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Mammalian Alkaline Phosphatases

From Biology to Applications in Medicine and Biotechnology

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Preface

Determinations of serum levels of alkaline phosphatase (AP) isozymes are perhaps the most widely used biochemical measurements in the clinical laboratory but, despite their widespread clinical utility, the structure and biological functions of these widely known enzymes have remained elusive for decades. However, great progress is now being made in this area of research that justifies the writing of this monograph summarizing major recent advances. This book is not intended to be an exhaustive treatise on the subject. A superb compilation on APs, summarizing knowledge in this field up to the late 70s, was published by Drs Robert McComb, George Bowers and Solomon Posen (McComb et al., 1979) for references published before 1979, but a few papers published in the 1960s and 1970s are specifically cited here because of their unique relevance to some of our discussions. Two subsequent reports, much smaller in scope relative to McComb et al.'s impressive book, have also been published: the first (Human Alkaline Phosphatases, in *Progress in Clinical and Biological Research*, Vol. 166, Editors Torgny Stigbrand and William H. Fishman, Alan R. Liss, New York, 1984), reported the Proceedings of the First Symposium on Alkaline Phosphatases held in Umeå, Sweden, September 16–17, 1983. The second, published as a special issue of *Clinica Chimica Acta* [Vol 186 (2), pp. 125–320, 1990; Guest Editor José Luis Millán], presented selected papers from the Third Alkaline Phosphatase Symposium that took place at the La Jolla Cancer Research Foundation on February 1–3, 1989, on the occasion of Dr William H. Fishman's retirement. I will refer to individual papers in these two small volumes when needed.

The present work is intended to highlight and discuss key new information obtained in the last 25 years (1980–2005), with special emphasis on the structure of the proteins and genes, the function of the isozymes as revealed from the study of the individual mouse knockout strains and new uses of these isozymes in the clinical setting and in biotechnology. In this period, approximately 5000 papers have been published containing alkaline phosphatase in the title. If one searches for papers using alkaline phosphatase as a keyword, the number climbs to 36 000. Hence it would have been impossible to cite each of these references in a small treatise such as this and I apologize to those researchers whose work is either not cited or not presented in as much detail as I would have liked. Owing to space considerations, I have chosen to cite those references that, in my opinion, best illustrate new findings, a novel property or a function that advances the field.

Many confirmatory papers are not cited. Furthermore, this book is intended to discuss the structure and function of "mammalian" APs. Hence a considerable body of literature on bacterial APs is not covered here. Some papers on bacterial APs are cited only when there is a need to clarify a property or mechanism that has not been yet elucidated for the mammalian enzymes. The same is true for other nonmammalian APs. The book has been written with the aim of informing a wide spectrum of scientists and health care professionals who are not necessarily experts in this area of research. However, enough detail is provided so that investigators working on APs will find this to be an up-to-date account of the state-of-the-art in this field.

I would like to dedicate this book to the memory of two individuals who were responsible in large measure to my having dedicated most of my scientific career to the study of mammalian APs. To the late Dr Raúl Francisco Balado (1920–98), who mentored me through my first steps in clinical chemistry and in the use of APs in the clinical setting in my home town, Mar del Plata, Argentina, in the period 1974–77. His friendship, encouragement and support in obtaining a Rotary International Fellowship to travel abroad to initiate a research career represented a cornerstone of my scientific and personal life. That fellowship would take me, on August 4, 1977, to the laboratory of the late Professor William H. Fishman (1914–2001), who at that time had just moved from Tufts University in Boston to La Jolla, California, to found a new research institute, i.e. the La Jolla Cancer Research Foundation, now known as the Burnham Institute for Medical Research. His pioneering work on the Regan isozyme as a tumor marker had prompted him to create a research institution focused on oncodevelopmental biology and he was recruiting staff to build his new center. Fortunately, he did not turn away this young and inexperienced though aspiring trainee. Dr Fishman's mentoring and advice were paramount in my scientific career. He remained interested in advances in the field of APs to the day of his passing and continues to be a father figure to me and to many of the members of the institute that he created. I think he would have enjoyed this monograph.

I would like to thank Dr Andrea Pillmann of Wiley-VCH Verlag GmbH & Co., for suggesting writing this book after my lecture at the International Conference on Genes, Gene Families, and Isozymes, Berlin, 2003 and for her help and that of her staff in progressing the manuscript to final production. I want to thank my secretary Trixi Czink at the Burnham Institute for her invaluable help in compiling the bibliography for the book. I also want to express my sincere appreciation to all the members of my laboratory, past and present, who contributed much of the published data discussed and cited in this book. I have had the pleasure of working with many talented collaborators throughout the years. I would particularly like to thank Professor Marc F. Hoylaerts for the many years of extremely productive scientific interactions and friendship and for his many comments and suggestions on this book, particularly his input to the sections on enzyme kinetics and structure. I also want to thank Per Magnusson for his suggestions regarding the clinical sections of this book.

La Jolla, California
July 2005

José Luis Millán

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
ASO	Allele-specific oligonucleotide
bIAP	Bovine intestinal alkaline phosphatase
BsMAb	Bispecific monoclonal antibody
BMD	Bone mineral density
BMP	Bone morphogenetic protein
cAMP	Cyclic AMP
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CIS	Carcinoma-*in-situ*
CMV	Cytomegalovirus
CPPD	Calcium pyrophosphate dihydrate
EAP	Embryonic alkaline phosphatase
ECAP	*Escherichia coli* alkaline phosphatase
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EP	Etoposide phosphate
ES	Embryo-derived stem cells
FADP	Flavin adenine dinucleotide-3′-phosphate
GABA	γ-Aminobutyric acid
GCAP	Germ cell alkaline phosphatase
GPI	Glycosylphosphatidylinositol
GPI-PLC	GPI-specific phospholipase C
GPI-PLD	GPI-specific phospholipase D
HPAC	High-performance affinity chromatography
HPLC	High-performance liquid chromatography
Hyp	Hypophosphatasemic mice

IAP	Intestinal alkaline phosphatase
IIAC	Idiopathic infantile arterial calcification
iPGM	Cofactor-independent phosphoglycerate mutase
IRMA	Immunoradiometric assays
k_{cat}	Catalytic rate constant
K_i	Inhibition constant
K_m	Michaelis constant
KO	Knockout
L-hArg	L-Homoarginine
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MICA	Monoclonal antibody immunocatalytic assay
MOP	Mitomycin phosphate
MPLA	Monophosphoryl lipid A
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MV	Matrix vesicle
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NPP1	Nucleotidetriphosphate pyrophosphohydrolase-1
OPN	Osteopontin
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEA	Phosphoethanolamine
PGC	Primordial germ cells
P_i	Inorganic phosphate
PLAP	Placental alkaline phosphatase
PLP	Pyridoxal-5-phosphate
pNPP	*p*-Nitrophenylphosphate
PNPPate	*p*-Nitrophenylphosphonate
POMP	Phenol mustard phosphate
PP_i	Inorganic pyrophosphate
PSA	Prostate-specific antigen
QTL	Quantitative trait loci
RA	Retinoic acid
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SEAP	Secreted embryonic alkaline phosphatase
SLP	Surfactant-like particle
SNuPE	Single nucleotide primer extension
TGCTs	Testicular germ cell tumors
TNAP	Tissue-nonspecific alkaline phosphatase
TNFα	Tumor necrosis factor alpha

TOF	Time-of-flight
TSAPs	Tissue-specific alkaline phosphatases
UTR	Untranslated region
V_{max}	Maximum velocity
VSMC	Vascular smooth muscle cells
WGA	Wheat germ agglutinin
wt	Wild-type

Glossary

Allotypes	Allelic types. Synonymous with allozymes
Allozymes	Used as synonymous of allelic variants
Null	Used as synonymous of knockout or deficient, e.g. *Akp2* null mice, to refer to either $Akp2^{-/-}$ or $Akp2^{\beta geo/\beta geo}$ or TNAP-deficient mice
Biliary AP	An isoform of liver-derived TNAP
Bone AP	Used interchangeably with skeletal AP, bone-derived TNAP, bone-type TNAP, bone-specific TNAP isoform
Leukocyte AP	An isoform of TNAP
Neutrophil AP	Synonymous with leukocyte AP, an isoform of TNAP
Liver AP	Used interchangeably with liver-type TNAP or liver-specific TNAP isoform
Skeletal AP	Used interchangeably with bone AP, bone-derived TNAP, bone-specific TNAP isoform
Vitamer	Term used to refer to any of several chemical forms of a vitamin, e.g. pyridoxal is a vitamer of vitamin B_6

Introduction

Alkaline phosphatases (APs; EC 3.1.3.1) are ectoplasmic proteins that catalyze the following general reaction:

$$R\text{–}OP + H_2O \rightarrow R\text{–}OH + P_i$$

where the hydrolysis of R–OP gives rise to inorganic phosphate (P_i) and an alcohol, sugar, phenol, etc. (R–OH). As such, they are members of the class of enzymes known as phosphomonoesterases. They are unique in this class in the sense that they appear to be nonspecific and able to act on a wide variety of substrates, at least *in vitro*, and they are referred to as "alkaline" because of their ability to perform this reaction most efficiently at pH above neutral, e.g. pH 8–11. This propensity is somewhat surprising, since these enzymes are present *in vivo* in compartments not known to be particularly alkaline. Nevertheless, the properties of APs have made them useful for a variety of biotechnological applications ranging from dephosphorylating phosphoproteins and DNA fragments to using them for end-point detection in a wide variety of immunoassays and also as reporter molecules *in vivo*. In mammals, AP activity is found in a wide variety of tissues and organs and the enzymes are primarily ectoplasmic in location and attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. Hence most of the artificial or macromolecular substrates used in the laboratory are never in contact with APs *in vivo* and in fact, as we will discuss, only two natural substrates have been unequivocally confirmed to date for one of the AP isozymes. The analysis of an inborn error of metabolism and of knockout mouse models will be a major focus in this book as these studies have clarified the biological role for some of the isozymes and for the others we now have good hypotheses to guide us in our future work. The clarification of the number and structure of the genes encoding human APs has also helped in our ability to detect abnormal alleles during prenatal diagnosis of hypophosphatasia and recent genotype/phenotype correlations of hypophosphatasia mutations have helped us predict the severity of phenotypic abnormalities in affected patients. Advances are also being made in experimental therapeutic approaches of hypophosphatasia and other illnesses. Although AP activity has long been used as indicator of health or disease in a variety of conditions, the potential use of APs itself as therapeutic target has

only come into focus as a result of recent mechanistic studies. Efforts to develop pharmacological drugs able to modulate AP activity are being greatly helped by the wealth of new information gained in recent years on the three-dimensional structure and of the functional domains of mammalian APs. APs are also expressed in a wide variety of tumors and serve as tumor markers for immunodetection and immunolocalization of malignancies. Hence tremendous progress has been made in the last 25 years and this book will attempt to describe the most significant advances in each of these areas. The book is divided into four parts, i.e. Part I: Gene and Protein Structure; Part II: *In vivo* Functions; Part III: AP Expression in Health and Disease; and Part IV: Uses of APs in Industry and Biotechnology. Chapters 1–3 summarize current knowledge of the structure of the genes and their expression and regulation and serve as preparatory material for what I consider the core of this book, i.e. Chapters 4–8. Chapters 4–6 describe the structure of mammalian APs, their structural/functional domains and *in vitro* properties, and Chapters 6–8 elaborate on what we know about the biological function of each AP isozyme both in humans and in mice, through the study of hypophosphatasia and also of each mouse model of AP deficiency. Chapters 9 and 10 summarize a great deal of literature on the clinical utility of AP determinations in health and disease, particularly as cancer markers. Chapters 11–15 describe some exciting developments in the use of APs in industry and biotechnology, but also in the use of APs both as therapeutic agents and as therapeutic targets for some conditions. Finally, Chapter 16 gives an overview of the methodologies that have been improved or developed in the last 25 years. The reference list includes 1027 publications that are cited in this monograph. Readers are encouraged to consult the original sources for details beyond what I was able to include in this book, given the inherent limitations of space.

Part I
Gene and Protein Structure

1
Gene Structure

1.1
Genomic Organization and Complexity

In humans, alkaline phosphatases (APs) (EC 3.1.3.1) are encoded by four genes traditionally named after the tissues where they are predominantly expressed, although the gene nomenclature is now gaining wider use (Table 1). The tissue-nonspecific AP (TNAP) gene (*ALPL*) is expressed at highest levels in liver, bone and kidney (hence the alternative name "L/B/K" AP), in the placenta during the first trimester of pregnancy and at lower levels in numerous other tissues (McComb et al., 1979). The other three isozymes, i.e. placental AP (PLAP), placental-like or germ cell AP (GCAP) and intestinal AP (IAP), show much more restricted tissue expression, hence the general term tissue-specific APs (TSAPs). Within the short span of 2 years, several groups working independently published the sequences of the cDNAs of human PLAP (Kam et al., 1985; Millán, 1986; Henthorn et al., 1986), human IAP (Berger et al., 1987a; Henthorn et al., 1987) and human TNAP (Weiss et al., 1986; Terao and Mintz, 1987). The sequences of the four human AP genes were all published within months of each other in the following year, i.e. human TNAP (Weiss et al., 1988), IAP (Henthorn et al., 1988a), PLAP (Knoll et al., 1988) and GCAP (Millán and Manes, 1988).

The TNAP gene (*ALPL*; NM_000478), maps to the short arm of chromosome 1 (1p36–p34) (Swallow et al., 1986; Smith et al., 1988) and is at least five times larger that the TSAP genes, due in part to the first intron (at least 20 kb long) which separates exon I, which contains only 5′-untranslated (UTR) sequences, from exon II, which contains part of the 5′ UTR and also the start codon for translation. The *ALPL* gene is located on the distal short arm of chromosome 1, band 1p36.12, specifically at position chr1: 21581175–21650208, thus occupying a length of 69 034 bp. Subsequently it was demonstrated, first for the rat homolog (*Alp1*) (Toh et al., 1989) and subsequently for the human *ALPL* and the mouse *Akp2* genes (Matsuura et al., 1990; Toh et al., 1990; Studer et al., 1991), that the TNAP genes are really composed of 13 exons; the first two exons (Ia and Ib) are noncoding and are separated from one another and from the exon that contains the ATG translation initiation site (exon II) by relatively large introns. The last exon contains the termination codon and the 3′-untranslated region of the mRNA. The rest of the exonic sequences are interrupted by introns at positions analogous to those of the

Table 1 Nomenclature of the human, mouse, and rat AP isozymes and genes, including chromosomal location, gene size, and accession numbers.

Gene	Protein name	Common name	Chromosomal location	Accession No.
Human genes				
ALPL	TNAP	Tissue-nonspecific alkaline phosphatase; TNSALP; "liver–bone–kidney type" AP	chr1:21581174–21650208	NM_000478
ALPP	PLAP	Placental alkaline phosphatase; PLALP	chr2:233068964–233073097	NM_001632
ALPP2	GCAP	Germ cell alkaline phosphatase, GCALP	chr2:233097057–233100922	NM_031313
ALPI	IAP	Intestinal alkaline phosphatase, IALP	chr2:233146369–233150245	NM_001631
Mouse genes				
Akp2	TNAP	Tissue-nonspecific alkaline phosphatase; TNSALP; "liver–bone–kidney type" AP	chr4:136199753–136254338	NM_007431
Akp3	IAP	Intestinal alkaline phosphatase, IALP	chr1:87031694–87555136	NM_007432
Akp5	EAP	Embryonic alkaline phosphatase	chr1:86990248–86993641	NM_007433
Akp-ps1	N/a	AP pseudogene, pseudoAP	chr1:86968828–86972484	NG 001340
*Akp6**	AKP6*	RIKEN sequence, new AP locus	chr1:87002298–87005230	AK008000
Rat genes				
Alp1	TNAP	Tissue-nonspecific alkaline phosphatase; TNSALP; "liver–bone–kidney type" AP	chr 5: 156511778–156568766	NM_013059
Alpi	IAPI	Intestinal alkaline phosphatase I	chr 9: 86076305–86079772	NM_022665
Alpi2	IAPII	Intestinal alkaline phosphatase II	chr9: 86107650–86110746	NM_022680

* tentative designation by the author

TSAP genes, but the introns are generally much larger (Fig. 1). Exons Ia in humans and rats have ~66% identical bases whereas sequence homology cannot be detected between the Ib exons in these two species (Toh et al., 1989). The major transcription start site and surrounding sequences of the 5′-most promoter Ia of the TNAP gene have been determined for humans (Weiss et al., 1988; Toh et al., 1989; Zernik et al., 1990) and mouse (Terao et al., 1990). Importantly, exons Ia and Ib are incorporated into the mRNA in a mutually exclusive fashion that results from the fact that each exon has its own promoter sequence. This results in two types of mRNAs, each encoding an identical polypeptide, but having different 5′-untranslated sequences (Kishi et al., 1989; Toh et al., 1989; Studer et al., 1991; Zernik et al., 1991). A 2127 bp cDNA encoding a functional feline TNAP of 524 amino acids has also been isolated (Ghosh and Mullins, 1995).

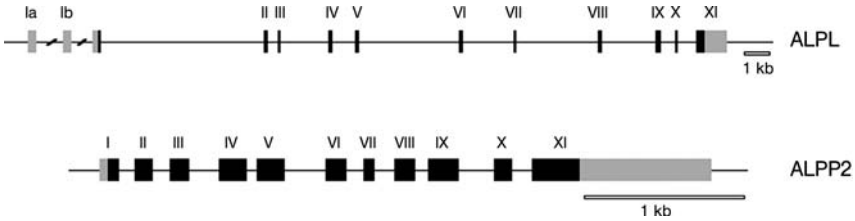

Fig. 1 Genomic organization of the human *ALPL* and *ALPP2* genes. Solid black boxes indicate coding exons and gray boxes mark the 5′ and 3′ untranslated regions (UTR).

The human TSAP genes, *ALPP*, *ALPP2* and *ALPI*, are clustered on human chromosome 2, bands q34–q37 (Griffin et al., 1987; Martin et al., 1987b) and are closely related to one another (Table 1). Their structures are almost identical, consisting of 11 exons interrupted by small introns (74–425 bp) at analogous positions all compressed in less than 5 kb of genomic DNA (Henthorn et al., 1988a; Knoll et al., 1988; Millán and Manes, 1988). Figure 1 shows the structure of the *ALPP2* gene as an example, in relation to the *ALPL* gene. The similarity in structure between all three TSAP genes suggests a divergent evolution for these genes since the chromosome mapping results show that the three related loci – *ALPP*, *ALPP2* and ALPI – are located in this order from centromere to telomere in the same region of the long arm of chromosome 2 (2q34–q37) (Griffin et al., 1987; Martin et al., 1987b). Each gene is comprised of 11 exons and 10 small introns contained within 4.5 kb of DNA. Specific regions of the introns, and also in the 3′-untranslated region of exon XI, show major differences in sequence and these regions have proven useful in the development of gene-specific probes. The PLAP gene also contains an Alu repeat sequence inserted in exon XI (Knoll et al., 1988). This Alu repeat sequence creates a new polyadenylation signal that may be responsible for alternative usage and consequently alternative size PLAP mRNA molecules approximately 300 bp shorter. Alternative PLAP mRNA molecules have already been observed in choriocarcinoma cells (Ovitt et al., 1986), colonic adenocarcinoma cells (Gum et al., 1987) and Hela cells (Chou and Takahashi, 1987). The

intron–exon junctions in the coding region are, however, remarkably similar for all four genes. Knoll et al. have proposed that insertion/deletion events in the promoters of these three isozyme genes may partially explain evolution of promoter activity (Knoll et al., 1988).

In mice, besides the *Akp2* gene, which maps to chromosome 4 (Terao et al., 1988), four additional AP loci are found clustered on chromosome 1 (Table 1); the *Akp3* locus that encodes the IAP isozyme (Manes et al., 1990); the *Akp5* gene that encodes the embryonic AP (EAP) isozyme (Manes et al., 1990); the *Akp-ps1* non-transcribed, intron-containing, pseudogene (Manes et al., 1990); and finally a new locus recently identified in the databases after completion of the mouse genome project (Fig. 2). We tentatively have named this gene *Akp6* and have determined that it codes for an IAP-like isozyme albeit it has catalytic properties distinct from IAP (S. Narisawa et al., personal communication). Furthermore, with the caveat that negative data are seldom conclusive, to date there is no confirmation in the Mouse Genome Databases that the *Akp1* or *Akp4* loci actually exist. These loci were postulated based on hybrid maps and biochemical data (Wilcox et al., 1979), but no sequences have been posted to date for either of these two proposed loci. The *Akp3*, *Akp5* and *Akp-ps1* are around 5.0 kb in length and are composed of 11 exons interrupted by 10 small introns with an organization remarkably similar to that of the human tissue-specific TSAP genes. The smallest exon in all cases is exon VII (73 bp) and the largest in all cases is exon XI, which codes for the C-terminal end of the molecule and also contains the 3′-UTR (640 bp in length for *Akp5* and 1180 bp for *Akp3*). The introns are amongst the smallest reported with the largest one, splitting exons V and VI, being only 214 and 261 bp in *Akp5* and *Akp3*, respectively. The mRNA molecules for all AP isozymes, human or mouse, are of the order of 2.4–3.0 kb in length and encode peptides ranging from 518 to 535 amino acids. The rat genome harbors the TNAP gene (*Alp1*) (Toh et al., 1989) that produces an mRNA of around 2.5 kb (Noda et al., 1987b; Thiede et al., 1988) but reportedly also has two IAP genes, i.e. *Alpi* and *Alpi2*, based on cDNA (Lowe et al., 1990; Strom et al., 1991; Engle and Alpers, 1992) and genomic cloning (Xie

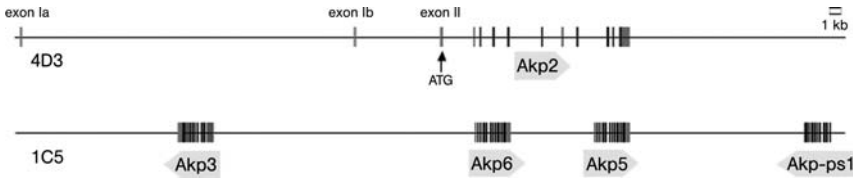

Fig. 2 Genomic organization of the murine AP loci. The mouse TNAP gene (*Akp2*) is located at 4D3 in chromosome 4. It stretches for 55 kb and consists of 12 exons and 11 introns including an alternative exon (exon 1b), located ~30 kb downstream of exon 1a. The mouse TSAP genes (*Akp3*, *Akp5*, *Akp6* and the *Akp-ps1* pseudogene) are closely linked at the 1C5 site in chromosome 1. The size of each TSAP gene is ~3.5 kb and they contain 11 exons and 10 introns. The direction of the *Akp3* gene and the *Akp-ps1* pseudogene is opposite to that of *Akp5* and *Akp6* genes. In the active AP genes, translation starts from the ATG site in the exon 2 and ends at the stop codon within the exon 11. Sequence numbers indicated beneath each gene are the actual location in the chromosome.

and Alpers, 2000). These genes produce mRNAs of 2.7 kb (IAP I) and 3.0 kb (IAP II) (Eliakim et al., 1990b).

After humans and rodents, *Bos taurus* is the species that has been studied the most in terms of its AP gene family. Besman and Coleman (1985) described two different IAP isozymes in the bovine intestine by C-terminal sequencing of chromatographically purified AP fractions. One of these was restricted to intestines of calves whereas the other was present in both calves and adults. Using the mouse IAP cDNA as probe, Weissig et al. (1993) cloned a gene that matched the amino terminus of the adult bovine AP isoform or bIAP I, and also an AP pseudogene. Subsequently, using a combination of protein sequencing and cDNA cloning, Manes et al. (1998) discovered an unprecedented level of complexity for the bovine AP family of genes, obtaining either full or partial cDNA evidence for up to seven IAP-like genes, i.e. bIAP II, III, IV, V, VI and VII. Garattini et al. (1987) cloned and sequenced a bovine kidney TNAP that displayed 90% homology with the human enzyme at both the nucleotide and amino acid levels. Manes et al. (1998) also found evidence for the existence of a second TNAP gene (TNAP-2). In the same year, another group reported the presence of two additional AP cDNAs present in bovine blastocysts (TSAP2 and TSAP3) (McDougall et al., 1998). Hence it seems that *Bos taurus* to date stands alone as the species with the highest degree of complexity in the AP family of genes.

Figure 3 shows a phylogenetic tree constructed from the homology of all the mammalian APs for which we have complete nucleotide or protein sequence. This cladogram was produced with the Clustal W algorithm of the DNASTAR software package. One can clearly see the clustering of the TNAP genes from different species as being considerably different from the TSAP isozymes that also cluster. Note that the duplication of the PLAP and GCAP genes appears to have occurred fairly recently based on the very limited number of substitutions. It has

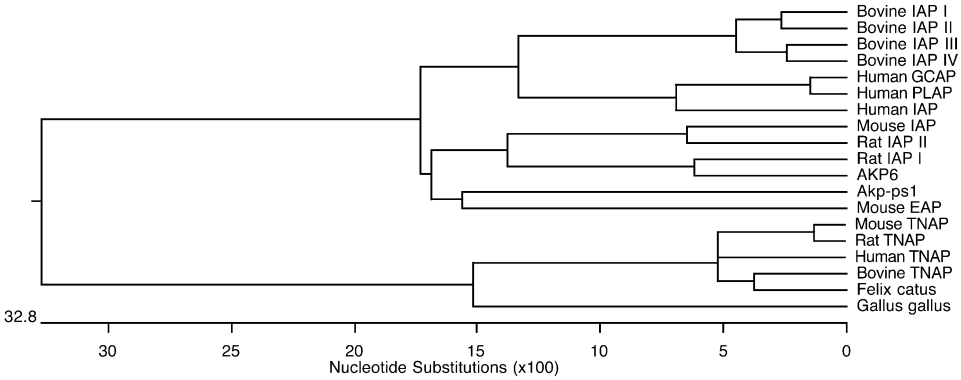

Fig. 3 Phylogenetic tree of all mammalian APs sequenced to date. The chicken TNAP (*Gallus gallus*) has also been included given that the chicken is a species used routinely for studies of bone mineralization. The sequences were aligned using the Clustal W method of the DNASTAR software package. The length of each pair of branches represents the distance between sequence pairs. The scale beneath the tree measures the distance between sequences.

been generally accepted that the appearance of the PLAP gene represents a late evolutionary event. Enzymes with the properties of PLAP were found expressed in the placenta of chimpanzee, orangutan and humans, but not in lower species (Doellgast and Benirschke, 1979; Goldstein and Harris, 1979). Doellgast et al. (1981) extended these observations to show that the placental tissue of the squirrel and spider monkey contain low but measurable levels of a heat-stable AP that resembled the human testis PLAP-like enzyme, that we now call GCAP.

In the late 1980s, we also had an opportunity to analyze genomic DNA samples from a number of Old and New World monkeys kindly provided by Dr Oliver A. Ryder (Center for Reproduction of Endangered Species, San Diego Zoo, San Diego, CA, USA) using the PLAP cDNA as a probe. As can be observed in Fig. 4, a complex hybridization pattern is observed in all the primate species indicating the probable existence of more than on PLAP-related gene. In order to attempt to quantify the number of genes present in each species, we rehybridized the blots using either a 144 bp *Sph–Sma*I or a 227 bp *Pst*I fragment containing the highly conserved exons V or IX, respectively, of the PLAP gene. The results enabled us to count the number of hybridizing bands and correlate them with the number of genes present in the different species. One duplication event seems to have occurred after the bifurcation of Prosimians and New World monkeys, very likely representing the duplication of the IAP gene. A second duplication event precedes the divergence of Old World monkeys and apes, where the precursor of the GCAP

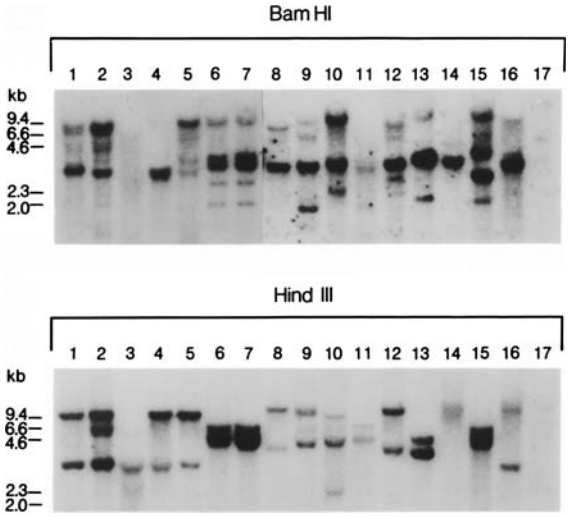

Fig. 4 Probing a Zoo blot for *ALPP*-like sequences. Southern blot analysis of genomic DNA from Old and New World monkey probed with the PLAP cDNA. The DNAs were digested with *Bam*HI and *Hin*dIII, respectively, and probed with the 2.0 kb *Eco*RI–*Kpn*I fragment of the PLAP cDNA. The lanes were loaded with digested genomic DNA from the following species: 1, human; 2, chimpanzee; 3, pygmy chimpanzee; 4, orang-utan; 5, lowland gorilla; 6, rhesus monkey; 7, lion tail macaque; 8, titi monkey; 9, red howler; 10, colobus monkey; 11, black spider monkey; 12, drill baboon; 13, douc langur; 14, ruffed lemur; 15, Allen's monkey; 16, siamanga; and 17, potto monkey.

gene may have duplicated to generate the PLAP gene (unpublished results). Doellgast and Wei (1984) had used extensively absorbed rabbit antisera to PLAP to investigate the presence of three distinct epitopes in old and New World monkeys. They concluded that the presence of two epitopes characteristic of the Nagao isozyme (see Chapter 10) in spider monkeys suggested that this gene product was closely related to the enzyme present in the primate placenta at the time of species divergence (humans and New World monkeys). Hence GCAP appears to be more ancestral than PLAP. However, definitive statements in this regard will have to await confirmation based on actual sequence data from the corresponding species.

1.2
Restriction Fragment Length Polymorphisms

Only two reports have been published concerning restriction fragment length polymorphisms (RFLPs) in the human *ALPL* gene (Weiss et al., 1987; Ray et al., 1988). *Bcl*I digestion of genomic DNA identified a two-allele polymorphism with either a single band of 7.4 kb or two bands of 4.3 and 3.1 kb (Weiss et al., 1987), whereas *Sst*I digestion identified two alleles of either 6.0 or 9.0 kb, respectively (Ray et al., 1988). In contrast, the orthologous mouse *Akp2* gene displays a considerably larger degree of genetic variability (Fröhlander and Millán, 2001). Differences between the *Akp2* alleles can be explained in some instances by simple mutations affecting a restriction site and in others by insertions or deletions affecting intron sizes. Single point mutations account for the *Apa*I 1.2 and 2.2 kb fragments in Balb/c and the *Pst*I site in intron 6 in 129/J and C57Bl/6J that generates 2.9 and 1.2 kb allelic fragments. Intron-size differences were also demonstrated through *Apa*I, *Bam*HI and *Hin*dII digestions. Sequencing of 17.5 kb of the *Akp2* gene also revealed an over-representation of repeats in the 5′ half of the gene, especially in introns 4 and 5, of possible relevance for the intron size differences. Di- and trinucleotide repeats, e.g. d(CA)n, d(GT)n, d(GT)n d(CCA)n, d(GGT)n, are most frequent but tetranucleotides, e.g. d(GGGC)n and d(GGGA)n, and also longer repeats are also present in the *Akp2* gene (Fröhlander and Millán, 2001). Finally, one RFLP has been reported for the bovine TNAP gene (Beever and Lewin, 1992) and a *Hin*dIII polymorphism for the porcine TNAP locus (Shalhevet et al., 1993).

Two RFLPs in the human *ALPP* gene were found using the restriction enzymes *Rsa*I (Martin, et al., 1987a) and *Pst*I (Tsavaler et al., 1987; Tsavaler et al., 1988). Both of these RFLPs correlated with electrophoretic polymorphisms (Beckman et al., 1989; Beckman et al., 1991). Further evidence, however, indicated that despite the correlation with PLAP electrophoretic types, *Pst*I(b) is an RFLP of the *ALPP2* locus, not *ALPP* (Beckman et al., 1992). Linkage disequilibria indicated close linkage between the *ALPP* and *ALPP2* loci, a situation now amply confirmed by the genome project data. *ALPP* and *ALPP2* RFLPs and haplotypes were found to show highly significant associations with spontaneous abortions in the Finnish

and Swedish populations (Wennberg et al., 1995). The Finnish abortions were associated with the *ALPP2* allele *Pst*I(b)2 and the Swedish abortions with the *ALPP* allele *Pst*I(a)2. The authors discuss that a possible mechanism behind the associations may be linkage disequilibria with deleterious alleles within or close to the AP gene complex. The existence of only limited RFLPs in the *ALPP* gene contrast with the high degree of "electrophoretic" variability found at this locus (Section 4.2).