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Original Article

Evidence that the Protein Tyrosine Phosphatase (PC12,Br7,Sl) Gamma (-) isoform modulates chondrogenic patterning and growth

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ABSTRACT One of the earliest events in bone morphogenesis is the condensation of embryonic mesenchymal cells into chondroblasts and their subsequent proliferation and differentiation into chondrocytes. During this time, certain signaling cascades operate to establish proper patterning and differentiation of the cartilaginous skeleton. Characterization of the signaling pathways involved in these processes remains to be accomplished. We have identified a novel murine cytosolic tyrosine phosphatase termed PTPPBS gamma (+/-) which is a member of the PTP PC12,Br7,Sl (PTPPBS) family. Spatiao-temporal expression analysis of the members of this tyrosine phosphatase family demonstrates significant expression of the gamma (-) splice variant in the cartilaginous skeleton. Using an embryonic mandibular explant culture system to serve as a model for cartilage formation, we examined the potential roles of the PTPPBS gamma phosphatase by loss-of-function studies achieved with antisense oligodeoxynucleotides. These studies demonstrated that loss of expression of the PTPPBS gamma (-) isoform resulted in abnormal patterning of Meckel's cartilage and an increase in the size of the chondrogenic regions. In gamma antisense-treated explants, bromodeoxyuridine-pulse labeling studies revealed increased proliferation of chondroblasts bordering along precartilaginous condensations and bordering populations of maturing chondrocytes. These studies provide evidence that in early skeletal development, PTPPBS gamma may regulate the rate of chondroblast proliferation in the cartilaginous skeleton.

KEY WORDS: *tyrosine phosphatase, chondrogenesis, chondroblast, Meckel's cartilage, embryo, antisense oligodeoxynucleotides.*

Introduction

Cells responsible for formation of the skeleton during embryo-fetal development are from neural crest-derived mesenchyme in the orofacial region or from mesodermal mesenchymal cells located in the limbs and along the axis. Two primary modes of bone formation exist which result in the transformation of preexisting connective tissue. One mode involves the conversion of primitive connective tissue into bone by intramembranous ossification. In this process, mesenchymal cells proliferate and condense into compact nodes where certain cells differentiate into capillaries while others form osteoblasts capable of secreting bone matrix (Gilbert, 1988). Flat bones of the skull and some facial bones are formed by intramembranous ossification. Alternatively, bones of the vertebral column, the pelvis and short and long bones are formed by endochondral ossification. Endochondral ossification occurs within hyaline cartilage whose shape resembles a small model of the bones to be formed. Prior to endochondral ossification, mesenchymal cells proliferate, condense and undergo differentiation into chondroblasts,

Abbreviations used in this paper: PTP/ PTPases, protein tyrosine phosphatase(s); PTPPBS, Protein Tyrosine Phosphatase (PC12,Br7,Sl); ODNs, oligodeoxynucleotides; BrdU, bromodeoxyuridine , DNA, deoxyribonucleic acid, cDNA, copy DNA; RNA, ribonucleic acid; mRNA, messenger RNA; bp, base pairs; nt, nucleotides; GD, gestational day; UTR, untranslated region; PCR, polymerase chain reaction; EST, extended sequence tag; GAPDH, glyceraldehyde phosphate dehydrogenase; FGF, fibroblast growth factor.

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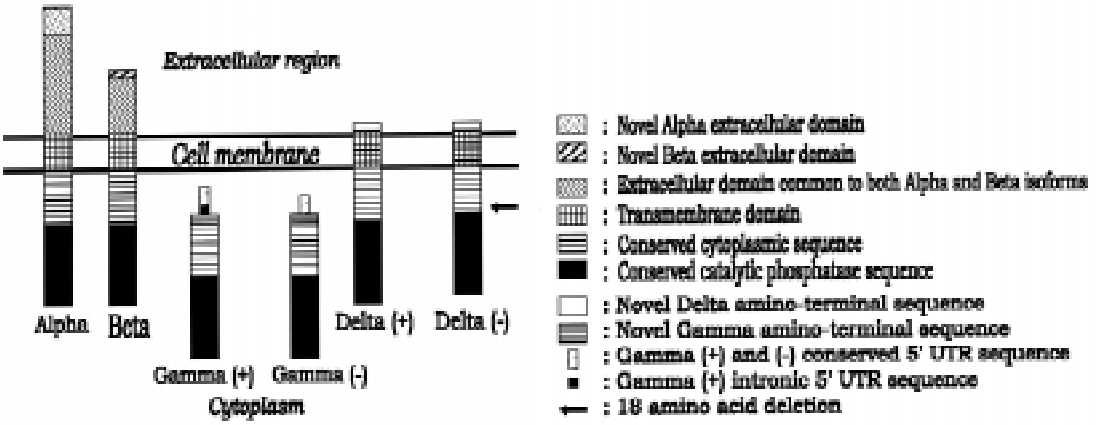

Fig. 1. Diagram of the PTPPBS family. *PTPPBS alpha and beta are putative receptor phosphatases. Messenger RNA encoding the PTPPBS gamma and delta isoforms are alternatively spliced into transcript variants. However, each pair of splice variants are predicted to encode the same proteins, although an 18 amino acid deletion occurs in the cytoplasmic domain of the delta (-) isoform (arrow). PTPPBS delta is predicted to be a membrane-anchored protein, whereas PTPPBS gamma is the only cytosolic phosphatase currently identified in this family. Human and rodent sequences have been identified for all PTPPBS members with the exception of the delta variants, which have been identified solely in human tissues.*

which form the precartilaginous pattern of the hyaline cartilage (Junqueira et al., 1989).

In both modes of bone formation, mesenchymal condensation and differentiation are likely initiated by various signaling pathways which function to establish structural patterning and development of the primitive skeleton. Although many studies have defined cellular processes in early bone formation, a detailed understanding of the molecular and cellular activities integral to this developmental process are less well understood.

In order to provide insight into this process, we have analyzed the developmental expressions of murine members of the tyrosine phosphatase family termed PTP(PC12,Br7,Sl), hence abbreviated as PTPPBS. Additionally, we have identified and characterized a novel member of this family, termed the PTPPBS gamma (+/-) isoform. The PTPPBS gamma (-) splice variant was found to be expressed during embryo-fetal development with significant expression in developing skeletal tissues. We studied the role(s) of the PTPPBS gamma (+/-) isoform in early cartilage formation *in vitro* by loss-of-function studies using antisense oligodeoxynucleotides (ODNs) in mouse mandibular explant cultures. The data strongly suggest that reduction of PTPPBS gamma (-) expression results in abnormal patterning of Meckel's cartilage and an increase in the size of the chondrogenic regions. Our studies indicate that PTPPBS gamma may be important in modulating chondroblast proliferation, including modulating the rate of chondroblast proliferation as the precartilaginous mass enters into chondrogenic differentiation.

Results

The PTPPBS Gamma +/- isoform

The PTP(PC12,Br7,Sl) family was identified as six distinct transcripts which encode a family of four protein tyrosine phosphatases (PTPases). Two of the PTPases are receptor PTPases and have been previously identified as the murine alpha isoform, PTP-Br7 (Ogata *et al.*, 1995; Augustine *et al.*, 2000), the rat alpha isoform, PTP-PC12 (Sharma and Lombroso, 1995), and the murine beta isoform, PTP-Sl (Hendriks *et al.*, 1995; Augustine *et al.*, 2000) (Fig. 1). For consistency, we have consolidated previous names of this family resulting in PTP(PC12,Br7,Sl), abbreviated as PTPPBS. We have additionally identified 4 PTPPBS isoforms, including mouse and human forms of PTPPBS gamma (+) and gamma (-) isoforms and the PTPPBS delta (+) and delta (-) isoforms, the later isoforms have been identified exclusively from human tissues (Fig. 1).

All predicted protein and amino acid sequences downstream from the transmembrane domain are conserved among the identified PTPPBS members (Augustine *et al.*, 2000). In addition, all members of the PTPPBS family possess a single catalytic region, while a majority of receptor-like PTPases contain two tandem repeats of the phosphatase catalytic region. The PTPPBS catalytic region is most similar to STEP and HePTP (Sharma and Lombroso, 1995; Lechleider *et al.*, 1993), sharing approximately 69% amino acid similarity and 55% amino acid identity, respectively (Augustine *et al.*, 2000).

The PTPPBS delta splice variants maintain the transmembrane domain along with a short amino acid sequence amino-terminal to this domain, suggesting that these isoforms are membrane-anchored phosphatases (Fig. 1). In contrast, the predicted amino acid sequence of the PTPPBS gamma variants contain no signal sequence or transmembrane domain, suggesting that PTPPBS gamma is a cytosolic phosphatase (Figs. 1, 2). The PTPPBS gamma variants share a 175 base pair (bp) sequence in the 5' untranslated region (UTR) (Figs. 1, 2). The gamma (+) variant contains an additional 117 bp sequence adjacent to a spliced acceptor site (Figs. 1, 2), however both variants are predicted to encode the same protein.

PTPPBS Gamma expression during gestation

In order to determine the localization of the PTPPBS isoforms, *in situ* hybridization demonstrated that each isoform possessed distinct expression patterns in adult tissues (Augustine *et al.*, 2000) but only the PTPPBS gamma (-) transcript was detected in the embryo.

Using a probe which recognized a sequence shared by both gamma variants, *in situ* hybridization of gestational day (GD) 15.5 embryos revealed diffuse expression of PTPPBS gamma throughout the brain and strong expression in cranial nerve roots and ganglia (Fig. 3 A,C). Strong expression was also detected throughout the skeletal tissues including neural crest-derived facial bones, bones of the skull, and at the periphery of cartilaginous skeletal

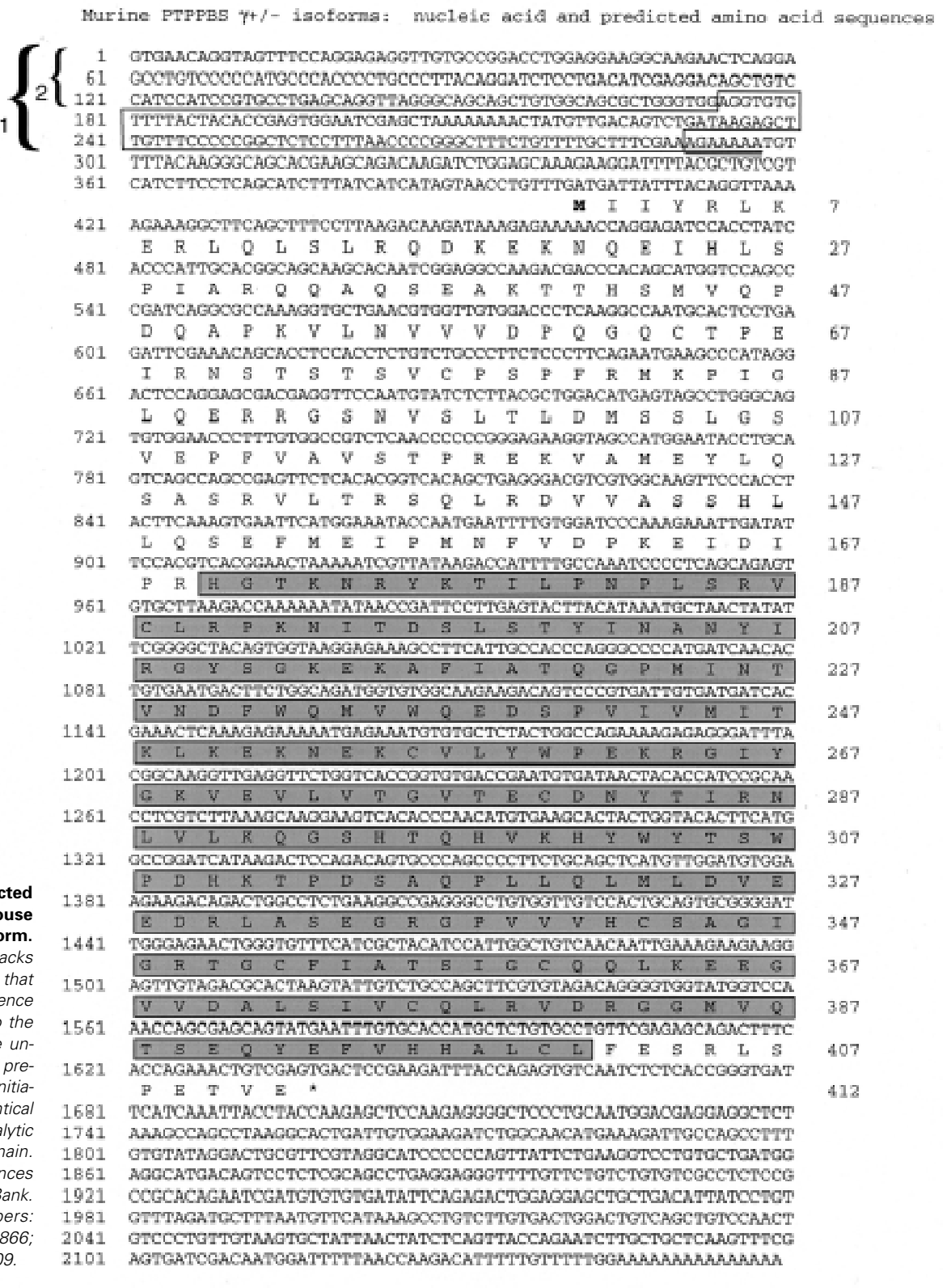

Fig. 2. Nucleotide and predicted amino acid sequence of mouse PTPPBS gamma (+/-) isoform. *The gamma (-) splice variant lacks the 117 bp intron sequence that is boxed. The upstream sequence (in bracket 2) is joined 5' to the splice acceptor site (double underlined). Both variants are predicted to share the same initiation codon and encode identical proteins. Shaded region: catalytic tyrosine phosphatase domain. The gamma isoform sequences have been submitted to GenBank. GenBank accession numbers: gamma (-) variant: AF041866; gamma (+) variant: AF129509.*

elements including the rib and vertebrae anlage (Fig. 3 A,B,D). In contrast, when using a probe which recognized specifically the gamma (+) variant, no hybridization was detected (Fig. 3 E,F), although the same probe hybridized with adult tissues (Augustine *et al.*, 2000). Thus, the data suggests that the observed gamma isoform expression in the embryo is predominantly that of the gamma (-) variant.

By the late fetal stage (gestational day 17.5), hybridization was detected throughout the brain, trigeminal ganglion, as well as in craniofacial bones and cervical vertebrae (Fig. 3 G-I). PTPPBS

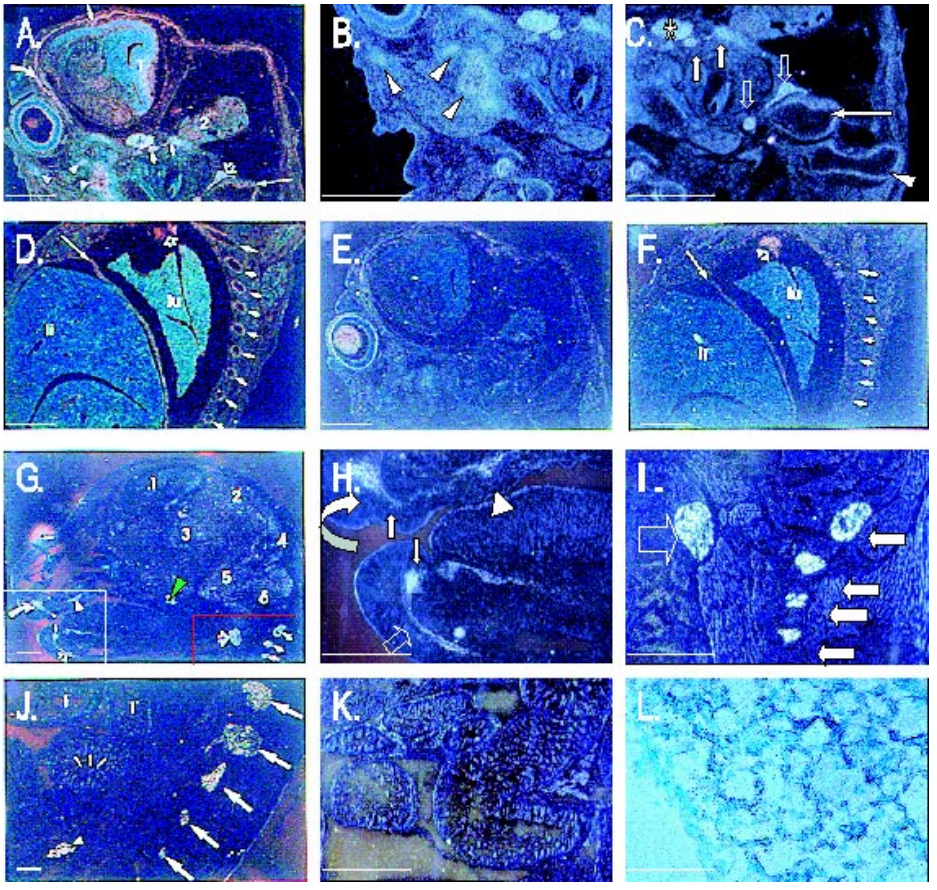

Fig. 3. PTPPBS gamma expression during gestation. (A) *Sagittal section of the cranial region of a GD 15.5 mouse fetus hybridized with the PTPPBS gamma (+/-) probe, exhibiting PTPPBS gamma(+/-) expression in the brain, cranial nerves and facial bones. Scale bar: 500 μm.* **(B)** *Closer view of PTPPBS gamma (+/-) hybridization in GD 15.5 facial bones. Scale bar: 330 μm.* **(C)** *Closer view of PTPPBS gamma (+/-) expression in GD 15.5. cranial nerves and bones. Scale bar: 330 μm. (Symbols in A-C: 1 and 2, brain; asterisk, trigeminal ganglion; small arrows, facial ganglion/nerve rootlets; open arrows, inferior ganglion and associated rootlets; arrowheads, maxilla and mandible; curved arrow, roof of the left orbit; midsize arrow, calvarium; long arrow, basioccipital bone; large arrowhead in plate C, C1 vertebrae (atlas).* **(D)** *Sagittal section of the thoracic region of the GD 15.5 pc fetus. Scale bar: 500 μm.* **(E, F)** *Sagittal section of the cranial region (E) and thoracic region (F) of the GD 15.5 mouse fetus hybridized with a PTPPBS gamma (+) variant-specific probe. Lack of signal indicated that most expression observed in plates A-D represented expression of the gamma (-) variant. Scale bar: 500 μm. (Plates D and F: Long arrow: diaphragm, open arrow: heart; lu: lung; li: liver; short arrows: cartilage primordium of the ribs and vertebrae).* **(G)** *Sagittal section of the cranial region of a GD 17.5 mouse fetus hybridized with the PTPPBS gamma (+/-) probe. Scale bar: 1 mm.* **(H)** *Closer view of oral structures within white box of plate G. Scale bar: 500 μm* **(I)** *Closer view of structures within red box of plate G. Scale bar: 500 μm (Symbols in G-I: Small open arrow, mandible; curved arrow, maxilla; small arrows, upper and lower incisors; arrowhead, palatal shelve; green arrowhead, trigeminal ganglion; 1-6, brain; large open arrow, anterior arch of C1 vertebrae; arrows, cervical vertebrae).* **(J)** *Sagittal section of the lumbar region of a GD 17.5 mouse fetus hybridized with the PTPPBS gamma (+/-) probe. (i, intestine; small arrows, intestinal villi; arrowhead, ilium; arrows, vertebrae).* **(K)** *Closer view of PTPPBS gamma (+/-) hybridization in the small intestine. Scale bar: 500 μm.* **(L)** *Brightfield view of lumbar vertebrae from plate J. Hybridization granules observed on cells within ossification centers of the vertebrae. Scale bar: 50 μm.*

gamma expression was also apparent in the oral-facial structures, including the mandible, palate, and incisors (Fig. 3 G,H) as well as along the axis and the ilium (Fig. 3J). Because spinal ganglion and vertebrae are closely associated along the axis, the *in situ* slides were also evaluated in brightfield to identify the cell population which expressed the strong PTPPBS signal in the axial region. Hybridization signal was observed on cells within the ossification centers of the axial vertebrae, indicating that PTPPBS gamma (-) expression continued to be expressed in the vertebral column during ossification (Fig. 3L). PTPPBS gamma expression was also detected in the small intestine (Fig. 3 J,K) which is an organ strongly positive for gamma isoform expression in the adult (Augustine *et al.*, 2000).

As a more sensitive means to assess PTPPBS gamma (+/-) isoform expression in mouse development, PCR analysis of embryonic mouse cDNA, made from whole embryos at various gestational stages, was undertaken. Expression of the PTPPBS gamma (-) variants was detected by PCR analysis on the four gestational days analyzed which included: GD 7 (the primitive streak stage), GD 11 (early organogenesis), GD 15 (late organogenesis) and GD 17 (fetal stage) (Fig. 4A). On these gestational days, PCR amplification was not detected of the PTPPBS gamma (+) splice variant (Fig. 4A) or of the other PTPPBS isoforms (data not shown).

Functional studies with antisense oligodeoxynucleotides

Because PTPPBS gamma (-) expression was significant in the developing skeleton, functional studies were designed using cultured mandibles as a model of early bone and cartilage formation. The mandible was selected because this structure can be isolated and cultured, enabling the earliest processes of bone development, particularly the processes of precartilaginous condensation and chondrogenic differentiation, to be studied *in vitro*.

PCR analysis was undertaken as a sensitive method to characterize PTPPBS gamma expression during mandibular differentiation (Fig. 4B). Complementary DNA was produced from RNA isolated from the mandibular arch/mandibles isolated from gestational days 10-14 embryos. PTPPBS gamma (-) was detected in the tissue on all days analyzed, although expression was lower on day 14. Expression of the gamma (+) variant was detected in only GD 12 mandibles (Fig. 4B).

To determine the relevance of PTPPBS gamma in mandibular development, loss-of-function studies were undertaken in cultured mandibular explants (Fig. 5). Antisense oligodeoxynucleotides (ODNs) designed against the PTPPBS gamma (+/-) isoform were administered to cultured mandibles and effects on chondrogenesis of Meckel's cartilage were evaluated. Mixed-phosphorothioate/phosphodiester antisense ODNs were designed to the PTPPBS gamma isoform. PTPPBS gamma scrambled antisense and 4-

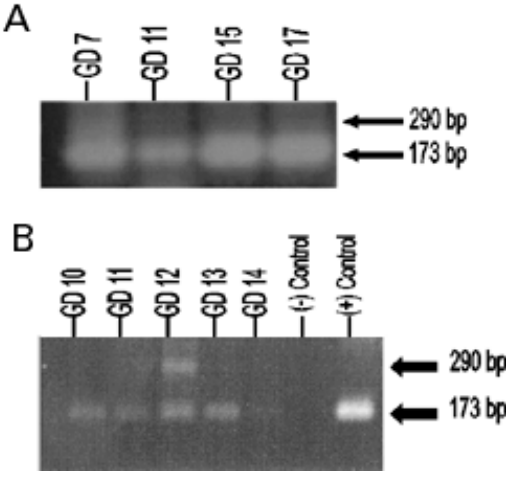

Fig. 4. PCR panels of PTPPBS gamma (+/-) expression during (A) embryonic gestation and (B) mandibular development. *The 290 bp and 173 bp bands represent PTPPBS gamma (+) versus (-) variant expression, respectively. (***A***) PCR panel of embryonic and fetal cDNA from various gestational days amplified for PTPPBS gamma. (***B***) PCR panel of PTPPBS gamma (+/-) expression during mandibular development. The higher band observed in the GD 12 sample indicates that both variants were expressed in the mandible at that time of development. (-) Control, PCR mastermix without cDNA; (+) Control, PCR amplification from plasmid containing PTPPBS gamma (-) variant.*

base mismatched antisense ODNs were used as ODN sequence-specificity controls for the study.

In order to verify that the ODNs were being incorporated by the explants, BrdU-labeled antisense ODNs were added to the media and the tissues were analyzed at various time points. Artifactual staining was not detected in mandibles cultured without labeled oligo (Fig. 5D). BrdU staining was detected at the periphery of the tissues within 48 hours (Fig. 5E) and was distributed throughout the tissue by 4 days (Fig. 5F).

To evaluate the effects of PTPPBS gamma expression inhibition on formation of Meckel's cartilage, mandibular explants were analyzed by staining the explants as wholemounts with alcian blue. Alcian blue is a dye which stains chondroitin-4- and chondroitin-6-sulphate, which are two proteoglycans found in the ground matrix surrounding maturing chondrocytes. When viewed as a wholemount, Meckel's cartilage from explants established from first branchial arch on GD 11.5 (40-45 somite pair) and grown for 9 days *in vitro,* appeared as a symmetrical wishbone-shaped structure (Fig. 6A). The precartilaginous condensations were viewed as opaque structures while regions undergoing advanced chondrogenic differentiation stained with alcian blue. All other soft tissues appeared transparent in the wholemount.

The morphology of Meckel's cartilage in most explants treated with scrambled or 4- base mismatched antisense ODNs was comparable to untreated controls (Fig. 6B) (Table 1). A small percentage of the scrambled and mismatched antisense ODN treated mandibles did exhibit minor shape changes in Meckel's cartilage morphology (Table 1). Inhibition of PTPPBS gamma (+/-) expression resulted in abnormal morphology, where the resulting Meckel's cartilage, in the bilateral and dorsal segments, were enlarged and stained strongly with alcian blue (Fig. 6C). The ventral medial segments were also absent in many affected mandibles (Fig. 6C, Table 1). The ventral to dorsal gradient of dysmorphology, ranging from lack of chondrogenesis in the ventral segment to enlarged chondrogenic dorsal segments, provided evidence that abnormal pattern formation of Meckle's cartilage occurred following PTPPBS antisense ODN treatment.

To determine whether the increased size of chondrogenic regions was related to cellular proliferation, a BrdU-pulse study was undertaken. Mandibular explants were grown in culture in the presence of no ODNs, scrambled antisense ODNs, or gamma antisense ODNs for 8 days. On the 8th day, the explants were incubated in the presence of BrdU for two hours and then removed for histological processing. The mandibles were sectioned and processed for BrdU detection as well as stained with safranin O, a dye which stains proteoglycans in the ground substance surrounding maturing chondrocytes (Carson, 1997). Precartilaginous condensations (Fig. 7 A,C) and sites containing maturing chondrocytes (Fig. 7 E,G) were initially identified by examining the morphology and staining characteristics of cell populations stained with safranin O. Cell proliferation in these areas was then evaluated by viewing the same regions in the BrdU-stained sections (Fig. 7 B,D,F,H).

Mandibles cultured with PTPPBS gamma antisense ODNs exhibited increased positive BrdU staining which was detected in chondroblasts located at the outer margins of precartilaginous condensations (Fig. 7D) and in chondroblasts bordering maturing chondrocyte populations (Fig. 7H). The increase in rates of

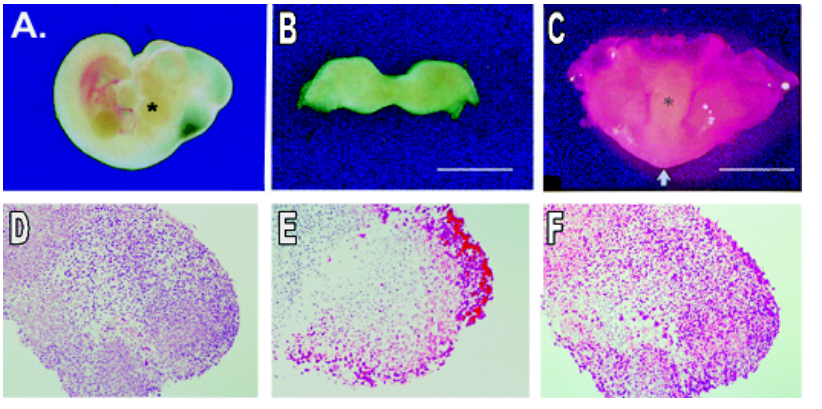

Fig. 5. *In vitro* growth and differentiation (A-C) and ODN uptake studies (D-F) in mouse first branchial arch-derived mandibles grown in serum-free defined medium. **(A)** *Day 11.5 pc (44-48 somite pair, Theiler Stage 19) mouse embryo. Arrow points to the first branchial arch, which gives rise to the mandible.* **(B)** *Day 11.5 pc mandibular explants were placed in 0.4 μm Transwell dishes (Costar Inc.) and cultured in serum-free defined media in these studies. Scale bar: 500 μm.* **(C)** *The gross morphology of a day 11.5 pc mandible grown in vitro for 9 days exhibited marked mandibular (arrow) and tongue morphogenesis (asterisk). Bar = 1 mm.* **(D)** *Histological section of a mandible explant stained for the presence of BrdU. The mandible explant was cultured for 4 days without BrdU-labeled ODN.* **(E)** *Section of a mandible explant cultured for 48 hours in the presence of BrdU-labeled antisense ODN.* **(F)** *Section of a mandibular explant cultured for 4 days in the presence of BrdU-labeled antisense ODN.*

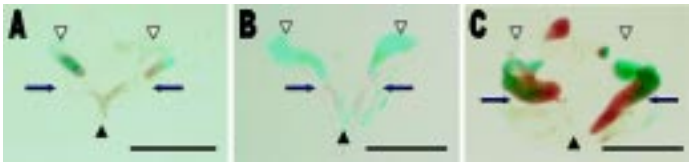

Fig. 6. Wholemount alcian blue staining and histology of Meckel's cartilage and surrounding cells after 9 days in culture. *Inhibition of PTPPBS gamma expression resulted in dysmorphogenesis of Meckel's cartilage and larger regions of chondrogenesis.Mandibles were dissected and plated on GD 11.5 and cultured for 9 days.* **(A)** *Untreated control.* **(B)** *Scrambled antisense ODN-treated.* **(C)** *Gamma antisense ODN-treated. Meckel's cartilage: Black arrowhead, ventral medial segment; blue arrows, lateral segments; white arrowheads, dorsal extensions. Scale bar: 750 μm.*

chondroblast proliferation was more moderate in chondroblasts which bordered precartilaginous condensations than in those bordering sites of maturing chondrocytes (Fig. 8 A,C). In the gamma antisense-treated mandibles, the rate of chondroblast proliferation bordering precartilaginous condensations increased by 22% and 41% compared to scrambled ODN-treated and no ODN-treated controls, respectively (Fig. 8A). In chondroblasts bordering regions of maturing chondrocytes, the rate of chondroblast proliferation in the gamma antisense-treated mandibles increased by 73% and 53% compared to scrambled ODN-treated and no ODN-treated controls, respectively (Fig. 8C). In contrast, there were no significant differences in the rates of chondroblast proliferation in chondroblasts located within the precartilaginous condensations (Fig. 8B) or of maturing chondrocytes within chondrogenic regions (Fig. 8D).

Real-time PCR (Heid *et al.*, 1996) was used as a quantitative method to analyze normalized percent reductions of PTPPBS gamma RNA levels in non-treated and ODN-treated explants. Following 7 days of culture in the presence or absence of oligonucleotides, the mandibles were collected and prepared for PCR analysis. Gamma isoform antisense ODNs were administered to determine whether the observed biological effects on chondrogenesis were directly related to transcriptional reductions of PTPPBS gamma expression. The PTPPBS gamma scrambled antisense ODN and a nonsense ODN were used as sequence-specificity and ODN-treatment controls, respectively.

Transcript levels of PTPPBS gamma isoform were reduced by 72% of control transcript levels following gamma antisense treatment as determined by amplification with gamma isoform-specific primers (Fig. 9). The nonsense ODN and scrambled antisense ODN treatments moderately affected the levels of gamma isoform PTPPBS isoform expression, where transcript levels were reduced by 22% and 42% of untreated control levels, respectively (Fig. 9).

Discussion

We have recently identified PTPPBS gamma, a novel member of the PTPPBS protein tyrosine phosphatase family. We have cloned PTPPBS gamma from human and mouse cDNA libraries and have confirmed its expression in embryonic and adult tissues. Although all members of the PTPPBS family have been found to be expressed in adult tissues, the gamma isoform was the only family member found to be developmentally expressed. In this study, we evaluated the expression and roles of the PTPPBS gamma (+/-) tyrosine phosphatase in embryonic development.

PTPPBS (+/-) isoform expression during gestation

In situ hybridization studies revealed PTPPBS gamma (-) expression in the skeletal, nervous and intestinal systems in mouse embryo-fetal development. PCR analysis of cDNA collected from embryos and fetuses (GD 11-17) and amplified to determine expressions the PTPPBS gamma (+) and (-) variants, as well as of the other PTPPBS isoforms, revealed positive expression of only the gamma (-) isoform, suggesting that it is the sole PTPPBS isoform which is developmentally expressed. However, further characterization of PTPPBS gamma (+/-) expression in the developing mandible revealed that the PTPPBS gamma (+) variant was expressed but only at restricted times. The gamma (+) variant, which is predicted to encode the same protein as gamma (-), displayed expression in the mandibular arch restricted to GD12.

Relevance of PTPPBS gamma splice variants

It remains to be determined why gamma (-) exhibited predominant expression in comparison to its splice variant. Alternative splicing of the 5' UTR, reflected in the sequence variations of the PTPPBS gamma variants, may regulate expression levels of these cytosolic molecules. This mechanism has been previously observed in rat and mouse β enolase (Oliva *et al.*, 1995). In another study, we demonstrated using an *in vitro* translation assay, that the unspliced gamma (+) isoform exhibited much greater translation efficiency than the spliced gamma (-) isoform (Augustine *et al.*, 2000). Thus, differential expression of the two splice variants may be related to modulation of the isoform's translation, hence regulating the amount of active protein present within the cell at a given time. Discrete expression of the gamma (+) isoform may occur as a means to supplement the levels of gamma protein product in the mandible during key time periods such as during GD12, when the mandibular arch undergoes extensive shape changes, resulting in its condensation and regression to form the lower part of the oral cavity (Kaufman, 1992).

Functional studies with antisense oligodeoxynucleotides

Because significant PTPPBS gamma expression was observed in developing skeletal elements, we carried out experiments focused on investigating roles of this phosphatase in early skeletal development. Using an embryonic mandibular explant culture

TABLE 1

MORPHOLOGY OF MECKEL'S CARTILAGE

evaluated by alcian blue staining from mandibular explants cultured for 9 days *in vitro.*

Treatment	*N*	*% Normal*	*% Affected*	*N Mild Dysplasia*	*N Asymmetric Dysmorpho-genesis*	*N Increased Size of chondrogenic regions*	*N Absent Ventral Region*
No ODN	21	95	5	1	0	0	0
Scrambled AS	13	77	23	3	0	0	0
PTPPBS-gamma AS 4 base mismatch	11	73	27	2	0	1	0
PTPPBS gamma AS	21	9.5	**90.5**	1	5[1]	5	8[2]

[1] **Affected mandibles also exhibited increased size of chondrogenic regions**
[2] **Affected mandibles also shared asymmetric dysmorphology and increased size of chondrogenic regions**
Underlined bold: Significant difference between antisense ODN effects versus scrambled and mismatched control ODN effects on Meckle's cartilage morphology: p<0.05

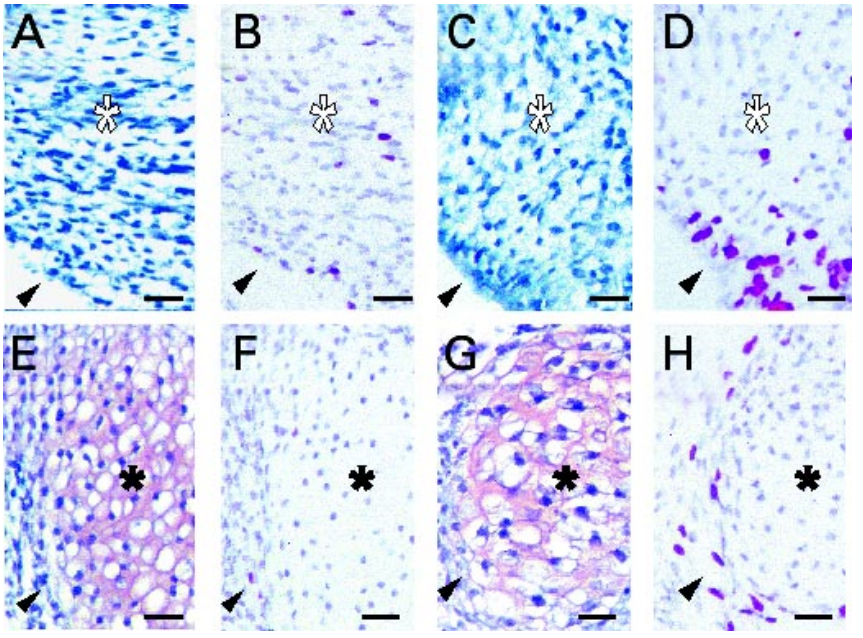

Fig. 7. Representative histology observed in BrdU cell proliferation assays of untreated, scrambled ODN-treated and PTPPBS gamma antisense ODN-treated mandibular explants. *Mandibles were cultured for 8 days and then pulsed for 2 hours with BrdU prior to termination. Mandibles were serial sectioned and stained with safranin O, to identify precartilaginous condensations (A,C) and chondrogenic centers (E,H), as well as immunostained with an antibody which detected BrdU incorporation (B,D,F,H).* **(A,B)** *Precartilaginous condensation from an untreated mandibular explant.* **(C,D)** *Precartilaginous condensation from a PTPPBS gamma antisense ODN-treated explant.* **(E,F)** *Chondrogenic site from a PTPPBS scrambled antisense ODN-treated explant.* **(G,H)** *Chondrogenic site from a PTPPBS gamma antisense ODN-treated explant. Scale bar: 30 μm.*

system to serve as a model for cartilage formation, we examined roles of the PTPPBS gamma phosphatase by loss-of-function studies achieved with antisense oligodeoxynucleotides.

Antisense targeting of PTPPBS gamma resulted in pattern loss of Meckel's cartilage, which included disruption of formation of the ventral segment and asymmetrical dysmorphogenesis of the lateral segments. Furthermore, the overall size of lateral and dorsal chondrogenic regions of Meckel's cartilage was larger in the gamma antisense ODN-treated explants.

The gradient of cartilage dysmorphogenesis ranging from absence of chondrogenesis in the ventral medial segment to progressive enlargement of the dorsal regions, suggested that the PTPPBS gamma phosphatase may be involved in pathways which are regulated by patterning genes. Putative morphogens, such as retinoic acid, are expressed along a gradient and are important in establishing pattern formation, such as in limb development (Tickle, 1991). Although future studies are essential to investigate this hypothesis, it is possible that PTPPBS gamma may be involved in a pathway which responds to morphogenic signals. In addition, the frequent absence of the ventral medial segment of Meckle's cartilage suggests that the phosphatase could have involvement with segment-specification genes. Both variants are expressed in the mandibular arch mesenchyme during the time when important mandibular patterning genes, such as Hoxa-7, -a-3, and -c-8 are also expressed (Chisaka and Capecchi, 1991; Le Moulleic *et al.*, 1992; Chisaka *et al.*, 1992).

Pulse studies using BrdU in antisense-treated and control mandibles were undertaken to determine whether changes in rates of cellular proliferation contributed to the enlarged size and abnormal shape of Meckel's cartilage. BrdU detection revealed no differences in cellular proliferation of the chondroblasts within precartilaginous condensations or of the maturing chondrocytes. However, the BrdU pulse studies did reveal increased proliferation of chondroblasts positioned at the outer margin of the precartilaginous condensations and of those bordering maturing chondrocytes. This response suggested that there could be an increased proliferation of peripheral chondroblasts which may give rise to an increased number of chondrocytes. Thus, the PTPPBS gamma isoform may be

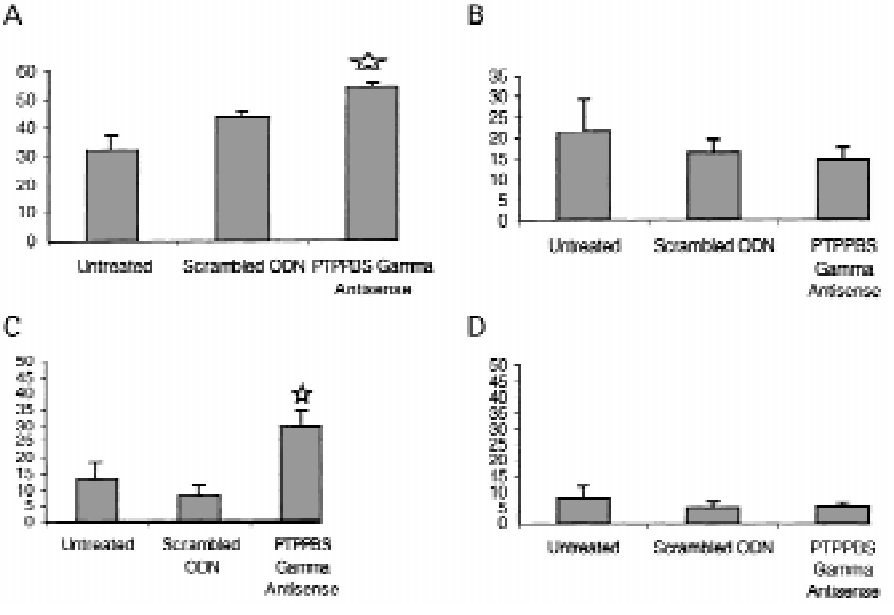

Fig. 8. Percent proliferation of chondroblasts and chondrocytes in mandibular explants cultured with PTPPBS gamma antisense ODN, scrambled antisense ODN or without ODNs. *The percentage of proliferating chondroblasts* **(A)** *bordering precartilaginous condensations or* **(C)** *bordering chondrogenic regions and the percentage of proliferation within* **(B)** *precartilaginous condensations or within* **(D)** *chondrogenic regions.*

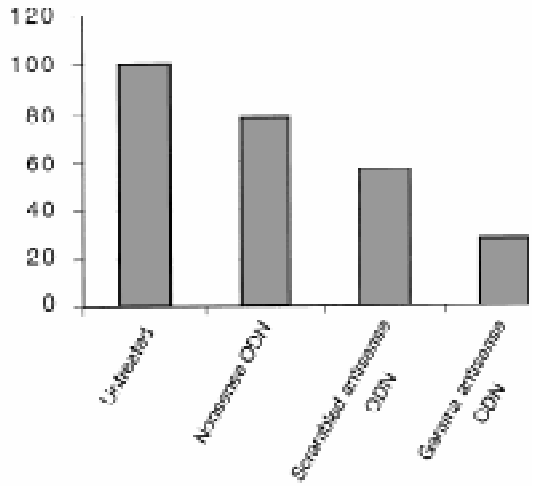

Fig. 9. Changes in PTPPBS gamma isoform expression in response to ODN treatment, as detected by quantitative real-time PCR. *Isoform levels from each treatment were normalized against endogenous GAPDH transcript levels in the cultured mandibles. Explants were grown in culture media containing ODN for 7 days prior to analysis. Fresh media and ODN were administered every 48 hours.*

important in regulating chondroblast proliferation and thereby play an early role in defining the overall size of the cartilage.

Structural patterning and cellular proliferation are suspected to have functionally associated roles in defining the size and shape of the skeletal anlage. The observation that reduction in PTPPBS gamma expression resulted in dysmorphogenesis and increased size of Meckel's cartilage supports the speculation that the phosphatase may play a role in both processes. In the limb bud, certain FGFs and *sonic hedgehog* form a genetic cascade essential for establishing the proper proximal-distal outgrowth and pattern of the limb and its skeletal elements (Niswander and Martin, 1993; Laufer *et al.*, 1994). Continued studies which focus on craniofacial skeletal patterning are necessary to provide insights on the genetic cascades involved in establishing the skeletal elements in this region.

The PTPPBS gamma phosphatase may also be involved in mesenchymal activities such as mesenchymal recruitment, aggregation and differentiation into precartilaginous condensations. The observation that PTPPBS gamma (-) expression was observed at the peripheral borders of the axial skeletal elements on GD 15 (Fig. 3), provides circumstantial evidence that the phosphatase could play a role in conducting signals between mesenchymal populations and regions of cellular differentiation. This suggestion is further supported by the observation that increased chondroblast proliferation was limited to the outer borders of precartilaginous condensations and maturing chondrogenic regions in gamma antisense-treated explants.

Addressing general criticisms of the use of oligodeoxynucleotides

Antisense ODN applications in *in vitro* culture systems have been beneficial for examining functional roles of genes in embryonic and adult biological processes. The biological data gained from *in vitro* assays is limited to the extent that such assays accurately reflect *in vivo* biological processes. In this study, we used mandibular explant culture as a model to study functions of PTPPBS in early chondrogenic events. Although this system has been previously demonstrated to serve as a model to study these events, we are limited in our scope of focus and additional studies will be required to determine whether this phosphatase has functional roles in earlier as well as more advanced stages of skeletal formation.

Real-time quantitative PCR demonstrated that the gamma antisense ODNs reduced gamma transcript by 72% of non-treated control levels, whereas nonsense and scrambled gamma antisense ODNs reduced gamma transcript by 22% and 42%, respectively. The reduction of PTPPBS gamma transcript produced in the nonsense and scrambled antisense ODN treatments reflects a common occurrence observed in many antisense studies (Shum *et al.*, 1993; Augustine *et al.,*1993; Augustine, 1997; Branch 1998). The exact mechanism for this ODN effect remains to be determined, although cellular uptake and distribution may be sequence dependent and might play a role in this effect (Etore *et al.*, 1998). Although the nonsense and scrambled antisense ODNs reduced PTPPBS transcript, these transcript levels remained greater than 50% of the control levels. The moderate reduction of transcript produced by the scrambled antisense ODNs serves as a possible explanation for why a small percentage of the scrambled-treated mandibles exhibited abnormal Meckel's cartilage formation (Table 1). Reflecting transgenic mouse studies which have shown that a majority of heterozygous null mutants exhibit normal phenotypes, it is possible that greater than a 50% reduction of PTPPBS gamma transcript was necessary to produce an overt biological phenotype from the antisense ODN treatment. This possibility has been demonstrated in previous antisense studies that have shown that a greater than a 50% reduction in targeted transcript level was necessary for producing a biological effect in embryonic and tissue culture systems (Augustine *et al.*, 1993; Monia, 1997; Branch 1998).

Although a sequence-specific biological effect was achieved from the gamma antisense ODNs, the PCR data indicate that the transcript was attenuated but not abolished. It is possible that a more pronounced effect would have been achieved if the gene was completely inactive, as would be achieved in transgenic homozygous null mutants. Altogether, the use of antisense ODNs in mandibular explants have provided a glimpse of the function of PTPPBS gamma in embryonic development. Additional studies which target PTPPBS gamma in transgenic overexpressing and/or knockout mice will likely provide additional functional data.

Future prospects

Currently, little is known regarding roles of tyrosine kinases and phosphatases in early skeletal development. In control explants, we observed a higher rate of chondroblast proliferation at the margins of precartilaginous condensations compared to those bordering chondrogenic centers. These observations suggest that a down-regulatory signaling cascade may be important in modulating the size of the skeletal elements as the precartilaginous condensations begin to undergo chondrogenic differentiation. In contrast to the controls, gamma antisense-treated mandibular explants exhibited increased proliferation of chondroblasts which bordered populations of maturing chondrocytes, suggesting that the reduction of gamma phosphatase activity may have disrupted the down-regulatory proliferative response of chondroblasts surrounding centers of maturing chondrocytes. This speculation supports the presumed function that the PTPPBS gamma phos-

phatase would normally down-regulate catalytic activity of a tyrosine kinase. Therefore, such loss of phosphatase activity could result in certain kinases to remain activated in signal transduction pathways leading to continued cellular proliferation. Future experiments which characterize protein-protein interactions of the PTPPBS gamma isoform are necessary to begin to identify the molecules which serve as substrates for this phosphatase, thus begin to elucidate its signal transduction pathway.

Altogether, these studies indicate that PTPPBS gamma may have roles in patterning and cellular proliferation of skeletal elements in the precartilaginous /cartilaginous skeleton. It is important to note that this phosphatase expression was not restricted to the cartilaginous skeleton but was also strongly expressed in regions of ossification and in the brain and intestinal track. Such expression patterns suggest that this gene may have functional roles at later stages in skeletal development as well as in other organ systems. Also, our PCR analysis detected expression of this phosphatase early in embryogenesis, prior the onset of precartilaginous skeletal formation. It is possible that this phosphatase may have roles in very early patterning and fate-determining processes of skeletal development and/or in other biological processes, although we have not investigated such roles in the present study. Future *in vitro* and *in vivo* functional studies will be of value for gaining additional knowledge regarding the function of PTPPBS gamma throughout embryo-fetal development.

Materials and Methods

Identification of the PTPPBS gamma (+/-) isoform

The PTPPBS gamma (+/-) variants were amplified from the CM-1 mouse colonic library cDNA (Amgen, Inc.) using primers 971-5 and 971-6 which were derived from a rat colonic expressed sequence tag (EST) designated Cr1-00124-d4-a and corresponded to base pairs (bp) 1292-1319 and 1640-1610 of the mouse PTPBr7 (PTPPBS alpha) sequence, respectively (Ogata *et al.*, 1995). These primers were used in combination with the library vector primers to amplify the sequences. PCR amplification was accomplished using Boehringer Mannheim (Indianapolis, In) extended length PCR reagents. The conditions were as follows: 1 cycle 94°C for 1 min., 35 cycles of 94ºC for 30 sec, 50ºC for 45 seconds, and 68ºC for 2 minutes and a final extension of 72ºC for 10 minutes. The sequence data resulting from the PCR products were used to design a probe specific to the 5' UTR of the gamma (+/-) isoform. This probe was used in plasmid pool screening (Sambrook *et al.*, 1989) of the CM-1 library to verify the entire coding sequence of the gamma (+/-) variants. DNA and deduced amino acid sequence analyses was accomplished using the GCG sequence analysis software package from the Genetic Computer Group, Inc. (Madison, Wisconsin)

In situ hybridization

PTPPBS isoform-specific probes were amplified by PCR from mouse brain cDNA (Clontech, San Francisco, CA), verified by sequencing and made into riboprobes by using the Promega *in vitro* transcription kit with ^{35}S incorporation (Wilkinson,1992). *In situ* hybridization was accomplished on cryostat sections of GD15 and 17.5 mouse fetuses using standard methods (Wilkinson,1992) with high stringency hybridization and wash conditions (hybridization and first wash were at 60ºC). Sections were autoradiographed for 2-3 weeks prior to development.

PCR analysis for PTPPBS transcripts

Mandibular explants were collected from culture, placed in Tri Reagent RNA lysis buffer (Molecular Research Center, Inc. Cincinnati, OH), and homogenized by passing the tissue through an 18-gauge syringe three times. Total RNA was extracted using the protocol provided by Molecular Research Center.

PCR panel analysis

Embryo-fetal expressions were analyzed by PCR using days 7, 11, 15, and 17 pc cDNA (Clontech) with Perkin Elmer Gene Amp PCR reagents or by RT-PCR analysis using rTth enzyme (Perkin Elmer) of RNA isolated from day 10-14 mandibular arch tissue according to standard kit methods (Perkin Elmer). Isoform-specific primers were designed to amplify a portion of each unique 5' UTR region. Primers were also designed to separately amplify the cytoplasmic consensus and catalytic regions. Cycle amplification of the cDNAs were as follows: 1 cycle: 94ºC 1 min, 35 cycles: 94ºC 30 sec, 50ºC 30 sec, 72º 45 sec and a final extension at 72ºC 10 min.

Real-time quantitative PCR

Mandibular explants were grown *in vitro* for 7 days in the presence of ODN. Fresh media and ODN were administered every 48 hours. On the 7th day of culture, fresh media and ODN were administered in the morning and the explants were removed from culture 6 hours later, total RNA was isolated and then treated with DNAse (Q1 DNAse; Promega) to eliminate genomic contamination. Copy DNA was made from mandibular RNA pooled from each treatment using random hexamers and Superscript II reverse transcriptase according to protocol supplied by manufacturer (Gibco BRL, Grand Island N.Y.). Real time PCR was conducted according to the protocol provided by Perkin Elmer (Norwood, CT) using the relative expression change normalization method (described in User Bulletin #2, ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosystems). As a reference house keeping control for expression normalization, GAPDH expression levels were evaluated in all treatments using the rodent GAPDH primer set and control FAM-probe kit (Perkin Elmer). Taqman primers and probes specific for PTPPBS gamma isoform expression were designed using the Prism Primer Express Software (Perkin Elmer). Selected probes were synthesized with the FAM and TAMRA fluorescent label and quencher, respectively (Amgen, DNA and Peptide Technology group). The primer and probes sequences were as follows:

Gamma (+/-) forward primer:
GAC CTG GAG GAA GGC AAG AA
Gamma (+/-) reverse primer:
TGT CAG GAG ATC CTG TAA GG
Gamma probe:
6-FAM-CA GGA GCC TGT CCC CCA TGC CCA CC-TAMRA

PCR amplification was carried out using the Taq Gold Taqman PCR reagent kit (Perkin Elmer) and standard PCR conditions (as described in User Bulletin #2, Perkin Elmer). For evaluation of results, standard curves were generated for each primer using non-treated day 14 mandibular cDNA as well as plasmid template containing the PTPPBS gamma isoform sequence. Ratios of the delta delta CT values for gamma isoform expression were normalized to GAPDH levels in each treatment by ratio and then the normalized values were evaluated as percent PTPPBS isoform levels compared to untreated control levels.

Mandibular explant culture

Mouse mandibles were cultured in serum-free media according to methods described by Slavkin *et al.*(1989) with minor protocol modifications. Briefly, gestational day 11 or 12.5 C1 mouse embryos were isolated and the mandibular divisions of the first branchial arch were microdissected and explanted. Explants were supported on the membranous support in 0.4 µm Transwell dishes (Costar Inc.) and cultured in BGJb medium (Gibco-BRL, Grand Island, N.Y.) buffered with 3.5 mg/ml bicarbonate. Cultures were maintained at 37ºC and 5% carbon dioxide with medium change every two days. All experiments were performed in a minimum of triplicate.

Antisense oligodeoxynucleotide design

Antisense oligodeoxynucleotides were designed against areas within the cytoplasmic region or against the 5' UTR region of PTPPBS gamma (+/-).

Control ODNs included the design of antisense sequences possessing 4 or 6 mismatched bases and scrambled antisense sequences. Sequences were selected based upon criteria described by Augustine (1997) and uniqueness of target sequence was verified using GenBank/EMBO FastA analysis with GCG software. All ODNs were 19-20 bases and were synthesized with phosphorothioate modifications on the first two 5' bases, the last four 3' bases, and on every third base and all remaining bases possessed phosphodiester bonds. This chimeric internucleotide bond chemistry minimizes the non-specific affinity of fully modified phosphorothioates to proteins, but maintains protection of the ODN against nuclease activity (Marshall *et al.*, 1992; Cummins *et al.*, 1996). ODNs were synthesized and deprotected using standard protocols. Purification was accomplished by reverse phase cartridge (Hamilton PRP-1), followed by ethanol precipitation (Augustine *et al.*, 1993, Marshall and Boymel, 1998). Product identity and purity was verified by capillary gel electrophoresis and mass spectrometry (Van Ausdall and Marshall, 1998). Purified ODNs were diluted in fresh BGJb media with a final concentration of 25 μM. Fresh ODN-containing media was replaced in cultures every two days. ODN sequences were as follows:

Nonsense ODN:
CTG GCC GAG AGC TGC AAT GC
Gamma (+/-): mid-sequence region:
GAG GTG GAG GTG CTG TTT CG
Scrambled gamma (+/-) mid-sequence region:
TTG GAC GGT CGG TTT GGA GG
Gamma (+/-) antisense-1: 5' region:
TCG ATG TCA GGA GAT CCT G
Gamma (+/-) 4-base mismatched antisense-1: 5' region:
CCG ATG TCA AGA GAT CGT T
Gamma (+/-) antisense-2: 5' region:
CCA CCC AGC GCT GCC ACA GC
Gamma (+/-) 4-base mismatched antisense-2: 5' region:
CCA ACC AGC TCC GCC GCA GC

ODN uptake studies

Gamma antisense ODNs (mid-sequence region) were synthesized with three bases containing bromodeoxyuridine (BrdU). This ODN was purified and placed in BGJb media at a final concentration of 25 μM. Media was replaced every two days. Mandible explants were collected on day 2,4, and 7, fixed in 10% zinc-formalin, embedded in paraffin and then transverse sectioned. BrdU was detected using an anti-BrdU antibody (Dako Diagnostica, Hamburg Germany) and detected by the avidin-biotin staining method (Carson, 1997).

Histology

Mandibular explants were collected and fixed in 10% zinc formalin and embedded in paraffin. Transverse sections (6 μm) were cut through the tissues and then stained with hematoxylin and eosin and alkaline phosphatase according to methods described by Carson (1997).

Cell proliferation study

GD 11.5 mandibular explants were dissected and then cultured for 8 days in the presence of 25 μM gamma 5' antisense ODN 1, 25 μM scrambled antisense or no ODN. On day 8 of culture, 16.2 μM of BrdU was added to the media and the culture continued for 2 hours. Mandibles were then removed from the media, fixed in 10% zinc-formalin fixative, embedded in paraffin, and then sectioned. BrdU was detected using an anti-BrdU antibody and detected by the avidin-biotin staining method. BrdU-pulsed mandibles were also sectioned and stained with safranin O (Carson, 1997) for identification of precartilaginous condensations and sites of active chondrogenic differentiation. Cell proliferation was assessed by first identifying the chondrogenic regions and precartilaginous condensations by evaluating the slides stained with safranin O. Precartilaginous condensations, which are comprised of chondroblasts, were identified by cellular morphology and by staining characteristics (*i.e.* larger cells with basophic nuclei and unstained cytoplasm). Regions of chondrogenic differentiation were identified by cellular morphology and by positive safranin O staining of the ground substance surrounding the chondrocytes. The chondroblasts were identified as the cells which bordered the outer peripheries of these areas. The identified precartilaginous condensations and chondrogenic regions were then evaluated on sides stained for BrdU. Chondroblasts were counted using a cytometer which tallied the BrdU positive cells from the negative cells. At least 3 chondrogenic areas and 1 precartilaginous condensation were evaluated in each specimen and at least 3 mandibles were evaluated per treatment group. Statistical analysis was carried out using Sigma Plot software and statistical significance was determined by the use of a two-tailed Student's *t*-test.

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