

https://dspace.org/

The isolation, cloning and characterisation of the Xenopus Laevis alkaline phosphatase gene from the neurala stage.

Constantinou, Constantinos.

1993
http://hdl.handle.net/10539/22943

Downloaded from a DSpace repository. https://dspace.org/

THE ISOLATION, CLONING AND CHARACTERISATION OF THE *XENOPUS LAEVIS* ALKALINE PHOSPHATASE GENE FROM THE NEURULA STAGE

Constantinos Constantinou

A thesis submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfilment of the requirements for the Degree of Master of Science in Biotechnology.

Johannesburg, 1993

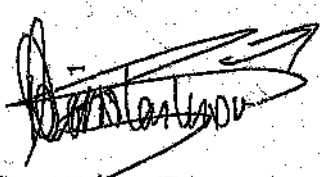
Abstract

Alkaline phosphatases (AP's) comprise a family of isozymes which are distributed widely in nature. Their function is unknown. There is evidence that in *Xenopus* tissue non-specific AP (TNAP) plays some role in differentiation and morphogenesis during embryological development. The isolation and elucidation of the primary structure of the TNAP gene is one aspect in the study of the function of TNAP in the embryo and is the primary aim of this work. A λgt10 cDNA library constructed with mRNA from the neurula stage of the *Xenopus* embryo, when AP activity is at its highest, was obtained and screened for a cDNA corresponding to the TNAP mRNA using mouse and chicken TNAP cDNA's as probes. A single positive clone was isolated and the insert found to be 700 base pairs long. The insert, termed *XC1*, was sequenced and found to correspond to the 5' region of the TNAP mRNA. *XC1* includes 250 nucleotides of the 5' untranslated region of the TNAP mRNA. This is followed by an open reading frame that encodes a polypeptide of 155 amino acids. The deduced amino acid sequence of this polypeptide is 74% homologous to the corresponding region in the mouse TNAP, and about 50% and 20% homologous to the corresponding regions in the human PLAP and *E. coli* AP, respectively. This is analagous to the homology with other TNAP's. The polypeptide includes a putative signal peptide at the N-terminus of the TNAP of 17 amino acids. Catalytically important regions included in *XC1*, *viz.* the active site, one N-glycosylation signal, metal ligand sites, and five alpha-helical and two beta-sheet domains are all highly conserved when compared to all known AP sequences. The 5' untranslated region of *XC1* shows no homology to other AP's which is as expected. In Northern blot analysis, *XC1* hybridised to a single band of 2.5 kilobase mRNA from neurula embryos under highly stringent conditions. Hybridisation patterns obtained from Southern blot analysis of *Xenopus* chromosomal DNA digested with eight restriction endonucleases, offered molecular

evidence that the TNAP expressed in neurula and other stages is the product of a single copy gene. Further screenings of the library failed to give the rest of the *Xenopus* TNAP sequence. *XCI* can be used as a specific probe to screen alternative libraries to obtain the whole TNAP gene sequence, and to study TNAP expression in *Xenopus* tissues and cells.

Declaration

I hereby declare that this dissertation is my own, unaided work. It is being submitted to the University of the Witwatersrand, Johannesburg, for the degree of Master of Science in Biotechnology. It has not been submitted before for any degree or examination in any other university, nor has it been prepared under the aegis or with the assistance of any other body or organisation or person outside the University of the Witwatersrand, Johannesburg.



Constantinos Constantinou

1ST day of July, 1993.

Acknowledgements

I wish to acknowledge a senior bursary from the University of the Witwatersrand, and a Masters bursary from the Foundation for Research and Development.

I would like to extend my gratitude to my supervisor Professor Barry Fabian, for his continued guidance and support. I am indebted to him for providing me the opportunity to travel to La Jolla where I obtained invaluable experience and where I was able to work with, and learn a great deal from some of the best scientists.

I am particularly grateful to Dr. José Luis Millán for having me in his laboratory. He also provided the nucleic acid probes used in this project. I am deeply grateful to Thomas Manes who spent a lot of his valuable time introducing me to everyone at the Foundation and for providing his continued assistance and for sharing his expert knowledge with me.

I want to thank Drs. Nils Fröhlander, Sonoko Narisawa and Marie-Claude Hofmann for all their assistance and for their expert advice. Finally, I'd like to thank Dr. Hazel Sive for the cDNA library, Mrs Lore Pillman for her assistance in South Africa, Pam Manning and the staff of the Central Graphics Units of the University of the Witwatersrand for their services, and Larry le Roux for his assistance in printing this thesis.

Contents

Abstract i

Declaration iii

Acknowledgements iv

Contents v

List of Abbreviations xi

List of Figures xiv

1 INTRODUCTION 1

1.1 ALKALINE PHOSPHATASES 1

1.1.1 HISTORICAL PERSPECTIVE 1

1.1.2 GENETICS OF ALKALINE PHOSPHATASES 2

1.1.2.1 Isozymes and evolution 2

1.1.2.2 Molecular Biology of Alkaline Phosphatases 8

1.1.3 BIOCHEMISTRY OF ALKALINE PHOSPHATASES 13

1.1.3.1 General Structure 13

1.1.3.2 Mechanism of Catalysis 16

1.1.3.3 Kinetics of Catalysis 17

1.1.3.4 The Inhibition Pattern of Alkaline Phosphatases . . . 21

1.1.4 PHYSIOLOGY OF ALKALINE PHOSPHATASES 24

1.1.4.1 Distribution and Function 24
1.1.4.2 Alkaline Phosphatases in Malignancies 27
1.1.4.3 Alkaline Phosphatases and Disease 29
1.1.4.4 Alkaline Phosphatases and Development 30
1.1.5 ALKALINE PHOSPHATASES IN XENOPUS 34
1.1.6 SUMMARY 35

1.2 THE ISOLATION AND CHARACTERISATION OF GENES 36
1.2.1 GENE LIBRARIES 36
1.2.1.1 Cloning Vectors 36
1.2.1.2 Genomic Libraries 38
1.2.1.3 cDNA Libraries 39
1.2.1.4 Library Screening 42
1.2.2 GENE SEQUENCING 42

1.3 ISOLATION OF THE *XENOPUS* TNAP GENE 44
1.3.1 POSSIBLE STRATEGIES 44

1.4 AIMS OF THE PROJECT 45

2 MATERIALS AND METHODS 47

2.1 THE cDNA LIBRARY 47

2.2 EVALUATION OF THE LIBRARY 47

2.3 PROBE PREPARATION 48
2.3.1 PROBE ISOLATION 48
2.3.2 RADIOACTIVE LABELLING OF PROBE 49

2.4 LIBRARY SCREENING 50
2.4.1 PLAQUE TRANSFER TO NYLON MEMBRANES 50

2.4.2 PROBE HYBRIDISATION 50
2.4.3 ISOLATION AND PURIFICATION OF POSITIVES 51
2.4.4 STRIPPING OF FILTERS 52

2.5 PHAGE DNA EXTRACTION 52

2.6 PCR AMPLIFICATION OF POSITIVE INSERTS 53

2.7 NEURULA STAGE EMBRYOS 54

2.8 RNA EXTRACTION 55
2.8.1 TOTAL RNA EXTRACTION 55
2.8.2 SELECTION OF POLY(A)$^+$ RNA 56

2.9 DNA EXTRACTION 57

2.10 HYBRIDISATION ANALYSIS 57
2.10.1 RESTRICTION OF GENOMIC DNA 57
2.10.2 AGAROSE GEL ELECTROPHORESIS OF DNA 58
2.10.3 SOUTHERN BLOTTING 58
2.10.4 FORMALDEHYDE GEL ELECTROPHORESIS OF RNA 59
2.10.5 STAINING OF FORMALDEHYDE GELS FOR RNA 60
2.10.6 NORTHERN BLOTTING 60
2.10.6.1 RNA Alkali Blotting 60
2.10.7 METHYLENE BLUE STAINING OF THE NORTHERN BLOTS 61
2.10.8 HYBRIDISATION OF PROBE 61
2.10.8.1 Stripping of Southern Blots 62

2.11 GENE SEQUENCING 63
2.11.1 PCR FRAGMENT CLONING 63

2.11.1.1 T-vector Construction 63
2.11.1.2 PCR Fragment Ligation 63
2.11.1.3 Transformation of Competent Cells 64
2.11.2 SMALL-SCALE PLASMID PREPARATION 64
2.11.3 LARGE-SCALE PLASMID PREPARATION 65
2.11.4 SEQUENCING OF CLONED INSERT 66
2.11.4.1 Set-up of Gel Apparatus 67
2.11.4.2 Annealing Template and Primer 67
2.11.4.3 The Labelling Reactions 68
2.11.4.4 The Termination Reactions 69
2.11.4.5 Denaturing Gel Electrophoresis 69
2.11.4.6 Reading the DNA Sequence 70

3 RESULTS 71

3.1 THE cDNA LIBRARY TITRE 71

3.2 SCREENING OF THE LIBRARY 71
3.2.1 SUITABILITY OF THE CHICKEN AND MOUSE TNAP PROBES 71
3.2.2 PRIMARY SCREENING OF THE LIBRARY 73
3.2.3 SECONDARY SCREENING OF THE LIBRARY 74

3.3 AMPLIFICATION OF POSITIVE INSERTS 74
3.3.1 AMPLIFICATION BY PHAGE LYSATE PREPARATION . . 74
3.3.2 PCR AMPLIFICATION OF POSITIVE INSERTS 76

3.4 THE SEQUENCE OF THE *XENOPUS* TNAP cDNA 76
3.4.1 CLONING OF *XCI* 77
3.4.2 THE *XCI* SEQUENCE 78
3.4.3 SEQUENCING OF OTHER POSITIVE INSERTS 82

3.5 HYBRIDISATION ANALYSIS 83
3.5.1 SOUTHERN BLOT ANALYSIS 83
3.5.2 NORTHERN BLOT ANALYSIS 85

4 DISCUSSION 89

4.1 THE SUITABILITY OF THE cDNA LIBRARY 89

4.2 PREPARATION OF POSITIVE INSERT *XCI* FOR SEQUENCING 91
4.2.1 PHAGE DNA EXTRACTION 91
4.2.2 T-VECTOR CONSTRUCTION 91

4.3 SCREENING OF THE LIBRARY 92
4.3.1 SUITABILITY OF THE CHICKEN AND MOUSE TNAP PROBES 92
4.3.2 ISOLATION OF PART OF THE *XENOPUS* TNAP cDNA AND COMPARISON WITH THE MOUSE TNAP cDNA . . 93
4.3.2.1 Authenticity of *XCI* 93
4.3.2.2 Failure to Isolate the Full-length TNAP cDNA 96
4.3.2.3 The Amino Acid Sequence Encoded by *XCI* 97

4.4 HYBRIDISATION ANALYSIS 100
4.4.1 SOUTHERN BLOT ANALYSIS 100
4.4.2 NORTHERN BLOT ANALYSIS 101

4.5 CONCLUSION 101

APPENDIX A: Media and Solutions 104

APPENDIX B: Bacterial Strains and Vectors 112

APPENDIX C: List of Suppliers 116

APPENDIX D: Published Amino Acid Sequences of Various Alkaline Phosphatases 117

APPENDIX E: Amino Acid Abbreviations 119

APPENDIX F: The Genetic Code 120

REFERENCES 121

List of Abbreviations

Aa	amino acid
AP	alkaline phosphatase
ATP	adenosine-5'-triphosphate
B. subtilis	*Bacillus subtilis*
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
C-terminal	carboxyl amino acid terminal
cDNA	complementary DNA
CGAM	cell guidance associated molecule
cTNAP	chicken TNAP
CTP	cytidine-5' triphosphate
ddATP	dideoxyATP
ddCTP	dideoxyCTP
ddGTP	dideoxyGTP
ddTTP	dideoxyTTP
dATP	deoxyATP
dCTP	deoxyCTP
dGTP	deoxyGTP
dTTP	deoxyTTP
DEPC	diethyl pyrocarbonate
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EAP	embryonic alkaline phosphatase
E. coli	*Escherichia coli*
EcoRI	Restriction enzyme G/AATTC

EcoRV	Restriction enzyme GAT/ATC
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
FSH	follicle stimulating hormone
GCAP	germ cell alkaline phosphatase
GTP	guanosine-5'-triphosphate
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
Hfl	high frequency of lysogeny
IAP	intestinal alkaline phosphatase
IgG	immunoglobulin G
IPTG	isopropyl-ß-D-thiogalactopyranoside
IU	international units
kbp	kilobase pairs
λgt10	bacteriophage lambda gt10
λgt11	bacteriophage lambda gt11
LB	Luria-Bertani medium
MBS	modified Barth's saline
MOPS	3-morpholinopropansulfonic acid
mRNA	messenger RNA
mTNAP	mouse TNAP
N-terminal	amino-amino acid terminal
NMR	nuclear magnetic resonance
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PIG	phosphatidylinositol glycan
PIPLC	phosphatidylinositol-specific phospholipase C
PLAP	term placental alkaline phosphatase
poly(A)$^+$ RNA	polyadenylated mRNA

RNA	ribonucleic acid
RNase	ribonuclease
rTNAP	rabbit TNAP
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TBE	tris-borate-EDTA
TCA	trichloroacetic acid
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TNAP	tissue non-specific alkaline phosphatase
Tris	tris(hydroxymethyl)aminomethane
TSAP	tissue-specific alkaline phosphatase
UV	ultraviolet
X. laevis	*Xenopus laevis*
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

List of Figures

1.1 A tentative model of the evolutionary relationship of the human AP isozymes 7
1.2 The C-terminal structure proposed to link proteins to phosphatidyl-inositol in the plasma membrane 12
1.3 The alpha-carbon trace of the complete dimer of *E. coli* alkaline phosphatase 15
Scheme 1 Proposed mechanisms of AP catalysis 16
1.4 Stereo drawing of the active site of the *E. coli* alkaline phosphatase . . 19
3.1 Isolation of the chicken and mouse TNAP cDNA probes from the vector pBluescript II KS^+ 72
3.2 Autoradiographs of filters from a primary screening of the library . . . 73
3.3 Autoradiographs of secondary screening filters 75
3.4 PCR amplification of the insert in a positive clone 76
3.5 EcoRI digests of small-scale phagemid preparations 77
3.6 Autoradiographs of sections of some of the sequencing gels used to sequence *XC1* 78
3.7 The nucleotide and deduced amino acid sequences of *XC1* 79
3.8 PCR amplification of other positive inserts 82
3.9 Southern blot analysis of other positive inserts 83
3.10 Agarose gel of *Xenopus* genomic DNA 84
3.11 Southern blot analysis 84
3.12 Southern blot analysis 84
3.13 Formaldehyde-agarose gel of total neurula RNA 86
3.14 Formaldehyde-agarose gel of total neurula RNA 87
3.15 Formaldehyde-agarose gel of total neurula RNA 87
3.16 Northern blot analysis 88

3.17 Northern blot analysis 88
4.1 Computer-assisted nucleotide alignment of *XC1* with the corresponding region of the mouse TNAP cDNA 94
4.2 Comparison of the deduced amino acid sequences of *XC1* and the corresponding region of the mouse TNAP 99

1 INTRODUCTION

1.1 ALKALINE PHOSPHATASES

1.1.1 HISTORICAL PERSPECTIVE

The existence of alkaline phosphatases (AP's) was first described more than a century ago by Tamman (1885) when extracts from various animal tissues were shown to possess phosphatase activity, *i.e.* were able to catalyze the hydrolysis of phosphate monoesters and convert organic phosphates into inorganic form. Suzuki *et al* (1907) first suggested that such enzymes constituted a separate class of eukaryotic enzymes. By 1913, the knowledge on this enzyme was extended by the work of Grosser & Husler (1912), von Euler (1912) and Plimmer (1913) who showed that, while this enzyme was present in a variety of tissues, it was present in highest amount in intestinal mucosa and that activity was optimal at alkali pH. Much of the early work on phosphatases was done on the enzyme from mammalian intestines, particularly calf intestine (Coleman, 1992).

The enzyme was first termed "phosphatase" in 1912 (von Euler & Funke, 1912). The International Union of Biochemistry (IUB) catalogued alkaline phosphatases as "orthophosphoric-monoester phosphohydrolases" with the index number EC 3.1.3.1 (International Union of Biochemistry, 1972).

It was as early as 1961 that Engstrom (1961, 1962) showed that enzymatic catalysis involved the formation of phosphatidylserine when intestinal alkaline phosphatase (IAP) was incubated with phosphate esters. Around the same time it became evident that *E. coli* possesses an enzyme similar to the mammalian IAP that is derepressible

by phosphate starvation (Horiuchi *et al*, 1959; Torriani, 1960) and which has similar catalytic properties, similar pH-profile and which also forms the same phosphoseryl intermediate as the intestinal enzyme (Coleman, 1992). In 1962, Plocke et al (1962) showed that alkaline phosphatases require divalent cations, *viz.* zinc and magnesium for full activity. It was shown that these metal ions are essential for initial phosphate binding and for the formation of the phosphoseryl intermediate (Applebury *et al*, 1970) and for the dephosphorylation of this intermediate (Chlebowski *et al*, 1976).

Since these early discoveries, it has become apparent that AP's are part of an enzyme system that has evolved substantial complexity. This system has been studied at various levels and has not only led to a greater understanding of these enzymes, their structural and functional properties, but has also contributed significantly to enzymology in general. Great progress has been made in molecular biology, and new sophisticated technologies are continually adding to our understanding of these important enzymes.

1.1.2 GENETICS OF ALKALINE PHOSPHATASES

1.1.2.1 Isozymes and evolution

In the seventies, several studies involving immunochemical characterisation, two-dimensional electrophoresis, partial proteolytic peptide mapping and amino acid sequencing, indicated that AP's exist in multiple forms, *i.e.* are coded for by multi-locus genes or exist as allelic variants or as hybrid polymers, that result from intragenic or intergenic complementation (Greene & Sussman, 1973; Badger & Sussman, 1976; McKenna *et al*, 1979; Sergeant & Stinson, 1979; Komoda *et al*, 1981). In humans, it has been found that several isozymes of AP exist which are encoded by a multilocus family comprising of four different genes (Millán, 1988). Three genes code for tissue-specific alkaline phosphatases (TSAP's) and these include the genes coding for intestinal AP (IAP), placental AP (PLAP) and testicular germ-cell AP (GCAP). A single gene codes for a tissue non-specific AP

(TNAP) that is expressed in the liver, bone, kidney, fibroblasts, leukocytes and early placenta, and in a variety of other tissues at low levels (Hummer & Millán, 1991). The structure and sequence of AP genes have recently been determined in several laboratories (Kam *et al*, 1985; Hua *et al*, 1986; Millán, 1986; 1987; Henthorn *et al*, 1986; 1987; 1988; Weiss *et al*, 1986; 1988; Berger *et al*, 1987; Knoll *et al*, 1987; 1988; Millán & Manes, 1988; Hsu & Anderson, 1989; Hsu, 1991). This has enabled comparisons to be made of the structures of the various isozymes in different animals and to determine the evolutionary relationships of these enzymes.

Most vertebrates have at least two AP isozymes and it appears that all animals possess an IAP (Hua *et al*, 1986). The isozymes can be distinguished by their thermostability, inhibition kinetics (see section 1.1.3.4), electrophoretic mobilities and immunoreactivity (Harris, 1989). Human PLAP and GCAP are very thermostable, surviving a temperature of 65°C for an hour, while TNAP and IAP are inactivated under such conditions (Moss and Whitby, 1975; Whitby & Moss, 1975). This property is often used to distinguish between TNAP and the tissue-specific isozymes and has helped in determining in which tissues the various isozymes are expressed.

The three human TSAP genes all map to bands q34-q37 on the long arm of chromosome 2 (Kam *et al*, 1985; Griffin *et al*, 1987; Martin *et al*, 1987). Each is composed of 11 exons and 10 introns occupying less than 5.0 kb of DNA (Hoylaerts *et al*, 1992; Watanabe *et al*, 1992). The gene for TNAP is located at the end of the short arm of chromosome 1, mapping to bands p34-p36.1 (Smith *et al*, 1988). This gene is encoded by 13 exons (*i.e.* contains two additional differentially spliced exons in the 5' region compared to the TSAP genes) and possesses significantly larger introns extending over 56 kb of DNA (Weiss *et al*, 1988; Hsu & Anderson, 1989). A similar genomic organisation has also been reported for the rat TNAP gene (Toh *et al*, 1989b).

The coding regions of the three TSAP genes show a high degree of homology

corresponding to 90-98% identity at the protein level (Millán, 1990; Watanabe *et al*, 1992). The mature TSAP's possess similar immunological and physicochemical properties (Watanabe *et al*, 1992). The amino acid sequence of the TNAP is, however, only 50-60% identical with either of the TSAP's (Hoylaerts *et al*, 1992). Immunological cross-reactivity does not occur between TNAP in rat, mouse or human, and any of the TSAP's from these respective animals (Misumi *et al*, 1988). This confirms the structural difference between TNAP and the TSAP isozymes. Partial cross-reactivity occurs between the TSAP's from different animals. Thus, one can infer that the TSAP's are more closely related immunologically to each other than to TNAP (Goldstein *et al*, 1982a).

It is interesting and of significance to this work that the TNAP sequences from different vertebrates, including mouse, rat, rabbit, chicken, bovine and human, have been determined and all show a high degree of homology, *i.e.* greater than 80% (Hua *et al*, 1986). It appears that the TNAP gene has been fairly well conserved from the chicken and murine to the bovine to the human enzyme (Hua *et al*, 1986). This is probably due to its very early divergence from the ancestral precursor gene, and that the early form of the TNAP gene was well conserved during subsequent evolution due perhaps to a strong selective pressure to maintain function.

The *E. coli* AP has been characterised by Bradshaw *et al* (1981). The gene *pho A* coding for this phosphatase is part of the *pho* regulon consisting of at least 18 genes coding for products involved in phosphate transport and metabolism (Coleman & Besman, 1987; Gettins *et al*, 1985; Ludke *et al*, 1984). The homology between the human AP's and *E. coli* AP is only about 33-35% (Hoylaerts *et al*, 1992). A direct comparison of the amino acid sequences of the human and *E. coli* AP's can be found in appendix D. *E. coli* possesses three different isozymes of AP. However, these differ only by the presence of an NH_2-terminal arginine residue on the subunits of isozyme 1 and the absence of the same on isozyme 3, with isozyme 2 being a heterodimer of the two types of subunit (Bradshaw *et al*, 1981). The relative proportions of each isozyme depends on the growth conditions of the cells (Bradshaw *et al*, 1981). This variety is clearly due to post-translational

modifications in *E. coli*. Post-translational processing is also responsible for the production of active AP's in *Saccaromyces cerevisiae* (Klionsky & Erm, 1989).

Bacillus subtilis possesses a similar regulon to the one in *E. coli* which codes for several species of AP, the major species expressed depending on the growth phase and whether the cell is sporulating or is in a vegetative state (Hulett *et al*, 1991). The DNA sequences of the two most important AP genes in *B. subtilis* show 64% identity, and 63% identity at the primary amino acid sequence level (Hulett *et al*, 1991). It appears that the various AP genes in *B. subtilis* arose by duplication of an ancestral gene. The duplicates then diverged to produce species of AP somehow better suited to perform specific functions in the cell undergoing certain growth changes.

From an evolutionary point of view, functional diversity can be created by several mechanisms. These include the duplication of an ancestral gene, differential mRNA splicing, allelic polymorphism and post-translational modifications. In the case of AP's, a combination of these processes seems to apply (Fröhlander & Millán, 1991). Differential splicing has been observed for TNAP (Matsuura *et al*, 1990). Differential post-translational modifications of carbohydrate moieties are responsible for the different tissue isoforms (liver, bone, kidney) of TNAP (Lehman, 1980). Some degree of carbohydrate heterogeneity is also observed in the TSAP isozymes (Fröhlander & Millán, 1991).

Considering the sequence homology and functional relatedness of the human TNAP and TSAP isozymes, it has been suggested that these enzymes represent a "multilocus enzyme system" and that the loci coding for the different isozymes were derived, in the course of evolution, from a common ancestral gene which underwent successive duplications (Harris, 1979). At present it is believed that the IAP was the first tissue-specific isozyme to evolve from the hypothetical precursor gene (Fishman, 1990). It appears that the IAP in fish, amphibians and reptiles all have the gene evolved from this precursor, but which evolved further to give rise to the "modern" form found in the higher vertebrates, birds and mammals

(Fishman, 1990). The protein coding regions of all the AP genes are interrupted at analagous positions by introns with splice junctions at identical positions within the triplet codons, and this also indicates that the AP multigene family originated from a single ancestral gene by a series of duplications (Weiss *et al*, 1988). The fact that the TNAP gene is on a separate chromosome to that of the TSAP genes indicates that the former gene diverged from the ancestral precursor gene much earlier than the TSAP genes.

There are three common alleles of PLAP and at least fifteen rare forms (Fröhlander & Millán, 1991). In fact, PLAP shows the highest degree of allelic variation of any enzyme studied to date (Millán & Manes, 1988) and this may correlate with a late appearance in evolution (Donald & Robson, 1974). Similar enzyme polymorphism is observed for GCAP (Millán & Stigbrand, 1983; Millán & Manes, 1988; Watanabe *et al*, 1989; Lowe & Strauss, 1990; Hendrix *et al*, 1990). Also supporting the idea that PLAP appeared only recently in evolution is the fact that this isozyme is only detectable in the placenta of man, and in the great apes, *i.e.* the orangutan and chimpanzee (Doellgast & Benirschke, 1979; Goldstein & Harris, 1979; Goldstein *et al*, 1982a). GCAP is only found in humans (Manes *et al*, 1990). No enzyme polymorphism has been observed for IAP or TNAP. This may be due to the fact that very few electrophoretic studies of these enzymes have been performed (Fröhlander & Millán, 1991). All other vertebrates appear to express a TNAP in the placenta (Knoll *et al*, 1988). Figure 1.1 illustrates a tentative model of the evolution of the human AP gene family.

Probing mouse genomic libraries with a full-length human PLAP cDNA revealed that the mouse has three TSAP genes with an organisation analogous to that of the three human TSAP genes (Manes *et al*, 1990). The three genes are the mouse embryonic AP (EAP) gene, intestinal AP (IAP) gene and a putative pseudogene. Reverse-transcriptase polymerase chain reaction of RNA revealed that the EAP gene is expressed in the two-cell to blastocyst stage preimplantation embryo and is re-expressed in the testis, thymus and intestine in the adult (Hahnel *et al*, 1990).

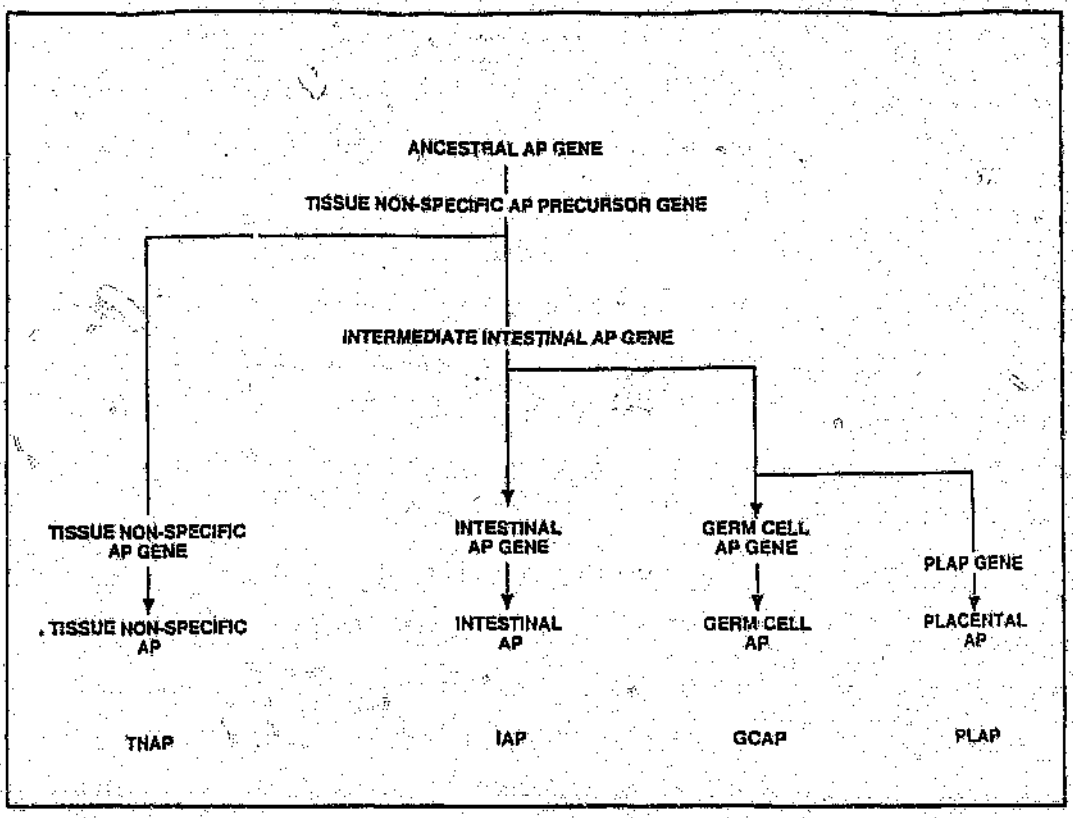


Figure I.1 A tentative model of the evolutionary relationship of the human AP isozymes (from Fröhlander & Millán, 1991).

Based on correlated tissue-specific expression, it would appear that the mouse embryonic AP (EAP) gene, is a direct ancestor of the human GCAP, and that the mouse IAP is a direct ancestor of the human IAP gene (Narisawa *et al*, 1992). A histochemical study of two- to eight-cell stage embryo also indicated that EAP has characteristics similar to PLAP and GCAP, including high heat stability and cross-reactivity with anti-PLAP polyclonal antisera (Lepire & Ziomek, 1989). However, comparing protein and DNA sequences indicates that the human and mouse AP genes diversified *after* rodent and primate radiation, *i.e.* the human and mouse AP genes form two distinct isozyme clusters that evolved separately (Manes *et al*, 1990). Moreover, the high degree of homology between mouse and human IAP (80.3%), and phylogenetic analysis, suggests that the mouse IAP and the human TSAP gene family are descended from the same ancestral gene (Manes *et al*, 1990). The third gene found in the mouse appears not to be expressed. The above arguments for a taxon-specific evolution of the TSAP gene family also explains why

such a pseudogene is not found in humans (Manes *et al*, 1990). Similarly, it also explains why humans have a PLAP gene not found in mice since, as discussed above, this gene is apparently a very late evolutionary product.

The degree of allelism observed in the human PLAP and GCAP and the similar diversity in number and types of AP genes in mouse and humans are not understood but indicate that unusual genetic mechanisms are functioning in the AP gene family (Manes *et al*, 1990). The purpose of this diversity may become clear when the specific functions of these enzymes are determined.

The molecular basis for the differences between isozymes, *viz.* the observed differences in immunoreactivity and also the possible differences in substrate specificity, is presently being studied - chiefly by site-directed mutagenesis. Studies of PLAP allozymes and PLAP and GCAP isozymes are showing which individual amino acid residues in the primary structure are chiefly responsible for the specific 3-D conformation of the specific enzyme which determines its immune and functional specificity. Substitution of even one of these residues can have drastic effects, for example the effect on amino acid inhibition (see section 1.1.3.4) (Hoylaerts & Millán, 1991). The substitution of a single amino acid converts GCAP to an enzyme with all the properties of PLAP (Watanabe *et al*, 1991). The human TNAP differs from the TSAP's mainly in a loop region (see section 1.1.3.1) defined by amino acid residues 400 - 430 (Millán, 1990). Clearly, these aa differences have a great impact on the overall 3-D conformation of these enzymes resulting in the observed differences.

1.1.2.2 Molecular Biology of Alkaline Phosphatases

In humans, IAP is normally expressed in foetal, neonatal and adult intestine, more specifically in the microvillous border of the epithelial cells of the intestinal mucosa (Hugon & Borgers, 1966; Millán, 1990). PLAP is detected in the syncytiotrophoblast cells of the placenta from about the eighth week of gestation throughout pregnancy (Fishman *et al*, 1976). GCAP is expressed in primordial

germ cells during their migration down the genital ridges (Hustin *et al*, 1987), and is found in trace amounts during the first stages of germ cell maturation in normal adult testis (Chang *et al*, 1980), and is also expressed in the thymus (Goldstein *et al*, 1982b). Besides its expression in such tissues as the liver, bone, kidney, lung and placenta (until about the twelfth week of pregnancy), TNAP is also found in high amounts in the osteoblasts of mineralising bone (McComb *et al*, 1979).

The two active mouse TSAP genes (EAP and IAP) and the three human TSAP genes all contain three very well conserved proximal promoter elements, *i.e.* an SP1 binding site within a 25 bp sequence, a GGGAGGG box, and a TATA (ATTTAAA) box within a conserved sequence of 35 nucleotides (Millán, 1987; Manes *et al*, 1990). Clearly, these elements must play an important role in ensuring the tissue-specific expression of these genes. Other work has indicated that these proximal promoter elements are sufficient to account for expressic ... human GCAP gene in tissue culture cells as well as in the tissues of transgenic mice (Manes *et al*, 1990). Similarly, upstream and downstream regulatory elements in the IAP genes of different animals appear to be conserved and are responsible for intestinal-specific expression (Manes *et al*, 1990).

The mouse TSAP genes also have consensus binding sites for the nuclear factors AP1 (Angel *et al*, 1987) and AP2 (Imagawa *et al*, 1987), as well as a conserved CACCC box (Myers *et al*, 1986) and sites for C/EBP (Graves *et al*, 1986) and the cAMP responsive element (Montminy *et al*, 1986; Manes *et al*, 1990). The EAP gene possesses a sequence of 35 consecutive guanine residues at the 3' untranslated region which has the potential to form triple-helical DNA structures and may play a regulatory role (Kohwi & Kohwi-Shigematsu, 1988; Manes *et al*, 1990). The mouse IAP gene also possesses t .o regions, one a 65 bp stretch of alternating guanines and adenines, and the other a 50 bp stretch of alternating cytosines and thymidines, which are able to form triple helical DNA structures which may also be involved in the regulation of expression of this gene (Kohwi & Kohwi-Shigematsu, 1988; Manes *et al*, 1990). Further work is needed to establish the significance of these conserved sequences in tissue-specific expression.

The cloning and sequencing of the various AP genes from different animals is mostly recent work (late 1980's). Only with further research and comparisons of forthcoming information about the structure and functions of these genes and their variant alleles will it become clear how the specific expression of these genes is brought about and controlled (Hahnel & Schultz, 1989).

The additional exons in the TNAP gene has drawn much interest, since two different promoter regions and two alternative mRNA's have been found in rat, mouse and human (Toh *et al*, 1989a; Toh *et al*, 1989b; Toh *et al*, 1990; Matsuura *et al*, 1990). These two RNA's differ at their 5' ends, specifically at a position 88 nucleotides upstream of the initiation codon ATG. Exons 3 to 13 are common to both mRNA's. The one form of RNA (E1) has the first exon, which is 36 kbp upstream from the 5' end of exon 3. The other TNAP mRNA (E2) has exon 2, which occurs 10 kbp from exon 3 in the TNAP gene. S1 nuclease mapping and primer extension showed that there are two independent promoters specific to each of the E1 and E2 RNA's. The possible roles of the two promoters in gene regulation, and of the differential splicing, remain to be proved. It has been suggested that the extra non-coding exon together with the adjacent TNAP gene promoter may have been acquired later in evolution as a distinct unit, perhaps in a DNA transposition event. An 11bp direct repeat found flanking the promoter and first exon of fully sequenced TNAP genes supports this hypothesis (Weiss *et al*, 1988). It is possible that the newly acquired unit is what conferred the widespread pattern of tissue expression characteristic of TNAP genes, unlike the other genes that are tissue-specific (Weiss *et al*, 1988).

All newly translated AP's contain both amino- and carboxyl-terminal signal peptide chains which are responsible for transport of the AP to the cell membrane (Watanabe *et al*, 1992). The amino-terminal signal peptide is found to be leucine-rich, is mainly hydrophobic, and is similar to the signal peptides of other secretory proteins such as mouse epidermal growth factor precursor and human pancreatic polypeptide hormone precursor (Scott *et al*, 1983; Schwartz *et al*, 1984). The signal peptides of all AP's have a significant degree of homology, *i.e.* about 30% (Kam

et al, 1985). These signal peptides are removed from the nascent protein during post-translational processing (Ogata *et al*, 1988).

Post-translational modification plays an important role in producing active AP. The major form of modification is glycosylation (Hua *et al*, 1986). A consensus sequence that defines a glycosylation signal has been identified as Asn-Xaa-Thr/Ser (Millán, 1988). The TSAP's have two such sites, whereas TNAP's in mouse, rat, bovine, and humans have five potential glycosylation sites. These sites occur at the same positions in all these enzymes, except in the mouse TSAP's where the positions differ slightly due to mutations (Manes *et al*, 1990). The most well studied AP in this regard is the human PLAP, probably because it was the first eukaryotic AP to be extracted in pure form and then fully sequenced (Millán, 1986). In the mature PLAP, only the Asn residue at position 249 is glycosylated (Millán, 1986). It has been shown that the glycan moieties play important roles, such as maintenance of polypeptide conformation and solubility, protection from proteolytic degradation, production of signals for intracellular sorting and externalisation, and for catalytic activity of glycoproteins (Watanabe *et al*, 1992). However, recent work has shown that AP mutants lacking N-linked oligosaccharide side chains are stable, are enzymatically active and are still anchored to the plasma membrane (Watanabe *et al*, 1992). TNAP's isolated from different tissues from the same animal often have slightly different electrophoretic mobility and thermostability (Weiss *et al*, 1988). From comparisons of Southern blots and cloned genomic DNA's, it appears that only one gene codes for TNAP in humans (Weiss *et al* 1988). It is possible that these differences are partly due to differential glycosylation of the five potential sites. More detailed analysis of TNAP's extracted from different tissues should clarify whether this or other post-translational modifications are responsible, and whether they are important factors in TNAP function in the different tissues.

Another important post-translational modification of AP's is the process which anchores them into the cell membrane. Initially, the precursor enzyme is glycosylated at Asp^{484} with high-mannose-type oligosaccharides and this

intermediate form of the enzyme is further processed by proteolysis which removes the hydrophobic peptide domain present at the C-terminus (making Asp^{484} the C-terminal aa). This peptide is replaced with glycolipid (Takami *et al*, 1988). This glycolipid is comprised of the following components (Cross, 1987): palmitic acid and stearic acid both of which constitute phosphatidylinositol, of which the inositol is glycosidically linked to an oligosaccharide of glucosamine and mannose. This oligosaccharide is, in turn, linked to ethanolamine which is then linked to the C-terminal amino acid (Asp^{484}) of the PLAP. In this way the PLAP is anchored to the plasma membrane via a phosphatidylinositol glycan (PIG) anchor attached to Asp^{484} (Micanovic *et al*, 1988). It appears that all other AP's are membrane bound in much the same way (Ogata *et al*, 1988; Watanabe *et al*, 1992) except the prokaryotic enzymes which are periplasmic in location (Millán, 1990), and have neither carbohydrate nor a fatty acid tail (Kim & Wyckoff, 1991). The way AP's are anchored in the plasma membrane is schematically represented in figure 1.2.

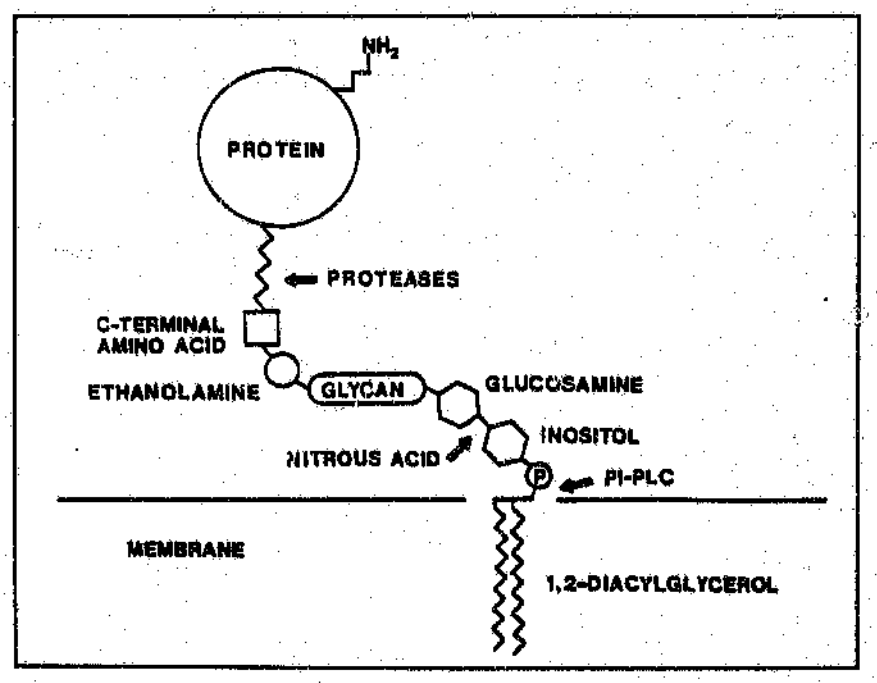


Figure 1.2 The C-terminal structure proposed to link proteins to phosphatidylinositol in the plasma membrane. This general structure is largely based on information derived from the variable surface glycoprotein of *Trypanosome brucei*. Data available on other proteins that are anchored to PI suggests that they are attached by a similar structure. The actual composition and arrangement of sugars in the glycan probably vary. The sites of action of various agents used in the determination of this structure are shown (Adapted from Low *et al*, 1986).

Intrachain disulfide bonds are also formed during transport of the enzyme to the cell membrane (Derman & Beckwith, 1991). Thus, externalisation of AP is coupled to other processes which are necessary for production of active enzyme.

1.1.3 BIOCHEMISTRY OF ALKALINE PHOSPHATASES

1.1.3.1 General Structure

Extraction of AP was initially difficult, mainly due to a lack of quick and reliable methods to measure phosphatase activity, and due to difficulty in solubilising the enzyme. In 1933, Bodansky (1933) developed colorimetric assays for measuring phosphatase activity. However, it was only in 1950 that Morton (1950) was able to extract AP in a relatively pure form using n-butanol. This clearly indicated that the enzyme was membrane bound. Since then, the enzyme has been isolated from various tissues including placenta, liver, bone, kidney and intestine and studies revealed these to be all glycoproteins (Treparier *et al*, 1976). More recently, it was found that AP's could be extracted from various mammalian tissues using phosphatidylinositol-specific phospholipase C (PI-PLC) which indicated that the AP was anchored to the plasma membrane via a covalent attachment to phosphatidylinositol (Hawrylak & Stinson, 1988).

In *E. coli*, the AP exists in the periplasmic space as a homodimer, each subunit containing 449 (or 450 if a variable Arg residue at the amino terminus is included) amino acids (Bradshaw *et al*, 1981). Four Cys residues are present as two intrachain disulphides (Coleman, 1992). The human AP's are also homodimeric glycoproteins in their active form, with molecular weights ranging between 120 and 140 kD (Kam *et al*, 1985). The monomeric subunits appear to be inactive (Takami *et al*, 1988). The monomers show a small variability in their amino acid chain length: TNAP monomers are each composed of 507 amino acids (Weiss *et al*, 1986), IAP of 509 (Henthorn *et al*, 1987), PLAP and GCAP of 513 each (Millán, 1986; Millán and Manes, 1988).

At present, the crystallographic structure of native *E. coli* AP has been studied at a resolution of 2.0 Å (Kim & Wyckoff, 1991). The overall shape and polypeptide conformation found from this structure is shown in figure 1.3A. The secondary structure of a monomer is represented as a ribbon diagram in figure 1.3B. The enzyme is basically a 2-fold symmetric dimer (100 Å x 50 Å x 50 Å) with two active sites located about 30 Å from each other. Each subunit shows typical α/β topology. The overall structure consists of a central ten-stranded β-sheet, flanked by 15 α-helices, and a minor three-stranded β-sheet with a helix on the top. The active centre is located at the carboxyl end of the central β-sheet, and one monomer provides all the ligands to the three metal ions (Coleman 1992). Together with the metal triplet and their ligands, the three residues Asp^{101}-Ser^{102}-Ala^{103} (a sequence that is found in most serine proteases and all eukaryotic AP's (Chaidaroglou & Kantrowitz, 1989)), Arg^{166} and some other amino acids comprise the active site region (Coleman, 1992). The residue at position 101 interacts electrostatically with Arg^{166} which is believed to constrain Ser^{102} in the proper position important for catalytic activity of the enzyme (Sowadsky *et al*, 1985).

Sequence alignment and conservation of important residues indicate that all AP's share much the same structure described above (Millán, 1988). The areas known to adopt α-helix or β-strand conformation show a much higher percentage of homology than do the more relaxed loop areas, and the regions that adopt β-strand conformation are more conserved than the α-helix regions - the coplanar β-sheets are 80-88% homologous including conservative replacements (Millán, 1990).

The key residues that constitute the catalytic site are conserved in all isozymes sequenced thus far (Millán, 1990). Each active site in each subunit of AP contains two zinc ions and one magnesium ion (Coleman, 1992). The amino acid residues that act as ligands to these metal-containing sites, provide coordination of the ions in the enzyme (Millán, 1990). A very high degree of ligand conservation is observed in all AP's so far sequenced and this suggests the important functional role of the metal sites (Millán, 1990).

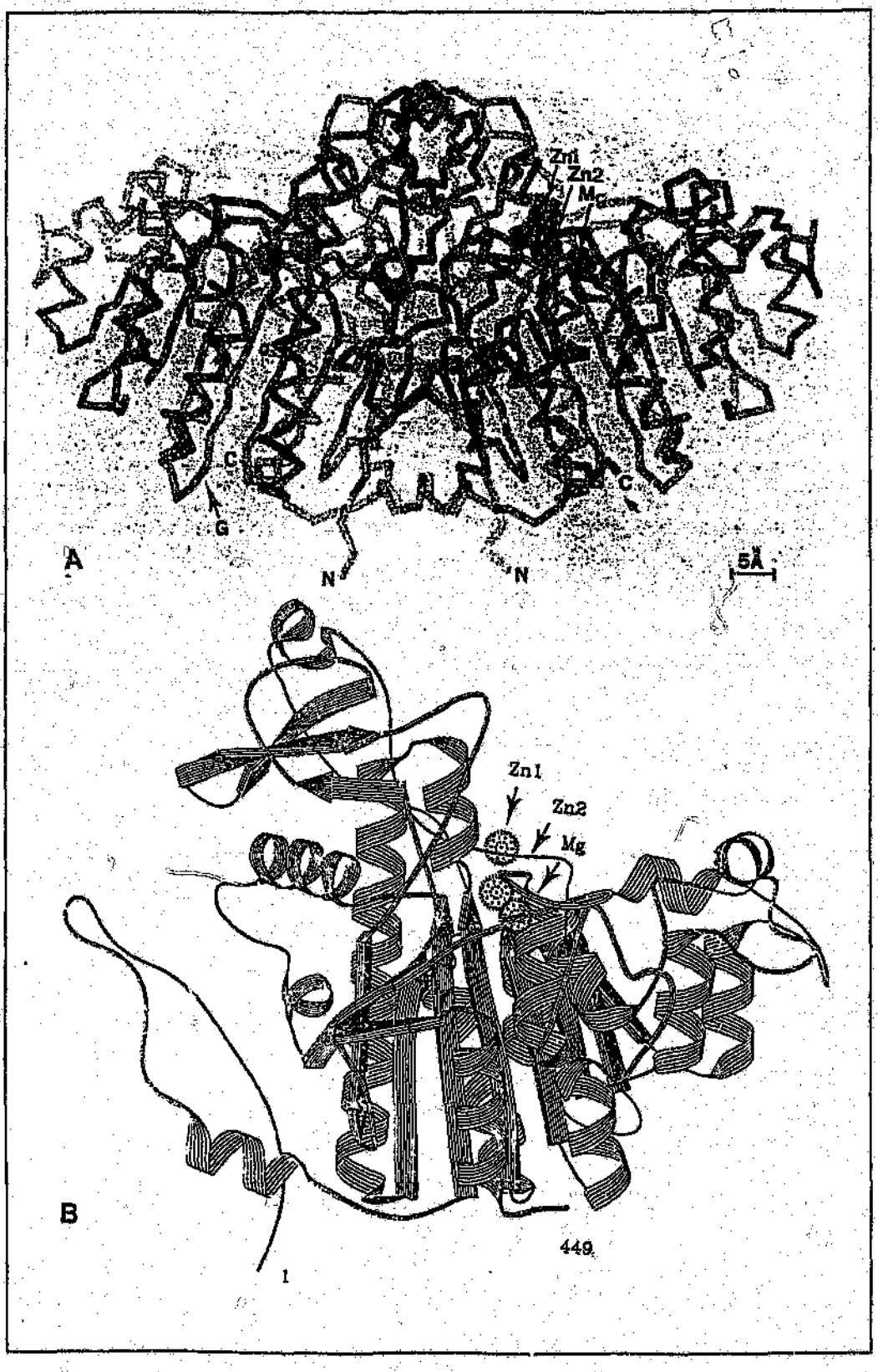
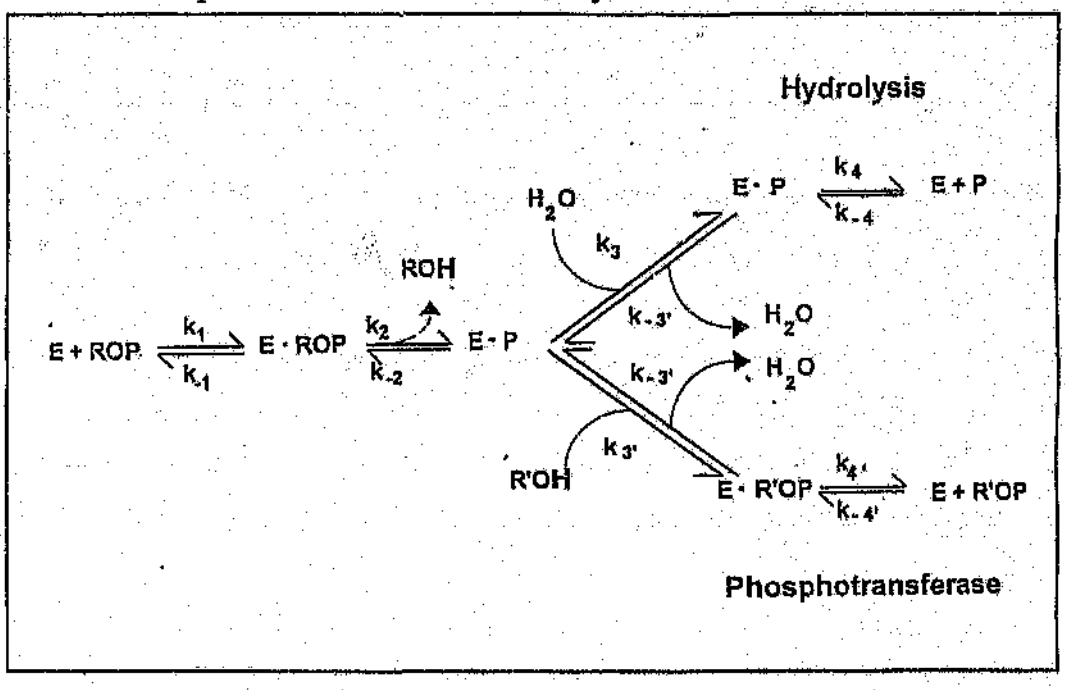


Figure 1.3 (A) The alpha-carbon trace of the complete dimer of *E. coli* alkaline phosphatase viewed with its non-crystallographic two-fold axis vertical and its maximum dimension horizontal. The tube radius is 0.5Å and the 3 metal ions are shown as spheres. The 2 active sites are about 30Å apart. (B) A ribbon drawing of a monomer of alkaline phosphatase. The stippled spheres represent the 3 metal sites (From Kim & Wyckoff, 1991).

1.1.3.2 Mechanism of Catalysis

The catalytic mechanism of AP's has been investigated in many chemical, kinetic (Bradshaw *et al*, 1981; Coleman & Gettins, 1983; Coleman, 1992), and structural (Sowadski *et al*, 1985) studies. The generally accepted catalytic mechanism, including both hydrolysis and phosphotransferase activities, is illustrated in scheme 1, first proposed by Wilson & Dayan (1965). This scheme includes the formation of a covalent phosphoenzyme complex, E-P, where the substrate is ROP (any phosphate monoester), ROH is the alcohol product, R'OH is the phosphate acceptor and R'OP is the phosphorylated acceptor. The hydrolysis of E-P involves both formation and dissociation of the non-covalent E·P complex.

Scheme 1 Proposed mechanisms of AP catalysis.



It is important to note that the Ser residue at position 102 in the *E. coli* AP and in homologous positions in other AP's (mammalian TSAP's have Ser^{92}, TNAP's have Ser^{93} (Engstrom, 1961, 1962; Garattini *et al*, 1987; Hoylaerts *et al*, 1992; Watanabe *et al*, 1992)) is the residue that binds phosphate (Watanabe *et al*, 1992). The Ser residue is responsible for a nucleophilic attack on the phosphorus of the substrate and covalent binding of the phosphate group forming the phosphoryl-

enzyme intermediate (E-P). This was already illustrated in the early 1960's when intermediates with Ser-102 phosphorylated (in *E. coli*) were isolated and studied (Schwartz & Lipman, 1961; Engstrom, 1962; Schwartz *et al*, 1963). Water (hydrolysis), or an alternative nucleophilic acceptor (transphosphorylation), dephosphorylates the phosphoryl-enzyme intermediate. Amino alcohols such as ethanolamine and Tris (hydroxymethyl)aminomethane which carry the charged amino group on the carbon adjacent to that carrying the OH acceptor are most effective phosphate acceptors in transphosphorylation reactions (Dayan & Wilson, 1964; Wilson *et al*, 1964; Coleman & Gettins, 1983).

Since the structure of the active site region of AP is found to be highly conserved in AP's from mammals and other vertebrates, the same reaction mechanism is believed to apply to all AP's so far studied (Kim & Wyckoff,1991).

1.1.3.3 Kinetics of Catalysis

As mentioned earlier, assays for AP activity were first developed by Bodansky (1933). Using these assays, the kinetics of AP activity under different conditions could be studied. It was found AP obeyed Michaelis-Menten kinetics (Folley & Kay, 1935). More recently, multinuclear NMR has been applied to study the kinetics of AP activity (Coleman & Gettins, 1983). In particular, ^{31}P NMR signals from substrates and products of AP have been used to measure the rates and product ratios for the hydrolysis and phosphotransferase reactions (Gettins *et al*, 1985). The *E. coli* enzyme has been studied most extensively and has thus served as a prototype for the study of other AP's present in prokaryotic and eukaryotic organisms (Chaidaroglou & Kantrowitz, 1989).

The rate of catalysis follows an approximately sigmoid pH-rate profile with an apparent pK_a of about 7.5 (Reid & Wilson, 1971; Coleman & Gettins, 1983). The wild-type enzyme generally exhibits maximal activity around pH 8, and the rate-limiting step is the release of the tightly bound inorganic phosphate (k_4 in scheme 1) from the enzyme product complex (Butler-Ransohoff *et al*, 1992). It is also

found that the enzymatic rate is independent of the pK_a of the substrate leaving group and inorganic phosphate is a competitive inhibitor (Coleman & Gettins, 1983). At low pH (<5.5), the phosphoserinyl intermediate is stable, *i.e.* k_3 in scheme 1 is rate limiting, and this has also been observed by ^{31}P NMR (Hull *et al*, 1976). At pH 8, k_3 exceeds the rate of dissociation of the product phosphate, k_4, by 10-fold (Coleman, 1992). At pH 6.3, k_4 is 26-fold greater than k_3 (Coleman, 1992). At this pH, k_3 is not as slow as k_{-3} and only at pH 5 is $k_3 = k_{-3}$ and [E-P] = [E·P] at equilibrium (Coleman, 1992). It is significant that if there is a phosphate acceptor present, then the overall rate of the reaction is accelerated (Chaidaroglou & Kantrowitz, 1989).

It is seen from X-ray crystal structures of E·P that the inorganic phosphate completely fills the active site. Figure 1.4 shows the active-site region of the wild-type *E. coli* AP based on NMR investigations on the (unstable penta-coordinate) E·P non-covalent intermediate. It appears that there is no binding pocket for the R group of the phosphate monoester which is consistent with the fact that AP hydrolyzes phosphate monoesters, ROP, at similar rates regardless of the size or chemical nature of R (Reid & Wilson, 1971). It is interesting that Ser^{102}→Cys^{102} AP mutants (which only differ by the presence of the thiol (SH) group instead of the hydroxyl of Ser) are sensitive to the leaving group of the phosphate ester substrate (Ghosh *et al*, 1986). The reason for this is not yet understood. The phosphate moiety is very closely associated with all three metals in the active site of the E·P intermediate together with several water molecules that form an extensive hydrogen-bonding network: the phosphate is coordinated to both zinc ions by hydrogen bonds, and the other two phosphate oxygens are tightly held by the two amino groups of the guanidinium group of Arg^{166}, which is in turn hydrogen bonded to Asp^{101} and a water molecule that is held by Asp^{151} and possibly Tyr^{109} (Coleman, 1992). Of the two phosphate oxygens involved with the guanidinium group, one is further hydrogen bonded to the amide of Ser^{102}, and the second is hydrogen bonded to two water molecules - one that is coordinated to the Mg^{2+} ion and the other forms a bridge to Lys^{328}. These positions (particularly that of the ions) are such that they activate Ser (the Ser hydroxyl to Ser-O$^-$) and water for the two nucleophilic

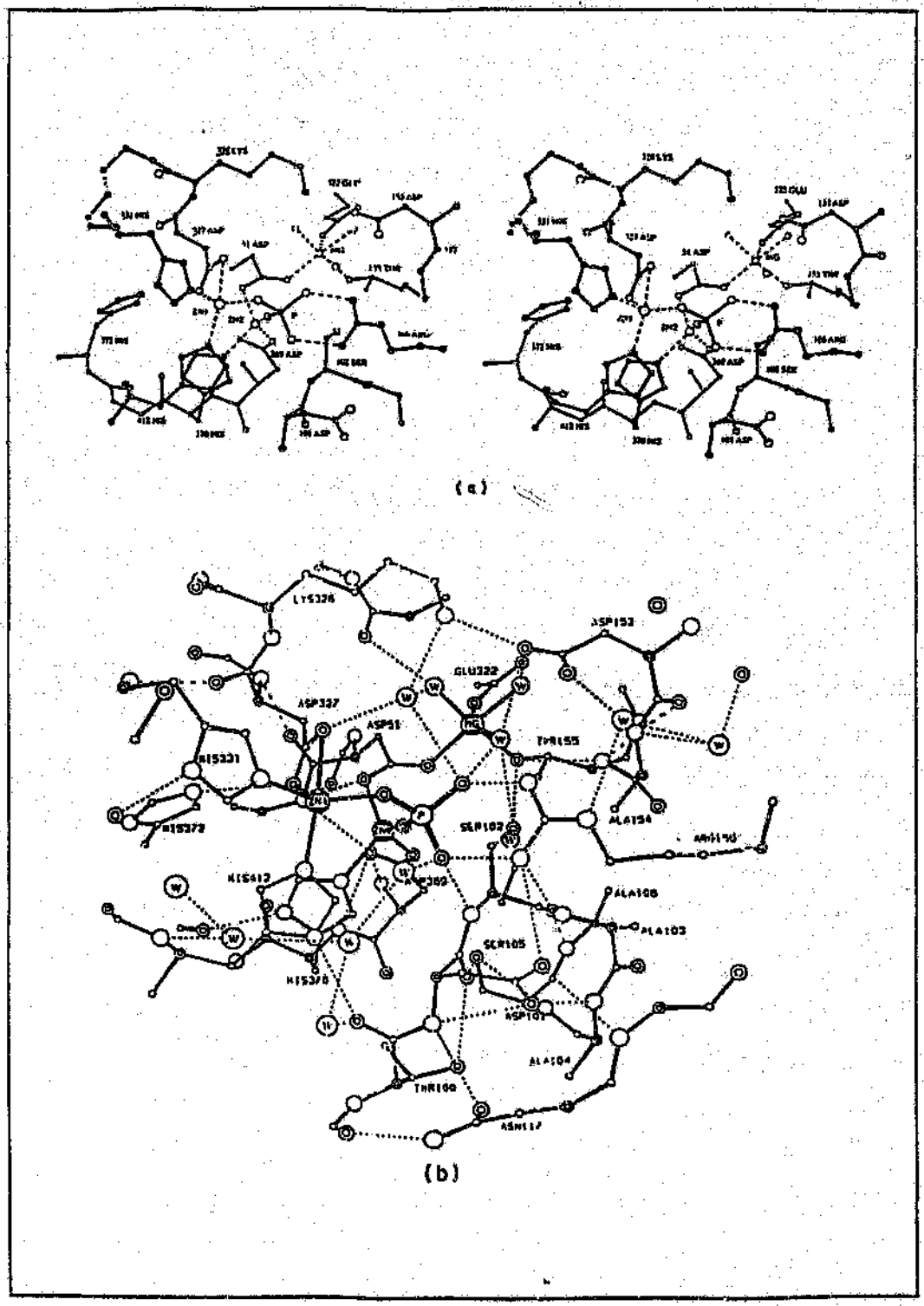


Figure 1.4 (a) Stereo drawing of the active site of the *E. coli* alkaline phosphatase, $(Zn1Zn2Mg3)_2AP$ in the presence of excess HPO_4^{2-} at pH 7.5. The co-ordination of the metals by the specific amino acid residues is illustrated. Atoms are shaded by atom type. Some residues and water molecules are omitted for clarity. (b) The active-site region of the E·P complex (2Å resolution) including all the atoms within 10Å of the phosphorous atom. Water molecules are labelled *W*. Dotted lines show the hydrogen bonds (From Coleman, 1992).

acks required for catalysis and for stabilisation of the alkoxide (RO) leaving group (Butler-Ransohoff *et al*, 1992). The zinc ion 1 is of primary importance in catalysis. Removal of it results in complete loss of activity (Trotman & Greenwood, 1971). This ion, by hydrogen bonding as described above, positions the phosphate moiety of the substrate and it also participates in the formation and hydrolysis of the phosphoenzyme complex during catalysis, since correct positioning of P is important for charge neutralisation needed for nucleophilic attack by serine (Coleman, 1992).

The roles that specific residues play in the catalytic mechanism of AP's have been elucidated largely by means of site-directed mutagenesis studies. This, combined with high-resolution X-ray crystallography, has greatly facilitated the study of structure-function relationships and the kinetics of reactions involving these enzymes (Chaidaroglou *et al*, 1988). Studies with human isozymes, show that the residues Asp^{91} and Arg^{166} in the human PLAP and GCAP serve the analagous active-site functions to Asp^{101} and Arg^{166} in *E. coli* (Hoylaerts *et al*, 1992). Different effects are observed with different mutations at these sites, reflecting different effects on the active site. Substitutions of Asp^{91} have little impact on K_m but decrease the turnover rate by 15-fold. Although it was found that Arg^{166} assists in substrate/phosphate binding, neither the residue itself nor the positive charge are essential for catalysis of substrate hydrolysis by the enzyme (Butler-Ransohoff *et al*, 1988; Chaidaroglou *et al*, 1988). However, substitutions of Arg^{166} do have a dramatic kinetic effect: k_{cat} decreases 20-fold and K_m increases by a factor of 6-10 (Hoylaerts *et al*, 1992). Substitutions of Asp^{91} (Asp^{91}→Ala^{91}/Asn^{91}) cause a defective positioning of the active-site Ser^{92} resulting in a low k_{cat} (Hoylaerts *et al*,1992). Arg^{166}→Ala^{166}/Lys^{166} substitutions result in a defective positioning of P in the active site which leads to ineffective charge neutralisation of the P which is needed to facilitate nucleophilic attack by serine during phosphorylation and also by water or R'OH during dephosphorylation (Hoylaerts *et al*, 1992).

1.1.3.4 The Inhibition Pattern of Alkaline Phosphatases

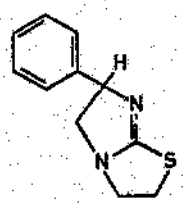
In the early seventies, when little was known about the molecular structure of AP's, but the existence of different AP isozymes was well recognised, it became evident that these isozymes could be selectively inhibited by amino acids and short peptides (Fishman & Sie, 1971). This unique kinetic property occurs only in eukaryotic AP's (Fishman, 1974). The inhibition occurs by an uncompetitive mechanism (Ghosh & Fishman, 1966; Byers *et al*, 1972). Only L- and not D-enantiomers of amino acids are effective at inhibition, *i.e.* inhibition is stereospecific (Fishman & Sie, 1971; Fishman, 1989), and it is organ-specific (Fishman & Sie, 1971).

TNAP from a variety of species is inhibited specifically by L-homoarginine, L-His, levamisole[1] and its analogue bromotetramizole[2], but not by L-Phe, L-Leu or the related peptides L-Phe-Gly-Gly and L-Leu-Gly-Gly (Fishman & Sie, 1971; Lin & Fishman, 1972; Mulivor *et al*, 1978). The three human TSAP isoenzymes, on the other hand, are (equally) inhibited by L-Phe but not by L-homoarginine or any of the other TNAP-specific inhibitors (Fishman & Sie, 1971; Fishman *et al*, 1963). Furthermore, GCAP is inhibited by L-Leu with a 15-20 fold higher efficiency than is PLAP (Doellgast & Fishman, 1976). The organ-specific sensitivity of AP isoenzymes to the different uncompetitive inhibitors has been used in clinical quantification and identification of AP isozymes (Green *et al*, 1971; Mulivor *et al*, 1978; Millán *et al*, 1980).

The mechanism of this uncompetitive inhibition by amino acids has only recently begun to be elucidated. An "homosteric mechanism" appears to be responsible

---

[1]Levamisole: L(-)-2,3,5,6-tetrahydro-6-phenylimidazo(2,1-b)thiazole



[2]Bromotetramizole: (+)-6(4-bromophenyl)-2,3,5,6-tetrahydroimidazo(2,1-b)thiazole ethanedioate

whereby a conformational change occurs in the protein upon binding of the specific amino acid *to the active site* which then leads to a modification of the reaction of the bound intermediate (Lin *et al*, 1971). Results from kinetic and thermodynamic studies are consistent with such an homosteric mechanism rather than an allosteric mechanism where the amino acid would bind to a site away from the active centre (Ghosh & Fishman, 1966; Lin *et al*, 1971). In these studies, it was shown that although inhibition by L-Phe of rat IAP and inhibition by L-Try of human PLAP and IAP is homosteric, it is also uncompetitive (Ghosh & Fishman, 1966; Lin *et al*, 1971).

Studies employing site-directed mutagenesis, reveal that specific residues act as major determinants of the inhibition. For example, Gly^{429} of GCAP is essential for the inhibition of this isoenzyme by L-Leu (Hummer & Millán, 1991). The Gly^{429} residue is found in a loop region (residues 400-430) that is unique to eukaryotic AP's and absent from the *E. coli* AP molecule (Hummer & Millán, 1991). Furthermore, comparisons of GCAP with the *E. coli* AP sequence and its three-dimensional structure, predict that this residue lies in proximity to the entrance to the active site (Hummer & Millán, 1991). In a similar way, Glu^{429} is the determinant for inhibition of PLAP by L-Phe (Hummer & Millán, 1991). Even though PLAP and GCAP share 98% homology at the protein level, the difference at position 429 of their primary structure results in a very different pattern of amino acid inhibition.

Further studies have shown that L-Leu inhibition of GCAP and L-Phe inhibition of PLAP occur as follows: when a phosphorylated substrate is bound to the active site, the guanidinium group of Arg^{166} (normally involved in positioning the phosphate as described above) is redirected to the carboxy group of L-Leu or L-Phe and this stabilises the inhibitor in the active site. In this way, the inhibitor's amino acid side group is positioned in the loop that contains either Gly^{429} (GCAP) or Glu^{429} (PLAP) and this further stabilises the inhibitor. This explains why inhibition occurs only at a stage in the enzyme cycle after the reversible substrate binding and positioning (Byers *et al*, 1972). When the phosphate is reversibly bound in the active site, the

Arg^{166} residue has not yet been positioned by the resulting conformational change necessary for catalysis to allow the carboxy group of the inhibitor (or any other ligand) to interact with Arg^{166} (Kim & Wyckoff, 1991). Thus, stable binding of the amino acid inhibitor and effective inhibition only occur when the phosphate is covalently bound in the active-site pocket (Hoylaerts *et al*, 1992). The inhibitor's unprotonated amino group co-ordinates with the Zn^{2+} ion 1 in the active site and this interferes with the hydrolysis (or transphosphorylation) of the phosphoenzyme intermediate (this phenomenon explains the uncompetitive nature of the inhibition). That the protonation state of the amino group is important, is clear from the observation that inhibition is strongly pH-dependent, being maximal at pH 9-10.5 and absent at pH 7.5 (Lin *et al*, 1971; Hoylaerts *et al*, 1992). Thus, all three functional groups of the inhibitor, *i.e.* its carboxy, amino and side groups participate in the process. Note that replacement of Glu^{429} by Gly^{429} in PLAP results in a conformational change in the loop region proximal to the active site of the enzyme (Hoylaerts & Millán, 1991). This eliminates steric constraints on the bulky L-Leu side group during its positioning which increases the active-site accessibility for the inhibitor. This explains the 17-fold greater inhibition efficiency in GCAP compared to that in PLAP (Hoylaerts *et al*, 1992). Thus, the conformation of the unique surface loop located in the vicinity of the active site influences the positioning of the amino acid inhibitor and its accessibility to the active site. Note that it has also been shown that Ser^{84} and Leu^{297} play a modulatory role in the inhibition of GCAP by L-Leu. However, the exact mechanism is unclear at present requiring further crystallographic analysis (Hummer & Millán, 1991).

At present, it is not known which residues confer L-homoarginine inhibition properties on TNAP isozymes (Hummer & Millán, 1991). Since the sequence homology between TNAP and the TSAP isozymes is only about 50% it will be more difficult to identify these residues (Hummer & Millán, 1991).

The biological role of inhibition is not known. It is significant that AP inhibitors are naturally occurring L-amino acids and peptides containing L-amino acids. It has been suggested that free amino acids in the tissues where AP isozymes are

expressed, through their action as inhibitors, control the activity of AP's (Hummer & Millán, 1991). The fact that amino acid inhibition occurs only in animals that possess different isozymes of AP's may also be significant. It is possible that the inhibitory site (which is in the vicinity of the active site) recognises the specific L-amino acids in the context of a peptide sequence and any substrates containing such a cognate sequence would not be acted upon by the catalytic site of the AP isozyme (Hummer & Millán, 1991). In this way only certain phosphorylated proteins act as substrates for the various AP isozymes which explains isozyme specificity of AP function.

1.1.4 PHYSIOLOGY OF ALKALINE PHOSPHATASES

1.1.4.1 Distribution and Function

AP's are highly ubiquitous enzymes and are found in all species ranging from bacteria to man (McComb *et al*, 1979). This wide distribution in nature indicates that these enzymes are involved in fundamental biochemical processes. However, the *in vivo* functions of AP's are not yet known and the physiological substrates have not been identified (Narisawa *et al*, 1992).

There is an inborn error of metabolism, called "hypophosphatasia", which is characterised by subnormal or absent TNAP activity, primarily in bone tissue and in plasma, and the resulting symptoms clearly demonstrate the crucial importance of TNAP in normal bone mineralisation (Millán *et al*, 1980). The severity of this disease varies, ranging from early loss of teeth and recurrent fractures in adults, to gross abnormalities of the skeleton in children, to severe prenatal hypomineralisation and death *in utero* (McFarlane *et al*, 1986; Weinstein & Whyte, 1981; Wolff & Zabransky, 1982). It is interesting that other tissues that normally express high TNAP activity, such as the liver and kidney, do not appear to be significantly impaired in hypophosphatasia patients (Fröhlander & Millán, 1991). Patients commonly have high serum levels of phosphoethanolamine, pyridoxine-5'-phosphate and inorganic pyrophosphate, and it has been suggested that these

compounds are the physiological substrates of TNAP, but precisely how these biochemical abnormalities are interrelated in adult hypophosphatasia is unknown (Fedde & Whyte, 1990).

The common evolutionary origin and the structural similarities of the four human AP's suggest that they all perform similar functions *in vivo*. Studies of hypophosphatasia may help to elucidate some of the functions of IAP, PLAP and GCAP but even the exact mode of action of the TNAP in bone mineralisation is far from clear. Moreover, other tissues that express high levels of TNAP do not calcify. This suggests that AP's may serve many different functions. IAP, present in vast amounts in the brush border of the intestinal mucosa, appears to be involved in the transport of metabolites, such as inorganic phosphate, from the gut. IAP could also serve to catalyse the hydrolysis of phosphate esters, making the products available for absorption. The *E. coli* enzyme is involved in the acquisition of phosphate from esters when free inorganic phosphate is depleted (Kim & Wyckoff, 1991). Similarly, the large amounts of PLAP in the syncytiotrophoblast lining, which represents the interphase between the foetal and maternal bloodstreams, also suggests that the AP is involved in transport (Fröhlander & Millán, 1991). There is, in fact, one case reported of PLAP deficiency in a dizygotic twin pair with Crouzon syndrome (Fröhlander & Millán, 1991) but it is not yet known whether the lack of PLAP activity is directly the cause of the syndrome or whether it is just linked to another element that causes the syndrome. No case of IAP or GCAP deficiency has been reported, and this could imply that such deficiencies are incompatible with normal development (Fröhlander & Millán, 1991).

There have been several reports associating AP activity with cell division. An ultrastructural histochemical study in synchronised HeLa cells showed that AP activity during the cell cycle was mostly associated with early and middle G1, there being much less activity in late G2 and undetectable activity in S phase (Vorbrodt & Borun, 1979). High levels of AP have been associated with early stage embryo cells, uterine reserve cells, intestinal epithelial cells and cancer cells, all having a high mitotic index (Narisawa *et al*, 1992). Since phosphorylation is a major

regulatory mechanism of the cell cycle (Cyert & Thorner, 1989), it is possible that AP's are involved in cell-cycle controlled dephosphorylation or transphosphorylation reactions (Narisawa *et al*, 1992). Enoch and Nurse (1991) have shown that mitosis in yeast and some fungi is controlled by several genes coding for protein kinases and phosphoprotein phosphatases. Axton *et al* (1990) found a serine/threonine phosphatase isozyme that is essential for mitosis in *Drosophila*. Many oncogene products and growth factor receptors have tyrosine kinase activity and tyrosine phosphatases appear to have important roles in cell growth and intercellular communication (Narisawa *et al*, 1992). Narisawa *et al* (1992) cite reports that suggest that AP's could act as protein tyrosine phosphatases but they consider it unlikely due to insignificant homology between AP and other protein phosphatase genes. Furthermore, AP's do not have their catalytic domain facing the cytoplasm. There is evidence that AP in mouse blastocysts does not have a PIG moiety and thus it is possible that in some developmental stages, EAP may function as a PIG-anchored enzyme, whereas in others it functions as a non-PIG-anchored enzyme, affecting its subcellular localisation and thus also the target substrates (Narisawa *et al*, 1992). Gene targeting experiments are being carried out to abolish expression of certain AP's, such as EAP (Narisawa *et al*, 1992), to determine the consequences of the absence of these AP's. Such and other work should elucidate the specific reactions that AP's perform in relation to their specific functions.

Recently, it has been suggested that AP's may play an important role, not necessarily as enzymes, but as structural proteins based on their biophysical properties (Millán, 1990). Some observations (such as the fact that the three TSAP isozymes are almost identical with only very subtle differences) suggest that additional domains of AP mediate protein-protein interactions that may regulate, complement, or give specificity to their catalytic activity (Millán, 1990). For example, TNAP expression in the bone may be modulated by hormonal influences, and the specific substrates of TNAP (related to bone mineralisation) occur only in this tissue, but, furthermore, there may be specific protein-protein interactions that are only present in the bone matrix which results in a non-specific enzyme such as TNAP, that is found all over the body, to perform a very specific and highly

specialised function, *viz.* bone mineralisation (Millán, 1990). It is clearly observed that TNAP interacts specifically with collagen type II, which is found mainly in mineralising cartilages (Vittur *et al*, 1984). It also interacts with collagen type X and weakly with type I, indicating that these interactions are essential for normal bone mineralisation (Wu *et al*, 1991a; 1991b; 1992). It is also found that mineralisation can be induced in cartilage by any AP, including bovine IAP (Beertsen & van de Bos, 1992). Tsonis *et al* (1988) have reported that PLAP and GCAP are homologous with cartilage matrix protein (CMP), von Willebrand factor (VWF), complement factor B, and the adhesion receptors Mac-1 and P150 - all proteins with protein binding capabilities. The area of homology shared by these proteins corresponds to the binding site of collagen or integrins.

These observations clearly suggest that AP's do play a structural role and now that the structures of the different isozymes are known, the importance of this role should be elucidated in the near future.

1.1.4.2 Alkaline Phosphatases in Malignancies

There have been many reports of an association between the (abnormal) expression of TSAP's and malignancy. AP's have been found to be overexpressed in cancerous tissues (eutopic expression) or are found in tissues which do not normally express them (ectopic expression). Most malignancies show an ectopic expression (Fröhlander & Millán, 1991) and are able to coexpress several isozymes at the same time, which is in clear contrast to normal differentiated tissues which normally express only one AP isozyme (Hirano *et al*, 1987; Hofmann *et al*, 1989).

Griffin and Cox (1966) demonstrated the presence of an AP in a HeLa cell line that appeared to be a PLAP isoform (was heat- stable and inhibited by L-phenylalanine). Fishman *et al* (1968) discovered that a patient with epidermoid carcinoma of the lung had high serum levels of an AP which was termed "Regan isozyme" after the patient's name. The enzyme was shown to be of neoplastic origin. The AP was identified as a PLAP isoform due to its sensitivity to L-phenylalanine, heat stability,

electrophoretic mobility through starch gels and its susceptibility to neuraminidase treatment. A different isozyme ("Nagao isozyme") was found in the serum and pleural fluid of a patient with "pleuritis carcinomatosa" which was L-leucine inhibitable (Nakayama *et al*, 1970). Later, gene cloning identified the Nagao isozyme as GCAP and not an allelic variant of PLAP (Millán & Manes, 1988; Hummer & Millán, 1991).

AP's with characteristics similar or identical to PLAP and GCAP isozymes have been shown to be expressed in a wide variety of malignant cell lines (Benham *et al*, 1981; McLaughlin *et al*, 1982). The ectopic expression of PLAP has been observed in malignancies of the lung, ovary, uterus and the gastrointestinal tract, among others (Nathanson & Fishman, 1971; Jacoby & Bagashawe, 1971; Fishman *et al*, 1976). GCAP is eutopically expressed in testicular seminomas (Jeppsson *et al*, 1984; Koide *et al*, 1987) and in ovarian cancer (Vergote *et al*, 1987). IAP has been found to be expressed mainly in hepatomas (Higashino *et al*, 1972).

Most of the malignancies studied to date show an abnormal expression of either PLAP or GCAP. Indeed, PLAP and GCAP are useful tumour markers in the management of testicular cancer and ovarian cancer patients (Millán, 1990). Clearly, these malignancies represent a condition where genes that are normally expressed only during early development are somehow turned on and inappropriately expressed. This phenomenon has been observed with other genes. For example, re-expression of α-foetoprotein has been found in mouse and human hepatomas, and in teratocarcinomas of the ovary and testis (Abelev, 1971). Gold and Freedman (1965) found cell surface glycoproteins, normally expressed and secreted into the lumen by epithelial cells of the foetal gastrointestinal tract, in the serum of patients with carcinoma of the gastrointestinal tract. These observations have led to the concept of oncodevelopmental biology: re-expression of "oncodevelopmental genes", normally expressed during early development, play an important role in tumorigenesis, *i.e.* cancer is seen as a developmental disease due to misregulation of gene expression (Fishman & Sell, 1976).

It is at present unclear what role AP's play in tumorigenesis. Several mechanisms are envisioned (Fröhlander & Millán, 1991) and different mechanisms may apply in different cases. It is possible that derepression of the AP gene itself is enough to cause a tumour, although this appears unlikely since transfection of AP cDNA's into normal cells and subsequent overexpression fails to elicit a transformed phenotype (Fröhlander & Millán, 1991). The AP gene may act in concert with other genes in tumorigenesis. The AP gene may simply be closely linked to a disease-causing gene. Another mechanism could involve the simultaneous activation or deregulation of AP genes and an oncogene. It has been shown that a tumour suppressor locus functionally regulates both IAP expression and tumorigenesis in HeLa/fibroblast hybrids (Latham & Stanbridge, 1990).

Further work is needed to elucidate which mechanisms apply. It is also not clear whether particular PLAP or GCAP alleles or phenotypes are responsible for tumour development and/or tumour progression (Fröhlander & Millán, 1991). Now that all the human AP genes have been characterised and allelic variation and RFLP's identified, finding the specific associations and mechanisms involved in tumorigenesis is possible.

1.1.4.3 Alkaline Phosphatases and Disease

It has been known since the early 1930's that various pathological conditions are associated with large increases in levels of serum AP (for a good review see Gutman, 1959) and the determination of serum AP activity has been widely used in clinical biochemical tests and used to monitor treatment (Fishman *et al*, 1965). Besides the association with cancer discussed in the previous section, other pathological conditions associated with elevated levels of serum AP include such skeletal disorders as rickets, hyperthyroidism, osteogenic sarcoma and others (McComb, 1979). Other disorders include obstructive jaundice, hepatitis, cirrhosis and other hepatobiliary disorders (McComb, 1979). AP levels are greatly increased in patients with AIDS (Payne *et al*, 1991). AP has also served as a marker for endothelial cells in several degenerative neuropathological diseases (Butterworth,

1986). Butterworth (1986) could show that AP activity is normal in Huntington's disease caudate nucleus whereas other enzymes, particularly in neurons and glia, were found to be markedly reduced. This kind of study helps to delineate the types of cells that are altered in any specific neurological disease.

In most cases the level of TNAP is increased in various bone and liver disorders, and raised IAP levels have been associated in some cases of cirrhosis (Fishman *et al*, 1965). Augmented levels of GCAP and PLAP have only been associated with various cancers as was discussed in section 1.1.4.2.

Thus, although the precise functions of AP's are not yet known, they are useful in the diagnosis of a wide variety of conditions and their association with these conditions may help to elucidate their functions.

1.1.4.4 Alkaline Phosphatases and Development

There have been many reports where an increase in AP activity has been correlated with normal embryological development. For example, Moog (1944) examined the spacial and temporal distribution of AP in the chick embryo and noted that during organogenesis a number of tissues, such as brain and lung, show intense AP activity which diminishes at later stages. This indicates that AP is closely associated with the initial stages of tissue differentiation. Brachet (1946) and Krugelis (1950) associated AP with organogenesis, and Lindeman (1949) with maturation of the retina. Karczmar and Berg (1951) in a histochemical study of the development of limbs in urodeles, found that AP is first detected just prior to the appearance of the limb rudiment, increases gradually during growth of the limb bud, and reaches a maximum immediately prior to organogenesis. In annelids, AP is expressed in the "endodermic mass" and the primordia of the nephridia after the initial cleavage stages (McComb *et al*, 1979). Some studies have also shown a correlation between AP activity and development in molluscs (McComb *et al*, 1979). AP activity is high during the active larval and early adult stages of insects and is generally low during pupation (McComb *et al*, 1979). Using an antipeptide antiserum that was developed

to react specifically with mouse EAP, it has been shown that, in addition to expression in the pre-implantation mouse embryo, EAP is expressed in spermatogenic cells in the testis that are in mid or late prophase of meiotic division I (Narisawa *et al*, 1992). This suggests that EAP may play an important role in preimplantation development and in meiosis. Transgenic mice with human GCAP, expressed GCAP in equivalent cells in the testis, as well as in the same stages in the mouse embryo as EAP expression (Narisawa *et al*, 1992). This suggests that EAP and GCAP have equivalent functions during development. The particular functions are not known but the association of AP activity with cell-division, particularly mitosis, discussed in section 1.1.4.1, may provide an important clue.

It is clear that the spatiotemporally regulated presence of AP in embryos has been noted for a long time but, as yet, no specific role for AP in development has been identified (Zackson & Steinberg, 1988). Brachet and Jeener (1948, cited in Osawa, 1951) found that AP in the nucleus of adult rat tissues is related to the turnover of phosphorus of DNA. This, and the observation that AP phosphatase activity is linked closely with nucleoproteins, suggests that one role is in the phosphorylation and dephosphorylation of DNA (Osawa, 1951).

In most animals studied, AP is detectable from fertilisation and is generally present at low levels through the blastula stages (Osawa, 1951). These levels increase gradually with a sharp, linear increase in AP activity during, or shortly after, gastrulation (Krugelis, 1950). In amphibia, the highest levels of AP can be found in the ectoderm, with less in the mesoderm and very low levels in the endoderm (Osawa, 1951). In the 8-cell stage of the ascidian embryo (*Ciona intestinalis*) only the four vegetal blastomeres give rise to tissues expressing AP, which indicates that localisation of AP expression can be predetermined by cytoplasmic segregation in the first cleavages of the egg (Whittaker, 1990). Pfoul and Guidice (1967) found that if embryonic cells of the sea urchin *Arabacia punctulata* are dissociated and reaggregated at the early pluteus stage, the increase in AP activity is not affected. However, if this treatment is performed at an earlier stage, the expression of AP is greatly diminished (Pfoul & Guidice, 1967). Similarly, Abe *et al* (1972) found

that the developmental increase of AP activity in early-blastula cells is sensitive to the disruption of cell-to-cell contact. If the dissociation of the embryo's is carried out at the *late*-blastula stage however, AP activity increases normally (Miyahara *et al*, 1982). From these observations it appears that cellular commitment to AP expression is already established by the end of the blastula stage. It is still not known, however, how cell interaction, an important process in morphogenesis, regulates gene expression, resulting in appropriate differentiation of cells (Miyahara *et al*, 1982).

An important feature of embryonic morphogenesis are the migrations and rearrangements that cells and cell groups partake in. Zackson and Steinberg (1988) have shown evidence that AP is directly responsible for such directed cell movements. In the embryos of the axolotl *Ambystoma mexicanum*, the pronephric duct (PND) primordium first appears as a nest of cells in the mesoderm, immediately ventral to the anterior somites, shortly after neurulation. The anterior end remains anchored to the developing pronephros, while the posterior tip migrates caudally along the boundary between the developing somites and the lateral mesoderm, until it reaches the cloacal wall with which it finally unites (Poole & Steinberg, 1981). This elongation involves a rearrangement of PND cells, and not cell division or the elongation of individual cells. Studies involving PND primordium grafts to various places in the embryo, and grafts performed well in advance of the migrating PND tip, has shown where the guidance information is, and when it appears, and also indicate that the PND guidance information that directs these cell movements consists of "a gradient of adhesiveness of PND cells to their substratum" (Zackson & Steinberg, 1986; 1988). Zackson and Steinberg (1988) proposed that a "molecular prepattern" guided the morphogenetic movements of cells of the developing PND. Using the trajectories of cells migrating in response to PND guidance information, a map was constructed that showed the expected distribution of a "cell guidance associated molecule" (CGAM). It was found that the distribution of AP corresponds with this expected distribution and gradient of a CGAM. Transplantation experiments, where other cells that exhibit directed migration along the PND pathway (cranial neural crest cells which normally

migrate in a different region of the embryo) were ectopically grafted in regions of the embryo with high levels of AP, with neighbouring regions low in AP, confirmed the role of AP in guided cell movement (Zackson & Steinberg, 1988). Furthermore, levamisole, an inhibitor of AP, is found to prevent PND migration (Zackson & Steinberg, 1989). The enzyme phosphatidylinositol-specific phospholipase C (PIPLC, releases AP, which is anchored to cell surfaces by phosphatidylinositol glycan (see section 1.1.3.1) and this enzyme also blocked PND cell migration without interfering with other morphogenetic cell rearrangements which occur in the absence of AP (Zackson & Steinberg, 1989).

From these studies, Zackson and Steinberg (1988) have suggested four possibilities for the role of AP in the embryo. It is possible that AP plays no role, that its distribution simply correlates with that of some other molecule that does guide cell migration. A second possibility is that AP does act directly, perhaps as a cell recognition or adhesion molecule, either by binding a phosphate-bearing ligand in a lock-and-key fashion or through other binding capabilities (AP is able to bind structural proteins as discussed in section 1.1.3.2). AP may, through hydrolysis or transferase activities, modulate the activity of molecules that are active in cell guidance. AP may modulate cell behaviour via its linkage to phosphatidylinositol by means of transmembrane signal transduction processes, much like insulin action which mediates some intracellular effects by interaction of the hormone with cell-surface receptors (Low & Saltiel, 1988). There are at present no new reports concerning the exact role of AP in cell movement or rearrangement, but investigations are continuing.

As already mentioned in section 1.1.4.1 the fact that there are no known cases of IAP and GCAP deficiencies and only one case of PLAP deficiency, which is associated with the severe abnormalities characterising Crouzon syndrome, indicates that these enzymes must play a vital role during the development of an organism. There are at least two developmental systems in humans that display switching or modulation of AP isozyme expression during differentiation and maturation (Millán, 1990). During early pregnancy, only the TNAP isozyme is expressed in the

trophoblast until about the eighth week of trophoblast development (Fishman *et al*, 1976; Sakiyama *et al*, 1979). Between 8 and 12 weeks, TNAP activity decreases and expression of the PLAP gene increases. PLAP is then the only AP isozyme expressed in the placenta (Fishman *et al*, 1976). During foetal gut differentiation both IAP and PLAP genes are active (Behrens *et al*, 1983; Sussman *et al*, 1986). After birth, PLAP activity rapidly diminishes, and in the adult only IAP is expressed in the jejunum and duodenum (Millán, 1990). It is not known what differentiation events or hormonal messages signal these changes in AP expression but they must be significant (Millán, 1990). It is possible that GCAP is involved in guiding the extra-embryonic primordial germ cells as they migrate to the developing gonad through the genital ridge during embryogenesis (Millán, 1990). The trace amounts of GCAP found expressed in the thymus could be from primordial germ cells trapped in the thymus during their migration (Millán, 1990). Research is needed to confirm these theories.

It is interesting that the F allele of PLAP has been associated with spontaneous abortion, and the rare D phenotype has also been implicated in women with a history of pregnancy complications and spontaneous abortions (Beckman & Beckman, 1975). These observations are at present not understood but also suggest that the appropriate expression of AP, in this case expression of the common S allele of PLAP, plays an important role in development.

1.1.5 ALKALINE PHOSPHATASES IN XENOPUS

In embryo's of *Xenopus laevis*, as in other amphibians, AP activity begins to increase at the gastrula stage (Krugelis, 1950; Abe *et al*, 1972). The level of AP activity increases sharply and linearly throughout gastrulation and is very high in the neurula stages and plateaus at the tail-bud stage after an 18-fold increase in activity (Maekawa & Yamana, 1975). Maekawa and Yamana (1975) could chromatographically separate AP activity from *X. laevis* embryo's into two peaks, corresponding to isozymes with molecular weights of approximately 200 000 and 400 000. The two isozymes are also electrophoretically distinguishable (Maekawa

& Yamana, 1975). Evidence is presented in this work suggesting that the two groups of isozymes correspond to TNAP and IAP.

There are some tentative data that suggest that the AP that is important during normal embryological development in the frog is the TNAP. The inability to isolate an AP clone using a probe based on the conserved 3' end of the human PLAP gene, even under low stringency conditions (T. Spieker, unpublished results), and that levamisole, a TNAP inhibitor, disrupts embryological development in *Xenopus* (B. Fabian, personal communication) all indicate the importance of TNAP in the embryo. Isolation and characterisation of TNAP genes has proven to be more difficult than with the specific types due to the greater complexity of the former.

Histochemical studies in *Xenopus* have shown that AP is found mainly in the kidney, heart, digestive tract, nervous system, retina, mesenchyme and endodermal yolk mass, both in the adult organ and its embryonic rudiment (Osawa, 1951). This distribution is similar to that in other amphibians (Maekawa & Yamana, 1975). Other data illustrate the possible roles of AP (TNAP) in the frog embryo. It is found that AP is preferentially expressed in the invaginating dorsal lip cells which invade the blastocoel to form the archenteron (C. Penny, unpublished results). There are also some tentative data that suggest that AP plays a role during neuronal pathfinding in *Xenopus* (T. Spieker, Honours report). Further work is needed to elucidate the precise functions of AP in the normal development of the frog embryo and the cloning and characterisation of the gene coding for TNAP will be a major contribution in this research.

1.1.6 SUMMARY

Alkaline phosphatases are a group of enzymes that catalyse the hydrolysis of inorganic and organic phosphate esters and show no substrate specificity. They have a high pH optimum, depend on zinc and magnesium ions for activity, and they are all dimeric in structure, with monomer molecular weights ranging between 40 000 and 70 000. These enzymes are glycosylated and, in eukaryotes, are membrane

bound. Although present in almost all living organisms, their precise physiological functions remain to be elucidated. They are closely associated with developmental processes, such as differentiation and morphogenesis, and augmented levels of AP are often associated with malignant tumours and various other diseases. Embryological and immunological studies have shown that there are at least two isoforms of AP in all animals, the TNAP and IAP. Humans and higher primates also have PLAP and humans have GCAP. All the isozymes are encoded for by a multilocus gene system. The evolutionary and functional relatedness of these isozymes in different animals is being elucidated based on comparisons of structure and tissue-specific expression.

1.2 THE ISOLATION AND CHARACTERISATION OF GENES

1.2.1 GENE LIBRARIES

The first step taken when a specific gene is to be studied, is its isolation from a library of genes using appropriate probes, and the cloning of the gene to produce a large number of copies of it, making the gene amenable to studies involving such manipulations as sequencing, mutagenesis and *in vitro* translation. The two types of library commonly used are the genomic library and the cDNA library. Both are constructed by attaching double-stranded DNA fragments to a suitable vector, and the recombinant DNA is introduced into a host cell, usually a bacterium such as *E. coli*, where it is replicated as the cell divides.

1.2.1.1 Cloning Vectors

When cloning any particular fragment of DNA it needs to be attached to a carrier DNA which contains a sequence that the host cell recognises as an origin of replication. In this way the fragment can be replicated as the host cell grows and divides. Small plasmids and bacteriophages were the first vectors used since they are able to replicate in host cells, are easily maintained in the cell without

necessarily being integrated into the genome of the host cell, and thus can be readily isolated in an intact form. This permits the easy bulk preparation of the cloned fragment of DNA, free from any host cell material.

An ideal cloning vector needs the following properties (Old & Primrose, 1989). First, it should be of low molecular weight. This renders the vector easy to handle. It is then resistant to damage by shearing, and it is readily isolated from host cells. Low molecular weight plasmids tend to occur in much higher copy numbers than large plasmids (Twigg & Sherratt, 1980), which also facilitates their isolation. With low molecular weight vectors, larger fragments of DNA can be cloned since there is a limit to the size of the recombinant vector that can be efficiently propagated in a host bacterium. Another advantage in smaller vectors, is that there is a greater chance that the vector will have unique substrate sites for restriction endonucleases which can then be used to cleave open the vector at the one site and to which the foreign fragment is then ligated.

Another important property in a vector, is its ability to confer readily selectable phenotypic traits on host cells. Usually, this is provided by a gene for antibiotic resistance on the vector and thus, only cells that are successfully transformed can be selected for. There are also markers that help to select for only cells that have been transformed with recombinant vector.

Many vectors have been developed having the abovementioned properties and any can be chosen to fulfil any particular requirement. Plasmids are being used less at present due to their relatively limited capacity for foreign DNA fragments. Vectors based on the λ bacteriophage are commonly used due to the many advances made that have greatly increased the carrying capacity of these vectors. In particular, there are the *replacement* vectors where two restriction sites define a non-essential region that can be removed and replaced with a foreign fragment. This deletion allows the phage to accomodate up to 25 kb of foreign DNA (Kaiser & Murray, 1985). Other improvements in λ-based vectors include the introduction of polylinkers which contain multiple unique restriction sites, the presence of markers

for selection against non-recombinants, and the introduction of suitable promoters that allow transcription of the insert which facilitates screening of libraries in chromosome walking proceedures. Vectors have also been developed where eukaryotic cDNA can be expressed in the form of a fusion polypeptide with the α-peptide of ß-galactosidase in *E. coli*. Such expression vectors are useful in antibody screening. Furthermore, ß-galactosidase expression can be used in a colour detection test to select for recombinants. In non-recombinants, the complete α-peptide that is expressed can associate with the ω-peptide, expressed by the host strain, to produce functional ß-galactosidase (α-complementation), and in the presence of the chromogenic substrate Xgal in the plating medium (plus IPTG, a gratuitous inducer of ß-galactosidase), dark-blue plaques on bacterial strains that lack the enzyme are produced. In recombinants the enzyme is not functional and white plaques result.

Certain bacterial strains have been developed to be used in conjunction with certain vectors. For example there are strains that are Lac⁻ due to a chromosomal deletion that are used in the colour test described above. There are strains designed to prevent restriction of the transforming vector, strains that prevent integration of the vector into the host genome, and strains where recombination between the host chromosome and vector is eliminated.

Another λ-derived vector is the cosmid. This cloning vector is a plasmid containing the λ *cos* site which results in its packaging into phage particles used to transfect bacteria. Cosmids are used to clone fragments up to 47 kb long and are thus often used in the construction of genomic libraries (Old & Primrose, 1989).

1.2.1.2 Genomic Libraries

The genomic library of an organism consists of a large number of recombinants, which together contain a complete collection of most, if not all, of the DNA sequences of the whole genome of that organism (Old & Primrose, 1989). Such a library is generally constructed by isolating the DNA of the organism, and

fragmenting it either by sonication, or restriction enzyme digestion. The larger the fragments, the fewer the number of clones required for a complete library. Cosmids are often used in genomic libraries since they can accomodate the largest fragments.

Several problems occur in the construction of genomic libraries. A gene of interest may be cut internally one or more times and is thus not obtained as a single fragment. The larger the gene, the more likely that this will occur. Extensive regions flanking the gene may also be desired. Another problem is that a gene can occur in a fragment too large to be accommodated by the vector and is then not cloned at all. To avoid these problems, the DNA is fragmented essentially randomly using sonication, or a restriction enzyme that cuts frequently, or a combination of restriction endonucleases with completely unrelated recognition sites (Maniatis *et al*, 1978). In this way, overlapping clones are produced, with no systematic exclusion of any sequence, and by "walking" from one clone to an adjacent one during screening, one can study whole genes and even clusters of genes.

A genomic library includes all the non-coding DNA of an organism such as introns and the extensive non-coding regions between genes that occurs in eukaryotes. Such a library is essential if one wants to study regions flanking genes, such as promoter and other regulatory sequences. However, the presence of the non-coding sequences results in many useless recombinants which often makes the screening process laborious and ineffective. A cDNA library is then much more useful since it renders the screening process much simpler and more efficient.

1.2.1.3 cDNA Libraries

A cDNA library is constructed from DNA reverse-transcribed from polyadenylated RNA (the messenger RNA) extracted from some tissue or cell type. The library is thus specific, not to the whole organism, but to a particular cell type. The genes represented in such a library will be those specifically expressed in some tissue at some time. A cDNA library is thus used when studying temporally regulated expression of a gene, during development, or tissue-specific gene expression.

An early proceedure for synthesising dsDNA from mRNA starts with the synthesis of the first strand of cDNA using oligo(dT) primers and reverse transcriptase (Higuchi *et al*, 1976). The single-stranded cDNA molecule is then obtained from the RNA-DNA hybrid by alkaline hydrolysis of the RNA. cDNA's are able to form a self-priming structure in which a hairpin loop at the 3'-terminus is stabilised by enough base-pairing and can then initialise second-strand synthesis with DNA polymerase (Higuchi *et al*, 1976). The hairpin is digested away by treatment with the single-strand-specific nuclease S1. cDNA's produced in this way are blunt-ended and cloning by blunt-ended ligation into a vector is inefficient (Sambrook, *et al*, 1989). To efficiently clone the cDNA, it and the vector are treated in various ways to introduce complementary ends. These treatments include ligation of linkers that contain sites for restriction enzymes that produce sticky ends, attachment of adaptors that have preformed cohesive ends, and by homopolymer tailing, *i.e.* adding complementary homopolymer sequences using terminal transferase (Huynh *et al*, 1985).

Nuclease S1 treatment generally leads to the loss of some sequence corresponding to the 5' end of the mRNA and often to adventitious damage to the duplex cDNA (Land *et al*, 1981). Thus, new methods have been developed that forego treatment with nuclease S1. Land *et al* (1981) used terminal transferase to add an oligo(dC) tail to the 3' end of the single-stranded cDNA thereby preventing hairpin formation. A full-length duplex cDNA could then be generated with an oligo(dG) primer. Two other methods devised by Okayama and Berg (1982) and Heidecker and Messing (1983) have also been used with great success. Both methods employ the oligo(dT) sequence for priming the first strand cDNA synthesis, but linked to the vector DNA. The mRNA is annealed to linearised and oligo(dT)-tailed vector DNA. Oligo(dG) tails are added to the cDNA first-strand. The cDNA-RNA hybrids are spun down an alkaline sucrose gradient which hydrolyses the RNA and enriches full-length molecules. Second-strand synthesis is primed with oligo(dC) linked to the same vector which is added and annealed to the vector molecule bearing the first-strand cDNA. Alternatively, the RNA strand is replaced in a DNA polymerase reaction that is primed by nicking the RNA with RNase H (Okayama and Berg,

1982). The second strand is thus synthesised by a nick-translation type of reaction. Several schemes based on this type of second-strand synthesis have been developed for efficient cDNA library cloning, *e.g.* those developed by Gubler and Hoffman (1983), and Lapeyre and Amalric (1985) which are presently widely used.

It must be noted that not all RNA's bear a 3'-terminal poly(A) sequence. Furthermore, the above methods result in 3' end bias since priming occurs at the 3' end of poly(A)$^+$ mRNA's, and cDNA synthesis is often incomplete. Thus, cDNA libraries are enriched in 3'-terminal sequences (Old & Primrose, 1989). Similarly, it is difficult to synthesise and clone full-length cDNA's from large mRNA's. This is often a problem when the cloned cDNA is to be expressed. These difficulties can be overcome by priming the first-strand cDNA synthesis with random oligonucleotide primers (usually a mixture of all possible chemically synthesised hexadeoxynucleotides) (Old & Primrose, 1989).

Two λ-based vectors are commonly used when constructing cDNA libraries: λgt10 and λgt11 (Huynh *et al*, 1985). λgt11 is an expression vector that is capable of producing a polypeptide coded by the cDNA insert fragment. Libraries constructed with this vector are thus termed "expression libraries". Such libraries can be screened with antibodies to isolate clones that carry an insert that codes for antigens which the antibodies recognise (Huynh *et al*, 1985). The site of insertion in λgt11 is within the *lacZ* gene that codes for the α-peptide of ß-galactosidase and thus the foreign insert can be expressed as a fusion protein with this peptide. This ensures that the foreign DNA will be expressed efficiently in *E. coli* (Huynh *et al*, 1985). A problem that is encountered with expression libraries is that correct expression of the insert is often not achieved, either because it is cloned in the wrong orientation, or that it is inserted in such a way that the wrong reading frame is transcribed during expression.

In the present work a λgt10 library was used where expression does not occur. Screening was done using nucleic acid probes and thus expression was not necessary. λgt10 is an extremely healthy bacteriophage, producing large recombi-

nant phage plaques of uniform size and shape (Huynh *et al*, 1985). This is thus the preferred vector when using nucleic acid probes to screen the library (see appendix B). Expression of any inserts that may be toxic to the cell and thereby prevent the isolation of the gene of interest is also avoided.

1.2.1.4 Library Screening

Once a suitable library has been prepared, it is screened to identify and isolate the clones that harbour the recombinant DNA molecule which carries the desired sequence, *i.e.* part or all of the gene to be studied. There are a number of different screening proceedures that can be employed, depending on the type of library used and the type of vector used. A few procedures are based on detection of a translation product of the cloned gene with an antibody to the product. Clearly, this requires that the cloned gene is properly expressed and is thus often inefficient. Screening based on complementary hybridisation of a nucleic acid molecule to the sequence of interest is usually easier. Hybridisation probing is normally performed on bacterial colonies containing plasmid or cosmid vectors, or on plaques with bacteriophage vectors. The bacterial colonies or plaques are transferred from an agar plate to a nitrocellulose or nylon membrane and the blots treated to remove all material except the DNA. The DNA molecules are denatured and fixed to the filter to allow for hybridisation with a nucleic acid probe. Usually the probe is radioactively labelled by nick-translation or end-filling so that any clones that bind the probe are identified by autoradiography. The probe is allowed to hybridise for a period, the filter washed to remove unbound probe, and then dried and autoradiographed.

1.2.2 GENE SEQUENCING

DNA sequencing, where the precise order of nucleotides in a piece of DNA is determined, is one of the most important and widely used techniques in molecular biology today. It may be used as an end in its own right, as when wanting to elucidate the structure of a gene, or to understand what makes certain genes non-

functioning, as in the study of inherited human disorders. Knowing the sequence of a gene is needed when planning any experimental manipulation of the gene. Restriction maps illustrating the positions of all restriction endonuclease sites can be drawn using computers if the sequence is known.

Two different sequencing techniques were developed independently and at about the same time. The two techniques are very different but are equally valuable. In the Maxam and Gilbert (1977) method, a defined DNA restriction fragment is treated with various chemical reagents that results in base-specific cleavage of the DNA. When the fragment of DNA is end-labelled and is exposed to each of these reagents in a limited reaction, a nested set of end-labelled fragments of different lengths is generated. The limited reaction is such that there are only a few cleavages per DNA molecule. The fragments thus generated are denatured and fractionated on the basis of their length on a gel containing 6-20% polyacrylamide (high resolution) and 7M urea (denaturant). The sequence can then be read off the sequencing ladders that result from autoradiography of the gels.

The second method is the chain termination method developed by Sanger *et al* (1977). This method is preferred for large-scale DNA sequencing (Old & Primrose, 1989). The DNA fragment to be sequenced is first denatured. A suitable primer is added and the DNA copied by a DNA polymerase (usually the Klenow fragment of DNA polymerase 1). This synthesis is carried out in the presence of the four deoxynucleotides one of which is usually radioactively labelled. Four separate reactions are carried out. In addition to the above components, each includes a low concentration of one of each of the four dideoxynucleoside triphosphate analogues. When an analogue is incorporated, DNA synthesis cannot proceed due to the absence of the 3' hydroxyl group to which the Klenow fragment usually catalyses the addition of the next deoxynucleotide. In this way, DNA synthesis is halted in every position along the DNA fragment being copied. Thus, each of the four reaction mixtures generates a population of partially synthesised radioactive DNA molecules, each having a common 5' end, but each varying in length to a specific base at the 3' end. Each reaction mixture is incubated for a suitable time and then

denatured and electrophoresed side-by-side. The sequence is read from the autoradiograph prepared from the gel.

Other enzymes that are currently more popular when using the chain-termination method are modified forms of the T7 DNA polymerase such as Sequenase, and the Taq DNA polymerase which allows the chain-termination reaction to be carried out at high temperatures which prevents chain-termination artefacts caused by secondary structure in the DNA. Special single-stranded vectors such as the M13mp series (Vieira & Messing, 1982) are often used, to facilitate the sequencing procedure, since the DNA to be sequenced can be inserted in single-stranded form adjacent to a specific primer-binding site to which a synthetic primer is annealed.

1.3 ISOLATION OF THE *XENOPUS* TNAP GENE

1.3.1 POSSIBLE STRATEGIES

As mentioned before, there are some tentative data that suggest that the isozyme important in *Xenopus* embryological development is a TNAP type. Until recently, no TNAP genes had been completely sequenced due to their large size. However, there are now several TNAP genes fully characterised and which could be used to probe cDNA libraries constructed from mRNA isolated from *Xenopus laevis* embryo's. There are, of course, several possible strategies that could be employed to isolate the *Xenopus* AP gene:

1) The probing of a library with a cDNA that codes for a previously characterised AP gene from other, possibly from closely related, animals. This strategy is based on the assumption that the homology shared by the AP genes should enable the identification of clones with part or all of the *Xenopus* TNAP sequence, under reasonably stringent conditions.
2) The probing of a library with a chemically synthesised oligonucleotide, based on the homology of previously sequenced

genes. The main disadvantage with this approach is the difficulty in labelling the short probes.

3) The probing of an expression library with an anti-alkaline phosphatase antibody. The success of this approach clearly depends on high expression of the cloned AP gene and that the product is essentially structurally the same as the active AP in *Xenopus*. A positive clone would contain most, if not all, of the cDNA for the AP, whereas in the first two strategies positive clones would not necessarily have full-length cDNA's and further screenings would be necessary.

4) By direct selection of AP activity in an expression library using the AP-specific chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). It is clearly necessary that the cloned AP be expressed and that the product is active.

5) By using the polymerase chain reaction (PCR) to generate a *Xenopus*-specific probe. Primers are needed that can bind to regions flanking part of the *Xenopus* AP gene. Such primers could be synthesised based on highly conserved regions in AP genes and the PCR product would then be highly specific for the *Xenopus* gene.

1.4 AIMS OF THE PROJECT

Previous histochemical studies of *Xenopus* embryos in this laboratory have partly elucidated the spatial distribution of the alkaline phosphatase in the developing embryo. Inhibition studies have indicated that the AP involved is a TNAP which also agrees with the spatial distribution found. These studies have also suggested that the TNAP plays an important role in the embryo which is supported by studies in other animals. The TNAP could be somehow involved in guiding cell movements as well as be an important enzyme in various processes that are essential for normal development of the embryo. Further work is necessary to elucidate the precise functions of AP in these early stages.

Until recently, it has proven difficult to study TNAP at the molecular level due to the lack of suitable probes. Eukaryotic AP gene structure and function has only been studied in the last decade and has only begun to be understood. It is thus now a good time to study the *Xenopus* gene at the molecular level. The molecular cloning of the *Xenopus* TNAP gene is the primary aim of this project. Any other AP genes that could be of interest will also be looked for since the necessary probes are available.

The search will begin with the screening of a cDNA library with TNAP-specific nucleic acid probes. Since AP expression is high during neurulation of the frog embryo (see section 1.1.5), a cDNA library constructed from mRNA from these stages will be screened. Other strategies may be employed if the first approach proves unsuccessful.

Once the TNAP cDNA clone is available, the sequence coding for the TNAP will be determined. The sequence obtained will then be compared to published TNAP sequences of other animals. The degree of homology will be essential in confirming the authenticity of the sequence obtained. Regions important for catalytic activity and sites of possible post-translational processing such as glycosylation will be identified.

The *Xenopus* TNAP cDNA will then be used in probing Southern blots of restricted genomic DNA in order to determine the complexity of the locus or loci, as the case may be. Similarly, Northern blot analysis will also be employed to study the size and expression of the TNAP mRNA.

2 MATERIALS AND METHODS

The composition of all media and solutions used in this work are given in appendix A. All probes used for screening and hybridisation analyses were kindly provided by Dr. José Millán of the La Jolla Cancer Research Foundation. Permission to perform the experiments involving *Xenopus laevis* described here was granted by the Animal Ethics Committee (clearance number 93 73 3).

2.1 THE cDNA LIBRARY

A cDNA library was donated by Dr. Hazel Sive of the Whitehead Institute for Biomedical Research (Cambridge, Massachusetts). The library was constructed from mRNA extracted from pooled *X. laevis* embryos in late neurula/ tailbud stages (stages 19 - 23, Niewkoop & Faber, 1967). Poly $(A)^+$ RNA was isolated and oligo d(T)-primed for first strand synthesis. The first strands were d(G) tailed and then oligo d(C) primed for second strand synthesis. All EcoRI sites were methylated and the methylation was confirmed. EcoRI linkers were attached and after EcoRI digestion, the cDNA fragments were inserted in the EcoRI site of the vector λgt10. The average insert size was found to be 1 kb and there were 4×10^6 independent inserts. The library was prepared in August 1986 and stored in 50% glycerol at -80°C.

2.2 EVALUATION OF THE LIBRARY

The library was titred to ensure that it was suitable for screening (not degraded and with a good number of plaque-forming units). *E. coli* strain C600 (Hfl) (appendix B) was grown in LB broth supplemented with maltose (0.2% final) and $MgSO_4$ (10

mM). A triplicate dilution series of the library was prepared ranging from 10^{-1} to 10^{-10} using salt-maltose (SM) phage buffer. 100 μl of the bacterial culture, in log phase, was added to each dilution and placed, gently shaking, at 37°C, for 20 minutes, to allow the phage to adsorb to the bacteria. Top agarose was melted by autoclaving and maintained at 47°C until needed. After supplementing the top agarose with 10mM $MgSO_4$, 3 ml were added to each adsorbed sample, inverted a few times and poured on prewarmed (37°C) LB agar plates. After 1 hour, when the top agarose set, the plates were incubated at 37°C for 6-8 hours till clear, well-defined plaques could be seen. All the clear plaques could be taken to represent recombinant plaques, since λgt10 is an immunity insertion vector (see appendix B). The number of plaques on each plate was counted where possible, and the number of plaque-forming units (PFU's) per ml of the library was determined.

2.3 PROBE PREPARATION

2.3.1 PROBE ISOLATION

The probes used for the primary screening were the chicken and mouse TNAP cDNA's. Both probes were received in plasmid pBluescript KS^+ (appendix B). The probes were isolated by digesting the plasmids with EcoRI and the digests electrophoresed at 100 V through a 0.67% agarose gel using 1X TBE with 0.5 μg/ml ethidium bromide (EtBr) as running buffer. This was generally how electrophoresis of DNA was performed.

All probe bands were cut out using a sharp razor blade. The small gel pieces carrying the probes were placed in small Beckman polycarbonate centrifuge tubes and spun at 55 000 RPM for 20 minutes at 15°C in a Beckman TL-100 ultracentrifuge. The supernatants carrying the probe DNA were transferred to eppendorfs and then washed once in 1 volume 1:1 phenol:chloroform and then in 1 volume of chloroform. The probes were precipitated with NaCl (0.3 M final) and 2.5 volumes of pure ethanol, and placed at -20°C for 1-2 hours. The pellets were

spun down, washed in 70% ethanol, vacuum dried, and then redissolved in TE to give 10-20 ng/μl solutions in which they were stored at 4°C.

Other probes were also used during screening and hybridisation analyses. These were the rabbit intestinal AP (rIAP), mouse intestinal AP (mIAP) and rabbit embryonic AP (rEAP) cDNA's. These were also provided ligated in pBluescript and were prepared in the same way as the chicken and mouse probes.

2.3.2 RADIOACTIVE LABELLING OF PROBE

Probe labelling was performed using a Random Primed DNA labelling kit (Boehringer Mannheim). Generally, the DNA probe would be radiolabelled with [α-^{32}P]dCTP (Du Pont) to give at least 1 x 10^6 CPM per ml of hybridisation solution. The procedure was as follows. 20-40 ng of the probe DNA was dissolved in 20 μl of TE and denatured by placing in boiling water for 5 minutes, and then transferred directly to ice. The labelling solution was prepared to 20 μl with reaction buffer, which included random sequence hexanucleotides which bind to the denatured probe DNA, and a solution containing a mixture of the deoxynucleotides dATP, dGTP and dTTP. After adding the denatured probe, the [α-^{32}P]dCTP and finally the Klenow enzyme were added. The mixture was incubated at 37°C for 1 hour. Unincorporated nucleotides were separated out using mini-spin column exclusion chromatography on Sephadex G50 (Worthington). The void volume of the labelling reaction mixture after exclusion chromatography, was brought up to 200 μl with TE buffer. To quantify the incorporation of the radioactive nucleotides, 1 μl of the labelled reaction mixture was placed in 2 ml scintillation fluid in scintillation vials, and the Cherenkov radiation measured with a scintillation counter (Beckmann LS 5000 CE). The labelled probe was denatured again by boiling for 10 minutes and immediately transferring to ice. The labelled and denatured probe was then ready for screening or hybridisation.

2.4 LIBRARY SCREENING

2.4.1 PLAQUE TRANSFER TO NYLON MEMBRANES

For screening of the library, an overnight culture of *E. coli*, in LB broth supplemented with maltose and $MgSO_4$ was prepared. For the primary screening, the library (1/10 dilution) was plated on petri dishes with diameter of 14 cm, such that there would be 35 000 PFU per plate. This was to ensure the maximum number of clear, non-confluent plaques on each plate. For each plate, 300 µl bacterial culture was mixed with 3.5 µl 1/10 diluted library phage, incubated at 37°C for 20 minutes for adsorption, mixed with 10 ml top agarose supplemented with 10 mM $MgSO_4$, and evenly poured over the LB agar plate. After setting, plates were incubated at 37°C for ≈ 4 hours to allow for large, clear plaques to grow. Each plate was blotted, in duplicate, onto Biotrans nylon membranes. Each plate was cooled to 4°C for 30 minutes. Each filter was evenly laid on the plate - the first filter was left on for 1 minute, the second for 15 minutes to ensure equivalent blotting. Each filter was marked with three assymetric marks using needle and india ink. This served to identify any positive clones later. After blotting, each filter was placed on Whatmann filter paper soaked with denaturation solution for 5 minutes. After blotting on dry filter paper, the filters were transferred to filter paper soaked with neutralising solution and left for 5 minutes. After air-drying each filter thoroughly, they were fixed by baking in a vacuum oven at 80°C for 1-2 hours.

2.4.2 PROBE HYBRIDISATION

The fixed filters were thoroughly prewetted in 2X SSC. The wet filters were placed in plastic sealable bags and prehybridisation solution added. Prehybridisation solution was always preheated to 56°C to ensure that the herring sperm DNA was denatured. Filters were prehybridised in a gently rotating water bath at 42°C for a minimum of 1 hour.

Labelled probe was mixed with the prehybridisation solution in the plastic bag containing the filters to be screened. After resealing, the filters were incubated at 42°C in a slowly rotating waterbath for 14-18 hours to allow hybridisation to occur.

2.4.3 ISOLATION AND PURIFICATION OF POSITIVES

Probed filters were removed from the hybridisation bags and placed in a tray containing a 2X SSC, 0.1% sodium dodecyl sulphate (SDS) solution for the first low stringency wash. This wash was repeated twice, each time shaking gently for 10 minutes. This was followed with a high stringency wash in 0.2X SSC, 0.1% SDS at room temperature, for 30 minutes. If the filters exhibited a high background signal, as determined with a Geiger counter, another wash was carried out with 0.2X SSC, 0.1% SDS at 47°C for 15-30 minutes. The filters were then placed between two layers of saran wrap and exposed to X-ray film overnight at -70°C. Films were developed in an X-ray developer.

Any dark spots with a slight tail (an artifact caused by the lifting off of the filters from the plates) on the autoradiograph were taken as putative positives. The clones that corresponded with these spots were marked off with pen on the plates. These plaques were then cut out of the agar plates and transferred by means of micro-pipette tips into 1ml SM buffer containing 10 μl chloroform. After vortexing thoroughly for a few seconds, these were left at room temperature, for about an hour, to allow the phage to diffuse into the buffer. These solutions, bearing the phage from the putative positive plaques were then prepared for secondary screening. Each plug generally yielded 10^3 - 10^4 PFU/μl of SM solution. 1:100, 1:1000 and 3:1000 dilutions of each positive plaque were made, to result in 10 - 100 PFU, 1 - 10 PFU and 3 - 30 PFU per plate, respectively. The diluted phage was plated on *E. coli* C600 (Hfl) and the screening process repeated.

Non-confluent, single positive plaques were transferred to SM buffer containing chloroform. Phage from such positives were then amplified, either by the growing

of lysates or by means of the polymerase chain reaction (PCR). This was done so that the DNA inserts from the positive plaques could be sequenced.

2.4.4 STRIPPING OF FILTERS

Filters from the first primary screening were stripped of the mouse and chicken TNAP probes to rescreen with alternative probes, *viz.* rabbit intestinal and rabbit embryonic AP cDNA's. To strip, filters were placed in a tray containing a hot (≈ 85°C) solution of 0.1X SSC, 0.1% SDS. The filters were washed for 15 minutes and exposed overnight to X-ray film, before being reprobed.

2.5 PHAGE DNA EXTRACTION

The method used here is a modified one from Sambrook *et al* (1989). For each positive plaque, six cultures of *E. coli* C600 were prepared. To 50 ml LB broth, 0.2% maltose, 0.2% glucose, 10 mM $MgSO_4$, 100 µl of an overnight culture of *E. coli* C600 was added. Each of the six preparations were given different inoculations of phage, ranging from 0 µl to 250 µl of the SM buffer containing the phage from the positive plaques. This was done to determine which size inoculum gave the maximum amount of lysis (maximum phage growth) after an overnight incubation at 37°C. Two lysates with the most lysis (cell debris/clumps) observed were chosen for phage DNA extraction. To each lysate, 500 µl chloroform was added and placed on a shaker for 30 minutes to release phage from the bacterial cells. All cell debris was spun down at 11 000 G, for 10 minutes at 4°C. The supernatant was carefully removed to a clean beaker. An equal volume of 2 M NaCl, 20% polyethylene glycol (PEG-8000) was added and the mixture allowed to stand on ice for 1 - 2 hours. Precipitated phage was then recovered by a 20 000 G centrifugation for 15 minutes at 4°C. Each pellet was resuspended in 1 ml SM buffer, the tube walls were washed well with the buffer to recover most of the phage. An equal volume of chloroform was added and the mixture vortexed for 30 seconds. The organic phase (bearing the PEG and cell debris) and the aqueous phase (the bacteriophage suspension) were separated by centrifuging at 3 000 G for 15 minutes at 15°C. A

glycerol step gradient was prepared: 750 μl 40% glycerol (in SM buffer) was placed at the bottom of an ultraclear centrifuge tube, and 750 μl 5% glycerol was carefully layered on the first layer. The aqueous supernatant was then layered on top of the 5% glycerol and the tube filled to the top with buffer. This was centrifuged in an SW40 rotor (swing bucket) at 55 000 RPM for one hour at 4°C. The supernatant was discarded, the walls of the tube dried, and the phage pellet resuspended in 50 μl SM and placed at 4°C overnight. Phage DNA was then extracted by phenol/chloroform, chloroform and ethanol washes. The DNA was then redissolved in TE buffer.

An alternative method was used to prepare a large-scale phage lysate. A 250 ml culture was prepared as above, but glucose was excluded. A constant bacterial inoculum size of 10^{10} cells was used. The appropriate volume of a starter culture was added to the 250 ml LB broth as determined by the fact that 1 OD_{600} unit = 8 X 10^8 cells/ml of culture (Sambrook *et al*, 1989). A range of phage inocula was also used, ranging from 5 X 10^6 to 5 X 10^{10} PFU's per 250 ml culture. These inocula were determined from titres determined from lysates prepared before.

2.6 PCR AMPLIFICATION OF POSITIVE INSERTS

Agar plugs carrying a single TNAP positive plaque were placed in 400 μl double-distilled water and vortexed to release the phage into the water. 100 μl of the phage suspension was then treated to release the DNA from the phage particles. The suspension was frozen rapidly to -20°C in a dry-ice-ethanol mixture, for 3 minutes, followed by a 3 minute thaw at 37°C. This freeze-thaw cycle was carried out twice. The released DNA was then denatured by placing in boiling water for 5 minutes and then transferred to ice immediately. The PCR reaction mixture contained 10 mM Tris-HCl (pH, 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin (Clontech), 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 1.0 μM of both the forward and reverse primers (5' and 3' amplimers that bind to sites flanking the cloning sites of λ and face each other from opposite strands), and 0.03 i.u./μl Taq polymerase (Perkin Elmer Cetus). The reaction mixture was layered

over with mineral oil to prevent evaporation, and then incubated in a thermal controller (MJ Research Inc.). There was an initial preparatory cycle consisting of denaturing at 94°C for 30 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 1 minute. This was followed by 30 cycles of denaturing at 94°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 1 minute. The PCR was concluded with a 7 minute chase reaction at 72°C followed by a holding temperature of 4°C. 20 μl of each reaction was loaded on a 1.8% agarose gel and electrophoresed.

When the PCR was completed, 1 volume of chloroform was added to each sample to get rid of the mineral oil. After vortexing briefly and centrifuging in a microfuge, the chloroform/oil sank to the bottom of the eppendorf. The supernatant, carrying the PCR amplified inserts, were transferred to new eppendorfs. To 30 μl of each sample, 6 μl of loading buffer was added. 18 μl of each sample was loaded into 2 adjacent wells on a 1% agarose gel with ethidium bromide and electrophoresed at 100 V for 15 minutes. The bands representing the amplified inserts were cut out and the DNA isolated as described in section 2.3.1. After a phenol/chloroform wash, and precipitation with 0.3 M NaCl/ethanol, the DNA was redissolved in 25 μl of TE. This DNA was then ligated into a phagemid vector for sequencing (2.11.1).

2.7 NEURULA STAGE EMBRYOS

To obtain eggs, female *Xenopus laevis* were injected subcutaneously with 700 IU follicle stimulating hormone (FSH) 3-4 days before the eggs were needed. The day before the eggs were to be laid, 200 IU pregnyl was injected. Eggs were collected by stripping (gentle massaging) and then placed in full-strength Modified Barth's Saline (MBS) for an hour. A testis was removed from a male frog which was sacrificed by a lethal injection of sodium pentobarbitone (0.08 ml per 200 g frog). The second testis was stored at 4°C in MBS for later use. The testis was macerated in MBS. After draining of the MBS solution from the eggs, the macerated testis was pipetted over the eggs and 1/10 MBS added to activate the sperm cells and to

allow fertilisation of the oocytes (left for 15 minutes). Fertilised eggs were then left at room temperature to develop and the stages determined microscopically as described by Niewkoop and Faber (1967). After the first few divisions, the eggs were dejellied by treatment with a 2.5% cysteine-HCl solution (pH 7.8 - 8.0). After washing thoroughly with 1/10 MBS, the embryos were left to develop further to the required stages. With the aid of a dissecting microscope (Wild Heerbrugg) and watchmakers forceps, embryos in stages 17 to 21, *i.e.* middle to late neurula (Niewkoop & Faber, 1967), were isolated and placed in a Petri dish filled with MBS.

2.8 RNA EXTRACTION

All glassware was rendered ribonuclease free by overnight treatment at 180°C. Sterile, disposable plasticware was used wherever possible. All solutions, except Tris-containing solutions, were treated with 0.1% diethyl pyrocarbonate (DEPC) overnight at 37°C and then autoclaved for 15 minutes to remove all the DEPC. All water used was double-distilled and DEPC treated. Gloves were worn throughout the work and changed frequently.

2.8.1 TOTAL RNA EXTRACTION

RNA was extracted from *Xenopus laevis* embryos in the neurula stage for Northern blot analysis using the TNAP cDNA as probe that would be isolated. In each extraction, approximately 5 ml of packed embryo was placed in a Dounce homogeniser and the MBS replaced with 10X volume of solution D. After homogenisation, proteins, lipids and large DNA molecules were precipitated with 1/10 volume 2 M sodium acetate (pH 4). 1 volume phenol (containing 0.1% hydroxyquinoline) and 1/5 volume chloroform, were added and mixed by vigorous shaking. After leaving on ice for 15 minutes, the aqueous and organic phases were separated by centrifugation at 10 000 G for 20 minutes at 4°C using a Sorvall RC5 centrifuge. All the precipitate formed a stringy white mass in the organic phase and

interface. The aqueous phase was isolated and 2 volumes 100% ethanol added. The total RNA was then allowed to precipitate at -20°C overnight.

The precipitated RNA was spun down at 10 000 G for 30 minutes at 4°C. The supernatant was discarded and the pellet blot-dried. The total RNA was redissolved in 250 μl 1 mM EDTA. The concentration of RNA was determined spectrophotometrically using a Beckman DU-64 spectrophotometer. The concentration was determined based on the fact that for RNA 1 OD_{260} unit = 40 μg. ml^{-1}.

2.8.2 SELECTION OF POLY(A)$^+$ RNA

mRNA was separated from some of the total RNA samples by affinity chromatography on oligo d(T)-cellulose. The RNA was heated to 65°C for 10 minutes and then placed on ice for 5 minutes. 0.5 g dry oligo d(T)-cellulose powder was used for each sample. The cellulose was equilibrated with 2 ml loading buffer in a Correx tube. 2 ml of denatured RNA was added to the equilibrated cellulose. Poly(A)$^-$ RNA was isolated by washing with binding buffer twice. After each wash and centrifugation at 1 500 G for 4 minutes at 15°C, the supernatant was collected. The poly(A)$^-$ RNA was precipitated with cold ethanol and stored at -20°C.

The cellulose beads were then washed four times with 5 ml binding buffer and once with 5 ml middle binding buffer to elute any more contaminating poly(A)$^-$ RNA. The supernatant was discarded after each wash. The poly(A)$^+$ RNA was then eluted from the cellulose beads by washing with 5 ml elution buffer. After centrifugation, the mRNA was precipitated by adding $^1/_{10}$ volume 3 M sodium acetate and 2.5 volumes ethanol and stored at -20°C.

Precipitated poly(A)$^+$ RNA was spun down at 10 000 G for 30 minutes at 4°C and redissolved in 250 μl water. After a further spin to get rid of any residual cellulose beads, the concentration of the mRNA was determined spectrophotometrically.

2.9 DNA EXTRACTION

DNA was extracted from *Xenopus laevis* for Southern blot analysis. The protocol used was modified from Müllenbach *et al* (1989). Blood was collected from sacrificed male frogs and centrifuged briefly in a microfuge. Red blood cells were washed in 6 ml phosphate buffered saline (PBS), pelleted and lysed by resuspending in 12 ml SE buffer. Proteinase K was added to a final concentration of 200 $\mu g.ml^{-1}$, and SDS was added to 1%. The solution was incubated at 55°C for 1 hour. After complete digestion, a prewarmed saturated NaCl solution (6 M) was added and gently mixed to a final concentration of 1.5 M. Proteins were then extracted by adding 1 volume of chloroform and mixing for 1 hour by gentle rotation. After a 10 minute centrifugation at 2 000 RPM, the aqueous phase was transferred to a fresh, sterile tube and mixed with 1 volume of a solution of phenol:chloroform:isoamylalcohol (24:24:1). After another spin, the aqueous phase was washed with 1 volume of chloroform and then the DNA was precipitated on a glass rod in 2.5 volumes of pure ethanol. The DNA was vacuum dried and redissolved in TE. The purity and yield of DNA was determined spectrophotometrically (for DNA, 1 OD_{260} unit = 50 $\mu g.\ ml^{-1}$).

2.10 HYBRIDISATION ANALYSIS

2.10.1 RESTRICTION OF GENOMIC DNA

The eight restriction endonucleases chosen to digest *Xenopus* genomic DNA were BamH I, EcoR I, Hind III, Pst I, Pvu II, Sac I, Xba I and Xho I. Each digest was performed on 10 μg DNA in a 100 μl volume, including the DNA dissolved in TE, 100 U of restriction enzyme, and appropriate enzyme buffer. The volume was made up to 100 μl with distilled water. Digestion was carried out at the optimum temperature for each enzyme overnight.

2.10.2 AGAROSE GEL ELECTROPHORESIS OF DNA

A 0.8% gel was prepared in 1X TBE buffer and 0.5 μg/ml ethidium bromide. The gel could be stored, at room temperature, under TBE, until ready for electrophoresis.

Before electrophoresis, each DNA digest was precipitated, washed and redissolved in 25 μl TE. 5 μl loading buffer was added and then each sample loaded into adjacent wells of the gel. Lambda molecular weight marker II (Boehringer Mannheim) was also loaded into remaining wells. The DNA was electrophoresed at 100 V in 1X TBE running buffer containing 0.5 μg/ml ethidium bromide. When the dye front reached a little more than half way down the gel, the power supply was switched off and the gel examined under UV transillumination and the completeness of the digestion ascertained. The gel was then photographed.

2.10.3 SOUTHERN BLOTTING

Blotting of the electrophoresed DNA was carried out by the capillary transfer method as described by Southern (1975). After electrophoresis, the gel was acid treated in 0.25 M HCl for 15 minutes on an orbital shaker. After rinsing with distilled water, the gel was placed in a high salt denaturing solution shaking for 1 hour. A sheet of Whatman 3MM paper was cut to size and placed over a glass plate with the ends of the paper immersed in a glass dish with 20X SSC. Any air bubbles trapped between the wet paper (wick) and plate were removed by rolling a glass rod over the wet paper. The gel was placed upside down onto the wick and again all air bubbles removed. A sheet of "Hybond" nylon membrane (Amersham), slightly larger than the dimensions of the gel, was immersed in 20X SSC for 5 minutes. The nylon filter was placed on top of the gel, again all air bubbles smoothed out. Two sheets of 3MM paper, the same size as the gel, were soaked in 20X SSC and layered onto the nylon, air bubbles eliminated. Three additional dry sheets of 3MM paper were placed on top. Finally, a stack of paper towels (about 8 cm high) was placed on the 3MM paper. A glass plate was placed on top of the stack and

weighed down with a 500 g weight. Short-circuiting was prevented by wrapping parafilm between the nylon and wick. Blotting was carried out for 1 to 3 days. The paper towels and 3MM paper were then discarded and the positions of the wells marked on the nylon filter with pen. The filter was placed between two sheets of Whatman paper and baked for 2 hours at 80°C in a vacuum oven. UV transillumination of the gel indicated the efficiency of transfer.

2.10.4 FORMALDEHYDE GEL ELECTROPHORESIS OF RNA

A 1% agarose gel was prepared in a fume hood. 1 g agarose was dissolved in 74 ml of RNase-free water by boiling in a microwave oven and then cooled to 65°C. 10 ml 10X MOPS, pH 7.0 and 16 ml filtered, deionised 37% formaldehyde were added. The formaldehyde was deionised by adding 50 ml formaldehyde to 5 g of a mixed-bed resin (Bio-Rad AG 501-X8) and stirred gently overnight at 4°C. After filtering twice through Whatman No. 1 filter paper, the formaldehyde was aliquoted and stored at -20°C. The final concentration of the formaldehyde in the gel was about 6%. The gel solution was mixed thoroughly and poured into the gel apparatus that was pretreated with 30% H_2O_2 for 30 minutes and then thoroughly rinsed with DEPC-treated water and finally with 1X MOPS. The slot former was inserted and the gel allowed to set.

RNA samples containing 20 μg and 10 μg (1:1 dilution) of total RNA were prepared for loading. To 12 μl of diluted or undiluted sample was added 5 μl 10X MOPS, 8 μl 37% formaldehyde and 25 μl formamide (deionised as for the formaldehyde). After heating the RNA samples to 65°C for 5 minutes and then rapidly cooled on ice to prevent renaturation of the RNA, 5 μl of sterile loading buffer was added to each sample and loaded onto the gel. The gel was run with 1X MOPS buffer. The level of the running buffer was just below that of the top of the gel. About 20 μl of 1X MOPS was added to each well before loading the samples to facilitate an even distribution of the samples in each well. The RNA was electrophoresed at 80 V for 4 hours till the dye-front was near the far edge of the gel. The running buffer was replaced with fresh buffer every hour to avoid

depletion of electrolytes. In some cases, EtBr was added to the running buffer and to the gel before it set to enable visualisation of the RNA.

2.10.5 STAINING OF FORMALDEHYDE GELS FOR RNA

If ethidium bromide was not included in the running buffer and gel solutions, the gels were stained with EtBr to visualise the electrophoresed RNA. After electrophoresis was complete, the gels were washed in 4 - 5 changes of water for 2 hours. The gels were then washed in 2 changes of 0.1 M ammonium acetate for 30 minutes each. Each gel was placed in a 0.005 mg/ml solution of EtBr made up in 0.1 M ammonium acetate. Each gel was stained for 1 hour. The gels were then destained with 0.1 M ammonium acetate for 45 minutes and the stained RNA visualised by UV transillumination.

2.10.6 NORTHERN BLOTTING

Northern blotting was employed to transfer the RNA from the formaldehyde-agarose gel to a nylon filter. After soaking the gel for 5 minutes in several changes of DEPC-treated water to wash away the formaldehyde and formamide, the gel was placed in 10X RNase-free SSC for 1 hour with gentle shaking. The Northern apparatus was set up as for the Southern blotting procedure (2.10.3). The RNA was blotted onto the nylon filter overnight using 10X RNase-free SSC as transfer buffer. The nylon filter was then removed and air-dried for an hour and then baked at 80°C for 3 hours. Alternatively, the wet nylon filter was fixed by means of a UV cross-linker.

2.10.6.1 RNA Alkali Blotting

An alternative, much more rapid procedure for Northern blotting of RNA was also employed. In this procedure, the blotting apparatus is the same as for the above method. However, the transfer buffer used is 0.05 M NaOH. The transfer time is then cut down, from 24 hours to 2 - 3 hours, and the blot sensitivity is much

higher. There is also no need to fix the RNA after alkali blotting. The nylon membrane was simply removed from the blotting apparatus and rinsed briefly in 2X SSPE buffer. It was then ready for hybridisation.

2.10.7 METHYLENE BLUE STAINING OF THE NORTHERN BLOTS

Two identical Northern blots, under identical conditions were prepared. One blot was hybridised to TNAP cDNA probe and the other was stained to determine the quality of the RNA and to locate the internal markers (rRNA).

For methylene blue staining, the dried and fixed nylon filter was soaked in 5% acetic acid for 15 minutes at room temperature. The filter was then transferred to the staining solution and left, with occasional shaking, for 5 minutes at room temperature. The stained filter was rinsed in water several times and air-dried on 3MM Whatman paper.

2.10.8 HYBRIDISATION OF PROBE

The baked membranes were thoroughly prewetted in 2X SSC (Southerns) or 6X SSC (Northerns). The blots were then transferred to plastic heat-sealable bags. Prehybridisation solution containing denatured, sonicated herring sperm DNA was added to the bags and the filters prehybridised at 42°C for 3 to 4 hours. Labelled probe was then prepared as described in section 2.3.2. In the case of Southern blots, denatured, labelled probe was added directly to the prehybridisation solution. For Northern blots, the prehybridisation solution was drained out of the bag and replaced with fresh hybridisation solution containing the labelled probe. For both Southerns and Northerns, probe was added such that there was always at least 2 X 10^6 CPM/ml hybridisation solution. Hybridisation was carried out at 42°C, gently shaking overnight. Both Northern and Southern blots were prepared to be probed with *Xenopus* specific probe. Southern blots were probed with a mouse intestinal AP (mIAP) cDNA to also elucidate the TSAP locus in *Xenopus*.

After hybridisation, Southern blots were washed at room temperature as follows:

2X SSC, 0.1% SDS for 10 minutes
2X SSC, 0.1% SDS for 10 minutes
0.2X SSC, 0.1% SDS for 30 minutes.

If there was a high background signal then the final wash was repeated at 45°C.

Northern blots were washed, mildly shaking, as follows:

2X SSPE, 0.1% SDS for 5 minutes at room temperature
2X SSPE, 0.1% SDS for 5 minutes at room temperature
2X SSPE, 0.1% SDS for 5 minutes at room temperature
0.1% SSPE, 0.1% SDS for 30 minutes at 45°C
0.1% SSPE, 0.1% SDS for 30 minutes at 45°C

The washed blots were placed between two sheets of saran wrap. In a darkroom, the blots were placed into an X-ray film cassette and covered with a sheet of X-ray film. Exposure was carried out overnight at -70°C. The film was developed in a developing machine.

2.10.8.1 Stripping of Southern Blots

Southern blots were stripped to be rehybridised with alternative probes. Stripping was carried out by placing the blots in a solution of 0.1X SSC, 0.1% SDS and incubated at 56°C for 3 hours. The blots were then exposed overnight (or for the same length of time that they were exposed with hybridised probe) to assess the efficiency of the stripping.

2.11 GENE SEQUENCING

The insert that was isolated from the *Xenopus* cDNA library using TNAP probes was amplified by the PCR (2.6). The fragment obtained, which represents part of the *Xenopus* TNAP gene, was then sequenced. To sequence, the amplified fragments were cloned into a Bluescript phagemid (Stratagene). The fragments produced by the PCR were clearly blunt-ended fragments and the oligonucleotide primers used in the PCR did not include any restriction endonuclease sites, *i.e.* cleavage of the PCR product could not be used to create any appropriate cohesive ends to facilitate ligation into an equivalently cut vector. As explained in the discussion, the vector used to clone the blunt-ended fragments needed to be treated as described in section 2.11.1.1. The fragments were then cloned and sequenced.

2.11.1 PCR FRAGMENT CLONING

2.11.1.1 T-vector Construction

The vector used was the phagemid pBluescript II KS^+. The vector was cleaved in the EcoR V site in the polylinker. The cleaved vector was then incubated with Taq polymerase (1 unit/μg phagemid/20 μl volume), reaction buffer, and 2 mM dTTP for 2 hours at 70°C. A single thymidine was thus added to the 3' ends of each fragment. After phenol extraction and precipitation, the "T-vector" was ready for cloning the PCR product. The method used here is from Marchuk *et al* (1991).

2.11.1.2 PCR Fragment Ligation

The PCR fragment was prepared as described in section 2.6. An excess of insert (7 μl of 0.5 μg/μl prep) was incubated with 1 μl (1 μg) of T-vector in the presence of T4 DNA ligase (1 unit/μg T-vector/10 μl volume) in T4 DNA ligase buffer diluted to 1X concentration. Ligation was carried out at 16°C in a 24 hour incubation. Appropriate controls were also set up. In one tube no insert was added (tests efficiency of ligation). In another tube, insert and ligase were excluded to see

if the vector was cleaved completely and if there was any residual uncut vector. In the last control, vector was excluded to verify that the insert alone was not contaminated with any vector DNA.

2.11.1.3 Transformation of Competent Cells

The recombinant phagemids prepared above were transformed into a bacterial host for amplification. High efficiency competent *Epicurian coli* strain XL 1-Blue was obtained from Stratagene as frozen cells (appendix B). For each transformation, 150 μl of competent cells was thawed on ice and added to each ligation tube. This was mixed by a brief and gentle shake and then kept on ice for 1 hour. At this time, agar plates were prepared for plating of transformed cells. For each experiment, 40 μl of a 2% solution of X-gal and 4 μl of a 1 M solution of IPTG were spread on an agar plate containing 100 μg/ml ampicillin. After an hour on ice, the cells with the ligates were heat-shocked (42°C, 1 minute), placed on ice for 1 minute and then plated directly on the prepared agar plates. These were incubated overnight at 37°C. Colonies of cells transformed with non-recombinant phagemid were differentiated from those with recombinant phagemid by their colour. The plates were incubated at 4°C for an hour to pronounce the colour of the colonies.

2.11.2 SMALL-SCALE PLASMID PREPARATION

A small-scale isolation of recombinant phagemid was carried out to determine whether the cloned phagemid indeed carried the insert that was amplified by the PCR. The procedure used was simple and rapid and was carried out as described by He *et al* (1990). Single white colonies were picked from the plates prepared as above and each used to inoculate 2 ml of LB broth containing 100 μg/ml ampicillin (to maintain the phagemid) and the cultures grown overnight at 37°C. Uninoculated controls were used to ensure no contamination with other bacteria, which would complicate the plasmid isolation procedure. 1.5 ml of each culture (the 0.5 ml remaining to be used in large scale preparations) was transferred to eppendorf tubes and the cells harvested by centrifugation at 10 000 G for 0.5 minutes. The cells

were resuspended in 100 µl lysis solution containing LiCl and Triton X-100. An equal volume of a phenol/chloroform mixture (1:1 (w/v)) was added, followed by vigorous vortexing for 15 seconds. The mixture was centrifuged for 1 minute at 15 000 G at 22°C. The upper phase of nucleic acids was collected, mixed with 2 volumes of absolute ethanol and the precipitated nucleic acids harvested by centrifugation at 15 000 G for 10 minutes at 4°C. The pellet was washed in 1 ml of absolute ethanol, vacuum-dried for 5 minutes, and the DNA dissolved in 50 µl TE containing 20 µg/ml RNase. 17 µl of each dissolved sample was incubated for 1 hour with EcoR I (10 units in 20 µl volume) to cleave out the PCR insert. 5 µl of 5X loading buffer was then added and then loaded onto a 1% gel for electrophoresis.

2.11.3 LARGE-SCALE PLASMID PREPARATION

The cultures that gave the highest yield of amplified fragment in the small-scale plasmid isolation, were grown into large-scale cultures. The 0.5 ml cultures left over from the small-scale preps (see above) were used to inoculate 250 ml LB broth containing 100 µg/ml ampicillin. These cultures were grown overnight. The procedure followed for the large-scale plasmid prep was modified from Sambrook *et al* (1989).

Each 250 ml culture was poured into a Sepcor bottle and the cells harvested by centrifugation for 8 minutes at 6 000 G (GSA rotor, Du Pont Sorvall RC-50). Each pellet of cells was resuspended in 8 ml of solution I, and 1 ml of a freshly prepared 50 mg/ml solution of lysozyme added. Each suspension was incubated at room temperature for 5 minutes. 18 ml of freshly prepared solution II was then added. After mixing by gently inverting each tube several times, they were incubated on ice for 10 minutes. 13.5 ml of cold solution III was added and the mixtures incubated on ice for a further 10 minutes. Each sample was transferred to clean Nalgene plastic centrifuge tubes and centrifuged at 4°C for 10 minutes at 20 000 G (SS 34 rotor). The supernatants, containing nucleic acids, were each transferred to new tubes. The nucleic acid was precipitated with 0.6 volume of isopropanol and

harvested by centrifugation at 10 000 G for 5 minutes. Each pellet was air dried and resuspended in 3 ml TE.

The phagemid was then isolated by isopycnic centrifugation in a CsCl-ethidium bromide gradient. Each 3 ml solution was transferred to clean 15 ml polypropylene tubes. 3.15 g CsCl was added to each sample. 400 μl of a 10 mg/ml stock solution of EtBr was also added. After balancing the tubes with TE, the samples were centrifuged at 15 000 G for 5 minutes. The supernatants were then transferred to polyallomer, bell-top, quick-seal centrifuge tubes (Beckmann) by means of pasteur pipettes. The filled tubes were balanced and sealed with a heat sealer. The tubes were placed in a fixed-angled rotor and ultracentifuged at 100 000 G at 15°C overnight.

Phagemid bands were extracted using a needle and syringe. The top of each tube was first punctured. A needle was inserted 4 mm below the phagemid band and the band was carefully sucked out. 1 volume of butanol was added, vortexed and the sample centrifuged. The phase bearing the butanol and EtBr mix was removed and the procedure repeated till all EtBr was extracted from the phagemid. Since this butanol extraction also removed water from the phagemid layer, distilled water was added to the original level to prevent the CsCl from precipitating out of solution. The phagemid was precipitated with 3 volumes 70% ethanol and harvested by a brief spin. After a wash with 70% ethanol, the phagemid was redissolved in 100 - 200 μl of TE.

2.11.4 SEQUENCING OF CLONED INSERT

DNA sequences were determined by the Sanger dideoxy chain-termination method (Sanger *et al*, 1977). All sequencing reactions were performed in 1.5 ml plastic centrifuge tubes and these were kept capped to minimize evaporation of the small volumes. All solutions were thoroughly mixed after each addition, typically by "pumping" the solution 2 or 3 times with the micropipette, avoiding the generation of bubbles. Brief spins were employed to prevent solution clinging to the walls of

the tubes. All materials used were from United States Biochemical unless otherwise mentioned.

2.11.4.1 Set-up of Gel Apparatus

The plates used to make the sequencing gels were thoroughly cleaned by wiping with ethanol and rinsing with deionised, distilled water. Plates were dried with lint-free tissues. One plate was siliconised by wiping with a 2% solution of dimethyldichlorosilane (in 1,1,1-trichloroethane). The plates were sealed together with 0.4 mm spacers using masking tape and fold-back spring clamps. The gel solution was prepared by adding 700 μl (10% (w/v)) ammonium persulphate and 35 μl TEMED (NNN'N'-tetramethylethylenediamine) to 70 ml of the acrylamide solution. After thorough mixing, the gel solution was poured into the plates. The flat end of a comb of sharp, V-shaped teeth was placed along the top of the gel. The gel was allowed to set in 20 minutes. Once set, the comb was turned around and inserted teeth-first into the top of the gel to form well-defined and uniform wells in the gel. The plates containing the set gel were clamped onto the electrophoresis apparatus, the chambers of the apparatus filled with running buffer (1X TBE), and the gel pre-electrophoresed at 60 Watts (W) for 15 minutes.

2.11.4.2 Annealing Template and Primer

For each set of four termination reactions, a single annealing (and subsequent labelling) reaction was set up. The annealing reaction was carried out in a final volume of 10 μl. 1 μl of primer (0.5 pmol) was mixed with 2 μl of (5X) annealing buffer and 7 μl (3 - 5 μg, slight excess) of denatured phagemid DNA. The double-stranded phagemid DNA was denatured by boiling for 5 minutes and transferring immediately to ice. After mixing thoroughly, the primer was annealed to the phagemid by incubating at room temperature for 5 minutes. The primers used were the universal KS, SK, T3, and T7 17-mer primers (Stratagene). Also used were the 5' and 3' amplimers that bind to the Lambda sequences flanking the insert. Finally, after preliminary sequencing, 18-mer oligonucleotides were custom-synthesised on

an Applied Biosystems DNA synthesizer. These were designed to bind to internal sites of the insert to facilitate sequencing of the whole insert. The one primer, called KS1 had the sequence

5'> ACCCAGGAACATGATGAC <3'

The second primer, used to sequence the second strand, was called SK1 and had the sequence

5'> AGGCCCAGGAGACGCTGA <3'

2.11.4.3 The Labelling Reactions

Sequenase version 2.0, and [α-^{35}S]dATP as tracer (Biggin *et al*, 1983) were used for sequencing. Two types of reactions were set up. Long reactions were set to read up to 5( )ses from the primer. Short reactions were set to accurately read the sequence within 30 bases of the primer. To the annealed template-primer the following were added:

For short reactions,

Dithiothreitol (DTT) (0.1 M)	1 μl
1:5 dil. of Labelling Nucleotide Mix	2 μl
[α-^{35}S]dATP (10 μCi/μl)	0.5 μl
Sequenase (1:8 dilution, 3 units/2 μl)	2 μl

For long reactions,

Dithiothreitol (DTT) (0.1 M)	1 μl
1:1 dil. of Labelling Nucleotide Mix	2 μl
[α-^{35}S]dATP (10 μCi/μl)	1 μl
Sequenase (1:8 dilution, 3 units/2 μl)	2 μl

Note that the above refer to the volumes added to each primer-annealed reaction. Usually, a pre-mix "cocktail" was freshly prepared with undiluted components and the appropriate amount of deionised, distilled water added to effect the correct dilutions. Sequenase was always added last. The right amount (5.5 μl) of "cocktail"

was then added to each annealed primer-template. For short reactions, the labelling reaction was incubated at room temperature for 3 - 4 minutes. For long reactions, labelling reaction was incubated at room temperature for 5 minutes.

2.11.4.4 The Termination Reactions

Termination reactions were carried out in microtitre plates (Corning) with wells labelled alphabetically in rows, and numerically in columns. One row was used for each primer-template reaction. Columns were designated a termination reaction, in the order A, C, G, T. In the wells of column A, 2.5 μl of ddATP was added; in column C, 2.5 μl of ddCTP; in column G, 2.5 μl of ddGTP; in column T, 2.5 μl of ddTTP. The tray was then warmed to 37°C for 1 minute. When the labelling reaction was complete, 3.5 μl of each labelled sample was added to the wells of the appropriate row in the microtitre plate. After mixing, the plate was incubated at 37°C for 5 minutes. 4 μl of stop solution was then added to each well, mixed thoroughly and stored, covered on ice till ready to load onto the sequencing gel.

2.11.4.5 Denaturing Gel Electrophoresis

Before loading, the microtitre plate with all the samples was placed in a tray of hot water (85°C) for 2 minutes to denature the DNA fragments. After flushing each well in the gel with the running buffer with a micropipette to remove unpolymerised acrylamide and urea, 2.5 μl of each sample were loaded into adjacent wells in the same order as in the microtitre plate. Gels were electrophoresed at a constant power of 60 W. Gels with the short reactions were electrophoresed for 2 - 3 hours. To read the maximum possible sequence from long gels, a batch of all reactions was run for 2 hours, and then a second batch loaded and electrophoresed a further 5 - 6 hours.

After electrophoresis, one glass plate was lifted off the gel by means of a spatula. The gel, remaining on the unsiliconised glass plate, was placed in fixing solution for 15 minutes. After fixing, the glass plate with gel was placed onto a sheet of

Whatman 3MM filter paper with gel facing up. A similar piece of paper was placed evenly over the gel and peeled off the glass plate. The gel was covered with a sheet of saran wrap and dried in a vacuum-drier at 80°C for 1 hour. The dried gel on the filter paper was placed in an X-ray cassette and exposed to X-ray film (Kodak XAR-5) overnight at room temperature before developing.

2.11.4.6 Reading the DNA Sequence

The sequences were read directly from the developed autoradiographs on a light box. The sequences for both strands were determined independently and in several repeated sequencing procedures, thereby confirming the accuracy of the sequence with no ambiguities. The procedure was repeated twice from the PCR stage, to eliminate errors introduced during PCR. The sequence so assembled was analysed using IBI's MacVector sequence analysis computer programme.

3 RESULTS

3.1 THE cDNA LIBRARY TITRE

The number of plaques was countable on the $1/10^4$ and $1/10^5$ dilution plates. The values obtained are shown in Table 3.1. The number obtained for each triplicate was averaged. The titre for the λgt10 *Xenopus* cDNA library was found to be 1.24 X 10^8 PFU/ml.

TABLE 3.1 DETERMINATION OF LIBRARY TITRE

DILUTION	NUMBER OF PLAQUES 1	NUMBER OF PLAQUES 2	NUMBER OF PLAQUES 3	AVERAGE
10^{-1}	TMTC**	TMTC	TMTC	
10^{-2}	TMTC	TMTC	TMTC	
10^{-3}	TMTC	TMTC	TMTC	
10^{-4}	118	123	134	125
10^{-5}	12	12	13	12.3
10^{-6}	3	0	1	1.3

**TMTC: Too many to count

3.2 SCREENING OF THE LIBRARY

3.2.1 SUITABILITY OF THE CHICKEN AND MOUSE TNAP PROBES

After the mouse and chicken TNAP cDNA's were excised from pBluescript KS^+,

the probes were separated from the vector by gel electrophoresis (figure 3.1 A, B). The probe bands were cut out and the cDNA eluted. Approximately 100 ng of each probe was obtained, dissolved in 100 μl TE buffer. Although the probe bands were close to the vector, the probes could be recovered with no contamination with vector (figure 3.1 C).

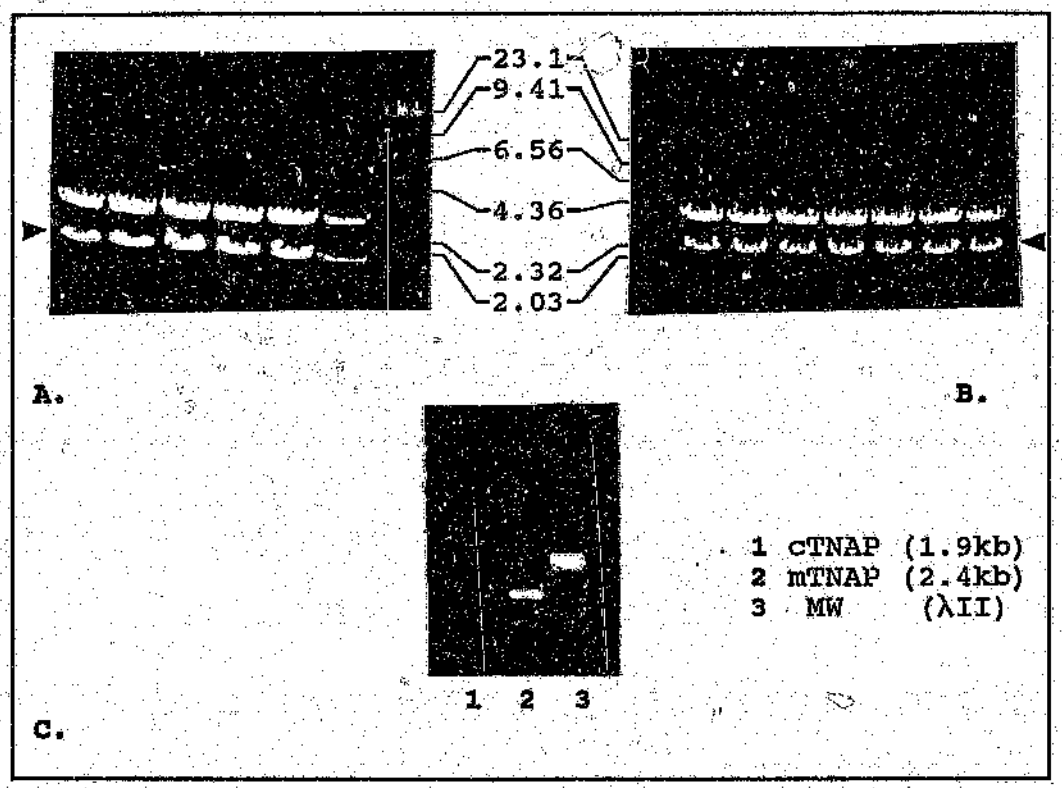


Figure 3.1 Isolation of the chicken and mouse TNAP cDNA probes from the vector pBluescript II KS$^+$. (A) The chicken TNAP and (B) The mouse TNAP cDNA excised from Bluescript with EcoRI. Molecular weight marker (λII) band sizes are indicated in kb pairs in the center. All 6 lanes on either side of the marker contain equivalent samples of EcoRI digested vector. Arrowheads indicate the probe bands. (C) Small aliquots of the probe preps were electrophoresed to assess their quality. The chicken sample (cTNAP) was diluted 1/50.

The probes were generally efficiently labelled. Comparing the radioactivity present before and after removal of unincorporated nucleotides, showed that there was always 75% - 90% incorporation of radioactive nucleotides. A count exceeding 2 X 10^6 CPM per 1 ml of hybridisation solution was always achieved and was found to give good signals during screening and hybridisation.

3.2.2 PRIMARY SCREENING OF THE LIBRARY

After the first screening with mouse and chicken TNAP probes, a very low number of plaques with a positive signal was obtained (figure 3.2). Only one positive plaque was common to both the mouse- and chicken- probed filters (found by aligning marks made on the duplicate filters when blotting the plaques).

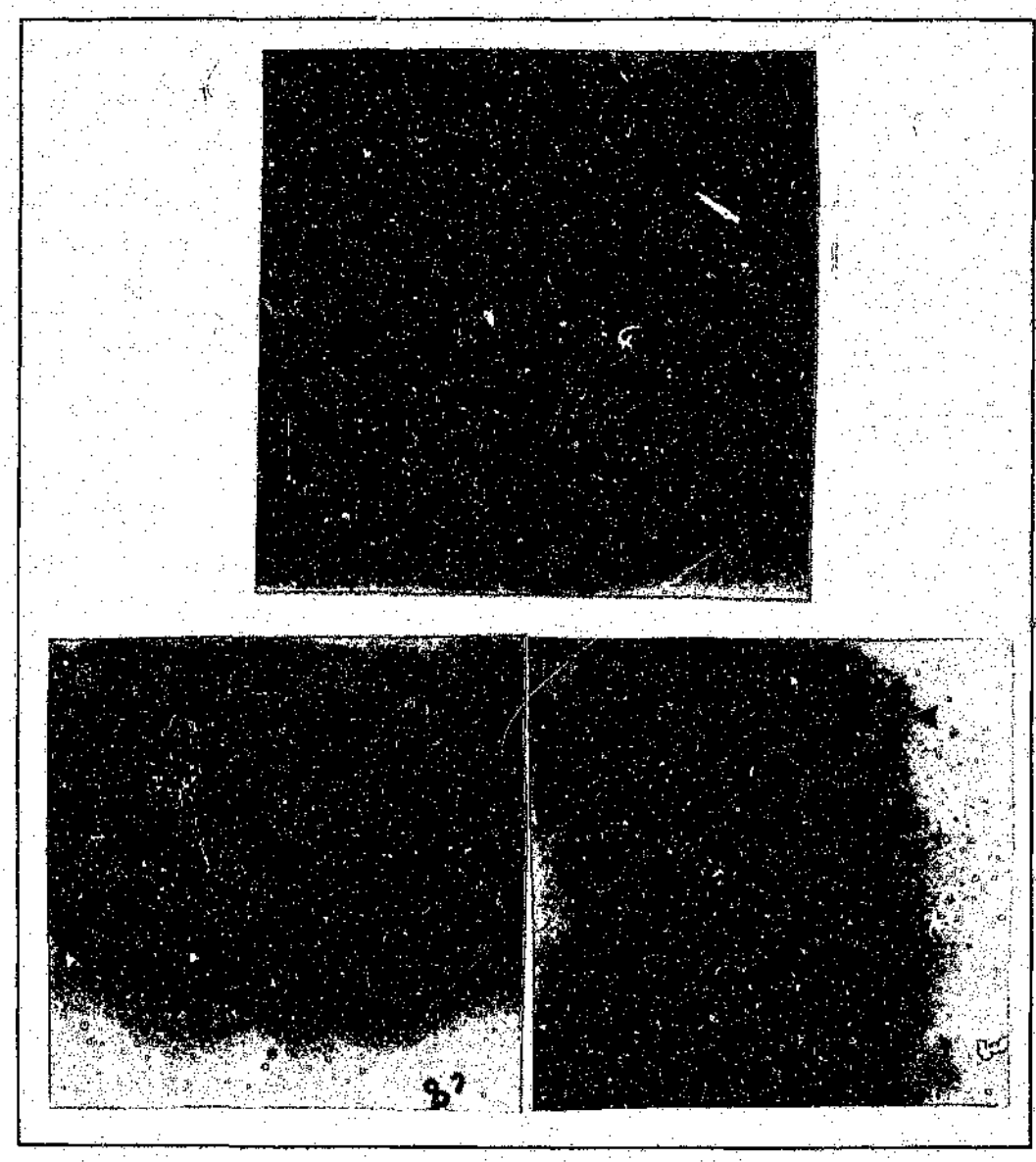


Figure 3.2 Autoradiographs of filters from a primary screening of the library. Filters were probed with labelled chicken (C) or mouse (M) TNAP. Positive plaques are clearly seen as dark spots and are ringed. The arrows indicate the 2 spots that represent the identical plaque on duplicate filters that hybridised both the mouse and chicken probes. Other spots on the filter probed with mouse TNAP are artefacts (are "perfectly round" compared to the "true" positives which possess a light "tail").

3.2.3 SECONDARY SCREENING OF THE LIBRARY

The plaques on the 1/100 dilution plates were generally much too confluent for screening (due to interphage recombination events) and thus only 1/1000 and 3/1000 plates were blotted for screening. Autoradiographs of the secondary screening filters typically had many positives (figure 3.3).

Not all putative positive plaques that were selected in the primary round of library screening turned out positive in the following round indicating that false positives were also selected. The plates that bore positive plaques in the second round were still too confluent to allow the picking of the plaques with no contamination with surrounding phage, and thus a third round of screening was often necessary. In the third round of screening, only the chicken TNAP probe was used for screening since at this stage all positives hybridised both the mouse and chicken probes. However, it was found that the chicken probe gave a stronger signal, either to it being more efficiently labelled, or due to stronger hybridisation due to a higher homology with the chicken TNAP probe. After the tertiary screening, positive plaques were clearly identified and could be isolated as single, non-confluent plaques with no contamination with other plaques. Only 1 single positive plaque was obtained in the first round of screening of the library.

3.3 AMPLIFICATION OF POSITIVE INSERTS

3.3.1 AMPLIFICATION BY PHAGE LYSATE PREPARATION

Considerable difficulty was encountered in the amplification and extraction of phage DNA. During the DNA extraction step (section 2.5) precipitation of phage particles from the phage lysate failed to occur - even after an overnight incubation of the lysate supernatant with the NaCl/PEG solution at 4°C. The method was modified in several ways, such as excluding glucose from the culture medium (glucose suppresses *lamB* which codes for the receptor to which λ binds (Sambrook *et al*, 1989)), but extraction of phage DNA could not be acheived. Thus, insufficient

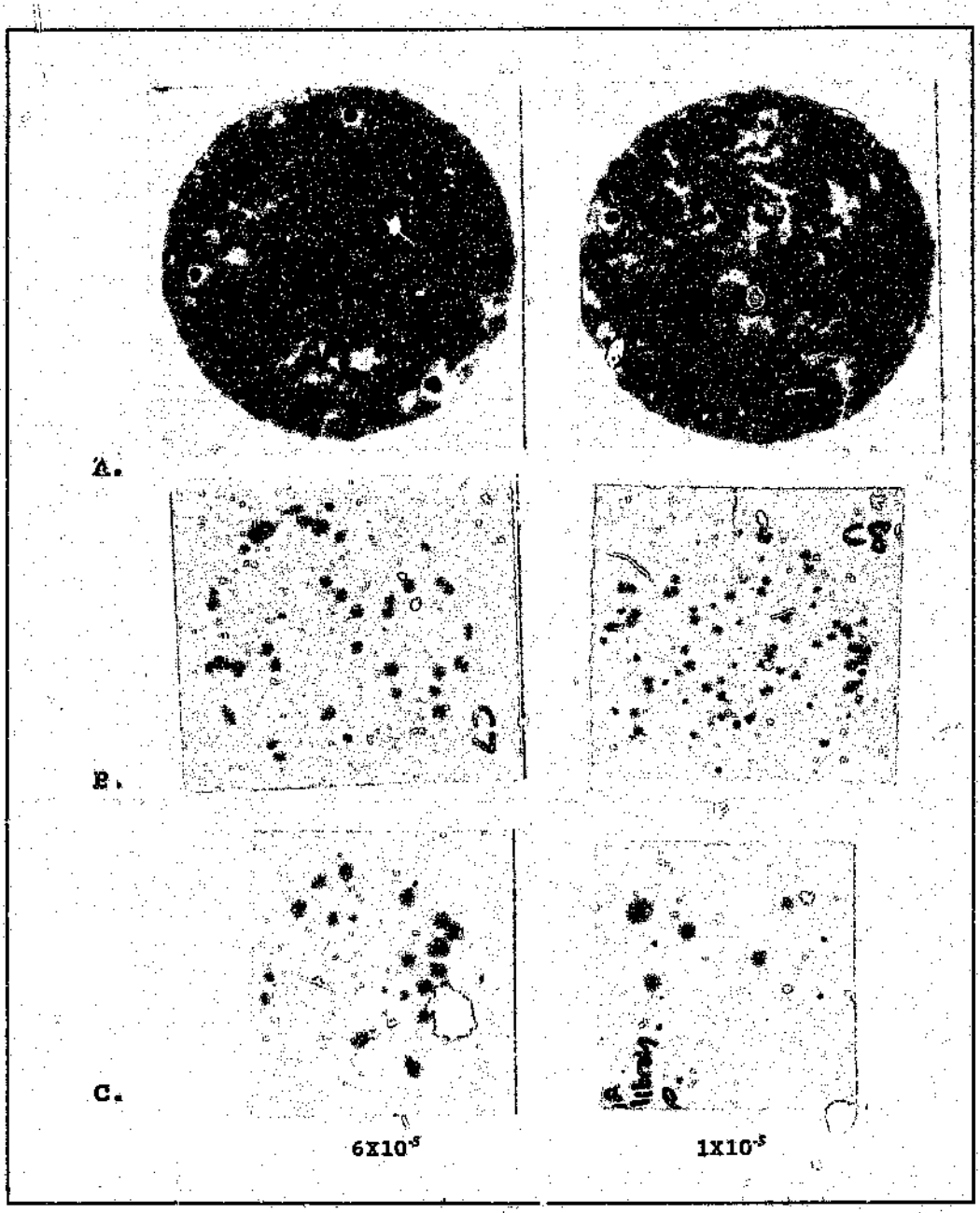


Figure 3.3 Autoradiographs of secondary screening filters. (A) Filters of plates containing a 3/1000 dilution of the positive clone that hybridised to both the mTNAP and cTNAP probes, shown in fig. 3.2. (B) Filters of plates of 1/1000 dilution of the same plaque as in A. (C) Filters from plates prepared by isolating a positive plaque (ringed in A) and rediluting as indicated to obtain single positive clones.

phage DNA was obtained for sequencing the positive inserts. This appears to be due to an unsuccessful method used to isolate phage DNA rather than lack of phage growth since bacterial lysis was clearly observed. Furthermore, the supernatants of the lysates was titred (using the same procedure for library titering). The phage titre was always found to be around 10^9 PFU's/ml which is high and should yield sufficient DNA for sequencing purposes (Sambrook *et al*, 1989).

3.3.2 PCR AMPLIFICATION OF POSITIVE INSERTS

The insert in positive clones was amplified by the PCR using primers that bind to regions flanking the insert site. A single product, corresponding to a fragment of DNA of approximately 700bp was obtained (figure 3.4). The lowest dilution of λ DNA yielded a low amount of product. It appears that the phage DNA solution added to the PCR reaction mixture contained some inhibitor of Taq polymerase, probably excess magnesium from the SM buffer used initially to dissolve the phage DNA, since the intermediate dilution gave the highest yield.

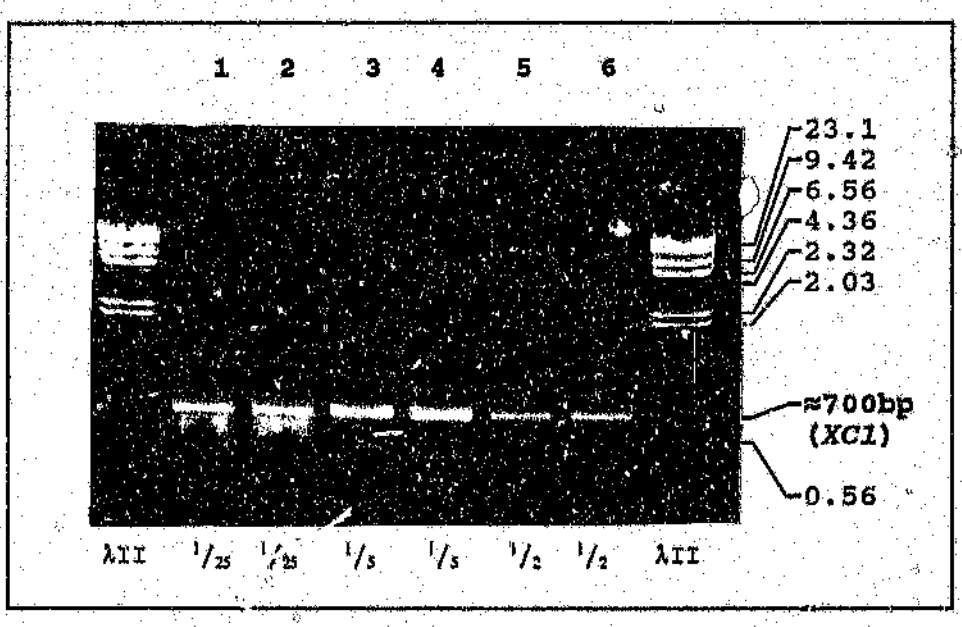


Figure 3.4 PCR amplification of the insert in a positive clone. The products from the PCR reactions were electrophoresed in a 1% gel together with marker λII whose bands are shown in kbp. Samples were loaded in duplicate. The dilution of the λ DNA from the positive plaque in the PCR reaction mixture is shown below each lane. The smears in lanes 1 and 2 are as a result of the vortexing during the isolation of the product DNA.

3.4 THE SEQUENCE OF THE *XENOPUS* TNAP cDNA

The 700bp fragment thus far obtained was expected to correspond to part of the *Xenopus* TNAP gene. It will henceforth be referred to as *XC1*. *X* indicates it is from *Xenopus*; *C* indicates it was obtained with the chicken TNAP probe (although

it also bound the mouse TNAP probe in the primary and secondary screenings of the library); *1* indicates it is the first fragment thus obtained.

3.4.1 CLONING OF *XC1*

Many white colonies were obtained after plating the bacteria transformed with the pBluescript phagemid ligated to *XC1*. This also showed that the degree of ligation of insert to vector was efficient, *i.e.* the ratio of 3.5:1 of insert:vector was found to be most suitable for the present purposes. Bacteria transformed with control ligates further confirmed the efficiency of ligation. There were also many small white satellite colonies. 12 large, white colonies were carefully picked for the small-scale phagemid prep. The phagemid from all 12 colonies contained the same 700bp fragment (figure 3.5). The colonies that gave the highest yield of *XC1* (colonies 1 and 6 in figure 3.5) were grown for large-scale plasmid preps. Each prep yielded approximately 1.6 - 2.4 mg of plasmid DNA.

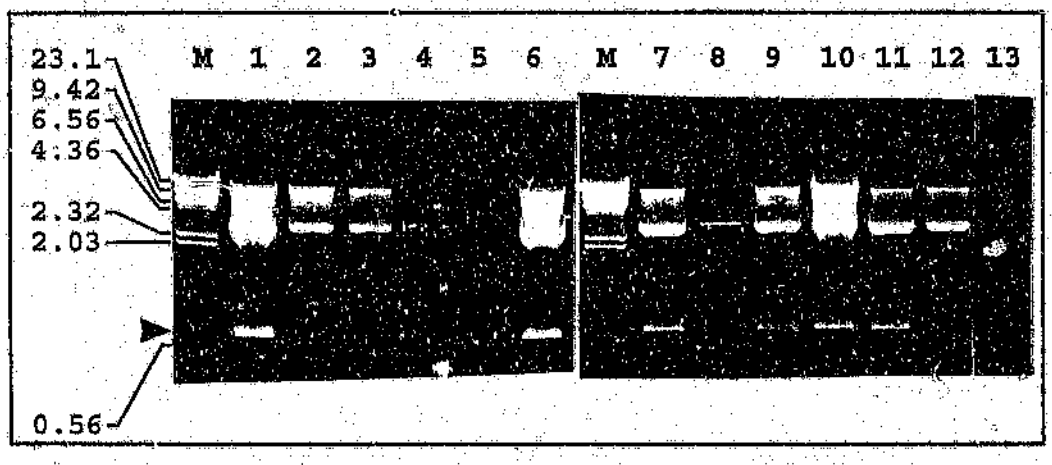


Figure 3.5 EcoRI digests of small-scale phagemid preparations. *XC1* (arrowhead) was recovered unfragmented in all preparations. Samples in lanes 1 and 6 gave the highest yield of *XC1*. In lane 13 where only PCR amplified *XC1* was loaded, the sample was overdiluted and thus only a very light band can be seen. All marker bands are shown in kbp. There is some contamination with chromosomal DNA.

3.4.2 THE *XCI* SEQUENCE

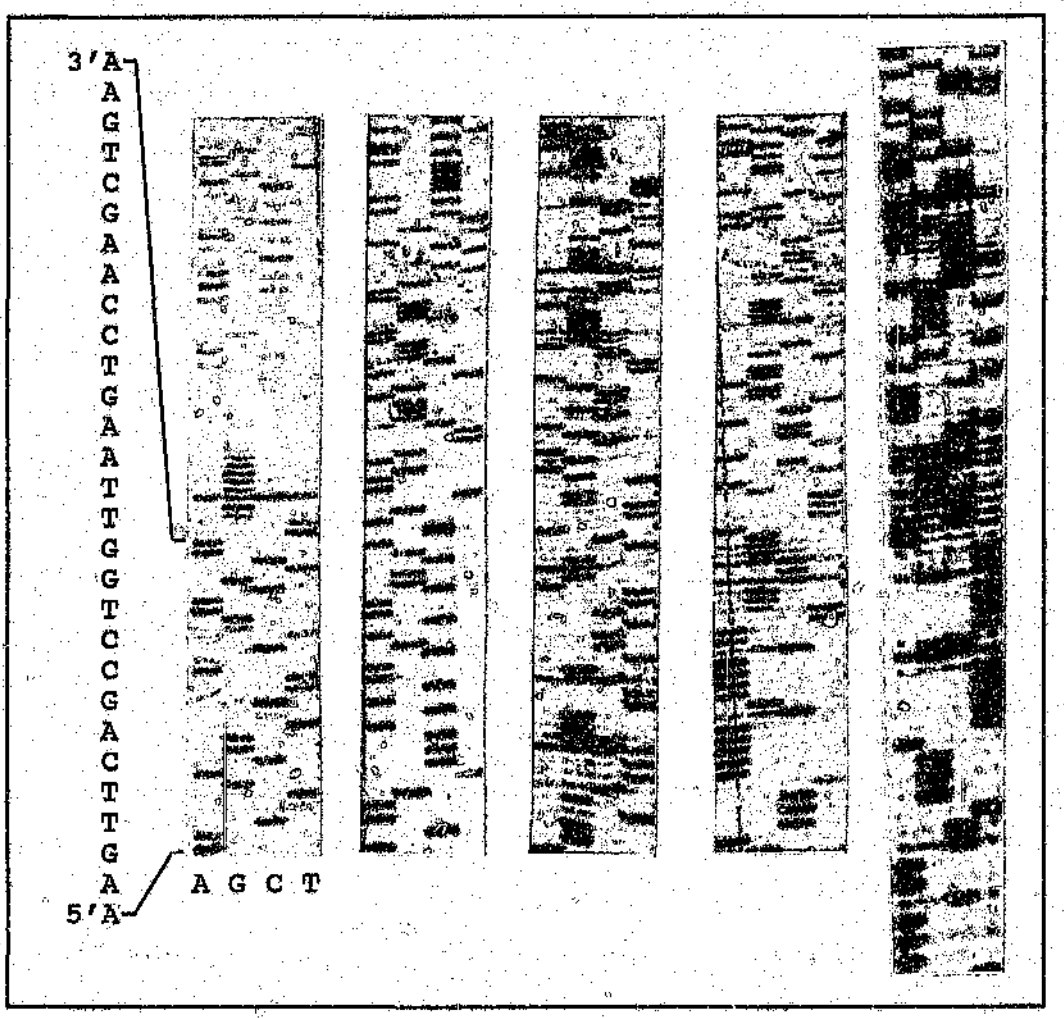
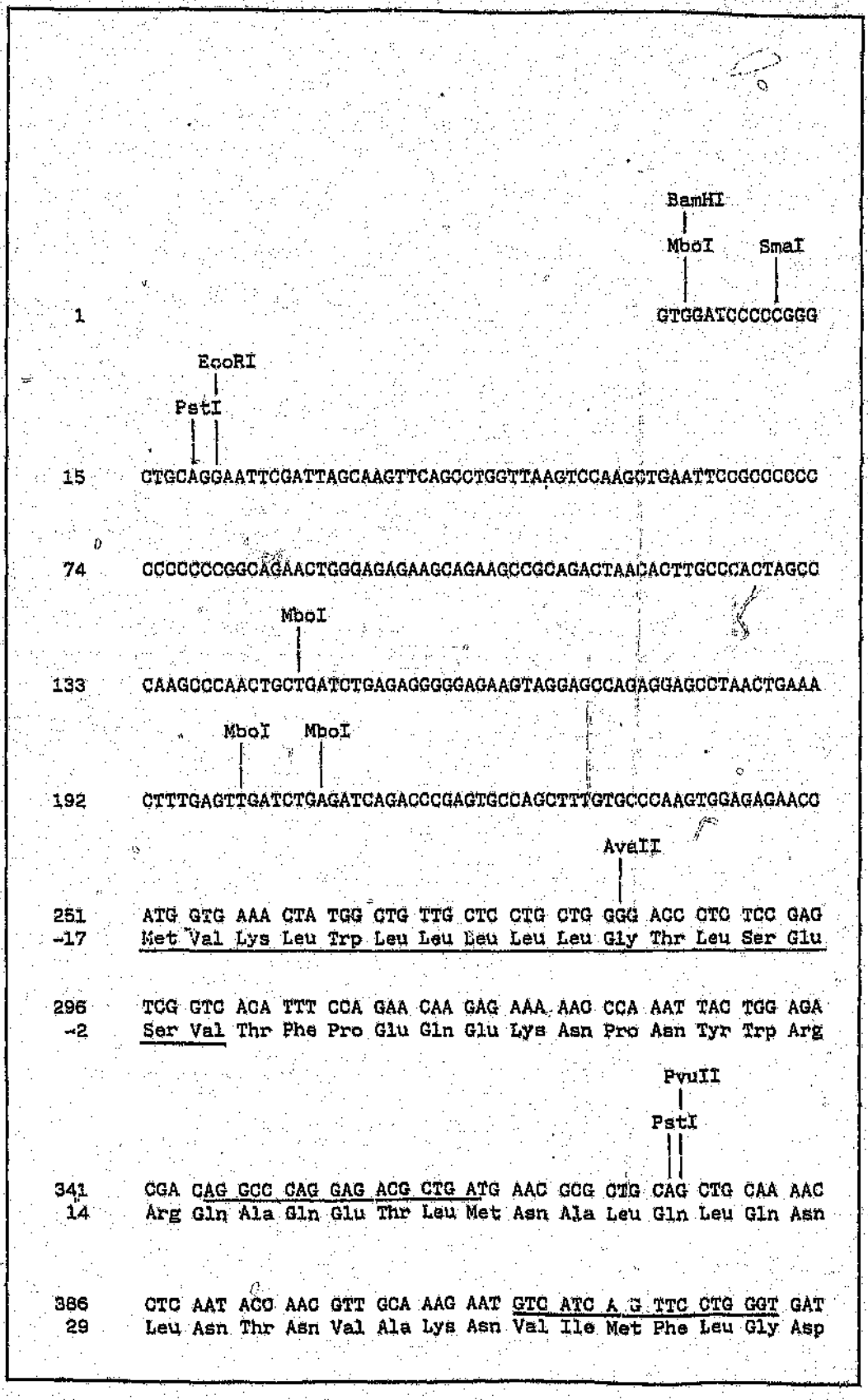


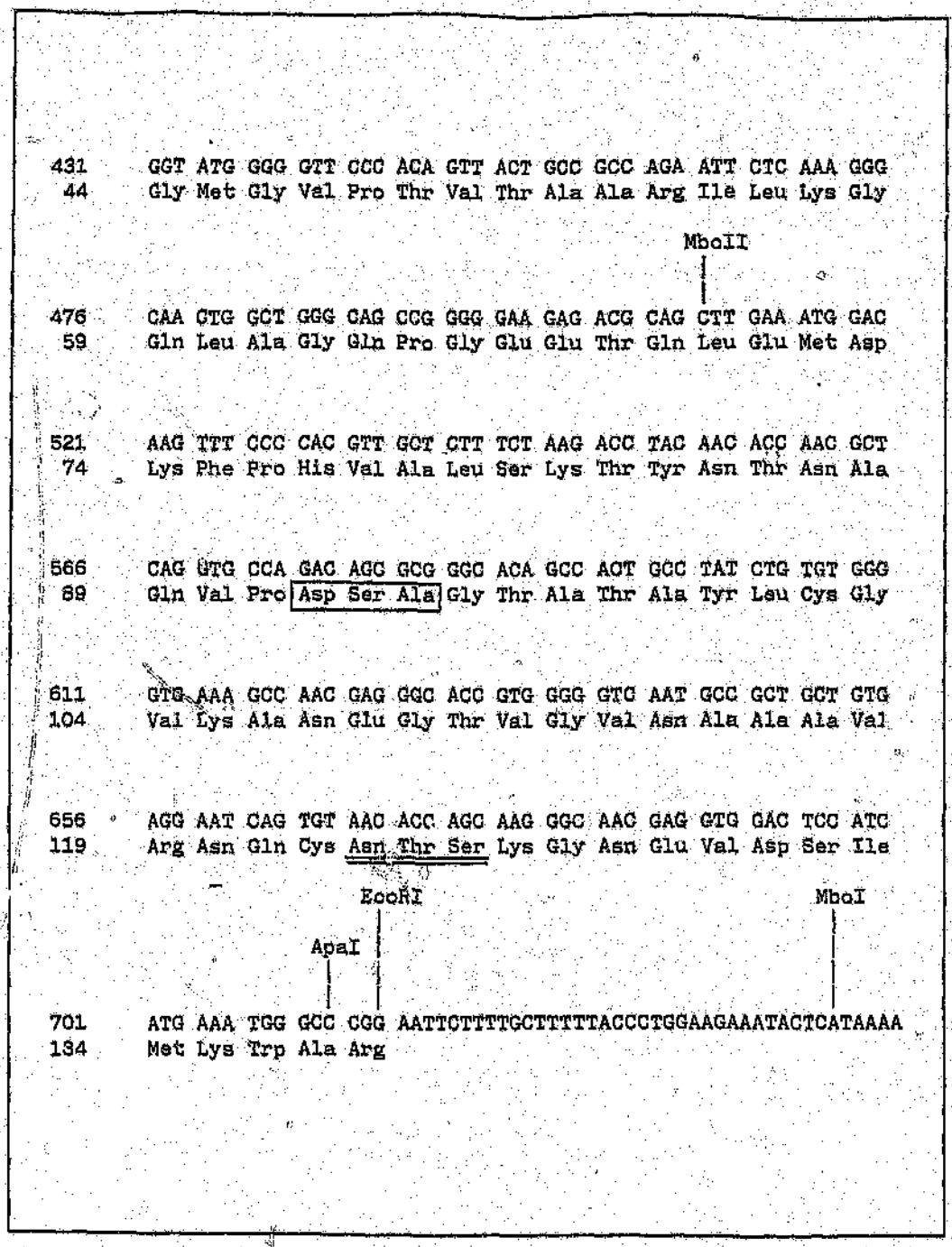
Figure 3.6 Autoradiographs of sections of some of the sequencing gels used to sequence *XCI*. A=ddATP, C=ddCTP, G=ddGTP, T=ddTTP. The same order of terminating dideoxynucleotides was used throughout. When using this order, the sequence read when turning the gel over gives the complementary sequence which can be used to confirm the sequence when reading the opposite strand. Part of the *XCI* sequence so obtained is shown.

By using various primers during sequencing, various lengths of the *XCI* fragment were sequenced, including the regions flanking the insert. Figure 3.6 shows some parts of the autoradiographs obtained of the sequencing gels. The complete sequence was put together by finding overlaps in the various sequences so obtained. After the preliminary sequencing procedure, the central region contained many ambiguities, *i.e.* the sequence could not be clearly determined from the sequencing

gels. This problem was overcome by using the 18 nucleotide oligomers that were designed to bind within the *Xenopus* fragment. These primers apparently bound efficiently since they produced clear, unambiguous results on the sequencing gels. The *XCI* insert was amplified 4 times in *different* PCR reactions and then sequenced independently. The sequences obtained differed in only 2 positions, illustrating the very low frequency of error introduced by the PCR. In this way, these errors could be detected and eliminated. The *XCI* sequence was read in both directions, *i.e.* both strands were sequenced, and, with the aid of a computer, all possible open reading frames (ORFs) were translated into the respective amino acid sequence. The sequences so obtained were compared to those of other TNAP sequences, *viz.* the mouse, rat, bovine and human TNAP sequences. In this way, the forward orientation of *XCI* and the position of the starting methionine residue were determined. Figure 3.7 shows the complete nucleotide sequence of the 700bp insert and the deduced amino acid sequence of the translated region. Some restriction endonuclease sites, including the EcoRI sites marking the sites of insertion of *XCI* in λgt10, were also determined by means of computer analysis. The sequence includes the BamHI, MboI, SmaI and PstI sites of the λgt10 polylinker that was included in the primer used for PCR amplification (nucleotides 1 - 20). The sequence from nucleotide 21 to 250 appears to be part of the 5'-untranslated region of the TNAP mRNA. This is followed by an ORF, starting with ATG at position 251. Nucleotides 251 to 305 code for the N-terminal signal peptide. The rest of the sequence, up to the EcoRI site at position 714, codes for 107 amino acid (aa) residues of the N terminus of the mature form of the *Xenopus* TNAP.

Figure 3.7 The nucleotide and deduced amino acid sequences of *XCI*. Both nucleotides and predicted amino acids are numbered on the left. Amino acids -17 to -1 comprise a putative signal peptide which is underlined. Also underlined are the two sites to which the 18-oligomers were designed to bind for sequencing. The phosphate-binding serine active site is boxed. The potential N-linked glycosylation signal (Asn-Xaa-Thr/Ser) is underlined by a double line. Restriction sites are indicated by the vertical dashes at the nucleotide immediately 5' to the site of cleavage.


BamHI
MboI
SmaI
1 GTGGATCCCCCGGG
EcoRI
PstI
15 CTGCAGGAATTCGATTAGCAAGTTCAGCCTGGTTAAGTCCAAGCTGAATTCGGCGCCCC
74 GCCCCCCGGCAGAACTGGGAGAGAAGCAGAAGCCGCAGACTAACACTTGCCCACTAGCC
MboI
133 CAAGCCCAACTGCTGATCTGAGAGGGGGAGAAGTAGGAGCCAGAGGAGCCTAACTGAAA
MboI MboI
192 CTTTGAGTTGATCTGAGATCAGACCCGAGTGCCAGCTTTGTGCCCAAGTGGAGAGAACC
AvaII
251 ATG GTG AAA CTA TGG CTG TTG CTC CTG CTG GGG ACC CTC TCC GAG
-17 Met Val Lys Leu Trp Leu Leu Leu Leu Gly Thr Leu Ser Glu
296 TCG GTC ACA TTT CCA GAA CAA GAG AAA AAG CCA AAT TAC TGG AGA
-2 Ser Val Thr Phe Pro Glu Gln Glu Lys Asn Pro Asn Tyr Trp Arg
PvuII
PstI
341 CGA CAG GCC CAG GAG ACG CTG ATG AAC GCG CTG CAG CTG CAA AAC
14 Arg Gln Ala Gln Glu Thr Leu Met Asn Ala Leu Gln Leu Gln Asn
386 CTC AAT ACC AAC GTT GCA AAG AAT GTC ATC A G TTC CTG GGT GAT
29 Leu Asn Thr Asn Val Ala Lys Asn Val Ile Met Phe Leu Gly Asp


431
GGT ATG GGG GTT CCC ACA GTT ACT GCC GCC AGA ATT CTC AAA GGG
44
Gly Met Gly Val Pro Thr Val Thr Ala Ala Arg Ile Leu Lys Gly
MboII
476
CAA CTG GCT GGG CAG CCG GGG GAA GAG ACG CAG CTT GAA ATG GAC
59
Gln Leu Ala Gly Gln Pro Gly Glu Glu Thr Gln Leu Glu Met Asp
521
AAG TTT CCC CAC GTT GCT CTT TCT AAG ACC TAC AAC ACC AAC GCT
74
Lys Phe Pro His Val Ala Leu Ser Lys Thr Tyr Asn Thr Asn Ala
566
CAG GTG CCA GAC AGC GCG GGC ACA GCC ACT GCC TAT CTG TGT GGG
89
Gln Val Pro Asp Ser Ala Gly Thr Ala Thr Ala Tyr Leu Cys Gly
611
GTG AAA GCC AAC GAG GGC ACC GTG GGG GTC AAT GCC GCT GCT GTG
104
Val Lys Ala Asn Glu Gly Thr Val Gly Val Asn Ala Ala Ala Val
656
AGG AAT CAG TGT AAC ACC AGC AAG GGC AAC GAG GTG GAC TCC ATC
119
Arg Asn Gln Cys Asn Thr Ser Lys Gly Asn Glu Val Asp Ser Ile
EcoRI
MboI
ApaI
701
ATG AAA TGG GCC CGG AATTCTTTTGCTTTTTACCCTGGAAGAAATACTCATAAAA
184
Met Lys Trp Ala Arg

3.4.3 SEQUENCING OF OTHER POSITIVE INSERTS

The cDNA library was screened several times using labelled cTNAP cDNA, *XC1*, rIAP cDNA and rEAP cDNA. It was found that the rEAP probe bound to exactly the same plaques to which *XC1* bound. There were no exceptions. Clones that hybridised to the other probes were isolated. 3 positive clones were isolated that bound *XC1* and/or the chicken probe. All 3 contained a 700bp size insert and sequencing confirmed that the same part of the *Xenopus* TNAP gene had been reisolated in every case. No new clones or any overlapping fragment of the TNAP could be obtained.

It was interesting to find 2 clones that bound rIAP exclusively. These 2 were isolated and their inserts amplified using PCR. Figure 3.8 shows the gel on which these PCR products were electrophoresed, together with PCR products of inserts isolated from 2 clones that were positive for the *XC1* probe (potentially different parts of the *Xenopus* TNAP cDNA and thus termed *XC2*).

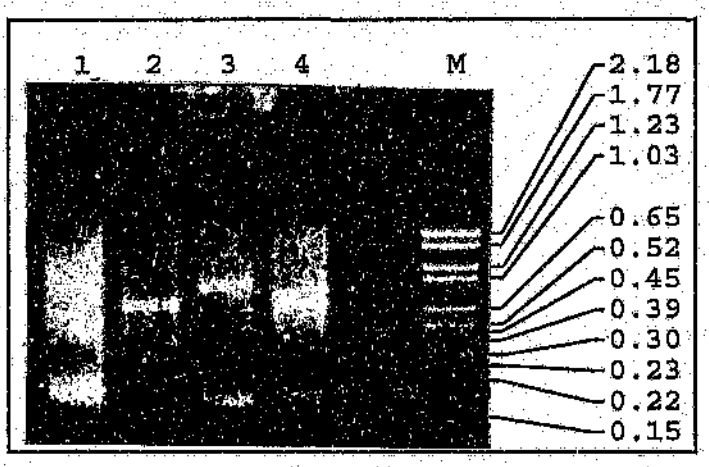


Figure 3.8 PCR amplification of other positive inserts. Agarose gel of PCR-amplified inserts isolated using labelled rIAP (lanes 1 and 3) and *XC1* (lanes 2 and 4). The inserts in lanes 2 and 4 are about 700bp and the insert in lane 3 is about 900bp. The smears indicate excessive shearing during isolation of the PCR products, particularly in lane 1 where a band is not clear. Lambda marker VI (M) was used and band sizes are shown in kbp.

This gel was blotted onto two nylon filters (by a two-way Southern blot procedure). One Southern blot was probed with labelled rIAP (figure 3.9A) and the other was

probed with labelled *XCl* (figure 3.9B). In both cases there was non-specific binding to the marker. There was no hybridisation to the rIAP and very strong binding to the *XCl* probe indicating that the chicken positive clones were both carrying the same insert which was essentially *XCl*.

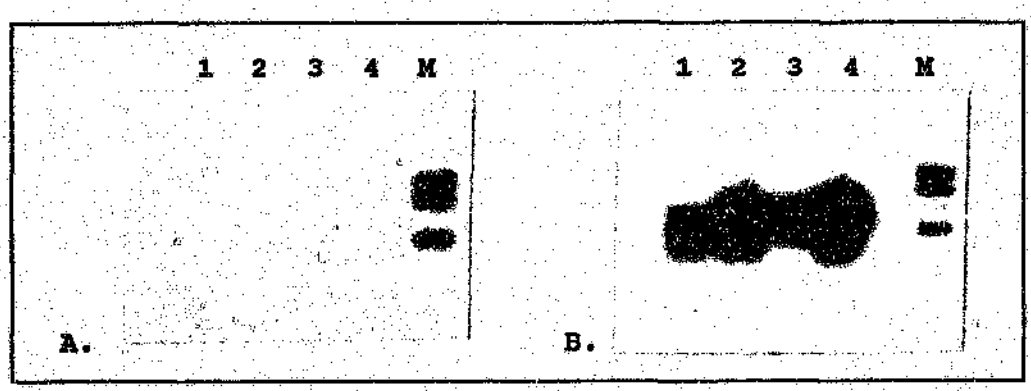


Figure 3.9 Southern blot analysis of other positive inserts. Southern blots of the gel in fig. 3.8 probed with labelled rIAP (A) and *XCl* (B). There is non-specific binding of *XCl* to the DNA in lanes 1 and 3 in B. The very high signal in lanes 2 and 4 (B) indicates that these lanes contain *XCl*.

3.5 HYBRIDISATION ANALYSIS

3.5.1 SOUTHERN BLOT ANALYSIS

The procedure used to extract DNA from *Xenopus* blood gave good yields of DNA with minimal contamination with protein. An average ratio for OD_{260}:OD_{280} of 1.88 was obtained for all samples. 15 ml of blood yielded 990 μg of genomic DNA. Figure 3.10 shows a gel of the *Xenopus* genomic DNA samples digested to completion with 8 restriction endonucleases. Southern blots of such gels were hybridised with labelled *XCl*. Clear band patterns were obtained (figure 3.11). Different Southerns prepared in the same way gave identical band patterns when hybridised with labelled *XCl*. Blots that were stripped could be reprobed since these were generally lacking any radioactive signal after an overnight exposure. The same band patterns were produced when stripped filters were probed. Stripped filters, as well as newly prepared ones, were hybridised to labelled mIAP cDNA probe. A different band pattern was obtained (figure 3.12).

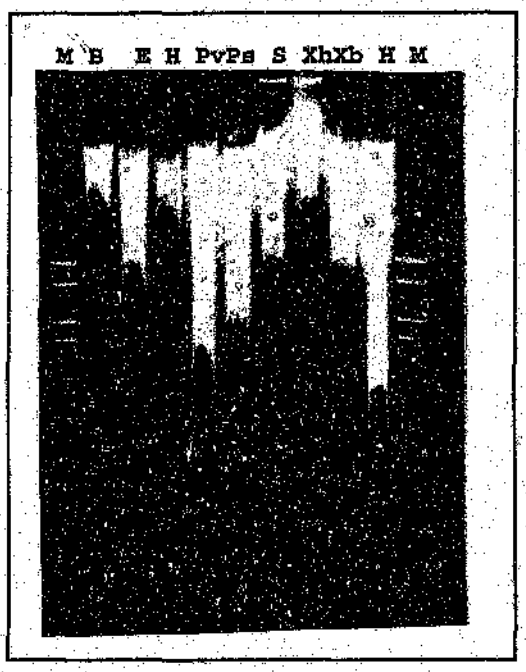


Figure 3.10 Agarose gel of *Xenopus* genomic DNA. The DNA was digested to completion with the following restriction endonucleases: B=BamHI, E=EcoRI, H=HindIII, Ps=PstI, Pv=PvuII, S=SacI, Xb=XbaI, Xh=XhoI. There are very few sites for XhoI, while HindIII yields fragments with an average size significantly lower than that produced by the other enzymes. Several bands resulting from medium and high repetitive sequences can be seen.



Figure 3.11 Southern blot analysis. A Southern blot of the gel in fig. 3.10 probed with labelled *XCI*. Lanes are indicated as in fig. 3.10.

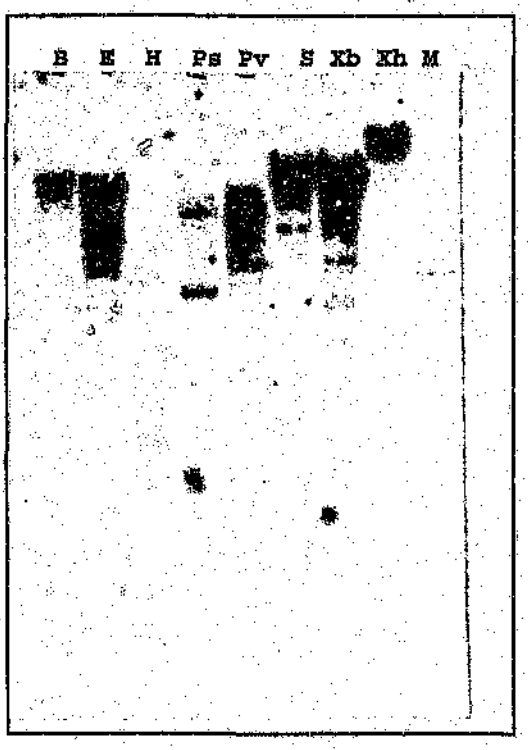


Figure 3.12 Southern blot analysis. The Southern blot of the gel in fig. 3.10 was probed with labelled mIAP. Comparison with fig. 3.10 shows that different band patterns are obtained with the tissue specific AP probe. Lanes are labelled as in fig. 3.10.

3.5.2 NORTHERN BLOT ANALYSIS

Five samples of RNA were prepared from neurula embryos. The results of the spectrophotometric analysis of these samples are shown in Table 3.2.

TABLE 3.2: SPECTROPHOTOMETRIC ANALYSIS OF RNA PREPS

SAMPLE :	1	2	3	4	5
OD_{260}	1.979	2.123	1.730	1.823	1.333
OD_{280}	1.193	1.320	1.075	1.095	0.787
OD_{260}/OD_{280}	1.659	1.608	1.609	1.665	1.694
[RNA]/$\mu g.ml^{-1}$	7.916	8.492	6.920	7.292	5.332
TOTAL RNA/μg	1 979	2 123	1 730	1 823	1 333

RNA in formaldehyde gels that contained ethidium bromide (EtBr), and where the running buffer included EtBr, could not be visualised by UV transillumination. There was a high background fluorescence due to non-specific binding of EtBr to the MOPS buffer. The fluorescence occurred as a gradient with a front that corresponded to the approximate distance that the bromophenol blue dye migrated (figure 3.13A). After destaining, background fluorescence was still high and RNA bands were only slightly discernable (figure 3.13B).

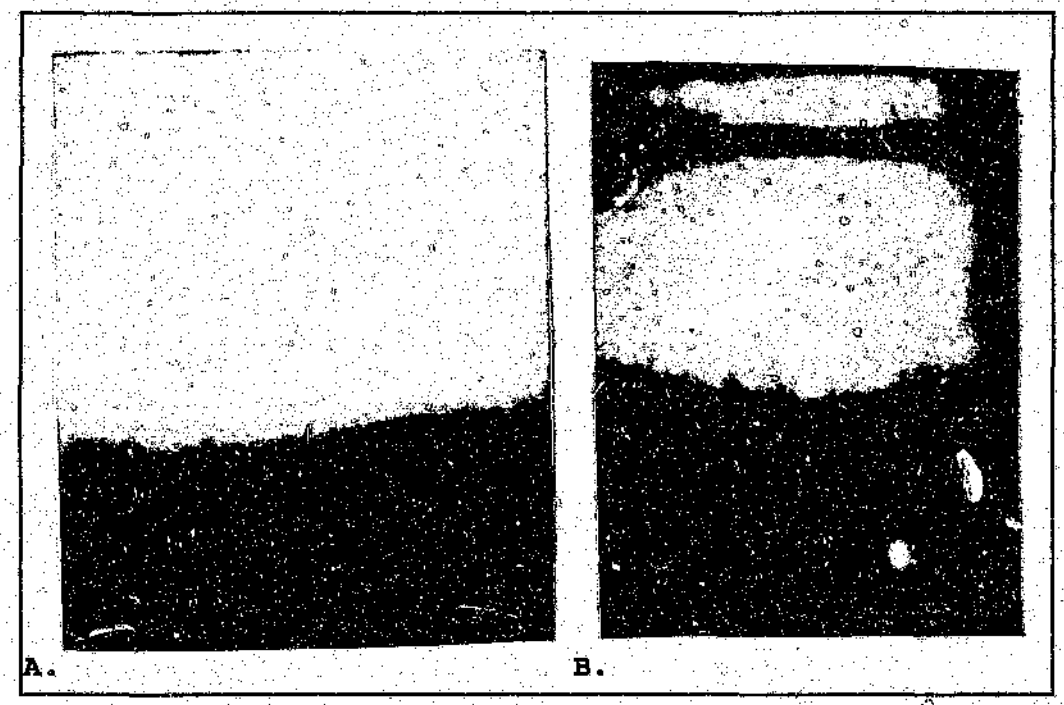


Figure 3.13 Formaldehyde-agarose gel of total neurula RNA. EtBr was included in the running buffer and the gel. (A) The gel prior to destaining. (B) The gel after destaining. RNA bands are not discernable.

It was thus impossible to use the internal RNA markers directly to determine the size of any positive bands that would be obtained on probed Northern blots. RNA staining was very poor in gels that were stained only after electrophoresis (figure 3.14).

It was thus decided to run identical gels with total RNA under the same conditions and to prepare two Northern blots from these gels. One was stained with methylene blue and the other was probed with *XCl*. In this way, the size of the *Xenopus* TNAP mRNA that would bind the labelled *XCl* probe could be approximated from the ribosomal RNA bands on the corresponding methylene blue-stained Northern blot. Gels were placed on an UV transilluminator to determine the efficiency of RNA transfer to the nylon filters after Northern blotting. It appeared that most, if not all, the RNA was transferred (figure 3.15).

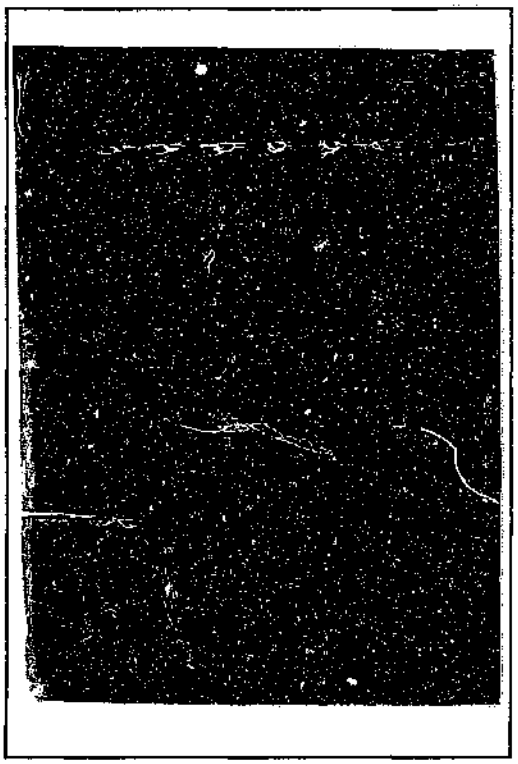


Figure 3.14 Formaldehyde-agarose gel of total neurula RNA. The gel was stained after electrophoresis. RNA was weakly stained.

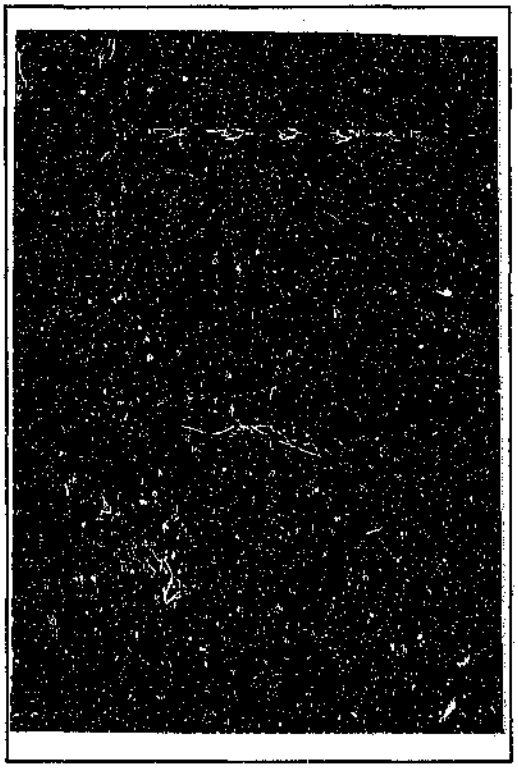


Figure 3.15 Formaldehyde-agarose gel of total neurula RNA. The gel in fig. 3.14 after alkali blotting. Some RNA remained in the gel.

Northern blots that were stained with methylene blue produced good clear ribosomal RNA bands which also showed that the RNA extracted from the embryos was clean and undegraded (figure 3.16). Northern blots that were probed with labelled *XCI* produced a single, clear band (figure 3.17). It was thus not necessary to prepare Northern blots with only mRNA since total RNA blots produced clear results. By comparing the distance that the *XCI* positive band migrated from the wells, with that of the rRNA bands in the stained blot, the size of the *Xenopus* TNAP mRNA was estimated as being 2.5kb.

Northern blots prepared using the alkali blotting procedure produced the same results but in half the time it took with the classical method.

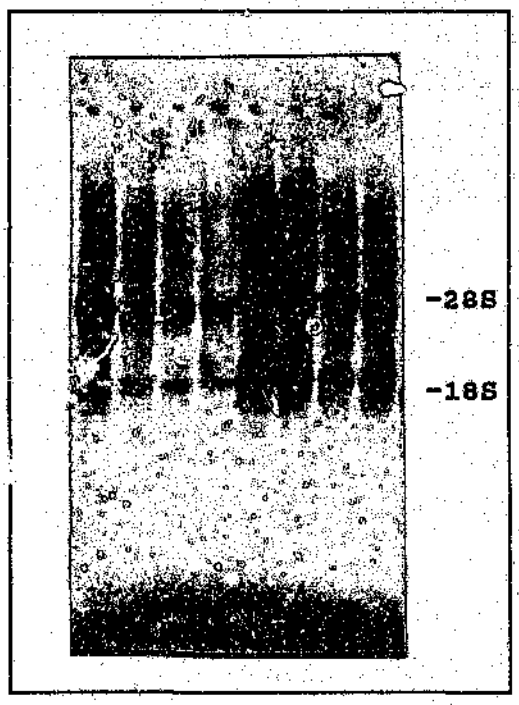


Figure 3.16 Northern blot analysis. The Northern blot of the gel in fig. 3.15 that was stained with methylene blue. RNA size markers are the internal 28S (5.5 kb) and 18S (2.1 kb) rRNA.

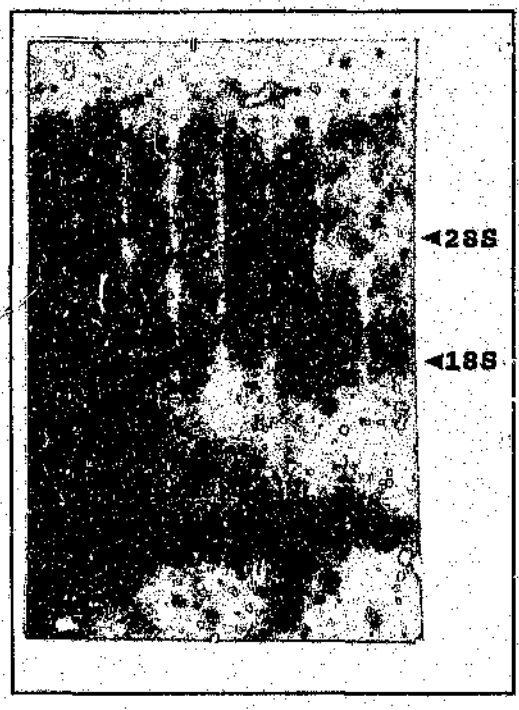


Figure 3.17 Northern blot analysis. Autoradiograph of a Northern blot as the one in fig. 3.16 probed with labelled *XCI*. The positions of the internal markers are indicated.

4 DISCUSSION

4.1 THE SUITABILITY OF THE cDNA LIBRARY

The titre found for the cDNA library that was screened was reasonably high, although it was 40X lower than the original titre. This showed that the library was still in good condition, that minimal degradation occured since its construction in 1986. The library was thus considered suitable for screening. However, since the average insert size was only 1 kb, and the full-length TNAP cDNA was expected to be at least 2.5 kb (comparable with other TNAP cDNA's), it was accepted that the library would have to be screened several times, and several positive clones be isolated to obtain the full-length sequence. The exact level to which the *Xenopus* TNAP mRNA is represented in the mRNA pool of neurula embryo cells is not known. For a mRNA in the lowest abundance class, in vertebrates, there are usually about 5 - 15 molecules of such a mRNA per cell (if that mRNA is expressed at all) and standard screening of a cDNA library prepared from the expressing tissues is usually sufficient to isolate the specific molecule (Axel *et al*, 1976; Young *et al*, 1976). Using the titre value obtained for the library, plating of the library was performed such that there were 35 000 plaques per plate. With 8 plates per primary screening, 2.8×10^5 recombinants were thus screened each time. The library used contains 4×10^6 independent inserts and thus even the rarest mRNA could be found in such a screening (Huynh *et al*, 1985).

Typically, the number of TNAP positive clones isolated in the first round of screening is very low. This is not surprising, since only a very small number of clones can be expected to have inserts that code for any particular part of a gene.

However, more positives were expected in the first round of screening than was obtained. One reason is the low insert size compared to the length of the full cDNA coding for TNAP. Each probe should be able to hybridise to different clones that although contain different inserts, these inserts would all be part of the TNAP sequence. Furthermore, the relatively high expression of AP in the neurula embryo (as discussed in section 1.1.5) should result in a cDNA library that has the TNAP sequence significantly represented, thereby decreasing the number of recombinants needed to be screened for its isolation. Only 0.27% of the mRNA from human placenta is PLAP mRNA (Kam *et al*, 1985). Nevertheless, out of only 150 000 recombinant phages in a λgt11 library, 68 clones reacted with PLAP antibody (Kam *et al*, 1985). This is significant since such a procedure also requires that the PLAP inserts be properly expressed, which as already discussed, decreases the chances of isolating a specific clone. Even after the polyclonal antibody was affinity purified, the number of positive clones remained high (23) (Kam *et al*, 1985). Similarly, Misumi *et al* (1988) found that the TNAP mRNA in rat liver is less than 0.005% of the mRNA population in the rat liver. Nevertheless, by screening 6 X 10^5 recombinant bacteriophages (without prior amplification) in a λgt11 cDNA library, constructed from rat liver mRNA, with anti-(liver AP) IgG, 6 positive clones were isolated. These led to the sequence of the full-length rat TNAP cDNA.

It is of course possible that the cDNA library is too small and does not adequately represent the mRNA pool of the neurula embryo. If necessary in the future construction of *Xenopus* cDNA libraries, it may be possible to increase expression of the TNAP gene in neurula and then construct a library from induced embryo's. Misumi *et al* (1988) were able to induce rat TNAP mRNA in the liver by bile-duct ligation and injecting with colchicine. The TNAP mRNA was increased 20-fold by this double treatment and this facilitated the isolation of the TNAP cDNA (Misumi *et al*, 1988).

It is not necessary that the average insert size of a library approximate the full-length cDNA. The probe that is obtained from a first screening can be used to screen the library for longer and overlapping cDNA clones. This step-wise

approach is usually sufficient to obtain the sequence of any full-length cDNA (Huynh *et al*, 1985).

4.2 PREPARATION OF POSITIVE INSERT *XCI* FOR SEQUENCING

4.2.1 PHAGE DNA EXTRACTION

It is not clear why the phage DNA extraction was not successful since the same method has been used successfully in other experiments unrelated to the present work. It is possible that the PEG used to precipitate the phage was spoiled. Fortunately, this problem was easily overcome by the use of PCR to amplify inserts from positive plaques. No difficulty was encountered with the PCR since in almost every case inserts were well amplified yielding uniform products uncontaminated with any other DNA.

4.2.2 T-VECTOR CONSTRUCTION

The construction of T-vectors for cloning of the PCR amplified inserts was necessary due to the template-independent terminal transferase activity of Taq polymerase that results in the addition of a single nucleotide at the 3' end of the amplified fragment (Clark, 1988; Mole *et al*, 1989). This nucleotide is almost always adenosine due to the strong preference of Taq polymerase for dATP (Clark, 1988). Clearly, this makes cloning blunt-ended fragments, such as PCR products, very inefficient. Alternative solutions to this problem include the addition of linkers, which have an internal restriction site, or adaptors, or by employing homopolymer tailing to create cohesive ends between vector and fragment to be cloned (Old & Primrose, 1989). These procedures are quite expensive, due to the cost of oligonucleotide synthesis, especially if done routinely. The primers employed in the PCR could have restriction sites incorporated in them, so that after cleavage the fragments have cohesive ends that can be ligated into an equivalently cut vector (Old & Primrose, 1989). A problem often encountered in this approach

is that many restriction endonucleases fail to cleave when their recognition sequences are located within a few base pairs of the end of a DNA fragment (Marchuk *et al*, 1991).

The procedure whereby single thymidines are added to the 3' ends of appropriately cleaved vectors was first described by Marchuk *et al* (1991). The so-called "T-vector", that has added thymidines, and the PCR products have complementary single base 3' overhangs. This clearly increased the efficiency of cloning the amplified fragments since a high number of bacteria transformed with recombinant vector showed that the ligation was efficient. Furthermore, under these conditions, vector self-ligation and concatamerisation of the inserts are prohibited.

4.3 SCREENING OF THE LIBRARY

4.3.1 SUITABILITY OF THE CHICKEN AND MOUSE TNAP PROBES

The *Xenopus* neurula λgt10 cDNA library was screened at high cell density with the chicken and mouse full-length TNAP cDNA probes. On the whole, both probes hybridised to the same clones. This is not surprising, since all TNAP's appear to share over 90% overall homology at the protein level (Garattini *et al*, 1987). Although both probes were efficiently labelled, the chicken probe always gave significantly darker spots in the autoradiographs. This was possibly due to higher homology of the former with the *Xenopus* TNAP. Alternatively, it is possible that the chicken was more efficiently labelled due to a higher content of cytosine residues. There was generally over 80% incorporation of radioactive nucleotides in the chicken probe but a precise comparison with incorporation in the mouse probe was not carried out. The chicken probe was 500 bp shorter than the mouse probe (figure 3.1) and this may have resulted in more efficient hybridisation of the former to the *Xenopus* sequence.

4.3.2 ISOLATION OF PART OF THE *XENOPUS* TNAP cDNA AND COMPARISON WITH THE MOUSE TNAP cDNA

4.3.2.1 Authenticity of *XC1*

Screening of 2.8 X 10^5 plaques, four times, led to the isolation of several clones that were found to contain the same insert, designated as *XC1*, that corresponds to part of the *Xenopus* TNAP gene. Several observations indicated that *XC1* is indeed an authentic TNAP cDNA probe. By means of computer-assisted analysis, the sequence of *XC1* was compared with that of the mouse TNAP cDNA. Significant homology was found between *XC1* and part of the mouse sequence that also confirmed that *XC1* corresponds to the 5' end of the TNAP, including part of the leader sequence. The comparison at the nucleotide level between the *Xenopus* and mouse cDNA sequences is shown in figure 4.1. Optimal alignment was achieved by introducing gaps in the sequence that are shown by the dashes in the aligned sequence. The overall homology was calculated to be approximately 63%. The degree of homology from the start codon to the end of the sequence, *i.e.* to the EcoRI site between the *Xenopus* sequence and the λ sequence, was found to be 71%. This is not surprising considering the fact that the 5' untranslated regions of the 2 cDNA's are expected to have less homology due to lower selective pressures for function. The 5' and 3' untranslated regions of all AP cDNA sequences are not homologous (Noda *et al*, 1987). This indicates that the non-coding regions of AP's are iso-specific and do appear to be conserved in evolution (Noda *et al*, 1987). A similar phenomenon is reported for the 3' noncoding region of the multigene actin family (Ponte *et al*, 1983). The degree of homology between the full-length *Xenopus* TNAP and other TNAP's should be higher.

The fact that *XC1* was isolated using TNAP-specific probes also suggests that *XC1* must be part of the *Xenopus* TNAP gene. A third observation that supports the authenticity of *XC1* is that it binds to an RNA species in Northern blots, prepared with *Xenopus* RNA, that corresponds to a size of 2.5kb which is the size of all other TNAP mRNA's studied thus far (see section 4.4.2).

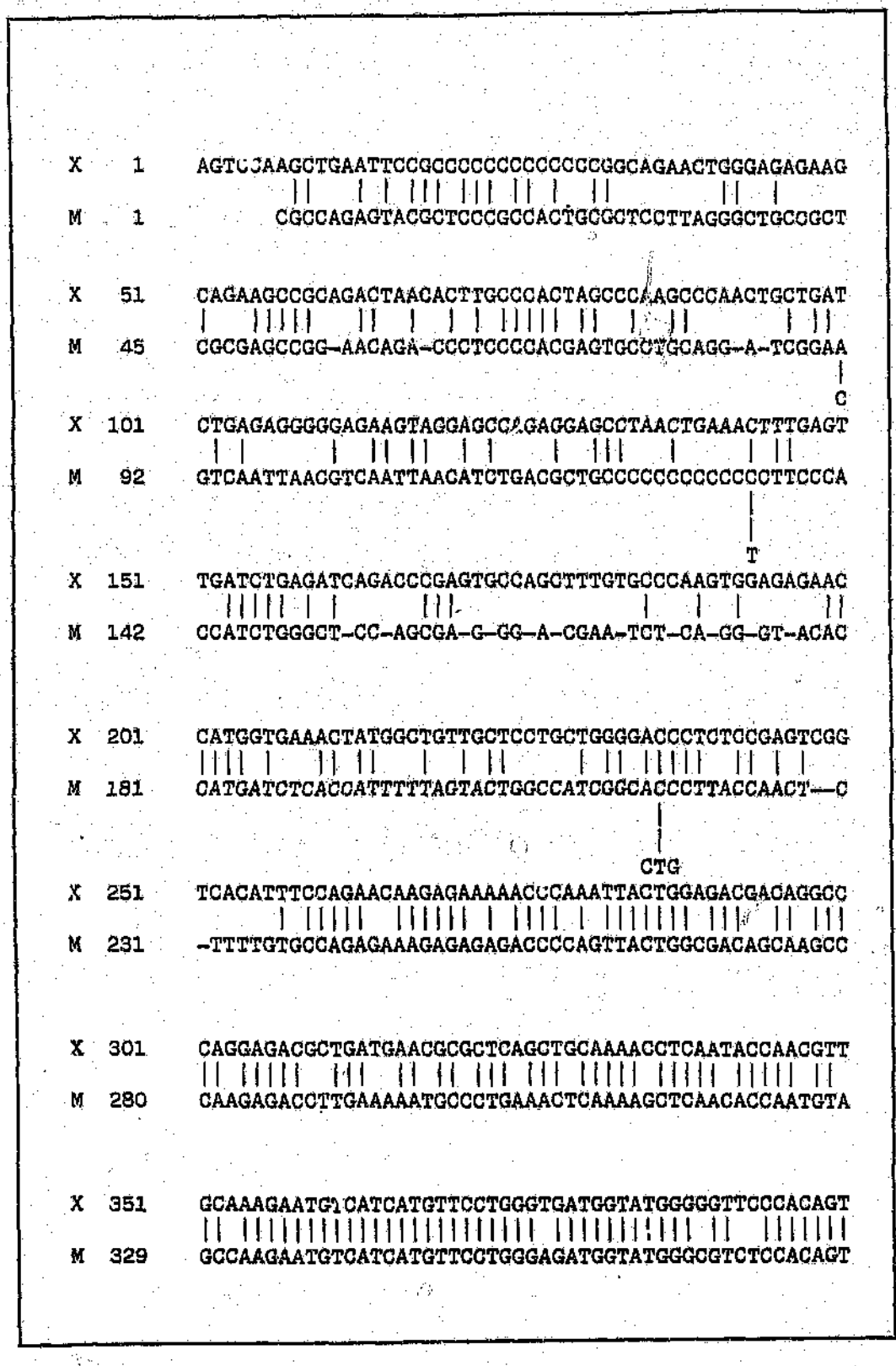
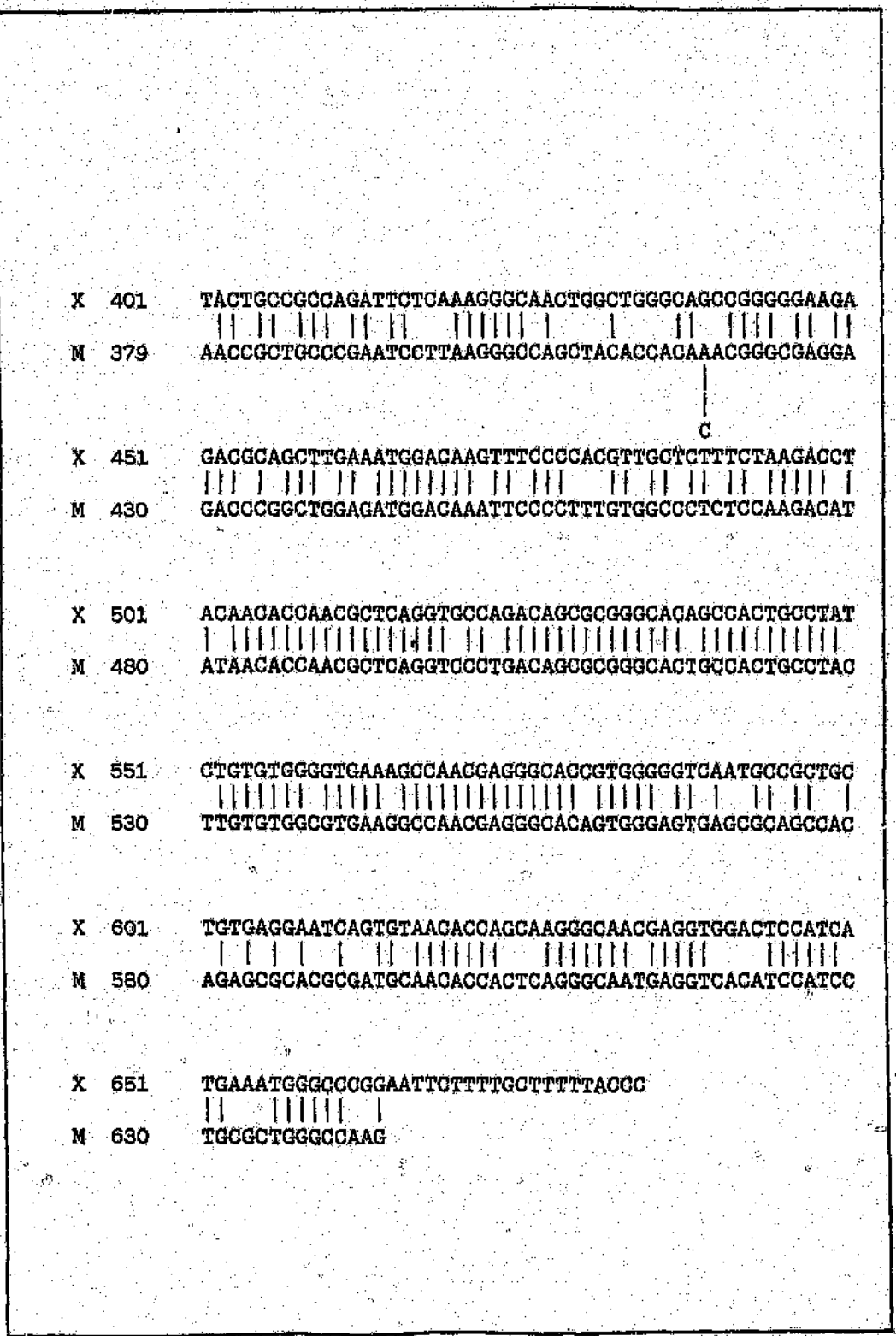


Figure 4.1 (Continues over) Computer-assisted nucleotide alignment of *XC1* (X) with the corresponding region of the mouse TNAP cDNA (M). The mouse sequence is taken from Hahnel and Schultz (1989). Nucleotide position numbers are at the left margin. The first nucleotide in the *Xenopus* sequence corresponds to nucleotide 50 in fig. 3.7.


X 401 TACTGCCGCCAGATTCTCAAAGGGCAACTGGCTGGGCAGCCGGGGGAAGA
M 379 AACCGCTGCCCGAATCCTTAAGGGCCAGCTACACCACAAACGGGCGAGGA
C
X 451 GACGCAGCTTGAAATGGACAAGTTTCCCCACGTTGCTCTTTCTAAGACCT
M 430 GACCCGGCTGGAGATGGAGAAATTCCCCTTTGTGGCCCTCTCCAAGACAT
X 501 ACAACACCAACGCTCAGGTGCCAGACAGCGCGGGCACAGCCACTGCCTAT
M 480 ATAACACCAACGCTCAGGTCCCTGACAGCGCGGGCACTGCCACTGCCTAC
X 551 CTGTGTGGGGTGAAAGCCAACGAGGGCACCGTGGGGGTCAATGCCGCTGC
M 530 TTGTGTGGCGTGAAGGCCAACGAGGGCACAGTGGGAGTGAGCGCAGCCAC
X 601 TGTGAGGAATCAGTGTAACACCAGCAAGGGCAACGAGGTGGACTCCATCA
M 580 AGAGCGCACGCGATGCAACACCACTCAGGGCAATGAGGTCACATCCATCC
X 651 TGAAATGGGCCCGGAATTCTTTTGCTTTTTACCC
M 630 TGCGCTGGGCCAAG

It may be considered unusual that the 5' end of a mRNA sequence was isolated. Since the synthesis of the first-strand of the cDNA was oligo dT-primed, the chances of under-representing the 5' end is increased due to incomplete synthesis of the first strand. However, the method used to construct the library was a modified version of the one described by Land *et al* (1981). In this method, the use of nuclease S1 is avoided by dG tailing first cDNA strands and oligo dC priming second strand synthesis. This method enriches for full-length cDNA clones. Other problems are also avoided which are associated with nuclease S1 treatment. 5' mRNA sequences are often lost when digesting with nuclease S1. Another anomaly is that occasionally a cDNA clone results which contains sequences at its 5' terminus which are not colinear with the mRNA due to incomplete digestion with the nuclease (Huynh *et al*, 1985). Strategies where nuclease S1 treatment is excluded are thus preferred.

4.3.2.2 Failure to Isolate the Full-length TNAP cDNA

It was surprising to find that, after several screenings, and sequencing of several positive clones, all isolates contained the same part of the TNAP gene. Overlapping regions were also not obtained. It appears that the rest of the TNAP mRNA was somehow lost during construction of the library. One possible explanation for this is that the EcoRI sites were not adequately protected during construction of the library and thus, when the attached EcoRI linkers were digested for ligation into the EcoRI site of λgt10, site(s) inside the TNAP mRNA were cleaved and part of the cDNA was accidentally lost. Clearly, the full-length TNAP cDNA will be isolated from alternative libraries, especially since there is now a *Xenopus*-specific probe. The full sequence should reveal any internal EcoRI sites that could support the above hypothesis.

4.3.2.3 The Amino Acid Sequence Encoded by *XCI*

The longest open reading frame was translated into the amino acid sequence (figure 3.7). This was used to identify which part of the TNAP gene *XCI* coded by comparison with the amino acid sequence of other TNAP's. The sequence obtained codes for a polypeptide of 155 amino acids with a calculated M_r of 16 919. The first methionine is part of the consensus sequence ACCAUGG which is considered optimal for the initiation of translation (Kozak, 1986). The initiating methionine codon is followed by a sequence encoding a predicted signal peptide. The putative signal peptide is 17 amino acids long, is mostly hydrophobic and is rich in leucine. This signal peptide is thus the same as that found in other cloned TNAP cDNA's, including the bovine, rat and human TNAP's (Kam *et al*, 1985; Weiss *et al*, 1986; Garattini *et al*, 1987; Misumi *et al*, 1988). However, the C terminus of the signal peptide in *Xenopus* can only be unequivocally defined by determining the N-terminal amino acid sequence of the mature frog TNAP. It is interesting to note that mouse epidermal growth factor precursor and human pancreatic polypeptide hormone precursor have similar signal peptides (Scott *et al*, 1983; Schwartz *et al*, 1984).

The first N-terminal amino acids are hydrophobic (Phe, Pro). It is common to find hydrophobic amino acids next to the signal peptide (Kam *et al*, 1985). The 155 amino acids encoded by *XCI* represents only 30% of the whole TNAP protein since all other TNAP's consist of 524 amino acids, including the N- and C-terminal signal peptides (Hua *et al*, 1986; Weiss *et al*, 1986, 1988; Garatinni *et al*, 1987; Misumi *et al*, 1988; Hsu, 1991). The proline in the third position after the N-terminal signal peptide is conserved in all AP's so far sequenced (Kam *et al*, 1985; Garatinni *et al*, 1987; Misumi *et al*, 1988; Weiss *et al*, 1988). The presence of this Pro residue also supports the predicted length of the signal peptide of 17 residues. This is a further confirmation that the sequence obtained is the *Xenopus* TNAP because all TNAP's so far sequenced have a signal peptide of 17 residues, whereas the TSAP's signal peptide ranges from 18 to 20 residues (see appendix D).

By comparing the amino acid sequence encoded by *XC1* with that of other TNAP's (such as those in appendix D) it can be seen that *XC1* includes the first four, and most of the fifth, alpha-helical domains which occur at the following positions (amino acid 1 being the first after the signal peptide): 19 - 25, 47 - 56, 62 - 68, 94 - 104, 132 - 138 (the fifth domain ends at residue 141). Similarly, *XC1* includes the first two beta-sheet domains (positions 35 - 42, 70 - 75).

Continuing with the structural comparisons, the active site sequence Asp^{92}-Ser^{93}-Ala^{94} is also included in *XC1* (figure 3.7). This active site sequence is found in the same position in all vertebrate AP's (in the human PLAP and GCAP Ala^{94} is replaced by Gly^{94} which supports the view that these isozymes are new evolutionary gene products (Goldstein *et al*, 1980)). Note the residues that interact with substrate phosphate; the Ser^{93} residue is the one that is phosphorylated during phosphatase activity. Arg^{167} is the other residue that interacts with substrate phosphate in the full-length TNAP and is not part of *XC1* (Weiss *et al*, 1986). The reactive serine is linked to the amino end of the α-helix, defined by residues 94 - 104.

Most of the metal ligand residues occur after the 300th amino acid (Leu^{300} in mouse and rat TNAP; Ile^{300} in bovine and human TNAP). Asp^{43} is one metal ligand site that is included in *XC1*. The other residues to be found in the full-length *Xenopus* TNAP (conserved in all other AP's) are His^{154}, Thr^{156}, Glu^{315}, Asp^{320}, His^{324}, Asp^{361}, His^{362} and His^{364}. It can be recalled that these sites are responsible for the precise coordination of the metal ions Zn1, Zn2 and Mg which is essential for enzyme function.

Another feature that can be found in the sequence encoded by *XC1* is a putative glycosylation signal site at Asn^{123}. Other such sites (defined by the sequence Asn-Xaa-Thr/Ser) should be found at Asn^{213}, Asn^{254}, Asn^{286} and Asn^{413} (Misumi *et al*, 1988). All TNAP's have these five potential glycosylation sites, whereas the other isozymes have only two, Asn^{122} and Asn^{249} (Millán, 1986).

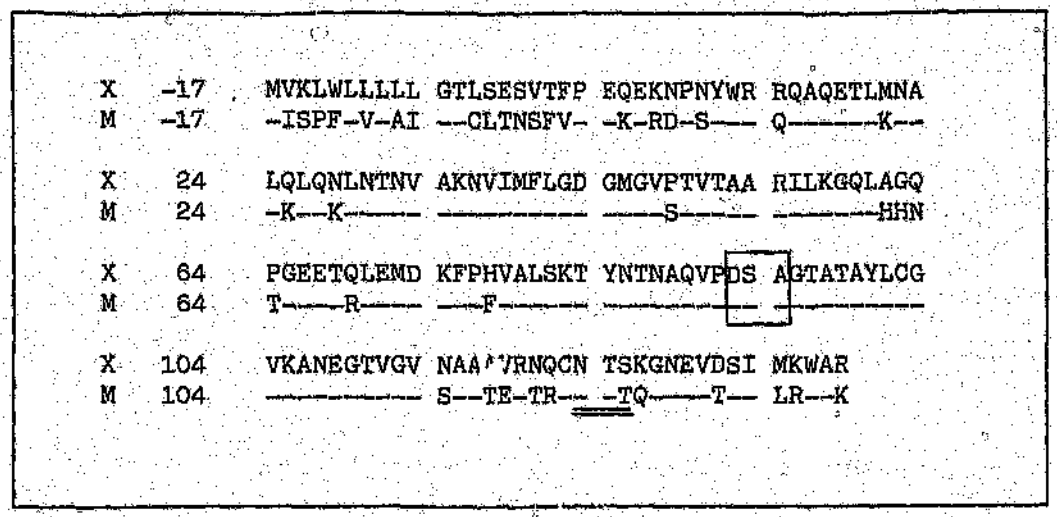


Figure 4.2 Comparison of the deduced amino acid sequences of *XCI* (X) and the corresponding region of the mouse (M) TNAP (Hahnel & Schultz, 1989). Amino acids are shown in the single-letter code (appendix B). The arrow indicates amino acid residue 1 of the mature form of the enzymes. The putative site of N-glycosylation is underlined with a double line and the putative active site is boxed. Numbering of amino acids corresponds to that used in figure 3.7). Amino acid residues in *XCI* that are identical with those of mouse TNAP are indicated by -.

Figure 4.2 shows the amino acid sequence encoded by *XCI* compared to that of the mouse TNAP sequence. The degree of homology is calculated to be 74%. The rat TNAP is 88% homologous to the human TNAP at the amino acid level (Misumi *et al*, 1988). The bovine enzyme is about 90% homologous with the human TNAP (Garatinni *et al*, 1987). Half of the non-homologous sites in the *Xenopus* sequence represent conservative changes, and it is expected that when the full-length sequence is known the degree of homology between the *Xenopus* and other TNAP's will be about 90%. It will then be found, just like the human TNAP, to be 52% homologous with the human PLAP (which reflects the evolutionary distance of TNAP from the other TSAP's), and 25% homologous with the *E. coli* AP (Weiss *et al*, 1986). It is the primary structure in the catalytically important regions that is conserved in all AP's, including the bacterial enzymes (Weiss *et al*, 1986; 1988).

4.4 HYBRIDISATION ANALYSIS

4.4.1 SOUTHERN BLOT ANALYSIS

It can be seen in figure 3.10 that the *Xenopus* genomic DNA has few sites for restriction enzyme XhoI and a relatively high number of HindIII sites. The completely digested DNA was blotted onto nylon filters and probed with labelled *XC1*. Clear, reproducible band patterns were obtained (figure 3.11) and a single-copy gene is indicated. The presence of a separate tissue-specific gene is evident from the different band patterns obtained when probing the same Southerns with labelled mouse IAP cDNA. It is not clear if both the TNAP and TSAP genes are expressed in the neurula embryo. Both isozymes are active in the mouse embryo (Hahnel *et al*, 1990). The presence of TNAP and IAP in the *Xenopus* embryo was tested by preparing Northern blots with mRNA from neurula tadpoles and probing with the mouse IAP. Preliminary results obtained suggested that only the TNAP gene is expressed. However, it seems likely that the TSAP gene is also expressed in the neurula stage since there is evidence that two isozymes occur in the *Xenopus* embryo (Maekawa & Yamana, 1975). The reason mouse probe failed to hybridise to the *Xenopus* TSAP mRNA is unclear and may simply be due to a too low a fraction of TSAP mRNA to be detected in the neurula stage. Further work is needed to determine whether an IAP gene is also expressed in the *Xenopus* embryo.

It is unlikely that there are any other AP genes in *Xenopus* analagous to the human PLAP and GCAP, since studies have shown that even in most mammalian species, including pigs, cats, dogs, hamsters, cows, sheep, guinea pigs and rats, only the TNAP (liver/bone/kidney) and the IAP occur (Goldstein *et al*, 1980). It is only in the higher primates, *viz.* chimpanzees, orangutans and humans that have other forms of AP's (Doellgast & Benirschke, 1979). It is interesting that mice and rabbits have also an embryonic form (EAP) that is closely related to the placental enzyme (Narisawa *et al*, 1992). This suggests that possibly the PLAP evolved from the EAP.

From figure 3.9B it is clear that the inserts that hybridised to *XCI* was not in fact a new or overlapping region of the *Xenopus* TNAP cDNA. This is because the signal obtained from the hybridised *XCI* is very high and indicates that the PCR amplified product is in fact *XCI* reisolated. RIAP failed to hybridise to the PCR amplified inserts that were initially isolated from screening the library with the rabbit probe. This indicates that the inserts were in fact false positives, *i.e.* that a TSAP gene was not obtained in this screening.

4.4.2 NORTHERN BLOT ANALYSIS

A clear, single band was obtained in Northern blots prepared with total neurula RNA, probed with labelled *XCI* (figure 3.17). The size of the mRNA hybridising to *XCI* could be estimated by comparisons of the 28S (5.5kb) and 18S (2.1kb) rRNA markers on the ethidium bromide stained blots that were prepared under identical conditions as those that were probed. These conditions included the voltage during RNA electrophoresis, the time of electrophoresis, the concentration of the formaldehyde-agarose gels, the physical dimensions of the gels and blots, and the amounts of RNA electrophoresed in each well. The size of the *Xenopus* TNAP mRNA is estimated to be about 2.5Kb which is the size of TNAP mRNA's from other animals. Although the TNAP mRNA is about five times larger than TSAP genes, the TNAP product is approximately the same size as the TSAP isozymes. The TNAP gene is large due mainly to intron size differences (Weiss *et al*, 1988).

4.5 CONCLUSION

There are several possible strategies that can be employed to isolate and characterise the *Xenopus* TNAP gene. In this work, previously isolated chicken and mouse TNAP cDNA's were chosen to be used as nucleic acid probes in the screening of a cDNA library. It was assumed that the *Xenopus* TNAP cDNA could be isolated on the basis of having a high degree of homology with these probes since all TNAP cDNA's so far sequenced share over 90% homology. With

reasonably high stringency conditions, the TNAP cDNA would be isolated and not any TSAP cDNA's. Several putative positive clones were obtained and generally both the chicken and mouse probes hybridised to the same clones. All true positives were found to contain the same part of the *Xenopus* TNAP cDNA. It seems that the rest of the mRNA was lost during construction of the library. The sequence obtained is considered to be part of the *Xenopus* TNAP gene on the basis that it was isolated with TNAP-specific probes, its high homology with the mouse TNAP, that it binds to mRNA in Northern blots that corresponds to the size of other TNAP mRNA's, the predicted signal peptide is analagous to that in other TNAP's and not to that in TSAP's, and the primary structure of all catalytically important regions included in the fragment isolated are conserved.

The fragment isolated here must now be used to screen alternative cDNA and genomic libraries to isolate the rest of the *Xenopus* TNAP cDNA and to characterise the entire gene. 5' and 3' fragments are available and can be used to ascertain whether the cDNA's obtained are sufficiently long to contain full-length message.

XC1 can now be used as a *Xenopus*-specific probe in the following studies:

1) Screening of genomic libraries to find the sequence of the whole gene, including introns and regulatory elements. What can be expected is a single major start for transcription approximately 25 nucleotides downstream of an A/T rich motif, a G/C-rich promoter region relatively abundant in the dinucleotide CpG, and three to four copies of the consensus sequence GGGCGG for SP1 binding (Weiss *et al*, 1988).
2) To investigate gene organisation and to identify and localise potential control elements that are relevant for the tissue-specific expression of the gene.
3) To employ Northern blot analysis to study the expression of the TNAP gene, both tissue- and temporally-specific.
4) To use the cDNA as a probe in *in situ* hybridisation studies in *Xenopus* embryos at different stages to elucidate the spatial and temporal distribution of AP

expression (presence of TNAP mRNA's), and possibly relate it to a developmental role.

5) To employ Southern blot analysis of restriction digests to analyse the complexity of the TNAP locus.
6) To investigate the function of TNAP by transfection of cDNA or genome clones into, for example, oocytes. The effect of overexpression by using strong expression vectors, and inhibition, such as by microinjecting antisense RNA into embryos, or by specifically abolishing TNAP expression in gene targeting experiments, could help elucidate the function of TNAP.

A cloned sequence allows for accurate manipulation of enzyme expression which could be used in studies of the enzyme's structure and function at the molecular level and its significance in morphogenesis. Other studies that could be carried out in the future include looking for restriction fragment length polymorphisms (RFLP) of the gene and to investigate TNAP polymorphism. One could also probe the chromosomal localisation of the TNAP gene and related sequences. The evolutionary relatedness of the *Xenopus* TNAP with other AP genes could also be studied.

APPENDIX A: Media and Solutions

Annealing Buffer (for sequencing)

Tris·HCl, pH 7.5	200 mM
$MgCl_2$	100 mM
NaCl	250 mM

Binding Buffer (for oligo(dT) columns)

Tris·HCl, pH 7.6	10 mM
LiCl	0.5 M
EDTA	1 mM
SDS	0.1%

Denaturing Gel Solution

Urea	46% (w/v)
TBE	1X
Acrylamide**	6%

** A solution of 38% acrylamide and 2% bisacrylamide was used when preparing the polyacrylamide gel.

Denaturation Solution (for screening and Southern blotting)

NaCl	1.5 M
NaOH	0.5 M

Denhardt's Solution (100X)

Polyvinylpyrrolidone	10 g
Bovine Serum Albumin	10 g
Ficoll 400	10 g
H_2O	To 500 ml
Filter Sterilise	

Elution Buffer (for oligo(dT) columns)

Tris·HCl, pH 7.6	10 mM
EDTA	1 mM
SDS	0.05%

Fixing Solution (for sequencing gels)

Methanol	12%
Glacial acetic acid	10%

Gel Loading Buffer (for DNA electrophoresis)

Glycerol	50%
TBE buffer	1X
Bromophenol blue	1%

Gel Loading Buffer (for RNA electrophoresis)

Glycerol	50%
Bromophenol blue	0.1%
Made up in DEPC-treated H_2O	

IPTG (isopropyl-ß-D-thiogalactopyranoside)

IPTG	100 mM
Made up in distilled H_2O	
Filter sterilised.	

Labelling Reaction Mix (5X)

Each of dCTP, dGTP, dTTP	1.5 μM

LB (Luria-Bertani) Broth

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g
H_2O	To 1L
pH	7.4

Autoclaved

LB Agar

Agar	15 g
LB Broth	To 1L

Autoclaved and poured into petri dishes.

LB Top Agarose

Agarose	7 g
LB Broth	To 1L

Autoclave

Ligase Buffer (10X)

Tris·HCl, pH 7.8	300 mM
$MgCl_2$	100 mM
DTT	100 mM
ATP	100 mM

Lysis Solution (for small-scale plasmid prep)

LiCl	2.5 M
Tris·HCl, pH 8.0	50 mM
Triton X-100	4% (v/v)
EDTA-Na_2	62.5 mM

Lysozyme Solution

Lysozyme	50 mg
Distilled H_2O	1 ml

Middle Binding Buffer (for oligo(dT) columns)

Tris·HCl, pH 7.6	10 mM
LiCl	100 mM
EDTA	1 mM
SDS	0.1%

Modified Barth's Saline

NaCl	88 mM
KCl	1 mM
$NaHCO_3$	2.4 mM
HEPES·NaOH	15 mM
$Ca(NO_3)_2 \cdot 4H_2O$	0.33 mM
$CaCl_2 \cdot 6H_2O$	0.41 mM
$MgSO_4 \cdot 7H_2O$	0.82 mM
pH	7.4

MOPS Buffer (10X)

EDTA	10 mM
Sodium acetate	50 mM
MOPS (3-[N-Morpholino]-propane-sulphonic acid	200 mM
pH	7.0

Neutralising Solution (used in screening)

NaCl	1.5 M
Tris·HCl (pH 7.4)	0.5 M

PBS (phosphate buffered saline)

NaCl	8 g
KCl	0.2 g
$Na_2HPO_4 \cdot 12H_2O$	2.9 g
KH_2PO_4	0.2 g
H_2O	To 1L
pH	7.4

Prehybridisation Solution (for screening)

Deionised formamide	50%
SSPE	5X
Denhardt's soln	5X
SDS	0.1%
Sonicated, denatured	
Herring Sperm DNA	100 μg/ml

Prehybridisation Solution (for Northern blots)

Deionised formamide	50%
SSPE	5X
Glycine	1%
Denhardt's soln	5X
Denatured, sonicated	
Herring Sperm DNA	0.4 mg/ml
SDS	0.1%

Made up in double-distilled H_2O

Prehybridisation Solution (for Southern blots)

Deionised formamide	50%
SSPE	5X
Denhardt's soln	5X
SDS	0.5%
Denatured, sonicated Herring Sperm DNA	20 μg/ml

SE Buffer

NaCl	75 mM
EDTA	25 mM
pH	8.0

SM Buffer

NaCl	100 mM
$MgSO_4 \cdot 7H_2O$	81.3 mM
Tris·HCl (pH 7.5)	50 mM
Gelatin	0.01%
H_2O	To 1L
Autoclave to sterilise	

Solution I (for large-scale plasmid prep)

Glucose	50 mM
Tris·HCl, pH 8.0	25 mM
EDTA-Na_2	10 mM

Solution II (for large-scale plasmid prep)

NaOH	0.2 N
SDS	1%

Solution III (for large-scale plasmid prep)

Potassium acetate (5 M)	60 ml
Glaciel acetic acid	11.5 ml
Distilled H_2O	28.5 ml
pH	4.8

Solution D (For RNA Extraction)

Guanidinium thiocyanate	4 M
Sodium citrate, pH 7.0	25 mM
Sarcosyl	0.5%
ß-mercaptoethanol	0.2 M

SSC Buffer (20X)

NaCl	3 M
$Na_3Citrate{\cdot}2H_2O$	0.3 M
pH	7.0

SSPE Buffer (20X)

NaCl	3.6 M
$NaH_2PO_4{\cdot}H_2O$	0.2 M
EDTA-Na_2	0.02 M
pH	7.4

Stop Solution

Formamide	95%
EDTA	20 mM
Bromophenol blue	0.05%
Xylene cyanol FF	0.05%

Supplemented LB medium

$MgSO_4$	10 mM
Maltose	0.2% (w/v)
Made up with LB broth	

Taq Polymerase Reaction Buffer (10X)

Tris, pH 8.3	100 mM
KCl	500 mM
$MgCl_2$	15 mM
Gelatin	0.1%

TBE Buffer (20X)

Tris·HCl, pH 7.4	1 M
Boric Acid	1 M
EDTA $Na \cdot 2H_2O$	20 mM

TE Buffer

Tris·HCl, pH 7.4	10 mM
EDTA, pH 8.0	0.1 mM

Termination Nucleotide Mixtures

Each dNTP	80 μM
NaCl	50 mM
One ddNTP	8 μM

X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)

X-gal	10%
Made up in dimethylformamide	

APPENDIX B: Bacterial Strains and Vectors

Bacterium *E. coli* C600 hflA (BNN 102)

Genotype: *e*14-(*mcrA*), *supE*44, *hsd*R, *thi*-1, *thr*-1, *leuB*6, *lacY*1, *tonA*21, *hflA*150[*chr*::Tn*10*(*tet*^{r})]

The construction of this strain is described in Young and Davis (1983). This strain was supplied by Invitrogen. It has a number of features which make it particularly useful in the present work, *viz.* in the screening of λgt10 libraries. It has long been considered an ideal *E. coli* host strain for use with λgt10 vectors since it lowers the frequency of background plaques (Young & Davis, 1983). It is a suppressing strain often used for making lysates (Sambrook *et al*, 1989) and for the propogation of λgt10 (Young & Davis, 1983). The *hflA* phenotype, useful for making lysates, may be maintained on medium containing 15 μg/ml tetracycline. Such an *hflA* strain is used to select λgt10 recombinants. The high frequency lysogeny mutation suppresses plaque formation by cI^+ bacteriophages but allows plaque formation by recombinant cI^- bacteriophages (Young & Davis, 1983). Since this strain is restriction minus (*hsdR*), cDNA inserts are not susceptible to host restriction enzymes.

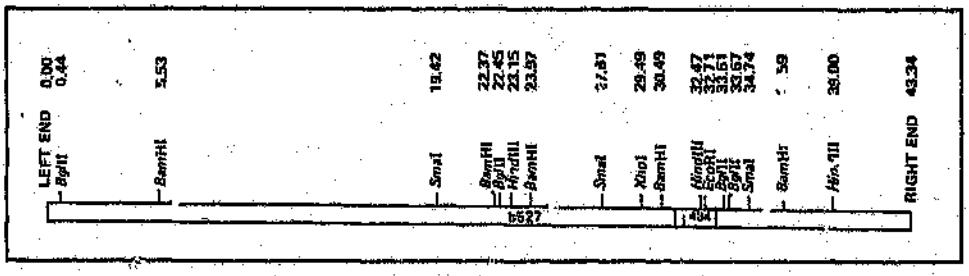
Bacterium *Epicurian coli* XL1-Blue

Genotype: Δ(*mcr*A)183, Δ(mcr CB-*hsd*SMR-*mrr*)173, recA$^-$ (*recA*1, *lac*$^-$, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, *supE*44, *relA*1, {F' proAB, *lacI*^{q}, *lacZ*ΔM15, Tn10})

This bacterial strain is currently the preferred host for pBluescript II vectors and

was developed at Stratagene (Bullock *et al*, 1987). Cells of this strain are deficient in all known restriction systems [Δ(*mcr*A)183, Δ(mcr CB-*hsd*SMR-*mrr*)173] and thus cloned cDNA that is cytosine- or adenine-methylated is not cleaved. The *end*A1 mutation renders the strain endonuclease-deficient and this greatly improves the quality of plasmid miniprep DNA (Sambrook *et al*, 1989). There is also little chance of there being any homologous recombination when cloning DNA in this strain; insert stability is ensured, due to the *rec*A mutation. The strain allows blue/white colour selection for pBluescript phagemids because of the *lac*ZΔM15 complementation on the F' episome. This colour selection is seen when plating on LB plates supplemented with ampicillin (to maintain the phagemid), X-gal and IPTG. Colonies containing phagemids without inserts will be blue after incubation for 12 - 18 hours at 37°C. Colonies with phagemids containing inserts remain white. The colour assay is not possible with strains that have lost the F' episome. The episome is maintained on media containing 12.5 μg/ml tetracycline. Plating on media with tetracycline and ampicillin selects for colonies that carry both the F' and the phagemid and this reduces the background of false positives. Another interesting feature is that the F' episome encodes for pili allowing infection by filamentous phage such as M13. Thus single-strand rescue from phagemid vectors is possible, which is useful for sequencing or mutagenesis studies.

Vector λgt10 (*imm*^{434}*b527*)

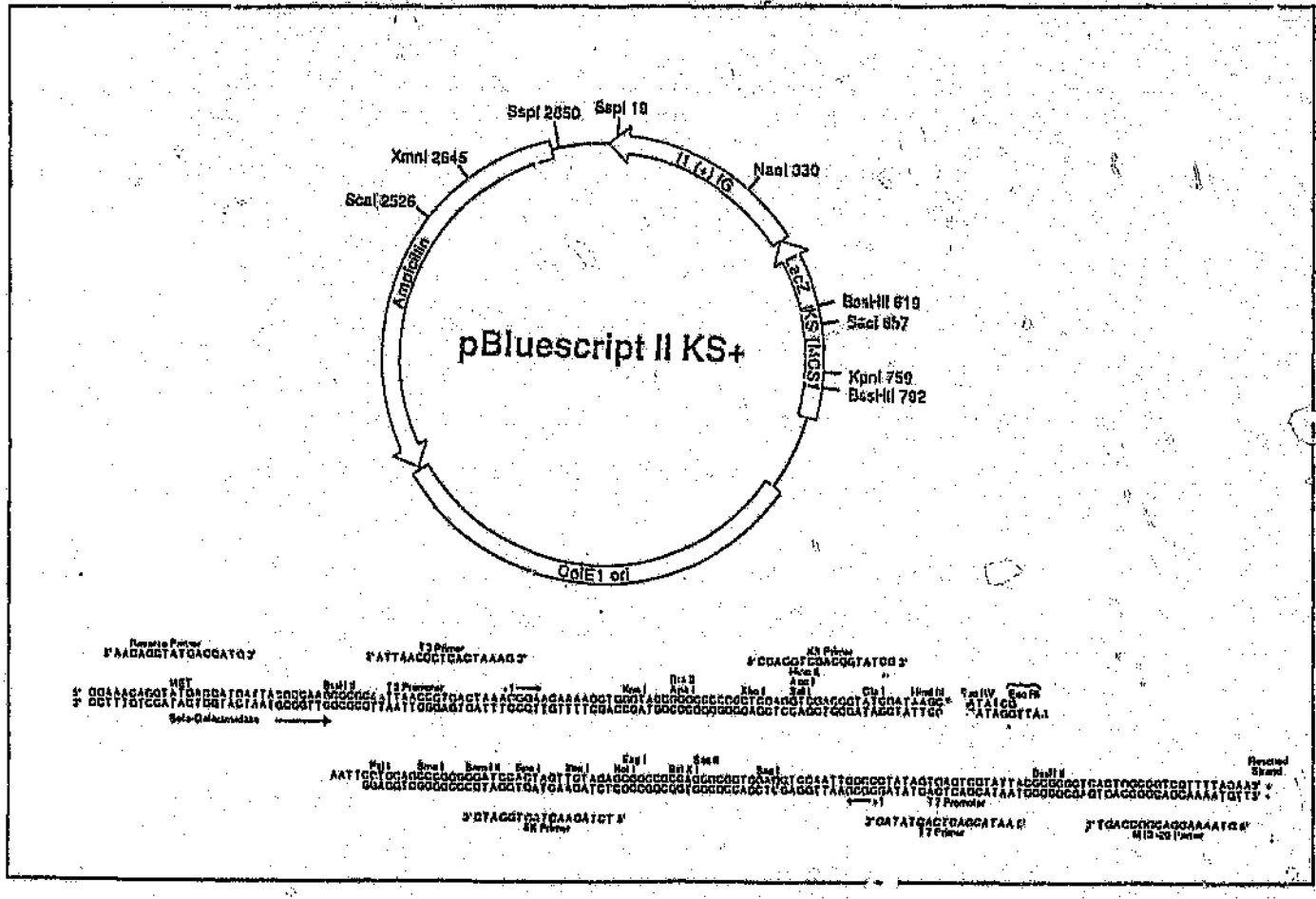


Map of λgt10 (*imm*^{434}*b527*). Restriction endonuclease sites are indicated in kbp's from the left end.

Bacteriophage λgt10 is widely used in the construction of libraries to be screened with nucleic acid probes. There is an EcoRI site within the phage repressor gene *c*I. Insertion of a DNA fragment into this site generates a *c*I$^-$ phage, which forms

a plaque with a clear center. A non-recombinant cI^+ phage forms a turbid plaque (Huynh *et al*, 1985). The use of such an immunity insertion vector enables the selection for recombinant phage. However, since λgt10 is still able to package its DNA without an insert, cDNA libraries cloned in λgt10 may initially consist of 70% to 99% parent phage lacking inserts (Huynh *et al*, 1985). This problem is eliminated by selecting against the non-recombinant parent phage during amplification of the library. A strain of *E. coli* that carries the *high frequency of lysogeny* mutation *hfl*A, such as C600, suppresses growth of non-recombinant cI^+ so that there is no plaque formation. cI^- phage with cDNA insert forms plaques efficiently on the *hfl*A strain. An advantage when using λgt10 vectors in constructing a library, is that it avoids the problem of loss of certain sequences due to expression that leads to a product toxic to phage or host cell growth.

Vector pBluescript II



pBluescript II phagemids (plasmids with a phage origin) are cloning vectors that have been designed by Stratagene to simplify commonly used cloning and sequencing procedures. Construction of nested deletions for DNA sequencing, generation of RNA transcripts *in vitro*, site specific mutagenesis and gene mapping are some of the procedures that can be performed with these vectors. These vectors have an extensive polylinker with 21 unique restriction sites. The polylinkers are flanked by functional T3 and T7 RNA polymerase promoters and either strand of a cloned insert can be transcribed *in vitro*. The polylinker and T3 and T7 RNA polymerase promoters are located at the N-terminal portion of a *Lac*Z gene fragment. Phagemids with no insert, when grown on strains of bacteria that lack the α-peptide portion of the ß-galactosidase gene, produce blue colonies, whereas recombinant phagemid, in such strains, results in white colonies.

pBluescript II contains the f1 origin, a 454 nucleotide intergenic region (M13 related) that contains all the *cis*-acting elements required for DNA replication and phage morphogenesis. This region allows pBluescript II phagemids to be rescued from F' bearing bacteria (superinfected with helper phage) as single-stranded DNA which is useful for dideoxynucleotide sequencing, or even site-specific mutagenesis. pBluescript II is available with two orientations of both the polylinker (SK and KS) and the f1 origin (+,-). The f1(+) orientation indicates the sense strand will be rescued upon superinfection with helper phage. (-) indicates the non-sense strand will be rescued (relative to the ß-galactosidase gene. The KS orientation indicates that the KpnI site is nearest to the *lac*Z promoter and the SacI site furthest from it. The vector used in this work is pBluescript II KS (+). (See figure). Note the primer binding sites used during sequencing. The BssHII sites can be used to cleave out insert plus T phage RNA promoters for gene mapping procedures.

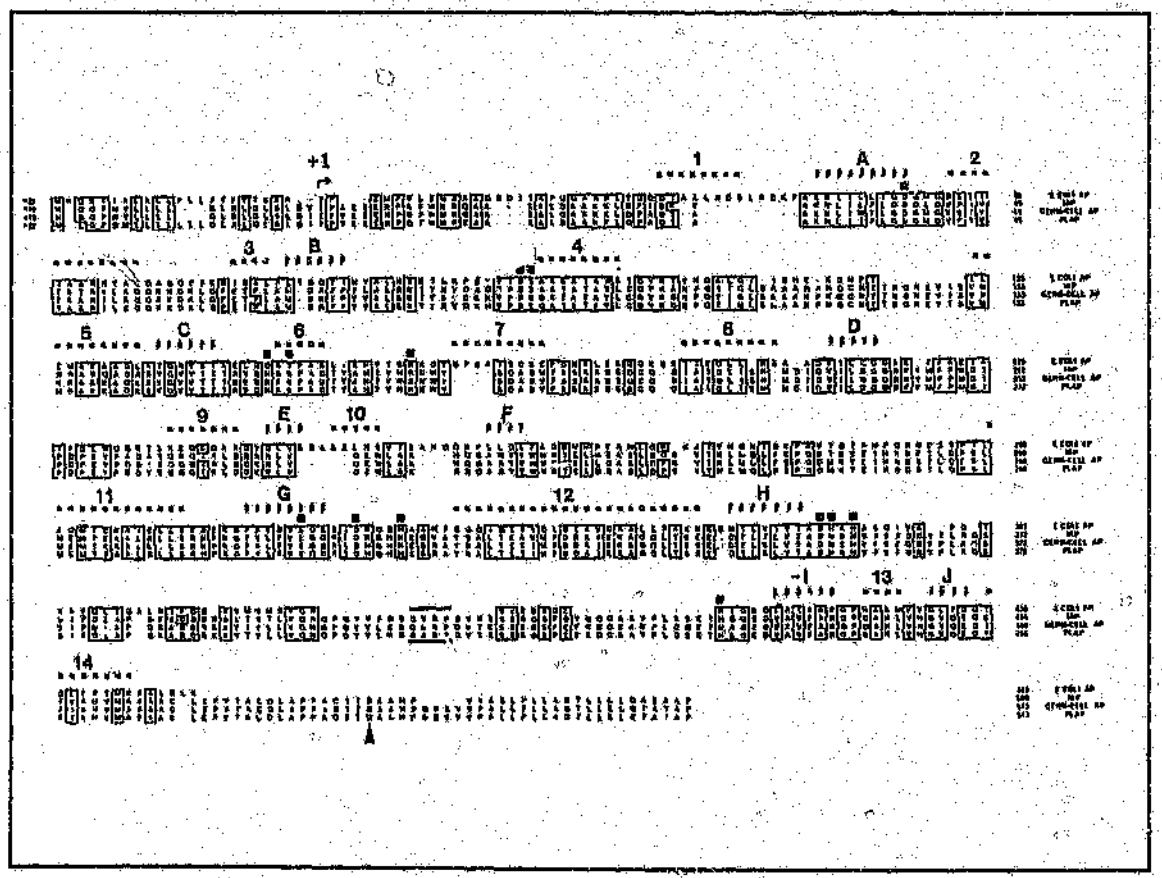
APPENDIX C: List of Suppliers

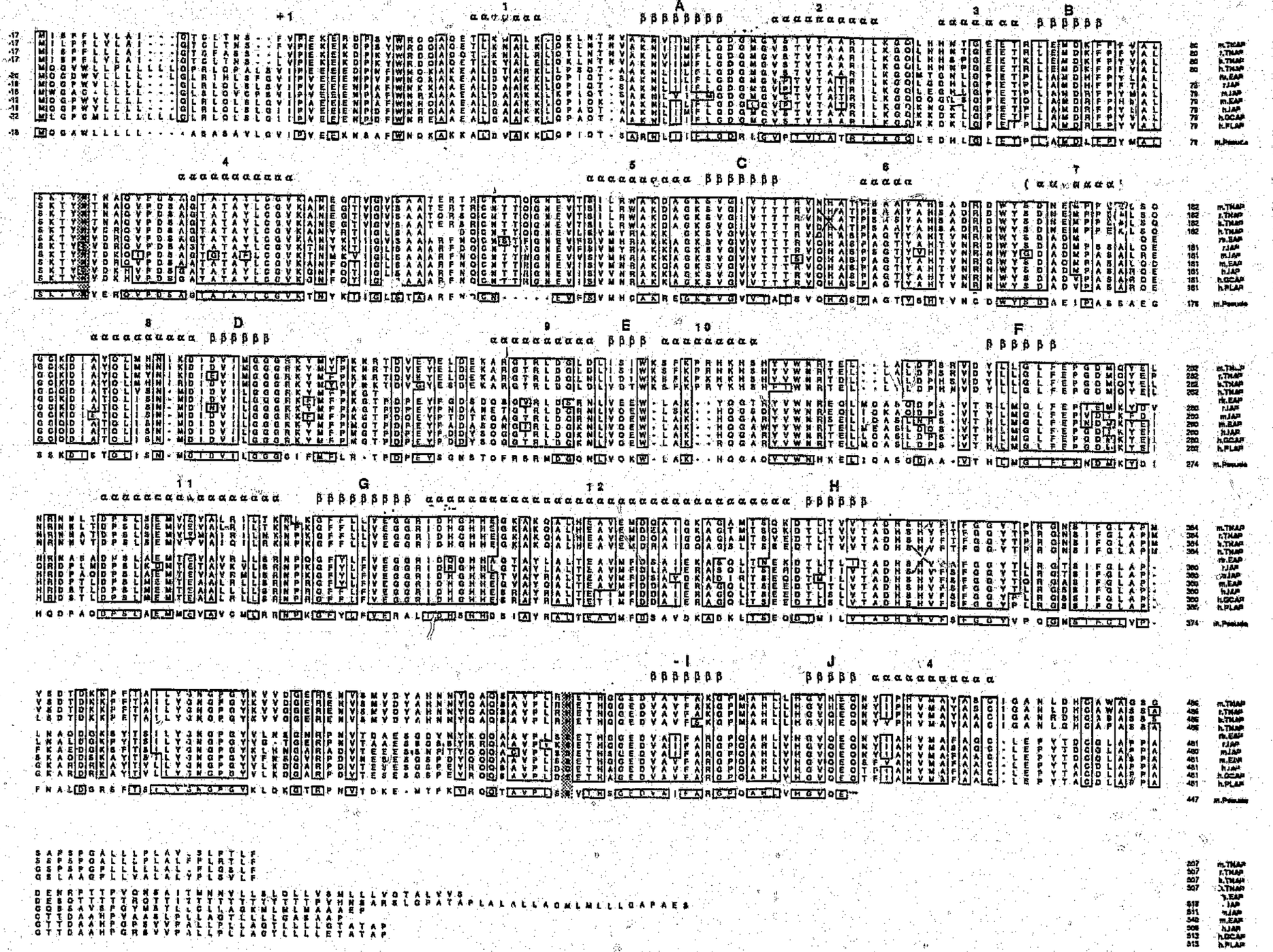
Company	Product
Amersham	Nylon Membranes
Beckman	Polyallomer centrifuge tubes
Bio-Rad	Mixed-bed resin AG 501-X8
Boehringer Mannheim	Molecular weight markers
	MOPS buffer
	Oligo(dT)-cellulose
	Restriction enclonucleases
	Random Primed DNA labelling kit
Clontech	Lambda gt10 cDNA insert screening amplimers
DuPont	[α-^{32}P]dCTP
Invitrogen	*E. coli* strain C600hfl
Kodak	XAR-5 X-ray film
Nalgene	Plastic centrifuge tubes
Promega	Herring sperm DNA
Perkin Elmer Cetus	Taq polymerase
Stratagene	*Epicurian coli* strain XL-1 Blue
	Vector pBluescript II KS^{+}
	KS, SK, T3, T7 primers
U S Biochemical	Sequenase version 2.0 dideoxynucleotide sequencing kit
Worthington Biochemical	Sephadex G50 minispin columns

APPENDIX D: Published Amino Acid Sequences of Various Alkaline Phosphatases

The chart on the next page has 12 mammalian AP's optimally aligned. All AP's, including the bacterial enzymes have been aligned in this way, illustrating the degree of homology at the amino acid level shared amongst them. Such alignment has shown that all conserved residues occur in the same position.

Below is a chart, from Millán (1990), that compares *E. coli* AP to the human isozymes.





APPENDIX E: Amino Acid Abbreviations

Amino Acid	3 Letter Abbreviation	1 Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Pyroglutamyl	<Glu	<E
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

APPENDIX F: The Genetic Code

First Position (5' end)	Second Position U	C	A	G	Third Position (3' end)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Ter*	Ter*	A
	Leu	Ser	Ter*	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Gly	Gly	A
	Val**	Ala	Glu	Gly	G

*Chain terminating

**Codes for Met if in the initiator position which is rare

REFERENCES

Abe, H., Miyahara, E. and Yamana, K. (1972). Synthesis of alkaline phosphatase in embryonic cells during aggregate formation. *Nature* **235**: 254.

Abelev, G. I. (1971). Alpha-fetoprotein in oncogenesis and its association with malignant tumours. *Adv. Cancer Res.* **14**: 295-558.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognised by a TPA-modulated *trans*-acting factor. *Cell* **49**: 729-739.

Applebury, M. L., Johnson, B. P. and Coleman, J. E. (1970). Phosphate binding to alkaline phosphatase. *J. Biol. Chem.* **245**: 4968-4976.

Axel, R., Fiegelson, P. and Schutz, G. (1976). Analysis of the complexity and diversity of mRNA from chicken oviduct and liver. *Cell* **11**: 247-254.

Axton, J. M., Dombreadi, V., Cohen, P. T. W. and Glover, D. M. (1990). One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell* **63**: 33-46.

Badger, K. S. and Sussman, H. H. (1976). Structural evidence that human liver and intestinal alkaline phosphatases are encoded by different genes. *Proc. Natl. Acad. Sci. USA* **73**: 2201-2205.

Beckman, G. and Beckman, L. (1975). The placental alkaline phosphatase variant D and natural selection. *Hereditas* **81**: 85-88.

Beertsen, W. and van de Bos, T. (1992). Alkaline phosphatase induces the mineralisation of sheets of collagen implanted subcutaneously in rat. *J. Clin. Invest.* **89**: 1974-1980.

Behrens, C. M., Enns, C. A. and Sussman, H. H. (1983). Characterisation of human fetal intestinal alkaline phosphatase. Comparison with the isozymes from the adult intestine and human tumour cell lines. *Biochem. J.* **211**: 553-558.

Benham, F. J., Fogh, J. and Harris, H. (1981). Alkaline phosphatase expression in human cell lines derived from various malignancies. *Int. J. Cancer* **27**: 637-644.

Berger, J., Garattini, E., Hua, J-C. and Udenfriend, S. (1987). Cloning and sequencing of human intestinal alkaline phosphatase cDNA. *Proc. Natl. Acad. Sci. USA* **84**: 695-698.

Biggin, M. D., Gibson, T. J. and Hong, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA* **80**: 3963-3965.

Bodansky, A. (1933). Phosphatase studies II. Determination of serum phosphatase. Factors influencing the accuracy of the determination. *J. Biol. Chem.* **101**: 93-104.

Brachet, J. (1946). Localisation de la phosphatase alcaline pendant le développement des Batraciens. *Experientia* **2**: 143.

Brachet, J. and Jeener, R. (1948). Recherches sur le rôle de la phosphatase alcalines des noyaux. *Biochim. Biophys. Acta* **2**: 423-430.

Bradshaw, R. A., Cancedda, F., Ericsson, L. H., Neumann, P. A., Piccoli, S. P., Schlesinger, M. J., Shriefer, K. and Walsh, K. A. (1981). Amino acid sequence of *Escherichia coli* phosphatase. *Proc. Natl. Acad. Sci. USA* **78**: 3473-3477.

Bullock, W. O., Fernandez, J. M. and Short, J. M. (1987). XL-1 Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5**: 376-379.

Butler-Ransohoff, J. E., Kendall, D. A. and Kaiser, E. T. (1988). Use of site-directed mutagenesis to elucidate the role of arginine-166 in the catalytic mechanism of alkaline phosphatase. *Proc. Natl. Acad. Sci. USA* **85**: 4276-4278.

Butler-Ransohoff, J. E., Rokita, S. E., Kendall, D. A., Banzon, J. A., Carano, K. S., Kaiser, E. T. and Matlin, A. R. (1992). Active-site mutagenesis of *E. coli* alkaline phosphatase: replacement of Serine-102 with nonnucleophilic amino acids. *J. Org. Chem.* **57**: 142-145.

Butterworth, J. (1986). Changes in nine enzyme markers for neurons, glia, and endothelial cells in agonal state and Huntington's disease caudate nucleus. *J. Neurochem.* **47**: 583-587.

Byers, D. A., Fernley, H. N. and Walker, P. G. (1972). Studies on alkaline phosphatase. Inhibition of human-placental phosphoryl phosphatase by L-phenylalanine. *Eur. J. Biochem.* **29**: 197-204.

Chaidaroglou, A., Brezinski, D. J., Middelton, S. A. and Kantrowitz, E. R. (1988). Function of Arginine-166 in the active site of *Escherichia coli* alkaline phosphatase. *Biochemistry* **27**: 8338-8343.

Chaidaroglou, A. and Kantrowitz, E. R. (1989). Alteration of aspartate 101 in the active site of *Escherichia coli* alkaline phosphatase enhances the catalytic activity. *Prot. Engin.* **3**: 127-132.

Chang, C. H., Angellis, D. and Fishman, W. H. (1980). Presence of the rare D-variant heat-stable, placental-type alkaline phosphatase in normal human testis. *Cancer Res.* **40**: 1506-1510.

Chlebowski, J. F., Armitage, I. M., Tusa, P. P, and Coleman, J. E. (1976). ^{31}P NMR of phosphate and phosphate complexes of metalloalkaline phosphatases. *J. Biol. Chem.* **251**: 1207-1216.

Clark, J. M. (1988). Novel non-templated nucleotide addition reactions catalysed by prokaryotic and eukaryotic DNA polymerases. *Nucl. Acids Res.* **16**: 9677-9686.

Coleman, J. E. (1992). Structure and mechanism of alkaline phosphatase. *Annu. Rev. Biophys. Biomol. Struct.* **21**: 441-483.

Coleman, J. E. and Besman, M. J. A. (1987). In *Hydrolytic Enzymes*, ed. Neuberger, A., Brocklehurst, K., pp. 377-406. Elsevier, New York.

Coleman, K. E. and Gettins, P. (1983). Alkaline phosphatase, solution structure, and mechanism. *Adv. Enzymol.* **55**: 381-452.

Cross, G. A. M. (1987). Eukaryotic protein modification and membrane attachment via phosphatidylinositol. *Cell* **48**: 179-181.

Cyert, M. S. and Thorner, J. (1989). Putting it on and taking it off: phosphoprotein phosphatase involvement in cell-cycle regulation. *Cell* **57**: 891-893.

Dayan, J. and Wilson, I. B. (1964). The phosphorylation of Tris by alkaline phosphatase. *Biochim. Biophys. Acta* **81**: 620-623.

Derman, A. I. and Beckwith, J. (1991). *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. *J. Bacteriology* **173**: 7719-7722.

Doellgast, D. J. and Benirschke, K. (1979). Placental alkaline phosphatase in Hominidae. *Nature* **280**: 601-602.

Doellgast, G. J. and Fishman, W. H. (1976). L-leucine. A specific inhibitor of a rare human placental alkaline phosphatase phenotype. *Nature* **259**: 49-51.

Donald, L. J. and Robson, E. B. (1974). Rare variants of placental alkaline phosphatase. *Ann. Hum. Genet.* **37**: 303-313.

Engstrom, L. (1961). Studies on calf-intestinal alkaline phosphatase, incorporation of inorganic phosphate into a highly purified enzyme preparation. *Biochim. Biophys. Acta* **52**: 49-59.

Engstrom, L. (1962). Incorporation of inorganic phosphate into alkaline phosphatase from *Escherichia coli*. *Biochim. Biophys. Acta* **56**: 606-609.

Enoch, T. and Nurse, P. (1991). Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. *Cell* **65**: 921-923.

Fedde, K. N. and Whyte, M. P. (1990). Alkaline phosphatase (tissue-nonspecific isoenzyme) is a phosphoethanolamine and pyridoxal-5'-phosphate ectophosphatase: normal and hypophosphatasia fibroblast study. *Am. J. Hum. Genet.* **47**: 767-775.

Fishman, L., Miyayama, H., Driscoll, S. G. and Fishman, W. H. (1976). Developmental phase specific alkaline phosphatase isoenzymes of human placenta and their occurence in human cancer. *Cancer Res.* **36**: 2268-2273.

Fishman, W. H. (1974). Perspectives on alkaline phosphatase isozymes. *Am. J. Med.* **56**: 617-650.

Fishman, W. H. (1989). On the importance of being (stereo) specific. *Clin. Chim. Acta* **186**: 129-132.

Fishman, W. H. (1990). Alkaline phosphatase isozymes: recent progress. *Clin. Biochem.* **23**: 99-104.

Fishman, W. H. and Sell, S. (eds.). (1976). *Oncodevelopmental Gene Expression*. Academic Press, Inc., New York, San Fransisco, London.

Fishman, W. H. and Sie, H. G. (1971). Organ-specific inhibition of human alkaline phosphatase isoenzymes of liver, bone, intestine and placenta; L-phenylalanine, L-tryptophan and L-homoarginine. *Enzymologia* **41**:141-167.

Fishman, W. H., Green, S. and Inglis, N. I. (1963). L-Phenylalanine: An organ specific, stereo-specific inhibitor of human intestinal alkaline phosphatase. *Nature* **198**: 685-686.

Fishman, W. H., Inglis, N. I. and Krant, M. J. (1965). Serum alkaline phosphatase of intestinal origin in patients with cancer and with cirrhosis of the liver. *Clin. Chim. Acta* 12 : 298-303.

Fishman, W. H., Inglis, N. I., Green, S., Antiss, C. L., Gosh, N. K., Reif, A. E., Rustigian, R., Krant, M. J. and Stolbach, L. L. (1968). Immunology and biochemistry of regan isozyme of alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res.* **28**: 150-154.

Folley, S. J. and Kay, H. D. (1935). The alkaline phosphomonoesterase of the mammary gland. *Biochem. J.* **29**: 1837-1850.

Fröhlander, N. and Millán, J. L. (1991). Alkaline phosphatases as reporters of developmental and cancerous events. *in vivo* **5**: 483-488.

Garattini, E., Hua, J. -C. and Udenfriend, S. (1987). Cloning and sequencing of bovine kidney alkaline phosphatase cDNA. *Gene* **59**: 41-46.

Gettins, P., Metzler, M. and Coleman, J. E. (1985). Alkaline phosphatase. ^{31}P NMR probes of the mechanism. *J. Biol. Chem.* **260**: 2875-2883.

Ghosh, N. K. and Fishman, W. H. (1966). On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. *J. Biol. Chem.* **241**: 2516-2522.

Ghosh, S. S., Bock, S. C., Rokita, S. E. and Kaiser, E. T. (1986). Modification of the active site of alkaline phosphatase by site-directed mutagenesis. *Science* **231**: 145-148.

Gold, P. and Freedman, S. O. (1965). Specific carcinoembryonic antigens of the human digestive system. *J. Exp. Med.* **122**: 467-481.

Goldstein, D. J. and Harris, H.(1979). Human placental alkaline phosphatase differs from that of other species. *Nature* **280**: 602-605.

Goldstein, D. J., Rogers, C. E. and Harris, H. (1980). Expression of alkaline phosphatase loci in mammalian tissues. *Proc. Natl. Acad. Sci. USA* **77**: 2857-2860.

Goldstein, D. J., Rogers, C. and Harris, H. (1982a). Evolution of alkaline phosphatases in primates. *Proc. Natl. Sci. USA* **79**: 879-883.

Goldstein, D. J., Rogers, C. E. and Harris, H. (1982b). A search for trace expression of placental-like alkaline phosphatase in non-malignant human tissues: demonstration of its occurance in lung, cervix, testis and thymus. *Clin. Chim. Acta* **125**: 63-75.

Graves, B. J., Johnson, P. F. and McKnight, S. L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**: 565-576.

Green, S., Anstiss, C. L. and Fishman, W. H. (1971). Automated differential isoenzyme analysis: II. The fractionation of serum alkaline phosphatases into "liver," "intestinal" and "other" components. *Enzymologia* **41**: 9-26.

Greene, P. J. and Sussman, H. H. (1973). Structural comparison of ectopic and normal alkaline phosphatase. *Proc. Natl. Acad. Sci. USA* **70**: 2936-2940.

Griffin, C. A., Smith, M., Henthorn, P. S., Harris, H., Weiss, M. J., Raducha, M. and Emanuel, B. S. (1987). Human placental and intestinal alkaline phosphatase genes map to 2q34-q37. *Amer. J. Hum. Genet.* **41**: 1025-1034.

Griffin, M. J. and Cox, R. R. (1966). Studies on the mechanism of hormone induction of alkaline phosphatase in human cell cultures II. Rate of enzyme synthesis and properties of base level and induced enzymes. *Proc. Natl. Acad. Sci. USA* **56**: 946-953.

Grosser, P. and Husler, J. (1912). Ueber das Vorkommen einer Glycerophosphatase in tierischen Organen. *Biochem. Z.* **39**: 1-5.

Gubler, U. and Hoffman, B. J. (1983). A simple and and very efficient method for generating cDNA libraries. *Gene* **26**: 263-269.

Gutman, A. B. (1959). Serum alkaline phosphatase activity in diseases of the skeletal and hepatobiliary systems. *Am. J. Med.* **27**: 875-901.

Hahnel, A. C. and Schultz, G. A. (1989). Cloning and characterisation of a cDNA encoding alkaline phosphatase in mouse embryonal carcinoma cells. *Clin. Chimi. Acta* **186**: 171-174.

Hahnel, A. C., Rappolee, D. A., Millán, J. L., Manes, T., Ziomek, C. A., Theodosiou, N. G., Werb, Z., Pedersen, R. A. and Schultz, G. A. (1990). Two alkaline phosphatase genes are expressed during early development in the mouse embryo. *Development* **110**: 555-564.

Harris, H. (1979). Multilocus enzymes in man. In, *Human Genetics: Possibilities and Realities*, ed. Porter, R. and O'Connor, M., pp. 187-204. (Ciba Foundation Symposium 66). Excerpta Medica, Amsterdam, Oxford, New York.

Harris, H. (1989). The human alkaline phosphatases: what we know and what we don't know. *Clin. Chim. Acta* **186**: 137-150.

Hawrylak, K. and Stinson, R. A. (1988). The solubilisation of tetrameric alkaline phosphatase from human liver and its conversion into various forms by phosphatidylinositol phospholipase C or proteolysis. *J. Biol. Chem.* **263**: 14368-14373.

He, M., Wilde, A. and Kaderbhai, M. A. (1990). A simple single-step procedure for small-scale preparation of *Escherichia coli* plasmids. *Nucl. Acids Res.* **18**: 1660.

Heidecker, G. and Messing, J. (1983). Sequence analysis of Zein cDNA's by an efficient mRNA cloning method. *Nucleic Acids Res.* **11**: 4891-4906.

Hendrix, P. G., Hoylaerts, M. F., Nouwen, E. J. and De Broe, M. E. (1990). Enzyme immunoassay of human placental and germ-cell alkaline phosphatase in serum. *Clin. Chem.* **36**: 1793-1799.

Henthorn, P. S., Knoll, B. J., Raducha, M., Rothblum, K. N., Slaughter, C., Weiss, M., Lafferty, M. A., Fischer, T. and Harris, H. (1986). Products of two common alleles at the locus for human placental alkaline phosphatase differ by seven amino acids. *Proc. Natl. Acad. Sci. USA* **83**: 5597-5601.

Henthorn, P. S., Raducha, M., Edwards, Y. H., Weiss, M. J., Slaughter, C., Lafferty, M. A. and Harris, H. (1987). Nucleotide and amino acid sequences of human intestinal alkaline phosphatase: Close homology to placental alkaline phosphatase. *Proc. Natl. Acad. Sci. USA* **84**: 1234-1238.

Henthorn, P. S., Raducha, M., Kadesch, T., Weiss, M. J. and Harris, H. (1988). Sequence and characterisation of the human intestinal alkaline phosphatase gene. *J. Biol. Chem.* **263**: 12011-12019.

Higashino, K., Hashinotsume, M., Yang, K. Y., Takahashi, Y. and Umamura, Y. (1972). Studies on a variant alkaline phosphatase in sera of patients with hepatocellular carcinoma. *Clin. Chim. Acta* **40**: 67-81.

Higuchi, R., Paddock, G. V., Wall, R. and Salser, W. (1976). A general method for cloning eukaryotic structural gene sequences. *Proc. Natl. Acad. Sci. USA* **73**: 3146-3150.

Hirano, K., Domar, U. M., Yamamoto, H., Brehmer-Anderson, E. E., Wahren, B. E. and Stigbrand, T. (1987). Levels of alkaline phosphatase isozymes in human seminoma tissue. *Cancer Res.* **47**: 2543-2546.

Hofmann, M. -C., Jeltsch, W., Brecher, J. and Walt, H. (1989). Alkaline phosphatase isozymes in human testicular germ cell tumours, their precancerous stage and three related lines. *Cancer Res.* **49**: 4696-4700.

Horiuchi, T., Horiuchi, S. and Mizunon, D. (1959). A possible negative feedback phenomenon controlling formation of alkaline phosphomonoesterase in *Escherichia coli*. *Nature* **183**: 1529-1530.

Hoylaerts, M. F. and Millán, J. L. (1991). Site-directed mutagenesis and epitope-mapped monoclonal antibodies define a catalytically important conformational

difference between human placental and germ cell alkaline phosphatase. *Eur. J. Biochem.* **202**: 605-616.

Hoylaerts, M. F., Manes, T. and Millán, J. L. (1992). Molecular mechanism of uncompetitive inhibition of human placental and germ-cell alkaline phosphatase. *Biochem. J.* **286**: 23-30.

Hsu, H. T. H. (1991). The partial sequencing of human non-specific (bone/liver/kidney) alkaline phosphatase gene. *Intl. J. Biochem.* **24**: 391-393.

Hsu, H. T. H. and Anderson, H. C. (1989). The isolation and partial sequencing of human bone alkaline phosphatase gene. *Intl. J. Biochem.* **21**: 847-851.

Hua, J-C., Berger, J., Pan, Y-C. E., Hulmes, J. D. and Udenfriend, S. (1986). Partial sequencing of human adult, human fetal, and bovine intestinal alkaline phosphatases: Comparison with the human placental and liver isozymes. *Proc. Natl. Acad. Sci. USA* **83**: 2368-2372.

Hugon, J. and Borgers, M. (1966). Ultrastructural localisation of alkaline phosphatase activity in the absorbing cells of the duodenum of mouse. *J. Histochem. Cytochem.* **14**: 629-635.

Hulett, F. M., Kim, E. E., Bookstein, C., Kapp, N. V., Edwards, C. W. and Wyckoff, H. W. (1991). *Bacillus subtilis* alkaline phosphatases III and IV. *J. Biol. Chem.* **266**: 1077-1084.

Hull, W. E., Halford, S. E., Gutfreund, H. and Sykes, B. D. (1976). ^{31}P nuclear magnetic resonance study of alkaline phosphatase: The role of inorganic phosphate in limiting the enzyme turnover rate at alkaline pH. *Biochemistry* **15**: 1547-1561.

Hummer, C. and Millán, J. L. (1991). Gly^{429} is the major determinant of uncompetitive inhibition of human germ cell alkaline phosphatase by L-leucine. *Biochem. J.* **274**: 91-95.

Hustin, J., Collette, J. and Franchimont, P. (1987). Immunohistochemical demonstration of placental alkaline phosphatase in various states of testicular development and in germ-cell tumors. *Int. J. Androl.* **10**: 29-35.

Huynh, T. V., Young, R. A. and Davis, R. W. (1985). Constructing and screening cDNA libraries in λgt10 and λgt11. In, *DNA Cloning: A Practical Approach*, ed. Glover, D. M., Vol 1, pp49-78. IRL Press, Oxford.

Imagawa, M., Chiu, R. and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C a. cAMP. *Cell* **51**: 251-260.

International Union of Biochemistry (1972). *Enzyme Nomenclature: Recommendations of the Commission on Biochemical Nomenclature on Nomenclature and Classification of Enzymes together with their Units and Symbols of Enzyme Kinetics*. Elsevier, Amsterdam, 1973.

Jacoby, B. and Bagashawe, K. D. (1971). Placental-type alkaline phosphatase from human tumor tissue. *Clin. Chim. Acta* **35**: 473-481.

Jeppsson, A., Wahren, B., Brehmer-Andersson, E., Silvfersward, C., Stigbrand, T. and Millán, J. L. (1984). Eutopic expression of placental-like alkaline phosphatase in testicular tumours. *Int. J. Cancer* **34**: 757-761.

Kaiser, K. and Murray, N. E. (1985). The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. In, *DNA Cloning: A Practical Approach*, ed. Glover, D. M., Vol. 1, pp. 1-47. IRL Press, Oxford.

Kam, W., Clauser, E., Kim, Y. S., Kan, Y. W. and Rutter, W. J. (1985). Cloning, sequencing and chromosomal localisation of human term placental alkaline phosphatase cDNA. *Proc. Natl. Acad. Sci. USA* **82**: 8715-8719.

Karczmar, A. G. and Berg, G. G. (1951). Alkaline phosphatase during limb development and regeneration of *Amblystoma opacum* and *Amblystoma punctatum*. *J. Exp. Zool.* **117**: 139-163.

Kim, E. E. and Wyckoff, H. W. (1989). Structure of alkaline phosphatases. *Clin. Chim. Acta* **186**: 175-187.

Kim, E. E. and Wyckoff, H. W. (1991). Reaction mechanism of alkaline phosphatase based on crystal structures. *J. Mol. Biol.* **218**: 449-464.

Klionsky, D. J. and Erm, S. D. (1989). Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. *EMBO J.* **8**: 2241-2250.

Knoll, B. J., Rothblum, K. N. and Longley, M. (1987). Two gene duplication events in the evolution of the human heat-stable alkaline phosphatase. *Gene* **60**: 267-276.

Knoll, B. J., Rothblum, K. N. and Longley, M. (1988). Nucleotide sequence of the human placental alkaline phosphatase gene. *J. Biol. Chem.* **263**: 12020-12027.

Kohwi, Y. and Kohwi-Shigematsu, T. (1988). Magnesium ion-dependent triple-helix structure formed by homopurine-homopyrimidine sequences in supercoiled plasmid DNA. *Proc. Natl. Acad. Sci. USA* **85**: 3781-3785.

Koide, O., Iwai, S., Baba, K. and Iri, H. (1987). Identification of testicular atypical germ cells by an immunohistochemical technique for placental alkaline phosphatase. *Cancer* **60**: 1325-1330.

Komoda, T., Sakagishi, Y. and Sekine, T. (1981). Multiple forms of human intestinal alkaline phosphatases. *Clin. Chim. Acta* **117**: 167-187.

Kozak, M. (1986). Point mutation defines a sequence flanking the AUG codon that modulates translation by eukaryotic ribosomes. *Cell* **44**: 283-292.

Krugelis, E. J. (1950). Properties and changes of alkaline phosphatase activity during amphibian development. *Comptes Rend. Lab. Carlsberg, Sér. Chim.* **27**: 273-290.

Land, H., Grey, M., Hanser, H., Lindenmaier, W. and Schutz, G. (1981). 5'-Terminal sequences of eukaryotic mRNA can be cloned with a high efficiency. *Nucleic Acids Res.* **9**: 2251-2266.

Lapeyre, B. and Amalric, F. (1985). A powerful method for the preparation of cDNA libraries: isolation of cDNA encoding a 100-kDal nucleolar protein. *Gene* **37**: 215-220.

Latham, K. M. and Stanbridge, E. J. (1990). Identification of the HeLa tumor-associated antigen, p75/150, as intestinal alkaline phosphatase and evidence for its transcriptional regulation. *Proc. Natl. Acad. Sci. USA* **87**: 1263-1267.

Lehman, F. G. (1980). Differentiation of human alkaline phosphatases by lectin binding affinity. *Klin. Wochensch* **58**: 947-951.

Lepire, M. L. and Ziomek, C. A. (1989). Preimplantation mouse embryos express a heat-stable alkaline phosphatase. *Biol. Reprod.* **41**: 464-473.

Lin, C. W. and Fishman, W. H. (1972). L-Homoarginine. An organ-specific, uncompetitive inhibitor of human liver and bone alkaline phosphohydrolases. *J. Biol. Chem.* **247**: 3082-3087.

Lin, C. W., Sie, H. G. and Fishman, W. H. (1971). L-Tryptophan: a non-allosteric organ-specific uncompetitive inhibitor of human placental alkaline phosphatase. *Biochem. J.* **124**: 509-516.

Lindeman, V. F. (1949). Alkaline and acid phosphatase activity of the embryonic chick retina. *Proc. Soc. Exp. Biol. and Med.* **71**: 435-437.

Low, M. G. and Saltiel, A. R. (1988). Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* **239**: 268-275.

Low, M. G., Ferguson, M. A. J., Futerman, A. H. and Silman, I. (1986). Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. *Trends in Biochemical Science* **11**: 212-215.

Lowe, M. E. and Strauss, A. W. (1990). Expression of a Nagao-type, phosphatidylinositol-glycan anchored alkaline phosphatase in human choriocarcinomas. *Cancer Res.* **50**: 3956-3962.

Ludke, D., Bernstein, J., Hamilton, C. and Torriani, A. (1984). Identification of the *phoM* gene product and its regulation in *Escherichia coli* K-12. *J. Biol. Chem.* **159**: 19-25.

MacFarlane, J. D., Kroon, H. M. and Cats, A. (1986). Ectopic calcifications in hypophosphatasia. *Eur. J. Radio.* **6**: 228-230.

Maekawa, H. and Yamana, K. (1975). Alkaline phosphatase isozymes of *Xenopus laevis* embryos and tissues. *J. Exp. Zool.* **192**: 155-164.

Manes, T., Glade, K., Ziomek, C. A. and Millán, J. L. (1990). Genomic structure and comparison of mouse tissue-specific alkaline phosphatase genes. *Genomics* **8**: 541-554.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eukaryotic DNA. *Cell* **15**: 687-701.

Marchuk, D., Drumm, M., Saulino, A. and Collins, F. S. (1991). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucl. Acids Res.* **19**: 1154.

Martin, D., Tucker, D. F., Gorman., Sheer, D., Spurr, N. K. and Trowsdale J. (1987). The human placental alkaline phosphatase gene and related sequences map to chromosome 2 band q37. *Ann. Hum. Genet.* **51**: 145-152.

Matsuura, S., Kishi, F. and Kajii, T. (1990). Characterisation of a 5'-flanking region of the human liver/bone/kidney alkaline phosphatase gene: two kinds of mRNA from a single gene. *Biochem. Biophys. Res. Commun.* **168**: 993-1000.

Maxam, A. M. and Gilbert, W. (1977). A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* **74**: 560-564.

McComb, R. B., Bowers, G. N. and Posen, S. (1979). *Alkaline Phosphatases.* Plenum, New York.

McKenna, M. J., Hamilton, T. A. and Sussman, H. H. (1979). Comparison of human alkaline phosphatase isoenzymes. *Biochem. J.* **181**: 67-73.

McLaughlin, P. J., Cheng, M. H. and Johnson, P. M. (1982). Expression on cultured human tumour cells of placental trophoblast membrane antigens and placental alkaline phosphatase defined by monoclonal antibodies. *I. t. J. Cancer* **30**: 21-26.

Micanovic, R., Bailey, C. A., Brink, L., Gerber, L., Pan, Y. C. E., Hulmes, J. D. and Udenfriend, S. (1988). Aspartic acid-484 of nascent placental alkaline

phosphatase condenses with a phosphatidylinositol glycan to become the carboxyl terminus of the mature enzyme. *Proc. Natl. Acad. Sci. USA* **85**: 1398-1402.

Millán, J. L. (1986). Molecular cloning and sequence analysis of human placental alkaline phosphatase. *J. Biol. Chem.* **261**: 3112-3115.

Millán, J. L. (1987). Promoter structure of the human intestinal alkaline phosphatase gene. *Nucl. Acids Res.* **15**: 10599.

Millán, J. L. (1988). Oncodevelopmental expression and structure of alkaline phosphatase genes. *Anticancer Research* **8**: 995-1004.

Millán, J. L. (1990). Oncodevelopmental alkaline phosphatase: in search for a function. *Prog. Clin. Res.* **344**: 453-475.

Millán, J. L. and Manes, T. (1988). Seminoma-derived nagao isozyme is encoded by a germ cell alkaline phosphatase gene. *Proc. Natl. Acad. Sci. USA* **85**: 3024-3028.

Millán, J. L. and Stigbrand, T. (1983). Antigenic determinants of human placental and testicular placental-like alkaline phosphatases as mapped by monoclonal antibodies. *Eur. J. Biochem.* **136**: 1-7.

Millán, J. L., Whyte, M. P., Avioli, L. V. and Fishman, W. H. Hypophosphatasia (adult form): Quantitation of serum alkaline phosphatase isoenzyme activity in a large kindred. *Clin. Chem.* **26**: 840-845.

Misumi, Y., Tashiro, K., Hattori, M., Sakaki, Y. and Ikehara, Y. (1988). Primary structure of rat liver alkaline phosphatase deduced from its cDNA. *Biochem. J.* **249**: 661-668.

Miyahara, K., Shiokawa, K. and Yamana, K. (1982). Cellular commitment for post-gastrular increase in alkaline phosphatase activity in *Xenopus laevis* development. *Differentiation* **21**: 45-49.

Mole, S. E., Iggo, R. D. and Lane, D. P. (1989). Using the polymerase chain reaction to modify expression plasmids for epitope mapping. *Nucl. Acids Res.* **17**: 3319.

Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mondel, G. and Goodman, R. H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **86**: 6682-6686.

Moog, F. (1944). Localisations of alkaline and acid phosphatases in the early embryogenesis of the chick. *Biol. Bull.* [illegible].

Morton, R. K. (1950). Separation and purification of enzymes associated with insoluble particles. *Nature* **166**: 1092-1095.

Moss, D. W. and Whitby, L. G. (1975). A simplified heat-inactivation method for investigating alkaline phosphatase isoenzymes in serum. *Clin. Chim. Acta.* **61**: 63-75.

Müllenbach, R., Lagoda, P. J. L. and Welter, C. (1989). An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends in Genetics* **5**: 391.

Myers, R. M., Tilly, K. and Maniatis, T. (1986). Fine structure genetic analysis of a ß-[illegible]obin promoter. *Science* **232**: 613-618.

Mulivor, R. A., Plotkin, L. I. and Harris, H. (1978). Differential inhibition of the human alkaline phosphatase loci. *Ann. Hum. Genet.* **42**: 1-13.

Nakayama, T., Yoshida, M. and Kitamura, M. (1970). L-Leucine sensitive, heat-stable alkaline phosphatase isoenzyme detected in a patient with pleuritis carcinomatosa. *Clin. Chim. Acta* **30**: 546-548.

Narisawa, S., Hofmann, M. -C., Ziomek, C. A. and Millán, J. L. (1992). Embryonic alkaline phosphatase is expressed at M-phase in the spermatogenic lineage of the mouse. *Development* **116**: 159-165.

Nathanson, L. and Fishman, W. H. (1971). New observations on the regan isoenzyme of alkaline phosphatase in cancer patients. *Cancer* **27**: 1388-1397.

Niewkoop, P. D. and Faber, J. (1967). *Normal Table of* Xenopus laevis *(Daudin)*. North-Holland Publishing Co., Amsterdam.

Noda, M., Yoon, K., Thiede, M., Buenaga, R., Weiss, P., Henthorn, P., Harris, H. and Rodan, G. A. (1987). cDNA cloning of alkaline phosphatase from rat osteosarcoma (ROS 17/2.8) cells. *J. Bone and Mineral Res.* **2**: 161-164.

Ogata, S., Hayashi, Y., Takami, N. and Ikehara, Y. (1988). Chemical character- tion of the membrane-anchoring domain of human placental alkaline phosphatase. *J. Biol. Chem.* **263**: 10489-10494.

Okayama, H. and Berg, P. (1982). High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* **2**: 161-170.

Old, R. W. and Primrose, S. B. (1989). *Principles of Gene Manipulation. An Introduction to Genetic Engineering*. Blackwell Scientific Publications, Oxford.

Osawa, S. (1951). Histochemical studies of alkaline phosphatase in the oogenesis and the early embryogenesis of the amphibia. *Embryologia* **1**: 1-20.

Payne, T. H., Cohn, D. L., Davidson, A. J., Henry, T. D., Schaefer, J. W. and Gabow, P. A. (1991). Marked elevations of serum alkaline phosphatase in patients with AIDS. *J. Acq. Imm. Def. Syn.* 4: 238-243.

Pfoul, R. J. and Giudice, G. (1967). The role of cell interactions in the control of enzyme activity during embryogenesis. *Biochim. Biophys. Acta* **142**: 263.

Plimmer, R. H. A. (1913). The metabolism of organic phosphorous compounds. Their hydrolysis by the action of enzymes. *Biochem. J.* 7: 43-71.

Plocke, D. J., Levinthal, C. and Vallee, B. L. (1962). Alkaline phosphatase of *Escherichia coli*: A zinc metalloenzyme. *Biochemistry* 1: 373-378.

Ponte, P., Gunning, P., Blau, H. and Kedes, L. (1983). Human actin genes are single copy for α-skeletal and α-cardiac actin but multicopy for ß- and α-cytoskeletal genes: 3' untranslated regions are isotype specific but are conserved in evolution. *Mol. Cell. Biol.* 3: 1783-1791.

Poole, T. J. and Steinberg, M. S. (1981). Amphibian pronephric duct morphogenesis: Segregation, cell rearrangement and directed migration of the *Ambystoma* duct rudiment. *J. Embryol. Exp. Morphol.* 63: 1-16.

Reid, T. W. and Wilson, I. B. (1971). *E. coli* alkaline phosphatase. In, *The Enzymes*, ed. Boyer, P. D., vol 4, pp. 373-416. Academic Press, New York.

Sakiyama, T., Robinson, J. C., Chou, J. Y. (1979). Characterisation of alkaline phosphatases from first trimester placentas. *J. Biol. Chem.* 254: 935-938.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Schwartz, J. H., Crestfield, A. M. and Lipmann, F. (1963). The amino acid sequence of a tetrapeptide containing the reactive serine in *E. coli* alkaline phosphatase. *Proc. Natl. Acad. Sci. USA* **49**: 722-729.

Schwartz, J. H. and Lipmann, F. (1961). Phosphate incorporation into alkaline phosphatase of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **47**: 1996-2005.

Schwartz, T. W., Hansen, H. F., HÅkanson, R., Sundler, F. and Tager, H. S. (1984). Human pancreatic icosapeptide: isolation, sequence, and immunocytochemical localisation of the COOH-terminal fragment of the pancreatic polypeptide precursor. *Proc. Natl. Acad. Sci. USA* **81**: 708-712.

Scott, J., Urdea, M., Quiroga, M., Sanchez-Pescador, R., Fong, N., Selby, M., Rutter, W. J. and Bell, G. I. (1983). Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins. *Science* **221**: 236-240.

Sergeant, L. E. and Stinson, R. A. (1979). Evidence that three structural genes code for human alkaline phosphatases. *Nature* **281**: 152-154.

Smith, M., Weiss, M. J., Griffin, C. A., Murray, J. C., Buetow, K. H., Emanuel, B. S., Henthorn, P. S. and Harris, H. (1988). Regional assignment of the gene for human liver/bone kidney alkaline phosphatase to chromosome 1p36.1-p34. *Genomics* **2**: 139-143.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-507.

Sowadsky, J. M., Handschumacher, M. D., Krishna Murthy, H. M., Foster, B. A. and Wyckoff, H. W. (1985). Refined structure of alkaline phosphatase from *Escherichia coli* at 2.8 Å resolution. *J. Mol. Biol.* **186**: 417-433.

Sussman, N. L., Seetharam, S., Blaufuss, M. C. and Alpers, D. H. (1986). Translation of rat intestinal RNA yields two alkaline phosphatases. *Biochem. J.* **234**: 563-568.

Suzuki, U., Yushimura, K. and Takaishi, M. (1907). *Bull. Coll. Argric. Tok. Imp. Univer.* **7**: 503.

Takami, N., Ogata, S., Oda, K., Misumi, Y. and Ikehara, Y. (1988). Biosynthesis of placental alkaline phosphatase and its post-translational modification by glycophospholipid for membrane-anchoring. *J. Biol. Chem.* **263**: 3016-3021.

Tamman, G. (1885). Ueber die Schicksale des Schwefels beim Keimen der Erbsen. *Z. Physiol. Chem.* **9**: 416-419.

Toh, Y., Yamamoto, M., Endo, H., Fujita,A., Misumi, Y. and Ikehara, Y. (1989a). Sequence divergence of 5' extremities in rat liver alkaline phosphatase mRNA's. *J. Biochem.* **105**: 61-65.

Toh, Y., Yamamoto, M., Endo, H., Misumi, Y. and Ikehara, Y. (1989b). Isolation and characterisation of a rat liver alkaline phosphatase gene: A single gene with two promoters. *Eur. J. Biochem.* **182**: 213-237.

Toh, Y., Yamamoto, M., Endo, H., Misumi, Y. and Ikehara, Y. (1990). Duplicate leader exons in the mouse liver-type alkaline phosphatase gene. *Biochem. International* **22**: 213-218.

Torriani, A. (1960). Influence of inorganic phosphate in the formation of phosphatases in *E. coli*. *Biochim. Biophys. Acta* **38**: 460-469.

Trepanier, J. M., Seargeant, L. E. and Stinson, R. A. (1976). Affinity purification and some molecular properties of human liver alkaline phosphatase. *Biochem. J.* **155**: 653-660.

Trotman, C. N. A. and Greenwood, C. (1971). Effects of zinc and other metal ions on the stability and activity of *Escherichia coli* alkaline phosphatase. *Biochem. J.* **124**: 25-30.

Tsonis, A., Argraves, W. S. and Millán, J. L. (1988). A putative functional domain of human placental alkaline phosphatase predicted from sequence comparisons. *Biochem. J.* **254**: 623-624.

Twigg, A. J. and Sherratt, D. (1980). Trans-complementable copy-number mutants of plasmid ColE1. *Nature* **204**: 216-218.

Vergote, I., Onsrud, M. and Nustad, K. (1987). Placental alkaline phosphatase as a tumour marker in ovarian cancer. *Obstet. Gynecol.* **69**: 228-232.

Vieira, J. and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259-268.

Vittur, F., Stagni, F. and de Bernard, B. (1984). Alkaline phosphatase binds to collagen; a possible mechanism of extravesicular mineralisation in epiphyseal cartilage. *Experientia* **40**: 836-837.

von Euler, H. (1912). Verhalten der Kohlenhydratphosphor-säureester im Tierkörper. *Z. Physiol. Chem.* **79**: 375-397.

von Euler, H. and Funke, Y. (1912). Ueber die Spaltung der Kohlenhydratphosphorsäureester. *Z. Physiol. Chem.* **77**: 488-496.

Vorbrodt, A. and Borun, T. W. (1979). Ultrastructural cytochemical studies of plasma membrane phosphatase activities during the HeLa S3 cell cycle. *J. Histochem. Cytochem.* **27**: 1596-1603.

Watanabe, S., Watanabe, T., Li, W. B., Soong, B-W. and Chou, J. Y. (1989). Expression of the germ-cell alkaline phosphatase gene in human choriocarcinoma cells. *J. Biol. Chem.* **264**: 12611-12619.

Watanabe, T., Wada, N. and Chou, J. Y. (1992). Structural and functional analysis of human germ cell alkaline phosphatase by site-specific mutagenesis. *Biochemistry* **31**: 3051-3058.

Watanabe, T., Wada, N., Kim, E. E., Wyckoff, H. W. and Chou, J. Y. (1991). Mutation of a single amino acid converts germ cell alkaline phosphatase to placental alkaline phosphatase. *J. Biol. Chem.* **266**: 1174-1178.

Weinstein, R. S. and Whyte, M. P. (1981). Heterogeneity of adult phosphatasia. *Arch. Intern. Med.* **141**: 727-731.

Weiss, M. J., Henthorn, P. S., Lafferty, M. A., Slaughter, C., Raducha, M. and Harris, H. (1986). Isolation and characterisation of a cDNA encoding a human liver/bone/kidney-type alkaline phosphatase. *Proc. Natl. Acad. Sci. USA* **83**: 7182-7186.

Weiss, M. J., Ray, K., Henthorn, P. S., Lamb, B., Kadesch, T. and Harris, H. (1988). Structure of the human liver/bone/kidney alkaline phosphatase gene. *J. Biol. Chem.* **263/264**: 12002-12010.

Whitby, L. G. and Moss, D. W (1975). Analysis of heat- inactivation of alkaline phosphatase isoenzymes in serum. *Clin. Chim. Acta* **59**: 361-367.

Whittaker, J. R. (1990). Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. *Biol. Bull.* **178**: 222-230.

Wilson, I. B., Dayan, J. (1965). The free energy of hydrolysis of phosphoryl-phosphatase. *Biochemistry* **4**: 645-649.

Wilson, I. B., Dayan, J. and Cyr, K. (1964). Some properties of alkaline phosphatase from *Escherichia coli*. Transphosphorylation. *J. Biol. Chem.* **239**: 4182-4185.

Wolff, C. and Zabransky, S. (1982). Hypophosphatasia congenita letalis. *Eur. J. Pediatr.* **138**: 197-199.

Wu, L. N. Y., Genge, B. R. and Wuthier, R. E. (1991a). Association between proteoglycans and matrix vesicles in the extracellular matrix of growth plate cartilage. *J. Biol. Chem.* **266**: 1187-1194.

Wu, L. N. Y., Genge, B. R., Lloyd, G. C. and Wuthier, R. E. (1991b). Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. *J. Biol. Chem.* **266**: 1195-1203.

Wu, L. N. Y., Genge, B. R. and Wuthier, R. E. (1992). Evidence for specific interaction between matrix vesicle proteins and the connective tissue matrix. *Bone and Mineral* **17**: 247-252.

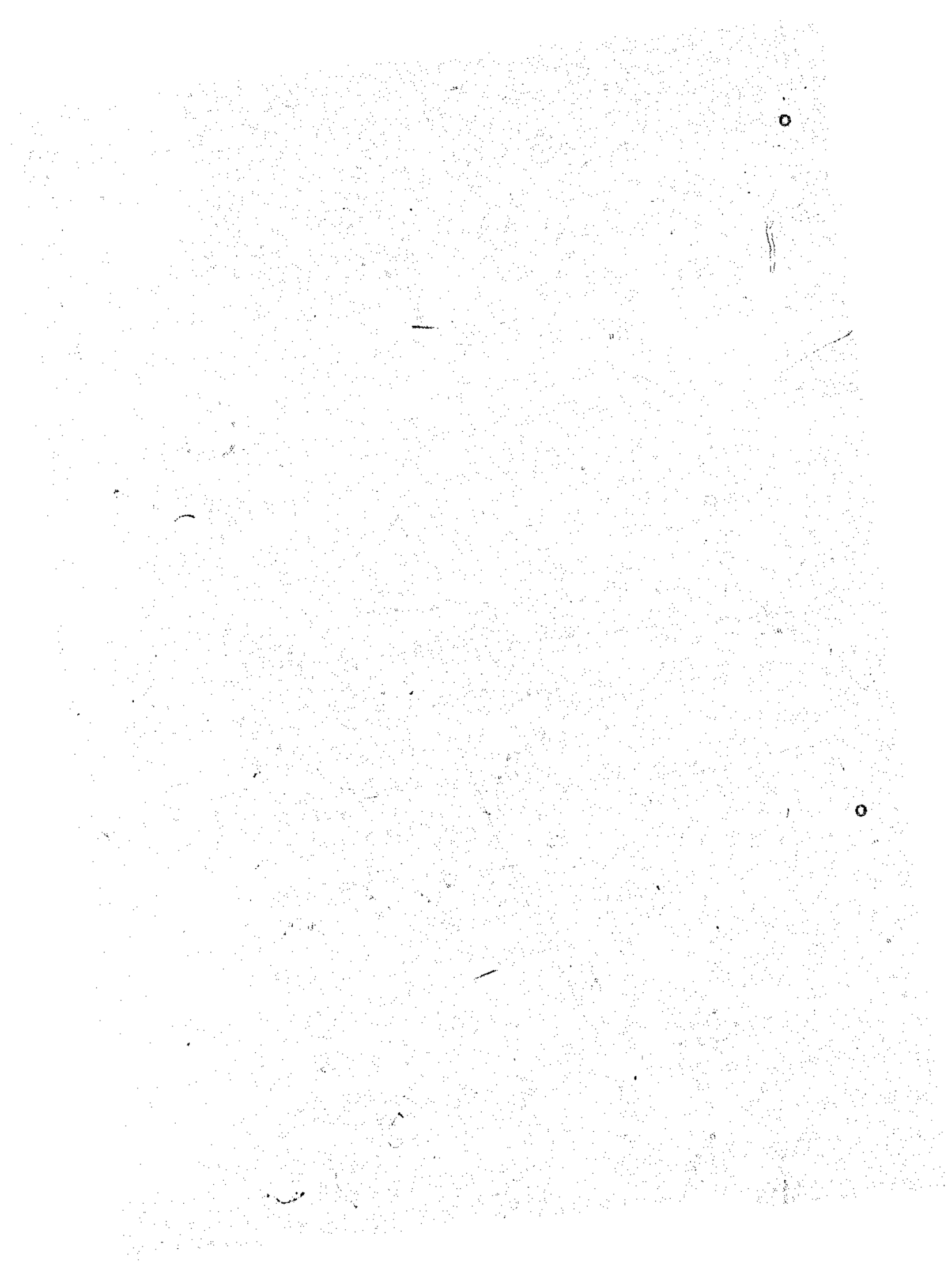
Young, B. D., Birnie, G. D. and Paul, J. (1976). Complexity and specificity of polysomal poly $(A)^+$ RNA in mouse tissues. *Biochemistry* **15**: 2823-2828.

Young, R. A. and Davis, R. W. (1983). Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* **80**: 1194-1198.

Zackson, S. L. and Steinberg, M. S. (1986). Cranial neural crest cells exhibit directed migration on the pronephric duct pathway: further evidence for an *in vivo* adhesion gradient. *Dev. Biol.* **117**: 342-353.

Zackson, S. L. and Steinberg, M. S. (1988). A molecular marker for cell guidance information in the axolotl embryo. *Dev. Biol.* **127**: 435-442.

Zackson, S. L. and Steinberg, M. S. (1989). Axolotl pronephric duct cell migration is sensitive to phosphatidylinositol-specific phospholipase C. *Development* **105**: 1-7.



Author: Constantinou Constantinos.
Name of thesis: The isolation, cloning and characterisation of the Xenopus Laevis alkaline phosphatase gene from the neurala stage.

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2015

LEGALNOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page)for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.