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The Dentin Matrix Protein 1 Gene of Prototherian and Metatherian Mammals

Satoru Toyosawa,* Colm O'hUigin, Jan Klein

Max-Planck-Institut für Biologie, Abteilung Immungenetik, Corrensstrasse 42, D-72076 Tübingen, Germany

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Abstract. Mineralization of tooth dentin (the deposition of hydroxyapatite crystals in and around collagen type I fibers of the extracellular matrix) requires the involvement of several genes, among them the gene coding for the dentin matrix protein 1, *DMP1*. We determined the exon–intron organization of the cattle *DMP1* gene and used this information to amplify by the polymerase chain reaction homologous gene fragments from the genomic DNA of two species of metatherian (marsupial) mammals and one prototherian (monotreme) species. The translated proto- and metatherian protein sequences are highly divergent from the eutherian sequences but retain the general characteristics of the DMP1 (high acidity, serine-richness, multiple glycosylation sites, and the presence of the RGD cell attachment tripeptide). They therefore appear to be functional even though, evolutionarily, teeth are in a regression phase in prototherians. It is possible, therefore, that *DMP1* is also involved in other functions besides dentinogenesis. The *DMP1* gene appears to evolve rapidly and apparently tolerates nonframe-shifting insertions/deletions throughout the coding sequence.

Key words: Dentin — Phosphoprotein — Dentin matrix protein 1 — Marsupials — Monotremes

Introduction

The two principal hard tissues of a mammalian tooth are dentin and enamel (Miles and Poole 1967). Dentin is a bonelike substance composed of mineralized extracellular matrix secreted by odontoblasts. The main component of the matrix is collagen, whereas the noncollagenous 10% of the matrix consists chiefly of acidic phosphoproteins (Linde and Goldberg 1993; Butler et al. 1997). While the fibrils of the collagenous part serve largely as a scaffolding for the deposition of the hydroxyapatite crystals, the components of the noncollagenous part are believed to be involved in the initiation of mineralization and rate of apatite growth, as well as functions not related to mineralization. The proteins comprising the noncollagenous part of the dentin matrix are only now beginning to be identified and characterized, primarily through DNA cloning.

One of the acidic phosphoproteins identified with the help of recombinant DNA techniques was originally termed AG1 (George et al. 1993) but later renamed dentin matrix protein 1, or DMP1 (George et al. 1994). The *DMP1* cDNA clone was obtained by screening a rat cDNA library prepared from odontoblast mRNA. The clone encoded a polypeptide chain comprised of 16 amino acid residues of a signal sequence and 473 residues of a mature protein with an estimated M_r of 53,000. The acidity of the protein was inferred from the preponderance of Asp and Glu residues, its phosphoprotein nature from the presence of >100 Ser residues forming sites for phosphorylation by casein kinases I and II. The presence of several *N*- and *O*-glycosylation sites indicated that DMP1 was a glycopro-

* *Permanent address:* Department of Oral Pathology, Osaka University Faculty of Dentistry, 1-8. Yamadaoka, Suita, Osaka 565, Japan
Correspondence to: C. O'hUigin; *e-mail:* colm.o'huigin@tuebingen.mpg.de

tein and this prediction was supported by the 61,000 M_r of the ^{32}P-labeled immunoprecipitated molecules. Transcripts of the *DMP1* gene were originally detected by northern blot hybridization in odontoblasts only (George et al. 1993) but were later shown by in situ hybridization to be present also in other mineralized tissues, including bone and cementum (D'Souza et al. 1997), as well as in fetal bovine brain (Hirst et al. 1997a).

Homologues of the *DMP1* gene or its transcripts have been cloned also in human (Hirst et al. 1997b), cattle (Hirst et al. 1997a), and mouse (MacDougall et al. 1998). Zoo blot analysis suggests that a *DMP1* homologue is also present in an unspecified monkey species, sheep, and dog (Hirst et al. 1997a). The rat and cattle DMP1 sequences show 67% identity at the nucleotide level and 79% identity at the protein level (Hirst et al. 1997a). The human *DMP1* gene consists of six exons separated by five introns and contains an open reading frame of 1539 base pairs (bp) (Hirst et al. 1997b). The human *DMP1* gene maps to the 4q21–4q22 region, in close proximity to the dentinogenesis imperfecta type 2 gene (MacDougall et al. 1996). However, the two genes are apparently not identical (Hirst et al. 1997b). The function of DMP1 is not known, but the protein is believed to bind to matrix fibrils after its delivery to the preformed collagen type I matrix and thus either to initiate nucleation or to facilitate mineralization through its high Ca^{2+}-binding capacity.

Dentin is believed to have a long evolutionary history that goes back all the way to the origin of vertebrates; in fact it is considered to be one of the distinguishing characteristics of the vertebrate subphylum (Smith and Hall 1990). The presence of the various types of dentin figures significantly in deductions concerning phylogenetic relationships among extant and particularly extinct vertebrate taxa (Ørvig 1967). The comparisons of dentin forms have, however, been based almost exclusively on morphological and microscopic–anatomical examinations, which could lead to erroneous conclusions as a result of frequent phenotypic convergences. The utility of dentin as a phylogenetic marker could greatly be increased by complementing the morphological by molecular studies. Moreover, the molecular evolution of the various dentin-forming proteins could be of great interest in its own right. Yet such studies have thus far been limited to the detection of serological cross-reactions by immunological methods (Wilson et al. 1977). Cross-reactions, however, do not establish homology of proteins, particularly those from highly divergent phyla. For all these reasons, we have initiated a search for the genes encoding dentin and other tooth-forming proteins in various vertebrate taxa. As an initial step in this search, we report here the results of an effort to detect the homologue of the *DMP1* gene in marsupials and monotremes.

Materials and Methods

Source and Isolation of DNA. Spleen of an adult red-necked wallaby (*Macropus rufogriseus*) was obtained from an animal that died in the Hamburg–Hagenbeck Zoological Garden. Tissues from the gray short-tailed opossum (*Monodelphis domestica*) were obtained from the colony maintained by Prof. W.H. Stone, Department of Biology, Trinity University, San Antonio, Texas. DNA from platypus (*Ornithorhynchus anatinus*) were provided by Dr. Robert W. Slade, Queensland Institute for Medical Research, Royal Brisbane Hospital, Australia. Tissues from the domestic cattle (*Bos taurus*) were obtained from Serva, Heidelberg, Germany. Platypus belongs to the order Monotremata, subclass Prototheria; wallaby and opossum are representatives of the order Marsupalia, infraclass Metatheria; and cattle represents the order Artiodactyla, infraclass Eutheria, class Mammalia. The tissues were kept frozen at −70°C until their use. Genomic DNA was isolated from the tissues by phenol–chloroform extraction.

Polymerase Chain Reaction (PCR). The *DMP1* genes of the marsupials and the monotreme were amplified using primers based on the cattle sequence. The primers were as follows: DEN-2 (antisense), 5′-ATCTTGGCAATCATTGTCATC-3′; DEN-8 (sense), 5′-ATAAAGACGATGATGAAGACG-3′; and DEN-12 (sense), 5′-GATGAAGACGACAGTGGAGATGACACCTT-3′. To determine the genomic organization of the cattle *DMP1*, primers were designed to amplify each region of the individual exon–intron boundaries. These primers were 5′-ATCCTGCTTATGTTCCTGTGG-3′, and 5′-CAAAATACTGAATCCAAGAGCTC-3′ for the boundary between exon 2 and exon 3, 5′-GTAGCCAGGTATCAAAATACTGA-3′ and 5′-TGTTCCTCTGAGCTAAGTTT-3′ for the boundary between exon 3 and exon 4, 5′-CATTTGGCTCAGACACCAAC-3′ and 5′-TGTTCCTCTGAGCTAAGTTT for the boundary between exon 4 and exon 5, and 5′-CATTTGGCTCAGACACCAAC-3′ and 5′-ATCGTCCCCAAAGGTGTCATCTCC-3′ for the boundary between exon 5 and exon 6. The 50-μl PCR mixes contained 100 ng/μl genomic DNA, 3.5 m*M* $MgCl_2$, 200 μ*M* dNTP, 10 m*M* Tris buffer, pH 8.5, and 2.5 U of *Taq* polymerase. Amplifications were performed in a PTC-100 Thermal Controller (Biozym, Oldendorf, Germany) in 35 cycles, each cycle consisting of 1 min of denaturation at 94°C, 60 s of annealing at the annealing temperature, and 3 min of extension at 72°C. The final extension was for 10 min at 72°C.

Subcloning and Sequencing. Twenty microliters of the PCR amplification product was purified by 2% low-melting point agarose (Gibco BRL, Eggenstein, Germany) gel electrophoresis. The bands were identified by ethidium bromide staining, excised, and isolated from the gel by DNA extraction kits (Qiagen, Hilden, Germany). The isolated DNA was blunt-ended, phosphorylated, and ligated to *Sma*I-digested pUC18 plasmid vector with the SurClone ligation kit (Pharmacia, Germany). The reactions were transformed into *E. coli* XL-1 blue component bacteria by standard methods and plated on LB agar containing ampicillin (50 μl/ml), X-Gal (5-bromo-4-choloro-3-indolyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalacto-pyranoside). Positive (white) colonies were picked and grown overnight in LB-ampicillin broth, and minipreps were prepared according to the standard protocol (Maniatis et al. 1991). The insert size was then checked by PCR.

DNA Sequencing. Double-stranded DNA was purified using the Qiagen plasmid kit. Two to five micrograms of DNA was used in the dideoxy sequencing reactions, carried out with the help of an AutoRead Sequencing kit (Pharmacia) which contained 5′ fluorescent-labeled sequencing primers and T7 DNA polymerase. The reactions were then processed by the Automated Laser Fluorescent (A.L.F.) Sequencer (Pharmacia). To determine the exon–intron organization of the cattle *DMP1* gene, the genomic sequence data were compared with the cDNA

sequences (George et al. 1993; Hirst et al. 1997a, b) and exon–intron boundaries were identified by comparison with the published consensus sequences (Breathnach and Chambon 1981).

Data Analysis. The nucleotide sequences and inferred protein sequences were aligned using the GCG computer program (Genetic Computer Group Inc., Madison, WI). The evolutionary relationships were then evaluated by the neighbor-joining algorithm (Saitou and Nei 1987) of the MEGA package (Kumar et al. 1993). Nucleotide distances were estimated using the method of Li et al. (1985).

Results

The aim of the study was to clone the *DMP1* gene of species representing meta- and prototherian mammals. Achieving this aim would have undoubtedly been facilitated by the use of cDNA libraries from appropriate tissues. Since, however, such tissues were not available to us, we had to make do with genomic DNA. Furthermore, since at the time when this project was initiated, the exon/intron organization was not known for the *DMP1* gene of any species, we first had to determine the exon boundaries of a known *DMP1* gene in order to design PCR primers for use on proto- and metatherian genomic DNA. We chose the cattle because in this species the full-length cDNA sequence of *DMP1* was known (Hirst et al. 1997a). Primer pairs were selected at intervals along the entire cDNA sequence and used to amplify the genomic cattle DNA. Products larger than predicted by the cDNA sequence were cloned and sequenced fully, or partially in the case of long introns. The primer pairs that in the end provided the desired information are listed under Materials and Methods. Using this approach, we were able to identify five exons in the coding sequence of the cattle *DMP1* gene (Fig. 1); they correspond to exons 2 through 6 of the human *DMP1* gene, whose exon–intron organization has been published in the meantime (Hirst et al. 1997b). (Since the 5′-most exon, now known to be exon 2, encompassed the 5′ end of the coding sequence, no attempt was made to identify the exon bearing the 5′ untranslated region, which is now known to be specified by exon 1.) From the size of the PCR-amplification products, we estimated the lengths of the four introns in the cattle *DMP1* gene to be approximately 800 bp (I2), 3 kb (I3), 590 bp (I4), and 3 kb (I5). For the human *DMP1* gene, lengths have been reported recently, but only for introns 2 (485 bp) and 4 (178 bp) (Hirst et al. 1997b). As in the human gene (Hirst et al. 1997b), in the cattle *DMP1* the donor and acceptor sequences, where they were determined, conform with the consensus sequences (Breathnach and Chambon 1981). They are as follows: GCT CTG CCA gtaagtatca (E2/I2 boundary), aaattttctag GTA GCC AGG (I2/E3 boundary), GAA TGG AAG gtgagtagaa (E3/I3 boundary), CCA CCT TTG gtaactatct (E4/I4 boundary), ttctttttag GAG AGC AGT (I4/E5 boundary), and caaactccag GCA AAT GAA (I5/E6 boundary). Also, as in the human gene, in the cattle sequence all the determined exon/intron boundaries are of type O, falling between codons.

Of the six *DMP1* exons, only exon 6 is suitable for phylogenetic analysis, all other exons being too short for identification in distant taxa: in the cattle, exons 2, 3, 4, and 5 code for protein stretches that are a mere 18, 16, 11, and 16 amino acid residues long, respectively. We therefore focused on exon 6, which specifies 449 amino acid residues. We chose primers annealing to the conserved regions at the opposite ends of exon 6. They were the primers DEN-8 and DEN-2, as well as DEN-12 and DEN-2 in the case of opossum and platypus genomic DNA, to which the seminested PCR approach was applied. The PCR amplification with these primers produced fragments 1200 bp (wallaby), 1218 bp (opossum), and 1176 bp (platypus) in length. The fragments were then cloned and sequenced.

An alignment of the nucleotide (Fig. 2) and translated amino acid (Fig. 3) sequences with the known *DMP1* sequences reveals that the amplification products are indeed *DMP1* homologues. The sequence similarities of the prototherian and metatherian proteins with the eutherian proteins range from 41 to 45 and from 42 to 46%, respectively (Table 1). The sequence similarity of the prototherian and metatherian proteins is approximately 48%. A dot-plot of human and platypus DMP1 protein (Fig. 4) indicates that the degree of sequence conservation is not very high. On an unrooted phylogenetic tree, the eutherian sequences cluster together, while the prototherian and metatherian sequences form separate branches (Fig. 5). In spite of the relatively high degree of divergence of the proto- and metatherian DMP1s from the eutherian DMP1s, certain features remain conserved among all the sequences. These include integrity of the longest exon, high acidity of the encoded protein, serine

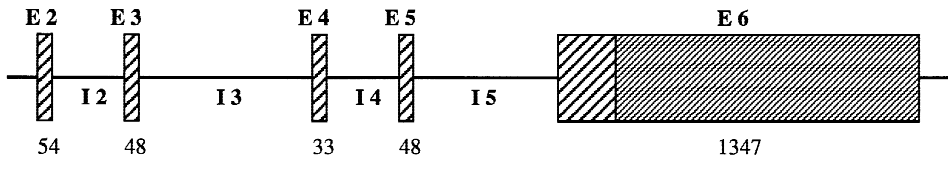

Fig. 1. Intron–exon organization of the cattle *DMP1* gene. The five protein-encoding exons are indicated by *striped boxes. Numbers below the boxes* indicate the size of coding regions within each exon. In other species we amplified fragments which correspond to the part of cattle exon 6, indicated by the *finely striped box.*

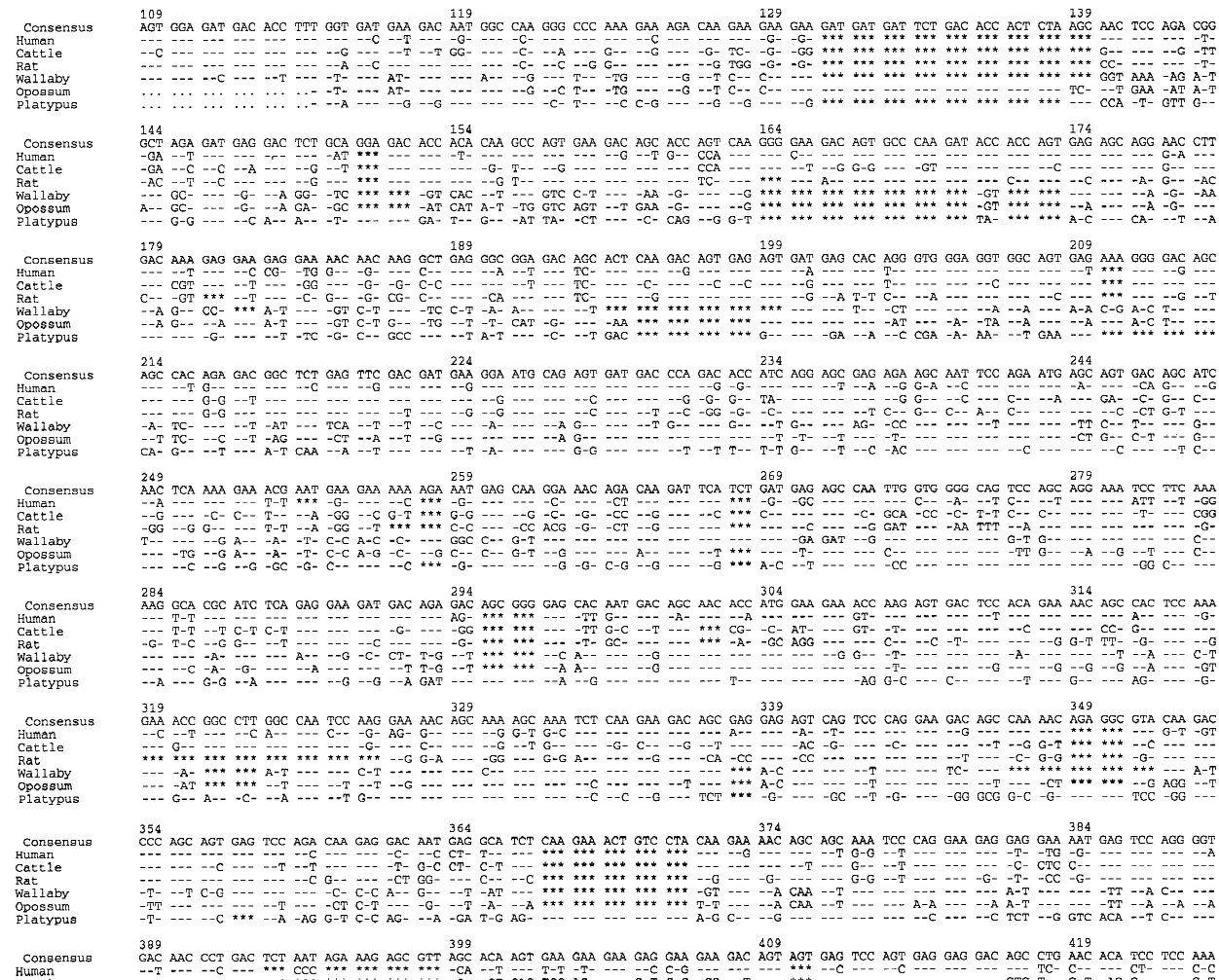

Fig. 2. Nucleotide *DMP1* sequences of the human, cattle, rat, wallaby, opossum, and platypus. The human sequence is from Hirst and co-workers (1997b), the cattle sequence from Hirst and co-workers (1997a), and the rat sequence from George and co-workers (1993). Identity with the simple majority consensus is indicated by *dashes*, unavailability of information by *dots*, and indels by *asterisks*. The *numbers above* the sequence indicate codon positions in the alignment, beginning with codon 109 of the human *DMP1* gene.

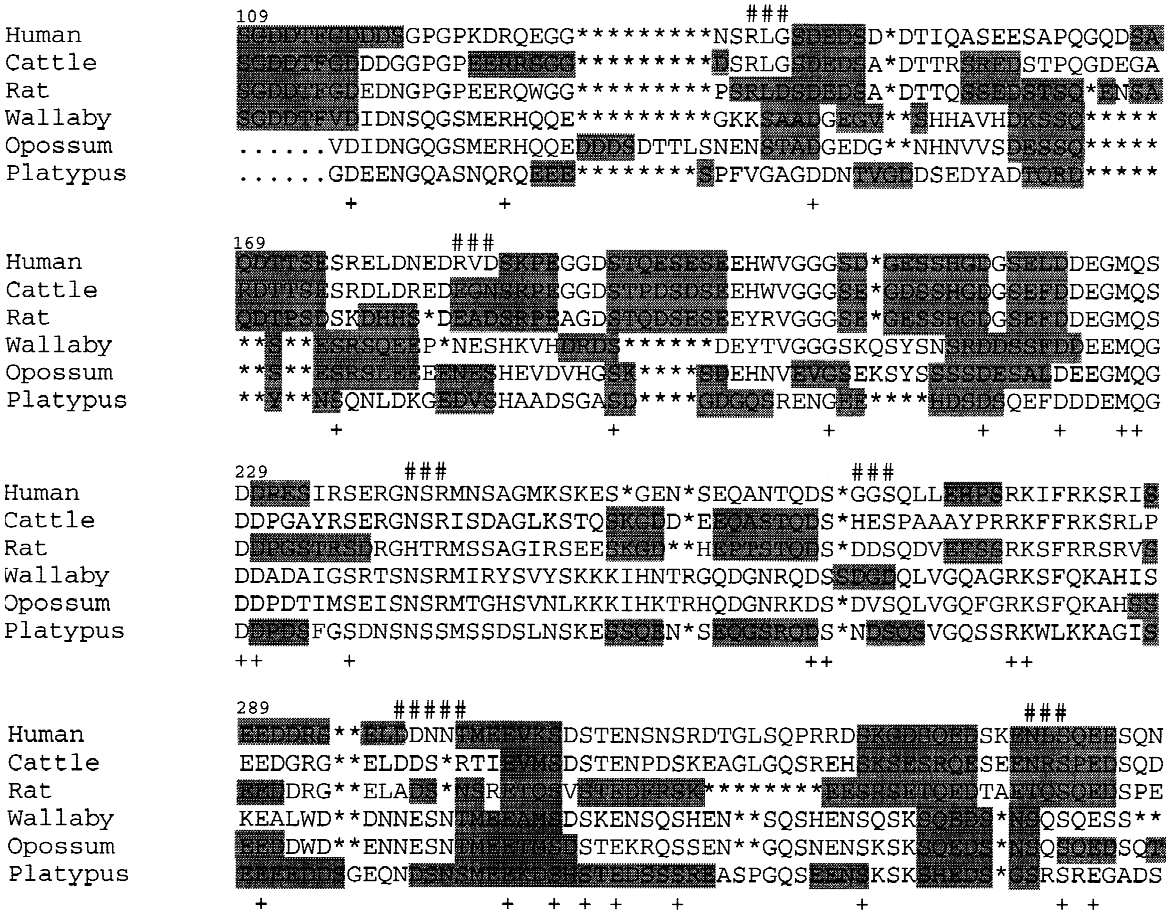

Fig. 3. Comparison of the deduced amino acid sequences translated from the human, cattle, rat, wallaby, opossum, and platypus *DMP1* genes. An *asterisk* indicates alignment gaps. Potential casein kinase I and II phosphorylation sites (i.e., Ser/Thr–X–X–Asp/Glu or Asp/Glu–X–X-Ser) are indicated by *filled boxes.* A # symbol above the alignment indicates potential *N*-glycosylation sites (i.e., Asn–X-Ser/Thr), while a + symbol under the alignment indicates amino acid residues conserved in all species. The *boxed* amino acid residues represent the RGD cell attachment motif.

richness, presence of a cell attachment motif, and multiple glycosylation sites.

Without a cDNA sequence, it is not possible to make inferences about the exon/intron organization of the proto- and metatherian *DMP1* genes. It is nevertheless clear that the eutherian exon 6 has remained intact in the evolution of proto- and metatherians—an interval that spans some 200 million years (my) (Carroll 1988). Presumably, exon 6, the longest of the six *DMP1* exons, is also functionally the most important. Southern blot analysis using an exon 6 cocktail of opossum, wallaby, and platypus probes is consistent with a single hybridizing locus in each species (not shown).

Acidity is imposed on the DMP1 molecule by the high content of aspartic and glutamic acids. The predicted platypus DMP1 fragment contains 11.5% Asp and 15.1% Glu; the opossum fragment, 10.1% Asp, 16.5% Glu; and the wallaby fragment, 9.8% Asp and 12.5% Glu. These values are comparable to those found in the eutherian DMP1 sequences (George et al. 1993; Hirst et al. 1997a, b). The conservation of high acidity supports the view that the Asp and Glu residues are required for the initiation and regulation of hydroxy apatite crystal growth during mineralization of the extracellular matrix.

Serine is an essential residue of the sequence motif recognized by the casein kinases I and II (Marshak and Carroll 1991) and the target of phosphorylation catalyzed by these enzymes. The predicted serine content of the sequenced fragments is high: 27.3, 23.9, and 23.5% of the platypus, opossum, and wallaby DMP1 fragments, respectively. Here, again, these values are similar to those reported for the eutherian proteins (George et al. 1993; Hirst et al. 1997a, b). Moreover, the serine positions and indeed the entire phosphorylation motifs are highly conserved among all the mammalian DMP1s thus far sequenced (Fig. 3).

The cell attachment peptide Arg–Gly–Asp, or RGD, is not specific for the DMP1 molecules but rather is found to be present in many unrelated proteins (Oldberg et al. 1986; Young et al. 1990; Fisher et al. 1990). In the DMP1s it occurs in the middle of the exon 6-encoded part and is strictly conserved among all the sequenced mammalian proteins (Fig. 3). Finally, all the known mammalian DMP1s contain multiple potential *N*-

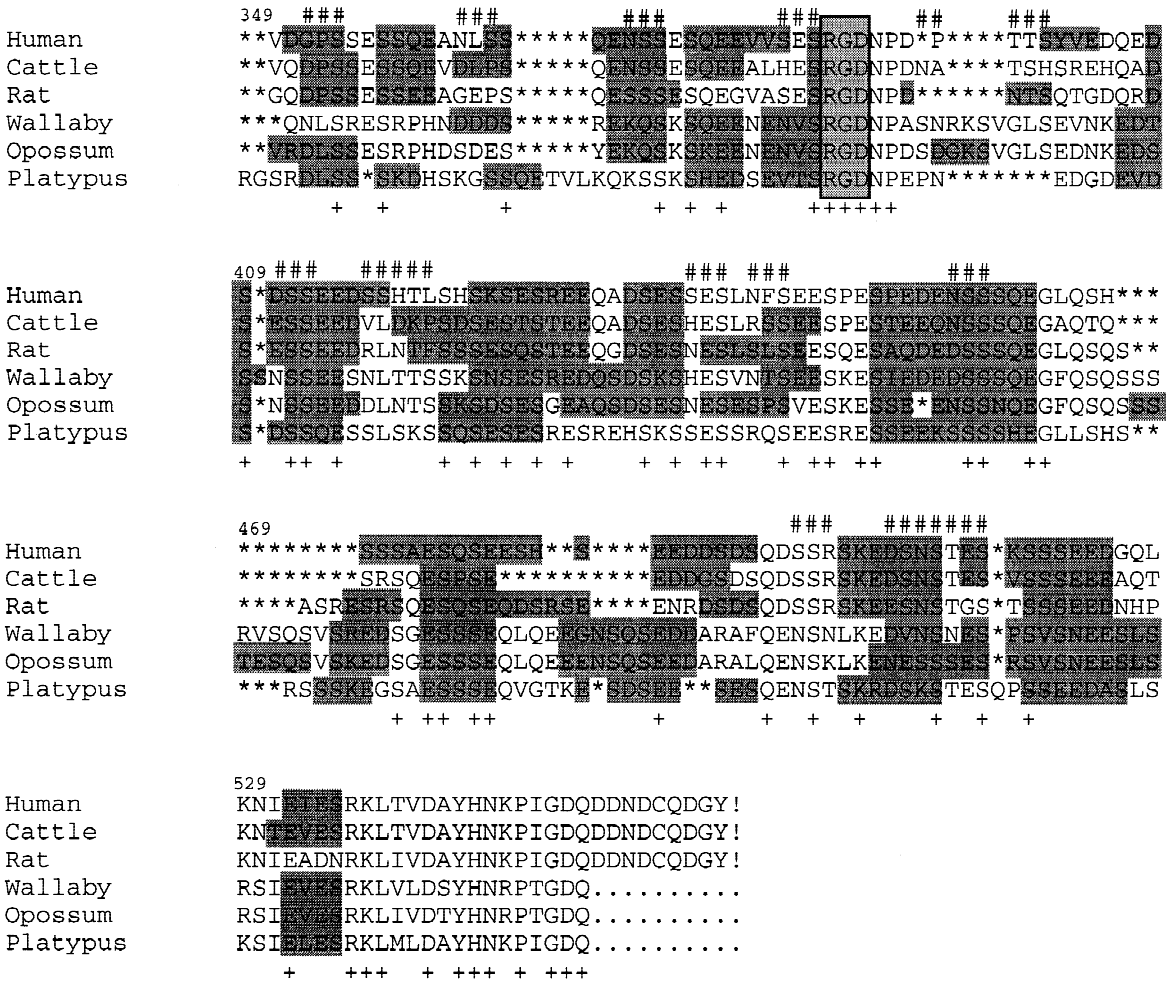

Fig. 3. Continued.

glycosylation sites, some of which are conserved among the various proteins (Fig. 3).

Notwithstanding the conservation of the various motifs, the sequence of the DMP1 is apparently quite tolerant of changes, both in terms of amino acid substitutions and of length of the molecule. The alignments of the sequences in Figs. 2 and 3 required quite a few insertions/deletions (indels), some of them as long as 16 amino acid residues (positions 131–146 in Fig. 3). Assuming that the sequenced pro- and metatherian fragments are derived from functional genes (for which assumption we have no evidence, however), it could be inferred that the DMP1 molecule is rather flexible in its tertiary structure.

Discussion

The four species used in the present study represent major branches in the evolution of mammals. The prototherians (monotremes), here represented by the platypus, have been, until recently, generally held to be the oldest branch, most closely related to the therapsid reptiles from which mammals are believed to have evolved (Carroll 1988). Recent comparisons of complete mitochondrial genome sequences (Janke et al. 1996, 1997), however, support the minority view (Gregory 1947), according to which the oldest branch are the metatherians (marsupials) and the prototherians are derived from them. The oldest metatherian fossils are from Late Cretaceous of North America (Clemens 1979), which would date the metatherian emergence to approximately 100 my ago. Molecular data (Hope et al. 1990) suggest, however, that meta- and eutherian mammals diverged at least 150 my ago. This, then, must also be the divergence time of the proto-/metatherian and eutherian DMP1 sequences in Figs. 2 and 3.

Comparisons of the sequences indicate that the *DMP1* gene is evolving rapidly. There are numerous indels required to optimally align even the eutherian sequences (the cattle–human alignment, for example, contains seven indels). An apparent flexibility in the length of internal segments is also seen in the degree of substitution of the aligned sequences. Pairwise comparisons of synonymous substitutions between species indicate that saturation has occurred, since in all instances the distance

Table 1. Percentage amino acid identity between aligned *dmp1* exon 6 sequences from different mammalian species (gapped sites were removed only in selected pairwise comparisons)

	Human	Cattle	Rat	Wallaby	Opossum	Platypus
Human		69	67	46	45	45
Cattle			67	42	43	41
Rat				42	43	42
Wallaby					73	47
Opossum						48

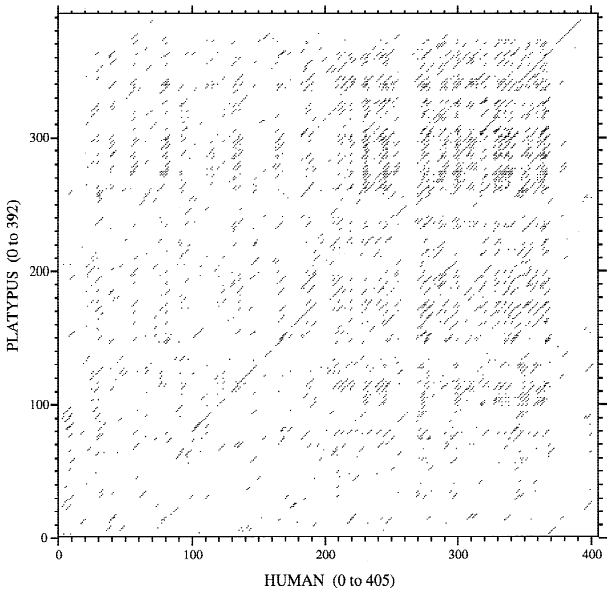

Fig. 4. Dot-plot of human DMP1 amino acids sequence (abscissa) compared to platypus DMP1 (ordinate). A dot indicates three matching residues in a window of size 6.

between the pairs is greater than 90%. Although it is difficult to estimate reliably the amount of synonymous substitution at this high level, the values obtained for human–rat comparison (96.5%) exceed values found for 27 of 28 human–rat gene pairs sampled by O'hUigin and Li (1992). Thus it appears that the underlying rate of synonymous substitutions (and hence mutations) is high at the *DMP1* locus. The same high degree of substitutions is seen at the nonsynonymous sites. Here the value of the human-rat comparison (17.9%) is exceeded by the values of only 2 of the 28 genes surveyed by O'hUigin and Li (1992). The human and rat *DMP1* genes appear to be representative of the *DMP1* genes we have sequenced and consequently probably accurately reflect the evolutionary rate of *DMP1*. The eutherian-to-metatherian or eutherian-to-prototherian comparisons indicate that nonsynonymous substitutions have occurred in excess of 40% over the 120–150 my, the interval within which these groups have diverged. This value is higher than the rate found in β-globin gene comparisons, where human and marsupial cat sequences differ by 23% nonsynonymous substitutions (Koop and Goodman 1988). Since the β-globin gene has an average evolutionary rate (Li et al. 1985), the *DMP1* gene must be evolving rapidly. Given the high nonsynonymous substitution rate, it may prove difficult to isolate and identify the gene in nonmammalian vertebrates using standard methods.

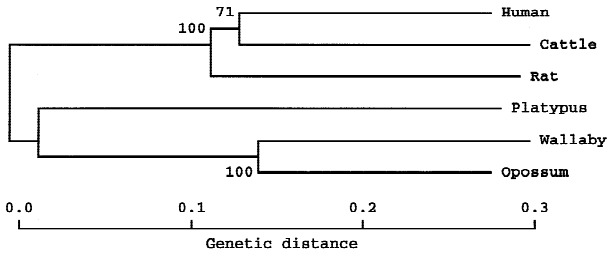

Fig. 5. Neighbor-joining tree of the deduced protein sequences of six *DMP1* genes. The scale bar represents the distance based on the proportion of amino acid differences. Gapped sites were removed from all sequences before distance estimates were made. The numbers on the nodes indicate the percentage recovery of that node in 500 bootstrap replications.

The synonymous distance found between marsupial mammals, the wallaby, and the opossum is 51.6%. This is considerably less than that found between the human, rodent, and bovine eutherian mammals, which averages 108%, with a range of 96.5 to 130%. The nonsynonymous distance between the two marsupials (14.5%) is also less than that found among the eutherian species, which averages 18.5%. Assuming a synonymous clock and a eutherian emergence time in the range 80–100 my ago, the degree of synonymous divergence places the wallaby–opossum split in the range 32–54 my ago, with some uncertainty due to saturation of synonymous sites.

In spite of its high evolutionary rate, the DMP1 molecule has retained its general character in terms of its high acidity, high serine content, multiple glycosylation sites, and the presence of the putative cell-attachment peptide. We interpret this conservation as an indication that the molecule serves a similar function in the various mammalian groups, even in the prototherians, in which the entire dentition is greatly reduced. There are three living species of prototherians: the short-beaked echidna (*Tachyglossus acculeatus*), found in Australia, Tasmania, and Papua New Guinea; the long-beaked echidna (*Zaglossus bruijni*) of Papua New Guinea; and the platypus, distributed through eastern Australia and Tasmania (Augee 1974). Adult echidnas lack teeth entirely, although they do develop an egg-tooth during their embryonic development (Hill and de Beer 1949); in the platypus, teeth are present in young animals only (Augee 1974; Poulton 1888). Platypus embryos develop the anlagen of five lower incisors, one canine, two premolars, and three molars, but all these, except one upper molar, two upper molars, and three lower molars, regress. Even the teeth that do actually erupt may not be functional and are ultimately replaced by horny pads (Augee 1984). Whether the teeth complete development before they

break through their enamel organ when erupting is uncertain. Interestingly, an extinct, middle Eocene platypus *Obduron insignis* had a similar dentition to the living platypus, except that the molars were fully calcified. Apparently, prototherian dentition has been in a declining phase for quite some time. It is, therefore, somewhat surprising that the platypus *DMP1* is recognizable at all. The fact that it is and that it still contains all the motifs that characterize eutherian *DMP1* genes supports the conclusion that it is still functional. Perhaps the gene is needed not only for dentinogenesis but also for other functions, possibly related to bone formation. Its expression in tissues other than teeth (D'Souza et al. 1997) would be consistent with this interpretation. It will be interesting to find out whether other genes involved in dentinogenesis have been conserved in the platypus and in the echidnas.

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