the appearance and evolution of the Gla proteins involved in the coagulation cascade, the most numerous representatives of the family of vitamin K-dependent proteins (see Table I.IV. on Chapter I, for more information on coagulation factors and other vitamin K-dependent proteins).

All high vertebrates, from fish to man, possess a blood clotting system, although with different degrees of complexity and with distinct proteins involved (129, 130, 174, 225, 321). Blood coagulation in mammals is a well known and extensively described process, involving several proteins, which play diverse roles, either promoting or inhibiting it, and possess a varied number of γ-carboxylated glutamic acid residues, through which they interact with Ca^{2+} and acidic phospholipid surfaces (174, 218, 369, 509, 518, 541).

Although less studied, we know now that blood coagulation in fish is essentially similar to clotting in mammals (e.g., 380, 464, 491, 549). Soluble fibrinogen molecules are transformed into an insoluble fibrin clot by thrombin, which is derived from a prothrombin molecule, under the mediation of a tissue factor (extrinsic) or thrombocyte factor(s) (intrinsic). Calcium is essential for both of these processes (129). The major differences in relation to mammalian blood clotting lie in the intrinsic conversion of prothrombin to thrombin, whereas the extrinsic systems appear to be more similar (129). Even among fishes, however, there is a high diversity in the blood clotting process. Elasmobranchs have been reported to be essentially hemorrhagic, and it has even been suggested that their blood contains little or no prothrombin (251, 551). Teleost fish, on the other hand, have a very rapidly clotting blood, although some reports indicate that prothrombin conversion is not always complete (551).

A group with chordate, and hence vertebrate, affinities are the echinoderms. They were the first deuterostomes (i.e., with indeterminated, radial segmentation and enterocoelic coelom) to evolve and constitute one of the main drives towards the evolution of the actual more evolved organisms. Although very little is known concerning clotting in echinoderms (e.g., 304), it is a fact that in this group divalent cations such as Ca^{2+} and Mg^{2+} are required for aggregation of blood cells (45). However, according to Boolootian (45), it is difficult to trace the origins of vertebrate clotting systems in the clotting reactions described for the echinoderms. It is more likely that the calcium-dependents clots of the echinoderms represent a case of parallel evolution with the calcium-dependent vertebrate clot, since little other resemblance between the two is found. This being true, the possibility that the main coagulation cascade factors (V, VII, VIII and X) may be present in all high and low vertebrates, raised by recent reviews on the evolution of blood clotting (e.g., 130, 174), lead us to conclude that these factors must have appeared over a relatively short period during the evolution of vertebrates from advanced invertebrate deuterostome ancestors that presumably did not have these *haemostatically active* factors.

BGP and MGP share some homology with the more diverged coagulation factors [see (218)]. In the case of MGP that homology occurs within the mature protein sequence, whereas for BGP the similarities are restricted to its leader peptide sequence and the Gla domain. Upon comparison, BGP seems more distant, in evolutionary terms, from the Gla-containing coagulation factors than from MGP. The search for a common ancestor for this entire group of proteins may lead us, therefore, to an ancestral protein, probably with a

totally different function, that used carboxylated glutamic acid residues to adsorb calcium in the fulfilment of its obscure function. This protein might have been playing its role in the cells of a revolutionary sea-urchin, unaware that its descendants would be involved, some million years later, in such extraordinary deeds as the conquering of the water and of the land.

The tale of the Gla proteins tells us, once again, that Nature avoids spending and always makes good use of what it already has. The picture that seems to arise, concerning this group of proteins, is that the ability to bind calcium (conferred by the γ-carboxyglutamic acid residues) has appeared somewhere in the past and was "fixed" in other proteins, either by gene duplication, gene modification, or exon shuffling (174 and references therein). These proteins remain a prime example of the development of a family of proteins with diverse functional properties but common, unified structural elements.

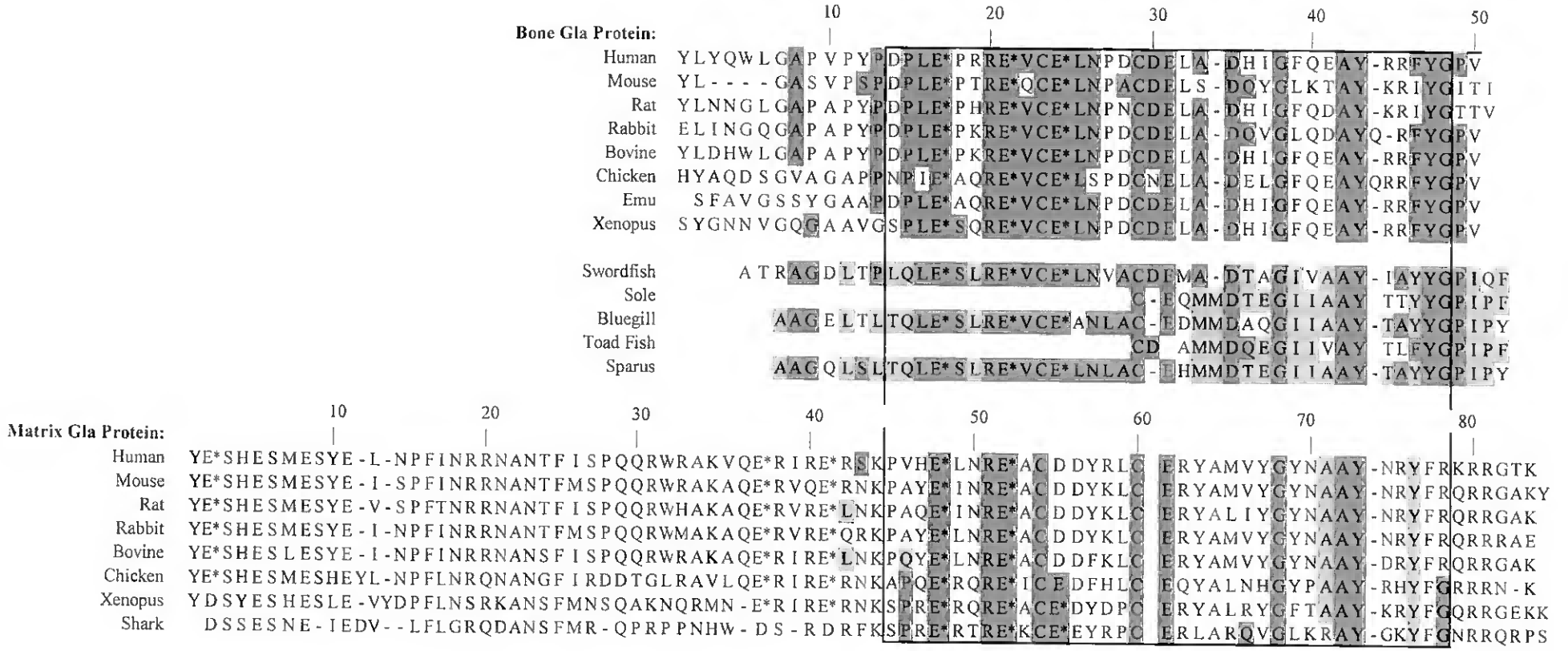


Figure IV-3. Protein sequence comparison between mature sequences from all known MGPs and selected BGPs representing different phylogenetic groups. Homologies between fish BGPs or fish BGPs/MGPs are highlited in pink; homologies between fish BGPs/other BGPs and/or MGPs are highlited in green. The positions of gamma-carboxyglutamate residues are indicated by (E*). Dashes indicated gaps in the sequence, introduced to increase homology. Residues are numbered according to the *Xenopus* MGP and BGP proteins. MGP sequences: *Xenopus* (65); human (63), mouse (235), rat (442), rabbit (496), cow (440), chicken (562) and shark[1] (453). BGP sequences: *Xenopus* (64), human and mouse (73), rat (399), rabbit (548), cow (421), chicken (70), emu (234), *Sparus* (64), swordfish (422) and bluegill (377).

[1] The shark MGP sequence extends beyond what is shown, but this region is omitted since there is no counterpart in any other species.

CHAPTER IV: GENERAL CONCLUSIONS AND PERSPECTIVES

More than half a century ago a Danish investigator, Henrik Dam, observed that feeding chicks an ether-extracted diet led to a bleeding tendency. From this chance discovery, he inferred that the cause of hemorrhagic tendency was the lack of a previously unrecognized fat soluble antihemorrhagic factor in the diet. This factor was named vitamin K (Koagulations-vitamin) and later shown to be required for the synthesis of specific blood coagulation factors by the liver. The discovery of vitamin K opened an exciting new field of research on biology and medicine, and many significant achievements were made in the following 50 years. These include, among others, the discovery of Dicumarol, the identifications of the unique amino acids γ-carboxyglutamic acid (Gla) and β-hydroxyaspartic acid, and the discovery of vitamin K-dependent carboxylase. Later, several proteins containing Gla residues were found in bone and kidney, indicating that the role of vitamin K is not restricted to blood coagulation, but is also associated with other biological phenomena.

One of the Gla proteins not associated with blood coagulation is Bone Gla protein (BGP, osteocalcin), a small protein which was isolated in 1976 from the mineralized phase of bovine bone by Dr. Paul Price and collaborators. BGP is the most abundant non-collagenous protein of bone but, despite the large number of studies involving this protein, little was known of its function until very recently. Almost simultaneously with the beginning of the present work, a study was published by Dr. Gerard Karsenty's group in which a putative function for mouse BGP as an intervenient in bone remodelling was advanced. This breakthrough marked a turning point for BGP studies, from a more "clinically orientated" phase to a more "functional" approach.

With the present study we intended to travel back in evolution, towards the origin of BGP. In fact, although this protein was probably invented long before the apperance of *Sparus aurata*, there are no reasons to think that its function has changed since the apearance of the first bony fishes, about 220 MY ago, to the extant teleosts. We intended to compare this "version" of the protein with its mammalian counterpart, in terms either of molecular structure, or tissue distribution and timing of appearance. This knowledge should allow us, not only to advance hypothesis concerning the evolutionary trajectory of this protein, but

also would give us the necessary tools to perform studies on the developmental appearance and regulation of this protein, namely by steroids such as vitamin D and the morphogenic hormone retinoic acid.

Sparus aurata BGP gene is the first gene coding for a Gla protein ever cloned in a fish, and bears many similarities with its mammalian homologs, at the nucleotide level as well as in tissue distribution and onset of expression during early development. Also as its mammalian counterpart, the spBGP gene promoter exhibits a modular organization, with a complex series of consensus sequences bearing homology to response elements for several physiologic mediators of BGP gene expression, including Steroid Response Element, OSE1 and OSE2. In addition, the unusually long intron II of spBGP bears a three-partite element which resemble a motif found in close proximity in the Vitamin D Responsive Element (VDRE) of the rat BGP gene, suggesting a role for this intron in the regulation of the spBGP gene.

Another necessary tool for further *in vitro* studies of BGP in fish is a bone-derived cell line, or an equivalent, which is required to perform studies on the regulation of the fish BGP gene. We obtained several isolates of fish-bone derived cells which presented several osteoblast-like characteristics, such as synthesis of alkaline phosphatase and BGP mRNA, and the capacity for nodule deposition and mineralization. These cells constitute already an invaluable tool to initiate regulation studies *in vitro*, and should permit to obtain fish bone-derived cell lines (Laizé *et al.*, work in progress).

If we assume that spBGP did not diverge significantly from the BGP of the earliest teleosts, we are led to conclude that this protein crossed more than 200 MY without significant changes, in particular in the region which is believed to be essential for the protein correct folding. The absence of BGP from the calcified tissues of cartilaginous fish (which skeleton is formed of calcified cartilage) supports the view that this small protein may not be essential for the deposition itself of Ca^{2+} ions but to control the way in which they are deposited (49), allowing for the formation of a more evolved tissue, a role so important that might have been essential for the success of the bony fishes, the colonization of land by vertebrates and, ultimately, the origin of man.

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APPENDIX I

PROTOCOLS USED THROUGHOUT THIS WORK

The following protocols, although extracted total or partially from other sources, were the ones more frequently used throughout this work, being described in this special section for ease-of-consultation purposes.

PROTOCOL 1

Isolation of plasmid DNA by the "boiling technique"

Reagents:
- Tris-Cl (Sigma, T-6066)
- EDTA (Fluka, 03680)
- Isopropanol (Sigma, I-9516)
- Ethanol (Ridel-de Haën, 32221)
- LB Broth (Sigma, L-3022)
- Ampicillin (Sigma, A-9393)
- Bidestilled, autoclaved H_2O
- Triton X-100 (USB, 22686)
- Sucrose (Sigma, S5016)

Solutions:

a) TE:
- 10mM Tris-Cl
- 1mM EDTA

b) STET:
- 8% Sucrose
- 5% Triton X-100
- 50mM EDTA
- 10mM Tris-Cl

c) Lisozyme solution freshly made: 10mg/ml lisozyme (Sigma, L6876) in 10mM Tris-Cl, pH 8.0.

Procedures:

1- Several white bacterial colonies were picked with a sterile toothpick and inoculated each in 2 ml LB + 50µg/ml ampicilin. Incubation took place O/N, with agitation, at 37 °C.

2- Next morning, 1.5 ml of each culture were transferred into a sterile 1.5 ml microcentrifuge tube and centrifuged in a microcentrifuge for 30 seconds at top speed (~ 13.000 rpm) to pellet the bacteria.

3- The culture medium was removed and the pellet ressuspended in 350µl STET, with vortex for ~ 1 minute.

4- 50 µl of lysozyme were added and mixed with Vortex.

5- The tubes were placed in a boiling water bath for 40 seconds and centrifuged immediatelly at top speed for 10 minutes.

6- The pellet was removed with a sterile toothpick and discarded. One vol. of ice-cold Isopropanol was added, mixed by inversion and the mixture was incubated at -30°C for 20 minutes.

7- The tubes were centrifuged at top speed for 15 minutes, the supernatant discarded and the obtained precipitated DNA washed with 350µl ice-cold 75% Ethanol.

8- The DNA was air-dried and dissolved in 20µl of bidestilled, autoclaved H_2O.

PROTOCOL 2

Reverse transcription of *Sparus* total RNA (spRNAt)

Procedures:

1- One microgram of spRNAt was added to 1.0 µl of 50µM Universal Adapter dt primer, in a sterile PCR tube (Perkin Elmer). Incubation was performed at 65°C for 3 minutes, followed by cooling in ice.

2- The following reagents were added, by this order:

- 4 µl $MgCl_2$ (25mM; Promega);
- 2 µl 10x PCR Buffer II (Promega);
- 8 µl dNTPs mix (2.5mM each; Perkin Elmer);
- 1.5 µl de DEPC-treated ddH_2O;
- 1 µl RNaseOUT inhibitor (GibcoBRL; 10777-019);
- 1 µl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) (Promega).

3- Incubation was performed at 42°C for 45 minutes.

4- The enzyme was inactivated by incubation at 99°C for 5 minutes and the reaction product was kept at -30°C until needed.

Note: All incubations were performed in a Perkin Elmer GeneAmp PCR System 2400.

PROTOCOL 3

Double-strand DNA sequence analysis using the Sanger's dideoxy-chain termination technique (^{T7}Sequencing Kit (Pharmacia)

Procedures:

1- Double strand DNA (dsDNA) was denatured by adding 0.1 vol. of a 2 M NaOH, 2 mM EDTA solution and incubating at 37°C for 30 minutes.

2- The pH was then neutralized by addition of 0.1 vol. $NaC_2H_3O_2$ and the DNA precipitated by adding 3 vol.s ice-cold 100% ethanol and incubated at -80°C for 15 minutes.

3- After centrifugation for 10 minutes at 13000 rpm, the pellet was washed with ice-cold 75% ethanol, air-dried and dissolved in the appropriate volume of sterile ddH_2O.

4- To each 10 µl of the denatured dsDNA were added 1 µl of the appropriate sequencing primer (5 µM) and 2 µl of annealing buffer (Pharmacia). The annealing was performed by heating in a water bath at 65°C and then allowing the temperature to slowly decrease to 30-35°C.

5- To the annealed DNA+primer were added 3 µl of Labelling Mix, 1 µl of [α-35S]dATPαS and 2 µl of diluted (1:5 in Dilution Buffer) T7 DNA Polymerase, and the mixture incubated for 5 minutes at R/T.

6- To each one of four microcentrifuge tubes containing 2.5 µl of each dideoxinucleotide were added 4.5 µl of the labelled DNA, followed by a 5 minute incubation at 37°C. The reaction was terminated by adding 5 µl of Stop solution.

PROTOCOL 4

Poly(A^{+}) RNA isolation from total RNA with the QuickPrep Micro mRNA Purification kit (Pharmacia)

Procedures:

A. Purification Step

1. Add DEPC-treated water to 200 µg of *S. aurata* total RNA (extracted from several *S. aurata*'s tissues) to reach the volume of 1 ml.

2. Add 1ml of oligo-dT resin (Pharmacia) in a sterile 1.5 ml centrifuge tube and centrifuge for 1 minute at 13000 rpm. Remove supernatant and add the diluted RNA to the oligo-dT pellet.

3. Mix for 3 minutes and centrifuge for 5 minutes at 13000 rpm.

4. Add 1 ml of High-Salt Buffer (Pharmacia), ressuspend the resin+RNA pellet and centrifuge for 1 minute at 13000 rpm. Repeat this washing step four times.

5. Add 1 ml of Low-Salt Buffer (Pharmacia), ressuspend pellet and centrifuge for 10 seconds at 13000 rpm. Remove supernatant and repeat this washing step once.

6. Ressuspend pellet in 300 µl of Low-Salt Buffer.

7. Add ressuspended resin+RNA to a MicroSpin column (Pharmacia), which was previously placed in a centrifuge tube. Centrifuge for 5 seconds at 13000 rpm.

8. Wash three times with 500 µl of Low-Salt Buffer, centrifuging for 5 seconds at 13000 rpm.

9. Replace collecting centrifuge tube for a sterile one, add 200 µl of pre-warmed (65°C) Elution Buffer (Pharmacia) to the column and centrifuge for 5 seconds at 13000 rpm. Repeat elution step once.

B. Concentration Step

1. To the 400 µl of RNA obtained previously add 10 µl of glycogen solution (Pharmacia), 40 µl of 2.5 M potassium acetate solution and 1 ml of pre-chilled (-20°C) 95% Ethanol.

2. Precipitate RNA by incubating at least 30 minutes at -20°C and centrifuge at 4°C, for 5 minutes at 13000 rpm.

3. Remove supernatant, dry for 10 minutes and add 5 µl of DEPC-treated ddH_2O.

PROTOCOL 5

Construction of a *Sparus* cDNA library, using the Marathon™ cDNA Amplification Kit (Clontech): Second-strand cDNA synthesis

Procedures:

To 10 µl of cDNA obtained with the "first strand reaction":

1. Add : 48.4 µl of sterile ddH_2O;
   16 µl of 5x second strand buffer;
   1.6 µl of dNTP mix;
   4 µl of 20x second strand enzyme cocktail[1]

   80 µl total volume

2. incubate at 16°C for 1.5 hours;
3. add 2 µl (10 U) of T4 DNA polymerase;
4. incubate at 16°C for 45 minutes;
5. add 4 µl of 20x EDTA/Glycogen mix (0.2 M EDTA, 2 mg/ml Glycogen) and 100 µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1);
6. centrifuge at 14000 rpm for 10 minutes;
7. transfer aqueous phase to a sterile 0.5 ml tube;
8. add 0.5 vol. of 4 M amonium acetate and 2.5 vol. of 95% ethanol at R/T;
9. centrifuge at 14000 rpm for 20 seconds;
10. remove supernatant and washing with 80% ethanol;
11. dry pellet for ~10 minutes and dissolving in 10 µl of sterile ddH_2O.

[1] The second-strand enzyme cocktail contains Rnase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase.

PROTOCOL 6

PCR amplification of the 5'-end of the sparus BGP gene, using the Universal Genome Walker™ Kit (Clontech)

Procedures:

Primary PCR:

In a 250 µl PCR tube were added:

- 37.8 µl H_2O (Sigma);
- 5 µl 10x *Tth* PCR Rx buffer (Clontech);
- 2.2 µl Magnesium Acetate (25 mM);
- 1 µl DNA template;
- 1 µl AP1 (10 µM);
- 1 µl Gene Specific Primer 1 (GSP1; SBG19R; 10 µM);
- 1 µl dNTP mix (10mM each);
- 1 µl Advantage Tth Polymerase Mix (50x).

The mixture was incubated in a Perkin Elmer's GeneAmp PCR System 2400, with the following program:

94°C - 2 seconds] x 7
72°C - 3 minutes]
94°C - 2 seconds] x 32
67°C - 3 minutes]
67°C - 4 minutes

Secondary PCR:

A secondary PCR Master Mix was prepared as follows:

- 37.8 µl ddH_2O;
- 5 µl 10x *Tth* PCR Rx Buffer;
- 1 µl dNTPs (10mM each);
- 2.2 µl Magnesium Acetate (25mM);
- 1 µl AP2 (10 µM);
- 1 µl Advantage *Tth* Polymerase Mix (50x)

Forty-eight microliters of the resulting solution were added to each one of two 250 µl PCR tubes, followed by 1 µl of 10 µM Gene Specific Primer 2 (GSP2; SBG20R) (see Table II.I for primer sequence) and 1 µl of a 1:50 dilution of each primary PCR reaction. Amplification was performed with the following conditions:

94°C - 2 seconds] x 5
72°C - 3 minutes]
94°C - 2 seconds] x 20
67°C - 3 minutes]
67°C - 4 minutes

Nested PCR:

In a 250 µl PCR tube were added:

- 1 µl of each secondary PCR reaction;
- 5 µl Mg-Free PCR Buffer (GibcoBRL);
- 4 µl dNTPs (2.5 mM each);
- 1.5 µl $MgCl_2$ (GibcoBRL);
- 2.5 µl SBG7R (10 µM);
- 2.5 µl AP2 (10 µM; Clontech);
- 0.25 µl Taq Polymerase (GibcoBRL);
- 33.25 µl ddH_2O.

The mixture was incubated in a Perkin Elmer's GeneAmp PCR System 2400, programmed as follows:

95°C - 2 minutes
95°C - 2 minutes]
63°C - 1 minute] 35 cycles
72°C - 2 minutes]
72°C - 10 minutes

PROTOCOL 7

Isolation of plasmid DNA by the alkaline lysis technique

Procedures:

1. 1.5 ml of bacteria grown on LB liquid medium were transferred to a 1.5 ml sterile centrifuge tube.

2. 100 µl of ice-cold Solution I [50 mM Tris.Cl (pH 8.0), 10 mM EDTA, 100 µg/ml Rnase A] were added, followed by vortexing.

3. 100 µl of R/T Solution II (200mM NaOH, 0.1% SDS) were added, followed by mixing by inversion and incubation for 5 minutes at R/T.

4. 100 µl of R/T Solution III (3 M $KC_2H_3O_2$, pH 5.5) were added, followed by mixing by inversion, incubation in ice for 10 minutes and centrifugation at 13000 rpm for 5 minutes.

5. Supernatant was transferred to a sterile 1.5 ml centrifuge tube and the DNA precipitated with 2 volumes of R/T Ethanol, followed by centrifugation at 13000 rpm, for 1 minute, at R/T.

6. The pelleted DNA was washed once with 75% ice-cold Ethanol, dried for ~ 10 minutes and dissolved on 50 µl of sterile ddH_2O.

PROTOCOL 8

Synthesis of a non-radioactivily labelled RNA probe, using the DIG RNA Labeling kit (Boehringer-Mannheim Biochemica)

Procedures:

1 µg Apa I - digested DNA	1 µg Pst I - digested DNA
2 µl dNTP Labelling Mix Dig (Boehringer)	2 µl dNTP Labelling Mix Dig
4 µl 5x Transcription Buffer (Boehringer)	4 µl 5x Transcription Buffer
2 µl Sp6 Polymerase (Promega)	2 µl T7 Polymerase (Promega)
1 µl RNA Guard (Pharmacia)	1 µl RNA Guard (Pharmacia)
DEPC-treated ddH_2O to fill 20 µl	DEPC-treated ddH_2O to fill 20 µl
Sense Probe	**Antisense Probe**

The mixtures were then:

- Incubated at 37°C for 2 hours.
- Treated with 2 µl of Rnase-free Dnase (RQ1, Promega), for 25 minutes, at 37°C.
- Precipitated with 0.1 vol. 3M $NaC_2H_3O_2$ (pH 4.0) / 1 vol. ice-cold 100% ethanol.
- Washed briefly with 75% ethanol and air-dried.
- Dissolved in 100 µl of DEPC-treated ddH_2O.

APPENDIX II

SOLUTIONS AND ADDITIONAL INFORMATION

Maleic acid buffer (MAB): 100mM maleic acid, 150mM NaCl (pH 7.5).

Guanidinium Isothiocyanate Solution (for 200 ml): 100 g Guanidine Isothiocyanate, 7.04 ml 0.75 M Sodium Citrate (pH 7.0), 10.56 ml 10% N-Lauroyl Sarcosine; DEPC-treated H_20 until 200 ml.

10x PBS: 80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4g KH_2PO_4, pH 7.4; add ddH_20 to fill 1000 ml.

20x SSC: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to 1 liter with H20. Sterilize by autoclaving.

SSPE (1 x): 180 mM NaCl, 10 mM $NaPO_4$ (pH 7.4), 1 mM EDTA.

TE (pH 7.4): 10 mM Tris.Cl (pH 7.4), 1mM EDTA (pH 8.0).

LB Medium: it is a widely used growing medium. It can be used in the liquid form or supplemented with 1.5% agar-agar to obtain a solid medium. It can be supplemented with antibiotics to allow for antibiotic resistance selection.

Composition: 10g/l bactopeptone; 5g/l yeast extract; 5g/l NaCl.

***Escherichia coli* DH-5α bacterial strain**: strain widely used for plasmid amplification. Allows for blue/white selection through α-complementation of β-galactosidase, and for ampicilin resistance.

Genotype: F^- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1.

pGEM®-T plasmid vector (Figure A2a): it is a multicopy phagemid with 3003 bp, derived from Promega's pGEM®-5Zf(+). It has 3'-T overhangs at the insertion site and the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA.

pGEM®-T Easy plasmid vector (Figure A2b): it is a multicopy phagemid with 3018 bp. It has 3'-T overhangs at the insertion site and the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA. The main difference to pGEM®-T plasmid vector resides in the restriction enzyme recognition sites that flank the multiple cloning site, which are *EcoR* I, *BstZ* I and *Not* I for pGEM®-T Easy and *BstZ* I for pGEM®-T.

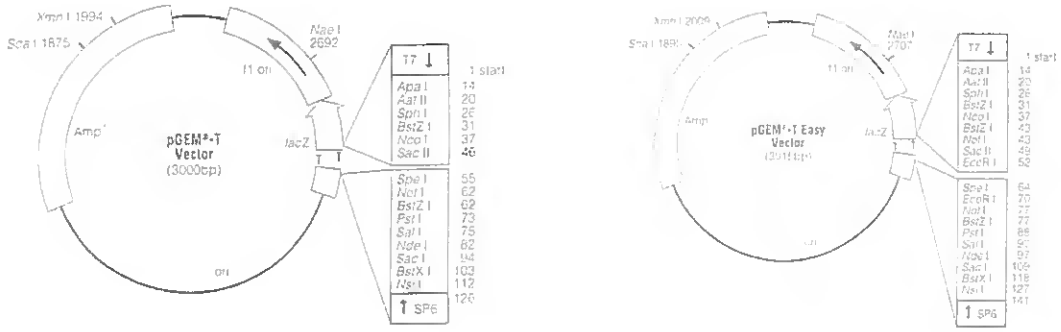

Figure A1. Schematic representation of pGEM®-T (A) and pGEM®-T Easy (B) plasmids used in this work as cloning vectors. Boxes in the right show the localization of the multiple cloning site restriction enzymes, as well as of the T7 and SP6 sequences. In the plasmid main body are represented the gene for ampicillin resistance (Amp^r), as well as the *lacZ* gene fragment and the f1 origin of replication (from Promega's technical manual).

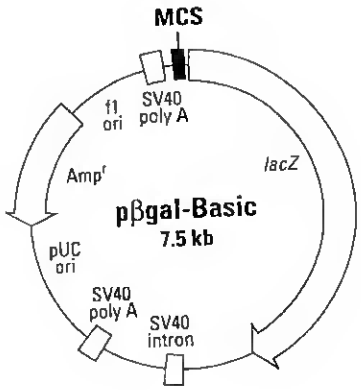

Figure A2. Schematic representation of pβgal-Basic plasmid vector, showing, among other features, the localization of the Multiple Cloning Site (MCS) the gene for ampicillin resistance (Amp^r), the *lacZ* gene, and the f1 and pUC sites for origin of replication; GenBank accession number U13184.

APPENDIX III

Cloning of the Bone Gla Protein Gene from the teleost fish Sparus aurata. Evidence for overall conservation in gene organization and bone-specific expression from fish to man.

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Cloning of the Bone Gla Protein Gene from the teleost fish *Sparus aurata*. Evidence for overall conservation in gene organization and bone-specific expression from fish to man

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Abstract

Bone Gla protein (BGP, Osteocalcin) is a bone-specific vitamin K-dependent protein which has been intensively studied in mammals. Although BGP is the most abundant non-collagenous protein of bone, its mode of action at the molecular level remains unclear. From an evolutionary point of view, the appearance of BGP seems to parallel the appearance of hydroxyapatite-containing bone structures since it has never been found in elasmobranchs, whose skeleton is composed of calcified cartilage. Accordingly, recent work indicates that, in mammalian bone, BGP is required for adequate maturation of the hydroxyapatite crystal. Taken together, these data suggest that teleost fishes, presumably the first vertebrates to develop a BGP-containing skeleton, may be a useful model to further investigate BGP function. In addition, fish offer several advantages over mammalian models, due to a large progeny, external embryonic development and transparency of larvae. In the present work, the BGP cDNA and gene were cloned from a teleost fish, *Sparus aurata*, and its tissue distribution, pattern of developmental expression and evolutionary pathways analyzed. The molecular organization of the *Sparus* BGP (spBGP) gene is similar to mammalian BGP genes, and its expression throughout development follows the onset of calcification. The spBGP gene encodes a pre-propeptide of 97 amino acid residues, expressed only in bone and showing extensive homology to its mammalian homologs. Phylogenetic analysis of the available BGP sequences supports the hypothesis that all BGPs have a single origin and share a common ancestor with a related vitamin K-dependent protein (Matrix Gla protein). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: BGP; Osteocalcin; Development; Tissue distribution; Evolution

1. Introduction

Bone Gla protein (BGP or Osteocalcin) belongs to the family of vitamin K-dependent Ca^{2+}-binding proteins and is found in bone of all vertebrates examined to date, from bony fish to mammals. In teleost fish BGP is present in the bone tissue at levels comparable to those found in mammalian bone (Cancela et al., 1995) but could not be found in the skeleton of cartilaginous fishes (i.e. shark vertebra), which is formed of calcified cartilage (Rice et al., 1994). These results suggest that BGP expression may be specific to hydroxyapatite-containing bone tissue.

All known BGPs are synthesized as pre-pro-precursors and their mature form is a small acidic protein (pI = 4.0) containing a unique sequence ranging from 46 to 50 amino acid residues, depending on the species (Cancela et al., 1995). The characteristic γ-carboxylated residues (Gla) are derived from a post-translation modification of selected glutamates (positions 17, 21 and 24 in the human BGP) through a vitamin K- and CO_2- requiring enzyme complex. They are located within a conserved motif in the central portion of the molecule, which also includes a disulfide loop (Cys-23-Cys-29 in the human BGP). The resulting tri-dimensional structure is thought to be responsible for the tight binding of BGP to hydroxyapatite. This is further corroborated by the finding that γ-carboxylation of residue 17 of human BGP is essential for the protein to exhibit its calcium-dependent conformational transition and its high affinity binding to hydroxyapatite crystals in bone (Nakao et al., 1994).

Abbreviations: aa, amino acid(s); bp, base pair(s); ORF, open reading frame; UTR, untranslated region(s); spBGP, sparus BGP; dph, days post-hatching

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In mammals, BGP is synthesized by osteoblasts and odontoblasts, being found in bone extracellular matrix and serum. Although high circulating levels of BGP are correlated with increased states of bone turnover *in vivo* (Price and Nishimoto, 1980; Tsuji et al., 1996), functional studies have failed to reveal a critical role for BGP in bone formation. In chronic treatments with the vitamin K antagonist warfarin, although the BGP levels in bone matrix were reduced to less than 2% of controls, this did not seem to affect bone formation or structure (Price et al., 1982). More recently, BGP-deficient mice were shown to develop a phenotype marked by higher bone mass, when compared to controls, due to increased bone formation (Ducy et al., 1996) and infrared micro-spectroscopy analysis has provided evidence that BGP is required for the correct maturation of the hydroxyapatite crystal in mammalian bone (Boskey et al., 1998). However, its mode of action at the molecular level remains unclear.

The amino acid sequence of BGP has been highly conserved from fish to man (Cancela et al., 1995), suggesting that its function has also been maintained, but nothing is known of its regulation of expression and appearance during development in species other than mammals and birds. Furthermore, there are no BGP cDNA or gene sequences available from lower vertebrates. In this work, we present the first DNA sequences for BGP from a lower vertebrate, the marine fish *Sparus aurata*, and compare it with those from higher vertebrates. The acquisition of these molecular tools allowed us to analyse its tissue distribution and expression throughout larval development of *Sparus*.

2. Materials and methods

2.1. The Sparus aurata model

The gilthead seabream *Sparus aurata* is a marine teleost, common in Mediterranean and Atlantic waters. It is a protandrous hermaphrodite, developing a testis during the first year of life and undergoing sex reversal to female between the second and the third years. The *Sparus* pelagic egg produces a 2 mm transparent larvae with the primordial fin, the notochord and the miomers completely developed. During the first 3–4 days of life after eclosion the pectoral fins start to develop, the eyes become functional, the brain is completely differentiated and the branchial arches are developing. Immediately after this period the mouth and the remaining parts of the digestive tract become functional and the primordial pectoral fins are completely formed. The larval stage ends when the specimens develop scales and fins are differentiated, which corresponds to the age of 40–45 dph. Calcified structures begin to appear at around 30 dph (or 70–80 mm in length), although a high variability has been observed, depending on a series of extrinsic and intrinsic factors which regulate individual development. Around 90 dph almost all calcified structures have developed, only the structures necessary for the growing of the fish remaining in the cartilaginous state (Arias, 1980, and references therein).

Due to its high commercial value, aquaculture techniques for *Sparus* have been developed since the late seventies and presently it is possible to control all stages of its life cycle (FEAP, 1997).

2.2. Cloning of a partial Sparus BGP cDNA

Total RNA from fully calcified *Sparus* juveniles was extracted using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacci, 1987). Total RNA (1 μg) was reverse transcribed at 42°C for 1 h using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) and an oligo(dT)-adapter (5′-ACGCGTCGACCTCGAGATCGATG$(T)_{18}$-3′), followed by amplification by the polymerase chain reaction (PCR), using a 5′ degenerated primer (SBG2F: 5′-TGC/TGAA/GCAC/TATGATGGAC/TACA/C/G/TGA-3′) designed according to the *Sparus* BGP (spBGP) mature protein sequence previously obtained (Cancela et al., 1995) and a reverse universal adapter (5′-ACGCGTCGACCTCGA-GATCGATG-3′). PCR reactions were conducted for 35 cycles (1 cycle: 40 s at 95°C, 30 s at 52°C and 45 s at 72°C) followed by a 10 min final extension at 72°C with AmpliTaq DNA polymerase (Perkin–Elmer). PCR products of the expected size were visualized by 1% agarose gel electrophoresis and ethidium bromide staining, excised from the gel, and eluted from the agarose slice using the Qiaex II Gel Extraction Kit (Qiagen). The resulting DNA fragments were cloned into the pGEM-T vector (Promega) and final identification was achieved by DNA sequence analysis using the Sequenase 2.0 kit (United States Biochemical Corporation) and the SP6 and T7 vector-specific primers, following established procedures (Sambrook et al., 1989).

2.3. Isolation of the full length spBGP cDNA

Poly A^+ RNA from *Sparus* was purified from 600 μg of total RNA (extracted from *Sparus* juveniles possessing a calcified skeleton, as assessed by alizarin red histological procedures; Gavaia et al., 2000) using the QuickPrep Micro mRNA Purification Kit (Pharmacia). This purified mRNA was used to identify the 5′-end of spBGP cDNA by 5′-RACE PCR with the Marathon™ cDNA amplification kit (Clontech). Amplification of the 5′-RACE-PCR products thus obtained was accomplished with Advantage Klen *Taq* polymerase, using the AP1 oligo (both from Clontech) and a specific reverse primer, SBG4R (5′-GTTAGGGGAAAT-GATCGAATCACAGTGGG-3′), designed according to the partial spBGP cDNA sequence previously obtained. Amplification conditions were those suggested by the supplier. The PCR product obtained was fractionated by agarose gel electrophoresis, purified from the gel and cloned

Table 1
Oligonucleotides used for PCR amplification of *Sparus* BGP cDNA and gene

Primer	Sequence[a]	Localization in the gene[b]
SBG2F	TGYGARCAYATGATGGAYACNGA[c]	+ 2453
SBG3R	GGGGATCGGTCCGTAGTAGG	+ 2518
SBG4R	GTTAGGGGAAATGATCGAATCACAGTGGG	+ 2733
SBG5F	TGCGAGCACATGATGGACACTGAGGGAATC	+ 2453
SBG7R	GAACCAGGAAGGCCAGAGTC	+ 124
SBG8F	TTCGTGGAGAGGGACCAGGC	+ 2121
SBG9R	GGGGATCGGTCCGTAGTAGG	+ 2518
SBG11R	CCATCAGCTGTCGTAGTAAGGC	+ 2548
SBG12F	GAGCTGGAAGTCTCCGGTCCG	+ 33
SBG13F	CCAGCCTGCCAGTGACAACCC	+ 371
SBG14R	GGTTGTCACTGGCAGGCTGG	+ 390
SBG15R	CCGCTCTCTTCTGTCTCACC	+ 2169
SBG18F	CGGTAAGTTGCATCAAACGG	+ 295
SBG19R	CCACTGCGGAGGCCTGGTCCCTCTCC	+ 2126
SBG20R	GTGGAGGCATCTGAGGGAAAACATCTCG	+ 364
SBG21R	CGGACCGGAGACTTCCAGCTCTGTCAC	+ 53
SBG22F	GACAAGGCACCAGCATTGACC	− 1119
SBG26R	GACGTTTCTATCGGCCAT	+ 1912
SBG27R	CCCGGGTTGTATGTGC	+ 1589
SBG28F	CGGAACACTGTTTGAAG	+ 573
SBG29F	CCCCAAACTACATAGTGC	+ 774
SBG30R	CAGAGTACAACTGAGCAC	+ 1385

[a] All sequences are described in the 5′ to 3′ direction.
[b] Corresponds to localization of the first nucleotide (5′ end of oligonucleotide) in the sequence of the spBGP gene.
[c] Y, pyrimidine; R, purine; N, G + a + T + c.

in pGEM-T Easy vector (Promega). Sequencing was performed as described above.

2.4. Cloning and sequencing of spBGP gene and 5′ flanking region

Genomic DNA was extracted from adult *Sparus* tissues as described (Sambrook et al., 1989) and its integrity checked by loading an aliquot onto a 0.8% agarose gel. Several oligonucleotide pairs were then used to selectively amplify overlapping regions of the BGP gene (see Table 1 for oligonucleotide sequence and localization). PCR reactions were performed using 0.4 μM of each primer, 1.5 mM $MgCl_2$, 0.05 mM of each nucleotide and 1 Unit of Taq DNA Polymerase (GibcoBRL). After an initial denaturation step (5 min, 95°C), amplification was performed for 35 cycles (one cycle: 2 min at 95°C, 1 min at 60°C, 2 min at 68°C) followed by a final elongation period of 12 min at 68°C. The resulting PCR products were cloned in pGEM-T Easy (Promega) and DNA sequenced. All exons and exon/intron borders were sequenced on both strands. The 5′ flanking region was obtained with the Universal Genome Walker™ Kit (Clontech) as specified by the manufacturer and the resulting genomic PCR products were cloned in pGEM-T Easy and sequenced as described.

2.5. Mapping of spBGP gene start site of transcription

The transcription start site of the spBGP gene was mapped by 5′-primer extension analysis. Fifteen micrograms of total RNA, extracted from the calcified jaw of a juvenile *Sparus* by established methods (Chomczynski and Sacci, 1987), were annealed to a reverse primer (SBG14R, Table 1) extending from +75 to +94 bp downstream from the initiation ATG codon (Fig. 1). The extension reaction was performed for 5 min using the M-MLV reverse transcriptase (Gibco BRL), and continued for an additional 60 min, after addition of 10 μCi [α-^{32}P] dCTP. The resulting single strand cDNA was RNase-treated for 15 min at 37°C and then phenol/chloroform-extracted and ethanol precipitated. Pellets were re-suspended in 5 μl of formamide-containing sequencing dye, heat-denatured (5 min at 65°C) and loaded onto a 6% bis-acrylamide, 7 M urea sequencing gel. Autoradiography was performed using Kodak X-Omat AR film (Sigma) with two intensifying screens at −30°C for 15 h. The size of the amplified fragments was determined from a known DNA sequence reaction loaded on adjacent lanes.

2.6. Northern blot analysis

Total RNA was extracted from *Sparus* tissues (vertebra, jaw, heart and liver) and from *Sparus* whole larvae collected at different developmental stages (27, 86 and 130 days post-hatching (dph)) and size-fractionated by electrophoresis on a 1.4% formaldehyde-containing agarose gel. The RNA in the gel was transferred onto N^+ Nylon membranes (Schleicher & Schuell) by a capillary method (Sambrook et al., 1989) and pre-hybridized at 42°C in 50%

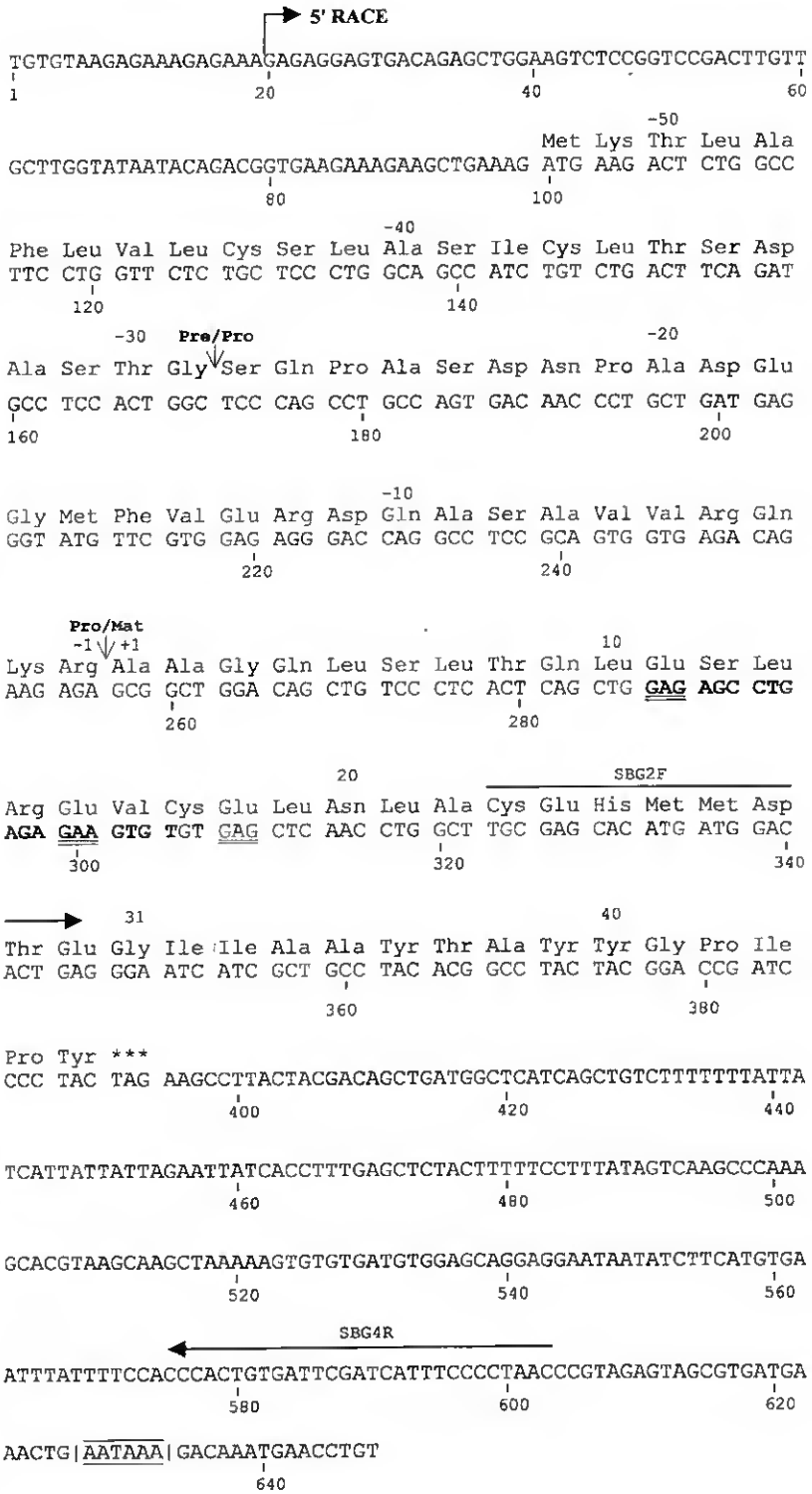

Fig. 1. Complete nucleotide sequence of the cDNA encoding *Sparus* BGP. Numerical positions in the nucleotide sequence beginning at the cap site (+1, as determined by 5′ primer extension), are noted below the sequence. The 5′ end obtained by 5′ RACE experiments is signalled. Amino acid residues are numbered according to residue 1 of the mature protein and are shown above the respective sequence. The stop codon is indicated by asterisks and the polyadenylation signal is boxed. The codons for the Gla residues are underlined twice. Sequences used for construction of SBG2F and SBG4R oligonucleotides are denoted by horizontal arrows. The Pre/Pro (deduced from comparison with other BGP sequences) and Pro/Mature (inferred from Cancela et al., 1995) cleavage sites are shown by vertical arrows and putative γ-carboxylase recognition site is highlighted in bold.

formamide, 5 × Denhardt's solution, 5 × SSPE and 50 μg/ml Calf Thymus DNA for 2–3 h. A spBGP cDNA probe (spanning from nucleotide 322 to 602 of the spBGP mRNA) was labelled with [α-^{32}P] dCTP using the Prime-It® II Random Primer Labeling Kit (Stratagene) and separated from unincorporated nucleotides on a NACS 52 PREPAC® column (GibcoBRL). The purified labelled probe was added to the pre-hybridization solution and incubated with the membrane overnight under the same conditions described for pre-hybridization. Blots were washed twice in 6 × SSPE [1 × SSPE is 180 mM NaCl, 10 mM $NaPO_4$ (pH 7.4), and 1 mM EDTA], 0.1% SDS at room temperature for 15 min and twice in 1 × SSPE, 0.1% SDS at 55°C for 30 min. Autoradiography was performed with Kodak X-Omat AR film with two intensifying screens at −30°C for up to one week.

2.7. RT-PCR amplification and Southern hybridization of BGP message in different tissues and developmental stages of Sparus

One microgram of total RNA extracted from tissues (vertebra, jaw, heart, liver, and muscle) and whole *Sparus* specimens from various developmental stages (neurula, 2, 3, 18, 27, 37, 47, 61, 75, 82, 91 and 130 dph) was treated with RNase-free DNase I for 3 h (37°C) and reverse transcribed using the same conditions described above. One twentieth of each reaction was amplified by PCR, using two specific oligonucleotide primers designed according to the spBGP cDNA sequence obtained previously (SBG8F and SBG11R). PCR reactions were carried out with Taq DNA polymerase (Promega) for 20 cycles (one cycle: 30 s at 95°C, 40 s at 60°C and 45 s at 68°C) followed by a final extension period of 10 min at 68°C. Positive and negative controls were made by amplifying, respectively, a clone of spBGP cDNA and a sample without DNA template with the same primers. As an internal control for the relative amount of RNA used for each sample, *Sparus* beta-actin was also amplified from the same amount of RT reaction, using two specific primers designed according to the published *Sparus* beta-actin cDNA (GenBank Accession # X89920; forward primer: 5′-TTCCTCGGTATGGAGTCC-3′; reverse primer: 5′-GGACAGGGAGGCCAGGA-3′). Resulting PCR products were Southern transferred onto a N^+ Nylon membrane (Schleicher & Schuell) and prehybridized at 42°C for 3 h using 5 × SSPE, 10 × Denhardt's, 0.5% SDS and 50 μg/ml heat denatured calf thymus DNA. Hybridization was performed for 15 h at 42°C in 50% formamide, 6 × SSPE, 0.5%SDS, 50 μg/ml heat-denatured calf thymus DNA and the *Sparus* cDNA fragment labelled with [α-^{32}P]-dCTP, as described above. Membranes were washed with 6 × SSC, 0.2% SDS (2 × 10 min, room temperature), 2 × SSC, 0.2% SDS (30 min., 42°C) and 0.1 × SSC, 0.2%SDS (60 min., 55°C). Autoradiography was performed with Kodak X-Omat AR film and two intensifying screens at −30°C.

2.8. Genomic Southern analysis

Aliquots (20 to 50 μg) of *Sparus* genomic DNA were digested with different restriction enzymes then ethanol precipitated, re-suspended in sample loading buffer and size-fractionated by electrophoresis on a 0.8% agarose gel for 9 h at 35 V. DNA markers (lambda DNA-HindIII digested and 1.0 kb ladder from GibcoBRL) were loaded in adjacent lanes in the gel. Following electrophoresis, the DNA was transferred to an N^+-Nylon membrane (Schleicher & Schuell) by a capillary method (Sambrook et al., 1989) and pre-hybridized at 42°C for 3 h in 6 × SSPE, 10 × Denhardt's solution, 0.5% SDS and 50 μg/ml calf thymus DNA. Hybridization was carried for 24 h in a solution containing 6 × SSPE, 0.5% SDS, 50% formamide, 50 μg/ml calf thymus DNA and either the full length or a 280 bp (from nucleotides 322 to 602) spBGP cDNA probe, labelled with [α-^{32}P] dCTP, as described above. Washing was performed with 6 × SSPE, 0.1% SDS (2 × 15 min, room temperature), 1 × SSPE, 0.1% SDS (2 × 15 min, 37°C) and 0.1 × SSPE, 0.5% SDS (30 min, 65°C). Autoradiography was performed with Kodak X-Omat AR film and two intensifying screens at −80°C for 8 days.

2.9. Localization of BGP gene expression by in situ hybridization

2.9.1. Animals and tissue preparation:

Sparus specimens collected at various developmental stages were fixed overnight at 4°C in freshly made 1% paraformaldehyde solution, washed 3 × 10 min. in TBST buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 0.1% Triton X-100), and stored in methanol at 4°C. Samples were dehydrated by passing them through an increasing alcohol series and embedding in paraffin. Tissues were cut into longitudinal 5 μm thick sections and mounted on slides pre-coated with 10% poly-L Lysine (Sigma) or Vectabond® (Vector Laboratories), dried for 48 h at 42°C, and kept at room temperature until use.

2.9.2. In situ hybridization

Digoxigenin-11-UTP-labeled single stranded RNA probes were prepared with a DIG RNA Labeling kit (Boehringer-Mannheim Biochemica), according to the manufacturer's instructions. A 325 bp fragment of spBGP cDNA (spanning from nucleotide 322 to the 3′end of the cDNA; see Fig. 1) was used to generate sense and anti-sense riboprobes. Transcription products were treated with RNase-free DNase and recovered by ethanol precipitation. One tenth of the RNA obtained was analyzed by electrophoresis onto a formaldehyde-containing agarose gel in order to check for RNA size and quality. The tissue sections obtained as described above were treated to remove the paraffin, hydrated in a decreasing alcohol series and incubated with 40 μg/ml of Proteinase K in Tris–HCl (pH 8.0) for 15 min at room temperature. Sections were fixed again with 4%

formaldehyde for 30 min then washed twice with PTW (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 0.1% Tween-20) for 5 min.

Pre-hybridization was carried at 55–60°C in 50% formamide, 4× SSC, 1× Denhardt's solution, 1 μg/ml yeast tRNA, 0.04% CHAPS ((3-Cholamidopropyl) dimethylammonium-1-propanesulfonate; Sigma) and 0.1 mg/ml heparin for 3 h. Hybridization was performed in the same buffer after addition of 0.1–1 μg/ml of digoxigenin-UTP-labeled RNA probe, previously diluted with buffer and denatured at 80°C for 5 min. Approximately 50 μl of solution was added to each section prior to covering it with parafilm and incubating at 55–60°C overnight. Following hybridization, sections were washed in 2× SSC at 55–60°C for 3×20 min then digested with RNase A (10 μg/ml) for 3 min at 37°C. A second series of washings was performed for 2×20 min with SSC/CHAPS (1.4× SSC, 0.6% CHAPS) at 55°C, once with PTW for 5 min at room temperature, and once with 1:1 PTW/100 mM maleic acid, 150 mM NaCl, pH 7.5, for 10 min at room temperature. Blocking was carried out for 1 h with blocking buffer (Boehringer-Mannheim Biochemica) at room temperature, followed by three washes with PTW at room temperature. Washed sections were incubated for 10 min with colour buffer (50 mM Tris, pH 9.5; 50 mM $MgCl_2$; 100 mM NaCl; 0.1% Tween 20) and then for several hours to overnight with colour reagent (337.5 μg/ml of nitroblue tetrazolium salt, 165 μg/ml of 5-bromo-4-chloro-3-indolyl-phosphate in colour buffer), at room temperature, with constant agitation. Slides were examined for the appearance of maximal signal-to-noise results, then washed in distilled water and mounted with DPX (BDH Laboratory Supplies).

2.10. Alcian blue and alizarin red whole mount staining

Sparus specimens collected at different stages throughout development were fixed, washed and stored as described previously in Section 2.9.1. Whole specimens were hydrated through a decreasing alcohol series and stained for cartilage with Alcian blue 8GX (Sigma, CI 74240) for various periods of time, according to size (Gavaia et al., 2000). Specimens were removed from the staining solution immediately after visible stained structures were observed, in order to avoid decalcification of small structures by the glacial acetic acid-ethanol solution. The specimens were then once again hydrated by passing through a six step decreasing alcohol series and incubated in 1% KOH at room temperature until cleared. Staining with Alizarin red S (Sigma, CI 58005) was performed as with Alcian blue, followed by incubation in a series of glicerol-1%KOH baths (starting at 1:3 ratio and increasing until 3:1). Storage in absolute glycerol was only initiated when the specimens were completely clear, with all the internal structures clearly visible. Five to ten microlitres of alkaline-equilibrated phenol were added to glycerol to prevent contamination.

2.11. Evolutionary analysis of spBGP

Phylogenetic analysis of BGP evolution was performed using all available amino acid BGP sequences and using as outgroup all known amino acid Matrix Gla Protein (MGP) sequences, as well as Pacific hagfish prothrombin and human coagulation factor II sequences obtained from GenBank. Character state changes were all weighted equally and insertions/deletions were coded as missing data, in order to avoid including them as if they were many independent events. All analyses were performed with PAUP 4.0b4a software (Swofford, 1998). Phylogenetic inferences used maximum parsimony as optimization criterion, with the heuristic algorithm. Parsimony methods search for minimum length trees, i.e. trees that minimize the amount of evolutionary change needed to explain the available data under a pre-specified set of constraints and upon permissible character changes. Confidence limits of individual clades were estimated by bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

3. Results

3.1. Cloning of the S. aurata BGP cDNA

Based on the amino acid sequence of mature spBGP previously obtained (Cancela et al., 1995), an oligonucleotide (SBG2F, Table 1 and Fig. 1) was designed and used to amplify a partial BGP cDNA (325 bp) by RT-PCR, using total RNA extracted from *Sparus* juveniles with a calcified skeleton (as assessed by alizarin red staining). This DNA fragment extended from amino acid 23 of the mature protein to the stop codon and extended an additional 254 bp to the site of insertion of the poly-A tail, 15 bp after a consensus polyadenylation signal (Fig. 1). The 5′ end of spBGP cDNA was obtained by 5′ RACE-PCR using a reverse primer (SBG4R, Table 1 and Fig. 1), as described in Section 2. As deduced from the comparison with the complete cDNAs for mammalian and chicken BGPs (Fig. 2 and Table 2), the spBGP cDNA encodes a pre-peptide of 24 residues, a pro-peptide of 28 residues and a mature protein of 45 residues. The later is in full agreement with the protein sequence previously obtained for mature BGP following its purification from the *Sparus* vertebra (Cancela et al., 1995). Although the *Sparus* cDNA is longer than its mammalian and avian counterparts, this difference is due to longer 5′and 3′UTRs, while the coding region is of a size comparable to those from all known BGP cDNAs (Table 2).

3.2. Molecular organization of the Sparus BGP gene

The fish BGP gene spans 2778 bp (Fig. 3) and is organized into four exons and three introns (the first two of phase I while the third intron is of phase II, Table 3), in agreement with the structure of all known BGP genes (Table 4). Comparison with the cDNA sequence allowed the identifi-

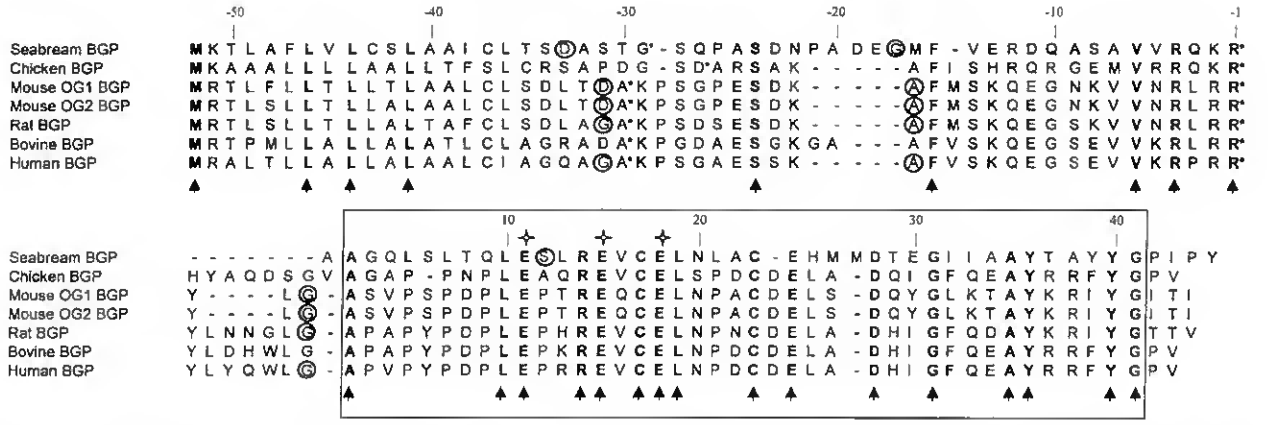

Fig. 2. Amino acid sequences of all known complete pre-proBGPs. Sequences were aligned and conserved residues marked by vertical arrows. A box encloses the most conserved region of the protein and a star is located above each gamma-carboxyglutamate residue. Dashes indicated gaps in the sequence, introduced to increase homology. A circle encloses amino acids corresponding to sites of intron insertion in the corresponding genes. Residues are numbered according to residue 1 of mature *Sparus* BGP protein. Sequence GenBank accession numbers are: AF048703 for *Sparus*; U10578 for chicken; X04142 for mouse OG1 and OG2; X04141 for rat; X53699 for bovine and X53698 for human.

cation of exon-intron splice junctions (Table 3), all of which conform to the AG/GT rule (Breathnach et al., 1978). The sites of insertion of the three introns within the protein coding sequence differ in *Sparus* as compared to the mammalian genes (Figs. 2 and 3), but each exon encodes roughly the same protein domain in both fish and human genes (Table 4). Primer extension analysis revealed two possible sites of transcription initiation, 99 and 78 bp upstream from the first ATG (Fig. 4). The site corresponding to the longest 5′UTR was found to be located, in the genomic DNA, 26 bp downstream from a consensus TATA box motif and was considered to correspond to the major start site of transcription of the spBGP gene (Fig. 3). According to these results, the size of the full length BGP cDNA was then redefined as 647 bp, numbered from the major cap site.

3.3. Analysis of the Sparus BGP gene promoter

In addition to the gene itself, 1145 bp of 5′-flanking DNA were sequenced and analyzed. The spBGP gene promoter has a modular organization with sequence motifs typical of a gene transcribed by the RNA polymerase II (Fig. 3), such as the canonical TATA box (TATAAA) located from −31 to −25 bp and putative CCAAT boxes found at positions −256 and −67 (Fig. 3). We have also identified a series of putative consensus sequences for steroid receptor binding sites and bone-specific transcription factors, known to be physiological mediators of BGP gene expression in higher organisms. Between positions −406 and −390, a sequence motif sharing homology with a steroid hormone response element (SRE) was identified. In addition, several OSE1 and OSE2-like motifs (Ducy and Karsenty, 1995) and one AP1-like motif (Ozono et al., 1990) in intron I were also identified. Interestingly, intron II was found to contain copies of many of the putative response elements already identified within the proximal promoter of the *Sparus* BGP gene, including a TATA consensus sequence and two CCAAT boxes.

3.4. Tissue distribution of BGP in Sparus

Of the different tissues analyzed by Northern hybridization, only calcified tissues (vertebra and jaw, Fig. 5A) showed the presence of BGP mRNA, with the strongest

Table 2
Characteristics of known BGP cDNAs and corresponding proteins. Sizes of all known BGP cDNAs, including 5′ and 3′ untranslated regions (UTR) and coding regions are indicated. Sparus 5′ UTR is according to site of transcription initiation, as determined by primer extension analysis. Comparison of sizes for pre-, pro- and mature forms of corresponding BGPs are also shown. GenBank accession numbers are provided in the right column (a.a = amino acid residues, bp = base pairs)

Species	cDNA size (bp)*	5′ UTR (bp)	Coding region (bp)	3′ UTR (bp)*	Protein Size (a.a.)			Accession numbers
					Pre	Pro	Mat.	
Homo sapiens	451	18	300	133	23	28	49	X53698
Bos taurus	437	27	300	110	23	28	49	X53699
Mus musculus	458	48	285	125	23	26	46	X04142
Rattus norvegicus	474	36	297	141	23	26	50	X04141
Gallus gallus	421	18	291	112	24	24	50	U10578
Sparus aurata	648	99	291	256	24	28	45	AF048703

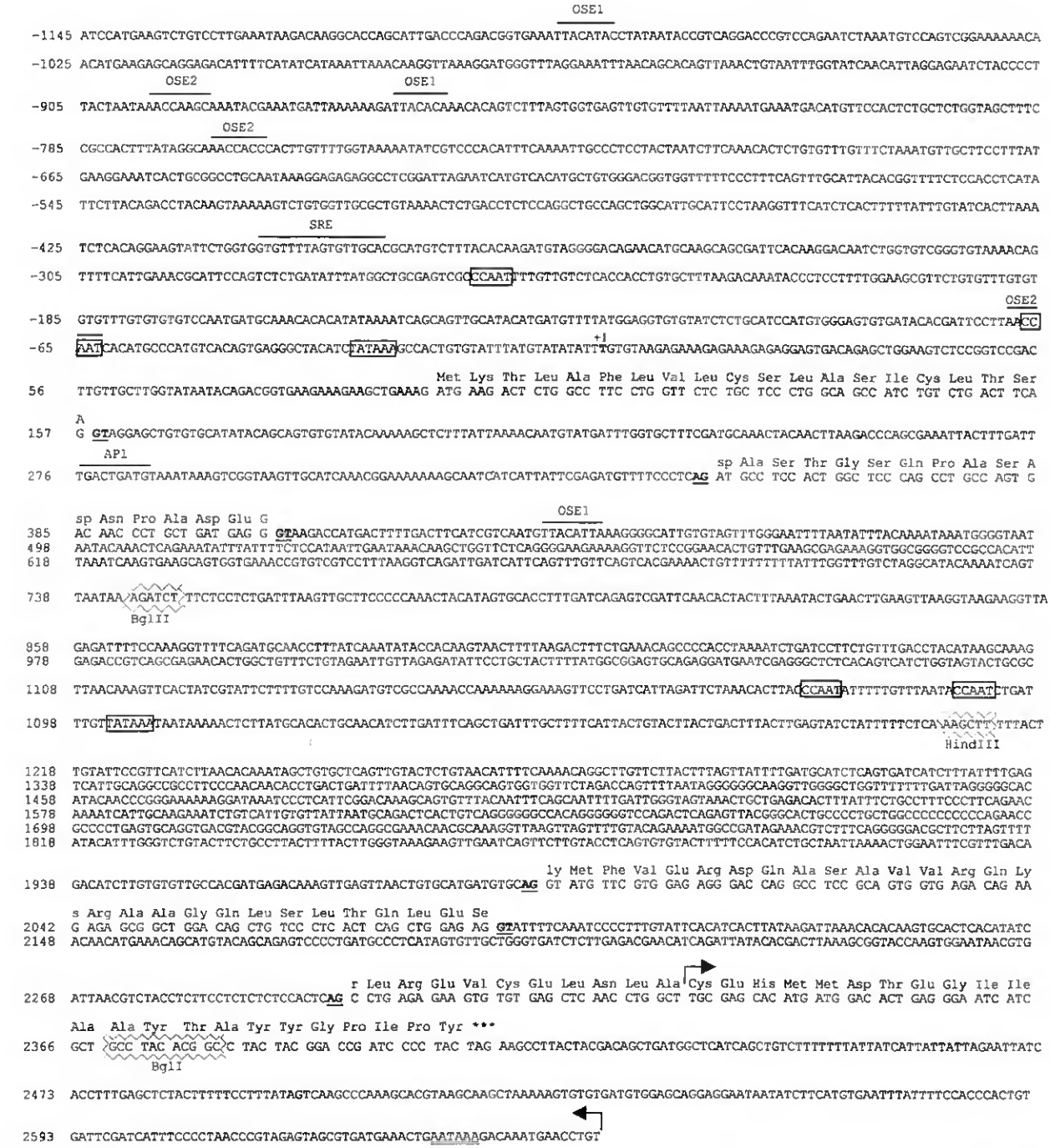

Fig. 3. Sequence of *Sparus* BGP gene and 5′-flanking region. The major site of transcription initiation is designated as +1 and the corresponding nucleotide is bold. Nucleotides are numbered in the left margin and the predicted amino acid sequence is shown above the coding sequence. The stop codon is indicated by asterisks and the polyadenylation signal is underlined twice. Consensus sequences at the intron borders are underlined and bold. Putative TATA and CCAAT motifs are boxed. The location of putative steroid-responsive elements (SRE) and transcription factor consensus sequences (OSE1, OSE2 and AP1) is indicated. Curved arrows mark the localization of the partial spBGP cDNA probe used in southern genomic hybridization.

signal observed in vertebra. No positive hybridization was seen in the other tissues analyzed (liver and heart, Fig. 5A; kidney, muscle and brain, results not shown), even after a longer exposure of the membrane (up to two weeks). The tissue distribution of BGP in *Sparus* was confirmed by a combination of a semi-quantitative assay (RT-PCR, 20 cycles) and Southern analysis. Amplification was performed using 1 μg of total RNA extracted from tissues showing a positive signal for spBGP mRNA by Northern analysis (vertebra, jaw) and from several of those showing no signal

Table 3
Exon-intron splice junctions and type of intron in *Sparus aurata* BGP gene. 5′ and 3′ borders for each intron are indicated. The consensus motif (5′gt...ag 3′), as described by Breathnach et al. (1978), is shown in bold. Type of intron is shown according to Patthy (1987)

Intron number	Intron borders		Type of intron
	5′ border	3′border	
Intron 1	..TCA.G gtag..	..tcag AT.GCC.	I
Intron 2	..GAG.G gtaa	..gcag GT.ATG.	I
Intron 3	.GAG.AG gtat	..tcag C.CTG.	II

(muscle, liver and heart). The presence of BGP mRNA was once again detected only in jaw and vertebra (Fig. 5B).

3.5. Expression of BGP mRNA throughout developmental stages of Sparus

Northern analysis showed that spBGP mRNA is abundant in fish larvae with 86 dph and older, which correspond to post-larval (juvenile) stages known to have a nearly fully mineralized skeleton (as detected by alcian blue/alizarin red staining; Fig. 5A). No BGP mRNA was detected at 27 dph with this method. Southern hybridization of spBGP mRNA amplified by RT-PCR (20 cycles, see Section 2 for details) produced positive signals in stages of *Sparus* development as early as 37 dph (Fig. 5B). Thereafter, a progressive increase in the strength of the signal detected for spBGP mRNA was observed as the specimens grew older (Fig. 5B). No signal was detected for 27 dph or younger, even after longer times of exposure of the autoradiography (results not shown).

3.6. Analysis of the BGP gene locus

Because evidence for BGP gene duplication exists in mammals (Desbois et al., 1994), we used genomic Southern analysis and gene amplification by PCR coupled with DNA sequence analysis to search for the possible existence of a cluster of BGP genes in the fish genome. Samples of *Sparus* genomic DNA were digested with restriction enzymes which, according to our sequence, should cut within the *Sparus* BGP gene (BglI, BglII and HindIII) or in its flanking DNA (EcoRI, BamHI and PstI), and analyzed by Southern hybridization using a specific probe spanning the full length spBGP cDNA. The positive signals observed for each restriction enzyme digestion (BglI, BglII, EcoRI, HindIII, and PstI) correspond to the expected number of fragments based on the known restriction map of the spBGP gene (Figs. 3 and 6A). A second genomic southern (using DNA digested with HindIII, BamHI, PstI and BglI) was hybridized with a partial spBGP cDNA (spanning from nucleotide 322 to 602). This clone was expected, from the known restriction map of the *Sparus* BGP gene, to hybridize only with the fragments located at the 3′end of the spBGP gene. The results obtained showed only the one expected fragment for each enzyme digestion (Figs. 3 and 6B), with the exception of BglI. In this case one site was located within the genomic DNA covered by the probe used, and, therefore, two genomic fragments should have hybridized to this probe. However, since this BglI site was located 43 bp from the 5′ end of the probe used (Fig. 3), the second genomic fragment (the top BglI fragment seen in Fig. 6A) would give only a very weak positive signal and was not detected in our autoradiography. In a second approach, we investigated the possibility of the existence of one or more homologous BGP genes with different intronic sequences. Since we found that the spBGP gene contains three introns, several primer sets were constructed, each set consisting of a pair of primers located in two consecutive exons and designed to amplify the intervening sequence located between them. Each intron was thus amplified using different pairs of oligonucleotide primers (Table 1). This resulted in the amplification, in each case, of a single PCR product of the expected size, as deduced from the molecular organization of the spBGP gene. After cloning and sequencing, each DNA fragment analyzed was shown to span the exon-intron border closest to the PCR primers used and to expand into the known intronic sequence. No differences were detected after comparison with our spBGP gene sequence.

A third approach consisted of amplifying the BGP cDNA by RT-PCR from different stages of development of *Sparus* and cloning and sequencing the resulting PCR products. Two internal primers were used (SBG5F and SBG4R; see Table 1) and spBGP mRNA was amplified from samples at 27 and 130 dph, corresponding to the appearance of first calcified structures and almost complete calcification (as detected by alizarin red staining; see Fig. 7B). All DNA sequences obtained were identical to the previously obtained spBGP cDNA (results not shown).

3.7. Detection of BGP mRNA in sagital sections of Sparus by in situ hybridization

Localization of specific sites of expression of the BGP gene was determined by *in situ* hybridization in *Sparus* sagital sections, using an antisense riboprobe specific to spBGP mRNA. The presence of BGP message was detected in vertebra, jaw, sites of fin insertion and dermis, where the scales are originated. Results obtained in vertebra and dermis are shown in Fig. 7A (panels A1–A3). These results indicate that the BGP gene is expressed in tissues undergoing mineralization. The control hybridization with sense spBGP riboprobe did not produce any positive signal (data not shown).

3.8. Evolutionary analysis of spBGP

The phylogenetic analysis performed using all known amino acid BGP sequences yielded thirty Maximum Parsimony (MP) trees, which were summarized using Strict Consensus (i.e. retaining only the clades that are common to all thirty MP trees). This can be considered to be the most conservative estimate of the evolutionary patterns. The

Table 4
Characteristics of known BGP genes structures. Gene, exon and intron sizes (in base pairs, bp) are indicated for all known BGP genes. Phase of intron is defined according to Patthy (1987). GenBank accession numbers for all gene sequences are indicated in the right column

Species	Gene size (bp)	Exon 1	Intron 1 (type)	Exon 2	Intron 2 (type)	Exon 3	Intron 3 (type)	Exon 4	Accession No.
Sparus aurata	2778	99 (5′ UTR) + 58	197 (I)	47	1713 (I)	86	221 (II)	100 + 257 (3′ UTR)	AF289506
Rattus norvegicus	1145	49 (5′ UTR) + 64	148 (I)	33	143 (I)	70	200 (II)	130 + 308 (3′ UTR)	M25490
Mus musculus	950	48 (5′ UTR) + 64	144 (I)	33	142 (I)	58	206 (II)	130 + 125 (3′ UTR)	L24429
Homo sapiens	1077	18 (5′ UTR) + 64	257 (I)	33	175 (I)	70	201 (II)	127 + 132 (3′ UTR)	X04143

Strict Consensus tree (Fig. 8) shows two clearly distinct clades assembling, respectively, BGPs *versus* MGPs (with the exception of shark MGP). Inside the BGP clade there is a clear separation between fish BGP and other BGPs, the same occurring with bird and amphibian BGPs.

4. Discussion

In the present work we describe the first DNA sequences (cDNA and gene) for Bone Gla Protein from a lower vertebrate species. In addition, we provide clear data on its tissue distribution and developmental appearance and comment on the conservation of BGP structure and function throughout evolution.

4.1. Analysis of the spBGP cDNA and corresponding protein

The *Sparus* BGP cDNA comprises 647 bp, and encodes a pre-propeptide of 97 amino acid residues. The pro-region contains a sequence motif homologous to the gamma carboxylase recognition site found in all other known BGPs and ends, as expected, with two basic residues, in this case Lys–Arg, as in the chicken protein (Neugebauer et al., 1995), and not Arg–Arg as in the mammalian BGPs

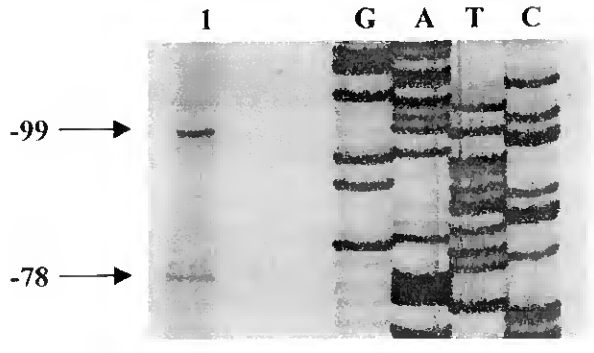

Fig. 4. Identification of transcription start site of the spBGP gene as determined by primer-extension. Poly $(A)^+$ RNA isolated from *Sparus* jaw was annealed to a spBGP reverse primer, reverse-transcribed and subjected to Rnase digestion, electrophoretic fractionation and autoradiography. The extension products are indicated in lane 1 and their sizes are shown on the left margin (lane 2 shows a fragment of known size). A sequencing ladder (lanes G, A, T and C) was fractionated in the same gel for assignment of transcription start sites at single nucleotide resolution.

(Fig. 2). Such dibasic residues are a common feature in propeptide sequences from proteins known to require proteolytic activation, such as peptide hormones and clotting factors (e.g. Choo et al., 1982).

Comparison of the protein sequences deduced from all available BGP cDNAs (Fig. 2) shows a conserved region spanning from amino acid 2 to 41 in the *Sparus* sequence (46% of identity at the amino acid level between *Sparus* and human in this region). This region encompasses the three glutamic acid residues that are γ-carboxylated in the mature protein (residues 11, 15 and 18) and the two invariable cysteine residues at positions 17 and 23 required for the disulphide bridge. In this region, a total of 16 invariant residues were identified. Their presence at those specific sites indicates that they must be required for adequate protein folding and function, since they have been fully conserved over more than 400 million years of evolution, the estimated time since divergency of fishes from higher vertebrates. When comparing the complete pre-prosequence of fish BGP with those from higher vertebrates, two regions in the fish sequence have either an insertion (from −21 to −17, Fig. 2) or a deletion (located at the N-terminus of the fish BGP, between residues −1 and +1, Fig. 2). Since these sites are the only two where significant changes also occur in some of the higher vertebrate sequences (Fig. 2), we conclude that they are most likely sites where changes affect neither the folding (and thus stability) nor the function of the protein. In addition, all BGP cDNAs, from fish to man, encode polypeptide chains with pre, pro and mature protein regions comparable in size (Table 2). These results suggest that some features in the protein structure and size must be conserved in order for BGP to adopt a functional three-dimensional structure in all species.

4.2. Bone specific expression of the spBGP gene

The results reported in this work show that in *Sparus*, as previously reported for other species (Price et al., 1988; Desbois et al., 1994), the expression of BGP is specific to bone tissues undergoing mineralization such as vertebra and jaw, as clearly seen by Northern blot and RT-PCR (Fig. 5). This conclusion is supported by *in situ* hybridization results, where specific message was detected exclusively in vertebra, jaw bone, sites of fin insertion and dermis, this last result being attributed to the production of BGP mRNA

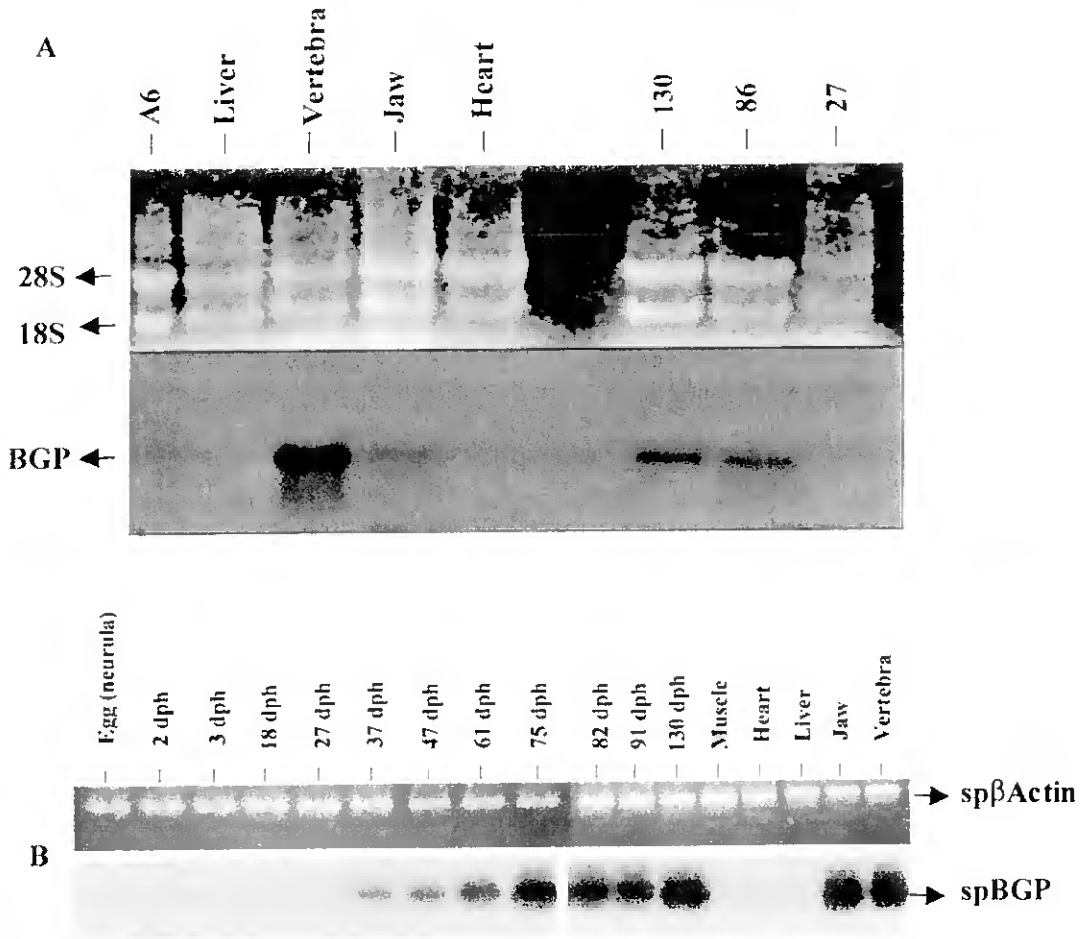

Fig. 5. (A) Analysis of tissue distribution and developmental expression of spBGP mRNA by Northern blotting. Total RNA was extracted from several tissues (liver, vertebra, jaw and heart) and developmental stages (27, 86 and 130 dph) of *Sparus*, size fractionated by denaturing agarose gel electrophoresis and transferred to a nylon membrane. RNA integrity was assessed by ethidium bromide staining of 28 and 18S ribosomal RNAs (top panel). Expression of spBGP was detected following hybridization with a ^{32}P-labelled spBGP cDNA (bottom panel). Total RNA from the *Xenopus laevis* cell line A6 was used as negative control. (B) Detection of spBGP mRNA by RT-PCR. Total RNA was extracted from developmental stages (neurula to 130 dph) and tissues (Muscle, Heart, Liver, Jaw and Vertebra) of *Sparus* and used to amplify spBGP mRNA by RT-PCR, using two specific primers (SBG8F and SBG4R, see Table 1), as described in Section 2. The resulting PCR products were size fractionated by agarose electrophoresis, transferred to a nylon membrane and hybridized with a ^{32}P-labelled spBGP cDNA. To check for RNA integrity, the same RT reactions were used to amplify spβActin mRNA using two specific primers based on the published sequence (see Section 2 for details).

by the scale-forming cells. Based on our results we conclude that, as in all other species analyzed, the spBGP gene is expressed only in bone tissue.

4.3. Molecular organization of the spBGP gene

The *Sparus* BGP gene spans 2778 bp of genomic DNA, from the major start site of transcription to the site of insertion of the poly(A) tail, and contains three introns which account for nearly 78% of the total DNA of this gene.

From all the regulatory sequences previously identified in the promoters of mammalian BGP genes, only OSE1 and OSE2-like sequence elements were readily identified within the spBGP gene promoter. These DNA motifs have been shown, in mammals, to mediate binding of bone-specific transcription factors. The OSE1 sequence binds OSF1, an osteoblast-specific transcription factor present in nuclear extracts of osteoblastic cell lines and primary osteoblasts, and is thought to be required for the early steps of osteoblast differentiation (Schinke and Karsenty, 1999). OSE2 binds CBFA1/OSF2, a transcription factor which, in mammals, is expressed only in osteoblastic cells and is responsible for the bone specific expression of BGP (Ducy et al., 1997). Since the fish BGP gene is also expressed specifically in bone tissues, as shown in this work, it is likely that its promoter is similarly regulated by bone specific transcription factors. This further suggests that fish homologs of OSF1 and OSF2 could be responsible for this tissue-specific regulation.

Vitamin D, through its active metabolite 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] plays a key role in the transcriptional regulation of BGP gene expression in mammalian osteoblasts, both *in vivo* and *in vitro* (Pan and Price, 1984;

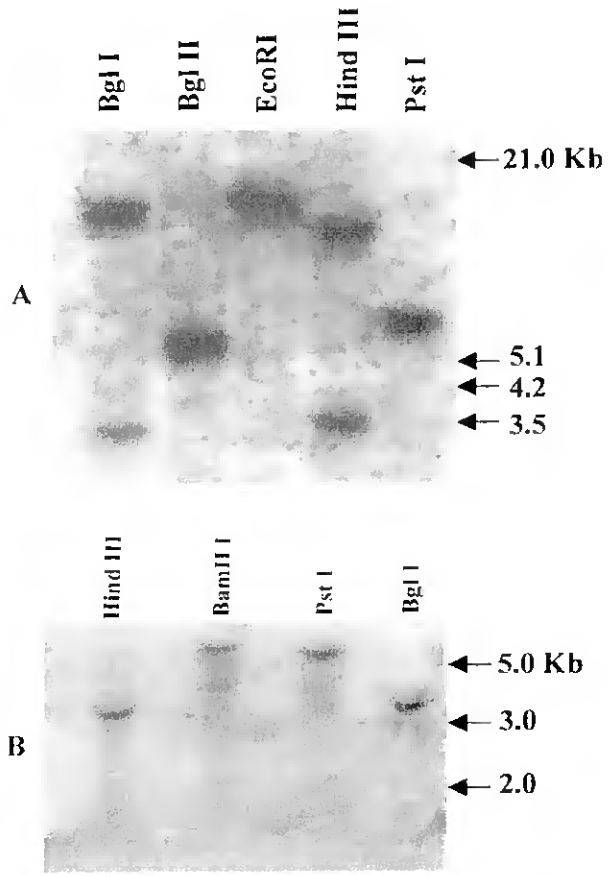

Fig. 6. Analysis of the *Sparus* BGP gene locus by Southern hybridization. Following restriction enzyme digestion with BglI, BglII, EcoRI, HindIII and PstI (panel A) or with HindIII, BamHI, PstI and BglI (panel B), genomic DNA samples were electrophoresed on a 0.8% agarose gel, transferred to a nylon membrane (Nytran +) and hybridized with either the complete (panel A) or a partial (panel B) spBGP cDNA, as described in Section 2. Sizes of the DNA markers are shown on the right margin.

Clemens et al., 1997), and specific vitamin D responsive elements (VDREs) were identified in BGP genes from different species, including rat (Yoon et al., 1988) and human (Ozono et al., 1990). In contrast with its mammalian counterparts, the proximal promoter of the spBGP gene contains no obvious VDRE regulatory elements. Fish are known to store vitamin D (Takeuchi et al., 1986), and in some species vitamin D or its metabolites can affect plasma calcium (Sundell et al., 1993) or phosphorous (Avila et al., 1999) levels, but until now, no VDRE has been identified from any fish gene. Furthermore, since even for relatively closely related species, such as human and rat, the conservation between regulatory elements can be low (Ozono et al., 1990), it may be the case that consensus sequences for regulatory elements are significantly different in fish gene promoters from those known in higher vertebrates. Therefore, work with deletion mutants from the spBGP gene promoter, gel mobility shift assays and DNase I footprinting analysis will be required to identify and characterize functional regulatory sequences within the fish BGP gene promoter.

4.4. Developmental expression of spBGP message

The expression of the BGP gene throughout *Sparus* development was studied by a combination of Northern, RT-PCR and Southern analysis in samples ranging from pre-feeding larval stages to juvenile fish. BGP mRNA was clearly present in fish at 37 dph while no signal could be detected at 27 dph. Since calcification was apparent at 30 dph (Fig. 7B), as determined by alizarin red-alcian blue histochemical staining, these results showed that the BGP gene was expressed in bone tissue at or around the same time that bone mineralization was occurring and the corresponding levels of mRNA increased progressively thereafter (Fig. 5B), as mineralization of the entire skeleton became effective (Fig. 7B). Therefore, the pattern of developmental expression of the BGP gene in *Sparus* parallels that previously observed for mammalian species (e.g. Desbois et al., 1994), with the onset of expression occurring shortly after or being concomitant with the appearance of calcified bone structures. These results strongly suggest that the function of BGP in fish is associated with bone mineralization, as previously seen in mammals, and indicate that bony fish may be a useful model, allowing insight into the evolution of BGP function and regulation at the molecular level.

4.5. Is the spBGP gene duplicated?

Our attempts to find more than one gene coding for *Sparus* BGP were unsuccessful. Whilst we cannot eliminate the possibility of gene duplication, we consider the available data to favour the existence of a single BGP gene in this species. These results are in contrast with those obtained in rodents where analysis of several mouse and rat strains have shown that, in these species, BGP is part of a gene cluster. While in the rat either one or multiple copies were detected depending on the strain (Rahman et al., 1993), in the mouse two BGP genes and one BGP-related gene were identified in all strains examined (Desbois et al., 1994). Our genomic southern approach followed the same conditions as in the mouse study, but our results point to the existence of a single spBGP gene, instead of the cluster found in the mouse genome. Given the very high degree of identity between the various mouse genes, it is possible that the duplication of the BGP gene in rodents occurred quite recently, after the branching of bony fish nearly 400 million years ago.

4.6. Evolution of spBGP gene

BGP is evolutionarily related to MGP, a 10 kDa vitamin K-dependent protein which, although also found in bone matrix, is produced by chondrocytes and has a wider tissue distribution than BGP. Our data suggest that all BGPs have a single origin and supports the previously stated hypothesis that they are derived, together with MGPs, from a common ancestor

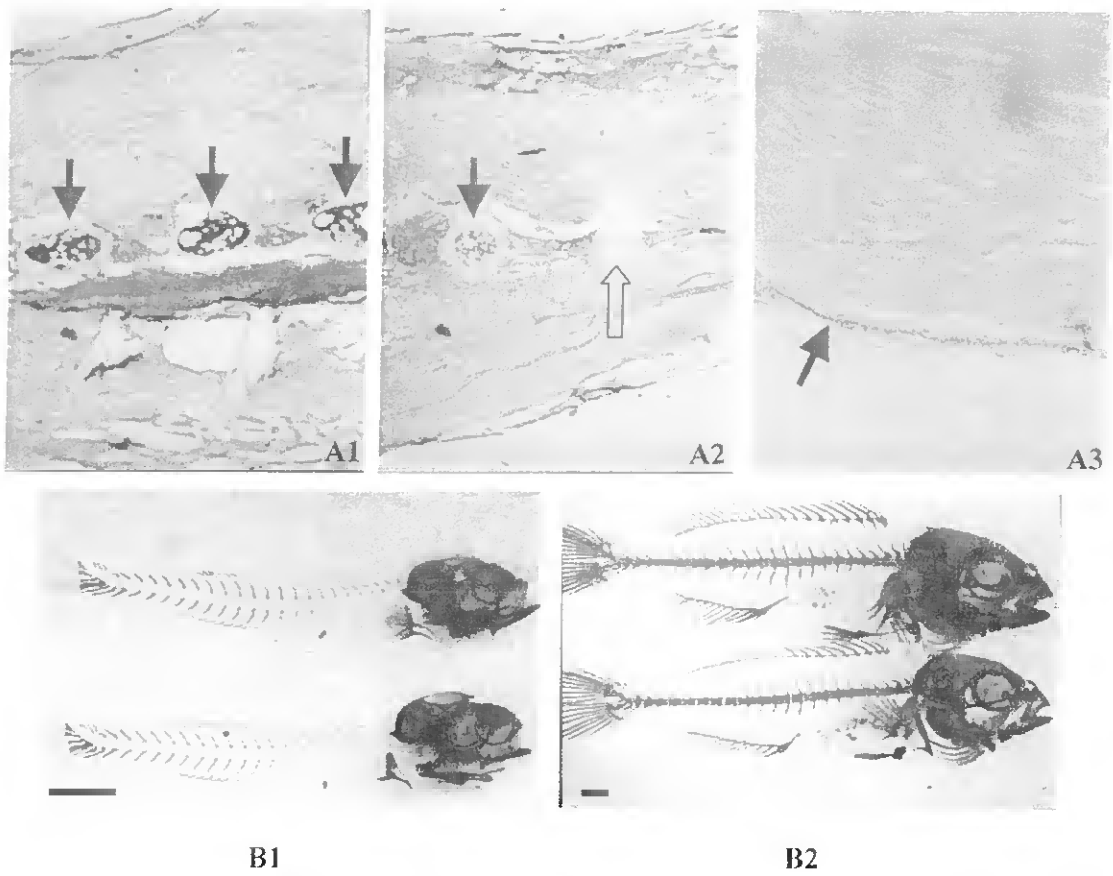

Fig. 7. (A). Localization of spBGP mRNA in *Sparus* tissues by *in situ* hybridization. Longitudinal 5 μm sections of whole undecalcified 90 dph *Sparus* were hybridized with an antisense digoxigenin-labelled spBGP riboprobe. Alkaline phosphatase-coupled antidigoxigenin antibody was used to detect specifically bound probe, as described in Section 2. Intense blue staining, corresponding to the presence of high quantities of spBGP mRNA, was detected (black arrows) in the medium vertebra (A1); less intense staining was observed in the vertebra localized towards the posterior end of the vertebral column (black arrow in A2), while no signal was detected in the more apical vertebrae (transparent arrow in A2); signal was also detected in dermis (arrow in A3). (B) Detection of cartilaginous and calcified structures in *Sparus*. Specimens with 30 (B1) and 90 dph (B2) were stained with Alcian Blue and Alizarin Red, as described in Section 2. Calcified structures are shown in red and non-mineralized cartilage is shown in blue. Scale bar represents 1 mm.

(Rice et al., 1994; Cancela et al., 1995). Both proteins also share some homology with the more diverged coagulation factors, which also belong to the same family of vitamin K-dependent proteins. Within the BGP clade, the fish form a clearly distinct group that might represent the most ancestral forms of BGP. Bird and amphibian BGPs are also more closely related among themselves than to all other vertebrates. No other patterns of evolution of BGPs within vertebrates could be unambiguously resolved with this data set.

In conclusion, we have shown in this work that BGP from a teleost fish has many similarities with its mammalian homologs, at the amino-acid level as well as in tissue distribution and onset of expression during early development. BGP has been reported to be absent from the calcified vertebra of cartilaginous fishes (Rice et al., 1994), indicating that the appearance of BGP may have been concomitant with the appearance of bone. Although earlier reports presented conflicting results on the presence or not of bone in chondrichthyans, recent work has extensively searched for bone-like structures in a variety of cartilaginous fishes and clearly identified all mineralized structures in these organisms as calcified cartilage (Clemens, 1992). Therefore, the presence of BGP in teleost fishes may result from the appearance, in these organisms, of an hydroxyapatite-rich matrix comparable to that found in bone of tetrapods. This further suggests that in fish, as proposed for mammals (Boskey et al., 1998), BGP may be required for the correct maturation of the hydroxyapatite crystal, an hypothesis that could explain the conservation of this protein over more than 400 million years of evolution. Therefore, and given the easy access to early embryos and transparent larvae, fish may be an adequate model to bring additional insight into the function of BGP, in particular for those aspects related to appearance, regulation and basic role during early development.

Acknowledgements

Sparus BGP cDNA and gene sequences were submitted to GenBank, being assigned the accession numbers

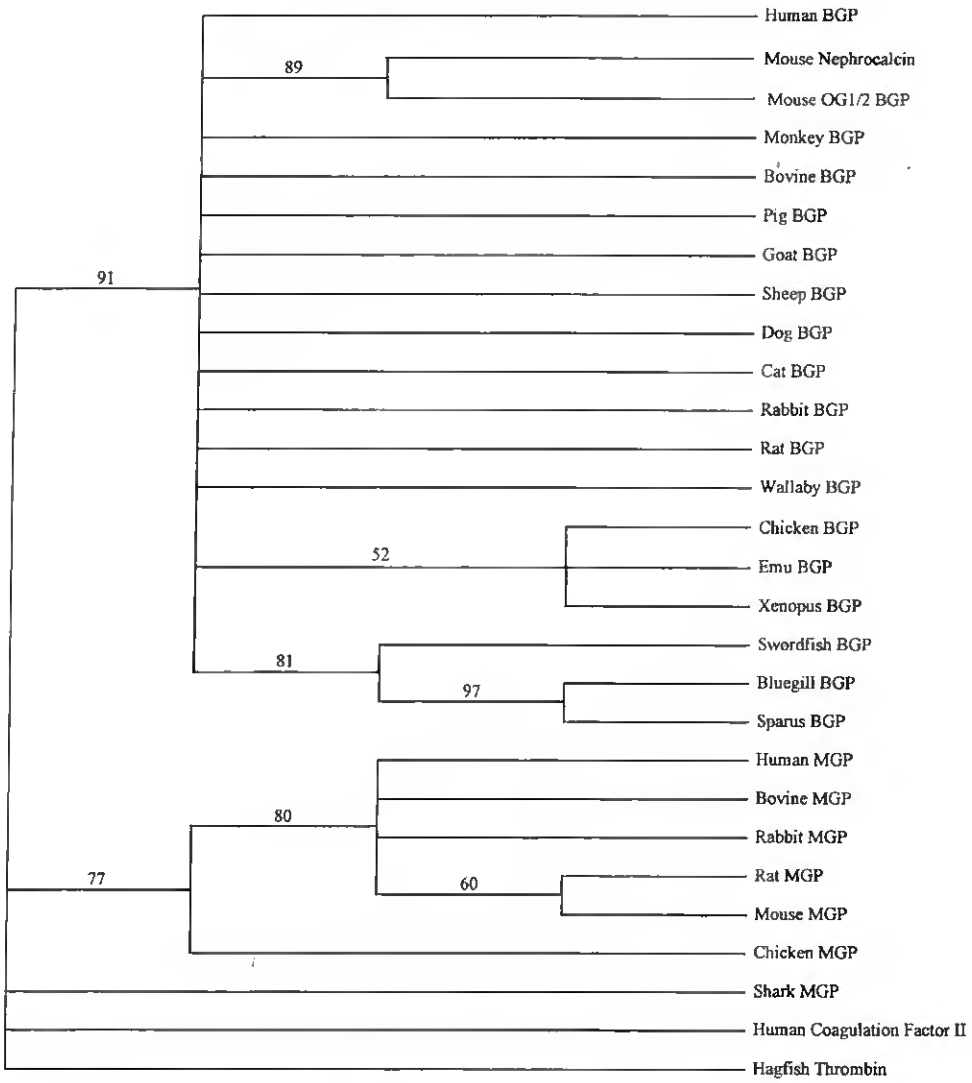

Fig. 8. Evolution of BGP in relation to other vitamin K-dependent proteins. Strict consensus tree of the 30 Maximum Parsimony Trees generated with the available data on BGPs, MGPs, Human Coagulation Factor II and Pacific Hagfish Thrombin. Numbers indicate confidence limits of individual clades, estimated by bootstrap analysis with 1000 replicates.

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E eu aqui fui ficando,
só para O poder ver.
E fui envelhecendo,
sem nunca O perceber.
O mar...

Pedro Ayres Magalhães