Molecular Cloning of Neuropathy Target Esterase

Thesis Submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by
Michael John Lush
Department of Biochemistry
University of Leicester

Submitted February 1998

UMI Number: U106116

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.

UMI U106116

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Acknowledgements

I am deeply grateful to Dr Paul Glynn for his help, encouragement and advice during the course of this project and his guidance in the preparation of this thesis.

I would also like to thank Kath Lilley and the Protein and Nucleic Acid Chemistry Laboratory (Leicester University) for oligonucleotide synthesis, protein and DNA sequencing. I would also like to thank David Read for technical assistance and Mr and Mrs Lush for their support during the writing of this thesis.

Finally I indebted to Dr Ruth Akhtar, for her support over the last four years and especially for putting up with me while I wrote this thesis.

Molecular Cloning of Neuropathy Target Esterase

Michael John Lush

Abstract

A single ingestion of certain organophosphorus esters (OPs) can cause a syndrome known as Organophosphate Induced Delayed Polyneuropathy (OPIDP), a paralysing neuropathy with degeneration of long axons, developing after a latent period of approximately one to three weeks. The primary target of these OPs has been shown to be a 155kDa neural protein designated Neuropathy Target Esterase (NTE), and the toxic effects apparently due to the covalent inhibition and subsequent secondary modification of this protein. Recently NTE has been purified to apparent homogeneity using a novel biotinylated OP and sufficient pure protein was produced for limited protein sequencing.

The aim of this project was to clone NTE cDNA using peptide sequence data. Initially, these sequences were used to design degenerate oligonucleotide primers for amplifying sections of brain cDNA by polymerase chain reaction (PCR). These approaches were unsuccessful. Subsequently, database searching with the peptide sequences identified a number of Expressed Sequence Tags (ESTs); these could be aligned to form an initial contig of 2.2kbp which encoded the 3' end of NTE cDNA. The 5' end of NTE cDNA, comprising a further 2.2kbp, was obtained by a PCR-based technique. The final 4.4kbp contig encoded a 1327 residue polypeptide predicted to have a molecular mass of 146kDa and at least one transmembrane domain. A novel serine esterase domain of approximately 200 residues was present near the C-terminus. NTE is unrelated to any known serine hydrolases but homologous proteins are predicted to be present in diverse prokaryotic and eukaryotic organisms. The homologue in *Drosophila* is associated with the swisscheese phenotype, an age-dependent neurodegeneration of the brain. NTE was also mapped to chromosome 19p13.3 between markers D19216 and D19S413 (using the UniGene database) and an OMIM search reveals that this is near the locus of cerebellar ataxia (Cayman type).

Abstract	ii
Table of contents	iii
List of Figures	v
Abbreviations	ix

Chapter 1 Introduction

	Page
1.1 Introduction	1
1.1.1 Organophosphate Induced Delayed Polyneuropathy	1
1.1.2 History of OPIDP	2
1.1.3 Organophosphate toxicology: acute effects	3
1.1.4 OPIDP is not an archetypal esterase inhibition syndrome	4
1.1.5 Biochemistry of NTE	7
1.1.6 Candidate proteins for NTE	9
1.2 Introduction to cloning	11
1.2.1 Positional cloning	11
1.2.2 Functional selection	12
1.2.3 Immunochemical selection	13
1.2.4 Nucleic acid hybridisation selection	14
1.2.5 'Virtual' cloning	14
1.3 PCR cloning of NTE	16
1.3.1 Degenerate PCR	16
1.3.2 Rapid Amplification of cDNA Ends	18
1.3.3 5' RACE	19
1.3.4 3' RACE	19
1.3.5 Other cloning work performed on NTE	20

Chapter 2 Methods and reagents

2.1 Protein methods	33
2.1.1 Preparation of microsomes	33
2.1.2 Protein Determination	34
2.1.3 S9B-labelling and solubilisation of microsomes	35
2.1.4 Solubilisation of microsomes and capture of NTE on avidin-Sepharose	36
2.1.5 Analysis of prep-PAGE fractions	37
2.1.6 V8 protease digest	38
2.1.7 SDS-Polyacrylamide Gel Electrophoresis	39
2.2 DNA purification and cloning	41
2.2.1 Agarose gel electrophoresis	41
2.2.2 PCR product purification by electroelution	42
2.2.3 PCR Product purification by QIAspin kit	43
2.2.4 Phenol extraction and ethanol precipitation	44
2.2.5 Ethanol precipitation of oligonucleotides	45

2.2.6	Purification of high molecular weight genomic DNA	45
2.2.7	DNA ligation with compatible sticky ends	48
2.2.8	pGEM-T cloning of PCR products	48
2.2.9	pCRscript cloning of PCR products	49
2.2.10	Transformation of ligated products	50
2.3	PCR Methods	52
2.3.1	General PCR and RACE methods	52
2.3.2	Direct PCR of bacterial colonies	53
2.3.2	Cycle sequencing	54
2.3.3	Preparation of a circular genomic DNA library for inverse PCR	55
2.3.4	Synthesis of cDNA for 3' RACE and RT-PCR	56
2.3.6	DEPC treatment of reagents and equipment	57
2.3.6	Amplification of cDNA bacteriophage library for use as PCR template	58
2.4	Hybridisation screening	60
2.4.1	Transfer of bacterial or bacteriophage colonies to solid support	60
2.4.2	Southern Blotting	61
2.4.3	Labeling DNA probes	62
2.2.4	Hybridisation conditions	63

Chapter 3 Isolation and protein sequencing of NTE

3.1	Introduction	64
3.2	Results and Discussion	65
3.2.1	Chicken brain NTE: Purification and protein sequencing	65
3.2.2	Pig brain NTE: Purification and protein sequencing	67
3.3	Significance of NTE peptide sequences in isolating the NTE cDNA	68

Chapter 4 Attempts to clone NTE cDNA using Rapid Amplification of cDNA Ends (RACE)

4.1	Introduction	81
4.2	Results and Discussion	81
4.2.1	Synthesis and quality control of cDNA	81
4.2.2	Refinement of 3' RACE protocol	82
4.2.3	3' RACE with degenerate primers designed to NTE peptides	83
4.2.4	Nested 3' RACE	84
4.3	Perspectives on Degenerate 3' RACE	87

Chapter 5 Attempts to clone NTE cDNA by PCR using degenerate primers of inferred relative position

5.1	Introduction	100
5.2	Results and discussion	100
5.2.1	Production and isolation of PCR-generated clone ORF1	100
5.2.2	Attempts to clone the ORF1 template	103
5.3	Retrospective view on the relative positions of LTNP, XGVP and the catalytic serine in NTE	105

Chapter 6 Cloning of NTE via homology to an expressed sequence tag

6.1	Introduction	118
6.2	Results and discussion	120
6.2.1	Sequencing the 3' end of NTE cDNA by alignment of ESTs	120
6.2.2	Subcloning of the 2 kbp insert from pDR2 into pBluescript SK+	121
6.2.3	Cloning of the 5' end of NTE by 5' RACE	122
6.2.4	Sequencing of the 5' RACE products	124
6.3	Review of gene cloning	125

Chapter 7 Analysis of the NTE cDNA and deduced protein sequence

7.1	Introduction	137
7.2	Results and Discussion	137
7.2.1	The NTE1 clones produced by the 5' RACE contain 11 out of 12 of the NTE peptide sequences.	137
7.2.2	NTE shares a putative novel esterase domain with proteins from both eukaryotes and prokaryotes.	139
7.2.3	Function of the N-terminal domain of NTE	140
7.2.4	Computer analyses of NTE and YOL4 sequences indicate NTE is a membrane bound protein.	142
7.2.5	Mutation to the swisscheese (sws) gene can produce a neurodegeneration syndrome or a specific anosmia to aldehydes (olfE).	143
7.2.6	The NTE gene is located on chromosome 19p13.3 near the mapped position of a recessive heritable ataxia.	144
7.3	Overview of predictions	147
7.4	Future work	148
7.4.1	What is the membrane topology of NTE and what organelles is it associated with?	148
7.4.2	What are the biological functions of the various domains of the NTE protein?	149
7.4.3	What is the biological role of NTE and what is its role in the development of OPIDP?	151
7.5	Conclusion	152

Appendix 1	EST sequencing of HOL4.3	163
Appendix 2	Sequence of NTE like proteins	175
Appendix 3	Gapped-BLAST search of SWISSPROT	187
Appendix 4	DNA sequence of a28, HOL4.2 and the a28HOL4 contig	209
References		219

List of Figures

Figure 1.1	General structure of organophosphorus esters	21
Figure 1.2	Catalytic mechanism of serine esterases.	22
Figure 1.3	Mechanism of OP inhibition and ageing in serine esterases.	23
Figure 1.4	Formation of saligenin cyclic o-tolyl phosphate from TOCP	24
Figure 1.5	Prediction of OP delayed neuropathic potential.	25
Figure 1.6	Tissue distribution of NTE activity as a percentage of adult hen brain NTE activity (reproduced from Johnson, 1992).	26
Figure 1.7	Putative N-terminal sequence around the NTE catalytic serine.	27
Figure 1.8	General structure of the biotinylated saligenin phosphoramidates used to purify NTE.	28
Figure 1.9	AChE-like enzymes which lack catalytic activity.	29
Figure 1.10	Synthesis of cDNA for EST sequencing produces a common 3' end sequence and many 5' end sequences from the same gene.	30
Figure 1.11	Amino acid degeneracy codes.	31
Figure 1.12	Synthesis of cDNA for 3'RACE.	32
Figure 3.1	Structure of 1, [Saligenin cyclic phosphoryl],-9,-biotinyl-diaminonoane (S9B).	71
Figure 3.2	Flow chart summarising the purification stages of NTE from chicken brain to purified NTE.	72
Figure 3.3	7.5% SDS-PAGE illustrating the two stages of NTE purification.	73
Figure 3.4	7.5% SDS-PAGE of aliquots from Preparative SDS-PAGE of Avidin eluate.	74
Figure 3.5	V8-digested NTE blotted on to Immobilon P prior to sequencing.	75
Figure 3.6	Chicken brain peptide sequence.	76
Figure 3.7	Pig NTE peptide sequence data.	77
Figure 3.8	Avidin-Sepharose extraction of V8 digest fragments containing the S9B binding site.	78

Figure 3.9	Peptide sequence derived from HPLC purified tryptic digest fragments from V8 digest fragments of pig NTE.	79
Figure 3.10	Sequence similarities between ADLT, VVPG and sequences linked to YOL4.	80
Figure 4.1	Pig NTE peptide sequence available for degenerate primer design.	90
Figure 4.2	Design of anchor primers for reverse transcription of poly-A RNA and 3' RACE.	91
Figure 4.3	Relative positions of trk1+, trk3-, trk5+ and trk9+ primers on pig trkC cDNA.	92
Figure 4.4	Quality control of BAT39-primed cDNA for 3' RACE	93
Figure 4.5	Ratification of 3' RACE protocol.	94
Figure 4.6	Design of degenerate primers from NTE peptides for 3' RACE experiment.	95
Figure 4.7	3' RACE with the F10I degenerate primer.	96
Figure 4.8	Translations of the DNA sequences from the 5' (F10I) end of 3' RACE products.	97
Figure 4.9	PCR amplification of BAT39-primed cDNA with degenerate primers F36IS, F36IT, F16I, F13IG and F13IY paired with reverse primers BAT117 and BAT318.	98
Figure 4.10	Typical example of 2nd round nested 3' RACE with degenerate primers.	99
Figure 5.1	Putative peptide map of the serine esterase domain of NTE.	107
Figure 5.2	Design of degenerate primers to the LTNP and XGVP peptide sequences	108
Figure 5.3	PCR amplification of oligo-dT primed pig brain cDNA using primers of putatively known position.	109
Figure 5.4	Translation of ORF1 and ORF2 sequence in three reading frames.	110
Figure 5.5	Alignment ORF1 with the N-termini of multiple serine esterase domains.	111
Figure 5.6	ORFA, ORFD, ORFE and ORRA primer locations on ORF1 sequence.	112
Figure 5.7	Inferred consensus sequence flanking the catalytic serine in NTE.	113
Figure 5.8	Inverse PCR allows amplification of DNA sequences flanking a small section of known sequence.	114
Figure 5.9	Inverse PCR on Mbol circularised pig genomic DNA with non-degenerate primers ORFA, ORFD and ORRA.	115
Figure 5.10	Alignment of ORF1 templates	116
Figure 5.11	Peptide map around the S9B-labelled serine of NTE, using sequence data from Figure 7.1.	117

Figure 6.1	Similarity between the pig NTE peptide sequence LAKL and YOL4_CAEEL, LAKL and RUBISCO, DLGL and YOL4_CAEEL, DLGL and Acetyl-CoA carboxylase.	126
Figure 6.2	Alignment between YCHK_ECOLI, YCHK_SHFTL, and the C-terminal domain of YOL4_CAEEL.	127
Figure 6.3	Similarity between translated EST sequences and the DLGL, DGHL, VVKS and FDQI peptide sequences.	128
Figure 6.4	Relative positions of primers in insert and pDR2 vector sequence at the 5' end of the HOL4.2 insert.	129
Figure 6.5	PCR of DRA/B primers vs NDR2/3/4 on IDR2 adult human brain cDNA library.	130
Figure 6.6	Synthesis of Marathon cDNA.	131
Figure 6.7	PCR amplification using NDR2 and NDR4 vs AP1 primers on Marathon cDNA.	132
Figure 6.8	PCR with RSP vs NDR4 sizing the clones.	133
Figure 6.9	5' end sequence from the NTE1 and NTE2 product groups.	134
Figure 6.10	Overlap between the NTE1 clone and HOL4.2 cDNA	135
Figure 6.11	Detection of the initiation codon of the NTE1 cDNA and alternate splice form NTE2 of the 5' end of the putative cDNA.	136
Figure 7.1	Positions of the known pig NTE peptide sequences on the sequence of human NTE cDNA.	153
Figure 7.2	Summary of the hypothetical proteins with a similarity to NTE's C-terminal esterase domain.	154
Figure 7.3	Comparison of NTE like proteins.	155
Figure 7.4	Multiple alignment of the putative esterase domain of proteins in Figure 7.3.	156
Figure 7.5	Similarity plot of data in Figure 7.4	157
Figure 7.6	Dotplot of YMF9 (vertical) vs NTE (horizontal)	158
Figure 7.7	Dotplot of NTE (horizontal) vs sws (A) and YOL4 (B).	159
Figure 7.8	Transmembrane predictions for NTE.	160
Figure 7.9	Putative secondary structure of NTE (not to scale).	161
Figure 7.10	Cytogenetic map of Chromosome 19 and genetic map of the ATCAY/NTE location.	162

Abbreviations

[³² P]DFP	³² P labelled diisopropyl phosphofluoridate
a28	5' RACE product encoding 5' end of NTE
a28HOL4	Sequences a28 and HOL4.2 joined together to form a 4.4kp sequence
aa	amino acid
ACh	acetylcholine
AChE	acetylcholinesterase
bp	base pair
BSA	Bovine Serum Albumin
BuChE	butyrylcholinesterase
cDNA	complementary DNA
cM	centiMorgan
cRS	cAMP-regulatory subunits
Da	Dalton
DFP	diisopropyl phosphofluoridate
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EST	Expressed Sequence Tag
FACS	fluorescence-activated cell sorter
GSP	Gene Specific Primer
HOL4.2	2.2kbp insert encoding 3' end of NTE
kb	kilobase (RNA)
kbp	kilobase pair
kDa	kiloDaltons
mRNA	messenger Ribonucleic Acid
NTE	Neuropathy Target Esterase
NTE1	5' end splice form of NTE
NTE2	5' end splice form of NTE
OP	Organophosphate
OPIDP	Organophosphate Induced Delayed Polyneuropathy
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PPA	phenyl phenylacetate
Prep SDS-PAGE	preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PSP	phenyl O, O' saligenin phosphate
PV	phenyl valerate
RACE	Rapid Amplification of cDNA Ends
S9B	1, [Saligenin cyclic phosphoryl],-9,-biotinyl-diaminonoane
S9D	digoxigenin- labelled version of S9B
S9F	fluorescein- labelled version of S9B
SDS	Sodium dodecyl sulphate
STS	Sequence Tagged Site
TFMK	trifluoromethyl ketone
TOCP	triorthocresyl phosphate
TSP	saligenin cyclic o-tolyl phosphate

Chapter 1 Introduction

1.1 Introduction

1.1.1 Organophosphate Induced Delayed Polyneuropathy

Organophosphate Induced Delayed Polyneuropathy (OPIDP) is a syndrome caused by certain organophosphate (OP) compounds and is characterised by a flaccid paralysis of the lower limbs developing 1 to 3 weeks after a single dose of OP. Clinically, OPIDP presents as initial sensory disorders (cramp and tingling), followed by weakness and ataxia in the lower extremities progressing to a flaccid paralysis; in severe cases upper limbs can also be affected. The symptoms are relatively permanent although some recovery is possible as peripheral nerves repair (Lotti, 1992).

Histological study of the damaged nerves shows the initial lesion manifesting at the same time as clinical signs (ie after 1 to 3 weeks), and expressed as a localised degeneration at the distal (though not terminal) end of individual nerve fibres. Large and long fibres (ie, those in the peripheral nerves and spinal cord) are the most susceptible to damage and as the syndrome progresses, an increasing proportion of the axons become involved and the already damaged axons undergo degeneration progressing up the axon but not affecting the cell body. Demyelination is also observed though this appears to be a secondary effect caused by the neuropathy (Bouldin and Cavanagh, 1979).

OPIDP is interesting from two view points: Firstly, study of the protein target of delayed neuropathic OPs has allowed the development of an in vitro testing system for the delayed neuropathic potential of many compounds; (this is important practically because hundreds of different OPs have been synthesised as candidate pesticides). Secondly, study of this syndrome has the potential to

elucidate part of the mechanism of axonal maintenance which is critically disrupted during OPIDP.

At the outset of this project, the protein target of the neuropathic OP, Neuropathy Target Esterase (NTE) had recently been isolated in this laboratory. The goal of this project was to use the purified NTE as a starting point to clone its cDNA. Possession of an NTE clone is an attractive proposition since, despite more than 60 years of research, almost all the data relating to NTE and OPIDP is essentially based on the inhibition of esterase activity of NTE, which is known to be unrelated to the actual genesis of the neuropathy (see Section 1.1.5). Therefore possession of NTE's DNA (and hence protein) sequence, would allow a number of approaches to the study of NTE, which have been hitherto impossible:

- Synthetic peptides or expressed NTE protein could be used to raise high titer antisera, making it possible to visualise NTE at a cellular level.
- NTE's protein sequence could be compared with that of already characterised proteins with similar sequence and might, in itself provide clues as to NTEs function.
- Expression, biochemical (and subsequently structural) study of catalytically active NTE would become feasible, since until now it has only proved possible to purify heavily denatured and inhibited NTE.

1.1.2 History of OPIDP

The earliest reported cases of OPIDP occurred during 1899 in France, as a result of using a phospho-creosote mixture in the treatment of pulmonary-tuberculosis, although it was not till the 1930s that it OPIDP was recognised as a syndrome (Inoue *et al*, 1988).

In the spring of 1930 in the midwestern and south-western states of the United States of America, an outbreak of a what is now known as OPIDP affected as many as 50,000 people (Inoue *et al*, 1988). The polyneuropathy was

eventually traced by Smith *et al*, (1930) to a popular drink called 'Ginger Jake' (an 80% alcoholic extract of Jamaica ginger, sold as a medical tonic to evade the Prohibition laws) which had been adulterated by the addition of approximately 2% triorthocresyl phosphate (TOCP). A 47 year follow up study on eleven survivors of the Ginger Jake poisoning still showed signs of abnormal reflexes, paralysis and spasticity (Morgan and Penoch, 1978). Another notable outbreak of OPIDP happened during 1959 in Morocco when 10,000 people were poisoned with cooking oil adulterated with jet engine oil (Inoue *et al*, 1988). In 1953 three research workers developed OPIDP after working on new insecticide called mipafox (Bidstrup *et al*, 1953) these were the first reported cases of OPIDP caused by compounds other than TOCP; since that time the list of OPIDP causing OPs has grown, thanks, in no small part to the routine screening of all new OP compounds for neuropathic potential.

1.1.3 Organophosphate toxicology: acute effects

Organophosphates are widely used as pesticides and also have limited medical applications (Figure 1.1). The acute neurotoxic action of many OPs is almost entirely due to their inactivation of acetylcholinesterase (AChE) by covalent reaction with its catalytic site. AChE is an important part of the nervous system, as it is responsible for inactivating the neurotransmitter acetylcholine (ACh) in the synapses of the brain and neuromuscular junction. Inhibition of AChE leads to a build up of the stimulatory ACh in the synapse leading to continual stimulation of the connecting neuron or muscle, ultimately causing paralysis and death by respiratory failure.

The hydrolytic mechanism of AChE utilises a charge relay system of a serine, histidine and aspartate to activate the serine and enable it to make a nucleophilic attack on the carbonyl group of the (non-covalently) bound substrate (Figure 1.2). The inhibitory power of the toxic OPs is partly due to the tetrahedral structure of the phosphate group, since it resembles the tetrahedral transition state Michaelis complex of the reacting ACh molecule (Figure 1.2). The other

factor in their toxicity is the permanence of the covalent linkage to the catalytic serine. This is dependent on the structure of the inhibiting OP: for example, a dimethyl phosphate adduct is removed by spontaneous hydrolysis in a matter of hours; in contrast inhibition by a diisopropyl phosphate group is essentially irreversible. The reason for this is a phenomenon known as 'ageing' in which a single group is spontaneously hydrolysed from the phosphate rendering the inhibition irreversible (Figure 1.3; Johnson, 1992).

1.1.4 OPIDP is not an archetypal esterase inhibition syndrome

The clinical syndrome known as Organophosphate Induced Delayed Polyneuropathy represents an unusual response to challenge by toxic OP compounds. Rather than an immediate onset of symptoms as is displayed in response to anti-AChE compounds, there is an asymptomatic period of 1 to 3 weeks prior to the development of the peripheral neuropathy

Originally, W. N. Aldridge (1954) suggested that, since the neuropathic agent was an OP, then the likely initiating stage in OPIDP would involve the OP phosphorylation of an esterase (now called Neuropathy Target Esterase; NTE). Initial theories posited inhibition of brain AChE or butyrylcholinesterase (BuChE), however these theories had to be discarded due to the existence of OPs which caused OPIDP but did not inhibit the suggested esterase activities (Abou-Donia, 1981).

TOCP is relatively inert compound (perhaps explaining why it was selected as an adulterant for Ginger Jake) and does not inhibit NTE in vitro. It requires bioactivation to saligenin cyclic o-tolyl phosphate (TSP; Figure 1.4) to both inhibit NTE and trigger OPIDP (Casida *et al*, 1961; Eto *et al*, 1962). This prompted the observation from Poulsen and Aldridge (1964) that there was a certain structural similarity between TSP, phenyl phenylacetate (PPA) and that the latter could be a substrate for NTE.

By screening for compounds that halted progressive labelling of the neuropathy target site by [32 P] diisopropyl phosphofluoridate ([32 P]DFP; an OP with both anti-AChE activity and also a delayed neuropathic effect (Johnson, 1969b), it was possible to dissect the various enzyme activities by differential inhibition using non-neuropathic and neuropathic compounds. Paraoxon a non-neuropathic OP was used to eliminate much of the non-NTE esterase activity; this left about 9-11% PPA esterase or [32 P]DFP labelling activity. The addition of mipafox (a potent neuropathic OP; Bidstrup *et al*, 1953) resulted in a further 3-4% loss of activity. This 3-4% PPA activity insensitive to paraoxon but sensitive to mipafox is the functional definition of NTE. Definitive assay conditions for NTE assay were laid down by Johnson (1978); the main alteration to the protocol was that substrate (PPA) was replaced by phenyl valerate (PV). The PV NTE assay has been successful in predicting the delayed neuropathic potential of more than 100 OPs and at least 20 organophosphinates, sulphonyl fluorides and carbamates (Johnson, 1975a and 1982). However, to achieve this level of success it is necessary to consider not only the inhibition of NTE but also the structure of the toxin.

As with OP inhibition of AChE, the OP group bound to NTE is faced with two possible fates; slow spontaneous hydrolysis from the enzyme, or ageing by loss of one of the residual groups from the phosphorus, rendering the inhibition irreversible and also introducing a negative charge to the active site serine (Figure 1.3). Curiously, unlike AChE, simple inhibition of NTE esterase activity, even for a period of weeks, does not necessarily lead to OPIDP (Johnson, 1970). It is clear that the non-neuropathic NTE inhibitors attack the same site in the enzyme as these reagents have a prophylactic effect preventing the action of neuropathic OPs by physically blocking the catalytic cleft. By examining a compound's ability to age and its activity against NTE it is possible to predict the neuropathic potential of an OP compound (Figure 1.5).

One particularly elegant demonstration of the necessity for ageing to trigger OPIDP was produced by Johnson and Read (1987). EPNO (Figure 1.1) is

an inhibitor of NTE and in vitro the potency of the L(-) and D(+) isomers are similar; however the L(-) isomer is rapidly aged in NTE whereas the D(+) isomer does not age, despite the similar structure and presence of a potentially cleavable R-O-P bond. When tested in vivo the L(-) isomer did induce OPIDP whereas the D(+) isomer was not only non-neuropathic, but also prophylactic if administered prior to a dose of phenyl O, O' saligenin phosphate (PSP; another member of the highly neuropathic saligenin phosphate family of NTE inhibitors; Figure 1.1).

Another of the interesting features of the ageing reaction in NTE, compared to that in AChE is that the leaving isopropyl moiety from DFP is retained within the enzyme with 100% efficiency (Clothier and Johnson, 1979; Williams and Johnson, 1981). This intramolecular transfer is not observed with all neuropathic OPs and thus is not critical for the initiation of the delayed neuropathy. For example, the highly neurotoxic octyl saligenin cyclic phosphate was found to age but does not display a similar intramolecular group transfer (Yoshida *et al*, 1995) indicating that it is the introduction of a negatively charged group at the catalytic site that initiates OPIDP.

The initiating events of OPIDP are well known: about 70 to 80% of NTE activity needs to be inhibited to trigger neuropathy and the inhibiting OP is aged within a few minutes (Clothier and Johnson, 1974); after this stage the actual physiological disturbance set up by the inhibition is unknown. What is known is that NTE has a half life of reappearance of 2 days in brain and 3.6 days in spinal cord after inhibition with a neuropathic OP (Meridith and Johnson, 1988). Thus NTE activity is completely restored by the time paralysis develops and it may be the disruption of NTE's function for a limited period that causes a knock-on effect on the stability of the axonal function. In addition there appears to be inhibition of retrograde axonal transport (of injected ¹²⁵I-labelled tetanus toxin) at five to seven days after OP challenge, although whether this represents cause or effect is unknown (Moretto *et al*, 1987).

1.1.5 Biochemistry of NTE

The molecular mass of denatured NTE was established to be approximately 155kDa by Williams and Johnson (1981); this was determined by [^3H]DFP-labelling of brain microsomes which had been pre-treated with paraoxon or paraoxon+mipafox, followed by solubilisation and SDS-PAGE. Various attempts have been made to measure the mass of catalytically active NTE by gel filtration: estimates have ranged from 1800kDa (Chemnitius *et al*, 1984) through 970kDa (Pope and Padilla, 1989) to 850kDa (Thomas *et al*, 1990). The discrepancy in size between the native and denatured forms of NTE is probably because NTE forms an aggregate which is apparently necessary for its activity. Pope and Padilla (1989) reported that their 970kDa fraction contained a 148 to 160 kDa polypeptide which possessed the appropriate [^3H]DFP binding characteristics for NTE.

Besides its size, little is known about NTE. It is a glycoprotein (P. Glynn, unpublished data) and it can hydrolyse several artificial ester substrates but appears inactive against several peptide substrates (Johnson, 1975b). However, its physiological substrate is unknown and it is even possible that it has no physiological substrate, especially since the near complete inhibition of NTE for a period of weeks appears to have no obvious adverse effect in adult chickens (Johnson, 1970). Figure 1.6 summarises the tissue distribution of NTE in the adult hen. It is worth noting that, even though NTE is present in non-neural tissue, the toxic effects of neuropathic OPs appear to be restricted to long axons in the spinal cord and peripheral nerves (Bouldin and Cavanagh, 1979).

Detergent solubilization studies show NTE is firmly associated with microsomal membranes and quite hydrophobic in nature (Davis and Richardson, 1987). Recent immunolocalisation studies have shown that NTE is located in neurons but not glia and the staining pattern indicates that NTE is located intracellularly, but not in the nucleus (Glynn *et al*, 1998). Carrington and Abou-Donia (1985) have shown that NTE undergoes fast axonal transport. These

findings are supported by immunohistochemical staining of NTE showing that it accumulates at a ligature on the sciatic nerve, mainly on the anterograde side and to a lesser extent the retrograde side of the blockage with staining building up after a period of 6 to 8 hours. The axonal transport of NTE is apparently independent of its modification by neuropathic OPs, as dosing chickens with a neuropathic OP has no gross qualitative or quantitative effects on the accumulation of NTE at the nerve ligature (Glynn *et al*, 1998).

A prerequisite for the goal of this project (ie, the cloning of NTE) is the ability to isolate the pure protein. However for many years NTE resisted purification attempts in several laboratories. This was primarily due to its low abundance ([³H]DFP labelling indicates that NTE comprises only 0.03% of brain microsomal protein; Williams and Johnson, 1981), and poor recovery of detergent solubilized [³H]DFP labelled NTE by conventional purification techniques such as ion-exchange and hydrophobic interaction chromatography gel filtration and sucrose gradient centrifugation. (Thomas *et al*, 1993). NTE activity is highly labile to even simple procedures such as dialysis and ultrafiltration, probably due to loss by surface adsorption (Pope and Padilla, 1989).

Previously the best purification protocol utilised Nonidet P40 solubilization of [³H]DFP-labelled brain microsomes, followed by detergent phase partitioning, anion exchange and preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Prep SDS-PAGE). This protocol yielded a fraction in which NTE comprised only 2.5% of the total protein (Rüffer-Turner *et al*, 1992). Sequential proteolytic digestion and purification stages on the enriched NTE fraction yielded a seven residue heterogeneous sequence within which the serine esterase consensus sequence could be discerned (Figure 1.7; Glynn *et al*, 1993).

The next generation of NTE purification attempts focused on using NTE inhibitors to bind and immobilise the NTE so it could be purified by affinity chromatography. One approach used a trifluoromethyl ketone (TFMK) liganded

onto Sepharose CL6B; the TFMK used (3-(9'-mercaptiononylthio)-1,1,1-trifluoropropan-2-one) is a reversible inhibitor for a wide range of serine hydrolases including NTE, and when used as an affinity ligand it was moderately successful in purifying NTE (Thomaes *et al*, 1993). However the technique was apparently not sufficient to purify NTE on a preparative scale as a later paper from the same research group did not report any N-terminal sequence data (Mackay *et al*, 1996)

The approach used in this project was to use one of the saligenin cyclic phosphoryl OPs (Figure 1.1). A series of novel biotinylated saligenin phosphoramidates which differed only by the length of the alkyldiamine spacer were synthesised (Figure 1.8) and their inhibitory potency assessed in the PV assay for NTE (see Section 1.1.4). The diaminonoane derivative (1, [Saligenin cyclic phosphoryl],-9,-biotinyl-diaminonoane (S9B)) was one of the two most potent inhibitors and was adopted as the main purification reagent (Glynn *et al*, 1994).

S9B proved to be able to specifically biotinylate NTE in a complex mixture of microsomal protein. A simple absorption/washing/elution of the biotinyl-NTE complex bound to an avidin-Sepharose affinity column allowed a 1000 fold enrichment of NTE with recoveries of 30%. A subsequent Preparative SDS-PAGE step removed contaminating endogenously biotinylated protein and yielded essentially pure NTE protein (Glynn *et al*, 1994). Chapter 3 describes purification of chicken and pig brain NTE, its digestion and N-terminal sequencing.

1.1.6 Candidate proteins for NTE

At the outset of the project it was thought that NTE was likely to be a member of the AChE family of serine esterases, which included enzymes capable of catalysing the hydrolysis of a wide variety of ester substrates (Krejci *et al*, 1991). Additionally there was a growing body of research showing that the AChE protein had significant non-catalytic function. Studies using antibodies

capable of discriminating between active and inactive AChE shows that 30% or the total AChE protein is inactive, which indicates a large pool of enzyme apparently uninvolved in its supposed primary physiological role (Chatel *et al*, 1993). Furthermore, inhibition of AChE can lead to a dose dependent decrease in chick neurite growth *in vitro*; however simple inhibition of the AChE appears to be insufficient to cause this effect since there is at least one AChE inhibitor which does not have this effect (Layer *et al*, 1993).

One group of proteins (neurotactin, glutactin, gliotactin and neuroligins 1 to 3; see Figure 1.9) within the AChE family are of particular interest. All are large glycoproteins which possess a domain with a significant sequence similarity to AChE, yet are enzymically inactive since they lack a catalytic serine. These enzymes appear to be involved in cell-cell interactions mediated through the extracellular AChE-like domain. Since the proteins have a comparable size and like NTE do not require catalytic activity for function, it was thought that NTE might be a new member of this protein family in which the catalytic serine had been retained, allowing NTE to keep a vestigial esterase activity unrelated to its physiological function. In this model inhibition and ageing of the AChE-like domain would disrupt the protein-protein interactions by which cells communicated. This line of thinking had a considerable influence on certain decisions taken during this project, particularly at the stages described in Chapters 5 and 6

1.2 Introduction to cloning

Gene cloning projects essentially rely on the selection of a DNA clone from a library of clones which represent all or most of the genes in a organism or a particular tissue or cell type.

Selection strategies for cloning an animal gene fall into 5 broad categories:-

1.2.1 Positional cloning

1.2.2 Functional selection

1.2.3 Immunochemical selection

1.2.4 Nucleic acid hybridisation selection

1.2.5 'Virtual' cloning

1.2.1 Positional cloning

It is possible to locate and clone genes responsible for genetic diseases simply on the basis of their position within the genome. Unless the disease is caused by large scale chromosomal rearrangements which are cytogenetically detectable, the gene localisation usually takes the form of linkage analysis (Wicking and Williamson, 1991).

Linkage analysis works by studying the way a disease or trait is inherited relative to DNA markers of known chromosomal location. Once the chromosomal location of the trait is determined, normally an interval of about 100 to 1000 kbp, it is then necessary to clone and search the genomic DNA for gene coding regions and, by a process of elimination, isolate the disease gene (Wicking and Williamson, 1991). However, since OPIDP does not closely resemble any known genetic disease and no strain polymorphisms are known which could be used to track NTE, linkage analysis is completely unsuitable as a possible cloning approach.

1.2.2 Functional selection

Functional selection exploits the biochemical properties of a protein to isolate the gene. Messenger RNA (mRNA) is isolated from the organ known to express the desired protein. It is then reverse transcribed to make cDNA and cloned into a plasmid with suitable promoter for expression in the vector's host. In theory, a cDNA expression library could be screened for the binding of S9B. However, there were a number of drawbacks to this approach. Due to NTE's large size and consequent high complexity, it seems unlikely that NTE would express as an active protein in a bacterial or yeast expression system, necessitating the use of more technically-challenging hosts such as insect or mammalian cell lines. Furthermore, since biotin is an endogenous cellular component it would probably cause an unacceptably-high background if a biotin-labelled probe was used to screen the library. Thus, a fluorescein- or digoxigenin-labelled version of S9B (S9F and S9D, respectively) would need to be synthesised.

One possible benefit to using a fluorescein-labelled saligenin phosphoramidate would be the ability to use a fluorescence-activated cell sorter (FACS) to screen and separate individual cells on the basis of S9F binding. This approach was used by Rice *et al* (1990) to clone a full length CD4 cDNA in a COS-7 host cell by screening 500,000 independent clones. FACS sorting of NTE would probably be a rather more demanding proposition. Rice *et al* already had a CD4 cDNA with which they had developed the system. Furthermore, CD4 is a cell surface protein and was detected by labelled antibody. Labelled saligenin phosphates are very hydrophobic and would permeate the entire cell with the S9F probably lodging in the cell membranes, leading to a high fluorescent background possibly masking any NTE signal.

The use of the traditional PV assay for NTE, with differential inhibition by paraoxon and mipafox (Section 1.1.4) would be too cumbersome and probably

too insensitive for this approach. Thus, functional cloning of NTE was not pursued as part of the cloning strategy.

1.2.3 Immunochemical selection

In antibody selection, cDNA is cloned into an expression vector and transferred into either a bacterial or bacteriophage host strain. The recombinant micro-organisms are grown to form colonies, then are transferred onto a suitable solid support (traditionally nitro-cellulose membrane). The immobilised colonies are then screened for binding of an antibody specific to the desired protein.

Antibody selection has the advantage over functional selection in that the protein need not be biochemically active to be detected. However, as with functional selection, the cDNA can be of different lengths (due to the reverse transcriptase failing to completely copy the mRNA). The cDNA is thus inserted into the vector in a random reading frame resulting in two thirds of the clones expressing in the wrong reading frame, effectively rendering them invisible to the antibody. Nevertheless, this approach has been successfully used to isolate over 200 genes and was not lightly dismissed (Sambrook *et al*, 1989b).

Rabbit antisera raised against a synthetic peptide designed from chicken brain NTE sequence provided the possibility of screening an expression library. However, these antisera were relatively low titre and on Western blots of microsomal protein stained not only NTE but several other polypeptides. Some experiments were performed to test the feasibility of antibody screening on colony lifts from a λ ZAP cDNA library. This showed that the serum had a significant amount of background reactivity to the lysed *E. coli* proteins present in the plaques. This background could not be satisfactorily removed by preabsorption with a lysed *E. coli* preparation and antibody selection was not pursued further.

1.2.4 Nucleic acid hybridisation selection

Nucleic acid hybridisation selection exploits the specific base pairing between complementary DNA strands. Conventionally this can be used in two ways:-

- A) Hybridisation screening of a DNA library. In which a radioactively- or fluorescently- labelled DNA probe is used to identify clones in a DNA library with matching sequence (Sambrook *et al*, 1989b).
- B) Polymerase chain reaction (PCR) in which two relatively short oligonucleotides are used to amplify regions of intervening DNA (Taylor, 1991).

Prior to the initiation of the project a commercial Clontech pig brain cDNA library was screened using two degenerate oligonucleotide probes designed using pig brain NTE sequence. This experiment failed to produce any positive clones, so a PCR based cloning strategy was pursued; this is discussed in Section 1.3.5.

1.2.5 'Virtual' cloning

One particularly attractive approach to cloning any gene is by searching the genetic databases for sequences similar to fragments of protein sequence determined by Edman degradation. There are two databases of particular relevance to gene cloning: EMBL and SWISSPROT databases.

The SWISSPROT protein database, though smaller than EMBL (the current releases have around 70,000 and 500,000 entries respectively), is more thoroughly annotated and cross-referenced. This makes it suitable for initial screening of peptide sequence to determine if the sequence is novel and also search for proteins which are similar to two or more NTE sequences. Discovery of such a protein would be useful to the project as it could provide a guide to the

relative positions of the peptide sequences and possibly provide clues as to NTE's function.

Although other genome databases are available (GenBank and DNA database of Japan) the exchange of data between them render them effectively identical and since EMBL was available on the local computing resources (Leicester University Computing Service and the Hixtons Human Genome Mapping Project computers) EMBL was used as the default database for almost all searches.

EMBL is a DNA database and contains DNA sequence from a variety of sources. Besides the cDNA sequences for already cloned genes, it also contains genomic sequence from the many genome sequencing projects currently in progress. Of particular interest is the Expressed Sequence Tag (EST) division of EMBL. ESTs are the result of picking and sequencing random clones from a directional cDNA library, often one that has been 'normalised', i.e. one that is synthesised to reduce the representation of highly expressed genes (Schuler *et al*, 1996). Each EST clone is sequenced once at the 5' end and once at the 3' end using standard primers flanking the plasmid's cloning site. Each sequencing run normally yields between 300-500 bp of DNA sequence. The clones are then propagated and usually made available via the IMAGE consortium (Lennon *et al*, 1996). Therefore any matches between a peptide sequence and an EST can be easily investigated either by obtaining the clone from IMAGE or by further research through the database.

One useful feature of the EST sequences is the asymmetry of the 5' and 3' end sequence results. Because the cDNA library was made using an oligo-dT primer, the overwhelming majority of 3' end sequences initiate from the very end of the 3' untranslated region and almost all of the 3' end sequences record the same data. Conversely the initiation point for the 5' end data is essentially random, determined by the termination of the reverse transcriptase. The 3' end sequence is useful to cluster ESTs by transcript and identify a group of

corresponding 5' end sequences even if these have no overlap (see Figure 1.10). This feature of EST is used in the UniGene project (Schuler *et al*, 1996) to produce Sequence Tagged Site markers (discussed in Section 7.2.6).

1.3 PCR cloning of NTE

1.3.1 Degenerate PCR

In conventional PCR, a unique pair of oligonucleotides are designed using known DNA sequence. These are used to amplify the region of DNA lying between them, normally producing a product of predictable size and often allowing the identification of the correct product by agarose gel electrophoresis.

When only peptide sequence is available, it is possible to deduce the DNA sequence which encodes the peptide sequence. However, due to the degeneracy of the genetic code a single amino acid can be coded for by up to six different codons (Figure 1.11). Thus, rather than synthesising a single oligonucleotide primer, a mixture of oligonucleotide primers are synthesised, each primer in the mix representing one possible permutation of DNA sequence which encodes the gene-specific peptide sequence. This degenerate sequence PCR can be used as an approach to gene cloning (McPherson *et al*, 1991).

An important consideration in designing degenerate primers is to keep the number of primers in the pool (the degeneracy) to a minimum. Since, as the degeneracy increases the proportion of primer identical to the desired gene drops, reducing the amount of product that can be produced as there is less primer to produce it. Secondly and more significantly, as the degeneracy increases, so does the number of primers that do not match the desired product; thus the chance of spurious product production increases, which can easily swamp any genuine signal.

The NTE peptide sequences available at the initiation of cloning of pig cDNA were assessed for reliability of the peptide sequence and their suitability for degenerate primer design (Figure 4.1). As a basis for a gene cloning project the sequences were less than optimal. Either the sequences were short, resulting in a short degenerate primer with a low melting temperature and high chance that the sequence is not unique in the genome, resulting in false positives. Alternately, the longer sequences contained the highly degenerate amino acids leucine, arginine or serine which radically increase the degeneracy of the primer pool and were also rich in fourfold degenerate residues.

The high overall degeneracy of the primer pools was in part addressed by introducing inosine to the 3rd position 'wobble' base if the position was fully degenerate (ie specified a A T G or C), but not within 3 bp of the 3' end of the primer as this is known to adversely affect priming. Inosine is known to base pair with all bases and has been often been used to reduce the degeneracy of a degenerate PCR primer pool (McPherson *et al*, 1991).

Degenerate PCR using primers derived from partial protein sequence often differs from conventional PCR in that the relative positions of the peptide sequence and hence the degenerate primers position is unknown. Such was the case with NTE and this significantly increased the complexity of the experiments, because two sets of PCR reactions needed to be performed to cover all possible permutations of the primer positions. In addition to the primer pairings, single primer controls are necessary since previous experience (as well as Hiltunen *et al*, 1994) has shown that degenerate primers can produce artifactual PCR products. Finally, negative controls omitting the cDNA are necessary for both the primer pair and single primer reactions to test for contamination and primer dimer formation.

The problem with the increased number of reactions in an experiment is not the large number of reactions, but the proportionally increased chance of spurious product formation. Without reliable information of how large a genuine

product should be, there is no simple method of choosing candidates for further investigation. Chapter 5 describes how two of the peptide sequences were putatively positioned relative to each other and used in a degenerate PCR experiment. Unfortunately there was little other positional information aside from the minimum distance that the sequence was from the C-terminal of NTE, provided by the fragment sizing that accompanied each peptide sequence determination (Figure 3.7). One method that could take advantage of this information was Rapid Amplification of cDNA Ends (RACE).

1.3.2 Rapid Amplification of cDNA Ends

RACE is a PCR-based method for the amplification and cloning of the 5' or 3' ends of a gene (called 5' RACE and 3' RACE respectively), using either DNA or degenerate DNA sequence for product selection (Frohman *et al*, 1990 and Lee and Caskey, 1990).

In RACE, cDNA is synthesised with a known DNA 'anchor' sequence attached to either the 3' or 5' end of the product. Then the desired gene is amplified using a Gene Specific Primer (GSP) derived from the known or inferred gene sequence and an anchor primer (Figure 1.12). The principle advantages of the RACE protocol are that only a single GSP is required for a reaction. In addition, since the peptide sequence and hence the degenerate primers are of known orientation relative to the mRNA, no extra reactions need to be run to test alternate orientations, unless a second round of RACE is performed to nest pairs of degenerate primers.

1.3.3 5' RACE

In 5' RACE (amplification of the 5' end of a cDNA) the cDNA is synthesised from RNA using reverse transcriptase and a poly-dT oligonucleotide primer. The anchor sequence can then be enzymatically attached to the 5' end of the cDNA (Frohman, 1990). This second stage in cDNA synthesis was the principal factor against using the 5' RACE method for cloning NTE, since the additional synthetic stages are poorly reproducible and can lead to truncated cDNAs which may omit the DNA sequence encoding the NTE peptides (Edwards *et al*, 1995).

The advantage of 5' RACE in the context of cloning NTE is that the more reliable N-terminal peptide sequence is used to design the 3' end of the degenerate primers; since the 3' end is most sensitive to mismatch, it is most important for the sequence in this region to be accurate. The principal disadvantage of 5' RACE is that the PCR product makes little use of any additional peptide sequence. In each Edman sequencing run there is normally sequence at the C-terminal which cannot be used to design degenerate primers because its either too heterogeneous or, more often, the Edman sequencing reaction is beginning to fail so the residues returned are unreliable. This sequence though too untrustworthy for primer design can be useful in rapidly identifying putative NTE clones since the sequence adjoining that produced by the degenerate primer should at least resemble the unused sequence.

1.3.4 3' RACE

3' RACE was used in the first attempt at cloning NTE using degenerate primers (see Chapter 4). The cDNA used in the 3' RACE protocol requires only a small change to the standard cDNA synthesis protocol; this involves attaching an adapter/anchor primer construct consisting of a poly-dT region (to anneal to the poly-A tail of mRNA) and a unique DNA sequence to 'tag' the 5' end of the cDNA and provide a target for a reverse anchor primer (Figure 1.12). One advantage of the 3' RACE method is it allows an estimate of the smallest PCR product that the

RACE reaction could theoretically produce, based on the size of the peptide fragments the original protein sequence came from (Figure 4.1). However, predicted product sizes only provide a rough guide to product size since 3' RACE is known to be able to produce truncated products if the poly-dT primer anneals to an adenine rich region within the mRNA sequence (Schmidt *et al*, 1993).

1.3.5 Other cloning work performed on NTE

Besides the cloning attempts described in Chapters 4 and 5, other methods were tried by this research group. The peptide sequences ADLT and DLGL (see Figure 3.7) were used to design three degenerate inosine-containing oligonucleotide probes. These were used to screen 1.6 million colonies from a Clontech pig brain cDNA library at low stringency but no clones were isolated. A second approach utilised the 26 amino acid ADLT (Figure 3.7) peptide sequence. Forward and reverse degenerate primers were designed to residues 1 to 7 and 15 to 21 respectively of the ADLT sequence. A PCR product produced by this primer pair would have produced a 63 bp product which could have been unambiguously identified as ADLT by translation of the 25bp DNA sequence that lay between the degenerate primers. However, despite careful optimisation, the degenerate primers failed to produce any products of the correct size.

Figure 1.1 General structure of organophosphorus esters

A) Various substitutions of this general structure are possible, R^1 and R^2 are usually alkyl or aryl groups bonded either directly to the phosphorous or via an oxygen, sulphur or nitrogen atom. The substituent X may be one of a wide variety of groups; either organic (aliphatic, aromatic or heterocyclic) bound to the phosphate via a labile group (usually oxygen or sulphur), or inorganic (halogen or cyanide groups). The common property of the X group is that it is the most labile of the four bonds attached to phosphorous.

Compound	R1	R2	X
Sarin	Me-	Pr ⁱ O-	-F
DFP	Pr ⁱ O-	Pr ⁱ O-	-F
Paraoxon	EtO-	EtO-	-OPh-4-NO ₂
Mipafox	(Me) ₂ CHNH-	(Me) ₂ CHNH-	-F
EPNO	Ph-	EtO-	-OPh-4-NO ₂

B) The saligenin cyclic phosphoryl OP compounds represent a special case of the OP form, in which the R^2 and X, positions are linked by a heterocyclic moiety. the CH_2O-P bond is broken when saligenin cyclic phosphoryl compounds form a covalent complex with the serine at the active site of NTE (see Figure 1.3).

Four saligenin phosphate compounds are mentioned in the text:-

Saligenin cyclic o-tolyl phosphate (TSP), in which the methyl group in the heterocyclic ring is hydroxylated and the leaving group is 2, methylphenol group (see Figure 1.4).

Octyl saligenin cyclic phosphate in which R^1 is octanol.

Phenyl O, O' saligenin phosphate (PSP) in which R^1 is phenol

Biotinylated saligenin phosphoramidates (ie S9B; see Figure 1.8).

Figure 1.2 Catalytic mechanism of serine esterases.

- 1) The oxygen of the catalytic serine makes a nucleophilic attack on the carbonyl atom in the ester, to form a tetrahedral Michaelis complex (the negative charge on the oxygen is stabilised by hydrogen bonding with other portions of the protein; not shown).
- 2) The Michaelis complex collapses ejecting the labile alcohol group (R'OH) leaving a bound acyl group (R-C=O).
- 3) The catalytic serine is regenerated by hydrolysis of the acyl group and the carboxylic acid is released from the catalytic cleft (adapted from Simmonds, 1992).

Figure 1.3 Mechanism of OP inhibition and ageing in serine esterases.

When a serine esterase (in blue) is inhibited by an OP, the tetrahedral OP-serine complex is stabilised by the hydrogen bonding that also stabilises the tetrahedral Michaelis complex formed during acylation of the enzyme (see Figure 1.2). The complex is not stable and can undergo one of two fates: 1) slow hydrolysis (over a period of hours) removing the OP and regenerating the enzyme, or, 2) 'Ageing' when one of the other phosphate groups are hydrolysed leaving a negative charge on the OP and rendering the inhibition permanent (adapted from Johnson, 1992).

Figure 1.4 Formation of saligenin cyclic o-tolyl phosphate from TOCP.
Adapted from Johnson, 1982

Neuropathic

Phosphate

Phosphonate

Non-neuropathic

Phosphinate

Carbamate

Figure 1.5 Prediction of OP delayed neuropathic potential.

All compounds are shown having covalently bound to the active site serine of NTE (see Figure 1.3). The phosphate and phosphonate both contain a relatively labile R-O-P group which can hydrolyse with the loss of the R group leaving a negatively charged group attached to the serine (Figure 1.3). In contrast the R-P and R-N bonds of the phosphinates and carbamates are resistant to such hydrolysis, do not age and do not cause OPIDP.

<u>Tissue</u>	<u>NTE activity</u> <u>% brain</u>	<u>Tissue</u>	<u>NTE activity</u> <u>% brain</u>
Brain	100	Muscle (pectoralis)	0
Spinal cord	23	Muscle (gastrocnemius)	0
Dorsal root ganglia	18	Liver	0
Spleen	60	Kidney	0
Spleen lymphocytes	26		
Blood lymphocytes	24	10-day embryo brain	26
Blood T cells	24	14-day embryo brain	21
Blood B cells	39	17-day embryo brain	64
Heart	14	20-day embryo brain	62
Thymus	69	6 week old brain	100-120
Ileum	94		

Figure 1.6 Tissue distribution of NTE activity as a percentage of adult hen brain NTE activity (reproduced from Johnson, 1992).

<u>Edman cycle number</u>	<u>Residue</u>
1	Lysine Isoleucine Valine Phenylalanine Alanine
2	Glutamic acid Valine Alanine
3	Lysine Alanine Glycine
4	Glutamic acid
5	Alanine Dehydroalanine
6	Methionine Lysine
7	Glycine

Figure 1.7 Putative N-terminal sequence around the NTE catalytic serine.

An enriched (2% pure) [^3H]DFP-labelled-NTE fraction was first digested with V8 protease and a 16kDa [^3H]DFP-labelled fragment was purified by preparative SDS-PAGE. This fragment was then thermolysin digested and a [^3H]DFP-labelled peptide fragment HPLC-purified. Edman degradation of this peptide fragment yielded a seven residue heterogeneous sequence. The dehydroalanine in cycle 5 reflects a modified serine; surrounding it can be discerned the serine esterase consensus sequence Gly-Glu-Ser-XXX-Gly (Glynn *et al*, 1993).

Figure 1.8 General structure of the biotinylated saligenin phosphoramidates used to purify NTE.

A total of six biotinylated saligenin phosphoramidates were synthesised with alkyldiamine spacers of 5, to 12 methyl carbon atoms in length. These were assayed for inhibition of NTEs phenyl valerate hydrolase activity and a rank order of potency was established to be $n=9, 10 > 8, 12 > 7 \gg 5$. The $n=9$ homologue was selected for subsequent work and designated S9B. Its I_{50} was determined to be 1.6 nM and it was shown that it could bind to a 155kDa protein (Glynn *et al*, 1994).

<u>Protein</u>	<u>Size (kDa)</u>	<u>Function</u>	<u>Tissue location</u>	<u>Reference</u>
Glutactin <i>Drosophila</i>	155	Cell-cell and cell- substrate interactions	Neural Basement membrane	Olson <i>et al</i> , (1990)
Neurotactin <i>Drosophila</i>	135	Cell adhesion	Neurons	Escalera <i>et al</i> , (1990)
Gliotactin <i>Drosophila</i>	109	Formation of blood nerve barrier	Peripheral glia	Auld <i>et al</i> (1995)
Neuroigin1 Rat	116	Cell-cell and cell- substrate interactions	Brain neurons especially synapses	Ichtchenko <i>et al</i> , (1995)
Neuroigin2 Rat	94	As neuroigin1	as neuroigin1	Ichtchenko <i>et al</i> , (1996)
Neuroigin3 Rat	100	As neuroigin1	as neuroigin1	Ichtchenko <i>et al</i> , (1996)

Figure 1.9 AChE-like enzymes which lack catalytic activity.

Figure 1.10 Synthesis of cDNA for EST sequencing produces a common 3' end sequence and many 5' end sequences from the same gene.

<u>Amino acid</u>	<u>symbol</u>	<u>Codons</u>	<u>Redundancy</u>
Alanine	A	GC[ATGC]	4
Arginine	R	CG[ATGC], AG[AG]	16
Asparagine	N	AA[TC]	2
Aspartic acid	D	GA[TC]	2
Cysteine	C	TG[TC]	2
Glutamine	Q	CA[AG]	2
Glutamic acid	E	GA[AG]	2
Glycine	G	GG[ATGC]	4
Histidine	H	CA[TC]	2
Isoleucine	I	AT[ATC]	3
Leucine	L	CT[ATGC], TT[AG]	16
Lysine	K	AA[AG]	2
Methionine	M	ATG	1
Phenylalanine	F	TT[TC]	2
Proline	P	CC[ATGC]	4
Serine	S	TC[ATGC], AG[TC]	32
Threonine	T	AC[ATGC]	4
Tryptophan	W	TGG	1
Tyrosine	Y	TA[TC]	2
Valine	V	GT[ATGC]	4

Figure 1.11 Amino acid degeneracy codes.

Figure 1.12 Synthesis of cDNA for 3' RACE.

A linker adapter designated BAT39 (see Figure 4.2) consisting of a poly-dT region at the 3' end of the primer followed by a unique anchor sequence (BAT39 5' GTTACGGCTGGATGAGTGACTTTTTTTTTTTTTTTTTT 3') was annealed onto poly-A RNA. Reverse transcriptase was then used to generate the cDNA and the RNA was then removed by digestion with RNase A.

The BAT39-primed cDNA was used in a series of 3' RACE reactions in which a degenerate Gene Specific Primer (GSP) was used in combination with a primer (BSP) made using the BAT anchor sequence (attached to the 5' end of all cDNA templates) as the reverse primer.

In 5' RACE the anchor primer is attached to the 3' end of cDNA (i.e the 5' end of the mRNA).

Chapter 2 Methods and reagents

2.1 Protein methods

2.1.1 Preparation of microsomes

Reagents

TE buffer

50mM TRIS-HCl pH8.0

1mM EDTA

DTT/TE

TE supplemented with 1mM dithiothreitol (DTT; Sigma)

0.9%NaCl/TE

0.9% Sodium Chloride in TE buffer

Protocol

A total of 150g of frozen brain tissue was thawed, chopped in to 1 litre of DTT/TE and then homogenised in a Waring blender set at low speed. Further DTT/TE was added to a final volume of 1500ml and distributed evenly in to six 250ml bottles for a JA-14 rotor (Beckman) and spun at 9000rpm (12,000xg) at 4°C for 15 minutes. The supernatant was decanted and transferred to six 250ml Type 19 bottles (Beckman), TE was added until the bottles were full whereupon they were spun at 19,000rpm (54,000g) at 4°C for 1 hour in a Type 19 Beckman rotor. The supernatant was discarded and the pellets were resuspended in 30ml 0.9%NaCl/TE using an Ultra-turrax set at medium speed.

The resuspended pellets from three 19k spins (ie 450g of processed brain) were placed in to six type 19 centrifuge bottles and topped up until full with additional 0.9% NaCl/TE, then spun at 19,000rpm at 4°C for 1 hour. The supernatant was discarded and each pellet was resuspended in 40ml TE, dispersing it by Ultra-turrax set at medium speed. A 1ml aliquot was taken from the resuspended pellets for protein determination (see section 2.1.2) and the

remaining microsomes were pooled and stored at -20°C in 25ml aliquots. Under these conditions NTE was stable for approximately two weeks. The typical yield was about 5mg of microsomal protein per gram of brain tissue processed; at least 3000mg of microsomal protein was required for the S9B labelling stage (Glynn *et al*, 1994).

2.1.2 Protein Determination

Reagents

DC protein assay kit (BioRad)

Reagent A an 'alkaline copper tartrate solution'

reagent B a 'dilute Folin Reagent'

Reagent S

Protocol

If the sample contained detergent 20µl of Reagent S was added per ml of Reagent A. A standard curve of 0, 250, 500, 750, 1000, 1500 and 2000µg/ml BSA was prepared and 100µl was pipetted into clean dry test tubes; a 5x dilution of sample was similarly prepared. To each tube 500µl of Reagent A was added and vortex mixed, then 4ml of reagent B was added and the tube was vortexed again. After 15 minutes the absorbance of each reaction was measured at 750nm. The standards typically produced ODs up to 0.6 (BioRad DC Protein Assay Kit).

2.1.3 S9B-labelling and solubilisation of microsomes

Reagents

TE buffer

50mM TRIS-HCl pH8.0

1mM EDTA

0.1%DTT/TE

TE supplemented with 0.1%DTT (Sigma)

160 μ M S9B in dry dimethylformamide (DMF) Supplied by D. Read (see Glynn *et al*, 1994)

10% SDS solution

Protocol

Microsomes equivalent to 750mg of protein were defrosted and diluted with TE to a final concentration of 2mg/ml and dispersed by Ultra turrax set at medium speed. The microsomes were then evenly split in to six Type 19 rotor bottles and warmed in a 37°C incubator for 15 minutes then 62.5 μ l of 160 μ M S9B in dry DMF was added to give a final concentration 160nM S9B. This was incubated for 20 minutes at 37°C after which the bottles were filled to the top with 180ml TE and then centrifuged at 19,000xg at 4°C for 1 hour. The supernatant was discarded and the pellet resuspended in 0.1%DTT/TE by Ultra-turrax. The resuspended microsomes were pooled in a 500ml Schott bottle and 5.7ml of 10%SDS solution was added to give a final SDS concentration of 0.15%. The bottle was then placed in a boiling waterbath for 7 minutes and subsequently cooled under a running tap. This process was repeated a further three times until a total of 3000mg of microsomal protein had been S9B labelled (Glynn *et al*, 1994).

2.1.4 Solubilisation of microsomes and capture of NTE on avidin-Sepharose

Reagents

TE buffer

50mM TRIS-HCl pH8.0

1mM EDTA

0.1%DTT/TE

TE supplemented with 0.1% DTT

DTT/SDS/TE

TE supplemented with 0.1% DTT and 0.15% SDS

10% SDS solution

50 ml Falcon tube

Centriprep100 spin column (Amicon)

Avidin-Sepharose -supplied by P. Glynn (Glynn *et al*, 1994)

Protocol

A 10ml column of avidin-Sepharose was packed in to a 2cm diameter chromatography column (Pharmacia) and pre-equilibrated by pumping 50ml of DTT/SDS/TE through at a rate of 80ml/hour. The SDS-solubilised S9B-labelled microsome extracts were then combined and were pumped through the column overnight at room temperature (a rate of approximately 80ml/hour). In the morning the remaining SDS extract was pumped through at a rate of 450ml/hour and the column was washed by pumping through 200ml 0.15%SDS/TE. The avidin-Sepharose slurry was transferred to a 50ml Falcon tube and washed a further four times by resuspending in 40 ml 0.15%SDS/TE, then pelleting by centrifugation at 1500rpm (400xg) for 2 minutes without braking in a benchtop centrifuge. The bound protein was then eluted by resuspending in 30ml of 1%SDS/1%DTT in 0.2x TE and then immersion in boiling water for 7 minutes. The extract was cooled to room temperature and the avidin-Sepharose removed, pelleting by centrifugation at 1500rpm (400xg) for 5 minutes after which the supernatant was decanted. Trace contamination from carried over avidin-

Sepharose was removed by a secondary spin. The supernatant was then concentrated down to a 2ml volume using a Centriprep100 spin column (Amicon; three 1600rpm spins were required: 45 minutes, 20 minutes and 10 minutes discarding filtrate between spins). A 50 μ l aliquot of the concentrate was taken (designated 'avidin eluate') and the rest of the concentrate was submitted to D. Read for preparative-PAGE to remove the remaining contaminant proteins (Glynn *et al*, 1994).

2.1.5 Analysis of prep-PAGE fractions

Reagents

SDS sample buffer

10% SDS

10% glycerol

62mM TRIS-HCl pH6.8

~0.1% bromophenol blue

0.25% Coomassie blue in 10% acetic acid/50% methanol

Microcon100 columns (Amicon)

Bovine Serum Albumin (BSA) concentration standards

2.5, 5, 7.5 or 10 μ g/ml BSA dissolved in 2% sample buffer and 0.1% DTT

Protocol

Fractions 20 to 32 from the prep-PAGE usually contained the 155kDa NTE protein band. An aliquot (40 μ l) from each fraction was mixed with 10 μ l of sample buffer and the mix boiled for 2 minutes, then 30 μ l was loaded on to a 7.5% SDS-PAGE gel along with 30 μ l the avidin eluate (40 μ l avidin eluate +10 μ l sample buffer). The SDS-PAGE gel (Section 2.1.7) was run at 200V until the bromomphenol blue marker had reached the bottom of the gel. The gel cassette was dismantled and the gel stained by immersion in 0.25% Coomassie blue for 20 minutes and destained as described in the SDS-PAGE protocol (Section 2.1.7). Fractions that contained the 155kDa NTE but no 120kDa polypeptide were selected (normally 4 fractions) and concentrated to 200-300 μ l using a

Microcon100 spin columns. 500µl of sample was spun in each column for 15 minutes at 3000rpm (720xg) in a benchtop microcentrifuge, the flowthrough was discarded and the retentates combined in a single column which was spun at 3000rpm for a further 10 minutes to give a final sample volume of 200-300µl.

The pure NTE solution was precipitated by adding 1.1 volumes of acetone and incubating overnight at -20°C, reserving 10µl for later analysis. The precipitate was pelleted by spinning at 14,000rpm (15,800xg) for 30 minutes in a microcentrifuge and the pellet dried by vacuum centrifuge for 20 minutes. The yield and purity of the pure NTE protein was assessed by running a 3µl aliquot of the microcon concentrate (diluted to 30µl with 2% sample buffer+0.1%DTT) on a 7.5% SDS-PAGE gel (Section 2.1.7 with 30µl concentration standard of 150, 300, 450 and 600ng BSA (Glynn *et al*, 1994)

2.1.6 V8 protease digest

Reagents

V8 protease (Sigma)

Novex 4-20% gradient gel (R and D systems)

Protocol

The precipitated NTE was redissolved in 40µl of 0.1%SDS/1% DTT in TE buffer and boiled for 3 minutes. A pilot digest of 4µl NTE + 1µl V8 protease solution was initially run with V8:NTE ratios of 1:6 and 1:18 (by mass) paired with a blank which contained only the V8 protease. The digests were incubated for 2 hours at 37°C and stopped by the addition of 15µl 2% sample buffer. The digests were then run on a Novex 4-20% gradient gel (150V for 1.5 hours) which was stained as per the SDS-PAGE protocol (Section 2.1.7). Once the appropriate ratio of NTE:V8 was determined the remaining NTE was digested under the same conditions then submitted to the Protein and Nucleic Acid (PNAC) sequencing laboratory for peptide sequencing by Edman degradation(Glynn *et al*, 1994).

2.1.7 SDS-Polyacrylamide Gel Electrophoresis

Reagents

Solution A (BioRad)

30% Acrylamide

0.8% Bis-acrylamide

Solution B

1.5M TRIS-HCl pH8.8

Solution C

10% SDS

Solution D

0.5M TRIS-HCl pH6.8

10% Ammonium persulphate (APS; must be made fresh)

SDS sample buffer

10% SDS

10% glycerol or sucrose

62mM TRIS-HCl pH6.8

~0.1% bromophenol blue

SDS-PAGE running buffer (x5 stock solution per litre)

15g TRIS (base)

72g glycine

5% SDS

TEMED (Sigma)

50ml syringe

Novex molecular weight size standard (R and D Systems)

Protocol

The gel plates were cleaned with methanol and assembled in the gel casting stand. To make 80ml of 7.5% acrylamide gel, 39ml distilled water, 20ml Solution A, 20ml of Solution B and 0.8ml of Solution C were mixed in a Buchner flask and degassed by sealing the flask with a rubber stopper and reducing the pressure for 10 minutes. The polymerisation reaction was initiated by adding

160 μ l 10% APS and 60 μ l TEMED, then the mixture was transferred to the gel cassette filling it to within 20mm of the upper edge of the smaller plate. The gel was then overlayed with butan-2-ol to exclude air and accelerate polymerisation.

After a maximum of 1 hour the butan-2-ol was poured off and the top of the gel was washed with several rinses of distilled water. The water was drained from cassette and a comb inserted in to the cassettes. Stacking gel was prepared by mixing 8.5ml Solution A, 12.5ml Solution B and 0.5ml Solution D with 28ml of distilled water in a clean Buchner flask. This mixture was degassed for 10 minutes and 500 μ l 10% APS and 50 μ l TEMED was added to the mix, which was then transferred to the gel cassette filling to the top of the smaller plate and taking care to ensure no bubbles were trapped by the comb. The stacking gel normally set within 1 hour and gels could be stored at 4°C for up to 10 days.

To run the gel, the comb was removed and the gel cassette fitted into a gel tank; this was filled with 1x SDS running buffer and samples applied to wells in 2% sample buffer. Novex molecular weight size standards were used throughout the project (Laemmli, 1971).

2.2 DNA purification and cloning

Unless otherwise stated the following techniques were based on ones presented in Sambrook *et al*, 1989a.

2.2.1 Agarose gel electrophoresis

Reagents

5xTBE

450mM TRIS-Borate pH8.0

10mM EDTA

Agarose electrophoresis grade (Sigma)

10mg/ml Ethidium bromide (Sigma)

1kb ladder (Gibco)

Loading buffer

50% glycerol

Trace ammounts of Bromophenol blue and Xylene cynol

(sufficent to allow visualisation of sample when loading gel)

Protocol

A 1% agarose gel in 0.5xTBE stained with 0.5µg/ml ethidium bromide was used to resolve DNA fragments throughout the project. 1% agarose was boiled in 0.5xTBE until the powder was completely dissolved. This was then allowed to cool to less than 45°C at which point ethidium bromide was added to a final concentration of 0.5µg/ml. The gel casting tray was then filled to a depth of 5-6mm and the well comb was then inserted. After the agarose gel had set the comb was removed the gel loaded into the electrophoresis tank and immersed in 0.5xTBE+0.5µg/ml ethidium bromide. The DNA sample was mixed with 0.1 volumes of loading buffer and pipetted into the wells. The gel was run at 2.5-5 V/cm, with the anode at the bottom the gel until the DNA bands of interest were satisfactorily resolved (monitoring by occasional viewing under UV light). Gels were photographed with Polaroid Type 53 film.

The Gibco 1kb ladder was used throughout the project for sizing DNA fragments. The ladder consists of bands of 75, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198 and 12216 bp in size. Figure 5.3 provides a good example of the first 12 bands, the bottom most being the 75bp in size.

2.2.2 PCR product purification by electroelution.

Reagents

Dialysis tubing

2% sodium bicarbonate

5xTBE

450mM TRIS-Borate pH8.0

10mM EDTA

TE buffer

10mM TRIS-HCl pH8.0

1mM EDTA

Protocol

The desired PCR product band was resolved by agarose gel electrophoresis and visualised with ethidium bromide under ultraviolet (UV) light (Section 2.2.1). A clean scalpel was used to cut the band from the gel (keeping the exposure to UV light to a minimum to reduce DNA crosslinking) and the agarose gel plug was placed along with 2-3ml 0.5xTBE in a short section of dialysis tubing (which was prepared by boiling for 10 minutes in a 2% solution of sodium bicarbonate, followed by thorough washing in distilled water). The dialysis tubing was sealed at each end by double knotting the tube and placed perpendicular to the current in an electrophoresis tank filled with 0.5xTBE and a voltage of 5V/cm placed across the tank for 2 hours. The DNA accumulated on the positively charged (anode) side of the dialysis tubing and was visualised under UV light. DNA bound to the dialysis bag was then dislodged by reversing the polarity for 1 minute then decanting the contents of the dialysis bag

(excluding the agarose gel plug). The DNA was recovered by phenol/chloroform extraction and ethanol precipitation (Section 2.2.4) and redissolved in 10mM TRIS-HCl pH8.0 or TE.

2.2.3 PCR Product purification by QIAspin kit

Reagents

Buffer PB (QIAGEN Proprietary buffer)

Buffer PE (QIAGEN Proprietary buffer)

Elution buffer

10mM TRIS-HCl pH8.0

QIAquick spin column (QIAGEN)

Protocol

Five volumes of buffer PB were added per 1 volume of PCR reaction, and the mixture was applied to a QIAquick spin column. After spinning at $\geq 10,000 \times g$ for 30-60 seconds in a 2ml collection tube, the flowthrough was discarded and 0.75ml buffer PE was applied to the column which was then spun as before, the flowthrough was again discarded. The empty spin column was spun briefly to remove residual buffer PE. Elution buffer (50 μ l) was then applied to the spin column which was placed in a sterile Eppendorf tube and elution buffer containing the DNA was spun out at $\geq 10,000 \times g$ (QIAGEN, 1997).

2.2.4 Phenol extraction and ethanol precipitation

Reagents

Phenol/chloroform

1 volume equilibrated phenol (Fisons)

1 volume chloroform (Fisons)

TE buffer

10mM TRIS-HCl pH8.0

1mM EDTA

3M Sodium Acetate pH5.2

Absolute ethanol

70% ethanol

Protocol

An equal volume of phenol/chloroform was added to the solution to be treated. The mixture was vortexed until the phenol/chloroform became emulsified and then spin at $\geq 10,000 \times g$ for 2 minutes until the phases separated. The upper aqueous phase was decanted and the procedure repeated prior to ethanol precipitation.

3M sodium acetate (0.1 volume) and 2 volumes of ethanol were mixed with the sample, and then incubated at -20°C for 1 hour. The sample was centrifuged at $>10,000 \times g$ for 30 minutes then the supernatant was decanted and the pellet rinsed in 500 μl 70% ethanol. After spinning for 5 minutes and removal of the ethanol the pellet was allowed to air dry prior to redissolving in TE or water.

2.2.5 Ethanol precipitation of oligonucleotides

Reagents

Absolute ethanol

3M Sodium acetate pH5.2

70% ethanol solution

10mM TRIS-HCl pH 8.0

Protocol

All oligonucleotides were synthesised by the Protein and Nucleic Acids (PNAC) laboratories and were precipitated by the addition of 0.1 volume 3M Sodium acetate, 2 volumes of absolute ethanol with 1 volume of primer stock solution and incubated for 1 hour at -20°C. After spinning at >10,000xg for 20 minutes the supernatant was decanted and the pellet washed with 70% ethanol. The pellet was then vacuum centrifuged until dry and redissolved in 50-200µl 10mM TRIS-HCl pH8.0. Primer concentration was determined by OD²⁶⁰ absorbance (OD²⁶⁰ 1 = 20µg/ml ≈ 3µM).

2.2.6 Purification of high molecular weight genomic DNA.

Reagents

Liquid Nitrogen (LN₂)

Extraction buffer

10mM TRIS-HCl pH8.0

100mM EDTA

20µg/ml Pancreatic RNase A (Gibco)

0.5% SDS

Proteinase K (Gibco)

Equilibrated Phenol (Fisons)

10M ammonium acetate

Absolute ethanol

70% ethanol

TE buffer

10mM TRIS-HCl pH8.0

1mM EDTA

General notes: To prevent premature melting of the pulverised brain powder any item which came into contact with the powder was exhaustively pre-chilled in LN₂ until almost no bubbles rose from the object.

Protocol

Approximately 20-40g of frozen (-20°C) pig brain was allowed to warm sufficiently to permit the tissue to be cut with difficulty, but it not allowed to fully defrost. The tissue was cut in to chunks of ~1-3 cm³ volume and immediately dropped into LN₂ and allowed to freeze completely (5-10 minutes). Meanwhile the stainless steel waring blender was carefully dried, filled 1/3rd full with LN₂ and the temperature allowed to equilibrate. The LN₂ and brain chunks were then poured in to the blender and LN₂ was added/removed to ensure the blades were covered to a depth of 1-2 inches (but no more as the nitrogen tends to splash out when the blender ran).

The lid was fitted to the blender and firmly held down (wearing insulating gloves and safety specs) then the blender was placed behind a screen and then run in short bursts (~1 second) holding the lid down and allowing time for gas pressure to release between runs. Homogenisation was continued until the grinding sound becomes more even, checking regularly to ensure the blades stayed well covered with LN₂ and topping it up when necessary.

Once the tissue was sufficiently pulverised (ie reduced to a powder with no large >2mm chunks) the LN₂ was transferred along with the powdered brain to a pre-chilled plastic beaker and the LN₂ was carefully allowed to boil off to leave a pink 'cake' (still moist with LN₂) which was transferred in to (pre-chilled) 50ml Falcon tubes. These could be stored almost indefinitely at -70°C, however the lids were punctured (with a 18 gauge needle) to allow residual LN₂ to boil off and

escape (the lids may be replaced after 2 weeks although samples have been successfully stored without this precaution for a year without obvious ill effect).

Seven grams of powdered brain was incrementally added to 70ml extraction buffer in a 250ml Schott bottle, stirring after each addition of powder to prevent the powder freezing into aggregates. The extraction mix was incubated for 1 hour at 37°C, swirling occasionally, then 7mg of proteinase K (for a final concentration of 100 µg/ml) was added and incubated at 50°C overnight, swirling periodically for the first few hours.

The proteinase K digest was cooled to room temperature and 1 volume of equilibrated phenol added. This was gently mixed by hand, turning the bottle end over end until the contents were thoroughly emulsified. The phenol emulsion was transferred to 50ml Falcon tubes and centrifuged at 3000xg for 45 minutes. The aqueous supernatant was gently decanted in to a clean 250ml Schott bottle. The phenol extraction was repeated a further two times prior to ethanol precipitation.

A total of 0.2 volumes for 10M ammonium acetate and 2 volumes of ethanol was added to the aqueous phase and swirled gently to mix. The floating precipitated DNA was fished with a bent glass rod and transferred in to 20ml of 70% ethanol. Then the precipitate was spun down at 3000xg for 5 to 10 minutes, the supernatant discarded and a further 70% ethanol solution added and the precipitate spun down again. As much ethanol as possible was removed and the pellet allowed to air dry (without allowing the DNA to dry out completely).

The pellet was covered with 3.5 ml of TE and the DNA redissolved by gently rocking at room temperature for at least 24 hours. The DNA concentration was determined by absorbance at OD²⁶⁰ (OD²⁶⁰ 1 = 50µg/ml) and protein contamination assessed by measuring OD²⁶⁰/OD²⁸⁰ and ensuring ratio was >1.8. The typical yield of DNA was approximately 1mg DNA per gram of tissue.

2.2.7 DNA ligation with compatible sticky ends

Reagents

5x ligation buffer (Gibco)

10mM ATP

T4 DNA ligase (Gibco)

20u/μl Calf intestinal alkaline phosphatase (CIAP; Gibco)

10x Dephosphorylation buffer

500mM TRIS-HCl pH 8.5

10mM EDTA

Protocol

pBluescript (Stratagene) was the standard vector for cloning and subcloning. After a double digestion the vector was gel-purified and dephosphorylated by incubation with 1 unit of CIAP in 1x Dephosphorylation buffer. The CIAP was subsequently heat inactivated at 65°C for 15 minutes. Of the CIAP-treated vector 50ng was incubated in a 3:1 insert:vector molar ratio with 1μl ligation buffer, 1μl ATP, 1u T4 DNA ligase in a 10μl reaction volume for either 3 hours at 15°C or overnight at 4°C subsequently 5μl of the ligation mix was used to transform supercompetent XL1-Blue cells (see Section 2.2.10).

2.2.8 pGEM-T cloning of PCR products

Reagents

10xT4 ligase buffer (Promega)

pGEM-T vector (50ng/μl)

T4 DNA ligase

distilled water

Protocol

PCR product was added to 50ng of pGEM-T vector in a molar ratio of 3:1, in a control reaction 2μl of control insert (supplied with kit) was added to 50ng

pGEM-T vector and a negative control was included which only contained pGEM-T vector. To each of the three reactions 1µl of T4 ligase buffer and distilled water was added for a final volume of 9µl. T4 (1µl) ligase was then added and the mixture was incubated at 15°C for three hours. An aliquot (5µl) was withdrawn for transformation in to high efficiency competent cells (see Section 2.2.10) and the remainder stored at 4°C (Promega, 1996).

2.2.9 pCRscript cloning of PCR products

Reagents

10mM dNTP mix (2.5mM of each nucleotide)

0.5units/µl Pfu polymerase

10x polishing buffer

100mM Potassium chloride

100mM Ammonium sulphate

200mM TRIS-HCl pH8.75

20mM Magnesium sulphate

1% Triton X-100

1mg/mlBSA

pCRscript vector (10ng/µl)

10x pCRscript reaction buffer

500mM TRIS-HCl pH 7.5

70mM Magnesium chloride

10mM DTT

10mM ATP

SrfI restriction enzyme (5u/µl)

Protocol

PCR products produced by non-proofreading polymerases were 'polished' to produce blunt ended products for pCRscript cloning by adding 1µl 10mM dNTP mix 1.3µl 10x polishing buffer and 1µl Pfu polymerase to 10µl purified PCR product, overlaying it with 20µl of mineral oil and incubating at 72°C for 30 minutes.

Once cool, 5.5µl of polished PCR products were mixed with 1µl pCRscript vector, 1µl pCRscript 10x reaction buffer, 1µl ATP and 1µl *SrfI* restriction enzyme. The mix was incubated at room temperature for 1 hour then the reaction was stopped by heating to 65°C for 10 minutes; 5µl of the reaction mix was then withdrawn for transformation in to supercompetent cells (see Section 2.2.10). A positive control using a supplied control insert and a negative control in which only pCRscript was added to the reaction mix were also included in each ligation experiment (Stratagene, 1997).

2.2.10 Transformation of ligated products

Reagents

NZY/Glucose broth (per litre)

5g Sodium chloride

2g Magnesium sulphate (hydrated)

5g bacto-yeast extract

10g casein hydrolysate

pH was adjusted to 7.5 with NaOH and then the solution was autoclaved and allowed to cool prior the addition of 20ml sterile 20% glucose solution.

Epicurian Coli XL1-Blue MRF' Kan supercompetent cells (Stratagene)

JM109 High efficiency competent cells (Promega)

15ml Falcon tubes

L-agar/50µg/ml ampicillin (per liter)

10g bacto-typtone

5g bacto-yeast extract

10g Sodium Chloride

15g bacto-agar

The pH was adjusted to 7.0 with NaOH and autoclaved; when cool (~45°C) ampicillin was added to a final concentration of 50µg/ml.

0.2M IPTG (Isopropyl-1-thio-β-D-galactopyranoside) in distilled water

10% X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) in dimethylformamide

Protocol

The JM109 cells were used to clone the pGEM-T vector, XL1-Blue were used for all transformations involving pBluescript and pCRscript.

The supercompetent cells were stored at -70°C, thawed on ice and 3.5µl of 1.44M β-mercaptoethanol was added to 200µl of the XL1-Blue cells. 50µl of competent cells were gently dispensed into four pre-chilled 15ml Falcon tubes and 5µl of the ligation mixes added (one sample tube, one positive control and one negative control; 0.1ng of control plasmid (pUC18) was added to the remaining tube). All four tubes were incubated on ice for 10 minutes, then placed in a 42°C water bath for 45 seconds and quickly returned to the ice to cool for 2 minutes. 450µl of NZY/glucose was then added and the transformation mix and then placed in a shaking (225-250rpm) incubator at 37°C for 1 hour.

The L-agar ampicillin plates were poured in 150mm petri dishes and allowed to set, then 60µl of 0.2M IPTG and 60µl of 10% X-gal was spread over the surface of the agar at least 30 minutes prior to the addition of the transformed cells. All of the sample transformation was spread on the L-agar/amp/Xgal/IPTG plates (~100µl per 15cm petri dish) and 50µl of the three control reactions were plated out and grown overnight at 37°C (Stratagene, 1997). Recombinant clones were selected by blue/white selection or hybridisation screening (section 2.2.4).

2.3 PCR Methods

These methods were based on McPherson *et al* 1993 and 1995.

2.3.1 General PCR and RACE methods

Due to the variable nature of the PCR protocol, several different cycling conditions and buffers were used. Rather than list the exact parameters for each experiment here, they are listed with the pertinent Figure, while the buffer recipe and equipment used are shown below.

Reagents

10x PCR buffer

- 250mM TRIS-HCl pH8.3
- 500mM Potassium chloride
- 1% Tween 20
- 1mg/ml gelatine
- Magnesium chloride added separately

10x Taq2000 buffer (Stratagene proprietary buffer)

10x Pfu polymerase buffer (Stratagene)

- 100mM Potassium chloride
- 100mM Ammonium sulphate
- 200mM TRIS-HCl pH8.75
- 20mM Magnesium sulphate
- 1% Triton X-100
- 1mg/mlBSA

10x KlenTaq buffer

- 400mM Tricine-KOH (pH9.2)
- 150mM Potassium Acetate
- 35mM Magnesium acetate

750ug/ml BSA
Pfu polymerase (Stratagene)
AmpliTaq (Perkin Elmer)
Taq2000 (Stratagene)
Advantage KlenTaq (Clontech)
Marathon-Ready cDNA, Human brain (Clontech)

Primers were dissolved in 10mM TRIS-HCl, Sterile 'Q' water (18M Ω) was used in all buffers and reagents were autoclaved. PCR reactions were covered with ~40 μ l of mineral oil (Sigma) and spun briefly at maximum speed to ensure complete mixing of reagents. PCR reactions were carried out in 0.5ml thin-walled PCR tubes (Alpha labs) in a Perkin Elmer Thermal cycler model 480.

2.3.2 Direct PCR of bacterial colonies

Reagents

10 μ l pipette tip
200 μ l pipette tip
L-broth 50 μ g/ml ampicillin (per liter
10g bacto-typtone
5g bacto-yeast extract
10g Sodium Chloride

The pH was adjusted to 7.0 with NaOH and autoclaved, when cool ampicillin was added to a final concentration of 50 μ g/ml.

LB agar/50 μ g/ml ampicillin

15g/liter bacto agar was added to L-broth and autoclaved; when cool (~45°C) ampicillin was added to a final concentration of 50 μ g/ml.

Sterile Q water

Protocol

Thin walled PCR tubes were labelled and charged with 10 μ l sterile Q water: for PCR and liquid culture, a sterile 10 μ l tip was fitted on to the end of a

200µl tip and used to pick a bacterial colony. The 10µl tip was dipped into the Q water then dropped in to L-Broth/50µg/ml ampicillin and incubated overnight, shaking at 37°C. The 200µl tip was refitted with a fresh 10µl tip and the process repeated.

For PCR and solid culture: PCR tubes were prepared as before, colonies were picked with a 200µl tip, which was dipped in to Q water then remaining bacteria were dabbed on to a gridded LB-agar plate (LB-agar+50µg/ml ampicillin) and incubated at 37°C overnight.

The PCR tubes were sealed and incubated (in a thermal cycler) at 100°C for 10 minutes, the other PCR reagents were then added and cycling commenced as normal.

2.3.2 Cycle sequencing

Reagents

Terminator ready reaction mix (ABI)

DNA template

Sequencing primer

Mineral oil (Sigma)

Absolute ethanol

3M Sodium Acetate pH4.6

70% ethanol

Protocol

All sequencing was performed with the ABI PRISM dye terminator system; 8µl of terminator ready reaction mix was mixed with 300-500ng of template DNA, 3.2pmol of sequencing primer and distilled water was added to a final volume of 20µl. The reaction was covered with mineral oil and thermal cycled through the following program (96°C, 30 sec; 50°C, 15 sec; 60°C 4min)x25. The 20µl reaction was separated from the oil and ethanol precipitated by the addition of 50µl absolute ethanol and 2µl 3M Sodium acetate,. The mixture was incubated on ice

for 10 minutes prior to spinning in a microcentrifuge at 14,000rpm (15,800xg) for 30 minutes,. The supernatant was removed and replaced with 250µl 70% ethanol, then the reactions were respun at 14,000rpm for 5 minutes. The supernatant was discarded and the reactions dried in a vacuum centrifuge, prior to submission to the PNAC laboratory for DNA sequencing.

2.3.3 Preparation of a circular genomic DNA library for inverse PCR

Reagents

High molecular weight genomic DNA (see Section 2.2.6)

Mbol (Promega)

TaqI (Promega)

Buffer C x1

50mM Sodium chloride

10mM TRIS-HCl pH7.9

10mM Magnesium chloride

1mM DTT

Buffer E x1

100mM Sodium chloride

6mM TRIS-HCl pH7.5

6mM Magnesium chloride

1mM DTT

5x ligation buffer (Gibco)

250mM TRIS-HCl pH 7.6

50mM Magnesium chloride

5mM ATP

25% polyethylene glycol

T4 Ligase (Gibco)

Protocol

Two circular genomic libraries were prepared using 100 units of either *Mbol* or *TaqI* to digest 70µg of high molecular weight genomic DNA (section

2.2.6) in a final volume of 700µl over a period of 5 hours. The two protocols were identical except for digestion conditions:-

Enzyme	Buffer	Temperature of digest
<i>MboI</i>	Promega Buffer C	37°C
<i>TaqI</i>	Promega Buffer E	65°C (under oil)

A 200µl aliquot of the digested DNA was phenol-chloroform extracted, ethanol precipitated and redissolved in 50µl of TE, 5µl of which was added to 781µl distilled water, 200µl of 5x ligation buffer, 14 units of T4 ligase and incubated at 4°C overnight. The ligated DNA was then ethanol precipitated and redissolved in 100µl of 10mM TRIS-HCl pH8.0, for use as a template in the inverse PCR reaction using the conditions described in Section 5.2.2 and 2.3.1.

2.3.4 Synthesis of cDNA for 3' RACE and RT-PCR

Reagents

Pig brain poly-A RNA (Gift from D. Read)

1mg/ml poly-dT₁₈ or BAT39 synthesis primers

BAT39 = 5' GTTACGGCTGGATGAGTGACTTTTTTTTTTTTTTTTTTTT 3'

5x 1st strand buffer (Gibco)

250mM TRIS-HCl pH8.3

375mM Potassium chloride

15mM Magnesium chloride

100mM DTT (Gibco)

20mM dNTP (Gibco)

1 volume of 25mM dATP

1 volume of 25mM dTTP

1 volume of 25mM dCTP

1 volume of 25mM dGTP

1 volume DEPC treated water (Section 2.3.6)

Superscript II reverse transcriptase (Gibco)

RNase A (Stratagene)

New sterile plastic wear were used throughout the protocol

Protocol

Two to three micrograms of pig brain poly-A RNA was added to either 0.5µg of poly-dT₁₈ or BAT39 primer and was made to a final volume of 11µl with DEPC-treated sterile Q water. The mixture was incubated at 70°C for 10 minutes, chilled on ice and then 6µl of 5x 1st strand buffer, 3µl 100mM DTT, 1.5µg 20mM dNTP and 1unit of Gibco Superscript II reverse transcriptase were mixed and DEPC-treated water was added to a final volume of 30µl. The reaction mix was incubated for 90 minutes at 42°C, then terminated by heating to 95°C for 5 minutes. The RNA template was then destroyed by the addition of 150ng RNase A. Product quality was assessed by PCR with a defined pair of primers directed to a low abundance transcript (see Chapter 4 for details).

2.3.6 DEPC treatment of reagents and equipment

Reagents

Diethyl pyrocarbamate (DEPC; Sigma)

Protocol

Sufficient DEPC was added for a final concentration of 0.1% (v/v), the bottle was sealed and stirred overnight at room temperature. The buffer was autoclaved to destroy residual DEPC. Most buffers can be treated excluding ones containing TRIS which prematurely breaks down the DEPC.

2.3.6 Amplification of cDNA bacteriophage library for use as PCR template

Reagents

L-broth/MgSO₄/Maltose

10g bacto-typtone

5g bacto-yeast extract

10g Sodium Chloride

10mM Magnesium sulphate

The pH was adjusted to 7.0 with NaOH, autoclaved and allowed to cool prior to the addition of 0.2% Maltose.

Top agar

10g bacto-typtone

5g bacto-yeast extract

10g Sodium Chloride

10mM Magnesium sulphate

7.5g bacto-agar

The pH was adjusted to 7.0 with NaOH, autoclaved and allowed to cool prior to the addition of 0.2% Maltose.

SM buffer

100mM sodium chloride

17mM Magnesium sulphate

50mM TRIS-HCl pH 7.5

0.01% gelatine

Protocol

An overnight culture of bacterial strain C600-hfl was used to inoculate an L-broth/MgSO₄/maltose culture, shaking at 200rpm at 37°C until the OD₆₀₀ reached between 1.8 and 2. The cells were then spun down at 3000rpm (1800xg) for 20 minutes in a bench top centrifuge, the supernatant discarded and the pellet resuspended in an equal volume of 10mM Magnesium sulphate to produce the competent cell stock.

Final dilution's of the phage library of between 10^{-5} and 10^{-10} were added to 200 μ l competent cells and the phage allowed to bind to the bacteria for 15 minutes at 37°C. Then 2-3 ml of molten top agar at 48°C was added and the mix poured on top of a 10cm L-agar plate and allowed to set for 20 minutes; the plates were then incubated overnight at 37°C. The number of plaques was assessed and the dilution that produced near confluent plaques across the top agar was selected. The transfection was then scaled up to grow at least 10^6 bacteriophage plaques, when they grew to about 1mm in diameter the plates were transferred to a cold room (4°C) and 10 μ l of SM buffer per cm² of petri dish was poured on to the plates and gently rocked overnight. In the morning the SM buffer was poured off, pooled and chloroform was added to a final concentration of 5%. This was shaken then the chloroform and cell debris removed by centrifugation 3000rpm (1800xg) in a benchtop centrifuge for 10 minutes. The supernatant was decanted and more chloroform was added to a final concentration of 0.3%, the amplified library was titered as before to determine the number of plaque forming units (pfu)/ml and the bacteriophage were stored in the short term at 4°C and for long term storage were frozen at -70°C.

2.4 Hybridisation screening

2.4.1 Transfer of bacterial or bacteriophage colonies to solid support

Reagents

Hybond N+ membranes in 15cm disks (Amersham)

10% SDS solution

Denaturing solution

0.5M Sodium hydroxide

1.5M Sodium chloride

Neutralising solution

0.5M TRIS-HCl pH 7.4

1.5M Sodium Chloride

20xSSC

3M Sodium chloride

0.3M Sodium citrate

pH was adjusted to 7.0 with NaOH

3MM paper (Whatman)

Protocol

Bacterial colonies or bacteriophage were grown over night on agar plates under the appropriate conditions until colonies were ~1mm in diameter. Three trays were then lined with 3MM paper which was saturated with either 10% SDS, Denaturing solution or Neutralising solution. The Hybond N+ disk was labelled in soft pencil then laid on top of the agar and left in place for 1 minute, 4 to 6 holes were punched asymmetrically in the membrane using a 19G needle dipped in permanent black ink to register the position of the membrane on the agar.

Then membrane was then laid colony side up, on to the SDS soaked 3MM paper for 3 minutes. It was then similarly transferred to the denaturing solution soaked paper and exposed for 5 minutes. Finally, it was exposed to neutralising buffer-soaked paper for 5 minutes. The membrane was rinsed briefly by dipping in a bath of 2xSSC, then laid on dry 3MM paper for 30 minutes until dry. The

DNA was fixed to the membrane by baking for 2 hours at 80°C and stored dry until needed.

2.4.2 Southern Blotting

Reagents

Hybond N+ membranes in 15cm disks (Amersham)

Denaturing solution

0.5M Sodium hydroxide

1.5M Sodium chloride

Neutralising solution

0.5M TRIS-HCl pH 7.4

1.5M Sodium Chloride

20xSSC

3M Sodium chloride

0.3M Sodium citrate

pH was adjusted to 7.0 with NaOH

Protocol

The agarose gel was immersed with gentle rocking in several gel volumes of denaturing solution for 45 minutes. The gel was then rinsed in distilled water and immersed in neutralising buffer for 30 minutes, at which point the buffer was changed for fresh neutralising buffer and the gel incubated for a further 15 minutes. The gel was placed upside down on a raised platform on top of two oblong strips of Whatman 3MM paper cut to the width of the gel, wetted in 10xSSC with the ends of the oblong immersed in a tray of 10xSSC (approximately 1litre). Taking care no bubbles were trapped between layers, the edges of the gel were masked with Saranwrap so that the paper towels (see below) would not contact the 10xSSC pool or the wetted 3MM paper. A piece of Hybond N+ membrane (which had been soaked in distilled water for 5 minutes) was placed on top of the gel and small holes were punched with a needle to mark the position of the sample origin. The Hybond was covered with two sheets of

3MM paper cut to fit the gel and this was covered with a 5-8 cm stack of paper towels surmounted by a 500g weight. This was left overnight for the DNA to transfer after which the Hybond N+ membrane was removed from the stack and fixed by baking at 80°C for two hours. The remains of the agarose gel were sometimes restained by immersion in 0.5xTBE and 0.5µg/ml ethidium bromide (see section 2.2.1) and checked under UV light to ensure the DNA had fully transferred.

2.4.3 Labeling DNA probes

Reagents

Labeling mix (25µl)

1x PCR buffer

150µM d[GAT] (2.5mM of dATP, dGTP, and dTTP)

2mM Magnesium chloride

0.5u AmpliTaq (Perkin Elmer)

5µl [³²P]dCTP labelled to >3000Ci/mmol; 10mCi/ml (Amersham)

PCR-produced DNA

Labeling primer

Protocol

All the DNA used as hybridisation probes were available as PCR products and were labelled by single strand PCR; the labelling mix above was prepared and 20ng of the PCR product to be labelled was added along with 0.2µM of forward primer for the product. The mix was covered with mineral oil and thermal cycled (94°C 5min, [94°C 30sec; 55°C 30 sec; 72°C 1min]x10). The labelled DNA was then purified by the QIAspin PCR purification kit (Section 2.2.3) and the labelling judged by measuring the counts per second of a 1µl aliquot in a scintillation counter.

2.2.4 Hybridisation conditions

Reagents

Hybridisation buffer

6x SSC

0.5%SDS

1x Denhardtts solution

100 μ g/ml denatured fragmented salmon sperm DNA (Sigma)

50x Denhardtts solution (per liter)

10g Ficol (Type 400, Pharmacia)

10g polyvinylpyrrolidone (Pharmacia)

10g BSA (Sigma)

20xSSC

3M Sodium chloride

0.3M Sodium citrate

pH was adjusted to 7.0 with NaOH

Protocol

Both probes used in this project were relatively short (<200bp) and identical conditions were used in both experiments.

The membrane was incubated with 10ml of hybridisation buffer for 1 hour at 65°C in a Techne hybridisation oven, then approximately 10⁵ cpm of labelled probe per cm² of membrane was boiled for 1 minute and applied to the membrane and allowed to hybridise for at least 1 hour at the same temperature.

The membranes were washed in 2xSSC+0.05% SDS at 65°C changing the buffer every 20 minutes for 80 minutes. The membrane was then washed in 0.2xSSC+0.05% SDS for 2-5 minutes at 65°C. The membrane was monitored with a hand-held Geiger counter throughout the washing process and if the radioactivity dropped below 10cps or the membrane had been washed in 0.2xSSC, the washing was stopped and the membrane wrapped in Sarenwrap and autoradiographed using Biomax MS film (Kodak) with intensifying screens.

Chapter 3 Isolation and protein sequencing of NTE

3.1 Introduction

The original aim of this project was to clone the NTE gene from chicken brain cDNA. The chicken has long been the test animal of choice for the study of Organophosphate Induced Delayed Polyneuropathy (OPIDP), since it is apparently more sensitive to OPIDP and due to its bipedal gait clinical symptoms are pronounced and thus can be easily and accurately assessed (Abou-Donia, 1981). Translation of the chicken cDNA sequence would allow synthesis of NTE peptides to which antibodies could be raised for use in immunohistochemistry. In addition chicken brain is known to be relatively rich in NTE compared with several mammalian species (for example pig and sheep brain have 20% less NTE and rat has 65% less; Meredith and Johnson, 1989).

The NTE purification protocol depends on the novel biotinylated organophosphate S9B (1,[Saligenin cyclic phosphoryl],-9,-biotinyl-diaminonoane; Figure 3.1). Incubation of S9B with brain microsomes leads to a specific labelling of NTE at the active site serine residue, producing a biotinyl-NTE complex which can be easily separated from almost all other proteins by avidin's extraordinary affinity for biotin (Glynn *et al*, 1994).

3.2 Results and Discussion

3.2.1 Chicken brain NTE: Purification and protein sequencing

A typical preparation of chicken brain NTE is summarised in Figure 3.2 and described in detail in Sections 2.1.1 to 2.1.6. Chicken brains were homogenised in TE containing 1mM DTT and the microsomes were prepared by differential centrifugation. The pelleted microsomes were resuspended in 0.9% NaCl/TE and stored at -20°C while the protein concentration of a sample was assayed using the BioRad DC reagent.

The microsomal protein solution was thawed and diluted to a protein concentration of 2 mg/ml with 0.9% NaCl/TE and S9B added to a final concentration of 160nM. After an incubation of 20 minutes at 37°C the microsomes were washed to remove unreacted S9B, then solubilized by boiling for 10 minutes in TE buffer plus 0.15% SDS, 0.1% DTT.

After a brief spin (2000xg; 10 min) the clear extract was passed through an avidin-Sepharose affinity column, and after washing, the biotinyl-NTE complex subsequently eluted by boiling the avidin-Sepharose slurry in 0.25% SDS, 0.25% DTT in 0.1xTE buffer (Figure 3.3). This avidin eluate was concentrated using a Centrspin 100 filter prior to preparative SDS-PAGE, which removed the endogenous biotinylated protein present in the avidin eluate (Figure 3.4).

A total of 200µg of pure chicken NTE was prepared from 1.8kg of fast frozen chicken brain using the methods described above. Of this pure NTE, 60µg was used as part of a group effort to produce a mouse monoclonal antibody. The remaining 140µg was used in protein sequencing experiments.

NTE has proven to be far from an ideal subject for peptide sequencing. Intact NTE protein was resistant to N-terminal sequencing by Edman degradation. Furthermore, CNBr cleavage, acid hydrolysis, chymotrypsin, thermolysin or

trypsin digestion were unsuitable for NTE digestion as they produced no discrete bands on SDS-PAGE which could be extracted and sequenced. Only partial digestion with *Staphylococcus aureus* V8 protease (EC:3.4.21.9) produced a selection of fragment bands with sizes of between 4 and 70kDa which were suitable for peptide sequencing.

Small scale experiments showed that the optimum digestion conditions were 1µg of V8 protease to 10µg of NTE, digesting for 2 hours at 37°C. The 140 µg of NTE was divided into several batches, digested and then submitted to the Protein and Nucleic Acid (PNAC) sequencing laboratory. Digested fragments were resolved on a 16.5% Tris-Tricine gel and Western blotted on to Immobilon-P membrane (Figure 3.5). Then individual bands were excised and sequenced by Edman degradation.

A total of five bands were cut from the Immobilon-P blots: of these, only three produced peptide sequences (this sort of peptide blockage was also experienced in sequencing pig NTE). A further four sequences had been produced in previous work by other members of the group (Figure 3.6). The sequence data produced by this work were not only heterogeneous but also the amount of derivatized amino acid produced by the Edman degradation was low. Thus, even when the sequencing indicated a single residue, there was a certain amount of suspicion as to its accuracy because the sequencing was operating at the limits of its sensitivity. These ambiguities severely limited the usefulness of the sequence data, as degenerate oligonucleotide probes and primers must be based on reliable sequence. This is because the specificity of a probe is related to its length and since the length is usually short (typically 21 bp or 7 residues) a single residue error can cause a large percentage error, further reducing the already limited specificity of a degenerate oligonucleotide pool.

After a few attempts to clone chicken brain NTE cDNA (data not shown) it became clear that producing sufficient reliable protein sequence data would be

prohibitively expensive and efforts were focused on obtaining pig brain NTE, since the source tissue was readily available at low cost.

3.2.2 Pig brain NTE: Purification and protein sequencing

The methods used to obtain pure NTE protein and sequence data from pig brain were identical to those described for chicken. A group effort over a period of 2 years and more than 50 Edman sequencing runs generated reliable N-terminal sequence for 8 NTE peptides from V8 digest fragments (Figure 3.7).

One modification to the purification protocol was developed to produce a cleaner set of V8 digest bands. Products from a V8 digestion of porcine NTE were passed through a second avidin-Sepharose column and eluted to extract the protein fragments bearing the S9B. Both the V8 avidin eluate and the flowthrough were run on SDS-PAGE, blotted and bands were excised for sequencing (Figure 3.8).

Two sequences were found in the V8 avidin eluate: LTNP and XGVP. The LTNP peptide sequence found in the V8 avidin eluate was associated with a 36kDa peptide fragment and indicated that the previously observed 72kDa fragment initiated by LTNP would also be S9B biotinylated. In the flowthrough, LTNP was found on a 20kDa peptide; because this peptide had failed to be bound by avidin, it was thought to be unbiotinylated, putatively placing the active site serine less than 36kDa and more than 20kDa from the LTNP sequence. The XGVP peptide sequence in the V8 avidin eluate was found on 32kDa and 23kDa fragments. In the flowthrough, XGVP was found on a 16kDa fragment, so was also thought to be unbiotinylated. This tentatively placed the active site serine less than 23kDa and more than 16kDa from the XGVP sequence. These sequence data were used to assemble a tentative peptide map around the active site serine which will be discussed in greater detail in Chapter 5 (Figure 5.1 and Section 5.1).

The final batch of peptide sequences were produced by excising V8 digested NTE fragments from a Western blot. A portion of each band was N-terminally sequenced, and the rest was redigested with trypsin. The trypsin digested fragments were fractionated by HPLC and then sequenced. This work (performed by Dr Tony Willis of the MRC Immunochemistry Unit, Oxford) produced one familiar sequence (DLGL) and five novel ones (Figure 3.9). The original aim of this work was to provide fresh sequence with some reliable evidence as to their relative positions within the NTE protein, to aid in design of degenerate PCR reactions for further cloning attempts. However it was found that DGHL, VVKS, FDQI and DLGL all had near perfect matches with sequences in the Expressed Sequence Tag (EST) division of EMBL. These sequence matches are discussed further in Chapter 6 (Section 6.1).

3.3 Significance of NTE peptide sequences in isolating the NTE cDNA

The pig brain NTE sequences were far more reliable than those produced from chicken NTE. The improvement in sequence quality was essentially due to the larger amounts of NTE it was possible to produce from pig brain. Firstly, it allowed more digested NTE to be submitted to PNAC which gave a stronger signal on the Edman degradation sequencing. Secondly, it made multiple sequencing runs possible and allowed detection of errors in detecting the residue and often resolved ambiguous residues.

Despite the relatively large quantities of pig brain NTE available for analysis, NTE still proved to be a challenging subject for sequencing. For unknown reasons, certain peptide fragments proved to be resistant to repeated Edman sequencing attempts (mirroring the experience with chicken NTE) and furthermore some turned out to be the result of contaminating avidin and carboxylesterase sequences (figure 3.7).

The avidin sequence contamination was easily detected because avidin's full protein sequence was available from SWISSPROT (sw:P02701) and a simple BLAST search was sufficient to detect any homology. However, the carboxylesterase contamination was considered a particularly insidious threat to the project. Since carboxylases are endogenously biotinylated (PROSITE: PS00188) they are greatly enriched by the avidin-Sepharose affinity protocol (see Figure 3.3). Because the carboxylase contamination was never fully characterised (Glynn *et al*, 1994), the concern was that the purification protocol could yield peptide sequence derived from a previously unknown carboxylesterase which copurified with NTE. Thus protein sequence which showed any similarity to carboxylase sequences was regarded with suspicion.

The routine database searching using the NTE peptide sequences was also an important part of the overall cloning strategy. Both the EMBL and SWISSPROT databases were regularly searched using the BLAST and BLITZ programs. BLITZ was found to be better at detecting similarities between short peptides and database entries but unfortunately it was limited to searching the smaller SWISSPORT database. BLAST was found to be less sensitive when using short peptides; however, it could be used to search EMBL DNA database, comparing the peptide sequence with each DNA sequence translated in all six reading frames.

It was hoped that sequence matches produced by the database searches could assist the project in two ways. First, it was possible that NTE had already been cloned as an EST (See Section 1.2.5); this could be obtained from the IMAGE consortium (Lennon *et al*, 1996) for further investigation. Secondly, if two NTE peptides were found to be similar to a single protein this could have given the relative position and separation of the NTE peptides; this information could then have been used to design degenerate oligonucleotides which would yield a PCR product of predictable size and only require a limited number of primer combinations to be investigated.

Up until the beginning of 1997, database searches using the eight older peptide sequences (Figure 3.7) had only produced two significant matches (between DLGL, LAKL and YOL4_CAEEL): these are discussed fully in Chapter 6 (Section 6.1). After this date, sequences began to appear linking YOL4_CAEEL with other NTE peptide sequences. Had the tryptic digest/HPLC sequence data been unavailable, either of these similarities would have refocused interest in YOL4 and lead to the cloning of NTE. In February 1997 the yeast sequence YMF9_YEAST (sw:Q04958; Devlin *et al*, unpublished) appeared in SWISSPROT which had a similarity with VVPG (Figure 3.10). YMF9 is a hypothetical 187kDa protein from *Saccharomyces cerevisiae* and was listed as being part of the YOL4/YCHK family of hypothetical proteins. YMF9 is discussed more fully in Chapter 7 (Sections 7.2.2 and 7.2.3). In June 1997 a 424 bp zebrafish EST clone 8K14 (em:AA494858) was submitted to EMBL (Clark *et al*, 1997) listed as being similar to both YOL4 and YMF9. The translated sequence contained two very close matches with both ADLT and VVPG (Figure 3.10). The 8K14 clone is an EST of unusual length since the sequence match is located within 210 amino acids of the N-terminal end of YOL4 (all the Human NTE ESTs in Chapter 6 are less than 2kbp long) and 8K14 probably represents a near full length NTE-like clone. This would be of great interest since full length sequence of another vertebrate NTE clone could highlight regions of functional importance in NTE, by looking at the regions of similarity between the sequences.

Figure 3.1 Structure of 1, [Saligenin cyclic phosphoryl],-9,-biotinyldiaminonoane (S9B).

Figure 3.2 Flow chart summarising the purification stages of NTE from chicken brain to purified NTE.

The amount of NTE in the microsomal fraction was estimated from the reported proportion of NTE in microsomal protein (0.03%; Rüffer-Turner *et al*, 1992). The other yields of NTE were estimated by comparison with standard quantities of protein on stained SDS-PAGE gels (See Figure 3.3 and Section 2.1.5).

Figure 3.3 7.5% SDS-PAGE illustrating the two stages of NTE purification.

In lane 6 band B represents the 155kDa NTE protein, bands A, C and D are endogenously biotinylated proteins (probably carboxylases). Band E represents a protein that binds S9B however it is not an NTE type activity as it is sensitive to paraoxon treatment. In lane 7 the major band is pure NTE.

Lane

- 1 150 ng BSA
- 2 300 ng BSA
- 3 450 ng BSA
- 4 600 ng BSA
- 5 Novex Molecular weight markers
- 6 Avidin eluate
- 7 155 kDa NTE protein band purified by Preparative SDS-PAGE

Figure 3.4 7.5% SDS-PAGE of aliquots from Preparative SDS-PAGE of Avidin eluate.

Preparative SDS-PAGE was used to separate the 155kDa NTE band from the contaminating endogenous biotinylated proteins. In this case fractions 26, 27 and 28 were judged to contain the bulk of the NTE protein and these three were pooled and acetone precipitated prior to use in sequencing or antibody production.

Careful examination reveals a faint band in lanes 21 22 23 and 24 located between the 120kDa carboxylase protein and the 155kDa NTE. In retrospect, knowing that NTE displays multiple splice forms (see Section 6.2.4) it is possible that this band represents an NTE isoform. However it could also represent a carboxylase or a proteolytic cleavage of the 155kDa NTE: further work would be needed to confirm this speculation.

Lane

M Novex Protein Molecular weight markers

A Avidin eluate

20 to 30 Aliquot number of preparative SDS-PAGE fractions

Figure 3.5 V8-digested NTE blotted on to Immobilon P prior to sequencing.

<u>Lane</u>	
1	Novex molecular weight markers
2	Blank lane
3	V8 protease
4	V8 digested NTE
5	V8 digested NTE

<u>Name</u>	<u>Sequence</u> <u>date and fragment size</u>	<u>Name</u>	<u>Sequence</u> <u>date and fragment size</u>
C18A	(ML) (QF) (PS) (LH) (RE) 3/12/93 18kDa	C29	VILPEXDR 28/3/94 29kDa
C18B	(SMKR) QPLRLFPQP 8/11/93 18kDa	C18C	(SAV)QLALEPQEE 6/4/94 18kDa
C21	GQSAW(PQ)P 14/12/93 21kDa	C8	(AML) DLTEPD 29/3/94 8kDa
C10	(AKRS) (DG) (LR) (TI) E (FV) DVAN 11/11/93 10kDa		

Figure 3.6 Chicken brain peptide sequence.

A total of 7 Edman degradations produced useful sequences from chicken brain NTE. Sequences C29, C18C and C8 were completed as part of this project. Brackets denote residues detected within a single Edman cycle. Residues shown in colour were duplicated in another sequencing run (C8 and C10; C18A and C18B).

The C18B sequence with an N-terminal methionine was likely to be correct since an antibody raised to a synthetic peptide of this sequence proved able to detect chicken brain NTE in a microsomal protein preparation.

<u>Name</u>	<u>Sequence</u>	<u>Date first observed</u>	<u>Fragment sizes</u>
ADLT	4444444443 3333222211 111111 <u>ADLTEGDLAN</u> <u>SHLPPEVLLY</u> MLNVRV	10/1/94	10 kDa
DLGL	1111111111 111 <u>DLGLPYFNVV</u> <u>TDI</u>	3/2/94	13kDa
IQPL	1111111111 1111111111 <u>IQPLRLFPSP</u> <u>GLPARTSPVR</u>	27/3/96	13kDa
LAKL	2222222222 2111111111 <u>LAKLPEGTLG</u> <u>HIYRRYPQVV</u>	6/9/95	4kDa
LTNP	7888867766 5442 <u>LTNPASNLAT</u> <u>VAVL</u>	21/1/94	70kDa (B) 36kDa (B) 20kDa
VILP	11111 <u>VILPE</u>	8/9/93	16kDa
VVPG	3333333232 122222211 11111 <u>VVPGDSVNSL</u> <u>LSILDVITG</u> XQAPQ	7/9/95	6.5kDa
XGVP	(4/2) 44442 2222 <u>(A/K)GVPVA</u> LVGP	15/3/94	32kDa (B) 23kDa (B) 16kDa

(B)= fragment known to be biotinylated

example contaminant sequences

(TVL) FQLRPAQAT	Pyruvate Carboxylase
KHFHPKALKS ?MQ?I	Pyruvate Carboxylase
KRKDVLTGGD	Avidin

Figure 3.7 Pig NTE peptide sequence data.

This figure summarises over 50 Edman sequencing runs (omitting sequences which were of insufficient quality to use in cloning). The digit above each residue represents the number of times the residue had been observed in that position in repeated protein sequencing runs. Thus ADLT has been observed four times and combines sequences of 9, 14, 18 and 26 amino acids. The underlined sequences indicate the extent of reliable sequence suitable for design of oligonucleotide probes. Reliability was assessed by considering the number of sequencing runs that had detected that residue or, if the sequence was unique, the yield of derivatized amino acid produced by the Edman degradation run.

The contaminant sequences were an important factor in the interpretation of the peptide sequence data, since they showed that the 'pure' NTE protein produced by the S9B protocol was susceptible to contamination and thus any protein sequence which had some similarity to any known endogenously biotinylated proteins was regarded with suspicion.

Figure 3.8 Avidin-Sepharose extraction of V8 digest fragments containing the S9B binding site.

The large band between 14 and 21kDa in the V8 avidin eluate represents the 16kDa avidin monomer, which was washed off the column when the digested biotinyl-NTE was eluted by boiling in 0.25% SDS, 0.25% DTT in 0.1xTE. The avidin masked any possible 16kDa biotinyl peptide fragments. This was not a problem in the original protocol since the undigested NTE was further purified by Preparative SDS-PAGE (picture courtesy of Dr P. Glynn).

<u>Name</u>	<u>Sequence</u>	<u>Date first observed</u>
DGHL	DGHLLTDGGY INNLP	2/10/96
VTFL	VTFLALHNYL GLTN	2/10/96
FDQI	FDQIYDVG YQ YGK	2/10/96
LPEG	LPEGLGHK	2/10/96
VVKS	VVKSSS?DG? LR??IDYFK	2/10/96

Figure 3.9 Peptide sequence derived from HPLC purified tryptic digest fragments from V8 digest fragments of pig NTE.

The VVKS sequence was originally a heterogeneous sequence; however, it was noted that the sequences could be separated by subtracting the already known LAKL sequence from the heterogeneous positions to yield the VVKS sequence. This processing was validated when the VVKS picked up an EST which also carried the FDQI sequence (see Figure 3.10).

A)

```

VVPG          1 VVPGDSVNSLLSILDVITGXQAPQ 24
      | |: ::||:||||:::|  ::
YMF9   328 LNTVKPGNPLTSLVSLNLFTHSMSSYGNS 357

```

B)

```

ADLT          1 ADLTEGDLANSHLPPEVL 18
      ||||| |: ||||| |||
8K14   31 LLEADLTEFDMQNSHLPSEVLYML 55

```

C)

```

VVPG          1 VVPGDSVNSLLSILDVITGXQAPQ 24
      |:|||||:|||||||
8K14   119 VKHVLPGDSVHSLLSILDVITGYP 142

```

Figure 3.10 Sequence similarities between ADLT, VVPG and sequences linked to YOL4.

A) VVPG shows a moderate similarity with the N-terminal domain of YMF9_YEAST (sw:Q04958).

B) ADLT shows a very close match with zebrafish clone 8K14 when translated in reading frame +3. This homology is of particular interest since ADLT sequence was used to produce a peptide antibody which is known to be specific to pig NTE, thus the ADLT sequence was known to represent a true NTE sequence.

C) VVPG shows a close match with zebrafish EST clone 8K14 (em:AA494858) when it is translated in reading frame +2. The frameshift between ADLT and VVPG is unsurprising since the DNA sequence the translation was made from was made from a 'single pass' sequencing run which can easily contain single base errors and frameshifts.

Chapter 4 Attempts to clone NTE cDNA using Rapid Amplification of cDNA Ends (RACE)

4.1 Introduction

The peptide sequences available at the initiation of the cloning attempt are shown in Figure 4.1. Every sequence except XGVP contained one or more amino acids encoded by highly redundant codons (see Section 1.3.1) and so did not favour design of oligonucleotides for use as specific probes for hybridisation to a cDNA library, or primers for PCR. Furthermore, until near the end of the project there were no clues to the relative position of these peptides within NTE. The general cloning strategies appropriate to cloning NTE and early attempts by other members of this laboratory in this endeavour have been discussed in Chapter 1. This chapter concentrates on the application of 3' RACE to the problem the background to this strategy is discussed in detail in Section 1.3.4.

4.2 Results and Discussion

4.2.1 Synthesis and quality control of cDNA

The 3' RACE protocol requires a population of cDNAs with a known anchor sequence attached to the poly-A tail end of the molecule. It is then possible to PCR amplify the desired cDNA using a degenerate primer based on known peptide sequence (Figure 1.12).

cDNA for 3' RACE was synthesised from pig brain poly-A RNA using Gibco Superscript II reverse transcriptase and the BAT39 synthesis primer (Figure 4.2) as described in Chapter 2 (Section 2.3.4). Since apparently identical cDNA synthesis reactions could produce batches of cDNA of variable quality (Edwards

et al. 1995), the quality of each batch of cDNA was assessed by PCR amplification of a known low abundance cDNA sequence, *trkC* (em:M80800). *TrkC* is a tyrosine protein kinase with a very superficial resemblance to NTE in that it is expressed at low levels, has a similar size (145kDa glycoprotein), and is preferentially expressed in neural tissue (Lamballe *et al.*, 1991). *TrkC* was selected as the control template as its cDNA was originally isolated from a Clontech pig brain cDNA library, an aliquot of which had been purchased for the NTE cloning project

Primers were designed which covered almost the full length of the published *trkC* cDNA sequence (Figure 4.3). Two 'forward' primers *trk1+* and *trk9+*, and one 'reverse' primer *trk3-* were amplified individually and as pairs (*trk1+* vs *trk3-* and *trk9+* vs *trk3-*) to show that the batch of cDNA contained full length copies of low abundance transcripts. A typical result is shown in Figure 4.4.

4.2.2 Ratification of 3' RACE protocol

To demonstrate that the 3' RACE protocol was viable, BAT39-primed cDNA was amplified using the forward primers *trk1+*, *trk5+* and *trk9+*, and the reverse BAT318 anchor primer (Figure 4.2 and 4.3). Of these primer combinations only *trk1+* produced a product band (of about 1.4kb; Figure 4.5). The published cDNA sequence (Figure 4.3) indicates that a 3' RACE product generated from the *trk1+* primer should be greater than 1024 bp long (the distance from the *trk1+* primer to the end of the published sequence). However, the published *trkC* cDNA sequence (2.5kbp) contains only 20 bp of 3' untranslated sequence and has no polyadenylation signal (Lamballe *et al.*, 1991). Taking this information into account, it was concluded that the 1.4kbp band observed in Figure 4.5 represented 1kbp of coding sequence plus a further 0.4kbp of untranslated sequence from the *trkC* mRNA. The failure of *trk5+* and *trk9+* to amplify is unclear. This may have been as a result of insufficient optimisation of PCR conditions, or because the primers were incompatible with

BAT318. However, since it appeared that the BAT39-primed cDNA was capable of sustaining a 3' RACE reaction, the investigation progressed to perform 3' RACE using degenerate primers derived from pig NTE peptide sequence.

4.2.3 3' RACE with degenerate primers designed to NTE peptides

The first series of attempts to clone NTE by 3' RACE used a single degenerate primer designated F10I which was designed using the pig ADLT peptide sequence (Figure 4.6). The degeneracy of the back translated DNA sequence was reduced from x2048 down to x32 by the introduction of inosine bases at the maximally degenerate positions (Ns) in the primer sequence. No inosines were introduced within three bases at the 3' end of the primer as this was known to adversely affect priming (McPherson *et al*, 1991).

In combination with BAT318, the F10I primer produced a diffuse but bright band of about 400 to 500 bp in size (Figure 4.7). Further PCR experiments showed that this band was specific to BAT39-primed cDNA, since poly-dT primed cDNA did not produce the band (data not shown). This indicated that a specific reaction with the BAT39 anchor primer was responsible for the product rather than the non-specific priming of BAT318. Titrations of magnesium chloride concentration and annealing temperatures (over the ranges of 1.5-3.0 mM Mg²⁺ and 55-60°C) failed to produce a more defined product band. Despite its small size (representing approximately 150 amino acids or 16kDa of protein sequence) the F10I product band was considered significant because poly-dT anchor primers can anneal within an mRNA, on to adenine rich regions of the sequence rather than the poly-A tail, thus yielding a truncated product (Schmidt *et al*, 1993).

To analyse the 400-500 bp band, it was excised from the agarose gel and the DNA purified by electroelution, before it was cloned into pGEM-T vector (see Section 2.2.2 and 2.2.8 respectively). A total of 18 recombinants were selected

by blue/white selection and each recombinant colony was propagated in L-Broth 50µg/ml Ampicillin. Plasmid DNA was purified (Section 2.2.3) and each end of the clone was partially sequenced by Dye Primer cycle sequencing (Section 2.3.2). Of the 18 recombinants, 13 contained inserts produced by F10I/BAT318 primer pair as they contained the F10I and BAT318 DNA sequence at the 3' and 5' ends, respectively. However, when the sequence from the F10I end of the insert was translated, it was found none of the clones possessed predicted peptide sequence matching the ADLT protein sequence known to lie beyond the seven residues used to design the F10I primer (Figures 4.8).

At this stage it was concluded that one of the F10I 3' RACE products which had not been cloned and sequenced might represent an amplified cDNA fragment from NTE, but would have to be separated from the numerous non-specific products also generated by the reaction. To this end a strategy of nested 3' RACE was developed and implemented.

4.2.4 Nested 3' RACE

In conventional PCR the two primers each exert a selective pressure on the final product, since for a template to amplify it must possess a binding site for both PCR primers. RACE lacks this double selection and any annealing and extension of the degenerate primer will lead to the formation of a PCR product as all templates possess the reverse anchor primer at the 5' end of the cDNA. This limitation could be in part overcome by nesting pairs of degenerate primers. Products from an initial 3' RACE reaction would be reamplified in a secondary RACE reaction using a different degenerate primer located downstream from the original primer, but the same anchor primer. In context of cloning NTE the major problem was that there was no reliable information as to the relative positions of the various peptide sequences within the NTE protein. Thus it was necessary to test all possible permutations of primer pairing to ensure that the correct arrangement was tested at least once.

Five more inosine-containing forward (sense) primers F36IS, F36IT, F16I, F13IG and F13IY were designed aiming to produce oligonucleotides with the lowest degeneracy possible from the peptide sequence available at the time (Figure 4.6). Due to the low annealing temperatures of the degenerate primer pools it was necessary to use a lower annealing temperature of 40°C during PCR cycling. Although such a low annealing temperature was liable to cause an increased level of false priming, it was decided that the second round of selection was likely to remove spurious products. Because of the uncertainty regarding the length of the 3' RACE product (the peptide sequences could be located anywhere in the NTE protein sequence) a very long extension time (7 minutes) was used to ensure even the largest RACE product would be completely extended. The 7 minute extension time was derived using the standard extension rate for Taq polymerase of 1kbp/minute (Kidd and Ruano, 1995) and the largest plausible template size (4.6 kbp coding sequence plus a large 3' untranslated region).

In the initial experiment BAT39-primed cDNA was amplified with either BAT117 or BAT318 reverse anchor primer paired with each of the new degenerate forward primers (Figure 4.9). Primers BAT117 and BAT318 produced a very similar pattern of bands when paired with NTE degenerate primers, while BAT primers and NTE primers used alone, produced very little product. BAT117 was adopted as the principal RACE anchor primer in the nested 3' RACE reactions since it appeared to give slightly cleaner results relative to the BAT318 and furthermore BAT318 could be used as a nested anchor primer in a possible second or third round of amplification (Figure 4.2).

The first round of 3' RACE produced a bank of eight products for amplification in the second round of RACE. Five templates were derived from degenerate NTE primer vs BAT117. The remaining three were controls comprising; BAT39-primed cDNA amplified with no primers (to show that residual cDNA carried over from the first round of PCR was not responsible for any product formation), BAT39-primed cDNA amplified with BAT117 alone (to control

for single primer amplification), and a water blank (to control against contamination). These samples were diluted and used as templates in the second round of RACE amplification.

In the second round of nested 3' RACE, all possible permutations of the degenerate primers were tested, even though there were two pairs of primers for which some positional information was available. From Figure 4.6 it can be seen that F13IY was located internally to F13IG and F36IS was internal to F36IT; however, this information was not used to reduce the number of reactions required as it would have complicated the assembling of the nesting reactions and, in any case, use of all primer permutations proved to be a valuable test of the specificity of the second round of PCR.

Each of the six primers (F36IS, F36IT, F16I, F13IG, F13IY and BAT117) were used in PCR reactions as single primers and as a pair with the BAT117 primer on each of the eight templates (in the case of the BAT117 this meant the templates were amplified with no primer at all and with a BAT117 alone). Thus each primer was used in 16 PCR reactions and the full experiment involved a total of 96 different reactions. A typical result and experimental layout for testing of the F36IT primer is described in Figure 4.10.

Several variations on this experiment were tried without success, including nesting the first round products with the F10I degenerate primer and varying the annealing temperature of the first round RACE amplification. The negative controls (single primer reactions and no template blanks) produced no products indicating that any products that were produced were a result of a RACE reaction between the degenerate primer and the anchor primer. However, the general outcome of these experiments was that first round of RACE produced a large number of products which in the second round of RACE, either failed to reamplify or produced products which were clearly a result of non-specific amplification.

At this stage in the 3' RACE experiments, the degenerate PCR using primers of inferred orientation which were being performed in parallel with the RACE (Chapter 5) started to show promising results and the 3' RACE experiments were discontinued.

4.3 Perspectives on Degenerate 3' RACE

The experiments described in this chapter were intended as a survey of the 3' RACE protocol. They concentrated on testing all the available degenerate primers to determine if any were capable of producing a defined product, either in a single round of amplification, or nested with another degenerate primer. Due to the complexity of assembling all the permutations only a single set of PCR conditions were investigated.

The second stage of the RACE experiments would have concentrated on double checking that the 3' RACE system was adequate for the task of amplifying a large rare transcript using degenerate primers made to trkC protein sequence. This was not attempted in the first series of experiments since it would have represented a task of equal magnitude to cloning NTE.

Once the viability of the system was reaffirmed, all the NTE based degenerate primers were to be extensively optimised with regard to annealing temperature, magnesium concentration, extension time and the effect of using other thermostable DNA polymerases. Additionally, limited nested 3' RACE was planned, using pairs of primers derived from the same peptide sequence (ie. F36IS being internal to F36IT and F13IY being internal to F13IG; see Figure 4.6), so only the appropriate reactions would need to be performed, thus allowing the second round of RACE to be optimised as the first was.

A third approach to the 3' RACE method would have been to Southern blot the products from the first round of RACE. The blot could be probed for hybridisation with each of the degenerate primers end labelled with ^{32}P . Any

band identified by this method could be cloned either by making a library from the 3' RACE products and rescreening it with the degenerate primer, or an antisense primer could be made and used to attempt to PCR amplify the intervening sequence from the cDNA. The advantage of the latter approach would be that the maximum size of the PCR product would be known as it could not be larger than the 3' RACE product it was originally detected in.

A survey of papers which successfully used a 3' RACE cloning approach with degenerate primers, reveals that the products produced by the reported reactions were relatively short and the approximate product size and primer position was known. For example Schmidt *et al*, (1994) used an N-terminal based inosine containing eight fold degenerate primer to amplify a 1.5kbp product from plastidial n-6 desaturase (a relatively abundant protein from spinach chloroplast). Similarly, Dry and Robinson (1994) reported cloning 1.65kbp of the grape berry polyphenol oxidase cDNA (again a relatively abundant protein) with a single primer in which all the degeneracy had been eliminated by substituting inosine in all degenerate positions within the backtranslated sequence.

In both cases there was information as to the approximate size of a product (both laboratories had N-terminal peptide sequence from the intact protein) and furthermore the proteins (and by implication the mRNA) were relatively abundant as only a 100 to 200 fold purification was required to extract the desired protein. The next largest 3' RACE reported was a ~500bp product amplified from the cDNA of a small GTP binding protein (Rab25) using a 256 fold degenerate primer (Goldenring *et al*, 1993).

In contrast, NTE is at least an order of magnitude less abundant than the plant proteins (no evidence as to Rab25's abundance was presented in the Goldenring paper). Furthermore it is interesting to look at the final peptide map of NTE in Figure 7.1. Of the four peptide sequences used to design primers for the 3' RACE (Figure 4.6), VILP does not appear in the peptide sequence at all

(See Figure 7.1 and Appendix 4), ADLT is located 3.9kbp from the 3' end of the cDNA sequence, LTNP is 2.0 kbp and DLGL is 1.1kbp from the 3' end (see Appendix 4). Thus the successful applications of degenerate 3' RACE were on more abundant templates and/or looking for shorter products, often with some indication of the product length to look for and lower degeneracy peptide sequence. It is possible therefore that degenerate 3' RACE on NTE would have failed regardless of the amount of optimisation, and it was fortunate that it was only one of several approaches to cloning NTE.

2 2 2 2 2 2 2 2 2 1 1 1 1 1 1 1 1 1 1 1 1
 ADLT A D **L** T E G D **L** A N **S** H **L** P P E V **L** **L** Y M **L**
 10kDa $\approx$ 100 amino acids $\approx$ 300bp

1 1 1 1 1 1 1 1 1 1 1 1 1
 DLGL D **L** G **L** P Y F N V V T D I
 13kDa $\approx$ 130 amino acids $\approx$ 400 bp

1 1 1 1 1
 XGVP (K/A)G V P V
 32kDa $\approx$ 320 amino acids $\approx$ 900bp
 S9B labelled fragment

6 6 6 6 5 5 5 5 5 4 3 3 2 1
 LTNP **L** T N P A **S** N **L** A T V A V **L**
 72kDa $\approx$ 700 amino acids $\approx$ 2100bp
 36kDa $\approx$ 330 amino acids $\approx$ 1000bp
 S9B labelled fragments

1 1 1 1 1
 VILP V I **L** P E
 16kDa $\approx$ 160 amino acids $\approx$ 450bp

Figure 4.1 Pig NTE peptide sequence available for degenerate primer design.

The number above each residue represents the number of times that residue had been observed in repeated protein sequencing runs. Below the sequence is the size of the V8 digest fragments which yielded the sequences along with the approximate amount of protein and DNA sequence required to encode the peptide. Conversion of molecular weight to DNA sequence assumes the average amino acid weighs 110 Daltons (Da).

Highly degenerate amino acids are highlighted in colour; leucine (green) is 16 fold degenerate, serine (red) is 32 fold.

5'		3'	
GTTACGGCTGGAT TGAGTGAC TTTTTTTTTTTTTTTTTTT			BAT39
GTTACGGCTGGAT TGAGT			BAT117 Td=54°C
TACGGCTGGAT TGAGTGAC			BAT318 Td=59°C
	Stop Stop		

Figure 4.2 Design of anchor primers for reverse transcription of poly-A RNA and 3' RACE.

A novel 20 bp sequence was designed using the program OLIGO for Windows Version 5.0. Primers were designed to have no predicted tendency to form primer dimers either with themselves or the degenerate primers to be used in the cloning attempt and also have maximum priming specificity according to OLIGO's primer annealing model. Stop codons were introduced at the 3' end to discourage anchor primers priming in an open reading frame.

Three oligonucleotides were made using this sequence. One synthesis primer, designated BAT39, had an oligo-dT tail to prime reverse transcription, and two anchor primers were for use as the reverse (antisense) primers in the 3' RACE reaction.

Figure 4.3 Relative positions of trk1+, trk3-, trk5+ and trk9+ primers on pig trkC cDNA.

Trk primers were designed to the published sequence of trkC cDNA (Lamballe *et al*, 1991. cDNA em:M80800).

Forward primers:-

5'----->3'

trk1+ ACCCTCATCACTGGA

trk5+ CCAGTCATTGAGAACCCC

trk9+ ATCACGGACATCTCAAGG

Reverse primer:-

5'----->3'

trk3- GATGTCCCAGGTAGATG

These forward and reverse primers were used as a positive control to test the integrity of the pig brain cDNA BAT39-primed templates used in the PCR and 3' RACE experiments. The products they produced would be of a predictable size (trk9+ vs trk3- = 2249 bp, trk1+ vs trk3- = 998 bp, trk5+ vs trk3- = 944 bp) and aid identification of correctly formed product.

Lane	Primer
1 kb	Clones 1 kb ladder
1	trk1+ alone
2	trk3- alone
3	trk3- alone
4	no primer blank
5	trk9+ vs trk3-
6	trk1+ vs trk3-

Figure 4.4 Quality control of BAT39-primed cDNA for 3' RACE.

PCR reaction conditions: (94°C, 5min, [94°C, 1min; 55°C, 1min; 72°C, 2min]x35) 1 μ M primer, 2mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 0.5u Taq2000 polymerase in 20 μ l reaction volume, 2.5 ng BAT39-primed cDNA).

Lane 5 shows a 2.2 kbp product produced by the trk9+ vs trk3- primer pair, lane 6 shows a 1 kbp product from the trk1+ vs trk3- primer pair, lanes 1, 2 and 3 show single primers are not responsible for the product bands.

Lane	Primers
1 kb	Gibco 1kbp ladder
1	trk1+ alone
2	trk9+ alone
3	trk3- alone
4	no primer blank
5	trk9+ vs trk3-
6	trk1+ vs trk3-

Figure 4.5 Ratification of 3' RACE protocol.

BAT39-primed cDNA was amplified with the forward primers trk9+, trk5+ and trk1+ against the anchor primer BAT318. Only the trk1+/BAT318 primer combination produces any products (shown above). (Conditions (94°C, 5min, [94°C, 1min; 55°C, 1min; 72°C 2min]x35) , 3ng BAT39-primed cDNA, 1μM each primer, 1.5mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 1u AmpliTaq polymerase in 30 μl reaction volume).

Lane 1 and 2 are duplicates of the 3' RACE reaction with a major band at about 1.4kbp (arrowed).

<u>Lane</u>	<u>Primers</u>
1 kb	Gibco 1kbp ladder
1	trk1+ vs BAT318
2	trk1+ vs BAT318
3	trk1+ alone
4	BAT318 alone

ADLT (Pig 10kDa) sequence

A D L T E G D L A N S H L P P E V L
GCNGAYYTNACNGARGGNGAYYTNGCNAAYWSNCAYYTNCNCNGARGTNYTN
F10I GCIGAYYTIACIGARGGNGA x32 Td=50-58°C

DLGL (Pig 13kDa) sequence

D L G L P Y F N V V T D I
GAYYTNGGNYTNCCNTAYTTYAAAGTNGTNACNGAYATH
F13IG GGIYTICCTAYTTYAA x8 Td=36-44°C
F13IY TAYTTYAAAGTIGTIACNGA x32 Td=44-52°C

VILP (Pig 16kDa) sequence

D E V I L P E
5' **G**ANGTNATHYTNCCNGAR 3'
F16I **G**AI GTIATHYTICNGA x24 Td=38-44°C

LTNP (Pig 36kDa) sequence

L T N P A S N L A T V A V L
5' YTNACNAAYCCNGCNWSNAAYYTNGCNACNGTNGCNGTNYTN 3'
F36IS WSIAAYYTIGCNACIGTNGC x64 Td=46-52°C
F36IT ACIAAYCCIGCIWSNAA x32 Td=42-44°C

Degeneracy codes: H=A,C or T; I=inosine; N=A,C,G or T;
R=A or G; S=C or G; W=A or T; Y=C or T

Figure 4.6 Design of degenerate primers from NTE peptides for 3' RACE experiment.

Codons shown in bold have been deduced from V8 specificity for peptide bonds on the C-terminal side of glutamic or aspartic acid residues (Drapeau, 1976). The 'x' values refer to the degeneracy of the primers. The temperature range shown next to the degeneracy is the lowest and highest Td value predicted for the primers in that pool. High and low Td values were calculated using the $2(A+T)+4(G+C)=Td$ formula rule (Kidd and Ruano, 1995) substituting A in all possible positions for the low Td calculation and substituting G when calculating the high Td. Inosine was deemed to add 0 to the Td.

Primer positions were selected to make use of the most reliable portions of the peptide sequence and to keep the degeneracy to a minimum.

Figure 4.7 3' RACE with the F10I degenerate primer.

BAT39-primed cDNA was PCR amplified with the degenerate primer F10I and RACE anchor primer BAT318. (Conditions (94°C, 5min, [94°C, 1min; 55°C, 1min; 72°C, 3min]x35), 3ng BAT39 cDNA 1.7μM F10I, 0.1μM BAT318, 1.5mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 1u AmpliTaq polymerase in 30 μl reaction volume).

The reaction produced a broad band of 400 to 500 bp. This band though relatively small was interesting as it was known that the RACE protocol could produce truncated products if the BAT39 annealed within the mRNA, as opposed to the poly-A tail (Schmidt *et al*, 1993).

Lane	Primers
1 kb	Gibco 1 kbp ladder
1	BAT318 alone
2	F10I vs BAT318
3	F10I alone

Clone ID	Residue number																									
	1	2	3	4	5	6	7	8	9	0	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2
	A	D	L	T	E	G	D	L	A	N	S	H	L	P	P	E	V	L	L	Y	M	L	N	V	R	V
r3-12	A	D	L	T	E	G	D	*	Q	V	K	M	Q	F	G	S	S	H	C	G	S	V	E	K	N	P
r2-13A	A	D	L	T	E	G	D	N	F	K	*	R	V	L	Y	N	*	F	K	F	V	K	G	S	R	V
r1-17A	A	D	L	T	E	G	D	*	Q	V	K	M	Q	F	G	S	S	H	C	G	S	V	E	K	N	P
r3-09A	A	D	L	T	E	G	E	E	D	*	T	K	P	N	L	E	X	S	*	Y	I	H	Q	S	H	T
r1-15	A	D	L	T	E	G	D	E	D	A	Y	K	K	Q	F	S	Q	X	I	K	N	N	V	T	P	D
r1-11	A	D	L	T	E	G	D	*	Q	V	K	M	Q	F	G	S	S	H	C	G	S	V	E	K	N	P
r3-05A	A	D	L	T	E	G	D	E	D	A	Y	K	X	Q	X	S	Q	H	I	K	N	T	V	T	P	D
r2-06A	A	D	L	T	E	G	E	E	D	*	T	K	P	X	S	X	K	S	*	H	I	H	X	S	H	T
r2-04A	A	D	L	T	E	G	D	*	Q	V	X	M	Q	F	G	S	S	H	C	X	S	V	E	K	N	P
r2-14A	A	D	L	T	E	G	D	K	C	X	S	S	H	D	L	T	L	E	R	K	X	E	X	R	R	V
r3-01A	A	D	L	T	E	G	E	E	D	*	P	T	P	X	X	E	K	S	*	Y	I	H	Q	S	H	T
r3-18A	A	D	L	T	E	G	E	E	D	*	I	K	P	N	S	D	I	S	X	Y	I	H	H	S	Y	T
r1-16A	A	D	L	T	E	G	E	R	S	X	X	X	X	X	G	R	A	R	G	*	K	K	M	W	X	T

Figure 4.8 Translations of the DNA sequences from the 5' (F10I) end of 3' RACE products.

The NTE sequence ADLT is shown in bold at the top of the table for comparison with the 13 F10I derived sequences arrayed below for comparison. The peptide sequence used to design the F10I degenerate primer is underlined. None of the F10I sequences show any significant similarity to the ADLT sequence beyond the 7th residue demonstrating they are not genuine NTE clones.

Figure 4.9 PCR amplification of BAT39-primed cDNA with degenerate primers F36IS, F36IT, F16I, F13IG and F13IY paired with reverse primers BAT117 and BAT318.

Amplification conditions (94°C, 5 min, [94°C, 30 sec; 40°C, 30 sec; 72°C, 7 min]x35), 3ng BAT39 cDNA, 1.7 μ M Degenerate primer, 0.1 μ M BAT318 or BAT117, 1.5mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 1u AmpliTaq polymerase in 30 μ l reaction volume).

<u>Lane</u>	<u>Primers</u>	<u>Lane</u>	<u>Primers</u>
1	BAT318 alone	9	F16I vs BAT318
2	BAT117 alone	10	F16I vs BAT117
3	F36IS vs BAT318	11	F16I alone
4	F36IS vs BAT117	12	F13IG vs BAT318
5	F36IS alone	13	F13IG vs BAT 117
6	F36IT vs BAT318	14	F13IG alone
7	F36IT vs BAT117	15	F13IY vs BAT318
8	F36IT alone	16	F13IY vs BAT117
1kb	Gibco 1kbp ladder	17	F13IY alone

Figure 4.10 Typical example of 2nd round nested 3' RACE with degenerate primers.

Conditions (94°C 5min, [94°C 30 sec, 40°C 30 sec, 72°C 7 min]x35) 1.7μM F36IT, 0.1μM BAT117, 1.5mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 1u AmpliTaq polymerase in 30 μl reaction volume, 5μl 1:10000 diluted template from 1st round amplification).

Template codes:

First round PCR reaction products amplified with BAT117 paired with; G, F13IG; Y, F13IY; S, F36IS; T, F36IT; I, F16I.

Negative control templates were; C, BAT39 cDNA diluted 1:10000; B, first round PCR amplified by BAT117 alone; H, water blank.

Gel A shows each template does not amplify in the presence of the F36IT alone. The BAT117 primer also shows no amplification on these templates (Data not shown).

Gel B shows the products produced by the F36IT/BAT117 primer pair. The products in lane S were potentially interesting, as a specific product of about 500bp was produced. However the template for that reaction was made using F36IS/BAT117 primer pair and since F36IS is located internally to F36IT (Figure 4.6), the 500bp product must be the result of non-specific hybridisation. Lane T shows the non-specific amplification resulting from F36IT reamplifying F36IT derived template.

Chapter 5 Attempts to clone NTE cDNA by PCR using degenerate primers of inferred relative position

5.1 Introduction

Chapter 3 described experiments in which S9B-labelled purified NTE, was digested with V8 protease before it was refractionated on an avidin-Sepharose column (Section 3.2.2). This allowed the separation of fragments bearing the (biotinylated) active site, from the complex mixture of NTE fragments. Fragments which passed through the column were deemed to be unbiotinylated (Figure 3.8). Only two sequences, LTNP and XGVP were found in the avidin repurified fraction and they were associated with protein fragments of 16 to 72kDa in size (Figure 3.7). The simplest organisation of the two peptides (ie, the one which required the minimum number of V8-mediated bond cleavages) resulted in the map depicted in Figure 5.1. In terms of this scheme, a PCR using forward primers designed to the LTNP sequence and reverse primers designed using XGVP sequence should produce a PCR product of approximately 100bp (~4kDa of peptide sequence). As discussed in Chapter 1 (Section 1.3.1) this is a useful observation as gave some clue as to what primers to use and what product size to expect.

5.2 Results and discussion

5.2.1 Production and isolation of PCR-generated clone ORF1

Two forward primers were designed to the LTNP peptide sequence and two reverse primers were designed to the XGVP peptide sequence using inosine to reduce the overall degeneracy of the primers; these primers were designated

F36IT, F36IS, R32IA and R32IK (Figure 5.2). All permutations of forward and reverse primers were tested in PCR reactions with oligo-dT primed pig brain cDNA at magnesium chloride concentrations of 1.5mM to 4mM; it was found that certain primer pairs produced product bands of about 170, 300 and 400 bp with 3mM magnesium chloride (Figure 5.3).

The 170 bp product band, which was the closest to the predicted size, was excised from the agarose gel and reamplified, using the same reaction conditions. This produced sufficient DNA to clone into the pGEM-T vector system (Section 2.2.8). Eighteen recombinants were picked by blue/white selection, then propagated in L-Broth containing 50µg/ml Ampicillin. Plasmid DNA was isolated and the inserts sequenced on both strands by Dye Primer cycle sequencing (see Section 2.3.2).

Out of the eighteen transformants, six did not contain a 170 bp insert and were discarded, four clones contained an identical sequence which was dubbed ORF1. A further two clones contained a different sequence named ORF2. The remaining six clones each contained different sequences, each one with a stop codon in the LTNP primers reading frame, thus they were eliminated as candidate clones possessing NTE cDNA sequence. The ORF1 and ORF2 sequences were translated in all 3 reading frames defining the F36IS sequence as the 5' end (Figure 5.4). The ORF2 sequence was dismissed as a candidate since, when translated, the F36IS and F32IK peptide sequences occurred in different reading frames. This frameshift was unlikely to be a result of a Taq polymerase-mediated mutation since two identical ORF2 clones were observed and, furthermore, Taq polymerase generates relatively few frameshift mutations (Tindall and Kunkel, 1988); thus investigation of ORF2 was suspended in favour of the ORF1 clone.

The ORF1 sequence was thought to be potentially interesting since both known NTE peptide sequences were in the same reading frame and were linked by ORF1's only open reading frame. The mismatch between the predicted

residues at the C-terminal of LTNP (VL) and the observed ones (TT), was thought to be a result of errors in protein sequencing. Both serine and threonine residues are difficult to detect by Edman sequencing as the anilinothiazolinone-amino acids (AZT-amino acids) cleaved from the peptide chain undergo a β -elimination process resulting in derivatised amino acids. When yields of amino acids are low (as was the case in the sequencing of NTE) the derivatives are hard to detect: serine can be detected as dehydroalanine; threonine co-elutes with proline, methionine and valine derivatives, effectively concealing it from detection (K. Lilley, Personal communication). The mismatch of the first residue (S vs R) was a result of the degenerate codon used in that position (WSN) as it codes for both serine and arginine. Extensive database searches were carried out using the BLAST, FASTA and BLITZ programs on both the DNA and deduced peptide sequence of ORF1, but produced no significant matches in either EMBL or SWISSPROT.

It was noticed that one of the predictions made by the putative peptide map (Figure 5.1) placed the LTNP sequence 20 to 27 kDa (180-250 amino acids) from the catalytic serine. Interestingly, the N-terminus of the acetylcholinesterase (AChE) family of serine esterases also lies approximately 200 residues from the catalytic serine (Krejci *et al*, 1991). This prompted an attempt to align the deduced ORF1 peptide sequence with the N-terminal region of the serine esterase alignment. (Figure 5.5). The degree of similarity revealed by the alignment between ORF1 and a portion of a known serine esterase family was thought sufficient evidence to justify cloning the flanking DNA sequence and determine if similarity really extended through and beyond the primer sequence.

5.2.2 Attempts to clone the ORF1 template

Various approaches were taken to clone the template which produced ORF1.

1) cDNA library screening

Hybridisation screening of 1 million plaques from a Clontech pig brain cDNA library using ³²P labelled ORF1 probe, yielded no genuine positive clones (Section 2.4.1).

2) 3' RACE

Non-degenerate forward primers were designed using ORF1 DNA sequence lying between the degenerate primers (two of these primers are shown in Figure 5.6). The primers were used in an unsuccessful attempt at 3' RACE on BAT39-primed cDNA which failed to produce any specific products despite optimisation and the use of nested primers.

3) 'hemi-degenerate' PCR

Previous work by the group (Section 1.1.5) had produced a limited amount of heterogeneous sequence, flanking the catalytic serine (see Figure 1.7; Glynn *et al*, 1993). In an attempt to elucidate the genuine sequence, SWISSPROT was BLITZ searched using the acetylcholinesterase active site sequence (VTLFGE\$AG). Twentyone different active sites were observed and the sequences combined into a catalytic site motif which was compared with this heterogeneous NTE sequence (Figure 5.7). The tentative consensus sequence residues was used to design two degenerate primers, denoted F-SECI and R-SECI. These primers were hardly used during the NTE cloning project, due to the highly speculative nature of their generation. However the R-SECI (the reverse primer) was tested with the nondegenerate ORF1 based forward primers (Figure 5.6) but produced no product (data not shown).

4) Inverse PCR

Inverse PCR is a method which allows the sequence flanking a short section of known DNA sequence to be specifically amplified (Ochman *et al*, 1990). The technique requires genomic DNA digested with a restriction enzyme to produce a population of DNA fragments each terminating in compatible sticky ends. The digested DNA is religated at a low DNA concentration (<2 µg/ml). This promotes intramolecular ligation over intermolecular ligation, thus the fragments self ligate to form circles rather than randomly reassociate with other fragments. PCR with non-degenerate primers pointing outwards from the known sequence amplifies the bulk of the circle resulting in novel sequence flanked by known (ORF1) sequence (Figure 5.8).

High molecular weight pig genomic DNA was prepared from frozen pig brain tissue (see Section 2.2.6 and 2.3.4). Two circularised genomic DNA (cgDNA) libraries were prepared using *TaqI*- and *MboI*-digested DNA. Restriction enzymes were selected on the basis that they produced 4bp sticky ends and that they did not cut the known ORF1 sequence. Two cgDNA libraries were constructed to guard against an enzyme that produced an ORF1 bearing fragments which were too large (>3kbp) to easily amplify from a complex mix, or too small (<200-300 bp) to readily circularise (Silver, 1991).

The circularised template was amplified with the ORFA vs ORRA as a positive control for the presence of the ORF1 sequence, as well as the inverse primers ORFD vs ORRA (Figures 5.6 and 5.9). Despite the very poor quality of the single primer controls and the ORFA vs ORRA positive control, an 800 bp band was considered worth pursuing and the gel was Southern blotted (see Section 2.4.2). When the blot was probed with ³²P-labelled ORF1 (see Section 2.4.3 and 2.4.4) a hybridisation signal was detected around the 800 bp band, so the entire PCR reaction which produced the band was cloned into pCR-script vector (Section 2.2.9).

Twenty recombinant colonies were selected on the basis of Blue/White selection and the ORF1 inserts were selected for by a diagnostic PCR between the ORFE vs vector primer. This produced a PCR product of predicted size (data not shown). On the basis of this experiment six colonies were selected (clones 996A 1, 3, 5, 18, 19 and 20) and the inserts sequenced by Dye Primer cycle sequencing (Section 2.3.2). All six clones were found to possess a 100% DNA match with the ORF1 sequence located between the degenerate primers, but only the first 9 bases of the primer were homologous to the ORF1 sequence; beyond that the known NTE peptide sequences and the translated sequence of the genomic clone diverged, proving conclusively that ORF1 did not represent a part of the NTE cDNA sequence and was merely the product of non-specific amplification (Figure 5.10).

5.3 Retrospective view on the relative positions of LTNP, XGVP and the catalytic serine in NTE

Now that NTE has been cloned and sequenced (Figure 7.1) it is possible to construct a more realistic peptide map surrounding the S9B-labelled serine residue (Figure 5.11). Most significantly, LTNP is 228 residues (~25kDa) upstream of XGVP, rather than the 4kDa predicted by the map in Figure 5.1. Part of the reason for this margin of error was the mistaken impression that the 16kDa XGVP fragment was not biotinylated, when, in fact, it was, but its presence in the V8 avidin eluate fraction was masked by the avidin monomer (Figure 3.8). A certain percentage of the 16kDa XGVP fragment was always detected in the avidin flowthrough fraction because, under the experimental conditions used (0.15% SDS), avidin-biotin binding is not totally efficient. In the light of the complete sequence data (Figure 7.1) it is clear that a PCR reaction between primers corresponding to LTNP and XGVP should yield a product of about 680bp. However, the largest discernible product of the degenerate PCR was only about 400bp (Figure 5.3), thus it is probable that this approach would have still failed.

More importantly, since the LTNP is 25kDa upstream of XGVP and the 36kDa LTNP fragment contains the S9B labelling site, then the catalytic serine must lie within the 11kDa (100 residues) downstream of the XGVP sequence.

Figure 5.1 Putative peptide map of the serine esterase domain of NTE.

Originally the map only consisted of the 32kDa XGVP sequence and the 36 and 72kDa LTNP peptide sequences; later sequencing work provided the other biotinyl and nonbiotinyl sequences which expanded and apparently confirmed the map.

The LTNP peptide sequence was eventually found to be associated with protein fragments of 72, 36 and 20kDa. The 72 and 36 kDa fragments were biotinylated by S9B, the 20kDa was possibly unbiotinylated. Similarly XGVP sequence was associated with two biotinylated fragments (32 and 23kDa) plus one 16kDa protein fragment which also appeared not to be biotinylated.

This information was used to construct the map shown above and infer the position of the catalytic serine (marked Ser) to between 20 to 27kDa (180-250 amino acids) from the LTNP peptide sequence. The map also predicted a 4kDa distance between the LTNP and XGVP sequences. This information was used to estimate a range of plausible product sizes from a PCR reaction using degenerate primers designed to the peptide sequence.

L T N P A S N L A T V A V L

F36IS WSIAAYYTIGCNACIGTNGC (x64) Td=46-52°C

F36IT ACIAAYCCIGCIWSNAA (x32) Td=42-44°C

(D/E) (A/K) GVPV

+ve 5' **GCN**AARGGNGTNCCNGTN 3'

R32IK ACIGGIACICCCIGC**NGC** (x4) Td=46-48°C

+ve 5' **GCN**GCNGGNGTNCCNGTN 3'

R32IA ACIGGIACICCYTTNGC (x8) Td=44-48°C

The LTNP sequence posed no unusual problems in selecting PCR primers (aside from the degeneracy of the sequence).

The XGVP peptide sequence posed two problems when designing reverse degenerate primers.

First, the Edman sequencing was unable to determine if the first residue in the sequence was an alanine or a lysine so two primers were designed, one assuming the 1st position was an alanine (R32IA) the other assuming it was a lysine (R32IK).

Secondly the sequence was too short to produce PCR primers with a reasonable Td value so the residue preceding the N-terminal was inferred from the specificity of V8 protease for glutamate and aspartate which combined to form the codon GCN (shown in bold) increasing the annealing temperature of the primers by 8-10°C.

Figure 5.3 PCR amplification of oligo-dT primed pig brain cDNA using primers of putatively known position.

All combinations of forward vs reverse primers were tested (Conditions (94°C 5min, [94°C 1min; 55°C 1min; 72°C 2min]x35), 3ng pig brain oligo-dT primed cDNA 1μM primer, 3mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 0.5u Taq2000 polymerase in 20 μl reaction volume).

Three faint bands (arrowed a, b and c) were evident in lanes 6 and 7, of sizes a ~170bp, b ~300bp, c ~400bp.

<u>Lane</u>	<u>Primers</u>	<u>Lane</u>	<u>Primers</u>
1	R32IA alone	5	F36IS vs R32IA
2	R32IK alone	6	F36IT vs R32IA
3	F36IS alone	7	F36IS vs R32IK
4	F36IT alone	8	F36IT vs R32IK
1 kb	Gibco 1 kb ladder		

ORF1

LTNP (Pig 36kDa) sequence residues 6-14

S N L A T V A V L

5' AGGAACCTGGCGACGGTGGCCACTACCACAGGTCAGGAAATGGGACTGAATATTGGGCCT

1 **R N L A T V A** T T T G Q E M G L N I G P

2 G T W R R W P L P Q V R K W D * I L G L

3 E P G D G G H Y H R S G N G T E Y W A F

TCCATGTGTTCTGTATTGCTCATTATCTATTTAAAGCTTAGAAGTGTTTTTCTTATTTTC

1 S M C S V L L I I Y L K L R S V F L I F

2 P C V L Y C S L S I * S L E V F F L F S

3 H V F C I A H Y L F K A * K C F S Y F P

XGVP (Pig 32kDa) sequence

D K G V P V

CTGCAGGTGGTTATCACTAGACTAAAACTGGACAAGGGCGTCCCCGT 3'

1 L Q V V I T R L K L **D K G V P V**

2 C R W L S L D * N W T R A S P

3 A G G Y H * T K T G Q G R P R

ORF2

LTNP (Pig 36kDa) sequence residues 6-14

S N L A T V A V L

5' AGGAACCTGGCGACGGTGGCCCATCTGGTAAATGAAGATCCCCTCCAAAAGTCATTATTT

1 **R N L A T V A** H L V N E D P L Q K S L F

2 G T W R R W P I W * M K I P S K S H Y L

3 E P G D G G P S G K * R S P P K V I I C

GTAGATTATATGATCACATACAGCTTTGGGTCTGGAACAGTCTTTGCCTTTTCATTCTCAG

1 V D Y M I T Y S F G S G T V F A F H S Q

2 * I I * S H T A L G L E Q S L P F I L S

3 R L Y D H I Q L W V W N S L C L S F S A

XGVP (Pig 32kDa) sequence

D K G V P V

CGCTTCCACCTGTCCCATGTTTCCTGGACAAAGGCGTCCCCGT 3'

1 R F H L S H V P G Q R R P R

2 A S T C P M F L **D K G V P V**

3 L P P V P C S W T K A S P

*= stop codon

Figure 5.4 Translation of ORF1 and ORF2 sequence in three reading frames.

```

LTNP/XGVP      SNLATVA                                     XKGVPV
ORF1            RNLATVATTTGQEM-GLNIGPSMCSVLLIIYLKLRSVFLIFLQVVITRLKLDKGVPV
Gliotactin      TLERDVVFVTTNY---GQVQGFKVYMYDNPDPKSFYRPHYSTVDRVMGECVFLGIPY
Lipase          AAALNVVGTLAQAPTAVLNGNEVISGVLEG-----KVDTFKGIPF
Neuroigin1      DDVDPLVTTNF---GKIRGIKKELNNEILG-----PVIQFLGVPY
Neuroigin2      EERFPVNTAY---GRVRGVRRELNNEILG-----PVVQFLGVPY
Neuroigin3      QAPAPTNTHTF---GKLRGARVPLPSEILG-----PVDQYLGVPY
Neurotactin     EGRYIMAVTGC---GPVEGV-----KEDGAFAFRGIPY
Glutactin       PQEAVVQAPEV---GQILGISGHKTIA-----NRPVNAFLGIRY
JHE             NSRSVVAHLDG---GIIRGVPRSADG-----IKFASFLGVPY
DroEst6         TDDPLLVQLPQ---GKLGRD-----NGSYYSYESIPY
DroAChE         VIDRLVVQTSS---GPVGRSVTVQG-----REVHVYGIPY
TorpAChE        DHSSELLVNTKS---GKVMGTRVPVLS-----SHISAFLLGIPF
HumBChE         IEDDIIIATKN---GKVRGMNLTVEFG-----GTVTAFLGIPY
Thyroglob       ISTPSVHIDSF---GQLQGGSQVVKVGTAW-----KQVYQFLGVPY
Carbox Est      PSSPPVVDTTK---GKVLGKYVSLEGFTQ-----PVAVFLGVPF
MsomEst1        PSAPPVVDTVK---GKVLGKFVSLEGFAQ-----PVAVFLGVPF

```

Figure 5.5 Alignment ORF1 with the N-termini of multiple serine esterase domains.

Residues identical to ORF1 are highlighted in red, those similar to ORF1 are marked in green. Similarity was defined as the amino acid pair having a score of 0 or greater on the BLOSUM62 protein comparison matrix (Henikoff and Henikoff, 1992).

The original alignment (Krejci *et al*, 1991) did not contain the sequence for neuroigin1, 2 or 3, or gliotactin, these were added to the alignment later. The addition of gliotactin was considered particularly auspicious, since it is an inactive serine esterase protein which possessed a 16 residue insertion relative to the other sequences much like the one present in ORF1.

Key to abbreviations

Gliotactin	<i>Drosophila</i> gliotactin	Auld <i>et al</i> , 1995
Lipase	<i>Geotrichum</i> lipase 1	sw:P17573
Neuroigin1	<i>Drosophila</i> neuroigin1	lichtchenko <i>et al</i> , 1995
Neuroigin2	<i>Drosophila</i> neuroigin2	lichtchenko <i>et al</i> , 1996
Neuroigin3	<i>Drosophila</i> neuroigin3	lichtchenko <i>et al</i> , 1996
Neurotactin	<i>Drosophila</i> neurotactin	Escalera <i>et al</i> , 1990
Glutactin	<i>Drosophila</i> glutactin	Olson <i>et al</i> , 1990
JHE	insect juvenile hormone esterase	sw:P12992
DroEst6	<i>Drosophila</i> esterase 6	sw:P08171
DroAChE	<i>Drosophila</i> AChE	sw:P07140
TorpAChE	Torpedo fish AChE	sw:P04058
HumBChE	Human Butyrlcholine esterase	sw:P06276
Thyroglob	Bovine thyroglobulin	sw:P01267
Carbox Est	rat carboxylesterase 3	sw:Q63108
MsomEst1	rabbit microsomal esterase 2	pir1:A34329

5' ORFA \
AGGAACCTGGCGACGGTGGCCACTACCACAGGTCAGGAAATGGGACTGAATAT
ORFE /
 TGGGCCTTCCATGTGTTCTGTATTGCTCATTATCTATTTAAAGCTTAGAAGTG

ORFD \
 TTTTTCCTATTTTCCTGCAGGTGGTTATCACTAGACTAAACTG**GACAAGGGC**
ORRA \
 3' GTCCCCGT

Figure 5.6 ORFA, ORFD, ORFE and ORRA primer locations on ORF1 sequence.

The DNA sequence derived from the degenerate primers is shown in bold. ORFA and ORFE are two of the primers used in the unsuccessful 3' RACE attempts at cloning more of the cDNA template which gave rise to ORF1.

The ORFD and ORRA were used in the successful inverse PCR of the ORF1 template.

Residue number	1 2 3 4 5 6 7	<u>Primer design</u>
Serine esterase	I F G E S A G	
consensus	V Y Q S	I/V V/A G E S M G
sequence	L A H H	5' RTN GYN GGN GAR WSN ATG GGN 3'
	T S S M	
	S W D	Forward primer
	V Y	F-SECI
		5' GYI GGI GAR WSI ATG GG 3'
Sequence from	K E K E A M G	
Glynn et al,	I V A S K	
1993	V A G	Reverse primer
	F	R-SECI
	A	5' CC CAT ISW YTC ICC NRC 3'
Common	V A G E S M G	
Residues	I V	
	1 2 3 4 5 6 7	

Figure 5.7 Inferred consensus sequence flanking the catalytic serine in NTE.

Figure 5.8 Inverse PCR allows amplification of DNA sequences flanking a small section of known sequence.

1) Genomic DNA is digested with a restriction enzyme (in this case *MboI*) to produce a population of DNA fragments with identical sticky ends.

2) The digested DNA is religated at high dilution to ensure the fragments self ligate rather than randomly reassociate with other fragments.

3) PCR with primers pointing outwards towards the unknown (flanking) sequence amplifies the bulk of the circle resulting in a novel sequence flanked by the known (ORF1) sequence.

Figure 5.9 Inverse PCR on *Mbol* circularised pig genomic DNA with non-degenerate primers ORFA, ORFD and ORRA.

Conditions: (94°C, 5min, [94°C, 1min; 55°C, 1min; 72°C, 2min]x35), 300 ng *Mbol*-circularised pig genomic DNA, 1μM each primer, 3mM MgCl₂, 0.2 mM dNTP, 1xPCR buffer, 1u AmpliTaq polymerase in 30 μl reaction volume.

Although all three primers produced significant non-specific amplification when alone (lanes 1, 2 and 3), and the ORFA vs ORRA positive control pair also produced a large smear, the ORFD and ORRA pair produce a discrete band of 800bp in lane 6 (arrowed).

<u>Lane</u>	<u>Primers</u>	<u>Lane</u>	<u>Primers</u>
1	ORFA alone	4	No primer blank
2	ORRA alone	5	ORFA vs ORRA
3	ORFD alone	6	ORFD vs ORRA
1kb	Gibco 1kb ladder		

A)	LTNPASNLATVAVL	EA
B)	RNLATVA TTTGQEMGLNIGPSMCSVLLIIYKLRSVFLIFLQVVITRLKLD DKGV PV	DKGV
C)	SPTLWHWCVTVATTGQEMGLNVGPSMCSVLLIIYKLRSVFLIFLQVVITRLKLDKDRKKIL	

Figure 5.10 Alignment of ORF1 templates.

Only the translated peptide sequence is shown in this figure as it conveniently summarises the relationship between the original NTE peptide sequences LTNP and XGVP, the translated ORF1 sequence, and the translated genomic clone.

A) Represents the original peptide sequences used to design the degenerate primers.

B) Represents the deduced ORF1 peptide sequence. Peptide sequenced derived from the degenerate primers is shown in bold.

C) Represents the section of the inverse PCR product homologous to the deduced ORF1 protein sequence. The inverse PCR product has been reassembled into the original templates configuration and translated to show that the homology between ORF1 and the template that gave rise to it only extends to the first two or three residues (6 to 9 base pairs) of the degenerate primers that produced it.

Chapter 5 Cloning of NTE via homology to an expressed sequence tag

6.1 Introduction

Figure 5.11 Peptide map around the S9B-labelled serine of NTE, using sequence data from Figure 7.1.

The S9B-labelled serine must lie in an 11kDa (100aa) interval (shown in black) upstream of the C-terminal end of the 36kDa fragments and downstream of the XGVP sequence.

(Figure 5.1). The DLGL sequence had shown similarity with a group of five overlapping human Expressed Sequence Tag (EST) sequences (R17727, H10459, T10292, H56504 and F51195). Translations of these ESTs were in turn similar to the C-terminal region of YOL4_CABEL. However DLGL also had a similarity with *Escherichia coli* acetyl-coenzyme A carboxylase (sw:P08193), a 345 residue biotinylated protein. This match suggested DLGL may not have derived from NTE but a contaminant sequence in the NTE protein preparation (see Section 3.3 and Figure 6.1).

YOL4 is a 152kDa (1351 residue) hypothetical protein from *C. elegans* chromosome III (Wilson et al., 1994). At the time there were only two similar database records: YCHK_ECOLI, an *Escherichia coli* 314 amino acid (34 kDa) hypothetical protein (sw:P37053) and a near identical 178 amino acid fragment from a partial hypothetical protein sequence from *Shigella flexneri* (YCHK_SHIFL

Chapter 6 Cloning of NTE via homology to an expressed sequence tag

6.1 Introduction

At the time the ORF1 sequence was being cloned (Chapter 5), ongoing studies in the laboratory revealed two peptide sequence similarities. Searches using the BLAST program on SWISSPROT revealed that the DLGL and the then new LAKL peptide sequence (Figure 3.7) were both associated with YOL4_CAEEL (sw:Q02331), a hypothetical protein which had been predicted to exist from genomic DNA sequence produced as part of the *Caenorhabditis elegans* genome sequencing project (Wilson *et al*, 1994).

The LAKL peptide sequence had a weak similarity with part of the N-terminal region of YOL4. It also had similarities with Ribulose Bisphosphate Carboxylase (RUBISCO) Large Chain, a 485 amino acid non-biotinylated plant carboxylase which showed that the LAKL/YOL4 match was not of unique quality (Figure 6.1). The DLGL sequence had shown similarity with a group of five overlapping human Expressed Sequence Tag (EST) sequences (R17727, H10469, T10299, H58564 and R51195). Translations of these ESTs were in turn similar to the C-terminal region of YOL4_CAEEL. However DLGL also had a similarity with *Escherichia coli* acetyl-coenzyme A carboxylase (sw:P08193), a 345 residue biotinylated protein. This match suggested DLGL may not have derived from NTE but a contaminant sequence in the NTE protein preparation (see Section 3.3 and Figure 6.1).

YOL4 is a 152kDa (1351 residue) hypothetical protein from *C. elegans* chromosome III (Wilson *et al*, 1994). At the time there were only two similar database records: YCHK_ECOLI, an *Escherichia coli* 314 amino acid (34 kDa) hypothetical protein (sw:P37053) and a near identical 178 amino acid fragment from a partial hypothetical protein sequence from *Shigella flexneri* YCHK_SHIFL

(sw:P37054; Figure 6.2). On examination, neither the YOL4 nor the YCHK sequences showed any significant similarity with any member of the AChE family of serine-esterases, or any other protein sequence in SWISSPROT or EMBL databases.

One of the ESTs that matched DLGL (em:H58564) was ordered from the IMAGE consortium cDNA collection at Research Genetics Inc, USA and the match was further investigated by the group in three ways:-

- 1) Deduced peptide sequence from the EST (Figure 6.1) was used to design a synthetic peptide which was coupled to Keyhole Limpet Haemocyanin (KLH) and used to immunise two rabbits. The antibody did not become available until after the peptide sequence produced by Tony Willis (Figure 3.9) had refocused interest in YOL4.
- 2) The EST was used to screen a λ DR2 adult human brain cDNA library and a single 2.2 kbp cDNA insert was isolated. This sequence was later named HOL4.2.
- 3) The EST was used to probe a human brain mRNA Northern blot which produced a very faint band at about 2 kb.

Combining the data from the similarity searches which indicated DLGL was similar to a known biotinylated protein (Figure 6.1) and the 2kb size of the mRNA measured on the Northern blot (much too small to encode the full NTE gene), it was concluded that the DLGL sequence was probably from a contaminating carboxylase protein and the similarity between DLGL, LAKL and YOL4 was just a coincidence and so work on YOL4-connection was suspended.

Interest in YOL4 was revived by the arrival of five new protein sequences (Section 3.3.2. and Figure 3.9). Similarity searches with the new peptides revealed striking links with the YOL4 hypothetical protein. DLGL was found to

be on the same EST (em:R51195) as the new DGHL sequence and the VVKS sequence was located right next to FDQI on another EST (em:R54769, Figure 6.3). In addition to the sequence similarities, the peptide antibody made using the EST based sequence (Figure 6.1) was found to be specific to partially-purified S9B-labelled NTE on a Western blot. These new data indicated that the human ESTs could code for part of the NTE gene.

The homologies between NTE, YOL4 and YCHK and their similarity with several other hypothetical proteins will be discussed in Chapter 7).

6.2 Results and discussion

6.2.1 Sequencing the 3' end of NTE cDNA by alignment of ESTs

As mentioned in Chapter 1 human ESTs normally come in pairs (one from the 5' and one from the 3' ends of the insert; see Figure 1.10 (and Section 1.2.5), the 3' ends of a particular transcript are normally the same because the cDNA was primed off the poly-A tail, thus each priming starts from more or less the same place in the transcript. This is useful as a single 3' EST sequence can be used to search the database and identify other clones from the same transcript, then the corresponding 5' sequence which contains DNA sequence from the coding region of the gene can be retrieved from the database and aligned to determine a preliminary consensus sequence for the gene without recourse to further DNA sequencing.

A total of 26 EST clones were recovered from the EST division of EMBL, in addition to this 3' and 5' sequence from the HOL4.2 (isolated from the λ DR2 human brain cDNA library) insert was also generated from cycle sequencing from vector primers. The 5'EST sequences were aligned by looking for sequence overlaps between sequences and also by reference similarities between YOL4 protein sequence and translations of the EST sequences. The completed 'virtual'

sequence covered a region of 2.2 kbp broken by a single gap of approximately 50bp for which no EST sequence was available. This preliminary DNA consensus sequence contained unavoidable insertions and deletions (despite careful alignment), as the sequences it was based on were derived from automatically generated single pass sequencing runs. However, it was possible to patch together parts of the reading frame by searching for parts of the translated sequence that matched the known NTE peptide sequences (see Figure 7.1) and/or YOL4 sequence (the full EST contig is listed in Appendix 1). This putative protein sequence allowed analysis of the C-terminus well before cycle sequencing of the clone was completed, and was instrumental in showing that the HOL4.2 clone was genuinely from NTE. Aside from allowing a preview of the protein sequence, the EST alignment allowed a series of dye terminator sequencing primers to be designed spaced throughout the HOL4.2 insert and directly confirm the EST based sequence. HOL4.2 is clearly a truncated NTE cDNA, 2200bp of DNA sequence is only enough to encode 730aa (~80kDa) of protein sequence as the polyadenylation signal and poly-A tail is present at the 3' end of HOL4.2, at least another 2kbp (~680aa, ~75kDa) of cDNA sequence would be required to complete the gene (cloning of the 5'end of NTE is described in Section 6.2.3).

6.2.2 Subcloning of the 2 kbp insert from pDR2 into pBluescript SK+

Due to its large size (9.1kbp) the pDR2 vector proved to be a poor subject for purification and sequencing. Thus the first stage in sequencing the gene was to subclone the HOL4.2 insert into a more tractable vector. The HOL4.2 insert was excised from pDR2 with a *Bam*HI/*Xba*I double digest and agarose gel purified, then subcloned into *Bam*HI/*Xba*I digested pBluescript SK+ (Section 2.2.7). One clone was selected and sequenced using both dye primer and dye terminator sequencing reactions using the primers based on the EST sequence; the final HOL4.2 sequence is shown in Appendix 4.

6.2.3 Cloning of the 5' end of NTE by 5' RACE

As mentioned in Section 6.2.1 the HOL4.2 insert was 2.2kbp long, enough to encode 730aa of proteins sequence. To clone the remaining sequence the initial cloning effort concentrated on demonstrating that the λ DR2 human brain cDNA library contained no larger NTE clones. Three antisense primers (NDR2, NDR3 and NDR4) were designed using version 5.0 OLIGO for Windows separated by approximately 100bp and selected for compatibility with a novel pair of forward primers designed to the pDR2 vector sequence, DRA and DRB (see Figure 6.4). The 100bp separation between the NDR primers was designed to help in identifying the correct products as genuine bands produced from NTE cDNA would differ in size depending on which primer was used. Most non-specific products would not display this feature and thus could be ignored.

The λ DR2 cDNA library was amplified (see Section 2.3.6) to produce sufficient amounts of library phage for repeated PCR experiments and approximately 10^7 plaque forming units (pfu) of phage was used in each reaction (boiled prior to the PCR to destroy DNase activity). Subsequently all permutations of gene specific (NDR2/3/4) and vector specific (DRA/B) primers were tested searching for PCR products larger than those produced by HOL4.2 insert in pDR2 vector (Figure 6.5).

Once it became clear that the λ DR2 cDNA library contained no full length inserts which could be amplified by PCR (a result later confirmed by conventional hybridisation screening performed by other members of the research group), 15 ng of Marathon human brain RACE ready cDNA was procured from Clontech and utilised in a series of 5' RACE reactions.

Figure 6.6 summarises the principal features of the Marathon cDNA system. Of particular note is the use of the T7 promoter sequence as the anchor primer (AP1). The pBluescript SK+ vector contains the T7 promoter and fortuitously it adjoined the 5' end of the HOL4.2 insert in the subcloned construct

described previously. Thus the NDR2 and 4 primers could be tested on a known template, and it was demonstrated that the NDR2/4 primers were compatible with the Marathon AP1 primer (data not shown).

Initial experiments used Pfu DNA polymerase because of its proof-reading activity and its ability to amplify large templates (Lundberg *et al*, 1991). Using the pBluescript/HOL4.2 construct as a primer positive control and a glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) specific primer as a cDNA template control, a set of PCR conditions were determined which would efficiently amplify both the control DNAs. However when these conditions were used on the Marathon cDNA only a smear was produced, even when nested PCR was attempted. The smearing of the product was finally overcome using by changing the polymerase to Clontech Advantage KlenTaq. This produced a discrete band of about 2.9kbp from the NDR4 primer and a slightly smaller band from the NDR2 primer (Figure 6.7).

Previously the Stratagene pCRscript cloning system was the method of choice for blunt end DNA cloning. However the pCRscript system was unsuitable for cloning the 5' RACE product since the Clontech linker adapter contained a *SrfI* restriction site. The pCRscript kit selects for recombinant plasmids by including *SrfI* (a blunt ending restriction enzyme with an 8bp recognition sequence 5' GCCC^yGGGC 3') into the ligation mixture. The pCRscript plasmid contains a *SrfI* restriction site which is also the insertion site for the PCR product, thus non-recombinant pCRscript is maintained in the linear state. When an insert ligates into the vector, the *SrfI* site is disrupted so the vector remains in a circular form which can transform the competent cells (Stratagene, 1996).

A certain amount of difficulty was encountered in cloning the 2.9kbp insert since limited amounts of the PCR product were available. The cloning problem was finally resolved by resynthesising the NDR2 and NDR4 primers and adding a *XhoI* site to the 5' end to make the primers XNDR2 and XNDR4 (Figure 6.4). The products from the 5' RACE reaction using these new primers were digested with

NotI/XhoI and ligated into *NotI/SalI* digested pBluescript SK+ vector, which had been dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP). Subsequently the ligation mix was transformed into Stratagene supercompetent XL1 Blue MRF' cells (see Sections 2.2.7 and 2.2.10).

Colony lifts were taken from recombinants and screened using the first 200bp of the HOL4.2 insert (from the 5' end to NDR4 primer) labelled with ³²P. One hundred and twenty two positive clones were selected for further investigation by PCR amplification between the vector primer RSP and NDR4 (Figure 6.8). The largest 14 clones were selected and dye primer sequenced (Section 2.3.2) from the vector sequences.

6.2.4 Sequencing of the 5' RACE products

Examination of the 14 5'-sequences showed that each was derived from independent cDNA transcripts as the initial DNA sequence was different in each clone (see Figure 6.9). However, when aligned they formed two groups of sequences which were named NTE1 and NTE2. Representatives from both these groups have been partially or fully sequenced using primers designed to the D16 NTE clone which had been produced other work by the research group (see Section 6.3).

Only one of the NTE1 clones has so far been sequenced in full (a28); it consists of a 2607bp sequence (Appendix 4) which has a 266bp overlap with the HOL4.2 DNA sequence (Figure 6.10). A contig of a28 and HOL4.2 (a28HOL4) contains one open reading frame (ORF) of 1342aa. The likely start codon is located 16aa into the ORF (Figure 6.11) to give a 1327aa protein (Appendix 4) with a predicted molecular weight of 146kDa, close to the 155kDa measured for NTE. Partial sequence data from the other NTE1 clone (a12) has been compared to the a28 sequence and found to be almost identical.

The NTE2 sequence group, contains three clones which have been partially sequenced and show evidence of alternate splicing at the 5' end (Figure 6.11). The remaining cDNAs are truncated and start more than 500bp from the beginning of the a28 clone and thus match both NTE1 and NTE2.

6.3 Review of gene cloning

Once it became clear that HOL4.2 insert encoded a portion of NTE cDNA sequence (see Section 6.1 and Figure 7.1) two approaches were taken to obtaining the 5' end of the gene: 5' RACE (described above) and Hybridisation screening of human brain cDNA libraries (undertaken by other members of the research group). The hybridisation screening started by rescreening the λ DR2 human brain which had originally yielded the HOL4.2 clone. No larger cDNA clones were detected (confirming the 5' RACE screening of the library; Section 6.2.3). Subsequent screening of a foetal brain cDNA library (kindly provided by W. Schwaeble, University of Leicester), produced four cDNA clones three of which have been partially sequenced and are not full length clones. The translation product of the remaining clone, D16, is identical to that of the contig a28HOL4. This sequence is analysed fully in Chapter7.

A)

```
LAKL          LAKLPEGTL GHIERRYPOV V
               ||::||| | : | : |||
YOL4  558 FS QLARVPEGLL NFIKMQYPQV GFRL 583
```

B)

```
1 LAKLP EGTLGHIWRR YPQVV 20
  : ::  |||| |::  ||
RUBISCO  392 DDSVLQFG GGTLGHPWEN APVVVANR 421
```

C)

```
yol4  1008 LLDLTYAHSA MFTGAQFNFS IKDLFEERLI EDLWISYFCI STDISTSEMR 1058
        :||| | : : ||| : || | | : : : : | ||| | | : : ||| : | ||
EST      VLDLTYPVTS MFTGSAFNRS IHRVFQDKQI EDLWLPYFNV TTDITASAMR
                        || ||||| :|||
DLGL                                DLGLPYFNV VTDI
```

D)

```
DLGL          1 DLGLPYFNVVTDI 14
               : ||| : | : ||
ACCD_ECOLI 184 LAKMQERGLPYISVLTDPMTG 195
```

Figure 6.1 Similarity between the pig NTE peptide sequence LAKL and YOL4_CAEEEL, LAKL and RUBISCO, DLGL and YOL4_CAEEEL, DLGL and Acetyl-CoA carboxylase.

A) The LAKL sequence was moderately similar to the hypothetical *C. elegans* protein YOL4. No human EST sequences were available for this region of the protein.

B) LAKL was also similar to RBL_HELAN (the large chain of Sunflower Ribulose biphosphate carboxylase (RUBISCO, sw:P45738).

C) The DLGL sequence was similar to the translated peptide sequence of EST H58564 and YOL4 hypothetical peptide sequence. Sequence shown in bold was used to make a peptide antibody (Section 6.1).

D) The DLGL sequence also has a similarity with *E. coli* acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (sw:P08193). This cast some doubt on the authenticity of the sequence.

| = identity, : = similarity (amino acid pair has a score of 0 or greater on the BLOSUM62 matrix. Henikoff and Henikoff, 1992).

	1					50
yol4_caeel	FWTPDRRSDF	SRLARILTGN	AIGLVLGSGG	ARGAAHVGVL	RALREEGIPV	
ychk_ecoli	MATIAFQGNL	AGIMRKI...	KIGLALGSGA	ARGWSHIGVI	NALKKVGIEI	
ychk_shifl	-----	-----	-- A -----	-----	----- C ----	
	51					100
yol4_caeel	DIVGGTSIGS	LIGGLYAETP	DDVVVETRAA	SWFNGM.SSL	WRKLLDLTYA	
ychk_ecoli	DIVAGCSIGS	LVGAAY....	.ACDRLSALE	DWVTSFSYWD	VLRLMDLSWQ	
ychk_shifl	-----	-----	-----	----- E -----	-----	
	101					150
yol4_caeel	HSAMFTGAQF	NFSIKDLFEE	RLIEDLWISY	FCISTDISTS	EMRVHRSGPL	
ychk_ecoli	RGGLLRGERV	FNQYREIMPE	TEIENCSRRF	AAVATNLSTG	RELWFTEGDL	
ychk_shifl	-----	-----	-----	-- P -----	-----	
	151					200
yol4_caeel	WAYCRASMSL	AGYLPPLCDP	QDGHLLLDGG	YVNNVPADVM	RNLGARCZIA	
ychk_ecoli	HLAIRASCSI	PGLMAPV..A	HNGYWLVDGA	VVNPIPISLT	RALGADIVIA	
ychk_shifl	-----	-----	-----	-----	-----	
	201					250
yol4_caeel	CDVG.SIEET	NLYDYG....	DSL	SGMWVLLKRL	NPFGTPPRIL	
ychk_ecoli	VDLQHDAHLM	QQDLLSFNVS	EENSENGDSL	PWHARLKERL	GSITTRRAVT	
ychk_shifl	--					
	251					300
yol4_caeel	NMEEIQSRLA	YVSCVRQLEV	VKKASYCRYL	RPPIEPFKTL	DFPKFQEIME	
ychk_ecoli	APTATEIMTT	SIQVLENR..	LKRNRMAGDP	PDILIQPVCP	QISTLDFHRA	
	301					350
yol4_caeel	LGLKYGREAC	HELITNDRAG	ILGDEKETR.			
ychk_ecoli	HAAIAAGQLA	VERKMDELLP	LVRTNI....			

Figure 6.2 Alignment between YCHK_ECOLI, YCHK_SHFTL, and the C-terminal domain of YOL4_CAEEL.

The fragmentary YCHK_SHFTL sequence was identical to YCHK_ECOLI (shown as - in the SHFTL sequence) except in 4 positions (shown in bold).

EST R51195 Reading frame +1

```
DLGLPYFNVVTDI                                DGHLLTDGGYINNLP
|| |||||:|||                                ||||| |||||
IEDLWLPYFNVTTDITASAMRVHKDGLWRYVRAXMDAVGLPAPAVXPKDGHLLMDGGYINNLPADI
1                                                                 67
```

EST R54769 Reading frame +3

```
VVKSSS?DG?LR??IDYFK                        FDQIYDVGYYQYQK
|||||  ||  ||  ||  ||  |||||
QLEVVKSSSYCEYLRPPIIDCFKTMDFGKFDQIYDVGYYQYQKAVFG
1                                                                 45
```

Figure 6.3 Similarity between translated EST sequences and the DLGL, DGHL, VVKS and FDQI peptide sequences.

Figure 6.4 Relative positions of primers in insert and pDR2 vector sequence at the 5' end of the HOL4.2 insert.

Primer DRA is located 493bp from the start of the published pDR sequence (em:U02428).

Primers XNDR2 and XNDR4 were used later in the project and include a *Xho*I site at the 5' end (shown in bold) to aid cloning.

Primer	sequence
	5'----->3'
DRA	CGTGCATGCCGATTGGTG
DRB	ATACAATAAACGCCATTTGA
NDR2	TTGGTGAGTTCCGAGTGTGG
NDR3	GCGTGAAGGCCACCA
NDR4	ACAGCCGGAACCTCTTGGATG
XNDR2	CCGCTCGAG TTGGTGAGTTCCGAGTGTGG
XNDR4	CCGCTCGAG ACAGCCGGAACCTCTTGGATG

Lane	Primers	Lane	Primers
1	DRA alone	7	DRA vs NDR2
2	DRB alone	8	DRA vs NDR3
3	NDR2 alone	9	DRA vs NDR4
4	NDR3 alone	10	DRB vs NDR2
5	NDR4 alone	11	DRB vs NDR3
6	no primer blank	12	DRB vs NDR4
1kb	Gibco 1kb ladder

Figure 6.5 PCR of DRA/B primers vs NDR2/3/4 on λ DR2 adult human brain cDNA library.

Conditions (94°C, 5min, [94°C, 1min; 68°C, 4min]x40), 10^7 pfu bacteriophage 2 μ M each primer, 0.2 mM dNTP, 1xTaq2000 buffer, 1u Taq2000 polymerase in 25 μ l reaction volume.

Lanes 7 and 9 show bands at about 300 and 500 bp as would be predicted from Figure 6.4. There also appears to be a faint band at about 800bp in both lanes (arrowed). Although this product could have represented a longer NTE clone, it could not have represented a full length clone (a further 2 to 3 kbp of DNA sequence would be needed to complete NTE cDNA), and so was not pursued.

Lane 8 (DRA vs NDR3) has failed to amplify properly; a similar pattern of products is seen in the negative control (data not shown) so the smear is presumably the result of primer dimer formation.

The DRB primer appears not to be as successful as DRA, only the DRB vs NDR3 primer pair has produced any suitable product and even that indicates the template was no larger than the one already known.

Lane	Primers	Lane	Primers
1	DRA alone	7	DRA vs NDR2
2	DRB alone	8	DRA vs NDR3
3	NDR2 alone	9	DRA vs NDR4
4	NDR3 alone	10	DRB vs NDR2
5	NDR4 alone	11	DRB vs NDR3
6	no primer blank	12	DRB vs NDR4
1kb	Gibco 1kb ladder		

A) Marathon cDNA synthesis primer

B) Marathon cDNA adaptor

Figure 6.6 Synthesis of Marathon cDNA.

Clontech synthesise the first strand of cDNA using the synthesis primer (A) and Moloney murine leukaemia virus reverse transcriptase. The second strand is made using a cocktail of *E. coli* DNA polymerase I, RNase H and *E. coli* DNA ligase. The double stranded cDNA is blunt ended with T4 DNA polymerase and the Marathon cDNA adapter (B) ligated onto both ends of the cDNA template using T4 DNA ligase (Clontech, 1997).

The amide blocking group attached to both 3' ends ensures that 5' overhang is not filled in by the polymerase; thus, when the strands are separated in the first round of PCR, each strand has only one copy of Adapter Primer 1 (AP1) region. Since the cDNA adapter does not have the AP1 complementary sequence, the AP1 primer alone is unable to anneal to the cDNA preventing non-specific amplification. When a gene specific primer (GSP) anneals to the cDNA and the strand complementary to the AP1 sequence is produced, the AP1 primer can anneal and PCR amplification can proceed.

Which end of the gene gets amplified is dependent on whether the GSP was designed to anneal to the first (anti-sense) or second strand (sense) cDNA strand; the former would amplify the 3' end of a gene, the latter works on the 5' end strand.

Figure 6.7 PCR amplification using NDR2 and NDR4 vs AP1 primers on Marathon cDNA.

Conditions (94°C, 5min, [94°C, 1min; 68°C, 4min]x30), 0.08 ng Marathon cDNA (2.5 µl), 0.2 µM AP1 primer, 0.2 µM NDR2/4 primer, 0.2 mM dNTP, 1xKlenTaq PCR buffer, 0.5 units Advantage KlenTaq polymerase in 25 µl reaction volume).

The NDR2/AP1 primer pair (lane 4) shows a major band at ~2.7 kbp (a), the NDR4/AP1 primer pair (lane 5) has a major band at ~2.9 kbp (b). These two bands were thought to be significant since they were about the correct size to encode the 5' end of the NTE cDNA. Secondly the size difference of the two bands was as predicted from the original design on the NDR primers (Figure 6.4).

Lane 6 shows the 1kbp band produced by the GAP-DH 5' RACE control primer (5'-TCCACCACCCTGTTGCTGTAG-3') supplied in the Marathon RACE ready cDNA kit.

<u>Lane</u>	<u>Primers</u>	<u>Lane</u>	<u>Primers</u>
1kb	Gibco 1kbp ladder	4	AP1 vs NDR2
1	AP1 alone	5	AP1 vs NDR4
2	NDR2 alone	6	AP1 vs GAP-DH
3	NDR4 alone	7	AP1 vs NDR4, no template blank

Figure 6.8 PCR with RSP vs NDR4 sizing the clones.

Conditions (94°C 5min, [94°C 1min, 68°C 4min]x25), 10 μ l boiled bacteria, 0.2 μ M RSP primer, 0.2 μ M NDR4 primer, 0.2 mM dNTP, 1xKlenTaq PCR buffer, 0.5 units Advantage KlenTaq polymerase in 25 μ l reaction volume).

The RSP primer (5'-GGAAACAGCTATGACCATG-3') is located 85bp from the *NotI* site used to clone the original PCR product, and pairs with the NDR4 primer to produce a product spanning the full insert length.

Colonies were picked using a 10 μ l pipette tip and the colony placed in 10 μ l of water; about 1 μ l of the water was then spotted out onto LB agar supplemented with 50 μ g/ml ampicillin. The bacterial suspension was heated to 100°C for 10 minutes then 15 μ l of PCR reaction mix was added to it.

After amplification the products were resolved on an agarose gel and clones of 2kbp or larger were identified and the corresponding colony was further cultured to produce sufficient DNA for sequencing. Twelve representative reactions out of 122 are shown in this figure.

NTE1

	1		50
a28		GTGGCGCCAC CATCGGTCCC GGAGTCCCAG TGATGCTCTG TGCCATAGAG	
d12			
	51		100
a28		CCCCATAAC TTCACTACTA CGTGATAGTA AATCCCCGGC AAAAACCAGC	
d12		CCAGC	
	101		150
a28		AGCGCCTTGC AAGCCCACGC CACCCCAAGC ATCCCAGGAC TCTTCTGAAA	
d12		AGCGCCTTGC AAGCCCACGC CACCCCAAGC ATCCCAGGAC TCTTCTGAAA	
	151		200
a28		CGACTCCGGG CTACCAGATC GGCCGTCCAG CTGGAATCAA CCGATGGAGG	
d12		CGACTCCGGG CTACCAGATC GGCCGTCCAG CTGGAATCAA CCGATGGAGG	

NTE2

	1		50
d41			
d43			
a27		GCCCGGGCAG GTCGAGTCAC GGGCTGGCTA CGAACTCCTC GGGGGCGAAG	
	51		100
d41		TT CCAGGACGTC CTGGCGCCCG GGGAAGGCTC	
d43		CGCCCG GGGAAGGCTC	
a27		GTGGCGGAGA GGGATGGGTT CCAGGACGTC CTGGCGCCCG GGGAAGGCTC	
	101		150
d41		GGCGGGACGG ATTTGCGGTG CGCAGCCAGT GCCGTTCGTC CCTCAGGTGC	
d43		GGCGGGACGG ATTTGCGGTG CGCAGCCAGT GCCGTTCGTC CCTCAGGTGC	
a27		GGCGGGACGG ATTTGCGGTG CGCAGCCAGT GCCGTTCGTC CCTCAGGTGC	
	151		200
d41		TTGGCGTGAT GATCGGGGCC GGAGTGGCGG TGGTGGTCAC GGCCGTGCTC	
d43		TTGGCGTGAT GATCGGGGCC GGAGTGGCGG TGGTGGTCAC GGCCGTGCTC	
a27		TTGGCGTGAT GATCGGGGCC GGAGTGGCGG TGGTGGTCAC GGCCGTGCTC	

Figure 6.9 5' end sequence from the NTE1 and NTE2 product groups.

Two clones match the NTE1 pattern and three clones match the NTE2 pattern; since the reverse transcription reaction has terminated at different points in the sequence, the clones are of different lengths and they must have been amplified from different templates and therefore independent clones.

	2320				2369
NTE1	CCACCTACTG	AGCCAGAAAA	TTCTAGGGAA	TTTGCAGCAG	CTGCAAGGAC
HOL4.2			..gcgGGGAA	TTTGCAGCAG	CTGCAAGGAC
	2370	↓ ↓↓			2419
NTE1	CCTTCCCAGC	AGGCTCTGGG	TTGGGTGTGC	CCCCACACTC	GGAACTCACC
HOL4.2	CCTTCCCAG.	..GCTCTGGG	TTGGGTGTGC	CCCCACACTC	GGAACTCACC
	2420				2469
NTE1	AACCCAGCCA	GCAACCTGGC	AACTGTGGCA	ATCCTGCCTG	TGTGTGCTGA
HOL4.2	AACCCAGCCA	GCAACCTGGC	AACTGTGGCA	ATCCTGCCTG	TGTgTGCTGA
	2470				2519
NTE1	GGTCCCCATG	GTGGCCTTCA	CGCTGGAGCT	GCAGCACGCC	CTGCAGGCCA
HOL4.2	gGTCCCCATG	GTGGCCTTCA	CGCTGGAGCT	GCAGCACGCC	CTGCAGGCCA
	2570				2619
NTE1	TCGGTCCGAC	GCTACTCCTT	AACAGTGACA	TCATCCGGGC	ACGCCTGGGG
HOL4.2	TCGGTCCGAC	GCTACTCCTT	AACAGTGACA	TCATCCGGGC	ACGCCTGGGG
	2620				2669
NTE1	GCCTCCGCAC	TGGATAGCAT	CCAAGAGTTC	CGGCTGTC..	
HOL4.2	GCCTCCGCAC	TGGATAgCAT	CCAAGAGTTC	CGGCTGTCAG	GGTGGCTGGC

Figure 6.10 Overlap between the NTE1 clone and HOL4.2 cDNA

The 3bp difference (arrowed) is probably a mutation in HOL4.2 sequence and as the deletion had no effect on the reading frame, the NTE1 sequence was used in the overlap zone.

	1				50
NTE1	AACAAAAGCT	GGAGTCCACC	GCGGTGGCGG	CCGCCCCGGG	AGGTGGCGCC
NTE1pep	.T..K..A..	G..V..H..R	..G..G..G.	.R..P..G..	R..W..R..H
NTE2					
NTE2pep					
	51				100
NTE1	ACCATCGGTC	CCGGAGTCCC	AGTGATGCTC	TGTGCCATAG	AGCCCCCATA
NTE1pep	..H..R..S.	.R..S..P..	S..D..A..L	..C..H..R.	.A..P..I..
NTE2					
NTE2pep					
	101				150
NTE1	ACTTCACTAC	TACGTGATAG	TAAATCCCCG	GCAAAAACCA	GCAGCGCCTT
NTE1pep	T..S..L..L	..R..D..S.	.K..S..P..	A..K..T..S	..S..A..L.
NTE2			..GCAGGTCG	AGTCACGGGC	TGGCTACGAA
NTE2pep			.X..R..S..	S..H..G..L	..A..T..N.
	151				200
NTE1	GCAAGCCCAC	GCCACCCCAA	GCATCCCAGG	ACTCTTCTGA	AACGACTCCG
NTE1pep	.Q..A..H..	A..T..P..S	..I..P..G.	.L..F..*..	N..D..S..G
NTE2	CTCCTCGGGG	GCGAAGGTGG	CGGAGAGGGA	TGGGTTCAG	GACGTCCTGG
NTE2pep	.S..S..G..	A..K..V..A	..E..R..D.	.G..F..Q..	D..V..L..A
	201			ACCATGG	250
NTE1	GGCTACCAGA	TCGGCCGTCC	AGCTGGAATC	AAC CGATGGA	GGCTCCGCTG
NTE1pep	..L..P..D.	.R..P..S..	S..W..N..Q	..P.. M ..E.	.A..P..L..
NTE2	CGCCCGGGGA	AGGCTCGGCG	GGACGGATTT	GCGGTGCGCA	GCCAGTGCCG
NTE2pep	..P..G..E.	.G..S..A..	G..R..I..C	..G..A..Q.	.P..V..P..
	251...ACCA	TGG.....	ACCATGG.....		300
NTE1	CAA ACTGGAA	TGGT GCTTGG	CGT GATG ATC	GGGGCCGAG	TGGCGGTGGT
NTE1pep	Q..T..G..M	..V..L..G.	.V..M..I..	G..A..G..V	..A..V..V.
NTE2	TTCGTCCCTC	AGGTGCTTGG	CGTGATGATC	GGGGCCGAG	TGGCGGTGGT
NTE2pep	F..V..P..Q	..V..L..G.	.V..M..I..	G..A..G..V	..A..V..V.
	301				350
NTE1	GGTCACGGCC	GTGCTCATCC	TCCTGGTGGT	GCGGAGGCTG	CGAGTGCCAA
NTE1pep	.V..T..A..	V..L..I..L	..L..V..V.	.R..R..L..	R..V..P..K
NTE2	GGTCACGGCC	GTGCTCATCC	TCCTGGTGGT	GCGGAGGCTG	CGAGTGCCAA
NTE2pep	.V..T..A..	V..L..I..L	..L..V..V.	.R..R..L..	R..V..P..K

Figure 6.11 Detection of the initiation codon of the NTE1 cDNA and alternate splice form NTE2 of the 5' end of the putative cDNA.

Reading frame 2 of the NTE1 clone gives an unbroken open reading frame starting from base 191, with the first methionine (starting at base 236) as the likely starting codon as it possesses the closest match to the optimum 'Initiator codon (ACCATGG; Kozak, 1986).

The NTE2 sequence diverges from NTE1 9 residues from the putative initiation codon and extends to the 5' end of NTE2 in a run of 37 residues unbroken by either initiation or stop codons, indicating NTE2 may represent a form of NTE substantially larger than that encoded by a28. It is unlikely that this is an artefact as the same sequence has been observed in six separate clones(Figure 6.9).

Chapter 7 Analysis of the NTE cDNA and deduced protein sequence

7.1 Introduction

Chapter 6 describes how the NTE cDNA was eventually cloned using EST data, 5' RACE and conventional hybridisation screening of a cDNA library and also how the open reading frame for NTE was located, this work has now been published in Lush *et al*, (1998). This chapter attempts to gather all the information relating to NTE (toxicology, biochemistry, protein sequence, peptide mapping and sequence similarities) and integrate it to produce a model of NTE on which future work can be based. Due to the inherent difficulty in predicting protein secondary structure from protein sequence and thus the consequent unreliability of these techniques when used in the absence of supporting data (Argos, 1989), the use of structural prediction programs has been kept to a minimum and, when used, the results treated with caution.

7.2 Results and Discussion

7.2.1 The NTE1 clones produced by the 5' RACE contain 11 out of 12 of the NTE peptide sequences.

The DNA sequence from the a28HOL4 contig was translated in all three reading frames. Reading frame +2 contained an ORF of 1342aa in size (Appendix 4) with the initiation codon located 16aa from the start of the ORF (Figure 6.11) to produce a predicted NTE protein sequence of 1327aa in length. Of the twelve pig NTE sequences (Figures 3.7 and 3.9), eleven found matches within the translated a28HOL4 cDNA sequence (NTE1; Figure 7.1). Only VILP failed to find a match within the sequence, the search was repeated with the available sequence from NTE2 but no further matches were found, indicating that

VILP was probably a contaminant sequence. The eleven pig NTE peptide sequences were widely dispersed throughout the translated human NTE clone from ADLT at residue 122 to FDQI at residue 1191. In addition antisera raised to a synthetic peptide of the final 13 C-terminal residues in Figure 7.1, reacted with pig NTE on Western blots (P. Glynn, personal communication). It was concluded that the a28HOL4 contig corresponded to one isoform of NTE.

The significance of the alternate splicing at the 5' end of NTE is unclear, the two forms seem to be expressed at roughly equal levels (two NTE1 sequences and only three NTE2 sequences were observed from a more or less random sample of NTE transcripts). Without an initiation codon for NTE2 it is possible that it is responsible for a much larger product (if NTE2 is a truncated cDNA) or much shorter (the next best Kozak sequence is located around base 1048 on the a28HOL4 sequence; see Appendix 4). Two approaches are applicable to determining the extent of alternate splicing in NTE: further 5' RACE using the 5' end sequence from NTE1 to amplify the missing sequence, or, a rather more ambitious approach would be to clone the entire genomic NTE sequence. This task could be approached by designing PCR primers spaced throughout the length of the NTE cDNA sequence and then used to amplify genomic DNA this could be sequenced and assembled to form the genomic clone. The promoter region and 5' end of NTE could be cloned by directed-inverse PCR, an inverse PCR library could be constructed using an enzyme known to cut the NTE cDNA near the 5' end, the inverse PCR primers could then be situated between the 5' end and the cut site thus they would amplify sequence upstream of the NTE gene. Once the genomic clone had been sequenced computer prediction of the exon-intron boundaries would allow permutations of splicing to be determined and then these predictions could be tested by PCR amplification of cDNA.

7.2.2 NTE shares a putative novel esterase domain with proteins from both eukaryotes and prokaryotes.

BLAST of searches SWISSPROT and EMBL using the deduced NTE peptide sequence have returned 11 protein sequences which have significant similarities with NTE; see Figure 7.2). With the exception of Swisscheese and YOL4, all are hypothetical proteins predicted to exist from genomic DNA sequence. The full database entry for each protein is reproduced in Appendix 2.

The common feature of all these proteins was a domain of about 200 amino acids, which, in NTE, was close to the C-terminal end (Figure 7.3). Interestingly, this region of NTE (residues 920 -1120) is precisely that in which the S9B-labelled serine was predicted to reside (See section 5.3). In fact, the data considered in section 5.3 indicate that the catalytic serine lies between NTE residues 955 and 1055; there are 10 serines in this region but particular attention is drawn to Ser966 which is located in the motif GX SXG, characteristic of many serine hydrolases (Brenner, 1988).

A multiple alignment of the regions similar to the NTE C-terminal from the other NTE like proteins was made using GCGs pileup program (Figure 7.4). Evolutionary theory predicts that regions of a protein that are important to its function are better conserved between species than non-critical regions and that the active site of a protein tends to be the best conserved between species as mutation to it is often deleterious to the protein's function (Brenner, 1988). With this in mind GCG program 'plotsimilarity' was used to plot the relative conservation throughout the multiple alignment (Figure 7.5).

On the graph the four regions had a high similarity score and correspond to NTE residues 929-945, 964-970, 1070-1075 and 1083-1087. Peak 964-970 is located on top of the highly conserved putative catalytic serine (Ser966). Given that Ser966 is the only serine located in a minimal serine hydrolase motif (GX SXG) and that the motif is conserved in all the sequences in the alignment,

Ser966 represents the best candidate for the catalytic serine in NTE. The peak at 929-945 is clearly caused by the near ubiquitous quartet of glycines and the surrounding conserved sequence. Six residues downstream (still within the zone of high conservation) lies a histidine (His945) which is conserved throughout all 11 sequences in the alignment. Since this was the only histidine so extensively conserved, it represents an excellent candidate to be involved in the catalytic triad. The third highest peak between 1083-1087, is located on a region that contains a 100% conserved aspartate residue in the alignment (Asp1086). Though there are other well conserved aspartates and glutamates in the alignment, Asp1086 is the only one that is flanked by well conserved sequence making it a good candidate to complete the triad.

7.2.3 Function of the N-terminal domain of NTE

Among all the proteins with any similarity to NTE only three have an extensive similarity with NTE beyond the 200 residue putative esterase domain: YMF9, sws and YOL4. YMF9 is the least like NTE as it is significantly longer (by 352 residues) and unlike YOL4 and sws; YMF9 has little similarity with the first 500 residues of NTE (see Figure 7.6) so has not been used in this analysis.

YOL4 and sws are better candidates as NTE homologues; both are known to be expressed in vivo (see Figure 7.2) and, as will be discussed later (see Section 7.2.5) mutations to sws produce a neurodegeneration in *Drosophila*. Therefore, assuming that NTE, YOL4 and sws are homologues which have similar function in all three species regions of similarity between them should delineate zones which are important to the function of the protein.

A gapped-BLAST search of SWISSPROT with NTE returns ambivalent results (gapped-BLAST is a modification of the BLAST algorithm which allows gaps to be introduced into both the database entry and query sequence permitting larger alignments between sequences; Altschul *et al*, 1997). Aside from strong matches with the putative esterase domain (significance 10^{-21} or

better), there are a number of weak matches with cAMP-regulatory subunits (cRS) which just border on the significant (10^{-7} or worse; see Appendix 3). In an attempt to gauge the relevance of the similarities between NTE and the cRS proteins a pair of dotplot graphs of NTE vs YOL4 and NTE vs sws were prepared using GCGs 'compare' and 'dotplot' programs (Figure 7.7). At the bottom of each of the plots the region of similarity between NTE and the 'KAP' proteins (a group of 17 cRS with the highest similarity scores with NTE on the gapped-BLAST search; see Appendix 3). This shows that the cRS similarities between residue 500 and 700 on NTE are located within a functionally significant region of the protein as the peptide sequence is conserved between the NTE homologues.

Direct evidence as to functional importance of this cRS-like domain is provided by Kretzschmar *et al* (1997) who characterised three of the swisscheese mutations (tabulated below).

Phenotype	Mutation	Days to 90% Mortality at 29°C
wildtype	na	18
sws ¹	Ser375 → stopcodon	9
sws ⁴	Gly648 → Arginine	7
sws ⁵	Gly965 → Aspartate	8

From a structural and mechanistic standpoint, there are four interesting points to these data: firstly, sws⁴ is a mutation at residue 648 which corresponds to part of the area of NTE similar to the KAP proteins, this would tend to confirm the presumption of importance for this section of sequence. Secondly sws⁵ is a Glycine to aspartate mutation and effectively adds a charge to the catalytic cleft in much the same way an aged OP does in NTE. Thirdly sws¹ eliminates 75% of the protein sequence but has a marginally less severe effect on mortality, relative to the other point mutations. Finally, despite the point mutations (sws⁴ and sws⁵) being well separated, they still have a similar gross effect on the insect: this could imply linkage between these two regions of the protein.

However, no definitive evidence exists and it is difficult to make any useful predictions about this N-terminal domain until more experimental evidence and/or better sequence similarity are available.

7.2.4 Computer analyses of NTE and YOL4 sequences indicate NTE is a membrane bound protein.

Transmembrane predictions were made for NTE by three programs: SAPS (Statistical Analysis of Protein Sequences; Brendel *et al*, 1992), TMpred (Hoffmann and Soffel, 1993) and SOSUI (Hirokawa *et al*, 1998). The results are summarised in Figure 7.8. The prediction of transmembrane (TM) domains depends on looking for runs of relatively hydrophobic residues, unfortunately it is not possible to discriminate between these segments and ones that occur in the interior of globular proteins (Creighton, 1993), thus TM prediction should always be treated with caution. The TM domain between residues 10-32 was detected by all three programs. The other TM domains predicted by TMpred have much lower scores and two are located near His945 and on Ser966 (Figure 7.1). In the past it has been noted that one of the common features of NTE inhibitors is that they tend to be hydrophobic with small leaving groups (Johnson, 1975b). This could suggest that the environment the catalytic triad is in is also hydrophobic and the hydrophobicity detected by TMpred is attributable to this rather than to any TM domains.

NTE is a membrane associated protein (Section 1.1.5) and the consensus prediction places a TM domain within 10 residues of the N-terminus, the PSORT package (Nakai and Kanehisa, 1992) detects no N-terminal cleavage signal suggesting that the TM domain is present *in vivo*. Combining the TM predictions and the inferred positions of the catalytic triad it is possible to construct a model of NTE which could be used as a basis for further investigation (Figure 7.9).

7.2.5 Mutation to the swisscheese (sws) gene can produce a neurodegeneration syndrome or a specific anosmia to aldehydes (olfE).

The swisscheese phenotype was first observed by Heisenberg and Böhl (1987) as part of a screening project for anatomical brain defects in *Drosophila*. Homozygous insects show reduced lifespan at elevated temperatures (29°C gives 90% mortality after 8.5 days in the average sws mutant, relative to an 18 day lifespan in the wild type). Affected individuals show formation of vacuoles in all regions of the brain; advancing age produces more vacuoles and also apoptotic cell death. Electron microscopy shows neurones enwrapped in multiple layers of membranes derived from nearby glia whereas, in the wild type, glia typically enwrap neurones in one or occasionally two processes (Kretzschmar *et al*, 1997).

Independent work by Ayyub *et al* (1990) on the genetics of olfactory behaviour resulted in the characterisation of five olfaction mutants (olfA, olfB, olfC, olfE and olfF) which are all recessive mutations which result in specific reduction (but not elimination) in response to aldehydes (olfA, B, E and F) or acetate esters (olfC). The interesting feature of these mutations is that olfE is produced by the same gene that is mutated in the swisscheese phenotype (Kretzschmar *et al*, 1997). The other olf genes have been mapped demonstrating that they are separate genes (for example; crossing olfA and olfB gives a near normal phenotype): this may imply that they are part of a signal transduction pathway since disruption to any one of the genes gives a similar phenotype.

Complementation studies of olfE and sws give conflicting results. Hasan showed that germ line transformation of olfE flies with a 14kbp stretch of genomic DNA containing the wildtype sws gene rescued the olfE phenotype (Hasan, 1990). However, a cross of the olfE/sws¹ strains show no signs of either the swisscheese or olfE phenotype (Kretzschmar *et al*, 1997). One possibility is that

the sws and olfE are separate genes; however, this seems unlikely as the region has been thoroughly searched for transcripts (Paterson and O'Hare, 1991, Kretzschmar *et al*, 1997). Alternatively it is not unknown for two mutations in a single gene to complement (certain combinations of para and smellblind mutations can complement even though they affect the same gene). Finally it is possible that the sws mutations affect only the 5.4kb transcript whereas the olfE mutations affect only the 1.7kb transcript (see Figure 7.2): there is a certain amount of evidence for this option since sequencing of the sws5.4 cDNA from one of the olfE insects failed to produce any mutations and it has been suggested that the olfE phenotype is a mutation affecting only the sws1.7 splice form (Kretzschmar *et al*, 1997).

7.2.6 The NTE gene is located on chromosome 19p13.3 near the mapped position of a recessive heritable ataxia.

The UniGene project is an international collaboration dedicated to genome mapping human EST sequences. The problem is approached using the 3' EST sequence to produce Sequence Tagged Site (STS) genetic markers, then map them relative to two Radiation Hybrid panels and a Yeast Artificial Chromosome library. As of October 1996 more than 16,000 genes have been localised by this method with a claimed reliability of 99% (Schuler *et al*, 1996). Over the past few years the STS has become a major genome mapping tool. Essentially an STS is a unique stretch of DNA sequence for which a diagnostic PCR reaction had been developed. By testing for the presence or absence of an STS in a panel of human genome fragments of previously mapped location, it is possible to assign a specific STS to a specific human genome fragment and thus determine its chromosomal location (Schuler *et al*, 1996).

A search of the UniGene database located ESTs from NTE between markers D19S216 and D19S413: this defines a region between 19.1 and 31.9 centiMorgans (cM) from the top of chromosome 19 in band p13.3. In physical terms this represents an interval of about 13Mbp (Davies and Read 1988);

however, it may well be a much smaller interval as Nystuen *et al* (1996) report that the region has an above average recombination rate. Other genes listed as being located in the D19S216-D19S413 interval include the human complement C3 precursor and, ironically, a human EST similar to the *Drosophila* glutactin precursor (Figure 1.9).

Once a chromosomal location was available, it was then possible to search the On-line Mendelian Inheritance in Man (OMIM, 1997) database for genetic diseases mapped to chromosome 19, looking for diseases which could plausibly be linked with defects in NTE. Of all the genes and diseases linked to chromosome 19, only cerebellar ataxia Cayman type (ATCAY; OMIM Entry Number: 601238; 30/4/1996) survived the most superficial inspection. ATCAY was identified by W. G. Johnson *et al.* (1978) in a highly inbred population on Grand Cayman Island, and is a rare (only 19 cases have been studied in Nystuen *et al.*, 1996) recessive disease characterised by marked psychomotor retardation and prominent nonprogressive cerebellar dysfunction including nystagmus, intention tremor, dysarthria, wide-based ataxic gait and hypotonia from early childhood. CT scans performed on two affected individuals showed a marked cerebellar hypoplasia (Nystuen *et al.*, 1996). ATCAY has been mapped by Nystuen *et al.* (1996) to chromosome 19p13.3 in a 9cM interval between markers D19S424 and GATA66B01. The most likely position for the disease is at or near marker D19S216, where the same allele was shared in all 38 chromosomes observed and generated a maximum lod score of 5.98 ($\theta=0$). The positions of ATCAY and NTE are summarised in Figure 7.10 indicating that it is possible that ATCAY is caused by a mutation to the NTE gene. However the relationship between ATCAY and NTE is tentative: the mutated gene could easily be the human homologue of *Drosophila* glutactin (see Section 1.1.6) which is also located in that interval.

To determine if ATCAY is caused by mutation to the NTE gene first it would be necessary to map the NTE gene to a much finer level than has been achieved by the UniGene project. This could be addressed by obtaining YACs

covering that area of chromosome 19 from the Human Genome Mapping Project resource centre, and determining by hybridisation which YACs NTE is located on, the YACs could be assembled into a contig by chromosome walking and the positions of D19S216, GATA66B01, and D19S413 could be determined by hybridisation. If NTE is located near D19S216 (and hence the ATCAY gene) it would be possible to collaborate with Nystuen *et al* (1996), assay NTE activity in affected individuals and ultimately clone and sequence the NTE cDNA (as NTE is expressed in blood lymphocytes, a relatively assessable source of RNA for use in cDNA synthesis and cloning) . Ultimately it seems unlikely that ATCAY mutation will yield critical information about NTE, however currently it would be the only human model of malfunction in NTE besides that caused by OP and may yield some insight.

7.3 Overview of predictions

The main findings of this chapter are that:-

- The cDNA clones discussed in Chapter 6 encode NTE and furthermore at least two alternately spliced versions of the NTE mRNAs exist.
- NTE is a novel serine esterase protein which has homologues in a wide range of species and the probable locations of the catalytic triad are His945, Ser966 and Asp1086.
- The esterase domain is apparently quite compact, possibly encoded by as few as 200 amino acids and similar sequences exist in a wide variety of organisms.
- NTE has one reasonably credible transmembrane domain located at the N-terminal.
- The *Drosophila* homologue of NTE (swisscheese) has a number of mutations which have more or less the same effect on survival and suggest close interactions between separate regions of the protein: all sws mutants show an age-dependant neurodegeneration.
- Mutations in NTE may cause a neurodegenerative disease; ESTs encoding NTE have been mapped to a region of chromosome 19p13.3 close to the positions of a recessive heritable ataxia.

These are a number of questions still unanswered:-

- What is the membrane topology of NTE and what organelles is it associated with?
- What are the biological functions of the various domains of the NTE protein?
- What is the biological role of NTE and what is its role in the development of OPIDP?

7.4 Future work

The first priority in continuing the research project into NTE is to fully sequence all the variants of NTE produced by the 5' RACE. Once this is complete, besides maintaining surveillance on the genetic databases with regular BLAST searches, the questions posed at the end of the previous section could be addressed thus.

7.4.1 What is the membrane topology of NTE and what organelles is it associated with?

Since NTE was cloned subsequent work has succeeded in transiently expressing catalytically active (D16) NTE in COS cells (Jane Atkins, personal communication). This work could provide the basis to test the structural predictions made in Figure 7.9 (ie that NTE has an N-terminal TM domain and almost all the protein is located on one side of the membrane). One method that has been used to study membrane topology was proteolytic digestion of microsomes which had been incubated in the presence of a *in vitro* transcription-translation system (Olender and Simoni, 1992). NTE expressed in such a system should be processed by the microsomes and, when the proteinase is added, the NTE should either be totally destroyed (if it has a cytosolic C-terminal) or left essentially untouched (if the N-terminal is cytosolic). If NTE has

more than one transmembrane domain a section of NTE should remain untouched as the cytosolic domains are digested by the proteinase.

For a detailed localisation of NTE to specific organelles the current generation of antisera raised to synthetic peptides is of limited use as their binding is sensitive to aldehyde fixation; this severely limits the microscopic resolution possible (though confocal microscopy is possible as this can be performed on unfixed or acetone-treated cells). However, a more robust polyclonal antisera could be raised to bacterially-expressed NTE domain to allow more extreme fixation procedures required for immunoelectron microscopy work, which would be able to resolve NTE's location and orientation in the cell.

7.4.2 What are the biological functions of the various domains of the NTE protein?

Recombinant forms of NTE offer one avenue to probing the functions of NTE; one useful modification would be a version of NTE starting at residue 34, omitting the transmembrane domain and incorporating a His tag to allow easy purification of the native enzyme. Assuming the single TM domain model is true, this should produce a more soluble form of NTE (presently NTE is soluble only in the presence of detergent; Davis and Richardson, 1987) which would be easier to study.

The suggestion that NTE binds cyclic nucleotides (Section 7.2.3) could then be tested by incubating purified NTE with radiolabelled cAMP or cGMP and assaying for binding. Should binding be detected it could be further localised by expressing portions of NTE in a suitable eukaryotic expression vector (ie one that has start and stop codons flanking the polycloning site as well as some form of purification system such as a His tag). This technology could be used to isolate and analyse the function of any part of NTE. Initially the main region of interest would be the 200 residue esterase domain (see Sections 7.2.2). If it could be

demonstrated that just the esterase domain was catalytically active then structural studies could commence using NMR and X-ray crystallography.

An 'esterase only' version of NTE could also be used to test the other prediction made in Figure 7.9, that the catalytic triad is composed of His945-Ser966-Asp1086 by mutating each of the three and determining the effect on catalysis, although the results would not be definitive as even if unrelated to the catalytic triad, mutation of these residues could have a severe effect on the protein as they are located in highly conserved regions. Only 3D structure (from NMR or X-ray crystallography) could ultimately resolve the residues comprising the catalytic triad.

The other question which could be addressed by protein expression is what other proteins does NTE interact with. There are two systems suitable for the task: phage display, in which an M13 phage cDNA library has been engineered to express the cDNAs as part of their coat protein; phage that do not bind immobilised NTE can be washed away, those that do bind can be eluted and propagated for further investigation (Dang *et al*, 1991). The other relevant system is the yeast two-hybrid system (Iwabuchi *et al*, 1993), in which part or all of the NTE protein would be fused with a DNA binding protein; interactions between NTE and a library of proteins activate the DNA binding protein (via an activator protein fused to the library proteins). The protein complex then activates a reporter gene, the activity of which is used to isolate possible positive interactions.

Of the two systems the yeast two hybrid seems the most likely to work as all the protein is expressed in a eukaryotic environment processing of the protein is more likely to be in the native form, additionally Clontech produce a mammalian version of the two-hybrid assay which would be useful if there were problems expressing NTE in a yeast host.

7.4.3 What is the biological role of NTE and what is its role in the development of OPIDP?

The NTE like genes in *Drosophila* (*sws*) may provide a useful model of OPIDP. When attempting to draw parallels between the swisscheese phenotype and OPIDP; the principal difference to consider (besides species) is that the *sws*⁵ mutation is effectively a constitutive ageing of the gene throughout the insect's development, whereas OPIDP is caused by a single transient event in an adult. To reconcile the two modes of action, two approaches are possible, production of an NTE knockout mouse would produce a constitutive loss of NTE activity more equivalent to *sws*¹. The other approach would be to collaborate with Kretzschmar *et al* (1997), in determining if *Drosophila sws* has any NTE like activities; such as binding of known neuropathic OPs (preferably ones not developed as insecticides). If a suitable OP can be found then it would be possible to start to dissect the role of *sws* in development by inhibiting *sws* (either transiently or constitutively) at various stages of the insect's life cycle to determine if there is any significant variation in the phenotype.

Similar work is possible with YOL4 although the background on it is less well developed (though Hope *et al*, (1996) have announced a '*C. elegans* expression pattern database' which may one day include YOL4 as part of the project involves reporter gene studies on the promoters of hypothetical proteins). Nevertheless, both *Drosophila* and *C. elegans* are easily genetically manipulated (due to their short lifecycle) and thus could be used to screen putative NTE binding proteins (produced by the yeast two hybrid system; Section 7.4.2) by knocking out the insect or worm homologue of the binding protein.

7.5 Conclusion

Over the duration of this project considerable advances have been made in the understanding of NTE. Not only has the cDNA been cloned, permitting experiments that directly address the initiation of OP induced-neuropathy at a molecular level, but also NTE has been established to be a novel serine esterase and with a homologue in *Drosophila* which has a key role in neuronal-glial cell interactions during development. With these advances it is possible to start to investigate the unknown events that still lie between ageing of NTE and neuropathy 1 to 3 weeks later. Or to put it another way, the smoking gun of OPIDP has been found, now all that remains to do is find out what the bullet looks like, where it went and who it hit.

```

1  MEAPLQTGMV LGVMIGAGVA VVVTAVLILL VVRRLRVPKT PAPDGPRYRF
51  RKRDKVLFYG RKIMRKVSQS TSSLVDTSVS ATSRPRMRKK LKMLNIAKKI
101 LRIQKETPTL QRKEPPPAVL EADLTEGDLA NSHLPSEVLY MLKNVRVLGH
      ADLTEGDLA NSHLPPEVL

151 FEKPLFLELC RHMVFQRLGQ GDYVFRPGQP DASIVVVDG LLELCLPGPD
201 GKECVVKEVV PGDSVNSLLS ILDVITGHQH PQRTVSARAA RDSTVLRLEPV
      VV PGDSVNSLLS ILDVITGXQA PQ

251 EAFSAVFTKY PESLVRVVQI IMVRLQRVTF LALHNYLGLT NELFSHEIQP
      IQP

301 LRLFPSPGLP TRTSPVRGSK RMVSTSATDE PRETPGRPPD PTGAPLPGPT
      LRLFPSPGLP ARTSPVR

351 GDPVKPTSLE TPSAPLLSRC VSMPGDISGL QGGPRSDFDL AYERGRISVS
401 LQEEASGGSL AAPARTPTQE PREQPAGACE YSYCEDESAT GGCPFGPYQG
451 RQTSSIFEAA KQELAKLMRI EDPSLLNSRV LLHHAKAGTI IARQGDQDVS
501 LHFVLWGCLH VYQRMIDKAE DVCLFVAQPG ELVGQLAVLT GEPLIFTLRA
551 QRDCTFLRIS KSDFYEIMRA QPSVVLAAH TVAARMSPFV ROMDFAIDWT
601 AVEAGRALYR QGDRSDCTYI VLNGRLRSVI QRGSGKKELV GEYGRGDLIG
651 VVEALTRQPR ATTVHAVRDT ELAKLPEGTL GHIKRRYPQV VTRLIHLLSQ
      LAKLPEGTL GHIWRRYPQV V

      ↓ start of HOL4.2 sequence
701 KILGNLQQLQ GPFPAAGSLG VPPHSELTNP ASNLATVAIL PVCAEVPMVA
      LTNP ASNLATVAVL

751 FTLELQHALQ AIGPTLLLNS DIIRARLGAS ALDSIQEFRL SGWLAQQEDA
801 HRIVLYQTDA SLTPWTVRCL RQADCILIVG LGDQEPTLQ LEQMLENTAV
851 RALKQLVLLH REEGAGPTRT VEWLNMRSWC SGHLHLRCPR RLFSRRSPAK
901 LHELYEKVFS RRADRHSDFS RLARVLGTNT IALVLGGGGA RGC SHIGVLK
951 ALEEAGVPVD LVGGTSIGSF IGALYAEERS ASRTKQRARE WAKSMTSVLE
      XGVPVA LVGP

1001 PVLDLTYPVT SMFTGSAFNR SIHRVFQDKQ IEDLWLPYFN VTTDITASAM
      DLGLPYFN VVTDI

1051 RVHKDGLWR YVRASMTLSG YLPPLCDPKD GHLMDGGYI NNLPADIARS
      D GHLTDGGYI NNLP

1101 MGAKTVIAID VGSQDETDL S TYGDSLSGWW LLWKRLNPWA DKVKVPDMAE
1151 IQSRLAYVSC VRQLEVVKSS SYCEYLRPPI DCFKTMDFGK FDQIYDVGYQ
      VVKSS SXDGXLRXXI DYFK FDQIYDVGYQ

1201 YGKAVFGGWS RGNVIEKMLT DRRSTDNLNES RRADVLAFFS SGFTDLAEIV
      YGK

1251 SRIEPPTSYV SDGCADGEES DCLTEYEEDA GPDCSRDEGG SPEGASPSTA
1301 SEMEEKSIL RQRRCLPQEP PGSATDA

```

Figure 7.1 Positions of the known pig NTE peptide sequences on the translated sequence of human NTE cDNA a28HOL4.

Identities between NTE peptides and translation are shown in bold. Transmembrane domains predicted by one program are shown single underlined, transmembrane domains predicted by three programs are shown double underlined (see Figure 7.8).

<u>Protein Name</u>	<u>Accession Number</u>	<u>Length (aa)</u>	<u>Origin</u>
YOL4_CAEEL	sw:Q02331	1351	<i>C. elegans</i>
Swisscheese	em: Z97187	1425	<i>Drosophila</i>
YCHK_ECOLI	sw:P37053	314	<i>E. coli</i>
YCHK_SHIFL	sw:P37054	200 (fragment)	<i>Shigella flexneri</i>
YLBK	em: Z98682	260	<i>Bacillus subtilis</i>
Mtcy20b11	tr:O05884	1048	<i>Mycobacterium tuberculosis</i>
Y0A3_MYCTU	sw:Q50733	583	<i>M. tuberculosis</i>
YVL7_CAEEL	sw:Q21534	880	<i>C. elegans</i>
YMF9_YEAST	sw:Q04958	1679	<i>Saccharomyces cerevisiae</i>
YK69_YEAST	sw:P36165	910	<i>S. cerevisiae</i>
Q12	pir:Q12043	749	<i>S. cerevisiae</i>

Figure 7.2 Summary of the hypothetical proteins with similarity to NTE.

Where possible the SWISSPROT name is used; the name is abbreviated in the main text by omitting species identifier. When the protein is not from SWISSPROT the most meaningful name was selected from the database entry (all of which are reproduced in full in Appendix 2).

Of these sequences two are known to be expressed in vivo: YOL4 has 17 matching *C. elegans* EST sequences registered in EMBL; swisscheese (sws) has been shown by Northern blotting to exist as 5.4kb and 1.7kb transcripts (sws5.4 and sws1.7). Sws5.4 has been cloned and sequenced and is expressed in *Drosophila* heads and bodies at all stages of development; in situ hybridisation shows, in brain, it is localised in neurons but not glia. Sws1.7 has not been cloned but is known to be derived from the 5' end of sws5.4 and is expressed weakly in *Drosophila* heads (Hasan, 1990 and Kretzschmar *et al*, 1997).

The eleven sequences listed above do not comprise an exhaustive list of all the known NTE-like sequences. BLAST searches of the pre-release DNA sequence data at TIGR (The Institute for Genomic Research, personal communication) shows that the genomes of *Neisseria meningitidis*, *Vibrio cholerae*, *Neisseria gonorrhoeae*, *Treponema pallidum*, *Deinococcus radioduran* and *Thermotoga maritima* all contain NTE homologies. All these organisms are important pathogens and it is just possible that one of the non-neuropathic NTE inhibitors could have an antibiotic effect on them.

Figure 7.3 Comparison of NTE like proteins.
The putative NTE esterase domain is marked in red.

Figure 7.4 Multiple alignment of the putative esterase domain of proteins in Figure 7.3.
The number at the right side of the alignment indicates the residue number for the appropriate sequence.

Figure 7.5 Similarity plot of data in Figure 7.4

The GCG program plotsimilarity was used to produce this graph. The program scores a 'window' of 10 residues assessing the overall similarity of all the columns of residues to determine a score based on the Dayhoff amino acid similarity matrix (Schwartz and Dayhoff, 1979). The score is plotted on a graph, the window is moved on one residue and the process is repeated. The higher the point on the graph, the better the conservation between sequences is in that region.

The graph represents the part of a multiple alignment in which all the sequences show significant similarity and has been renumbered to match the alignment shown in Figure 7.4. The dashed line represents the average peak height. The positions of the His, Ser and Asp are numbered relative to the start of the ungapped NTE sequence.

Figure 7.6 Dotplot of YMF9 (vertical) vs NTE (horizontal).

The red bar indicates the region of NTE sequence similar to the KAP group of proteins returned by the gapped-BLAST search. Window size 30 residues, stringency 17

Figure 7.7 Dotplot of NTE (horizontal) vs sws (A) and YOL4 (B).

The red bar indicates the region of NTE sequence similar to the KAP group of proteins returned by the gapped-BLAST search. The comparison was done using GCGs 'compare' program with a comparison window of 30 residues at a stringency of 17 identities.

<u>Residue No.</u>	<u>Predicted transmembrane region</u>	<u>Prediction program</u>
8 to 32	GMVLGVMIGAGVAVVVTAVLILLV	SAPS
10 to 32	VLGVMIGAGVAVVVTAVLILLV	SOSUI
11 to 32	LGVMIGAGVAVVVTAVLILLV	TMpred (score 2375)
734 to 753	LATVAILPVCAEVPMVAFTL	TMpred (score 958)
925 to 943	VLTGNTIALVLGGGGARGC	TMpred (score 1087)
956 to 976	GVPVDLVGGTSIGSFIGALYA	TMpred (score 1276)

Figure 7.8 Transmembrane predictions for NTE.

All three programs detect a possible transmembrane domain located close to the N-terminal of NTE, the other three hits registered by TMpred seem to be less convincing, as the score is lower (though still significant at >500) and the two highest are located near or over the putative catalytic domain (see Figure 7.1).

Figure 7.9 Putative secondary structure of NTE (not to scale).

The TM prediction suggests the transmembrane domain is at the N-terminus with a short span of protein sequence emerging from the membrane. To be functional the His, Ser and Asp residues of the catalytic triad must be in close proximity.

Figure 7.10 Cytogenetic map of Chromosome 19 and genetic map of the ATCAY/NTE location.

Appendix 1 EST based sequence of HOL4.2

This sequence alignment was produced from 23 human ESTs retrieved from the EMBL database these are denoted by a single letter followed by five digits. Each of these EST sequences represents a 'single pass' sequencing of the 5' end of a single EST clone. The corresponding 3' end sequences for the YOL4-like clones were separately assembled and combined into a single consensus sequence, the reversed and complemented 3' end sequence is included in the alignment as sequence 3prm. The 8b6a16 sequence represents the consensus sequence from three dye primer sequencing runs on the 5' end of the HOL4.2 insert.

The alignment was assembled by hand on GCGs 'lineup' sequence alignment editor. The relative positions of the ESTs was determined by converting the raw EMBL format datafiles into a mini database using the GCG 'toblast' program, then performing BLAST searches of that database using individual EST sequences. BLAST was able to detect any of overlap between the search sequence and any other EST. These data could then be assembled into the alignment below running from just beyond the LTNP sequence to the polyadenylation signal (shown in red) and poly-A tail.

The consensus sequence for the alignment (cons) was then translated in all three forward reading frames and the DNA sequence were also used to search SWISSPROT using BLAST. The correct reading frame for any given section of sequence was determined by comparison to the known NTE peptide sequences LTNP, XGVP, DLGL, DGHL, VVKS and FDQI. Regions in the alignment where these sequences match have an extra line 'nte-seq'. The YOL4 peptide sequence was also used to fix the reading frame where BLAST showed a significant similarity between the two sequences. For reasons of clarity these matches are not shown in this alignment.

Regions where the reading frame was fixed by comparison to homologous sequences are shown double underlined; regions where the sequence was merely inferred from the known reading frame are shown single underlined.

The first 66bp of H10469 and the first 19 bp of T10299 do not match the consensus, this may represent isoforms of NTE but it is more likely they represent immature mRNAs the regions of mismatch with the consensus have been blocked out with Ns.

This figure is based on the GCG .msf file format. However to save space, all the blank lines except the ones flanking the current sequence have been deleted.

List of EMBL/Genbank accession numbers used in alignment.

t09456	t66368
r87932	t10299
r89699	h58564
h05032	r51195
r56734	r15042
r13907	h61615
t23706	r51470
f12067	r45802
r25505	r54769
h09837	r48493
h10469	r47780
r17727	m78841

```

1
8b6a16 AATTTGCAGC AGCTGCAAGG ACCCTTCCCA GGCTCTGGGT TGGGTGTGCC
t09456 .....
cons AATTTGCAGC AGCTGCAAGG ACCCTTCCCA GGCTCTGGGT TGGGTGTGCC
frame1 ..F..A..A. .A..A..R.. T..L..P..R ..L..W..V. .G..C..A..
frame2 N..L..Q..Q ..L..Q..G. .P..F..P.. G..S..G..L ..G..V..P.
frame3 .I..C..S.. S..C..K..D ..P..S..Q. .A..L..G.. W..V..C..P

51
8b6a16 CCCCACTCG GAACTCACCA ACCCAGCCAG CAACCTGGCA ACTGTGGCAA
t09456 .....
cons CCCCACTCG GAACTCACCA ACCCAGCCAG CAACCTGGCA ACTGTGGCAA
frame1 P..T..L..G ..T..H..Q. .P..S..Q.. Q..P..G..N ..C..G..N.
frame2 .P..H..S.. E..L..T..N ..P..A..S. .N..L..A.. T..V..A..I
frame3 ..H..T..R. .N..S..P.. T..Q..P..A ..T..W..Q. .L..W..Q..
NTE-pep ..... ..L..T..N ..P..A..S. .N..L..A.. T..V..A..V

101
8b6a16 TCCTGCCTGT GTGTGCTGAG GTCCCCATGG TGGCCTTCAC GCTGGAGCTG
t09456 .....CTGAG GTCCCCATGG TGGCCTTCAC GCTGGAGCTG
r87932 .....
cons TCCTGCCTGT GTGTGCTGAG GTCCCCATGG TGGCCTTCAC GCTGGAGCTG
frame1 .P..A..C.. V..C..*..G ..P..H..G. .G..L..H.. A..G..A..A
frame2 ..L..P..V. .C..A..E.. V..P..M..V ..A..F..T. .L..E..L..
frame3 S..C..L..C ..V..L..R. .S..P..W.. W..P..S..R ..W..S..C.
NTE-pep ..L.....

151
8b6a16 CAGCACGCCC TGCAGGCCAT CGGTCCGACG CTA CTCTCTTA ACAGTGACAT
t09456 CAGCACGCCC TGCAGGCCAT CGGTCCGACG CTA CTCTCTTA ACAGTGACAT
r87932 .....
cons CAGCACGCCC TGCAGGCCAT CGGTCCGACG CTA CTCTCTTA ACAGTGACAT
frame1 ..A..R..P. .A..G..H.. R..S..D..A ..T..P..*.. Q..*..H..
frame2 Q..H..A..L ..Q..A..I. .G..P..T.. L..L..L..N ..S..D..I.
frame3 .S..T..P.. C..R..P..S ..V..R..R. .Y..S..L.. T..V..T..S

201
8b6a16 CATCCGGGCA CGCCTGGGGG CCTCCGCACT GGATAGCATC CAAGAGTTCC
t09456 CATCCGGGCA CGCCTGGGGG CCTCCGCACT GGATAGCATC CAAGAGTTCC
r87932 .....NATAGCATC CAAGAGTTCC
r89699 .....GTTCC
h05032 .....
cons CATCCGGGCA CGCCTGGGGG CCTCCGCACT GGATAGCATC CAAGAGTTCC
frame1 H..P..G..T ..P..G..G. .L..R..T.. G..*..H..P ..R..V..P.
frame2 .I..R..A.. R..L..G..A ..S..A..L. .D..S..I.. Q..E..F..R
frame3 ..S..G..H. .A..W..G.. P..P..H..W ..I..A..S. .K..S..S..

251
8b6a16 GGCTGTCAGG GTGGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC
t09456 GGCTGTCAGG GTGGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC
r87932 GGCTGTCAGG GTGGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC
r89699 GGCTGTCAGG GTNGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC
h05032 .....
cons GGCTGTCAGG GTGGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC
frame1 .A..V..R.. V..A..G..P ..A..G..G. .C..T..P.. Y..R..T..L
frame2 ..L..S..G. .W..L..A.. Q..Q..E..D ..A..H..R. .I..V..L..
frame3 G..C..Q..G ..G..W..P. .S..R..R.. M..H..T..V ..S..Y..S.

```

```

301                                     350
8b6a16 TACCAGACGG ACGCCTCGCT GACGCCCTGG ACCGTGCGCT GCCTGCGACA
t09456 TACCAGACGG ACGCCTCGCT GACGCCCTGG ACCGTGCGCT GCCTGCNACA
r87932 TACCAGACGG ACGCCTCGCT GACGCCCTGG ACCGTGCGCT GCCTGCGACA
r89699 TACCAGACGG ACGCCTCGCT GACGCCCTGG ACCGTGCGCT GCCTGCGACA
h05032 .....
cons TACCAGACGG ACGCCTCGCT GACGCCCTGG ACCGTGCGCT GCCTGCGACA
frame1 ..P..D..G. .R..L..A.. D..A..L..D ..R..A..L. .P..A..T..
frame2 Y..Q..T..D ..A..S..L. .T..P..W.. T..V..R..C ..L..R..Q.
frame3 .T..R..R.. T..P..R..* ..R..P..G. .P..C..A.. A..C..D..R

351                                     400
8b6a16 G.CCGACTGC ATCCTCATTG TGGGCCTGGG GGACCAGGAG CCTACCCTCG
t09456 GGCCGACTGC ATCCTCATTG TGGGCCTGGG GGACCAGGAG CCTACCCTCG
r87932 GGCCGACTGC ATCCTCATTG TGGGCCTGGG GGACAGGAGG CCTACCCTCG
r89699 GGCCGACTGC ATCCTCATTG TGGGCCTGGG GGACCAGGAG CCT.....
h05032 .....
cons GGCCGACTGC ATCCTCATTG TGGGCCTGGG GGACCAGGAG CCTACCCTCG
frame1 G..R..L..H ..P..H..C. .G..P..G.. G..P..G..A ..Y..P..R.
frame2 .A..D..C.. I..L..I..V ..G..L..G. .D..Q..E.. P..T..L..G
frame3 ..P..T..A. .S..S..L.. W..A..W..G ..T..R..S. .L..P..S..

401                                     450
8b6a16 GCCANCTGGA GCAGATGCTG GAGAACACGG CTGTGCGCGC CCTTAAGCAG
t09456 GCCAGCTGGA GCAGATGCTG GAGAACACGG CTNTTCGCGC CCTTAAGCAG
r87932 GCCAGCTGGA GCAGATGCTG GAGAACACGG CTGTGCGCGC CCTTAAGCAG
h05032 .....
cons GCCAGCTGGA GCAGATGCTG GAGAACACGG CTGTGCGCGC CCTTAAGCAG
frame1 .P..A..G.. A..D..A..G ..E..H..G. .C..A..R.. P..*..A..A
frame2 ..Q..L..E. .Q..M..L.. E..N..T..A ..V..R..A. .L..K..Q..
frame3 A..S..W..S ..R..C..W. .R..T..R.. L..C..A..P ..L..S..S.

451                                     500
8b6a16 CT.GTCCTGC TCCACCGAGA GGANGGCGCG GGCCCC....
t09456 CTAGTCCTTC TTCACCGAGA GGAAGGGCGC GGG.....
r87932 CTAGTCCTGC TCCACCGAGA GGAGGGCGCG GGCCCACGNA CTCGTTGGGA
h05032 .....
cons CTAGTCCTGC TCCACCGAGA GGANGGCGCG GGCCCNCGNA CTCGTTGGGA
frame1 ..S..P..A. .P..P..R.. G..X..R..G ..P..X..X. .S..L..G..
frame2 L..V..L..L ..H..R..E. .X..G..A.. G..P..R..T ..R..W..E.
frame3 .*..S..C.. S..T..E..R ..X..A..R. .A..X..X.. L..V..G..S

501                                     550
t09456 .....
r87932 GTGGCTAAAT ATGCGCATGG GTGCTCGGGG CACTGCACCT .....
r89699 .....
cons GTGGCTAAAT ATGCGCATGG GTGCTCGGGG CACTGCACCT NNNNNNNNNN
frame1 V..A..K..Y ..A..H..G. .C..S..G.. H..C..T..X ..X..X..X.
frame2 .W..L..N.. M..R..M..G ..A..R..G. .T..A..P.. X..X..X..X
frame3 ..G..*..I. .C..A..W.. V..L..G..A ..L..H..L. .X..X..X..

```

	551				600
r89699					
h05032				GGC	ACTGCACCTG
r56734				.GCTCGGGGC	ACTGCACCTG
r13907				.GCTCGGGGC	ACTGCACCTG
t23706					
cons	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NGCTCGGGGC	ACTGCACCTG
frame1	.X..X..X..	X..X..X..X	..X..X..X.	.A..R..G..	T..A..P..A
frame2	..X..X..X.	.X..X..X..	X..X..X..X	..L..G..A.	..L..H..L..
frame3	X..X..X..X	..X..X..X.	.X..X..X..	X..S..G..H	..C..T..C.
	601				650
r89699					
h05032	CGCTGTCCGC	GCGCCTNTTT	TCGCGCCGCA	GCCCTGCCAA	GCTGCATGAG
r56734	CGCTGTCCGC	GCGCCTCTTT	TCGCGCCGCA	GCCCTGNCAA	GCTGCATGAG
r13907	CGCTGTCCGC	GCGCCTCTTT	TCGCGCCGCA	GCCCTGCCAA	GCTGCATGAG
t23706					
f12067					
r25505			..CGCGCCGC	AGCCCTGCCA	AGCTGCATGA
h09837					CATGA
h10469				NNNNNN	NNNNNNNNNN
r17727					
cons	CGCTGTCCGC	GCGCCTCTTT	TCGCGCCGCA	GCCCTGNCAA	GCTGCATGAG
frame1	..L..S..A.	.R..L..F..	S..R..R..S	..P..X..K.	..L..H..E..
frame2	R..C..P..R	..A..S..F.	.R..A..A..	A..L..X..S	..C..M..S.
frame3	.A..V..R..	A..P..L..F	..A..P..Q.	.P..X..Q..	A..A..*..A
	651				700
r89699					
h05032	CTCTACGAGA	AGGTTTTNTC	CAGGCGNGCG	GACCGGCACA	GCGACTTCTC
r56734	CTCTACGAGA	AGGTTTTCTC	CAGGCGCGCG	GACCGGCACA	GCGACTTCTC
r13907	CTCTACGAGA	AGGTTTTCTC	CAGGCGCGCG	GACCGGCACA	GCGACTTCTC
t23706	AA	GCTTGGCACG	AGGGGCGCGC	GGACCGGCAC	AGCGACTTCT
f12067			GCGCGC	GGACCGGCAC	AGCGACTTCT
r25505	GCTCTACGAG	AAGGTTTTCT	CCAGGCGCGC	GGACCGGCAC	AGCGACTTCT
h09837	GCTCTACGAG	AAGGTTTTCT	CCAGGCGCGC	GGACCGGCAC	AGCGACTTCT
h10469	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN
r17727			GCGCGC	GGACCGGCAC	AGCGACTTCT
t66368					
cons	CTCTACGANA	AGGTTTTNNC	CAGGGCGCGC	GGACCGGCAC	AGCGACTTCT
frame1	L..Y..X..K	..V..X..X.	.R..A..R..	G..P..A..Q	..R..L..L.
frame2	.S..T..X..	R..F..X..P	..G..R..A.	.D..R..H..	S..D..F..S
frame3	..L..R..X.	.G..F..X..	Q..G..A..R	..T..G..T.	.A..T..S..

	701		750
r89699			
h05032	CC.....		
r56734	CCGCTTGGA	GGGTGCTCAC	GGGGAACACC ATTGCCCTTG TGCTAGGNGG
r13907	CCGCTTGGA	GGGTGCTCAC	GGGGAACACC ATTGCCCTTG TGCTAGGCGG
t23706	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGCG
f12067	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGCG
r25505	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGCG
h09837	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTTGGCG
h10469	CCCGCTTGCG	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGGG
r17727	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGNT
t66368		TGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGNG
t10299			
cons	CCCGCTTGGC	AGGGTGCTCA	CGGGGAACAC CATTGCCCTT GTGCTAGGCG
frame1	<u>.P..L..G..</u>	<u>R..V..L..T</u>	<u>..G..N..T..I..A..L..V..L..G..G</u>
frame2	<u>..R..L..A..</u>	<u>.G..C..S..</u>	<u>R..G..T..P..L..P..L..C..*..A..</u>
frame3	<u>P..A..W..Q</u>	<u>..G..A..H..</u>	<u>.G..E..H..H..C..P..C..A..R..R..</u>
	751		800
h05032			
r56734	GGGCGGGGNC	AGGGNTGCCT	CGCACATCGG AGTACTAAAG GCATTAGANG
r13907	GGGC.....		
t23706	GGGGCGGGGC	CAGGGGCTGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
f12067	GGGGCAGGGG	CTGC.....	TCGCACATCG GAGTACTAAA GGCATTAGAG
r25505	GGGGCGGGGC	CAGGGGCTGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
h09837	AGGGCGGGGC	CAGGG..TGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
h10469	GGGGCCGGGC	CAGGGGCTGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
r17727	GGGGCGGGG.	CAGGGC.TC.	TCGCACATCG GAGTACTAAA GGCATTAGAG
t66368	GGGNC.GGGG	CAGGGCGTGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
t10299			
cons	GGGGCGGGGC	CAGGGGCTGC	TCGCACATCG GAGTACTAAA GGCATTAGAG
frame1	<u>..G..G..A..</u>	<u>.R..G..C..</u>	<u>S..H..I..G..V..L..K..A..L..E..</u>
frame2	<u>G..A..G..P</u>	<u>..G..A..A..</u>	<u>R..T..S..E..Y..*..R..H..*..R..</u>
frame3	<u>.G..R..G..</u>	<u>Q..G..L..L</u>	<u>..A..H..R..S..T..K..G..I..R..G</u>
	801		850
h05032			
r56734	AGGCGGGGGT	NCCC..TNGA	CTNGTGGGCG GCACGTNCAT TGGCTCTNTC
r13907			
t23706	GAGGCGGGGG	TCCCCGTGGA	CCTGGTGGGC GGTACGTCCA TTGGCTCTTT
f12067	GAGGCGGGGG	TCCCCGTGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
r25505	GAGGCGGNNG	TCCCCGTGGA	CCTGGTGG.C GGCACGTCCA TTAGCTCTTT
h09837	GAGGCGGGGG	TCCCCGTGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
h10469	GAGGCGGGGG	TCCCC.TGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
r17727	GAGGCGGGGN	TCCCCGTGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
t66368	GAGGCGGGGT	TCCCCGTGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
t10299			NN
h58564			
cons	GAGGCGGGGG	TCCCCGTGGA	CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
frame1	<u>E..A..G..V</u>	<u>..P..V..D..</u>	<u>.L..V..G..G..T..S..I..G..S..F..</u>
frame2	<u>.R..R..G..</u>	<u>S..P..W..T</u>	<u>..W..W..A..A..R..P..L..A..L..S</u>
frame3	<u>..G..G..G..</u>	<u>.P..R..G..</u>	<u>P..G..G..R..H..V..H..W..L..F..</u>
NTE-pep	<u>...A..G..V</u>	<u>..P..V..A..</u>	<u>.L..V..G..P.....</u>

	851		900
h05032			
r56734	ATCGGAGCGT	TGTACGGTAG	G.....
r13907			
t23706	CATCGGAGCG	TTGTACGCGA	GGAG.....
f12067	CATCGGAGCG	TTGTACGCGG	AGAGCGCACC
r25505	CATCGGAGCG	TTGTACGCGA	GGAGCGCACT
h09837	CATCGGAGCG	TTGTACGCGA	GGAGCGCAGC
h10469	CATCGGAGCG	TTGTACGCGA	GGAGCGCAGC
r17727	CATCGGAGCG	TTGTACGGGA	GGAGCGCA.C
t66368	CATCGGAGCG	TTGTACGGNA	GGAGCGCAGG
t10299	NNNNNNNNNN	NNNNNNNGGA	GGAGCGCAGC
h58564			
cons	CATCGGAGCG	TTGTACGCGA	GGAGCGCAGC
frame1	<u>.I..G..A..</u>	<u>L..Y..A..R</u>	<u>..S..A..A.</u>
frame2	<u>..S..E..R.</u>	<u>.C..T..R..</u>	<u>G..A..Q..P</u>
frame3	<u>H..R..S..V</u>	<u>..V..R..E.</u>	<u>.E..R..S..</u>
	901		950
f12067	G.....		
r25505	GCCCCGGAGT	TGGCAAGAGC	ATGACTTCGG
h09837	GCCCCGGAGT	GGGCAAGAGC	ATGACTTCGG
h10469	GCCCCGGAGT	TGGCAAGAGC	ATGACTTCGG
r17727	GCCCCGGAGT	GGGCAAGAGC	ATGACTTCGG
t66368	GCCCCGGAGT	GGGCAAGAGC	ATGACTTCGG
t10299	GCCCCGGAGT	GGGCAAGAGC	ATGACTTCGG
h58564			
cons	GCCCCGGAGT	GGGCAAGAGC	ATGACTTCGG
frame1	<u>..P..G..V.</u>	<u>.G..K..S..</u>	<u>M..T..S..V</u>
frame2	<u>A..R..E..W</u>	<u>..A..R..A.</u>	<u>.*..L..R..</u>
frame3	<u>.P..G..S..</u>	<u>G..Q..E..H</u>	<u>..D..F..G.</u>
	951		1000
f12067			
r25505	CTCACGTACC	CAGTNAACTT	CATGTT....
h09837	CTCACGTACC	CAGTCACCTC	CATGTTCACT
h10469	CTCACGTACC	CAGTCACCTN	CATGTTCACT
r17727	CTCACGTACC	CAGTCACCTC	CATGTTCACT
t66368	CTCACGTACC	CAGTCACCTC	CATGTTCACT
t10299	CTCACGTACC	CAGTCACCTC	CATGTTCACT
h58564			
cons	CTCACGTACC	CAGTCACCTC	CATGTTCACT
frame1	<u>L..T..Y..P</u>	<u>..V..T..S.</u>	<u>.M..F..T..</u>
frame2	<u>.S..R..T..</u>	<u>Q..S..P..P</u>	<u>..C..S..L.</u>
frame3	<u>..H..V..P.</u>	<u>.S..H..L..</u>	<u>H..V..H..W</u>

	1001		1050
r25505			
h09837	CATCCATCGG	GT.....	
h10469	CATCCATCGG	GTCTTTCAGG	NTAAGCAGAT TNAGGAC.TG TTGCTGC.TT
r17727	CATCCATCGG	GTCTTCCAGG	ATAAGCAGAT TGAGGACCTG TGGCTGCCTT
t66368	CATCCATCGG	GTCTTTC..	
t10299	CATCCATCGG	GTCTTCCAGG	ATAAGCAGAT TGAGGACCTG TGGCTGCCTT
h58564		AGG	ATAAGCAGAT TGAGGACCTG TGGCTGCCTT
r51195			AT TGAGGACCTG TGGCTGCCTT
r15042			
cons	CATCCATCGG	GTCTTNCAGG	ATAAGCAGAT TGAGGACCTG TGGCTGCCTT
frame1	<u>.I..H..R..</u>	<u>V..X..O..D</u>	<u>..K..Q..I..</u> <u>.E..D..L..</u> <u>W..L..P..Y</u>
frame2	<u>..S..I..G.</u>	<u>.S..X..R..</u>	<u>I..S..R..L</u> <u>..R..T..C.</u> <u>.G..C..L..</u>
frame3	<u>H..P..S..G</u>	<u>..L..X..G.</u>	<u>.*..A..D..</u> <u>*..G..P..V</u> <u>..A..A..L.</u>
NTE-pep			D..L.. G..L..P..Y
	1051		1100
h09837			
h10469	ACTTAAACGT	.ANACACAGAT	A.....
r17727	ACTTAAACGT	GACCACAGAT	ATTAA.....
t66368			
t10299	ACTTCAACGT	GACCACAGAT	ATCACCGCCT CAGCCATGCG AGTCCACAAA
h58564	ACTTCAACGT	GACCACAGAT	ATCACCGCCT CAGCCATGCG AGTCCACAAA
r51195	ACTTCAACGT	GACCACAGAT	ATCACCGCCT CAGCCATGCG AGTCCACAAA
r15042			ACAAA
h61615			GCG AGTCCACAAA
r51470			
cons	ACTTCAACGT	GACCACAGAT	ATCACCGCCT CAGCCATGCG AGTCCACAAA
frame1	<u>..F..N..V.</u>	<u>.T..T..D..</u>	<u>I..T..A..S</u> <u>..A..M..R.</u> <u>.V..H..K..</u>
frame2	<u>T..S..T..*</u>	<u>..P..Q..I.</u>	<u>.S..P..P..</u> <u>Q..P..C..E</u> <u>..S..T..K.</u>
frame3	<u>.L..Q..R..</u>	<u>D..H..R..Y</u>	<u>..H..R..L.</u> <u>.S..H..A..</u> <u>S..P..Q..R</u>
NTE-pep	<u>..F..N..V.</u>	<u>.V..T..D..</u>	<u>I.....</u> <u>.....</u> <u>.....</u>
	1101		1150
t66368			
t10299	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC AGCATGACGC TGTCGGGCTA
h58564	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC ANATG.ACGC TGTCGGGCTA
r51195	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC ANATGGACGC TGTCGGGCTA
r15042	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC ANATNGACGC TGTCGGGCTA
h61615	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC ACATGGACGC TGTCGGGCTA
r51470			
cons	GATGGCTCCC	TGTGGCGGTA	CGTGCGCGCC ANATGGACGC TGTCGGGCTA
frame1	<u>D..G..S..L</u>	<u>..W..R..Y.</u>	<u>.V..R..A..</u> <u>X..W..T..L</u> <u>..S..G..Y.</u>
frame2	<u>.M..A..P..</u>	<u>C..G..G..T</u>	<u>..C..A..P.</u> <u>.X..G..R..</u> <u>C..R..A..T</u>
frame3	<u>..W..L..P.</u>	<u>.V..A..V..</u>	<u>R..A..R..X</u> <u>..M..D..A.</u> <u>.V..G..L..</u>
	1151		1200
t66368			
t10299	CCTGCCCC.G	CTGTGCGACC	CAAGGACGGG CACCTACTCA TGGATG....
h58564	CCTGCCCCCG	CTGTG.ACCC	CAAGGACGGG CACCTACTCA TGGATGGCGG
r51195	CCTGCCCCCG	CTGTGNACCC	CAAGGACGGG CACCTACTCA TGGATGGCGG
r15042	CCTGCCCCCG	CTGTGNACCC	CAAGGACGGG CACCTACTCA TGGATGGCGG
h61615	CCTGCCCCCG	CTGTGACCCC	CAAGGACGGG CACCTACTCA TGGATGGCGG
r51470			
cons	CCTGCCCCCG	CTGTGNACCC	CAAGGACGGG CACCTACTCA TGGATGGCGG
frame1	<u>.L..P..P..</u>	<u>L..X..T..P</u>	<u>..R..T..G.</u> <u>.T..Y..S..</u> <u>W..M..A..A</u>
frame2	<u>..C..P..R.</u>	<u>.C..X..P..</u>	<u>Q..G..R..A</u> <u>..P..T..H.</u> <u>.G..W..R..</u>
frame3	<u>P..A..P..A</u>	<u>..V..X..P.</u>	<u>.K..D..G..</u> <u>H..L..L..M</u> <u>..D..G..G.</u>
NTE-pep			D..G.. H..L..L..T ..D..G..G.

	1201		1250
t10299			
h58564	CTACATCAAC	AATCTGCCAG	CGGACATCGC CCGCAGCATG GGTGCCAAAA
r51195	CTACATCAAC	AATCTGCCAG	CGGACATCGC CCGCAGCATG GGTGCCAAAA
r15042	CTACATCAAC	AATCTGCCAG	CGGACATCGC CCGCAGCATG GGTGCCAAAA
h61615	CTACATCAAC	AATCTGCCAG	CGGACATCGC CCGCAGCATG GGTGCCAAAA
r51470			
cons	CTACATCAAC	AATCTGCCAG	CGGACATCGC CCGCAGCATG GGTGCCAAAA
frame1	..T..S..T.	.I..C..Q..	R..T..S..P ..A..A..W. .V..P..K..
frame2	L..H..Q..Q	..S..A..S.	.G..H..R.. P..Q..H..G ..C..Q..N.
frame3	<u>.Y..I..N..</u>	<u>N..L..P..A</u>	<u>..D..I..A. .R..S..M.. G..A..K..T</u>
NTE-pep	<u>.Y..I..N..</u>	<u>N..L..P...</u>	
	1251		1300
t10299			
h58564	CGGTCATCGC	CATTGACGTG	GGGAGCCAGG ATGAGACGGA CCTCAGCACC
r51195	CGGTCATCGC	CATTGACGTG	GGGAGCCAGG ATGAGACGGA CCTCAGCACC
r15042	CGGTCATCGC	CATTGACGTG	GGGAGCCAGG ATGAGACGGA CCTCAGCACC
h61615	CGGTCATCGC	CATTGACGTG	GGGAGCCAGG ATGAGACGGA CCTCAGCACC
r51470			..TGAGACGGA CCTCAGCACC
r45802			
cons	CGGTCATCGC	CATTGACGTG	GGGAGCCAGG ATGAGACGGA CCTCAGCACC
frame1	R..S..S..P	..L..T..W.	.G..A..R.. M..R..R..T ..S..A..P.
frame2	.G..H..R..	H..*..R..G	..E..P..G. .*..D..G.. P..Q..H..L
frame3	<u>..V..I..A.</u>	<u>.I..D..V..</u>	<u>G..S..Q..D ..E..T..D. .L..S..T..</u>
	1301		1350
t10299			
h58564	TACGGGGACA	GCC.....	
r51195	TACGGGGACA	GCCTGTTCGG	CTGGTGGCTG CTGTGGAAGC GG.....
r15042	TACGGGGACA	GCCTGTTCGG	CTGGTGGCTG CTGTGGAAGC GGCTGAATCC
h61615	TACGGGGACA	GCCTGTTCGG	CTGGTGGCTG CTGTGGAAGC GGCTGAATCC
r51470	TACGGGGACA	GCCTGTTCGG	CTGGTGGCTG CTGTGGAAGC GGCTGAATCC
r45802			
cons	TACGGGGACA	GCCTGTTCGG	CTGGTGGCTG CTGTGGAAGC GGCTGAATCC
frame1	.T..G..T..	A..C..P..A	..G..G..C. .C..G..S.. G..*..I..P
frame2	..R..G..Q.	.P..V..R..	L..V..A..A ..V..E..A. .A..E..S..
frame3	<u>Y..G..D..S</u>	<u>..L..S..G.</u>	<u>.W..W..L.. L..W..K..R ..L..N..P.</u>
	1351		1400
r51195			
r15042	CTGGGCTGAC	AAGGTAAAGG	TTCCAGACAT GGGTGAAATC CAGTTCCCGC
h61615	CTGGGCTGAC	AAGGTAAAGG	TTCCAGACAT GGCTGAAATC CAGTNCCGCC
r51470	CTGGGCTGAC	AAGGTAAAGG	NTNCAGACAT AGCTGAAATT CAGTTCCGC.
r45802			
cons	CTGGGCTGAC	AAGGTAAAGG	TTCCAGACAT GGCTGAAATC CAGTTCCGCC
frame1	..G..L..T.	.R..*..R..	F..Q..T..W ..L..K..S. .S..S..A..
frame2	L..G..*..Q	..G..K..G.	.S..R..H.. G..*..N..P ..V..P..P.
frame3	<u>.W..A..D..</u>	<u>K..V..K..V</u>	<u>..P..D..M. .A..E..I.. Q..F..R..L</u>

	1401		1450
r51195			
r15042	TGGCCTACGT	GTCCTG....	
h61615	TGGCCTACGT	GTCCTGTGTT	GCGGCATNAG AAGGTTGTCA AGTCCAGTTC
r51470			
r45802			
r54769			..GGCAGCTA GAGGTTGTCA AGTCCAGCTC
r48493			..GGGCAGCTA GAGGTTGTCA AGTCCAGCTC
r47780			..NGGCAGCTA GAGGTTGTCA AGTCCAGCTC
m78841			
cons	TGGCCTACGT	GTCCTGTGTT	GNGGCAGCTA GAGGTTGTCA AGTCCAGCTC
frame1	W..P..T..C	..P..V..L.	<u>..X..Q..L..</u> <u>E..V..V..K</u> <u>..S..S..S.</u>
frame2	..G..L..R..	V..L..C..X	..G..S..*.. <u>..R..L..S..</u> <u>S..P..A..P</u>
frame3	<u>..A..Y..V..</u> <u>..S..C..V..</u>	<u>X..A..A..R</u>	<u>..G..C..Q.</u> <u>..V..Q..L..</u>
NTE-pep			...V..V..K ..S..S..S.
	1451		1500
r15042			
h61615	CTACTGCGAG	TAC.....	
r51470			
r45802			
r54769	CTACTGCGAG	TACCTGCGCC	CGCCCATCGA CTGCTTCAAG ACCATGGACT
r48493	CTACTGCGAG	TACCTGCGNC	CGCCCATCGA CTGCTTCAAG ACCATGGACT
r47780	CTACTGCGAG	TACCTGCGCC	CGCCCATCGA CTGCTTCAAG ACCATGGACT
m78841			
cons	CTACTGCGAG	TACCTGCGCC	CGCCCATCGA CTGCTTCAAG ACCATGGACT
frame1	<u>..Y..C..E..</u> <u>Y..L..R..P</u> <u>..P..I..D.</u> <u>..C..F..K..</u> <u>T..M..D..F</u>		
frame2	..T..A..S.	..T..C..A..	R..P..S..T ..A..S..R. .P..W..T..
frame3	L..L..R..V	..P..A..P.	..A..H..R.. L..L..Q..D ..H..G..L.
NTE-pep	..?..D..G..	?..L..R..?	..?..I..D. .Y..F..K..
	1501		1550
r45802			
r54769	TTGGGAAGTT	CGACCAGATC	TATGATGTGG GCTACCAGTA CGGGAAGGCG
r48493	TTGGGAAGTT	CGACCAGATC	TATGATGTGG GCTACCAGTA CGGGAAGGCG
r47780	TTGGGAAGTT	CGACCAGATC	TATGATGTGG GCTACCAGTA CGGGAAGGCG
m78841			TA CGGGAAGGCG
3prm			
cons	TTGGGAAGTT	CGACCAGATC	TATGATGTGG GCTACCAGTA CGGGAAGGCG
frame1	<u>..G..K..F.</u> <u>..D..Q..I..</u> <u>Y..D..V..G</u> <u>..Y..Q..Y.</u> <u>..G..K..A..</u>		
frame2	L..G..S..S	..T..R..S.	..M..M..W.. A..T..S..T ..G..R..R.
frame3	..W..E..V..	R..P..D..L	..*..C..G. .L..P..V.. R..E..G..G
NTE-pep	F.	..D..Q..I..	Y..D..V..G ..Y..Q..Y. .G..K.....
	1551		1600
r51470			
r45802		ACGTC	ATTAGAAAA TGCTCACAGA
r54769	GTGTTTGGAG	GCTGGAGCCT	TGGCAACGTC ATTGAGAAAA TGCTCACAGA
r48493	GTGTTTGGAG	GCTGGAGCCG	TGGCAACGTC ATTGAGAAAA TGCTCACAGA
r47780	GTGTTTGGAG	GCTGGAGCNT	NGGCAACGTC ATTGAGAAAA TGCTCACAGA
m78841	GTGTTTGGAG	GCTGGAGCCG	TGGCAACGTC ATTGAGAAAA TGCTCACAGA
3prm			
cons	GTGTTTGGAG	GCTGGAGCCN	TGGCAACGTC ATTGAGAAAA TGCTCACAGA
frame1	<u>V..F..G..G</u> <u>..W..S..X.</u> <u>..G..N..V..</u> <u>I..E..K..M</u> <u>..L..T..D.</u>		
frame2	..C..L..E..	A..G..A..X	..A..T..S. .L..R..K.. C..S..Q..T
frame3	..V..W..R.	..L..E..P..	W..Q..R..H ..*..E..N. A..H..R..

	1601		1650
r51470			
r45802	CCGGCGG.CT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
r54769	CCGGCGGTCT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
r48493	CCGGCGGTCT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
r47780	CCGGCGGTTT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
m78841	CCGGCGGTCT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
3prm			
cons	CCGGCGGTCT	ACAGACCTTA	ATGAGAGCCG CCGTGCAGAC GTGCTTGCCT
frame1	.R..R..S..	T..D..L..N	..E..S..R. .R..A..D.. V..L..A..F
frame2	..G..G..L.	.Q..T..L..	M..R..A..A ..V..Q..T. .C..L..P..
frame3	P..A..V..Y	..R..P..*	*..E..P.. P..C..R..R ..A..C..L.

	1651		1700
r51470			
r45802	TCCTAAGCTC	TGGCTTCACT	GACTTGGCAT AGATTGTGTC CCGGATTGAG
r54769	TCCCAAGCTC	TGGCTTCACT	GACTTNGCAG AGATTGTGTC CCGGATTGAG
r48493	TCCCAAGCTC	TGGCTTCACT	GACTTGGCAG AGATTGTGTC CCGGATTGAG
r47780	TCCCAAGCTC	TGGCTTCACT	GACTTGGCAG AGATTGTGTC CCGGATTGAG
m78841	TCCCAAGCTC	TGGCTTCACT	GACTTGGCAG AGATTGTNTC CCGGATTGAG
3prm			
cons	TCCCAAGCTC	TGGCTTCACT	GACTTGGCAG AGATTGTGTC CCGGATTGAG
frame1	..P..S..S.	.G..F..T..	D..L..A..E ..I..V..S. .R..I..E..
frame2	S..Q..A..L	..A..S..L.	.T..W..Q.. R..L..C..P ..G..L..S.
frame3	.P..K..L..	W..L..H..*	..L..G..R. .D..C..V.. P..D..*..A

	1701		1750
r51470			
r45802	CCCCCACGA	GCTATGTCTC	TTTGGCTGT. CTG.ACGGAG AG.....
r54769	CCCCCACGA	GCTATGTCTC	TGTGGCTGTG CTTGACGGAG AGGAGTCAGA
r48493	CCCCCACGA	GCTATGTCTC	TGTGG.TGTG CTGGACGGAG AGGAGTCAGA
r47780	CCCCCACGA	GCTATGTCTC	TTTGGCTGTG CTT.ACGGAG AGG.....
m78841	CCCCCACGA	GCTATGTCTC	TNATGGCTGT GCTGACGGAG AGGAGTCAGA
3prm			
cons	CCCCCACGA	GCTATGTCTC	TNTGGCTGTG CTTGACGGAG AGGAGTCAGA
frame1	P..P..T..S	..Y..V..S.	.X..A..V.. L..D..G..E ..E..S..D.
frame2	.P..P..R..	A..M..S..L	..W..L..C. .L..T..E.. R..S..Q..I
frame3	..P..H..E.	.L..C..L..	X..G..C..A ..*..R..R. .G..V..R..

	1751		1800
r45802			
r54769	TTGTCTTGAA	GAGTATGAGG	AGGA.....
r48493	TTGTCT....		
r47780			
m78841	TTGTCTGACA	GAGTATGAGG	AGGACGCCGG ACCCGACTGC TCGAGGGATG
3prm			A
cons	TTGTCTNNA	GAGTATGAGG	AGGACGCCGG ACCCGACTGC TCGAGGGATN
frame1	.C..L..X..	E..Y..E..E	..D..A..G. .P..D..C.. S..R..D..X
frame2	..V..X..X.	.S..M..R..	R..T..P..D ..P..T..A. .R..G..X..
frame3	L..S..X..R	..V..*..G.	.G..R..R.. T..R..L..L ..E..G..X.

	1801		1850
r47780			
m78841	AAGGGGGGTN	CCCCGAGGGC	GCAA..CCCA GCACTGCCTC CGAGATGGAG
3prm	AGGGGGGTTC	CCCCGAAGGC	GCAANCCCCA GCACTGCCTC CGAGATGGAG
cons	ANGGGGGNTN	CCCCGANGGC	GCAANCCCCA GCACTGCCTC CGAGATGGAG
frame1	..G..G..X.	.P..X..G..	A..X..P..S ..T..A..S. .E..M..E..
frame2	X..G..X..X	..P..X..A.	.Q..X..P.. A..L..P..P ..R..W..R.
frame3	.X..G..X..	P..R..X..R	..X..P..Q. .H..C..L.. R..D..G..G

	1851		1900
r47780			
m78841	GAGGAGAAGT	CGATTCTCCG	GCAACGACGC
3prm	GAGGAGAAGT	CGATTCTCCG	GCAACGANGC
cons	GAGGAGAAGT	CGATTCTCCG	GCAACGANGC
frame1	E..E..K..S	..I..L..R.	.Q..R..X..
frame2	.R..R..S..	R..F..S..G	..N..X..A.
frame3	..G..E..V.	.D..S..P..	A..T..X..L

	1901		1950
r47780			
m78841	GGCTCAGCCA	CAGATGCCTN	AGGACCTCGA
3prm	GGCTCAGCCA	CAGATGCCTG	AGGACCTCGA
cons	GGCTCAGCCA	CAGATGCCTN	AGGACCTCGA
frame1	.A..Q..P..	Q..M..P..X	..D..L..X.
frame2	..L..S..H.	.R..C..L..	R..T..S..X
frame3	G..S..A..T	..D..A..X.	.G..P..R..

	1951		2000
r47780			
m78841	C.ACCC.TGG	ACTGGCT...	
3prm	CCACCCCTGG	ACTGGGCTGG	GGGTGNNCCC
cons	CCACCCCTGG	ACTGGNNTGG	GGGTGNNCCC
frame1	..T..P..G.	.L..X..W..	G..X..X..R
frame2	P..P..L..D	..W..X..G.	.G..X..P..
frame3	.H..P..W..	T..G..X..G	..V..X..P.

	2001		2050
m78841			
3prm	CTCCTGCTGC	TATGCCTGTG	ACCCCCGCGG
cons	CTCCTGCTGC	TATGCCTGTG	ACCCCCGCGG
frame1	L..L..L..L	..C..L..*	.P..P..R..
frame2	.S..C..C..	Y..A..C..D	..P..R..G.
frame3	..P..A..A.	.M..P..V..	T..P..A..A

	2051		2100
m78841			
3prm	GCCCTGAGCG	GGGATGCAGT	GTTGCACTGA
cons	GCCCTGAGCG	GGGATGCAGT	GTTGCACTGA
frame1	.P..*..A..	G..M..Q..C	..C..T..D.
frame2	..P..E..R.	.G..C..S..	V..A..L..M
frame3	A..L..S..G	..D..A..V.	.L..H..*..

	2101		2149
3prm	CCAATAAACT	CGCCTCTTGG	AAAAAAAAAA
cons	CCAATAAACT	CGCCTCTTGG	AAAAAAAAAA
frame1	..N..K..L.	.A..S..W..	K.....
frame2	P..I..N..S	..P..L..G.	
frame3	.Q..*..T..	R..L..L..E	

Appendix 2 NTE-like proteins and PROSITE entry

```
ID    UPF0028; PATTERN.
AC    PS01237;
DT    NOV-1997 (CREATED); NOV-1997 (DATA UPDATE); NOV-1997 (INFO UPDATE).
DE    Uncharacterized protein family UPF0028 signature.
PA    [GA]-[GS]-G-[GA]-A-R-G-x-[SA]-H-x-G-x(9)-[IV]-x-[IV]-D-x(2)-[GA]-G-
x-S-
PA    x-G.
NR    /RELEASE=35,69113;
NR    /TOTAL=6(6); /POSITIVE=6(6); /UNKNOWN=0(0); /FALSE_POS=0(0);
NR    /FALSE_NEG=0; /PARTIAL=0;
CC    /TAXO-RANGE=??EP?; /MAX-REPEAT=1;
DR    Q50733, Y0A3_MYCTU, T; P37053, YCHK_ECOLI, T; P37054, YCHK_SHIFL,
T;
DR    Q04958, YMF9_YEAST, T; Q02331, YOL4_CAEEL, T; Q21534, YVL7_CAEEL,
T;
DO    PDOC00951;
```

```
{PDOC00951}
{PS01237; UPF0028}
{BEGIN}
```

```
*****
* Uncharacterized protein family UPF0028 signature *
*****
```

The following uncharacterized proteins have been shown [1] to share regions of similarities:

- Yeast chromosome XIII hypothetical protein YMR059c (1679 aa).
- Caenorhabditis elegans hypothetical protein ZK370.4 (1351 aa).
- Caenorhabditis elegans hypothetical protein M110.7 (880 aa).
- Escherichia coli hypothetical protein ychK (314 aa).
- Mycobacterium tuberculosis hypothetical protein MtCY9C4.03c (583 aa)

These are proteins of variable size which share a domain of about 200 residues. This domain is located at the C-terminal of the eukaryotic members of this family. It can be picked up the database by the following glycine-rich pattern.

-Consensus pattern: [GA]-[GS]-G-[GA]-A-R-G-x-[SA]-H-x-G-x(9)-[IV]-x-[IV]-D-

x(2)-[GA]-G-x-S-x-G

-Sequences known to belong to this class detected by the pattern: ALL.

-Other sequence(s) detected in SWISS-PROT: NONE.

-Last update: November 1997 / First entry.

[1] Bairoch A.

Unpublished observations (1997).

Mtcy20b11

ID O05884 PRELIMINARY; PRT; 1048 AA.
AC O05884;
DT 01-JUL-1997 (TREMBLREL. 04, CREATED)
DT 01-JUL-1997 (TREMBLREL. 04, LAST SEQUENCE UPDATE)
DT 01-JUL-1997 (TREMBLREL. 04, LAST ANNOTATION UPDATE)
DE HYPOTHETICAL 110.2 KD PROTEIN.
GN MTCY20B11.14C.
OS MYCOBACTERIUM TUBERCULOSIS.
OC PROKARYOTA; FIRMICUTES; ACTINOMYCETALES; MYCOBACTERIACEAE.
RN [1]
RP SEQUENCE FROM N.A.
RC STRAIN=H37RV;
RA BADCOCK K., CHURCHER C.M.;
RL SUBMITTED (MAY-1997) TO EMBL/GENBANK/DBJ DATA BANKS.
RN [2]
RP SEQUENCE FROM N.A.
RC STRAIN=H37RV;
RA COLE S.T., BARRELL B.G., RAJANDREAM M.A.;
RL SUBMITTED (MAY-1997) TO EMBL/GENBANK/DBJ DATA BANKS.
RN [3]
RP SEQUENCE FROM N.A.
RC STRAIN=H37RV;
RX MEDLINE; 96181548.
RA PHILIPP W.J., POULET S., EIGLMEIER K., PASCOPELLA L.,
RA BALASUBRAMANIAN V., HEYM B., BERGH S., BLOOM B.R., JACOBS W.R. JR.,
RA COLE S.T.;
RL PROC. NATL. ACAD. SCI. U.S.A. 93:3132-3137(1996).
DR EMBL; Z95121; E314474; -.
KW HYPOTHETICAL PROTEIN.
SQ SEQUENCE 1048 AA; 110198 MW; 5481E282 CRC32;

The EMBL entry also had the following comments:-

complement(12274..15420)
/note="MTCY20B11.14c". Len: 1048. Function: unknown membrane protein organised in two domains. Domain comprising first ~500 aa residues is similar to various antibiotic resistance and efflux proteins and contains sugar transport proteins signature1 (PS00216). Part corresponding to last 550 aa residues very similar (2.1e-28; 36.5% identity in 572 aa overlap) to hypothetical 62.1 kd M. tuberculosis protein (Q50733; Y0A3_MYCTU) cy9c4.03 and contains cyclic nucleotide-binding domain signature 2 (PS00889). Similar to other M. tuberculosis proteins: MTCY3G12.01, 6.3e-32; MTCY98.0002c, 6.3e-32; MTCY9C4.03c, 1.5e-26; MTCY369.27c, 2.5e-26. FASTA best: MMR_BACSU Q00538 methylenomycin a resistance protein (466 aa) opt: 733 z-score: 719.4 E(): 4.4e-33; (31.0% identity in 429 aa overlap)"

O05884 Length: 1048 February 12, 1998 12:46 Type: P Check: 5529 ..

```
1  MHISLHGKGK FANLTRRRRP SSASVLLVAG FGAFLAFLDS TIVNIAFPDI
51  QRSFPSYDIG SLRWILNGYN IVFAAFMVAA GRLADLLGRR RTFLSGVLVF
101 TIASGLCAVA GSVEQLVAFR VLQIGGAAIL VPASLALVVE GFDAARRAHA
151 IGLWGAAGAAI AAGLGPPIGG LLVEWAGWRW VLLVNVPLGI VAAIATKRML
201 VESRASGRRR MPDLRGALLL AVTLGLVTLG LVKGPDWGWL SVATVGSFLA
251 SVLTSVGFVH SSRSHAPLV EPALLRSRSF VAGNLLTLVA AAGFYCYGLT
301 HVLYLNYVWH YSLLKAGFAI APAAVVAADV AAALGRVAGR HGHRVIVLVG
351 ALVWAGSLVW YLQRVGSEPD FLRVWLPQQL LQGIGVGATL PVLSSAALAE
401 VAKGGSYATS SAVVSTTRQL GAVLGVAVMV ILIGKPEHGT AEEALRRGWA
451 MAAICFIAVA VAAAVLGRTN RNPVQMPAPE PAIAPRLEPP IPQPAAPIE
501 HWAAGDADPL GNLPLFAGLD AATLAQLGEH VEDVELEAGC YLFHEGDPSD
551 SLYVIRTGRV QVLQDSIVLK ELGRGEVLGE LGLLIDAPRS ATVRALRDTK
```

```

601  LVRLTKAQFD EIADHGALAA LVKVLATRLR EAPPPATDST SPEVVVSVIG
651  VSGDAPVPAV AAGLLTALSA RLRVADPGRV DRDGLDRAER VADKVVLHAA
701  VEDAGWRDFC LRVADRIVLV AGDPNPQAAR LPARARGADL VLAGPAASRE
751  HRRQWEELIT PRSVHVHVR RILENVRPLA ARIAGRSIGL VLGGGGGARGF
801  AHLGVLDELE RVGVTIDRFA GTSMGAVIAV FGACGMDAAT ADAYAYEYFI
851  RHNPLSDYAF PVRGLVRGRR TLTLLEAAFG DRLVEELPKE FRCVSVDLLA
901  RRPVVHRRGR LVDVIGCSLR LPGIYPPQVY NGRLHVDGGV LDNLPVSTRA
951  SPDGPLIAVS IGLGGGGPGS ARQDGSPKVP GIGDTLMRTM TIGSQRGADA
1001 ALSLAQVVIR PDTGAVGLLE FHQIDAAREA GRVAAREAMP HIMALLNR

```

Q12

```

ID  Q12043      PRELIMINARY;  PRT;    749 AA.
AC  Q12043;
DT  01-NOV-1996 (TREMBLREL. 01, CREATED)
DT  01-NOV-1996 (TREMBLREL. 01, LAST SEQUENCE UPDATE)
DT  01-NOV-1996 (TREMBLREL. 01, LAST ANNOTATION UPDATE)
DE  CHROMOSOME XV READING FRAME ORF YOR081C.
GN  YOR2964C.
OS  SACCHAROMYCES CEREVISIAE (BAKER'S YEAST).
OC  EUKARYOTA; FUNGI; ASCOMYCOTINA; HEMIASCOMYCETES.
RN  [1]
RP  SEQUENCE OF 434-749 FROM N.A.
RA  BOHN C., BOLOTIN-FUKUHARA M., DAIGNAN-FORNIER B., DANG D.V.,
RA  VALENS M.;
RL  SUBMITTED (JUL-1996) TO EMBL/GENBANK/DDBJ DATA BANKS.
RN  [2]
RP  SEQUENCE FROM N.A.
RA  VOSS H., BENES V., RECHMANN S., TEODORU C., SCHWAGER C., PACES V.,
RA  ANSORGE W.;
RL  SUBMITTED (JUL-1996) TO EMBL/GENBANK/DDBJ DATA BANKS.
RN  [3]
RP  SEQUENCE FROM N.A.
RA  MIPS;
RL  SUBMITTED (JUL-1996) TO EMBL/GENBANK/DDBJ DATA BANKS.
RN  [4]
RP  SEQUENCE FROM N.A.
RA  BENES V., ANDRADE M.A., RECHMANN S., TEODORU C., BANREVI A.,
RA  SANDER C., VALENCIA A., ANSORGE W., VOSS H.;
RL  SUBMITTED (JAN-1996) TO EMBL/GENBANK/DDBJ DATA BANKS.
DR  EMBL; Z74989; E251992; -.
DR  EMBL; X94335; E217721; -.
SQ  SEQUENCE 749 AA; 84715 MW; 2CAFB911 CRC32;

```

Q12043 Length: 749 February 11, 1998 16:03 Type: P Check: 8887 ..

```

1  MSNTLPVTEF LLSKYYELSN TPATDSSSLF KWLYHKTLR QOLLISDLSS
51  QKKHAISYDQ WNDIASRLDD LTGLSEWKT I DESSLYNYKL LQDLTIRMHR
101 LRTTHDYHRL LYLIRTKWVR NLGNMNNVNL YRHSHTGTKQ IIHDYLEESQ
151 AVLTAIHQS NMNDHYLLGI LQQTRRNIGR TALVLSGGST FGLFHIGVLA
201 ALFESDLMPK VISGSSAGAI VASIFCVHTT QEIPSLLTNV LNMEFNIFND
251 DNSKSPNENL LIKISRFCQN GTWFNNQPLI NTMLSFLGNL TFREAYNKTG
301 KILNITVSPA SIYEQPKLLN NLTAPNVLIW SAVCASCSPG GVFPSTPLFE
351 KDPHTGKIKE WGATNLHLSN MKFMDGSVDN DMPISRLSEM FNVDHIIACQ
401 VNIHVFPLLK FSNTCVGGEI EKEITARFRN QVTKIFKFFS DETIHFLDIL
451 KELEFHPYLM TKLKLHFLQQ YSGNVTILPD LSMVGQFHEV LKNPSQLFLL
501 HQTTLGARAT WPKISMIQNN CGQEFALDKA ITFLKEKIII SSSIKNPLQF
551 YQPRFSEQIK SLSIMDADLP GVDLEESSN SLSIIKSPNK TAAPGRFPLO
601 PLPSPSSTFN KRKMDMLSPS PSPSTSPQRS KSSFTQQGTR QKANSLSFBI
651 GASSLRLLKKS PLKVPSPQF KKRSSYYNQ N MSAEMRKNRK KSGTISSYDV
701 QTNSDFPIPI AIENGSDNT LFNPSRFPMD AMSAATNDNF MNNSDIFQN

```


SWISSCHEESE

Currently no peptide sequence for swisscheese has been submitted any of the sequence databases, this 'database entry' consists of the header for the EMBL DNA entry and a translation based on the published paper.

```
ID   DMRNASCP   standard; RNA; INV; 5364 BP.
XX
AC   Z97187;
XX
NI   e1054009
XX
DT   25-JUN-1997 (Rel. 52, Created)
DT   05-JAN-1998 (Rel. 54, Last updated, Version 5)
XX
DE   Drosophila melanogaster mRNA for the swiss cheese protein
XX
KW   swiss cheese protein.
XX
OS   Drosophila melanogaster (fruit fly)
OC   Eukaryotae; mitochondrial eukaryotes; Metazoa; Arthropoda;
Tracheata;
OC   Hexapoda; Insecta; Pterygota; Diptera; Brachycera; Muscomorpha;
OC   Ephydroidea; Drosophilidae; Drosophila.
XX
RN   [1]
RP   1-5364
RA   Kretzschmar D.;
RT   ;
RL   Submitted (24-JUN-1997) to the EMBL/GenBank/DDBJ databases.
RL   Kretzschmar D., Institut fuer Entwicklungsbiologie, universitaet
RL   Regensburg, Universitaetsstrasse 31, Regensburg 93053 GERMANY.
XX
RN   [2]
RA   Kretzschmar D., Hasan G., Sharma S., Heisenberg M., Benzer S.;
RT   "The swiss cheese mutant causes glial hyperwrapping and brain
RT   degeneration in Drosophila";
RL   J. Neurosci. 17:7425-7432(1997).
XX
FH   Key          Location/Qualifiers
FH
FT   source        1. .5364
FT                  /organism="Drosophila melanogaster"
FT                  /sequenced_mol="cDNA to mRNA"
FT   mRNA          <1. .>5364
FT                  /product="swiss cheese protein"
XX
SQ   Sequence 5364 BP; 1399 A; 1421 C; 1406 G; 1138 T; 0 other;
```

swisscheese Length: 1425 July 4, 1997 14:25 Type: P Check: 1500 ..

```
1  MDVLEMLRAS ASGSYNTTFS DAWCQYVSKQ ITATVYMYFA LVMSLLFIA
51 WFLYFKRMAR LRLRDEIARS ISTVTNSSGD MRGLRFRKRD KMLFYGRMRL
101 RKMKNVSGQM YSSGKGYKRR AVMRFARRIL QLRRDNMPLE MRTVEPPAEY
151 LEETIEGSDR VPPDALYMLQ SIRIFGHFEK PVFLRLCKHT QLLELMAGDY
201 LFKITDPDDS VYIVQSGMIN VYISNADGST LSLKTVRKGE SVTSLLSFID
251 VLSGNPSYYK TVTAKAIEKS VVIRLPMQAF EEVFQDNPDV MIRVIQVIMI
301 RLQRVLEFAL RNYLGLNAEL VQNHMRYKSV STMSGPINSQ TSQSSRQAPN
351 GPPMVISQMN LMQSAVSGTG SSGVSVTVTR PPSSPSRHSR EEHTLSDPNP
401 NPDGSFHGTT NLFTEVHGDA PNADLFHQQQ QQHSVGNLST RRSSITLMAP
```

```

451  DPSHSCQLQTP  GVTTSIDMRL  VQSSAVDSLRL  KELGLSEEDS  HIIEPFVELR
501  ELEPNVTLLIT  EGNADDVCVW  FVMTGT LAVY  QSNQDATRAK  QDKSDMLIHF
551  VHPGEIVGGL  AMLTGEASAY  TIRSRITRI  AFIRRAAIYQ  IMRQRPRIVL
601  DLGNGVVRRL  SPLVRQCDYA  LDWIFLESGR  AVYRQDESSD  STYIVLSGRM
651  RSVITHPGGK  KEIVGEYGKG  DLVGIVEMIT  ETSRTTTVMA  VRDSELAKLP
701  EGLFNAIKLR  YPIVVTKLIS  FLSHRFLGSM  QTRSGSGAPG  APVEANPVTH
751  KYSTVALVPI  TDEVPMTPFT  YELYHSLCAI  GPVLHLTSDV  VRKQLGNSIF
801  EAANEYRLTS  WLAQQEDRNI  ITLYQCSSL  SAWTQRCMRQ  ADVILIVGLG
851  DRSHLVGKFE  REIDRLAMRT  QKELVLLYPE  ASNAKPANTL  SWLNARPWVT
901  KHHHVLGVK  IFTRKSQYRI  NDLYSRVLLS  EPNMHSDFSR  LARWLTGNSI
951  GLVLGGGGAR  GAAHIGMLKA  IQEAGIPVDM  VGGVVSIGALM  GALWCSEINI
1001 TTVTQKAREW  SKKMTKWFLQ  LLDLTYPITS  MFSGREFNKT  IHDTFGDVSI
1051 EDLWIPYFTL  TTDITASCHR  IHTNGSLWRY  VRSSMSLSGY  MPPLCDPKDG
1101 HLLLDGGYVN  NLPADVMHNL  GAAHIIAIDV  GSQDDTDLTN  YGDDLSGWWL
1151 LYKKWNPFST  PVKVPDLPI  QSRLAYVSCV  RQLEEVKNSD  YCEYIRPPID
1201 KYKTLAFGSF  DEIRDVG YVF  GKNYFESMAK  AGR LGRFNQW  FNKEPPKRVN
1251 HASLNEYTFI  DLAQIVCRLP  ETYAVNTAEL  FSEDEDCDGY  ISEPTTLNTD
1301 RRRIQVSRAG  NSLSFSETEM  DSDVELDLKL  ERKTDKSTQS  SPPSNSRSDM
1351 RGKEEARHMS  NWHWGVKHKD  ETGSGANEAT  KTQTGQEQL  QQEQQDQAT
1401 AEQLVDKDKE  ENKENRSSPN  NETKN

```

Y0A3_MYCTU

```

ID  Y0A3_MYCTU      STANDARD;      PRT;      583 AA.
AC  Q50733;
DT  01-NOV-1997 (REL. 35, CREATED)
DT  01-NOV-1997 (REL. 35, LAST SEQUENCE UPDATE)
DT  01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE  HYPOTHETICAL 62.1 KD PROTEIN CY9C4.03C.
GN  MTCY9C4.03C.
OS  MYCOBACTERIUM TUBERCULOSIS.
OC  PROKARYOTA; FIRMICUTES; ACTINOMYCETALES; MYCOBACTERIACEAE.
RN  [1]
RP  SEQUENCE FROM N.A.
RC  STRAIN=H37RV;
RA  CONNOR R., CHURCHER C.M., BARRELL B.G., RAJANDREAM M.A., WALSH
S.V.;
RL  SUBMITTED (JUL-1996) TO EMBL/GENBANK/DBJ DATA BANKS.
CC  -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
DR  EMBL; Z77250; E255311; -.
DR  PROSITE; PS01237; UPF0028; 1.
KW  HYPOTHETICAL PROTEIN.
SQ  SEQUENCE 583 AA; 62123 MW; 6B664CD3 CRC32;

```

Y0A3_MYCTU Length: 583 February 11, 1998 15:57 Type: P Check: 216

..

```

1  MTTARRRPKR  RGT DARTALR  NVPILADIDD  EQLERLATTV  ERRHV PANQW
51  LFHAGEPADS  IYIVDSGRFV  AVAPEGHVFA  EMASGDSIGD  LGVIAGAARS
101 AGVRALRDGV  VWRIAAETFT  DMLEATPLLQ  SAMLRAMARM  LRQSRPAKTA
151 RRP RVIGVVS  NGDTAAAPMV  DAIATSLDSH  GRTAVIAPPV  ETTSAVQEYD
201 ELVEAFSETL  DRAERSNDWV  LVVADRGAGD  LWRHYVSAQS  DRLVVLVDQR
251 YPPDAVDSL  A  TQRPVHLITC  LAEPDPSWWD  RLAPVSHHPA  NSDGFGALAR
301 RIAGRSGLLV  MAGGGARGLA  HFGVYQELTE  AGVVIDRFGG  TSSGAIASAA
351 FALGMDAGDA  IAAAREFIAG  SDPLGDYTIP  ISALTRGGRV  DRLVQGFFGN
401 TLIEHLPRGF  FSVSADMITG  DQIIHRRGSV  SGAVRASISI  PGLIPPVHNG
451 EQLLVDGGLL  NNL PANVMCA  DTDGEVICVD  LRRTFVPSKG  FGLLPPIVTP
501 PGLLRRLTG  TDNALPPLQE  TLLRAFDLAA  STANLRELPR  VAAIIEPDVS
551 KIGVLNFKQI  DAALEAGRMA  ARAALQAQPD  LVR

```

YCHK_ECOLI

ID YCHK ECOLI STANDARD; PRT; 314 AA.
AC P37053;
DT 01-JUN-1994 (REL. 29, CREATED)
DT 01-JUN-1994 (REL. 29, LAST SEQUENCE UPDATE)
DT 01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU INTERGENIC REGION.
GN YCHK.
OS ESCHERICHIA COLI.
OC PROKARYOTA; GRACILICUTES; SCOTOBACTERIA; FACULTATIVELY ANAEROBIC
RODS;
OC ENTEROBACTERIACEAE.
RN [1]
RP SEQUENCE FROM N.A.
RC STRAIN=K12;
RX MEDLINE; 94110230.
RA BOESL M., KERSTEN H.;
RL J. BACTERIOL. 176:221-231(1994).
RN [2]
RP SEQUENCE FROM N.A.
RC STRAIN=K12 / MG1655;
RA BLATTNER F.R., PLUNKETT G. III, MAYHEW G.F., PERNA N.T., GLASNER
F.D.;
RL SUBMITTED (JAN-1997) TO EMBL/GENBANK/DDBJ DATA BANKS.
RN [3]
RP SEQUENCE FROM N.A.
RC STRAIN=K12;
RX MEDLINE; 97251357.
RA AIBA H., BABA T., FUJITA K., HAYASHI K., INADA T., ISONO K.,
RA ITOH T., KASAI H., KASHIMOTO K., KIMURA S., KITAKAWA M.,
RA KITAGAWA M., MAKINO K., MIKI T., MIZOBUCHI K., MORI H., MORI T.,
RA MOTOMURA K., NAKADE S., NAKAMURA Y., NASHIMOTO H., NISHIO Y.,
RA OSHIMA T., SAITO N., SAMPEI G., SEKI Y., SIVASUNDARAM S.,
RA TAGAMI H., TAKEDA J., TAKEMOTO K., TAKEUCHI Y., WADA C.,
RA YAMAMOTO Y., HORIUCHI T.;
RL DNA RES. 3:363-377(1996).
CC -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
DR EMBL; M64675; -; NOT_ANNOTATED_CDS.
DR EMBL; AE000221; G1787485; -.
DR EMBL; D90852; G1805511; -.
DR PIR; B36871; B36871.
DR ECOGENE; EG12120; YCHK.
DR PROSITE; PS01237; UPF0028; 1.
KW HYPOTHETICAL PROTEIN.
SQ SEQUENCE 314 AA; 34355 MW; F3E918DB CRC32;

YCHK_ECOLI Length: 314 February 11, 1998 15:56 Type: P Check: 307

..

```

1  MATIAFQGNL AGIMRKIKIG LALGSGAARG WSHIGVINAL KKVGIIEIDIV
51 AGCSIGSLVG AAYACDRLSA LEDWVTSFSY WDVLRMLDLS WQRGGLLRGE
101 RVFNQYREIM PETEIENCSR RFAAVATNLS TGRELWFTEG DLHLAIRASC
151 SIPGLMAPVA HNGYWLV DGA VVNPIPISLT RALGADIVIA VDLQHDHLM
201 QQDLLSFNVS EENSEGDSL PW HARLKERL GSITTRRAVT APTATEIMTT
251 SIQVLENRLK RNR MAGDPPD ILIQPVCPQI STLD FHR AHA AIAAGQLAVE
301 RKMDELLPLV RTNI

```

YCHK_SHIFL

ID YCHK_SHIFL STANDARD; PRT; 192 AA.
AC P37054;
DT 01-JUN-1994 (REL. 29, CREATED)
DT 01-JUN-1994 (REL. 29, LAST SEQUENCE UPDATE)
DT 01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU INTERGENIC REGION
(FRAGMENT).
GN YCHK.
OS SHIGELLA FLEXNERI.
OC PROKARYOTA; GRACILICUTES; SCOTOBACTERIA; FACULTATIVELY ANAEROBIC
RODS;
OC ENTEROBACTERIACEAE.
RN [1]
RP SEQUENCE FROM N.A.
RC STRAIN=2A;
RX MEDLINE; 93023838.
RA HROMOCKYJ A.E., TUCKER S.C., MAURELLI A.T.;
RL MOL. MICROBIOL. 6:2113-2124(1992).
RN [2]
RP CONCEPTUAL TRANSLATION.
RA RUDD K.E.;
RL UNPUBLISHED OBSERVATIONS (FEB-1994).
CC -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
CC -!- CAUTION: THIS IS A CONCEPTUAL TRANSLATION; A FRAMESHIFT HAD TO
CC BE INTRODUCED IN POSITION 178 TO PRODUCE THIS ORF.
DR EMBL; X66849; -; NOT_ANNOTATED_CDS.
DR PROSITE; PS01237; UPF0028; 1.
KW HYPOTHETICAL PROTEIN.
FT NON_TER 192 192
SQ SEQUENCE 192 AA; 20831 MW; D02A513B CRC32;

YCHK_SHIFL Length: 192 February 11, 1998 15:56 Type: P Check: 2663

..

1 MATIAFQGNL AGIMRKIKIA LALGSGAARG WSHIGVINAL KKVCIEIDIV
51 AGCSIGSLVG AAYACDRLSA LEDWVTSFSY WELLRLMDLS WQRGGLLRGE
101 RVFNQYREIM PETEIENCSR RFAPVATNLS TGRELWFTEG DLHLAIRASC
151 SIPGLMAPVA HNGYWLVDGA VVNPIPISLT RALGADIVIA VD

YK69_YEAST

ID YK69_YEAST STANDARD; PRT; 910 AA.
AC P36165;
DT 01-JUN-1994 (REL. 29, CREATED)
DT 01-JUN-1994 (REL. 29, LAST SEQUENCE UPDATE)
DT 01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE HYPOTHETICAL 102.7 KD PROTEIN IN PRP16-SRP40 INTERGENIC REGION.
GN YKR089C OR YKR409.
OS SACCHAROMYCES CEREVISIAE (BAKER'S YEAST).
OC EUKARYOTA; FUNGI; ASCOMYCOTINA; HEMIASCOMYCETES.
RN [1]
RP SEQUENCE FROM N.A.
RX MEDLINE; 94262327.
RA GARCIA-CANTALEJO J., BALADRON V., ESTEBAN P.F., SANTOS M.A., BOU
G.,
RA REMACHA M.A., REVUELTA J.L., BALLESTA J.P.G., JIMENEZ A., DEL REY
F.;
RL YEAST 10:231-245(1994).
CC -!- SUBCELLULAR LOCATION: INTEGRAL MEMBRANE PROTEIN (POTENTIAL).
CC -!- SIMILARITY: TO YEAST YMR313C.
DR EMBL; Z27116; G415909; -.

DR EMBL; Z28314; G486575; -.
 DR PIR; S38167; S38167.
 DR PIR; S39130; S39130.
 KW HYPOTHETICAL PROTEIN; TRANSMEMBRANE.
 FT TRANSMEM 282 302 POTENTIAL.
 FT TRANSMEM 310 326 POTENTIAL.
 FT TRANSMEM 426 442 POTENTIAL.
 SQ SEQUENCE 910 AA; 102716 MW; 825AC76F CRC32;

YK69_YEAST Length: 910 February 11, 1998 15:55 Type: P Check: 8852

..

```

    1  MSSKISDLTS TQNKPLLVTQ QLIKYYEQI LGTSQNIPI LNPKNKFIRP
   51  SKDNSDVERV EEDAGKRLQT GKNKTTNKVN FNLDTGNEDEK LDDDQETVTE
  101  NENNDIEMVE TDEGEDERQG SSLASKCKSF LYNVFGVNGYE RDILIDKVCS
  151  QKQHAMSFEW WCSAGARLDD LTGKTEWKQK LESPLYDYKL IKDLTSRMRE
  201  ERLNRNYAQL LYIIRTNWVR NLGNMGVNL YRSHVGTGY LIDEYMMESR
  251  LALESLMESD LDDSYLLGIL QQTRRNIGRT ALVLSGGGTF GLFHIGVLGT
  301  LFELDLLPRV ISGSSAGAIV ASILSVHHKE EIPVLLNHIL DKEFNIFKDD
  351  KQKSESENLL IKISRFFKNG TWFDNKHLVN TMIEFLGDLT FREAYNRTGK
  401  ILNITVSPAS LFEQPRLLNN LTAPNVLIWS AVCASCSPG IFPSSPLYEK
  451  DPKTGERKPW TGSSSVKFVD GSVDNDLPIS RLSEMFNVDH IIAQOVNIHV
  501  FPFLKLSLSC VGGEIEDEFS ARLKQNLSSI YNFMANEAIH ILEIGSEMGI
  551  AKNALTKLRS VLSQQYSGDI TILPDMCMLE RIKELLSNPT KEFLREITN
  601  GAKATWPKVS IIQNHCGQEF ALDKAISYIK GRMIVTSSLK TPFQFADSVI
  651  GLIKAPEQTS DESKNPENST LLTRTPTKGD NHISNVLDDN LLESESTNSL
  701  LLLRENASTY GRSPSGFRPR YSITSASLNP RHQRRKSDTI STSRPFAKSF
  751  SFSVASPTSR MLRQSSKING HPPPILOKKT SMGRMLFPMO AKTYDPESHE
  801  LIPHSASIED PAMVDKKLHF GRKSRYLHRM NKKWVSSNI LYTDSDKEDH
  851  PTLRLISNFD SDAMIHSDLA GNFRHSIDG RPPSQATKSS PFRSRPSSST
  901  QHKSTTSFTQ

```

YLBK

LOCUS 2340007 260 aa 19-AUG-1997
 DEFINITION YlbK protein-
 ACCESSION 2340007
 PID g2340007
 DBSOURCE EMBL: locus BS16823KB, accession Z98682
 KEYWORDS -
 SOURCE Bacillus subtilis-
 ORGANISM Bacillus subtilis
 Eubacteria; Firmicutes; Low G+C gram-positive bacteria;
 Bacillaceae; Bacillus-
 REFERENCE 1 (residues 1 to 260)
 AUTHORS Bertero,M-, Presecan,E-, Glaser,P-, Richou,A- and Danchin,A-
 TITLE Bacillus subtilis chromosomal region downstream nprE
 JOURNAL Unpublished
 REFERENCE 2 (residues 1 to 260)
 AUTHORS Glaser,P-
 TITLE Direct Submission
 JOURNAL Submitted (18-AUG-1997) P- Glaser, Regulation de
 l'Expression
 Genetique, Institut Pasteur, 28 Rue du Dr Roux, Paris,75724,
 FRANCE
 COMMENT Related sequences X96983, D90852, X73124, U09529-
 FEATURES Location/Qualifiers
 source 1--260
 /organism="Bacillus subtilis"
 /strain="168"
 Protein 1--260
 /product="YlbK protein"

CDS 1--260
 /gene="ylbK"
 /coded_by="Z98682:17111--17893"
 /transl_table=11

YLBK.pep Length: 260 January 18, 1998 12:46 Type: P Check: 1679 ..

```

1 MAKPKIGLAL GSGGARGLAH LGVLSSLHKH QIEVDMIAGS SMGALVGSFY
51 AAGHDVATMK KVAKAFKRRL YADYTVPKLG FLKGDRVRQL VHAYTFGKPI
101 EELQIPLGIV ACDLQTGEKI VFRKGSVSDA VRASISIPGI FIPQRDLGRL
151 LVDGAVVDRI PVSVVKDMGA DIIIASDVSR VRKTETAVHI FDVIMQSMDI
201 LQNELVRHQT IAADIMIRPS LETYSSSSFA NIEEMISAGE EATNRMISKI
251 RKEIENWEGS

```

YMF9_YEAST

ID YMF9_YEAST STANDARD; PRT; 1679 AA.
 AC Q04958;
 DT 01-NOV-1997 (REL. 35, CREATED)
 DT 01-NOV-1997 (REL. 35, LAST SEQUENCE UPDATE)
 DT 01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
 DE HYPOTHETICAL 187.1 KD PROTEIN IN OGG1-CNA2 INTERGENIC REGION.
 GN YML059C OR YM9958.03C.
 OS SACCHAROMYCES CEREVISIAE (BAKER'S YEAST).
 OC EUKARYOTA; FUNGI; ASCOMYCOTINA; HEMIASCOMYCETES.
 RN [1]
 RP SEQUENCE FROM N.A.
 RC STRAIN=S288C / AB972;
 RA DEVLIN K., CHURCHER C., BARRELL B.G., RAJANDREAM M.A.;
 RL SUBMITTED (NOV-1994) TO EMBL/GENBANK/DBJ DATA BANKS.
 CC -!- SUBCELLULAR LOCATION: INTEGRAL MEMBRANE PROTEIN (POTENTIAL).
 CC -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
 DR EMBL; Z46729; G577137; -.
 DR PROSITE; PS01237; UPF0028; 1.
 KW HYPOTHETICAL PROTEIN; TRANSMEMBRANE.
 FT TRANSMEM 50 70 POTENTIAL.
 FT TRANSMEM 104 124 POTENTIAL.
 FT TRANSMEM 235 255 POTENTIAL.
 FT TRANSMEM 884 904 POTENTIAL.
 FT TRANSMEM 1127 1147 POTENTIAL.
 FT TRANSMEM 1369 1389 POTENTIAL.
 FT TRANSMEM 1396 1416 POTENTIAL.
 SQ SEQUENCE 1679 AA; 187132 MW; C229D462 CRC32;
 YMF9_YEAST Length: 1679 February 11, 1998 15:57 Type: P Check: 209

```

..
1 MRSMNCTTNN TNNTGQNTKN SLGSSFNSSN YTSYRFQTCL TDQIISEAQT
51 WSLSSLFNFS WVVSFYVMGA SRMIFRYGWY LATLSLLRIP KWIFFKLHHV
101 QFTLSFWLIL FALAVIVFVT YTIMKERILS QYKRLTPEFL PLENTGKSGS
151 SANINAASTQ SANAPPAIGS STTGASSIID SKKHSLKDGN ENETFLSSYL
201 DQFLSAIKIF GYLEKPVFHD LTKNMKTQKM DEGEILLLDS TIGFAIVVEG
251 TLQLYHEVDH SDKDHGDETD HSDTDGLDDQ DRDEEDEEED DDIDNYDTKS
301 CSSNLIDEED ESGVYIHLKN GLGNFQLLNT VKPGNPLTSL VSILNLFTHS
351 MSSYGNSNFP SELSSPIDTT VSVNNMFCSS EQNFSNTDSM TNSTNSFPTF
401 PSSMPKLVAR AATDCTIGII PPQSFAKLTA KYPRSASHII QMVLTKLYHV
451 TFQTAHDYLG LTKEIMDIEV LLNKSIVYEL PYLKEAVIR KFKTVDKSSG
501 SADLEPKPKN SNASSKLKPK PKAKPSDGII QSLKIANANA NTSSNSLSLK
551 PEFTHHPSSR HVVLGSRDQF NPGDLLSNVP LSRTMDILSP NPIHNNNRNK
601 SNGINTSTSN QHKRSSRSSS NNASVHKKF SSLSPELRNA QLSTSPLSLD
651 NTSVHDHIHP SPVHLKGRVS PRPNLLPTTS FSAAQEETED SALRMALVEA
701 MLTYLGVNKS NMSVSSSSIA NMSSLNSPQL NEMYSRRPSN ASFLMSPHCT
751 PSDISVASSF ASPQTQPTML RILPKEYTIS NKRHNKSKSQ DKKKPRAYKE
801 ELTPNLDFED VKKDFAQGIQ LKFFKKGTTI VEQNARGKGL FYIISGKVN
851 TTNSSSSSVS SMSKPEQVSA QSSHKGENPH HTQHLLYSVG SGGIVGYLSS

```

```

901  LIGYKSFVNI VAKSDVYVGF LSSATLERLF DKYFLIYLRI SDSLTKLLSS
951  RLLKLDALEH WVHLRASETL FSQGDSENGI YVVLNGRLRQ LQQQSLNSNS
1001 TSSEEVETQN IILGELAQGE SFGEVEVLTA MNRYSTIVAV RDSEARIPR
1051 TLFELLALEH PSIMIRVSRL VAKKIVGDRT VPALTGDPLS IKENDFTSLI
1101 PPTKASYSSS LSHKPQNITS GTITFRITIT LPITSGLPVE AFAMKLVQAF
1151 KQVGRTTIGL NQRTTLTHLG RHAFDRLSKL KQSGYFAELE EMYQTVVYIS
1201 DTPVKSNNWR TCIAQGCIL LLADARSPSA EIGEYKLLL NSKTTARTEL
1251 ILLHPERYVE PGLTHKWLRY RPWVHSHHHI QFSLTGTTLM NEGKMHVLNN
1301 GALALMDKLI QTEFSRKTQQ NISKLLPDSI KNTVENFSSR FMKSKRQYYT
1351 PVHRHKNDLF RLARILSGQA IGLVLGGGGA RGISHLGVIQ AIEEQGIPVD
1401 VIGGTSIGSF VGGLYAKDYD LVPIYGRVKK FAGRISSIWR MLTDLTWPVT
1451 SYTTGHEFNR GIWKTFGDTR IEDFWIQYYC NSTNITDSVQ EIHSFGYAWR
1501 YIRASMSLAG LLPPLEENG MLLDGGYVDN LPVTEMRARG CQTIFAVDVG
1551 SADDRTPMEY GDSLNGFWII FNRWNPFSH PNIPNMAEIQ VRLGYVASVN
1601 ALEKAKNTPG VVYVRPPIEE YATLDFSKFE EIHVGVVDYG RIFLQGLIDD
1651 DKMPYIPGSQ ETTLNSQVPE FLLHRRNSI

```

YOL4_CAEEL

```

ID  YOL4_CAEEL      STANDARD;      PRT;  1351 AA.
AC  Q02331;
DT  01-FEB-1994 (REL. 28, CREATED)
DT  01-FEB-1994 (REL. 28, LAST SEQUENCE UPDATE)
DT  01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE  HYPOTHETICAL 152.4 KD PROTEIN ZK370.4 IN CHROMOSOME III.
GN  ZK370.4.
OS  CAENORHABDITIS ELEGANS.
OC  EUKARYOTA; METAZOA; ACOELOMATES; NEMATODA; SECERNENTEA; RHABDITIDA.
RN  [1]
RP  SEQUENCE FROM N.A.
RC  STRAIN=BRISTOL N2;
RX  MEDLINE; 94150718.
RA  WILSON R., AINSCOUGH R., ANDERSON K., BAYNES C., BERKS M.,
RA  BONFIELD J., BURTON J., CONNELL M., COPSEY T., COOPER J., COULSON
A.,
RA  CRAXTON M., DEAR S., DU Z., DURBIN R., FAVELLO A., FRASER A.,
RA  FULTON L., GARDNER A., GREEN P., HAWKINS T., HILLIER L., JIER M.,
RA  JOHNSTON L., JONES M., KERSHAW J., KIRSTEN J., LAISSTER N.,
RA  LATREILLE P., LIGHTNING J., LLOYD C., MORTIMORE B., O'CALLAGHAN M.,
RA  PARSONS J., PERCY C., RIFKEN L., ROOPRA A., SAUNDERS D., SHOWNKEEN
R.,
RA  SIMS M., SMALDON N., SMITH A., SMITH M., SONNHAMMER E., STADEN R.,
RA  SULSTON J., THIERRY-MIEG J., THOMAS K., VAUDIN M., VAUGHAN K.,
RA  WATERSON R., WATSON A., WEINSTOCK L., WILKINSON-SPROAT J.,
RA  WOHLDMAN P.;
RL  NATURE 368:32-38(1994).
CC  -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
DR  EMBL; M98552; G156504; -.
DR  WORMPEP; ZK370.4; CE00396.
DR  PROSITE; PS01237; UPF0028; 1.
KW  HYPOTHETICAL PROTEIN.
SQ  SEQUENCE 1351 AA; 152392 MW; 8B1CCF16 CRC32;

```

YOL4_CAEEL Length: 1351 February 11, 1998 15:57 Type: P Check: 7001

..

```

1  MSFFYSLFAP LIFLVTFIYN HVLLILFTVC IIGAAFFVS YYLFGYSNTP
51  SSASSSATPS SRNSPNSSELF DSQYIIISCF DRITEERSKK VPTILETDNE
101 DDEIRVNGSP KSGTPTNTQT IEPPTSILNL MVNSASGSNL SGARRMRKRD
151 WAKKLYKSLV QDSPGRTPTD ESSDEENANV VMGGGSVPRR RSKHGNSSRR
201 RQSTAFQLAK DLIRGSRYSY FRQEQENNKD TRVRPPQEFF EPTDLPEIPQ
251 NLQPEIFYIL HNLKMLELPS EWKLDPREIE VRSFQAGDYI VKPGESDDAI
301 YVAIDGELTV HIRHMEGKEY LVKCIPAGGS FFSLLSMLDI LMDFPSIFRT

```

```

351 VALKAADPCR VAKFPITSFR ESYHKNPEAW IRPIQIVITR LLHVTLTTLH
401 QYMGLSSELM RRTTLPRFFF EKRKKISKFL VAYLFSYEQD QLIVARKWMA
451 EAFGLVADDV VSEQIGSKIH LESYEAGHVI IEQGAEVEVL MMVLHGNNLIL
501 AQESLFDEEN NEEDESAVIR VTARELVGGL QILTNEPSFY TIRAAVQTRV
551 AIMKKKDFSA FLEAHPEIYL PVAHSVLRRL SPFLRGVDFA LDWVLVDSGH
601 ACYRAGDMAD SLFVVLSGRL RSVEKKTVE EFGRGDVLGM MEVLTKKPRA
651 TTVLAVRFSQ LARVPEGLN FIKMQYPQVG FRLVQLLGQY YSQTNRAPF
701 AAPTIVRTNE LGATDPMSHI KNLHTIAVVP ASPDVPLVPF TCELYHALSS
751 NLRVLRLLSQ KVAACLDPSV LEKQADFRLM HWLNVQEDTY PLVIYECDF
801 QTNWTRRCLR QADAILVVAI GGKTPEKQTL MRELMNMNQD GVRTNKLIL
851 LWPESTKTPS GTIEWLKNSY FSGHHHIRAP KRMLQWNRKF RKMSRAEVM
901 PTSVENEVIE YYEKNVFWTP DRRSDFSRLA RILTGNAIGL VLGGGGGARGA
951 AHVGVLRALR EEGIPVDIVG GTSIGSLIGG LYAETPDDV VETRAASWFN
1001 GMSSLWRKLL DLTIAHSAMF TGAQFNFSIK DLFEERLIED LWISYFCIST
1051 DISTSEMRVH RSGPLWAYCR ASMSLAGYLP PLCDPQDGH LLDGGYVNNV
1101 PADVMRNLGA RCVIACDVGS IEETNLYDYG DSLSGMWVLL KRLNPFGTTP
1151 RILNMEEIQS RLAYVSCVRQ LEVVKKASYC RYLRPPIEPF KTLDFPKFQE
1201 IMELGLKYGR EACHELITND RAGILGDEKE TRKFKRQQSR REKPDVSRV
1251 SFTDLAAAMS KIPVVRPTLR HSMSLNPSAN GPVGRAGDHF LLDDDLFNDT
1301 DYYEEESSQS ENGNESTED EDLVIGPPSS SSSGGNVSEN PRGTTTPRPS
1351 S

```

YVL7_CAEEL

```

ID YVL7_CAEEL STANDARD; PRT; 880 AA.
AC Q21534; Q20023;
DT 01-NOV-1997 (REL. 35, CREATED)
DT 01-NOV-1997 (REL. 35, LAST SEQUENCE UPDATE)
DT 01-NOV-1997 (REL. 35, LAST ANNOTATION UPDATE)
DE HYPOTHETICAL 100.1 KD PROTEIN M110.7 IN CHROMOSOME II.
GN M110.7.
OS CAENORHABDITIS ELEGANS.
OC EUKARYOTA; METAZOA; ACOELOMATES; NEMATODA; SECERNENTEA; RHABDITIDA.
RN [1]
RP SEQUENCE OF 1-560 FROM N.A.
RC STRAIN=BRISTOL N2;
RA THOMAS K.;
RL SUBMITTED (JUN-1995) TO EMBL/GENBANK/DBJ DATA BANKS.
RN [2]
RP SEQUENCE OF 521-880 FROM N.A.
RC STRAIN=BRISTOL N2;
RA BAYNES C.;
RL SUBMITTED (JUN-1995) TO EMBL/GENBANK/DBJ DATA BANKS.
CC -!- SIMILARITY: BELONGS TO THE UPF0028 FAMILY.
DR EMBL; Z49968; G1067008; -.
DR EMBL; Z49966; G886444; -.
DR WORMPEP; M110.7; CE03510.
DR PROSITE; PS01237; UPF0028; 1.
KW HYPOTHETICAL PROTEIN.
SQ SEQUENCE 880 AA; 100053 MW; E4477FC7 CRC32;

```

YVL7_CAEEL Length: 880 February 11, 1998 15:57 Type: P Check: 2638

```

..
1 MTPDKKRDSS EKISKQPPRE LFEPNEQEQV PSHIKPEIFF VLKALEGLEL
51 PTTWQLDPKD VETLSIDTGS VVLSPPGRAND VIVVVISGEL GIFTNVSLNE
101 KPNNKYIHLK ALTSCRVATY HLTSFHTSFI ANPQQWIRTI QVVMTRLQOC
151 TLITCNMYLG IGGKCLNAGD LEHDDTGTSL TFDVYPGDML PSMQILTNEP
201 AMCSAKALEK TIYFKICRDE YIQFLFAHPV IYLRALAFHAL QFISPFARV
251 DMAVHWHRIE TGQALFRQGD KSDSMHIVMG GRLRAVDSTK IIEEYGRDL
301 IGITDMAEKR PRRNTVMAVR FSHIVCIPEN LLSFVKIRYP QVNSARWSRR
351 DFTEFSASRR VPLTEFTCEL YNQLSKHVKT LRLSSSVVEN YFESEVITKK
401 ADYGLMHWLN VQEIAYSLVL YQCDFHKTNW TRRCLRMADA ILMVALGTES

```


451 KEEQVLAEAL LSCNEKGVRO SKELVFLWPI DTPTPSGTAA WIKESYYSGY
501 HHLRAPNRLF SFPLKTREKK IVEYYETTVY GEISYQSDFS RLARILTGNA
551 IGIVFGGGGA RGAAGAGALR ALIEKKVQID MVGGTSIGAL FGSLYATTPD
601 IRAVGRMKDF FTDRLRNNIL DVVRDLTPY CGILTGHFRN LCVQRMNDV
651 NIEDCWVSFF CITDTLTSSS MRIHRNGIMW PVVRSSMSIA GYVPPICDPQ
701 DGHLLLDGAY VNNLPADIMR SLGANVVIAI DVGMSDDNTN LRNYGFSISG
751 TWCLFKRWWP FGEELRVLM NEVQNRLAYV CCVNQMEIVK NAQYCYVVKL
801 PIESFGIFDF SKFDQAAQIG YDITKQKMEE FFEDSVATRR KLLGCARNVR
851 QTPQKSKNDN ILSFVNMPVL PKPPSDIKSD

Appendix 3 Gapped BLAST search of Swissprot

This search used NTE1 to search SWISSPROT, it misses some of the sequences known to contain an NTE like esterase domain as they had not yet entered the SWISSPROT database.

BLAST Search Results

Commencing search, please wait for results.
BLASTP 2.0.3 [Nov-14-1997]

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schälffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= NTE (1327 letters)

Database: Non-redundant SwissProt sequences
68,512 sequences; 24,689,586 total letters

Searching.....done
Distribution of 100 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
Q02331 YOL4_CAEEL HYPOTHETICAL 152.4 KD PROTEIN ZK370.4 IN C...	680	0.0
Q21534 YVL7_CAEEL HYPOTHETICAL 100.1 KD PROTEIN M110.7 IN CH...	503	e-141
Q04958 YMF9_YEAST HYPOTHETICAL 187.1 KD PROTEIN IN OGG1-CNA2...	452	e-126
Q50733 Y0A3_MYCTU HYPOTHETICAL 62.1 KD PROTEIN CY9C4.03C	119	2e-26
P37053 YCHK_ECOLI HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU I...	103	2e-21
P37054 YCHK_SHIFL HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU I...	101	8e-21
P05987 KAPR_DICDI CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	57	1e-07
P31319 KAPR_APLCA CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	53	3e-06
P07278 KAPR_YEAST CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	52	6e-06
P31321 KAP1_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE I-BETA ...	51	1e-05
P09456 KAP0_RAT CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA R..	50	3e-05
P00514 KAP0_BOVIN CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA...	50	3e-05
P49605 KAPR_USTMA CAMP-DEPENDENT PROTEIN KINASE TYPE II REGU...	49	4e-05
P10644 KAP0_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA...	48	7e-05
P16905 KAPR_DROME CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	48	7e-05
P31322 KAP3_BOVIN AMP-DEPENDENT PROTEIN KINASE TYPE II-BETA...	47	2e-04
P07802 KAP0_PIG CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA R.	47	2e-04
P12849 KAP1_MOUSE AMP-DEPENDENT PROTEIN KINASE TYPE I-BETA ...	47	2e-04
P47200 FNRA_PSEST TRANSCRIPTIONAL ACTIVATOR PROTEIN FNRA	46	4e-04
P30625 KAPR_CAEEL CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	46	5e-04
P31323 KAP3_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE II-BETA...	46	5e-04
P23926 ANR_PSEAE TRANSCRIPTIONAL ACTIVATOR PROTEIN ANR	45	6e-04
P12369 KAP3_RAT CAMP-DEPENDENT PROTEIN KINASE TYPE II-BETA R.	45	6e-04
P37428 FNR_SALTY FUMARATE AND NITRATE REDUCTION REGULATORY P..	45	0.001
P03019 FNR_ECOLI FUMARATE AND NITRATE REDUCTION REGULATORY P..	45	0.001
Q90980 CNG3_CHICK CYCLIC NUCLEOTIDE GATED CHANNEL, ROD PHOTO...	44	0.001
P45199 FNR_HAEIN FUMARATE AND NITRATE REDUCTION REGULATORY P..	44	0.001
Q50734 Y0A4_MYCTU HYPOTHETICAL ABC TRANSPORTER ATP-BINDING P...	44	0.002
Q03041 CNG2_BOVIN CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL ...	43	0.004
Q00195 CNG2_RAT CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL (C.	43	0.004
Q28718 CNG2_RABIT CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL ...	43	0.004
P31320 KAPR_BLAEM CAMP-DEPENDENT PROTEIN KINASE REGULATORY C...	42	0.005
Q24278 CNG_DROME CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL (CNG..	42	0.005
Q29441 CNG3_BOVIN CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 3 (...	42	0.007
P13861 KAP2_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPH...	41	0.009
Q90805 CNG1_CHICK CYCLIC NUCLEOTIDE GATED CHANNEL, CONE PHOT...	41	0.009
Q62398 CNG2_MOUSE CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL ...	41	0.009
P29973 CNG1_HUMAN CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC ...	41	0.009
Q00194 CNG1_BOVIN CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC ...	41	0.012
P29974 CNG1_MOUSE CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC ...	41	0.012
Q62927 CNG1_RAT CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NU.	41	0.012
Q28279 CNG1_CANFA CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC ...	41	0.012
Q03043 KGP2_DROME CGMP-DEPENDENT PROTEIN KINASE, ISOZYME 2 F...	41	0.016

P46148 ETRA_SHEPU	ELECTRON TRANSPORT REGULATOR A	40	0.020
P32023 KGP3_DROME	CGMP-DEPENDENT PROTEIN KINASE, ISOZYME 2 F...	40	0.020
P23619 HLYX_ACTPL	REGULATORY PROTEIN HLYX	40	0.027
Q14028 CNG4_HUMAN	CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 4 (...)	40	0.027
Q28181 CNG4_BOVIN	240K PROTEIN OF ROD PHOTORECEPTOR CNG-CHAN...	40	0.027
Q03611 YN82_CAEEL	HYPOTHETICAL 88.6 KD PROTEIN ZC84.2 IN CHR...	39	0.035
P12368 KAP2_RAT	CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPHA...	39	0.046
P00515 KAP2_BOVIN	CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPH...	38	0.14
Q64359 CNGX_RAT	CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL OC.	37	0.18
P55934 CNG ICTPU	CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL	37	0.23
P36165 YK69_YEAST	HYPOTHETICAL 102.7 KD PROTEIN IN PRP16-SRP...	37	0.23
P12367 KAP2_MOUSE	CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPH...	37	0.23
P54513 YQHO_BACSU	HYPOTHETICAL 32.9 KD PROTEIN IN GCVT-SPOII...	36	0.30
P06170 CRP_SALTY	CATABOLITE GENE ACTIVATOR (CAMP RECEPTOR PR..	36	0.30
P24290 FX24_RHILV	PROBABLE TRANSCRIPTIONAL ACTIVATOR (ORF-240)	36	0.30
P29283 NTCA_SYN7	GLOBAL NITROGEN REGULATOR	36	0.30
Q16280 CNG2_HUMAN	CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL ...	36	0.30
P03020 CRP_ECOLI	CATABOLITE GENE ACTIVATOR (CAMP RECEPTOR PR..	36	0.40
P34578 YNX5_CAEEL	HYPOTHETICAL 139.4 KD PROTEIN T20G5.5 IN C...	36	0.40
P40308 YM93_YEAST	HYPOTHETICAL 73.6 KD PROTEIN IN GLC8-PRE5 ...	36	0.52
P46908 FNR_BACSU	ANAEROBIC REGULATORY PROTEIN	36	0.52
P43564 YFD4_YEAST	HYPOTHETICAL 119.5 KD PROTEIN IN MOB2-RIM1...	35	0.68
P33779 NTCA_SYN3	GLOBAL NITROGEN REGULATOR	35	0.90
Q10898 Y05N_MYCTU	HYPOTHETICAL 53.5 KD PROTEIN CY251.23	35	0.90
P12624 MACS_BOVIN	MYRISTOYLATED ALANINE-RICH C-KINASE SUBSTR...	34	1.2
P29281 CRP_HAEIN	CATABOLITE GENE ACTIVATOR (CAMP RECEPTOR PR..	34	1.5
P00516 KGPB_BOVIN	CGMP-DEPENDENT PROTEIN KINASE, ALPHA ISOZY...	34	1.5
P37882 CGB1_MESAU	G2/MITOTIC-SPECIFIC CYCLIN B1	33	2.6
P14619 KGPB_HUMAN	CGMP-DEPENDENT PROTEIN KINASE, BETA ISOZYM...	33	3.5
P22770 ACH7_CHICK	NEURONAL ACETYLCHOLINE RECEPTOR PROTEIN, A...	33	3.5
Q16281 CNG3_HUMAN	CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 3 (...)	33	3.5
P29286 FIXK_BRAJA	NITROGEN FIXATION REGULATION PROTEIN FIXK	32	4.5
P32492 MYS4_YEAST	MYOSIN-4 ISOFORM	32	4.5
Q08530 BTR_BORPE	TRANSCRIPTIONAL REGULATORY PROTEIN BTR	32	5.9
P55222 VFR_PSEAE	CYCLIC AMP RECEPTOR-LIKE PROTEIN	32	5.9
P20689 KMLC_RAT	MYOSIN LIGHT CHAIN KINASE, SKELETAL MUSCLE (.	32	5.9
P50576 HMD4_BRARE	HOMEODOMAIN PROTEIN DLX-4	32	5.9
Q28983 ZAN_FIG	ZONADHESIN PRECURSOR	32	5.9
P50577 DLX5_CHICK	HOMEODOMAIN PROTEIN DLX-5	32	7.8

sp|Q02331|YOL4_CAEEL HYPOTHETICAL 152.4 KD PROTEIN ZK370.4 IN CHROMOSOME III
Length = 1351

Score = 680 bits (1736), Expect = 0.0
Identities = 381/810 (47%), Positives = 515/810 (63%), Gaps = 44/810 (5%)

Query: 476 LNSRVLLHHAAGTIIARQGDQDVSLHFLVWGCLHVVYQRM-----DKAEDVCLFVAQPG 530
+ S++ L +AG +I QG ++ L VL G L + Q + ++ ++ +
Sbjct: 465 IGSKIHLSEYEAHVIEQGAEEVLMMLVHGNLILAQESLFDEENNEEDSAVIRVTAR 524

Query: 531 ELVGQLAVLTGEPLIFTLRAQRDCTFLRISKSDFYIEIMRAQPSVVLSSAAHTVAARMSPFV 590
ELVG L +LT EP +T+RA + K DF + A P + L AH+V R+SPF+
Sbjct: 525 ELVGGLQILTNEPSFYTIRAAVQTRVAIMKKKDFSAFLEAHPEIYLPVAHSVLRRLSPFL 584

Query: 591 RQMDFAIDWTAVEAGRALYRQGDSDCTYIVLNGRLRSVIQSGGKELVGEYGRGDLIG 650
R +DFA+DW V++G A YR GD +D ++VL+GRLRSV KK +V E+GRGD++G
Sbjct: 585 RGVDFALDWVLVDVSGHACYRAGDMADSLFVVLSGRLRSV-----EKKTVEEFGRGDVLG 639

Query: 651 VVEALTRQPRATTVHAVRDTLAKLPEGTGLHIKRRYPQVVTRLIHLSSQKILGNLQQLQ 710
++E LT++PRATTV AVR ++LA++PEG L IK +YPQV RL+ LL Q + +
Sbjct: 640 MMEVLTKKPRATTVLAVRFSQLARVPEGLNFIKMQYPQVGFRLVQLLGQYY--SQTNRR 697

Query: 711 GPFPAAGSLGVPPHSEL--TNPAS---NLATVAILPVCAEVPMAFTLELQHALQAIGPT 765
PF A + + +EL T+P S NL T+A++P +VP+V FT EL HAL +
Sbjct: 698 APFAAPTIVIRT---NELGATDPMISHIKNLHTIAVVPASPDVPLVPFTCELYHALSSNLRV 754

Query: 766 LLLNSDIIRARLGASALDSIQEFRLSGWLAQQEDAHRIVLYQTDASLTPWTVRCLRQADC 825
L L+S + A L S L+ +FRL WL QED + +V+Y+ D + T WT RCLRQAD
Sbjct: 755 LRLSSQKVAACLDPSVLEKQADFRMLMHWLNQEDTYPLVIYECDFQTQTNWTRRCLRQADA 814

Query: 826 ILIVGLGDQEPTLGQLEQMLENT---AVRALKQLVLLHREEGAGPTRTVEWLNMRSWCSG 882
 IL+V +G + P L + L N VR K+L+LL E P+ T+EWL S+ SG
 Sbjct: 815 ILVVAIGGKTPEKQTLMLRELMNMNQDGVRTNKEILLWPESTKTPSGTIEWLK-NSYFSG 873

Query: 883 HLHLRCPRRL-----FSRRSPAKLH-----ELYEKVFSRRADRHSDFSRLARVL 926
 H H+R P+R+ F + S A++ E YEK DR SDFSRLAR+L
 Sbjct: 874 HHHIRAPKRMLQWNRKFRKMSRAEVMTPTSVENEVIEYKENVFWTPDRRSDFSRLARIL 933

Query: 927 TGNTIALVLGGGGARGCSHIGVLKALEEAGVPVDLVGGTSIGSFIGALYAEERSASRTKQ 986
 TGN I LVLGGGGARG +H+GVL+AL E G+PVD+VGGTSIGS IG LYAE +
 Sbjct: 934 TGNAIGLVLGGGGARGAAHVGVLRALREEGIPVDIVGGTSIGSLIGGLYAE TPDDVVVET 993

Query: 987 RAREWAKSMTSVLEPVLDTYPTVSMFTGSAFNRSIHRVFQDKQIEDLWLPYFNVTTDIT 1046
 RA W M+S+ +LDLTY ++MFTG+ FN SI +F+++ IEDLW+ YF ++TDI+
 Sbjct: 994 RAASWFNGMSSLWRKLLDLTYAHSAMFTGAQFNFSIKDLFEERLIEDLWISYFCISTDIS 1053

Query: 1047 ASAMRVHKDGLWRYVRASMTLSGYLPPLCDPKDGHLLMDGGYINNLPADIARSMGAKTV 1106
 S MRVH+ G LW Y RASM+L+GYLPPLCDP+DGHLL+DGGY+NN+PAD+ R++GA+ V
 Sbjct: 1054 TSEMRVHRSGPLWAYCRASMSLAGYLPPLCDPQDGHLLLDGGYVNNVPADVMRNLGARC 1113

Query: 1107 IAIDVGSQDETDLSTYGDLSLWLLWKRLNPWADKVKVPDMAEIQSRLAYVSCVRQLEV 1166
 IA DVGS +ET+L YGDSLSG W+L KRLNP+ ++ +M EIQSRLAYVSCVRQLEV
 Sbjct: 1114 IACDVGSIEETNLYDYGDLSGMWVLLKRLNPFGTTPRIILMEEIQQSRLAYVSCVRQLEV 1173

Query: 1167 VKSSSYCEYLRPPIIDCFKTMDFGKFQDIYDVGYQYKAVFGGWSRGNVIEKMLTDRRST- 1225
 VK +SYC YLRPPI+ FKT+DF KF +I ++G +YG+ N +L D + T
 Sbjct: 1174 VKKASYCRYLRPPIEPFKTLDFPKFQEIIMELGLKYGREACHELIT-NDRAGILGDEKETR 1232

Query: 1226 --DLNESRRADVLAFFSSGFTDLAEIVSRI 1253
 +SRR + FTDLA +S+I
 Sbjct: 1233 KFKRQQSRREKPDVSRVSTDLAAAMSKI 1262

Score = 106 bits (262), Expect = 2e-22

Identities = 87/286 (30%), Positives = 134/286 (46%), Gaps = 40/286 (13%)

Query: 49 RFRKRDKVLFYGRKIMRKVSQSTSSLVDTSVSAT-----SRPRMRKK----- 90
 R RKRD + +K+ + + Q + T S+ S PR R K
 Sbjct: 145 RMRKR-----WAKKLYKSLVQDSPGRTPTESSDEENANVVMGGGSVPRRRSKHGNSSRR 200

Query: 91 --LKMLNIAKKILRIQKETPTLQKE-----PPPAVLE-ADLTEGDLANSHLPSEVL 139
 +AK ++R + Q +E PP E DL E +L E+
 Sbjct: 201 RQSTAFQLAKDLIRGRSRYFRQENNKDTRVRPPQEFFEPTDLPE---IPQNLQPEIF 257

Query: 140 YMLKNVRVLGHFEKPLFLELC-RHMFVQRLGQGDYVFRPGQPDASIYVVQDGLLELCLPG 198
 Y+L N+++L E P +L R + + GDY+ +PG+ D +IYV DG L + +
 Sbjct: 258 YILHNLKML---ELPSEWKLDPREIEVRSFQAGDYIVKPGESDDAIYVAIDGELTVHIRH 314

Query: 199 PDGKECVVKEVVPGDVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFT 258
 +GKE +VK + G S SLLS+LD++ RTV+ +AA V + P+ +F +
 Sbjct: 315 MEGKEYLVKICIPAGGSFFSLLSMLDILMDFPSIFRTVALKAADPCRVAKFPITSFRESYH 374

Query: 259 KPESLVRVVQIIMVRLQRTFLALHNYLGLTNEFSHEIQPLRLF 304
 K PE+ +R +QI++ RL VT LH Y+GL++EL P F
 Sbjct: 375 KNPEAWIRPIQIVITRLLHVTTLTHQYMGLSSELMRRFTLPRFFF 420

Score = 34.0 bits (76), Expect = 1.5

Identities = 28/116 (24%), Positives = 51/116 (43%), Gaps = 15/116 (12%)

Query: 597 IDWTAVEAGRALYRQGDSDCTYIVLNGRLRSVIQSGGKELVGEYGRG----DLIGVV 652
 I+ + +AG + + G+ D Y+ ++G L I+ GK+ LV G L+ ++
 Sbjct: 279 IEVRSFQAGDYIVKPGESDDAIYVAIDGELTVHIRHMEGKEYLVKICIPAGGSFFSLLSML 338

Query: 653 EALTRQP---RATTVHAVRDTELAKLPEGTLGHIKRRYPQ-----VVTRLIHL 697
 + L P R + A +AK P + + P+ V+TRL+H+
 Sbjct: 339 DILMDFPSIFRTVALKAADPCRVAKFPITSFRESYHKNPEAWIRPIQIVITRLLHV 394

Score = 34.0 bits (76), Expect = 1.5
Identities = 24/117 (20%), Positives = 52/117 (43%), Gaps = 6/117 (5%)

Query: 592 QMDFAIDWTAVEAGRALYRQGDSDCTYIVLNGRL----RSVIQRGSGKKE--LVGEYGR 645
Q+ I + EAG + QG + +VL+G L S+ + +++ V
Sbjct: 464 QIGSKIHLESYEAGHVIIIEQGAEVEVLMMVLHGNLILAQESLFDEENNEEDESAVIRVTA 523

Query: 646 GDLIGVVEALTRQPRATTVHAVRDTLAKLPEGTLGHIKRRYPQVVTRLIHLLSQKI 702
+L+G ++ LT +P T+ A T +A + + +P++ + H + +++
Sbjct: 524 RELVGGQLILTNEPSFYTIRAAVQTRVAIMKKKDFSAFLEAHPEIYLPVAHSVLRRL 580

Score = 32.8 bits (73), Expect = 3.5
Identities = 28/101 (27%), Positives = 48/101 (46%), Gaps = 12/101 (11%)

Query: 171 GDYVFRPGQPDASIYVVQDGLLELCLPGPDGKECVVKEVVPDGSVNSLLSILDVITGHQH 230
G +R G S++VV G L K+ VV+E GD +L +++V+T
Sbjct: 599 GHACYRAGDMADSLFVVLGRLRSV-----EKKTVEEFGRGD----VLGMEVLT---K 646

Query: 231 PQRVTSARAARDSTVLRRLPVEAFSAVFTKYPESLVRVQII 271
R + A R S + R+P + + +YP+ R+VQ++
Sbjct: 647 KPRATTVLAVRFSQLARVPEGLLNFIKMQYPQVGFRVLQLL 687

sp|Q21534|YVL7_CAEEL HYPOTHETICAL 100.1 KD PROTEIN M110.7 IN CHROMOSOME II
Length = 880

Score = 503 bits (1280), Expect = e-141
Identities = 279/706 (39%), Positives = 400/706 (56%), Gaps = 55/706 (7%)

Query: 508 CLHVVQRMIDKAEDVCLFVAQPGELVGLAVLTGEPLIFTLRAQRDCTFLRISKSDFYEI 567
CL+ D F PG+++ + +LT EP + + +A + +I + ++ +
Sbjct: 165 CLNAGDLEHDDTGTSLTFDVYPGDMPLPSMQILTNEPAMCSAKALEKTIYFKICRDEYIQF 224

Query: 568 MRAQPSVVLSSAHTVAARMSPFVRQMDFAIDWTAVEAGRALYRQGDSDCTYIVLNGRLR 627
+ A P + L A +SPF R D A+ W +E G+AL+RQGD+SD +IV+ GRRL
Sbjct: 225 LFAHPVIYLRALFAHALQFISPFARVFDMAVHWHRIETGQALFRQGDKSDSMHIVMGGRRL 284

Query: 628 SVIQRGSGKKELVGEYGRGDLIGVVEALTRQPRATTVHAVRDTLAKLPEGTLGHIKRRY 687
+V +++ EYGR DLIG+ + ++PR TV AVR + + +PE L +K RY
Sbjct: 285 AV-----DSTKIIIEYGRDLIGITDMAEKPRPRNTVMVRFSHIVCIPENLLSFVKIRY 339

Query: 688 PQVVTRLIHLLSQKILGNLQQLQGPFPAAGSLGVPPHSELTNPASNLATVAILPVCAEVP 747
PQV + + + F S VP
Sbjct: 340 PQV-----NSARWSRRDFTFSA-----SRRVP 362

Query: 748 MVAFTLELQHALQAIGPTLLLSNDIIRARLGASALDSIQEFRLSGWLAQQEDAHRIVLYQ 807
+ FT EL + L TL L+S ++ + + ++ L WL QE A+ +VLYQ
Sbjct: 363 LTFEFTCELYNQLSKHVKTLLRLSSSVVENYFESEVITKKADYGLMHWLNQEIAYSLLVLYQ 422

Query: 808 TDASLTPTVTRCLRQADCILIVGLGDQ---EPTLGQLEQMLENTAVRALKQLVLLHREEG 864
D T WT RCLR AD IL+V LG + E L + VR K+LV L +
Sbjct: 423 CDFHKTNWTRRCLRMADAILMVALGTESKEEQVLAEALLSCNEKGVRSKELVFLWPIDT 482

Query: 865 AGPTRTVEWLNMRWSCSGHLHLRCPRRLFS---RRSPAKLHELKVFSSRRADRHSDFSR 921
P+ T W+ S+ SG+ HLR P RLFS + K+ E YE SDFSR
Sbjct: 483 PTPSGTAAWIK-ESYYSYHHLRAPNRLFSFPLKTREKKIVEYYETTVYGEISYQSDFSR 541

Query: 922 LARVLTGNTIALVLGGGGARGCSHIGVLKALEEAGVPVDLVGGTSIGSFIGALYA---EE 978
LAR+LTGN I +V GGGGARG +H G L+AL E V +D+VGGTSIG+ G+LYA +
Sbjct: 542 LARILTGNAIGIVFGGGGARGAAHAGALRALIEKKVQIDMVGGTSIGALFGSLYATTPDI 601

Query: 979 RSASRTKQRRAREWAKSMTSVLEPVLDTYPVTSMTFTGSAFNRSIHRVFQDKQIEDLWLPY 1038
R+ R K + ++ ++L+ V DLT+P + TG FN + R+ D IED W+ +
Sbjct: 602 RAVGRMKDFFTDRLRN--NILDVVRDLTWPYCGILTGHFRNLQVQRLNDVNIEDCWVSF 659

Query: 1039 FNVTTDITASAMRVHKDGSLSWRYVRASMTLSGYLPPLCDPKDGHLLMDGGYINNLPADIA 1098
F +TTD+T+S+MR+H++G +W VR+SM+++GY+PP+CDP+DGHLL+DG Y+NNLPADI
Sbjct: 660 FCITTDLTSSSMRIHRNGIMWPVVRSSMSIAGYVPPICDPQDGHLLLDGAYVNNLPADIM 719

Query: 1099 RSMGAKTVIAIDVG-SQDETDLSTYGDSLGSWLLWKRLNPWADKVKVPDMAEIQSRLAY 1157
 RS+GA VIAIDVG S D T+L YG S+SG W L+KR P+ ++++V +M E+Q+RLAY
 Sbjct: 720 RSLGANVVIAIDVGMSDDNTNLRNYGFSISGTWCLFKRWPFGEELRVLMNEVQNRLAY 779

Query: 1158 VSCVRQLEVVKSSSYCEYLRPIDCFKTMDFGKFDQIYDVGYQYQK 1203
 V CV Q+E+VK++ YC Y++ PI+ F DF KFDQ +GY K
 Sbjct: 780 VCCVNQMEIVKNAQYCYVVKLPYESFGIFDFSQAAQIGYDITK 825

Score = 41.8 bits (96), Expect = 0.007
 Identities = 39/178 (21%), Positives = 71/178 (38%), Gaps = 32/178 (17%)

Query: 113 KEPPPAVLEADLTGDLANSHLPSEVLYMLKNVRVLGHFEKPLFLELC-RHMFVQRLGQG 171
 K+PP + E + E + SH+ E+ ++LK + L E P +L + + + G
 Sbjct: 15 KQPPRELFEPN--EQEQVPSHIKPEIFFVLKALEGL---ELPTTWQLDPKDVETLSIDTG 69

Query: 172 DYVFRPGQPDASIYVVQDGLLELCPLPGPDGKECVVKEVVPDGSVNSLLSILDVITGHQHP 231
 V PG+ + I VV G L + +V +
 Sbjct: 70 SVVLSPPGRANDVIVVVISGELGI-----FTNVSLNEKPN 103

Query: 232 QRTVSARAARDSTVLRPLVEAFSAVFTKYPESLVRVQIIMVRLQRVTFALHNYLGL 289
 + + +A V + +F F P+ +R +Q++M RLQ+ T + + YLG+
 Sbjct: 104 NKYIHLKALTSCRATYHLTSFHTSFIANPQQWIRTQVVMTRLQQCTLTICNMVYLG 161

**sp|Q04958|YMF9_YEAST HYPOTHETICAL 187.1 KD PROTEIN IN OGG1-CNA2 INTERGENIC
 REGION
 Length = 1679**

Score = 452 bits (1150), Expect = e-126
 Identities = 278/855 (32%), Positives = 440/855 (50%), Gaps = 110/855 (12%)

Query: 459 AAKQELAKLMRIEDPSL-LNSRVLLHHAAGTIIARQGDQDVSLHFLWGCLHVVYQRM-- 515
 A K+EL + ED + L K GT I Q + L +++ G ++V
 Sbjct: 797 AYKEELTPNLDLFEDVKKDFAQGIQLKFFKKTIVEQNARGKGLFYIISGKVNVTNSSS 856

Query: 516 -----IDKAEDVC-----LFVAQPGELVGQLAVLTGEPLIFTLRAQRDC 554
 + K E V L+ G +VG L+ L G + A+ D
 Sbjct: 857 SVVSSMSKPEQVSAQSSHKGENPHHTQHLLYSVSGSGGIVGYLSSSLIGYKSFVNIVAKSDV 916

Query: 555 TFLRISKSDFYEIMRAQPSVVLSAAHTVAARMSPFVRQMDFAIDWTAVEAGRALYRQGD 614
 +S + + L + ++ +S + ++D A++W + A L+ QGD
 Sbjct: 917 YVGFLLSATLERLFDKYFLIYLRISDSLTKLLSSRLKLKLDHALEWVHLRASETFLFSQGDS 976

Query: 615 SDCTYIVLNGRLRSVIQRGSGKKE-----LVGEYGRGDLIGVVEALTRQPRATT 663
 ++ Y+VLNGRLR + Q+ ++GE +G+ G VE LT R +T
 Sbjct: 977 ANGIYVVLNGRLRQLQQQSLSNSNTSSEEVETQNIILGELAQGESFGEVEVLTAMNRYST 1036

Query: 664 VHVRDTELAKLPEGLTGHKRRYPQVTRLIHLLSQKILGNLQQIQQGPFPAAGSLGV-- 721
 + AVRDELA++P + +P ++ R+ L+++KI+G+ P G L +
 Sbjct: 1037 IVAVRDESELARIPRTLFELLALEHPSIMIRVSRLVAKKIVGDRTV---PALTDGPLSIKE 1093

Query: 722 -----PP-----HSELTPASNLA-----TVAILPVCAEVPMAFTLELQHALQAI 762
 PP S L++ N+ T+ ILP+ + +P+ AF ++L A + +
 Sbjct: 1094 NDFTSILPPTKASYSSSLSHKPQNITSGTITFTITILPITSGLPVEAFAMKLVQAFKQV 1153

Query: 763 GPTLL-LNSDIIRARLGASALDSIQEFRLSGWLAQQEDAHRIVLYQTDASL-TPWTVRCL 820
 G T + LN LG A D + + + SG+ A+ E+ ++ V+Y +D + + WT C+
 Sbjct: 1154 GRTTIGLNQRTTLTHLGRHAFDRLSKLGSGYFAELEEMYQTVVYISDTPVKSNWTRTCI 1213

Query: 821 RQADCILIVG-LGDQEPGLGLEQMLENTAVRALKQLVLLHREAGPTRTVEWLNMRSW 879
 Q DCIL++ +G+ E++L N+ A +L+LLH E P T +WL R W
 Sbjct: 1214 AQGDCILLADARSPSAEIGEYKLLLSKTTARTELILLHPERYVEPGLTHKWLRYRPW 1273

Query: 880 CSGHLHLRCPRRLFSSRRSPAKLH-----ELYEKVFSRRADRH----- 916
 H H++ + + K+H +L + FSR+ ++
 Sbjct: 1274 VSHHHHIQFSLTGTTLMNEGKMHVLNNGALALMDKLIQTEFSRKTQQNISKLLPDSIKNT 1333

Query: 917 -----SDFSRLARVLTGNTIALVLGGGGARGCSHIGVLKALE 953
 +DF RLAR+L+G I LVLGGGGARG SH+GV++A+E
 Sbjct: 1334 VENFSSRFMKSRQYYTPVHRHKNDFLRLARILSGQAIGLVGGGGARGISHLGVIQAI 1393

Query: 954 EAGVPVDLVGGTSIGSFIGALYAEERSASRTKQRAREWAKSMTSVLEPVLDTYPVTSMF 1013
 E G+PVD++GGTSIGSF+G LYA++ R +++A ++S+ + DLT+PVTS
 Sbjct: 1394 EQGIPVDVIGGTSIGSFVGGLYAKDYDLVPIYGRVKKFAGRISSIWRMLTDLTWPVTSYT 1453

Query: 1014 TGSANRNSIHRVFQDKQIEDLWLPYFNVTDDITASAMRVHKDGLWRYVRASMTLSGYLP 1073
 TG FNR I + F D +IED W+ Y+ +T+IT S +H G WRY+RASM+L+G LP
 Sbjct: 1454 TGHEFNRIWKTFGDTRIEDFWIQYYCNSTNITDSVQEIHSFGYAWRYIRASMSLAGLLP 1513

Query: 1074 PLCDPKDGHLLMDGGYINNLPADIARSMGAKTVIAIDVGSQDETDLSTYGDSLSCGWLLW 1133
 PL ++G +L+DGGY++NLP R+ G +T+ A+DVGS D+ YGDSL+G+W+++
 Sbjct: 1514 PL--EENGSMLLDGGYVDNLPVTEMRARGCQTIFAVDVGSADDRTPMYEGDSLNGFWIIF 1571

Query: 1134 KRLNPWADKVKVPDMAEIQSRLAYVSCVRQLEVVKSSSYCEYLRPPIDCFKTMDFGKFDQ 1193
 R NP++ +P+MAEIQ RL YV+ V LE K++ Y+RPPI+ + T+DF KF++
 Sbjct: 1572 NRWNPFSSHPNIPNMAEIQVRLGYVASVNALEKAKNTPGVVYVRPPIEYATLDFSKEE 1631

Query: 1194 IYDVGYYGKAVFGG 1208
 IY VG YG+ G
 Sbjct: 1632 IYHVGVDYGRIFLQG 1646

Score = 58.2 bits (138), Expect = 8e-08
 Identities = 25/62 (40%), Positives = 40/62 (64%)

Query: 237 ARAARDSTVLRPVEAFSAVFTKYPESLVRVQIIMVRLQRVTFLALHNYLGLTNELFSH 296
 ARAA D T+ +P ++F+ + KYP S ++Q+++ +L VTF H+YLGLT E+
 Sbjct: 409 ARAATDCTIGIIPPQSFAKLTAKYPRASASHIIQMVLTKLYHVTFQTADHYLGLTKEIMDI 468

Query: 297 EI 298
 E+
 Sbjct: 469 EV 470

Score = 31.7 bits (70), Expect = 7.8
 Identities = 32/146 (21%), Positives = 61/146 (40%), Gaps = 13/146 (8%)

Query: 140 YMLKNVRVLGHFEKPL---FLELCRHMVFQRLQGQDYVFRPGQPDASIYVVQDGLLE--- 193
 Y L +R+ K L L+L + + L + +F G IYVV +G L
 Sbjct: 933 YFLIYLRISDSLTKLLSSRLKLDHALEWVHLRASETLEFSQGDSANGIYVVLNGLRLRLQ 992

Query: 194 ---LCLPGPDGKECVVKEVVPD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRP 249
 L +E + ++ G+ + ++V+T V A RDS + R+P
 Sbjct: 993 QQSLSNSNTSSEEVETQNIILGELAQGESFGEVEVLTAMNRYSTIV---AVRDESELARIP 1049

Query: 250 VEAFSAVFTKYPESLVRVQIIMVRL 275
 F + ++P ++RV +++ ++
 Sbjct: 1050 RTLFELLALEHPSIMIRVSRLVAKKI 1075

sp|Q50733|Y0A3_MYCTU HYPOTHETICAL 62.1 KD PROTEIN CY9C4.03C
Length = 583

Score = 119 bits (296), Expect = 2e-26
 Identities = 69/193 (35%), Positives = 109/193 (55%), Gaps = 6/193 (3%)

Query: 919 FSRLARVLTGNTIALVLGGGGARGCSHIGVLKALEEAGVPVDLVGGTSIGSFIGALYAE 978
 F LAR + G ++ LV+ GGGARG +H GV + L EAGV +D GGTG G+ A +A
 Sbjct: 295 FGALARRIAGRSGLVMAGGGARGLAHFGVYQELTEAGVVIDRFGGTSSGAIASAAFALG 354

Query: 979 RSASRTKQRAREWAKSMTSVLEPVLDTYPVTSMTGSAFNRSIHRVFQDKQIEDLWLPY 1038
 A ARE+ +P+ D T P++++ G +R+ F + IE L +
 Sbjct: 355 MDAGDAIAAAREFIAGS-----DPLGDTIPISALTRGGRVDRDLVQGGFGNTLIEHLPRGF 410

Query: 1039 FNVTTDDITASAMRVHKDGLWRYVRASMTLSGYLPPLCDPKDGHLLMDGGYINNLPADIA 1098
 F+V+ D+ +H+ GS+ VRAS+++ G +PP+ + + LL+DGG +NNLPA++
 Sbjct: 411 FSVSADMITGDQIIHRRGSVSGAVRASISIPGLIPPVHNGE--QLLDGGLLNNLPANVM 468

Query: 1099 RSMGAKTVIAIDV 1111
+ VI +D+
Sbjct: 469 CADTDGEVICVDL 481

Score = 43.8 bits (101), Expect = 0.002
Identities = 34/120 (28%), Positives = 51/120 (42%), Gaps = 11/120 (9%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVDGLLELCLPGPDG 201
L+NV +L + L + + + ++F G+P SIY+V G P+G
Sbjct: 19 LRNVPIADIDDEQLERLATTVERRHVPANQWLFHAGEPADSIYIVDSG--RFVAVAPEG 76

Query: 202 KECVVKEVVPGDSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKYP 261
V E+ GDS+ L VI G R+ RA RD V R+ E F+ + P
Sbjct: 77 H--VFAEMASGDSIGD----LGVIAG---AARSAGVRALRDGVVWRIAETFTDMLEATP 127

Score = 36.0 bits (81), Expect = 0.40
Identities = 24/101 (23%), Positives = 47/101 (45%), Gaps = 4/101 (3%)

Query: 602 VEAGRALYRQGDRSDCTIYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 661
V A + L+ G+ +D YIV +GR +V G + E GD IG + + R+
Sbjct: 45 VPANQWLFHAGEPADSIYIVDSGRFVAVAPEG---HVFAEMASGDSIGDLGVIAGAARS 100

Query: 662 TTVHAVRDTELAKLPEGTGLGHKRRYPQVVTRLIHLLSQKI 702
V A+RD + ++ T + P + + ++ +++ +
Sbjct: 101 AGVRALRDGVVWRIAETFTDMLEATPLLQSAMLRAMRML 141

sp|P37053|YCHK_ECOLI HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU INTERGENIC REGION

Length = 314

Score = 103 bits (255), Expect = 2e-21
Identities = 61/192 (31%), Positives = 104/192 (53%), Gaps = 10/192 (5%)

Query: 922 LARVLTGNTIALVLGGGGARGCSHIGVLKALEEAGVPVDLVGGTSIGSFIGALYAEERSA 981
LA ++ I L LG G ARG SHIGV+ AL++ G+ +D+V G SIGS +GA YA +R +
Sbjct: 10 LAGIMRKIKIGLALGSGAARGWSHIGVINALKKVGIEIDIVAGCSIGSLVGAAYACDRLS 69

Query: 982 SRTKQREAREWAKSMT--SVLEPVLDTYPVTSMTGSAFNRSIHRVFQDKQIEDLWLPHYF 1039
+ +W S + VL ++DL++ + G + + +IE+ +
Sbjct: 70 A-----LEDWVTSFSYWDVLR-LMDLSWQRGGLLRGERVFNQYREIMPETEIENCSSRRFA 123

Query: 1040 NVTTDITASAMRVHKDGLRWYVRASMTLSGYLPPLCDPKDGHLLMDGGYINNLPADIAR 1099
V T+++ +G L +RAS ++ G + P+ +G+ L+DG +N +P + R
Sbjct: 124 AVATNLSTGRELWFTEGDLHLAIRASCSSIPGLMAPVA--HNGYWLVDGAVVNPIPISLTR 181

Query: 1100 SMGAKTVIAIDV 1111
++GA VIA+D+
Sbjct: 182 ALGADIVIAVDL 193

sp|P37054|YCHK_SHIFL HYPOTHETICAL 34.4 KD PROTEIN IN HNR-PURU INTERGENIC REGION

Length = 192

Score = 101 bits (249), Expect = 8e-21
Identities = 60/190 (31%), Positives = 102/190 (53%), Gaps = 8/190 (4%)

Query: 922 LARVLTGNTIALVLGGGGARGCSHIGVLKALEEAGVPVDLVGGTSIGSFIGALYAEERSA 981
LA ++ IAL LG G ARG SHIGV+ AL++ + +D+V G SIGS +GA YA +R +
Sbjct: 10 LAGIMRKIKIALALGSGAARGWSHIGVINALKKVCIEIDIVAGCSIGSLVGAAYACDRLS 69

Query: 982 SRTKQREAREWAKSMTS-VLEPVLDTYPVTSMTGSAFNRSIHRVFQDKQIEDLWLPHYFN 1040
+ +W S + L ++DL++ + G + + +IE+ +
Sbjct: 70 A-----LEDWVTSFSYWELLRLMDLSWQRGGLLRGERVFNQYREIMPETEIENCSSRRFAP 124

Query: 1041 VTTDITASAMRVHKDGLWRYVRASMTLSGYLPPLCDPKDGHLLMDGGYINNLPADIARS 1100
V T+++ +G L +RAS ++ G + P+ +G+ L+DG +N +P + R+
Sbjct: 125 VATNLSTGRELWFTEGDLHLAIRASCSIPGLMAPVA--HNGYWLVDGAVVNPIPISLTRA 182

Query: 1101 MGAKTVIAID 1110
+GA VIA+D
Sbjct: 183 LGADIVIAVD 192

sp|P05987|KAPR_DICDI CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN
Length = 327

Score = 57.4 bits (136), Expect = 1e-07
Identities = 51/226 (22%), Positives = 96/226 (41%), Gaps = 13/226 (5%)

Query: 486 KAGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLI 545
KAG II +QGD+ + + G +Y + + + V + G G+LA++ G P
Sbjct: 88 KAGDIIIKQGDEGDLFFVIDSGICDIYVCQNGGSPTLVMEVFEGGSF-GELALIYGSPRA 146

Query: 546 FTLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQMD-----AI 597
T+ A+ D ++ + + I+ Q ++S +R +D A+
Sbjct: 147 ATVIARTDVLRLWALNGATYRRILMDQTIKKRKLVEEFLEKVS-ILRHIDKYERVSLADAL 205

Query: 598 DWTAVEAGRALYRQGDSDCTYIVLNGRL---RSVIQRGSGKKELVGEYGRGDLIGVVEA 654
+ + G + RQGD D YI++ G++ + + +V E D G +
Sbjct: 206 EPVNFQDGEVIVRQGDGDRFYIIVEGKVVTQETVPGDHSTSHVVSELHPSDYFGEIAL 265

Query: 655 LTRQPRATTVHAVRDTELAKLPEGTGLGHIKRRYPQVVTRLIHLLSQ 700
LT +PRA TV ++ T+ +L + Q++ R + +Q
Sbjct: 266 LTDRPRAATVTSIGYTKCVELDRQRFNRLCGPIDQMLRRNMETYNQ 311

sp|P31319|KAPR_APLCA CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN (N4
SUBUNIT

OFFPROTEIN KINASE A)
Length = 378

Score = 53.1 bits (125), Expect = 3e-06
Identities = 51/202 (25%), Positives = 87/202 (42%), Gaps = 18/202 (8%)

Query: 484 HAKAGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEP 543
H AG +I +QGD+ + + + G + VY +V + G G+LA++ G P
Sbjct: 154 HRHAGEVVIQQGDEGDNFYVIDQGEVDVY-----VNNVHVTSIGEGGSFGELALIYGTP 207

Query: 544 LIFTLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQMD-----F 595
T++A+ D I + + I+ +++S + +D
Sbjct: 208 RAATVKAKTDVKLWGIDRDSYRRILMGSTIRKRIYEDFLSKVS-ILENLDKWERLTVAD 266

Query: 596 AIDWTAVEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKE--LVGEYGRGDLIGVVE 653
A++ E + RQG+ + +I+L G +V+QR S +E VG G D G +
Sbjct: 267 ALEPVQFEDKEEIVRQGEPEGEDFFIILEGS-AAVLQRRSENEEPVEVGRGLGPSDYFGEIA 325

Query: 654 ALTRQPRATTVHAVRDTELAKL 675
L +PRA TV A + KL
Sbjct: 326 LLLDRPRAATVVARGPLKCVKL 347

sp|P07278|KAPR_YEAST CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN
Length = 416

Score = 51.9 bits (122), Expect = 6e-06
Identities = 47/195 (24%), Positives = 84/195 (42%), Gaps = 17/195 (8%)

Query: 488 GTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIFT 547
G I +QGDQ + V G + Y D + + PG G+LA++ P T
Sbjct: 208 GATIIKQGDQGDYFYVVEKGTVDYF-----VNDKNVNSSGPGSSFGELALMYNSPRAAT 261

Query: 548 LRAQRDCTFLRISKSDFYEIM-----RAQPSVVLSSAHTVAARMSPFVRQMDFAIDWT 600
 + A DC + + F +I+ R +L + + + + ++ A+D
 Sbjct: 262 VVATSDCLLWALDRLTFRKILLGSSFKRLMYDDLKSMFVLKSLTTYDRAKLADALDTK 321

Query: 601 AVEAGRALYRQGDRSDCTIYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 660
 + G + R+GD+ + Y++ G + V ++G G ++ + D G V L PR
 Sbjct: 322 IYQPGETIIREGDQGENFYLIEYGAV-DVSKKGQG---VINKLKDHDYFGEVALLNDLPR 377

Query: 661 ATTVHAVRDTLAKL 675
 TV A + T++A L
 Sbjct: 378 QATVTATKRTKVATL 392

Score = 32.8 bits (73), Expect = 3.5
 Identities = 24/83 (28%), Positives = 33/83 (38%), Gaps = 5/83 (6%)

Query: 601 AVEAGRALYRQGDRSDCTIYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 660
 +V G + +QGD+ D Y+V G + + V G G G + + PR
 Sbjct: 204 SVPKGATIIKQGDQGDYFYVVEKGTVDYFVNDNK-----VNSSGPGSSFGELALMYNSPR 258

Query: 661 ATTVHAVRDTLAKLPEGTLGHI 683
 A TV A D L L T I
 Sbjct: 259 AATVVATSDCLLWALDRLTFRKI 281

sp|P31321|KAP1_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE I-BETA REGULATORY CHAIN

Length = 381

Score = 50.8 bits (119), Expect = 1e-05
 Identities = 67/296 (22%), Positives = 117/296 (38%), Gaps = 41/296 (13%)

Query: 402 QEEASGGSLAAPARTPTQEPREQPAGACEYSYCEDESATGGCFPGPYQGRQTSSIFEAAK 461
 Q ++ ++ P + R + G Y E+++ + P + +++ +A
 Sbjct: 74 QSDSHDEEVSPTPPNPVVKARRRRGGVSAEVYTEEDAVSYVRKVIPKDYKTMTALAKAIS 133

Query: 462 QEL--AKLMRIEDPSLLNSRVLLHHAKAGTIIARQGDQDVS LHFVLWGCLHVVYQRMIDKA 519
 + + A L E + ++ + H AG + +QG++ + + V G + VY
 Sbjct: 134 KNVLFALHDDNERSDIFDAMFPVTHI-AGETVIQQGNEGDNFYVVDQGEVDVY-----V 186

Query: 520 EDVCLFVAQPGEVLGQLAVLTGEPLIFTLRAQRDCTFLRISKSDF-----Y 565
 + G G+LA++ G P T++A+ D I + + Y
 Sbjct: 187 NGEWVTNISEGGSFGELALIYGTTPRAATVKAKTDLKLWGIDRDSYRRILMGSTLRKRKMY 246

Query: 566 EIMRAQPSVVLSSAAH----TVAARMSPFVRQMDFAIDWTAVEAGRALYRQGDRSDCTIYIV 621
 E ++ S++ S TVA R+ P E G + QG+ D YI+
 Sbjct: 247 EEFLSKVSILESLEKWERLTVADRLEP-----VQFEDGEKIVVQGEPEGDDFYII 295

Query: 622 LNGRLRSVIQRGSGKKEL--VGEYGRGDLIGVVEALTRQPRATTVHAVRDTLAKL 675
 G SV+QR S +E VG G D G + L +PRA TV A + KL
 Sbjct: 296 TEG-TASVLQRRSPNEEYVEVGRGLGPSDYFGEIALLLNRPRAATVVARGPLKCVKL 350

sp|P09456|KAP0_RAT CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA REGULATORY CHAIN

Length = 381

Score = 49.6 bits (116), Expect = 3e-05
 Identities = 49/199 (24%), Positives = 86/199 (42%), Gaps = 18/199 (9%)

Query: 487 AGTIIARQGDQDVS LHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGEVLGQLAVLTGEPLIF 546
 AG + +QGD+ + + + G + VY + V + G G+LA++ G P
 Sbjct: 160 AGETVIQQGDEGDNFYVIDQGEVDVYVN-----NEWATSVGEGGSF-GELALIYGTTPRAA 213

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAHTVAARMSPFVRQMD-----FAID 598
 T++A+ + I + + I+ +++S + +D A++
 Sbjct: 214 TVKAKTNVKLWGIDRDSYRRILMGSTLRKRKMYEEFLSKVS-ILES LDKWERLTVADALE 272

Query: 599 WTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRSGGKEL--VGEYGRGDLIGVVEALT 656
 E G+ + QG+ D +I+L G +V+QR S +E VG G D G + L
 Sbjct: 273 PVQFEDGQKIVVQGEFPGDEFFIILEG-TAAVLQRRSENEEFVEVGRLGPSDYFGEIALLM 331

Query: 657 RQPRATTVHAVRDTLAKL 675
 +PRA TV A + KL
 Sbjct: 332 NRPRAA TVVARGPLKCVKL 350

sp|P00514|KAP0_BOVIN CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA REGULATORY CHAIN

Length = 379

Score = 49.6 bits (116), Expect = 3e-05
 Identities = 49/199 (24%), Positives = 86/199 (42%), Gaps = 18/199 (9%)

Query: 487 AGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
 AG + +QGD+ + + + G + VY + V + G G+LA++ G P
 Sbjct: 158 AGETVIOQQGDEGDNFYVIDQGEVDVYVN-----NEWATSVGEGGSF-GELALIYGTTPRAA 211

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAHTVAARMSPFVRQMD-----FAID 598
 T++A+ + I + + I+ +++S + +D A++
 Sbjct: 212 TVKAKTNVKLWGIDRDSYRRILMGSTLRKRKMYEEFLSKVS-ILES LDKWERLTVADALE 270

Query: 599 WTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRSGGKEL--VGEYGRGDLIGVVEALT 656
 E G+ + QG+ D +I+L G +V+QR S +E VG G D G + L
 Sbjct: 271 PVQFEDGQKIVVQGEFPGDEFFIILEGS-AAVLQRRSENEEFVEVGRLGPSDYFGEIALLM 329

Query: 657 RQPRATTVHAVRDTLAKL 675
 +PRA TV A + KL
 Sbjct: 330 NRPRAA TVVARGPLKCVKL 348

sp|P49605|KAPR_USTMA CAMP-DEPENDENT PROTEIN KINASE TYPE II REGULATORY CHAIN (PKA)

Length = 522

Score = 49.2 bits (115), Expect = 4e-05
 Identities = 51/206 (24%), Positives = 83/206 (39%), Gaps = 19/206 (9%)

Query: 487 AGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQ-----PGELVGQL 536
 A + QG Q + V +G L VY R D + + PG G+L
 Sbjct: 236 ANVTVIEQGAQGDYFYVVEFGTLDVYVRSPDAVSEGAPSASALLGDKKVSYPGSSFGEL 295

Query: 537 AVLTGEPLIFTLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAHTVAARMSPFVR----- 591
 A+L +P T+ + CT + + F I+ S + + F R
 Sbjct: 296 ALLYAQPRAATVLSTSACTLWALDRITFRSILMETNSRRRRLYKFLMDVPLFERLSAAE 355

Query: 592 --QMDFAIDWTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRSGGKELVGEYGRGDLI 649
 ++ +++ G A+ QG+R +I++ G V + G +E+VG+ RGD
 Sbjct: 356 RAKISDSLELREYSRGEAVISQGERGSEFFIIVEGD-AEVRKTKQGGEVVGKLSRGDYF 414

Query: 650 GVVEALTRQPRATTVHAVRDTLAKL 675
 G + AL A TV A T+ A+L
 Sbjct: 415 GEL-ALLNNRCAATVAAAGATDDARL 439

sp|P10644|KAP0_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA REGULATORY CHAIN

(TISSUE-SPECIFIC EXTINGUISHER-1) (TSE1)

Length = 381

Score = 48.4 bits (113), Expect = 7e-05
 Identities = 49/199 (24%), Positives = 85/199 (42%), Gaps = 18/199 (9%)

Query: 487 AGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
 AG + +QGD+ + + + G VY + V + G G+LA++ G P
 Sbjct: 160 AGETVIOQQGDEGDNFYVIDQGETDVYVN-----NEWATSVGEGGSF-GELALIYGTTPRAA 213

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQMD-----FAID 598
T++A+ + I + + I+ +++S + +D A++
Sbjct: 214 TVKAKTNVKLWGIDRDSYRRILMGSTLRKRKMYEEFLSKVS-ILES LDKWERLTVADALE 272

Query: 599 WTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRGSGKKEL--VGEYGRGDLIGVVEALT 656
E G+ + QG+ D +I+L G +V+QR S +E VG G D G + L
Sbjct: 273 PVQFEDGQKIVVQGEPEGDEFFIILEGS-AAVLQRRSENEEFVEVGRLGPSDYFGEIALLM 331

Query: 657 RQPRATTVHAVRDTLAKL 675
+PRA TV A + KL
Sbjct: 332 NRPRAAATVVARGPLKCVKL 350

sp|P16905|KAPR_DROME CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN TYPE I (DRI

CLASS I TO CLASS IV)
Length = 377

Score = 48.4 bits (113), Expect = 7e-05
Identities = 49/200 (24%), Positives = 87/200 (43%), Gaps = 19/200 (9%)

Query: 487 AGTIIARQGDQDVSLHFVWLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
AG I +QGD+ + + + G + V+ ++ +++ G G+LA++ G P
Sbjct: 155 AGENIIQQGDEGDNFYVIDVGEVDVFN-----SELVTTISEGGSF-GELALIYGTTPRAA 208

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQMD-----FAID 598
T+RA+ D I + + I+ +R+S + +D +++
Sbjct: 209 TVRAKTDVKLWGIDRDSYRRILMGSTIRKRKMYEEFLSRVS-ILES LDKWERLTVADSLE 267

Query: 599 WTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRGSGKKE---LVGEYGRGDLIGVVEAL 655
+ + G + +QG D YI+L G V+Q+ S + E VG G D G + L
Sbjct: 268 TCSFDDGETIVKQGAAGDDFYIILEG-CAVVLQQRSEQGEDPAEVGRLGSSDYFGEIALL 326

Query: 656 TRQPRATTVHAVRDTLAKL 675
+PRA TV A + KL
Sbjct: 327 LDRPRAATVVARGPLKCVKL 346

sp|P31322|KAP3_BOVIN CAMP-DEPENDENT PROTEIN KINASE TYPE II-BETA REGULATORY CHAIN

Length = 418

Score = 47.3 bits (110), Expect = 2e-04
Identities = 71/314 (22%), Positives = 117/314 (36%), Gaps = 25/314 (7%)

Query: 375 GDISGLQGG--PRSDFDMAYE-RGRISVSLQEEASGGSLAAPARTPTQEPREQPAGACEY 431
GD GG P + A E R S + +EE + A P + A C
Sbjct: 59 GDAGAAGGGGTPSKGVNFEEPRHSDSENGEEEEEEAADAGAFNAPVINRFTRRASVCAE 118

Query: 432 SYCEDESATGGCP--FGPYQGRQTSSIFEAAKQELA-KLMRIEDPSLLNSRVLLHHAKAG 488
+Y DE P Q + + EA K L K + E S + + K G
Sbjct: 119 AYNPDEEEDDAESRIIHPKTDDQRNRLQEACKDILLFKNLDPEQMSQVLDAMFEKLVKEG 178

Query: 489 TIIARQGDQDVSLHFVWLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIFTL 548
+ QGD + + + G +Y + C+ G+LA++ P T+
Sbjct: 179 EHVIDQGGDDGDNFYVIDRGTFDIYVKCDGVGR--CVGNYNDRGSFGELALMYNTPRAATI 236

Query: 549 RAQRD-----CTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQMDFAIDWT 600
A TF RI + + + S + S + +S ++ +D I
Sbjct: 237 TATSPGALWGLDRVTFERRIIVKNNAKKRKMYESFIESLPFLKSLEVSRRLKVVD-VIGTK 295

Query: 601 AVEAGRALYRQGDRSDCTYIVLNGRLRSVIQRGSGKKEL-----VGEYGRGDLIGVVE 653
G + QGD +D +IV +G ++ ++R GK E+ + RG G +
Sbjct: 296 VYNDGEQIIAQGDSADSFFIVESGEVKITMKR-KGKSEVEENGAVEIARCSRQYFGELA 354

Query: 654 ALTRQPRATTVHAV 667
+T +PRA + HA+
Sbjct: 355 LVTNKPRAASAHAI 368

sp|P07802|KAP0_PIG CAMP-DEPENDENT PROTEIN KINASE TYPE I-ALPHA REGULATORY CHAIN
Length = 380

Score = 47.3 bits (110), Expect = 2e-04
 Identities = 52/205 (25%), Positives = 89/205 (43%), Gaps = 30/205 (14%)

Query: 487 AGTIIARQGDQDVSLHFVLWGCLHVVQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
 AG + +QGD+ + + + G + VY + V + G G+LA++ G P
 Sbjct: 159 AGETVIOQQGDEGDNFYVIDQGEMDVYVN-----NEWATSVGEGGSF-GELALIYGT PRAA 212

Query: 547 TLRAQRDC-----TFLRI-----SKSDFYEIMRAQPSVVLSSAHTVAARMSPFVRQ 592
 T++A+ + ++ RI K YE ++ S++ S ++ +
 Sbjct: 213 TVKAKTNVCLKWGNDRDSYRRILMGSTLRKRKMYEEFLSKVSILES LDKWERLTVADALEP 272

Query: 593 MDFAIDWTAVEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKEL--VGEYGRGDLIG 650
 + F E G+ + QG+ D +I+L G +V+QR S +E VG G D G
 Sbjct: 273 VQF-----EDGQKIVVQGEPEGDEFFIILEGS-AAVLQRRSENEEFVEVGRLGPSDYFG 324

Query: 651 VVEALTRQPRATTVHAVRDTLAKL 675
 + L +PRA TV A + KL
 Sbjct: 325 EIALLMNRPRAA TVVARGPLKCVKL 349

sp|P12849|KAP1_MOUSE CAMP-DEPENDENT PROTEIN KINASE TYPE I-BETA REGULATORY CHAIN
Length = 381

Score = 46.9 bits (109), Expect = 2e-04
 Identities = 60/270 (22%), Positives = 109/270 (40%), Gaps = 19/270 (7%)

Query: 417 PTQEPREQPAGACEYSYCEDESATGGCPFGPYQGRQTSSIFEAAKQEL--AKLMRIEDPS 474
 P + R + G Y E+++ + P + +++ +A + + + L E
 Sbjct: 89 PVVKARRRRGGVSAEYVTEEDAVSYVRKVI PKDYKTMTALAKAISKNVLFSLDDNERSD 148

Query: 475 LLNSRVLLHHAAGTIIARQGDQDVSLHFVLWGCLHVVQRMIDKAEDVCLFVAQPGELVG 534
 + ++ + H T+I +QG++ + + + G + VY + G G
 Sbjct: 149 IFDAMFPVTHIGGETVI-QQNEGDNFYVIDQGEVDVY-----VNGEWTNISEGGSFG 201

Query: 535 QLAVLTGEPLIFTLRAQRDCTFLRISKSDFYEIM-----RAQPSVVLSSAHTVAARMS 587
 +LA++ G P T++A+ D I + + I+ R LS + +
 Sbjct: 202 ELALIYGT PRAATV KAKTDLKLWGIDRDSYRRILMGSTLRKRKMYEEFLSKVSILESLEK 261

Query: 588 PFVRQMDFAIDWTAVEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKEL--VGEYGR 645
 + A++ E G + QG+ D YI+ G SV+QR S +E VG G
 Sbjct: 262 WERLTVADALEPVQFEDGEKIVVQGEPEGDFYIITEG-TASVLQRRSPNEEYVEVGR LGP 320

Query: 646 GDLIGVVEALTRQPRATTVHAVRDTLAKL 675
 D G + L +PRA TV A + KL
 Sbjct: 321 SDYFGEIALLLNRPRAATV VARGPLKCVKL 350

sp|P47200|FNRA_PSEST TRANSCRIPTIONAL ACTIVATOR PROTEIN FNRA
Length = 244

Score = 46.1 bits (107), Expect = 4e-04
 Identities = 25/108 (23%), Positives = 55/108 (50%), Gaps = 1/108 (0%)

Query: 602 VEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 661
 ++ G L+RQGD + V +G LR+ G++++ G + +L+G + + +
 Sbjct: 47 LKKGETLFRQGD TFSVFAVRSGALRTFSVTDGGEEQITGFHLPSELVG-LSGMDTEMYP 105

Query: 662 TTVHAVRDTLAKLPEGTLGHIKRRYPQVVTRLIHLSSQKILGNLQQL 709
 T A+ T + ++P L + PQ+ +L+ ++S++I + Q +
 Sbjct: 106 VTAQALETTSVCEIPFERLDELSVLLPQLRRQLMRIMSREIRDDQQMM 153

sp|P30625|KAPR_CAEEL CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN
Length = 376

Score = 45.7 bits (106), Expect = 5e-04
 Identities = 52/217 (23%), Positives = 90/217 (40%), Gaps = 18/217 (8%)

Query: 487 AGTIIARQGDQDVSLEHFLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
 AG I QG++ + + + G + VY + L + + G G+LA++ G P
 Sbjct: 155 AGETIIEQGEEGDNFYVIDKGTVDVYVN-----HEYVLTINEGGSF-GELALIYGTTPRAA 208

Query: 547 TLRAQRDCTFLRISKSDFYEIM-----RAQPSVVLSSAHTVAARMSPFVR-QMDFRID 598
 T+ A+ D I + + I+ R LS + A + + R + A++
 Sbjct: 209 TVIAKIDVKLWLAIDRLTYRRILMGSVTKKRKMYDEFLSKVQ-ILADLDQWERNVADALE 267

Query: 599 WTAVEAGRALYRQGDSDCTIYVLNGLRLRSVIQRGSGKK--ELVGEYGRGDLIGVVEALT 656
 E G + QG D +I+L G +V+Q+ S ++VG G D G + L
 Sbjct: 268 RCDFFPGTHVVEQGPQDEFFIILEGEA-NVLQKRSDAPFDVVGHLGMSDYFGEIALLL 326

Query: 657 RQPRATTVHAVRDTLAKLPEGTGLGHKRRYPQVVTR 693
 +PRA TV A + KL + +++ R
 Sbjct: 327 DRPRAATVVAKTHLKCILDRNRFERVMGPFVREILKR 363

sp|P31323|KAP3_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE II-BETA REGULATORY CHAIN
Length = 418

Score = 45.7 bits (106), Expect = 5e-04
 Identities = 67/314 (21%), Positives = 118/314 (37%), Gaps = 25/314 (7%)

Query: 375 GDISGLQGG--PRSDFDMAAYERGRI-SVSLQEEASGGSLAAPARTPTQEPREQPAGACEY 431
 GD+ GG P + A E + S +EE + + A P + A C
 Sbjct: 59 GDLGAAAGGGTPSKGVNFAEPMQSDSEDEGEEEEEAPADAGAFNAPVINRFTRRASVCAE 118

Query: 432 SYCEDESATGGCP--FGPYQGRQTSSIFEAAKQELA-KLMRIEDPSLLNSRVLLHHAKAG 488
 +Y DE P Q + + EA K L K + E S + + K G
 Sbjct: 119 AYNPDEEEDDAESRIIHPKTDQNRNLQEACKDILLFKNLDPEQMSQVLDAMFEKLVKDG 178

Query: 489 TIIARQGDQDVSLEHFLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIFTL 548
 + QGD + + + G +Y + C+ G+LA++ P T+
 Sbjct: 179 EHVIDQGDDGDNFYVIDRGTFDIYVKCDGVGR--CVGNYNDRGSFGELALMYNTPRAATI 236

Query: 549 RAQRDCTFLRISKSDFYEIMRAQPSVVLSSAHTVAARMSPFVRQMDFAIDWTAVEA---- 604
 A + + F I+ + + + PF++ ++F+ V+
 Sbjct: 237 TATSPGALWGLDRVTFRRII-VKNNAKKRKMYESFIESLPFLKSLEFSERLKVVVDVIGTK 295

Query: 605 ----GRALYRQGDSDCTIYVLNGLRLRSVIQRGSGKKEL-----VGEYGRGDLIGVVE 653
 G + QGD +D +IV +G ++ ++R GK E+ + RG G +
 Sbjct: 296 VYNDGEQIIAQGDSADSFFIVESGEVKITMKR-KGKSEVEENGAVEMPRCSRQYFGEA 354

Query: 654 ALTRQPRATTVHAV 667
 +T +PRA + HA+
 Sbjct: 355 LVTNKPRAASAHAI 368

sp|P23926|ANR_PSEAE TRANSCRIPTIONAL ACTIVATOR PROTEIN ANR
Length = 244

Score = 45.3 bits (105), Expect = 6e-04
 Identities = 24/108 (22%), Positives = 57/108 (52%), Gaps = 1/108 (0%)

Query: 602 VEAGRALYRQGDSDCTIYVLNGLRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 661
 ++ G L+RQGD + V +G L++ +G++++ G + +L+G + + +
 Sbjct: 47 LKKGEFLFRQGDPFSGVFAVRSGALKTFSITDAGEEQITGFHLPSELVG-LSGMDTETYP 105

Query: 662 TTVHAVRDTLAKLPEGTGLGHKRRYPQVVTRLIHLSSQKILGNLQQL 709
 + A+ T + ++P L + + PQ+ +L+ L+S++I + Q +
 Sbjct: 106 VSAQALETTTSVCEIPFERLDELSEQLPQLRRQLMRLMSREIRDDQMM 153

Score = 36.7 bits (83), Expect = 0.23
Identities = 27/95 (28%), Positives = 48/95 (50%), Gaps = 8/95 (8%)

Query: 168 LGQGDYVFRPGQPDASIYVVQDGLLELCLPGPDGKECVVKEVVPGDVSNLLSILDVITG 227
L +G+++FR G P S++ V+ G L+ G+E + +P + V LS +D T
Sbjct: 47 LKKGEFLFRQGDPFQSVFAVRSGALKTFISITDAGEEQITGFHLPSELVG--LSGMDTET- 103

Query: 228 HQHPQRTVSARAARDSTVLRPLVEAFSAVFTKYPE 262
+P VSA+A ++V +P E + + P+
Sbjct: 104 --YP---VSAQALETTSVCEIPFERLDELSEQLPQ 133

sp|P12369|KAP3_RAT CAMP-DEPENDENT PROTEIN KINASE TYPE II-BETA REGULATORY CHAIN
Length = 416

Score = 45.3 bits (105), Expect = 6e-04
Identities = 72/314 (22%), Positives = 117/314 (36%), Gaps = 27/314 (8%)

Query: 375 GDISGLQGG--PRSDFDMAYERGRISVSLQEE---ASGGSLAAPARTPTQEPREPAGAC 429
GD GG P + A E R EE A G+ AP + R A
Sbjct: 59 GDAGAAAGGGTPSKGVNFEEPMRSDSENGEEEEAAEAGAFNAPVIN--RFTRRASVCAE 116

Query: 430 EYSYCEDESATGGCPFGPYQGRQTSSIFEAAKQELA-KLMRIEDPSLLNSRVLLHHAKAG 488
Y+ E+E P Q + + EA K L K + E S + + K G
Sbjct: 117 AYNPDEEEDDAESRIIHPKTDQNRNLQEACKDILLFKNLDPEQMSQVLDAMFEKLVKEG 176

Query: 489 TIIARQGDQDVSLEHVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGLAVLTGEPLIFTL 548
+ QGD + + + G +Y + C+ G+LA++ P T+
Sbjct: 177 EHVIDQGGDDGNFYVIDRGTFDIYVKCDGVGR--CVGNYNDRGSFGELALMYNTPRAATI 234

Query: 549 RAQRD-----CTFLRISKSDFYEIMRAQPSVVLSAAHTVAARMSPFVRQMDFAIDWT 600
A TF RI + + + S + S + +S ++ +D I
Sbjct: 235 TATSPGALWGLDRVTFRRRIIVKNNAKKRKMYESFIESLPFLKSLEVSRKLVVD-VIGTK 293

Query: 601 AVEAGRALYRQGDERSDCTIYIVLNGRLRSVIQRGSGKKEL-----VGEYGRGDLIGVVE 653
G + QGD +D +IV +G +R ++R GK ++ + RG G +
Sbjct: 294 VYNDGEQIIAQGDSADSFFIVESGEVRITMKR-KGKS DIEENGAVEIARCLRGQYFGELA 352

Query: 654 ALTRQPRATTVHAV 667
+T +PRA + HA+
Sbjct: 353 LVTNKPRAASAHAI 366

sp|P37428|FNR_SALTY FUMARATE AND NITRATE REDUCTION REGULATORY PROTEIN
Length = 250

Score = 44.5 bits (103), Expect = 0.001
Identities = 23/104 (22%), Positives = 54/104 (51%), Gaps = 1/104 (0%)

Query: 602 VEAGRALYRQGDERSDCTIYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 661
++ G+ L++ GD Y + +G ++S G +++ G + GDL+G +A+
Sbjct: 52 IQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAGDLVG-FDAIGSGHHP 110

Query: 662 TTVHAVRDTTELAKLPEGTLGHKRRYPQVVTRLIHLLSQKILGN 705
+ A+ + + ++P TL + + P + +++ L+S +I G+
Sbjct: 111 SFAQALETSMVCEIPFETLDDLSGKMPNLRQQMRLMSGEIKGD 154

sp|P03019|FNR_ECOLI FUMARATE AND NITRATE REDUCTION REGULATORY PROTEIN
Length = 250

Score = 44.5 bits (103), Expect = 0.001
Identities = 23/104 (22%), Positives = 54/104 (51%), Gaps = 1/104 (0%)

Query: 602 VEAGRALYRQGDERSDCTIYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPR 661
++ G+ L++ GD Y + +G ++S G +++ G + GDL+G +A+
Sbjct: 52 IQKGQTLFKAGDELKSLYAIRSGTIKSYTITEQGDEQITGFHLAGDLVG-FDAIGSGHHP 110

Query: 662 TTVHAVRDTELAKLPEGTGLGHIKRRYPQVVTRLIHLLSQKILGN 705
 + A+ + + ++P TL + + P + +++ L+S +I G+
 Sbjct: 111 SFAQALETSMVCEIPFETLDDLSGKMPNLRQQMMRLMSGEIKGD 154

**sp|Q90980|CNG3_CHICK CYCLIC NUCLEOTIDE GATED CHANNEL, ROD PHOTORECEPTOR, ALPHA
 SUBUNIT (CNG CHANNEL 3) (CNG-3) (CNG3)
 Length = 645**

Score = 44.1 bits (102), Expect = 0.001
 Identities = 36/126 (28%), Positives = 59/126 (46%), Gaps = 6/126 (4%)

Query: 139 LYMLKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCLPG 198
 L LK VR+ E L +EL + Q GDY+ R G +Y++++G +L +
 Sbjct: 423 LETLKKVRIFADCEAGLLVELVLKLPQVYSPGDYICRKGDIGREMYIIEG--KLAVVA 480
 Query: 199 PDGKECVVKEVVPD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVF 257
 DG V + VV D S +SIL++ +RT + R+ S + L +
 Sbjct: 481 DDG---VTQFVVLSDGSYFGEISILNIKSGKAGNRRTANIRSIGYSDLFCLSKDDLMEAL 537
 Query: 258 TKYPES 263
 T+YP++
 Sbjct: 538 TEYPDA 543

**sp|P45199|FNR_HAEIN FUMARATE AND NITRATE REDUCTION REGULATORY PROTEIN HOMOLOG
 Length = 257**

Score = 44.1 bits (102), Expect = 0.001
 Identities = 21/108 (19%), Positives = 57/108 (52%), Gaps = 1/108 (0%)

Query: 602 VEAGRALYRQGDSDCTYIVLNGRLRSVIQRSGGKELVGEYGRGDLIGVVEALTRQPPRA 661
 ++ + L++ GD + Y + +G ++S SG++++ + GDL+G +A+T+
 Sbjct: 55 IQKSQVLKAGDSLNSIYAIRSGTIKSYTISESGEEQITSFHLPGDLVG-FDAITQMHP 113
 Query: 662 TTVHAVRDTELAKLPEGTGLGHIKRRYPQVVTRLIHLLSQKILGNLQQL 709
 + A+ + ++P L + + P++ +++ L+S +I + + +
 Sbjct: 114 SFAQALETAMVCEIPFDILDDLAGKMPKLRQQILRLMSSEIKSDQEMI 161

Score = 36.0 bits (81), Expect = 0.40
 Identities = 24/98 (24%), Positives = 45/98 (45%), Gaps = 8/98 (8%)

Query: 174 VFRPGQPDASIYVVQDGLLELCLPGPDGKECVVKEVVPD-SVNSLLSILDVITGHQHPQR 233
 +F+ G SIY ++ G ++ G+E + +PGD V D IT QHP
 Sbjct: 61 LFKAGDSLNSIYAIRSGTIKSYTISESGEEQITSFHLPGDLVG-----FDAITQMHP- 114
 Query: 234 TVSARAARDSTVLRLPVEAFSAVFTKYPESLVRVQII 271
 A+A + V +P + + K P+ ++++++
 Sbjct: 115 --FAQALETAMVCEIPFDILDDLAGKMPKLRQQILRLM 150

**sp|Q50734|Y0A4_MYCTU HYPOTHETICAL ABC TRANSPORTER ATP-BINDING PROTEIN
 CY9C4.04C
 Length = 330**

Score = 43.8 bits (101), Expect = 0.002
 Identities = 24/68 (35%), Positives = 31/68 (45%)

Query: 602 VEAGRALYRQGDSDCTYIVLNGRLRSVIQRSGGKELVGEYGRGDLIGVVEALTRQPPRA 661
 V+AG L+ Q D Y+V G V + G +ELV GD G + L PR+
 Sbjct: 231 VKAGEVLFEQSTMGDLIYVSEGEFEIVRELADGGEELVKTAAPGDYFGEIGVLFLHPRS 290
 Query: 662 TTVHAVRD 669
 TV A D
 Sbjct: 291 ATVRARSD 298

Score = 37.9 bits (86), Expect = 0.10
Identities = 23/83 (27%), Positives = 40/83 (47%), Gaps = 1/83 (1%)

Query: 484 HAKAGTIIARQGDQDVSLSHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEP 543
H KAG ++ Q ++ V G + + + D E++ + A PG+ G++ VL P
Sbjct: 230 HVKAGEVLFEQSTMGDLIYVVSEGEFEIVRELADGGEEL-VKTAAPGDYFGEIGVLFHLP 288

Query: 544 LIFTLRAQRDCTFLRISKSDFYE 566
T+RA+ D T + + F E
Sbjct: 289 RSATVRARSDATAVGYTAQAFRE 311

Score = 37.1 bits (84), Expect = 0.18
Identities = 57/234 (24%), Positives = 90/234 (38%), Gaps = 39/234 (16%)

Query: 52 KRDKV--LFYGRKIMRKVSQSTSSSLVDTSVSATSRPRMRKKLMLNIAKKILRIQKETP- 108
+RDKV +F ++ ++ + +V + `SR RK+ + L I + K P
Sbjct: 83 RRDKVGIVFQAFNLVSSLTALENVMVPLRAAGVSRAAARKRAEDLLIRVNLGERMKHRPG 142

Query: 109 -----TLQRKEPPPAV-LEADLTEGDLANSHLP-----SEVLYMLKN-----VRVLGHF 151
QR A+ L+ L D +HL EVL ++++ V V H
Sbjct: 143 DMSGGQQQRVAVARAIALDPQLILADEPTAHLDFIQVEEVLRLIRSLAQGDRVVVATHD 202

Query: 152 EKPL-----FLELCRHMVFQR-----LGQGDYVFRPGQPDASIYVVQDGLLELCLPGP 199
+ L LEL V + G+ +F IYVV +G E+
Sbjct: 203 SRMLPLADRVLLELMPAQVSPNQPPETVHVKAGEVLFEQSTMGDLIYVVSEGEFEIVRELA 262

Query: 200 DGKECVVKEVPGDSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAF 253
DG E +VK PGD + + H R+ + RA D+T + +AF
Sbjct: 263 DGGEELVKTAAPGDYFGEIGVLF-----HLPRSATVRARSDATAVGYTAQAF 309

**sp|Q03041|CNG2_BOVIN CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL (CYCLIC-
NUCLEOTIDE-GATED CATION CHANNEL 2) (CNG CHANNEL 2) (CNG-2) (CNG2)
Length = 663**

Score = 42.6 bits (98), Expect = 0.004
Identities = 38/147 (25%), Positives = 65/147 (43%), Gaps = 13/147 (8%)

Query: 117 PAVLEADLTEGDLANSHLPSEVLYMLKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFR 176
PA L A++ N HL + LK VR+ E L +EL + Q GDY+ R
Sbjct: 433 PAKLRAEIA----INVHLST-----LKKVRIFQDCEAGLLVELVLKLRPQVFSFGDYICR 483

Query: 177 PGQPDASIYVVQDGLLELCLPGPDGKECVVKEVVPDGSVNSLLSILDVITGHQHPQRTVS 236
G +Y++++G +L + DG ++ S +SIL++ +RT +
Sbjct: 484 KGDIGKEMYIIKEG--KLAVVADDG--VTQYALLSAGSCFGEISILNIKSKMGNRRTAN 539

Query: 237 ARAARDSTVLRLPVEAFSAVFTKYPES 263
R+ S + L + T+YP++
Sbjct: 540 IRSLGYSDFCLSKDDLMEAVTEYPDA 566

**sp|Q00195|CNG2_RAT CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL (CYCLIC-
NUCLEOTIDE-
GATED CATION CHANNEL 2) (CNG CHANNEL 2) (CNG2) (CNG-2) (OCNC1)
Length = 664**

Score = 42.6 bits (98), Expect = 0.004
Identities = 38/147 (25%), Positives = 65/147 (43%), Gaps = 13/147 (8%)

Query: 117 PAVLEADLTEGDLANSHLPSEVLYMLKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFR 176
PA L A++ N HL + LK VR+ E L +EL + Q GDY+ R
Sbjct: 435 PAKLRAEIA----INVHLST-----LKKVRIFQDCEAGLLVELVLKLRPQVFSFGDYICR 485

Query: 177 PGQPDASIYVVQDGLLELCLPGPDGKECVVKEVVPDGSVNSLLSILDVITGHQHPQRTVS 236
G +Y++++G +L + DG ++ S +SIL++ +RT +
Sbjct: 486 KGDIGKEMYIIKEG--KLAVVADDG--VTQYALLSAGSCFGEISILNIKSKMGNRRTAN 541

Query: 237 ARAARDSTVLRLPVEAFSAVFTKYPES 263
R+ S + L + T+YP++
Sbjct: 542 IRSLGYSDFCLSKDDLMEAVTEYPD 568

sp|Q28718|CNG2_RABIT CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL
(CYCLINUCLEOTIDE-
GATED CATION CHANNEL 2) (CNG CHANNEL 2) (CNG-2) (CNG2) (AORTA
CNG CHANNEL) (RACNG)
Length = 664

Score = 42.6 bits (98), Expect = 0.004
Identities = 38/147 (25%), Positives = 65/147 (43%), Gaps = 13/147 (8%)

Query: 117 PAVLEADLTEGDLANSHLPSEVLYMLKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFR 176
PA L A++ N HL + LK VR+ E L +EL + Q GDY+ R
Sbjct: 433 PAKLRAEIA----INVHLST-----LKKVRIFQDCEAGLLVELVLKLRPQVFSFGDYICR 483

Query: 177 PGQPDASIYVVQDGLLELCLPGPDGKECVVKEVPGDSVNSLLSILDVITGHQHPQRTVS 236
G +Y++++G +L+ DG ++ S +SIL++ +RT +
Sbjct: 484 KGDIGKEMYIIKEG--KLAVVADDG--VTQYALLSAGSCFGEISILNIKSGKMGNNRRATAN 539

Query: 237 ARAARDSTVLRLPVEAFSAVFTKYPES 263
R+ S + L + T+YP++
Sbjct: 540 IRSLGYSDFCLSKDDLMEAVTEYPD 566

sp|P31320|KAPR_BLAEM CAMP-DEPENDENT PROTEIN KINASE REGULATORY CHAIN
Length = 403

Score = 42.2 bits (97), Expect = 0.005
Identities = 44/196 (22%), Positives = 79/196 (39%), Gaps = 8/196 (4%)

Query: 487 AGTIIARQGDQDVS LHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
AG ++ RQG + V G L V+ DV + G G+LA++ P
Sbjct: 179 AGEVVIRQGGVGDFYFVVETGALDVFVNRNGNG-DVKVTDYSAGGSFGELALMYNAPRAA 237

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPS-----VVL SAAHTVAARMSPFVR-QMDFAI DW 599
T+ A + + + F I+ S + + + P+ R ++ A++
Sbjct: 238 TVVATAESVLWALDRVTFRRILMDHTSRKRRMYEAFLEEVPLLSSLEPYERHKKIADALES 297

Query: 600 TAVEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQP 659
A G + RQG + YI+ G + +G++ +G+ G + L+ +P
Sbjct: 298 VAYADGDVVIRQGDVGENFYIIEAGDAEVIKIDENGEHHFRPLHKGNFYFELALLSDKP 357

Query: 660 RATTVHAVRDTELAKL 675
R T+ A + AKL
Sbjct: 358 RVATIRAKGKLKCAKL 373

Score = 41.0 bits (94), Expect = 0.012
Identities = 32/105 (30%), Positives = 42/105 (39%), Gaps = 7/105 (6%)

Query: 602 VEAGRALYRQGDSDCTYIVLNGRLRSVIQRGSGKKELVGEYGRGDLIGVVEALTRQPRA 661
V AG + RQG D Y+V G L + R V +Y G G + + PRA
Sbjct: 177 VAAGEVVIRQGGVGDFYFVVETGALDVFVNRNGNGDVKVT DYSAGGSFGELALMYNAPRA 236

Query: 662 TTVHAVRDTELAKLPEGTLGHI-----KRRYPQVVTRLIHLLS 699
TV A ++ L L T I KRR + + LLS
Sbjct: 237 ATVVATAESVLWALDRVTFRRILMDHTSRKRRMYEAFLEEVPLL 281

Score = 32.5 bits (72), Expect = 4.5
Identities = 19/81 (23%), Positives = 39/81 (47%), Gaps = 1/81 (1%)

Query: 488 GTIIARQGDQDVS LHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIFT 547
G ++ RQGD + + + G V ++ + E+ G G+LA+L+ +P + T
Sbjct: 303 GDVVIRQGDVGENFYIIEAGDAEVI-KIDENGEHHFRPLHKGNFYFELALLSDKPRVAT 361

Query: 548 LRAQRDCTFLRISKSDFYEIM 568
+RA+ ++ K F ++
Sbjct: 362 IRAKGKLGKCAKLGKKAFTRL 382

sp|Q24278|CNG_DROME CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL (CNG CHANNEL)
Length = 665

Score = 42.2 bits (97), Expect = 0.005
Identities = 34/128 (26%), Positives = 54/128 (41%), Gaps = 4/128 (3%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVDGLLELCPLPGPDG 201
LK VR+ E L L + Q GDY+ R G +Y+V+ G +L + G DG
Sbjct: 424 LKQVRIFHDTEPGLLEALVLKQLQVSPGDYICRKGVDVGKEMYIVKRG--KLSVVGDDG 481

Query: 202 KECVVKEVVPDGSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKYP 261
V + SV +S+L++ +RT + R+ S + L + YP
Sbjct: 482 --ITVLATLGAGSVFGEVSVLEIAGNRTGNRRRTANVRSLGYSDLFCLAKRDLWETLSDYP 539

Query: 262 ESLVRVVQ 269
E+ + Q
Sbjct: 540 EARSTLTQ 547

sp|Q29441|CNG3_BOVIN CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 3 (CNG CHANNEL 3)
(CNG-3) (CNG3)
Length = 706

Score = 41.8 bits (96), Expect = 0.007
Identities = 33/123 (26%), Positives = 58/123 (46%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVDGLLELCPLPGPDG 201
L+ VR+ E L +EL + GDY+ + G +Y++++G +L + DG
Sbjct: 496 LRKVRIFQDCEAGLLVELVLKLRPAVFPSPGDYICKKGDIGREMYIIEG--KLAVVAEDG 553

Query: 202 KECVVKEVVPDGSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
+ + VV GD S +SIL++ +RT + R+ S + L + T+Y
Sbjct: 554 ---ITQFVVLGDGSYFGEISILNIKSKSGNRRRTANIRSIGYSDLFCLSKDDLMEALTEY 610

Query: 261 PES 263
PE+
Sbjct: 611 PEA 613

sp|P13861|KAP2_HUMAN CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPHA REGULATORY
CHAIN
Length = 404

Score = 41.4 bits (95), Expect = 0.009
Identities = 69/317 (21%), Positives = 125/317 (38%), Gaps = 28/317 (8%)

Query: 417 PTQEPREQPAGACEYSYCEDESATGGCPFGPYQGRQTSSIFEAAKQELA-KLMRIEDPSL 475
P++ R A Y+ E+E T P Q + EA K L K + E S
Sbjct: 91 PSRFNRRVSVCAETYNPDEEEEDTDPRVIHPKTDEQRCRLQEACKDILLFKNLDQEQLSQ 150

Query: 476 LNSRVLLHHAAGTIIARQGDQDVSLSHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQ 535
+ + KA + QGD + + + G + ++ V + + G+
Sbjct: 151 VLDAMFERIVKADEHVIDQGGDGNFYVIERGTIDILVTKDNQTRSVGQYDNRGS--FGE 208

Query: 536 LAVLTGEPLIFTTLRAQRD-----CTFLRISKSDFYEIMRAQPSVVLSSAHTVAARMS 587
LA++ P T+ A + TF RI + + + S + S + +S
Sbjct: 209 LALMYNTPRAATIVATSEGLWGLDRVTFRIIVKNNAKKRKMFESFIESVPLLKSLEVS 268

Query: 588 PFVRQMDFAIDWTAVEAGRALYRQGDSDCTIYVLNG-----RLRSVIQRGSGKKEL-V 640
++ +D I + G + QG+++D YI+ +G R R+ + G +E+ +
Sbjct: 269 ERMKIVD-VIGEKIYKDGRIITQGEKADSFYIIESGEVSILIRSRKSNKDGNGQEVEI 327

Query: 641 GEYGRGDLIGVVEALTRQPRATTVHAVRDTTELAKLP-----EGTLGH----IKRRYPQVVT 692
 +G G + +T +PRA + +AV D + + E LG +KR
 Sbjct: 328 ARCHKGQYFGELALVTNKPRAASAYAVGDVKCLVMDVQAFERLLGPCMDIMKRNISHYEE 387

Query: 693 RLIHLLSQKI-LGNLQQ 708
 +L+ + + LGNL Q
 Sbjct: 388 QLVKMFSSVDLGNLQ 404

sp|Q90805|CNG1_CHICK CYCLIC NUCLEOTIDE GATED CHANNEL, CONE PHOTORECEPTOR, ALPHA

**SUBUNIT (CNG CHANNEL 1) (CNG-1)
 Length = 735**

Score = 41.4 bits (95), Expect = 0.009
 Identities = 33/123 (26%), Positives = 57/123 (45%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
 LK VR+ E L +EL + GDY+ + G +Y++++G +L + DG
 Sbjct: 519 LKKVRIFQDCEAGLLIELVLKLPKPTVFSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 576

Query: 202 KECVVKEVPGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
 + + VV D S +SIL++ +RT + R+ S + L + T+Y
 Sbjct: 577 ---ITQFVVLSDGSYFGEISILNIKSGSKGNRRRTANIRSIGYSDLFCLSKDDLMEALTEY 633

Query: 261 PES 263
 PE+
 Sbjct: 634 PEA 636

**sp|Q62398|CNG2_MOUSE CYCLIC-NUCLEOTIDE-GATED OLFACTORY CHANNEL (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 2) (CNG CHANNEL 2) (CNG-2) (CNG2)
 Length = 664**

Score = 41.4 bits (95), Expect = 0.009
 Identities = 38/147 (25%), Positives = 65/147 (43%), Gaps = 13/147 (8%)

Query: 117 PAVLEADLTEGDLANSHLPSEVLYMLKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFR 176
 PA L A++ N HL + LK VR+ E L +EL + Q GDY+ R
 Sbjct: 435 PAKLRAEIA----INVHLST-----LKKVRIFQDCEAGLLVELVLKLRPQVFSPGDYICR 485

Query: 177 PGQPDASIYVVQDGLLELCPLPGPDGKECVVKEVPGDSVNSLLSILDVITGHQHPQRTVS 236
 G +Y++++G +L + DG ++ S +SIL++ +RT +
 Sbjct: 486 KGDIGKEMYIIKEG--KLAVVADDG--VTQYALLSAGSCFGEISILNIKSGSKMGNRRRTGT 541

Query: 237 ARAARDSTVLRLPVEAFSAVFTKYPES 263
 R+ S + L + T+YP++
 Sbjct: 542 IRSLGYSDFCLSKDDLMEAVTEYPDA 568

sp|P29973|CNG1_HUMAN CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NUCLEOTIDE GATED

**CHANNEL, PHOTORECEPTOR) (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 1) (CNG CHANNEL 1) (CNG-1) (CNG1)
 Length = 686**

Score = 41.4 bits (95), Expect = 0.009
 Identities = 33/123 (26%), Positives = 58/123 (46%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
 LK VR+ E L +EL + Q GDY+ + G +Y++++G +L + DG
 Sbjct: 470 LKKVRIFADCEAGLLVELVLKLPQVYSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 527

Query: 202 KECVVKEVPGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
 V + VV D S +SIL++ +RT + ++ S + L + T+Y
 Sbjct: 528 ---VTQFVVLSDGSYFGEISILNIKSGKAGNRRRTANIKSIGYSDLFCLSKDDLMEALTEY 584

Query: 261 PES 263
P++
Sbjct: 585 PDA 587

sp|Q00194|CNG1_BOVIN CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NUCLEOTIDE GATED

CHANNEL, PHOTORECEPTOR) (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 1) (CNG CHANNEL 1) (CNG-1) (CNG1)
Length = 690

Score = 41.0 bits (94), Expect = 0.012
Identities = 32/123 (26%), Positives = 58/123 (47%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
LK VR+ E L +EL + Q GDY+ + G +Y++++G +L + DG
Sbjct: 472 LKKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 529

Query: 202 KECVVKEVVPDGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
+ + VV D S +SIL++ +RT + ++ S + L + T+Y
Sbjct: 530 ---ITQFVVLSDGSYFGEISILNIKSGKAGNRRTANIKSIGYSDFCLSKDDLMEALTEY 586

Query: 261 PES 263
P++
Sbjct: 587 PDA 589

sp|P29974|CNG1_MOUSE CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NUCLEOTIDE GATED

CHANNEL, PHOTORECEPTOR) (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 1) (CNG CHANNEL 1) (CNG-1) (CNG1)
Length = 684

Score = 41.0 bits (94), Expect = 0.012
Identities = 32/123 (26%), Positives = 58/123 (47%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
LK VR+ E L +EL + Q GDY+ + G +Y++++G +L + DG
Sbjct: 466 LKKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 523

Query: 202 KECVVKEVVPDGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
+ + VV D S +SIL++ +RT + ++ S + L + T+Y
Sbjct: 524 ---ITQFVVLSDGSYFGEISILNIKSGKAGNRRTANIKSIGYSDFCLSKDDLMEALTEY 580

Query: 261 PES 263
P++
Sbjct: 581 PDA 583

sp|Q62927|CNG1_RAT CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NUCLEOTIDE GATED

CHANNEL, PHOTORECEPTOR) (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 1) (CNG CHANNEL 1) (CNG-1) (CNG1)
Length = 683

Score = 41.0 bits (94), Expect = 0.012
Identities = 32/123 (26%), Positives = 58/123 (47%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
LK VR+ E L +EL + Q GDY+ + G +Y++++G +L + DG
Sbjct: 465 LKKVRIFADCEAGLLVELVLKLQPVYSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 522

Query: 202 KECVVKEVVPDGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVFTKY 260
+ + VV D S +SIL++ +RT + ++ S + L + T+Y
Sbjct: 523 ---ITQFVVLSDGSYFGEISILNIKSGKAGNRRTANIKSIGYSDFCLSKDDLMEALTEY 579

Query: 261 PES 263
P++
Sbjct: 580 PDA 582

sp|Q28279|CNG1_CANFA CGMP-GATED CATION CHANNEL PROTEIN (CYCLIC NUCLEOTIDE GATED

CHANNEL, PHOTORECEPTOR) (CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 1) (CNG CHANNEL 1) (CNG-1) (CNG1)

Length = 691

Score = 41.0 bits (94), Expect = 0.012

Identities = 32/123 (26%), Positives = 58/123 (47%), Gaps = 6/123 (4%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLGQGDYVFRPGQPDASIYVVQDGLLELCLPGPDG 201
LK VR+ E L +EL + Q GDY+ + G +Y++++G +L + DG

Sbjct: 473 LKKVRIFADCEAGLLVELVLKLQPQVYSPGDYICKKGDIGREMYIIKEG--KLAVVADDG 530

Query: 202 KECVVKEVVPGD-SVNSLLSILDVITGHQHPQRTVSARAARDSTVLRLPVEAFSAVF TKY 260
+ + VV D S +SIL++ +RT + ++ S + L + T+Y

Sbjct: 531 ---ITQFVVLSDGSYFGEISILNIKSGKAGNRRTANIKSIGYSDLFCLSKDDLMEALTEY 587

Query: 261 PES 263

P++

Sbjct: 588 PDA 590

sp|Q03043|KGP2_DROME CGMP-DEPENDENT PROTEIN KINASE, ISOZYME 2 FORMS T1/T3 (CGK)

(PROTEIN FORAGING)

Length = 1088

Score = 40.6 bits (93), Expect = 0.016

Identities = 37/173 (21%), Positives = 76/173 (43%), Gaps = 16/173 (9%)

Query: 487 AGTIIARQGDQDVSLHFVLWGCLHVVYQRMIDKAEDVCLFVAQPGELVGQLAVLTGEPLIF 546
A +I ++GD ++ + G + V + E L +++G+LA+L

Sbjct: 543 AKNLIIEKGDVGSIVYVMEDGRVEVSR-----EGKYLSTLSGAKVLGELAILYNCQRTA 596

Query: 547 TLRAQRDCTFLRISKSDFYEIMRAQPSVVLSSAAHTVAARMSPFVRQM-----DFAID 598
T+ A +C I + F IM + ++ A ++ + P + + ++

Sbjct: 597 TITAITECNLWAIERQCFQTIMM-RTGLIRQAEYSDFLKSVPFVKDLAEDTLIKISDVLE 655

Query: 599 WTAVEAGRALYRQGDRSDCTYIVLNGRLRSVI-QRSGGKKELVGEYGRGDLIG 650
T + G + RQG R D +I+ G++R I Q+ + +++ + G+GD G

Sbjct: 656 ETHYQRGDHIVRQGARGDTFFIISKGKVRVTIKQQDTQEEKFIRMLGKGDFFG 708

sp|P46148|ETRA_SHEPU ELECTRON TRANSPORT REGULATOR A

Length = 250

Score = 40.2 bits (92), Expect = 0.020

Identities = 19/108 (17%), Positives = 54/108 (49%), Gaps = 1/108 (0%)

Query: 602 VEAGRALYRQGDRSDCTYIVLNGRLRSVIQRSGGKKELVGEYGRGDLIGVVEALTRQPPRA 661
++ G +++ GD + + +G ++S G +++ G + GD+IG + + Q

Sbjct: 52 IQKGEQIFKSGDLLKSLFAIPSGTIKSYTITEQGDEQITGFHLAGDVIG-FDGIHAQSHQ 110

Query: 662 TTVHAVRDTELAKLPEGTLGHIKRRYPQVVTRLIHLLSQKILGNLQQL 709
+ A+ + + ++P L + P++ +++ L+S +I+ + + +

Sbjct: 111 SFAQALETSMVCEIPFNILDELSTMPKLRQQIMRLMSNEIMSDQEMI 158

sp|P32023|KGP3_DROME CGMP-DEPENDENT PROTEIN KINASE, ISOZYME 2 FORMS T2/CD5 (CGK)

(PROTEIN FORAGING)

Length = 933

Score = 40.2 bits (92), Expect = 0.020

Identities = 29/129 (22%), Positives = 60/129 (46%), Gaps = 10/129 (7%)

Query: 531 ELVGQLAVLTGEPLIFTLRAQRDCTFLRISKSDFYEMRAQPSVVLSSAHTVAARMSPFV 590
+++G+LA+L T+ A +C I + F IM + ++ A ++ + P

Sbjct: 426 KVLGELAILYNCQRTATITAITECNLWAIERQCFQTIMM-RTGLIRQAEYSDFLKSVPFI 484

Query: 591 RQM-----DFAIDWTAVEAGRALYRQGDRSDCTYIVLNGRLRSVI-QRGSKKELVG 641
+ + ++ T + G + RQG R D +I+ G++R I Q+ + +++ +

Sbjct: 485 KDLAEDTLIKISDVLEETHYQRGDHIVRQGARGDTFFIISKGKVRVTIKQQDTQEEKFIR 544

Query: 642 EYGRGDLIG 650

G+GD G

Sbjct: 545 MLGKGDFFG 553

sp|P23619|HLYX_ACTPL REGULATORY PROTEIN HLYX

Length = 240

Score = 39.9 bits (91), Expect = 0.027

Identities = 20/108 (18%), Positives = 54/108 (49%), Gaps = 1/108 (0%)

Query: 602 VEAGRALYRQGDRSDCTYIVLNGRLRSVIQRGSKKELVGEYGRGDLIGVVEALTRQPPA 661
V+ + +++ GD Y + +G ++S SG++++ + GDL+G +A+

Sbjct: 51 VQKSQIIFQSGDELRSIYAIRSGTIKSYTISESGEEQITAFHLPGLVGFDAIMNMKHV 109

Query: 662 TTVHAVRDTELAKLPEGTLGHIKRRYPQVVTRLIHLLSQILGNLQQL 709

A+ + + ++P L + + P++ +++ L+S +I + + +

Sbjct: 110 GFAQALETSMICEIPFDILDDLAKMPKIRHQIMRLMSNEIKSDQEMI 157

sp|Q14028|CNG4_HUMAN CYCLIC-NUCLEOTIDE-GATED CATION CHANNEL 4 (CNG CHANNEL 4)

(CNG-4) (CNG4) (CYCLIC NUCLEOTIDE-GATED CATION CHANNEL

MODULATORY SUBUNIT)

Length = 909

Score = 39.9 bits (91), Expect = 0.027

Identities = 34/117 (29%), Positives = 56/117 (47%), Gaps = 14/117 (11%)

Query: 155 LFLELCRHMVFQRLGQ-----GDYVFRPGQPDASIYVVQDGLLELCPLPGPDGKECVV 206
LF R M+F L + DYV + G+ +Y++Q G +++ L GPDGK +V

Sbjct: 620 LFQGCDRQMIFDMLKRLRSVVYLPNDYVCKKGEIGREMYIIQAGQVQV-LGGPDGKSVLV 678

Query: 207 KEVVPGDSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRPLPVEAFSAVFTKYPES 263

+ SV +S+L V G+ +RT + A + + L + + + YPES

Sbjct: 679 --TLKAGSVFGEISLLAVGGGN---RRTANVVAHGFTNLFILDKKDLNEILVHYPES 730

sp|Q28181|CNG4_BOVIN 240K PROTEIN OF ROD PHOTORECEPTOR CNG-CHANNEL (CONTAINS:

GLUTAMIC ACID-RICH PROTEIN (GARP) AND CYCLIC-NUCLEOTIDE-GATED

CATION CHANNEL 4 (CNG CHANNEL 4) (CNG-4) (CYCLIC

NUCLEOTIDE-GATED CATION CHANNEL MODULATORY SUBUNIT))

Length = 1394

Score = 39.9 bits (91), Expect = 0.027

Identities = 34/117 (29%), Positives = 56/117 (47%), Gaps = 14/117 (11%)

Query: 155 LFLELCRHMVFQRLGQ-----GDYVFRPGQPDASIYVVQDGLLELCPLPGPDGKECVV 206
LF R M+F L + DYV + G+ +Y++Q G +++ L GPDGK +V

Sbjct: 1076 LFQGCDRQMIFDMLKRLRSVVYLPNDYVCKKGEIGREMYIIQAGQVQV-LGGPDGKSVLV 1134

Query: 207 KEVVPGDSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRPLPVEAFSAVFTKYPES 263
 + SV +S+L V G+ +RT + A + + L + + + YPES
 Sbjct: 1135 --TLKAGSVFGEISLLAVGGGN---RRTANVVAHGFTNLFILDKKDLNEILVHYPES 1186

sp|Q03611|YN82_CAEEL HYPOTHETICAL 88.6 KD PROTEIN ZC84.2 IN CHROMOSOME IIII
Length = 772

Score = 39.5 bits (90), Expect = 0.035
 Identities = 31/122 (25%), Positives = 54/122 (43%), Gaps = 4/122 (3%)

Query: 142 LKNVRVLGHFEKPLFLELCRHMVFQRLQGQDYVFRPGQPDASIYVVQDGLLELCPLPGPDG 201
 L+ VR+ E L EL + Q GD++ + G +Y+V+ G L++ DG
 Sbjct: 519 LRKVRIFQDCEAGLLAELVLKLQLQVFSFGDFICKKGDIGREMYIVKRGRLQVV--DDD 576

Query: 202 KECVVKEVVPGDSVNSLLSILDVITGHQHPQRTVSARAARDSTVLRPLPVEAFSAVFTKYP 261
 K+ V + SV LSIL++ +RT + R+ + + L +YP
 Sbjct: 577 KKVFEV--TLQEGSVFGEISILNIAGSKNGNRRTANVRSVGYTDLFVLSKTDLWNALREYP 634

Query: 262 ES 263
 ++
 Sbjct: 635 DA 636

sp|P12368|KAP2_RAT CAMP-DEPENDENT PROTEIN KINASE TYPE II-ALPHA REGULATORY CHAIN
Length = 370

Score = 39.1 bits (89), Expect = 0.046
 Identities = 36/153 (23%), Positives = 69/153 (44%), Gaps = 16/153 (10%)

Query: 534 GQLAVLTGEPLIFTLRAQRD-----CTFLRISKSDFYEMRAQPSVVLSSAAHTVAAR 585
 G+LA++ P T+ A D TF RI + + + S + S +
 Sbjct: 173 GELALMYNTPRAATIVATSDGSLWGLDRVTFRRRIIVKNNAKKRKMFESFIESVPLFKSLE 232

Query: 586 MSPFVRQMDFAIDWTAVEAGRALYRQGDRSDCTYIVLNGRLRSVIQ-----RSGGKKE 639
 MS ++ +D I + G + QG+++D YI+ +G + +I+ + G +E+
 Sbjct: 233 MSERMKIVD-VIGEKIYKDGRIITQGEKADSFYIIESGEVSILIRSKTKTKNNGGNQEV 291

Query: 640 -VGEYGRGDLIGVVEALTRQPRATTVHAVRDTE 671
 + +G G + +T +PRA + +AV D +
 Sbjct: 292 EIAHCHKQYFGELALVTNKPRASAYAVGDVK 324

Appendix 4 5' RACE and HOL4.2 DNA sequences

a28

1 AACAAAAGCT GGAGTCCACC GCGGTGGCGG CCGCCCGGGC AGGTGGCGCC
 51 ACCATCGGTC CCGGAGTCCC AGTGATGCTC TGTGCCATAG AGCCCCCATA
 101 ACTTCACTAC TACGTGATAG TAAATCCCCG GCAAAAACCA GCAGCGCCTT
 151 GCAAGCCCAC GCCACCCCAA GCATCCCAGG ACTCTTCTGA AACGACTCCG
 201 GGCTACCAGA TCGGCCGTCC AGCTGGAATC AACCGATGGA GGCTCCGCTG
 251 CAAACTGGAA TGGTGCTTGG CGTGATGATC GGGGCCGAG TGGCGGTGGT
 301 GGTCACGGCC GTGCTCATCC TCCTGGTGGT GCGGAGGCTG CGAGTGCCAA
 351 AAACCCCAGC CCCGGATGGC CCCCGGTATC GGTTCGGAA GAGGGACAAA

401 GTGCTCTTCT ATGGCCGGAA GATTATGCGG AAGGTGTCAC AATCCACCTC
 451 CTCCCTCGTG GATACCTCTG TCTCCGCCAC CTCCCGGCCA CGCATGAGGA
 501 AGAAACTGAA GATGCTCAAC ATTGCCAAGA AGATCCTGCG CATCCAGAAA
 551 GAGACGCCCCA CGCTGCAGCG GAAGGAGCCC CCGCCCCGAG TGCTAGAAGC
 601 TGACCTGACC GAGGGCGACC TGGCTAACTC CCATCTGCCC TCTGAAGTGC
 651 TTTATATGCT CAAGAACGTC CGGGTGCTGG GCCACTTCGA GAAGCCACTC
 701 TTCCTGGAGC TCTGCCGCCA CATGGTCTTC CAGCGGCTGG GCCAGGGTGA
 751 CTACGTCTTC CGGCCGGGCC AGCCAGATGC CAGCATCTAC GTGGTGCAGG
 801 ACGGGCTGCT GGAGCTCTGT CTGCCAGGGC CTGACGGGAA GGAGTGTGTG
 851 GTGAAGGAAG TGGTTCCTGG GGACAGCGTC AACAGCCTTC TCAGCATCCT
 901 GGATGTCATC ACCGGTCAAC AGCATCCCCA GCGGACCGTG TCTGCCCGGG
 951 CGGCCCGGGA CTCCACGGTG CTGCGCCTGC CGGTGGAAGC ATTCTCCGCG
 1001 GTCTTCACCA AGTACCCGGA GAGCTTGGTG CGGGTCGTGC AGATCATCAT
 1051 GGTGCGGCTG CAGCGAGTCA CCTTACTGGC ACTGCACAAC TACCTGGGTC
 1101 TGACCAATGA GCTCTTCAGC CACGAGATCC AGCCCCTGCG TCTGTTCCCC
 1151 AGCCCCGGCC TCCCAACTCG CACCAGCCCT GTGCGGGGCT CCAAGAGAAT
 1201 GGTCAGCACC TCAGCTACAG ACGAGCCCAG GGAGACCCCA GGGCGGCCAC
 1251 CCGATCCCAC CGGGGCCCCG CTGCCTGGAC CTACAGGGGA CCCTGTGAAG
 1301 CCCACATCCC TGGAAACCCC CTCGGCCCCCT CTGCTGAGCC GCTGCGTCTC
 1351 CATGCCAGGG GACATCTCAG GCTTGAGGG TGGCCCCCGC TCCGACTTCG
 1401 ACATGGCCTA TGAGCGTGGC CGGATCTCCG TGTCCCTGCA GGAAGAGGCC
 1451 TCCGGGGGGT CCCTGGCAGC CCCCCTCGG ACCCCCCACTC AGGAGCCTCG
 1501 TGAGCAGCCG GCAGGCGCCT GTGAATACAG CTACTGTGAG GATGAGTCGG
 1551 CCACTGGTGG CTGCCCTTTC GGGCCCTACC AGGGCCGCCA GACCAGCAGC
 1601 ATCTTCGAGG CAGCAAAGCA GGAGCTGGCC AAGCTGATGC GGATTGAGGA
 1651 CCCCTCCCTC CTGAACAGCA GAGTCTTGCT GCACCACGCC AAAGCTGGCA
 1701 CCATCATTGC CCGCCAGGGA GACCAGGACG TGAGCCTGCA CTTCTGTGCTC
 1751 TGGGGCTGCC TGCACGTGTA CCAGCGCATG ATCGACAAGG CGGAGGACGT
 1801 GTGCCTGTTC GTAGCGCAGC CCGGGGAACT GGTGGGGCAG CTGGCGGTGC
 1851 TCACTGGCGA ACCTCTCATC TTCACACTGC GAGCCCCAAG CGACTGCACC
 1901 TTCCTGCGGA TCTCCAAGTC CGACTTCTAT GAGATCATGC GCGCACAGCC
 1951 CAGTGTGGTG CTGAGTGCGG CGCACACGGT GGCAGCCAGG ATGTCGCCCT

2001 TCGTGCGCCA GATGGACTTC GCCATCGACT GGACTGCAGT GGAGGCGGGA
 2051 CGCGCGCTGT ACAGGCAGGG CGACCGCTCC GACTGCACTT ACATCGTGCT
 2101 CAATGGGCGG CTGCGTAGCG TGATCCAGCG AGGCAGTGGC AAGAAGGAGC
 2151 TGGTGGGCGA GTACGGCCGC GGCACCTCA TCGGCGTGGT GGAGGCACTG
 2201 ACCCGGCAGC CGCGAGCCAC GACGGTGAC GCGGTGCGCG ACACGGAGCT
 2251 GGCCAAGCTT CCCGAGGGCA CCTTGGGTCA CATCAAACGC CGGTACCCGC
 2301 AGGTCGTGAC CCGCCTTATC CACCTACTGA GCCAGAAAAT TCTAGGGAAT
 2351 TTGCAGCAGC TGCAAGGACC CTTCCCAGCA GGCTCTGGGT TGGGTGTGCC
 2401 CCCACACTCG GAACTCACCA ACCCAGCCAG CAACCTGGCA ACTGTGGCAA
 2451 TCCTGCCTGT GTGTGCTGAG GTCCCCATGG TGGCCTTCAC GCTGGAGCTG
 2501 CAGCACGCCC TGCAGGCCAT CGGTCCGACG CTACTCCTTA ACAGTGACAT
 2551 CATCCGGGCA CGCCTGGGGG CCTCCGCACT GGATAGCATC CAAGAGTTCC
 2601 GGCTGTC

HOL4.2

1 GCGGGGAATT TGCAGCAGCT GCAAGGACCC TTCCCAGGCT CTGGGTGGG
 51 TGTGCCCCCA CACTCGGAAC TCACCAACCC AGCCAGCAAC CTGGCAACTG
 101 TGGCAATCCT GCCTGTGTGT GCTGAGGTCC CCATGGTGGC CTTACGCTG
 151 GAGCTGCAGC ACGCCCTGCA GGCCATCGGT CCGACGCTAC TCCTTAACAG
 201 TGACATCATC CGGGCACGCC TGGGGGCCCTC CGCACTGGAT AGCATCCAAG
 251 AGTTCCGGCT GTCAGGGTGG CTGGCCCAGC AGGAGGATGC ACACCGTATC
 301 GTACTCTACC AGACGGACGC CTCGCTGACG CCCTGGACCG TGCCTGCCT
 351 GCGACAGGCC GACTGCATCC TCATTGTGGG CCTGGGGGAC CAGGAGCCTA
 401 CCCTCGGCCA GCTGGAGCAG ATGCTGGAGA ACACGGCTGT GCGCGCCCTT
 451 AAGCAGCTAG TCCTGCTCCA CCGAGAGGAG GGCGCGGGCC CCACGCGCAC
 501 CGTGGAGTGG CTAAATATGC GCAGCTGGTG CTCGGGGCAC CTGCACCTGC
 551 GCTGTCCGCG CCGCCTCTTT TCGCGCCGCA GCCCTGCCAA GCTGCATGAG
 601 CTCTTCGACA AGGTTTTCTC CAGGCGCGCG GACCGGCACA GCGACTTCTC
 701 CCGCTTGGCG AGGGTGCTCA CGGGGAACAC CATTGCCCTT GTGCTAGGCG
 751 GGGGCGGGGC CAGGGGCTGC TCGCACATCG GAGTACTAAA GGCATTAGAG
 801 GAGGCGGGGG TCCCCGTGGA CCTGGTGGGC GGCACGTCCA TTGGCTCTTT
 901 CATCGGAGCG TTGTACGCGG AGGAGCGCAG CGCCAGCCGC ACGAAGCAGC

951 GGGCCCCGGA GTGGGCCAAG AGCATGACTT CGGTGCTGGA ACCTGTGTTG
 1001 GACCTCACGT ACCCAGTCAC CTCCATGTTT ACTGGGTCTG CCTTTAACCG
 1051 CAGCATTTCAT CGGGTCTTCC AGGATAAGCA GATTGAGGAC CTGTGGCTGC
 1101 CTTACTTCAA CGTGACCACA GATATCACCG CCTCAGCCAT GCGAGTCCAC
 1151 AAAGATGGCT CCCTGTGGCG GTACCTGCGC GCCAGCATGA CGCTGTCGGG
 1201 CTACCTGCCC CCGNTGTGCG ACCCCAAGGA CGGGCACCTA CTCATGGATG
 1251 GCGGCTACAT CAACAATCTG CCAGCGGACA TCGCCCGCAG CATGGGTGCC
 1301 AAAACGGTCA TCGCCATTGA CGTGGGRAGC CAGGATGAGA CGGACCTCAG
 1351 CACCTACGGG GACAGCCTGT CCGGCTGGTG GCTGCTGTGG AAGCGGCTGA
 1401 ATCCCTGGGC TGACAAGGTA AAGGTTCCAG ACATGGCTGA AATCCAGTCC
 1451 CGCCTGGCCT ACGTGTCTTG TGTGCGGCAG CTAGAGGTTG TCAAGTCCAG
 1501 CTCCTACTGC GAGTACCTGC GCCCGCCCAT CGACTGCTTC AAGACCATGG
 1551 ACTTTGGGAA GTTCGACCAG ATCTATGATG TGGGCTACCA GTACGGGAAG
 1601 GCGGTGTTTG GAGGCTGGAG CCGTGGCAAC GTCATTGAGA AAATGCTCAC
 1651 AGACCGGCGG TCTACAGACC TTAATGAGAG CCGCCGTGCA GACGTGCTTG
 1701 CCTTCCCAAG CTCTGGCTTC ACTGACTTGG CAGAGATTGT GTCCCGGATT
 1751 GAGCCCCCCA CGAGCTATGT CTCTGATGGC TGTGCTGACG GAGAGGAGTC
 1801 AGATTGTCTG ACAGAGTATG AGGAGGACGC CGGACCCGAC TGCTCGAGGG
 1851 ATGAAGGGGG GTCCCCGAG GGC GCAAGCC CCAGCACTGC CTCCGAGATG
 1901 GAGGARGAGA AGTCGATTCT CCGGCAACGA CGCTGTCTGC CCCAGGAGCC
 1951 GCCCGGCTCA GCCACAGATG CCTGAGGACC TCGACAGGGG TCACCCCTC
 2001 CCTCCCACCC CTGGACTGGG CTGGGGGTGG CCCCCTGGGG GTAGCTCACT
 2051 CCCCCTCCTG CTGCTATGCC TGTGACCCCC GCGGCCACA CACTGGACTG
 2101 ACCTGCCCTG AGCGGGGATG CAGTGTTGCA CTGATGACTT GACCAGCCCC
 2150 TCCCCAATA AACTCGCCTC TTGGAAAAA AAAAAAAAAA AAAAAA

Translation of the full a28-HOL4.2 sequence

(residues mentioned in Chapter 4 shown in bold)

```

1                                                    50
a28HOL4 AACAAAAGCT GGAGTCCACC GCGGTGGCGG CCGCCCGGGC AGGTGGCGCC
ntelpep .T..K..A.. G..V..H..R ..G..G..G. .R..P..G.. R..W..R..H

51                                                    100
a28HOL4 ACCATCGGTC CCGGAGTCCC AGTGATGCTC TGTGCCATAG AGCCCCCATA
ntelpep ..H..R..S. .R..S..P.. S..D..A..L ..C..H..R. .A..P..I..

101                                                    150
a28HOL4 ACTTCACTAC TACGTGATAG TAAATCCCCG GCAAAAACCA GCAGCGCCTT
ntelpep T..S..L..L ..R..D..S. .K..S..P.. A..K..T..S ..S..A..L.

151                                                    200
a28HOL4 GCAAGCCCAC GCCACCCCAA GCATCCCAGG ACTCTTCTGA AACGACTCCG
ntelpep .Q..A..H.. A..T..P..S ..I..P..G. .L..F..*.. N..D..S..G

201                                                    250
a28HOL4 GGCTACCAGA TCGGCCGTCC AGCTGGAATC AACCGATGGA GGCTCCGCTG
ntelpep ..L..P..D. .R..P..S.. S..W..N..Q ..P..M..E. .A..P..L..

251                                                    300
a28HOL4 CAAACTGGAA TGGTGCTTGG CGTGATGATC GGGGCCGGAG TGGCGGTGGT
ntelpep Q..T..G..M ..V..L..G. .V..M..I.. G..A..G..V ..A..V..V.

301                                                    350
a28HOL4 GGTCACGGCC GTGCTCATCC TCCTGGTGGT GCGGAGGCTG CGAGTGCCAA
ntelpep .V..T..A.. V..L..I..L ..L..V..V. .R..R..L.. R..V..P..K

351                                                    400
a28HOL4 AAACCCCAGC CCCGGATGGC CCCCGGTATC GGTTCGGGAA GAGGGACAAA
ntelpep ..T..P..A. .P..D..G.. P..R..Y..R ..F..R..K. .R..D..K..

401                                                    450
a28HOL4 GTGCTCTTCT ATGGCCGGAA GATTATGCGG AAGGTGTCAC AATCCACCTC
ntelpep V..L..F..Y ..G..R..K. .I..M..R.. K..V..S..Q ..S..T..S.

451                                                    500
a28HOL4 CTCCCTCGTG GATACCTCTG TCTCCGCCAC CTCCCGGCCA CGCATGAGGA
ntelpep .S..L..V.. D..T..S..V ..S..A..T. .S..R..P.. R..M..R..K

501                                                    550
a28HOL4 AGAAACTGAA GATGCTCAAC ATTGCCAAGA AGATCCTGCG CATCCAGAAA
ntelpep ..K..L..K. .M..L..N.. I..A..K..K ..I..L..R. .I..Q..K..

551                                                    600
a28HOL4 GAGACGCCCA CGCTGCAGCG GAAGGAGCCC CCGCCCGCAG TGCTAGAAGC
ntelpep E..T..P..T ..L..Q..R. .K..E..P.. P..P..A..V ..L..E..A.

601                                                    650
a28HOL4 TGACCTGACC GAGGGCGACC TGGCTAACTC CCATCTGCCC TCTGAAGTGC
ntelpep .D..L..T.. E..G..D..L ..A..N..S. .H..L..P.. S..E..V..L

651                                                    700
a28HOL4 TTTATATGCT CAAGAACGTC CGGGTGCTGG GCCACTTCGA GAAGCCACTC
ntelpep ..Y..M..L. .K..N..V.. R..V..L..G ..H..F..E. .K..P..L..

701                                                    750
a28HOL4 TTCCTGGAGC TCTGCCGCCA CATGGTCTTC CAGCGGCTGG GCCAGGGTGA
ntelpep F..L..E..L ..C..R..H. .M..V..F.. Q..R..L..G ..Q..G..D.

```

	751		800
a28HOL4	CTACGTCTTC CGGCCGGGCC AGCCAGATGC CAGCATCTAC GTGGTGCAGG		
ntelpep	.Y..V..F.. R..P..G..Q ..P..D..A. .S..I..Y.. V..V..Q..D		
	801		850
a28HOL4	ACGGGCTGCT GGAGCTCTGT CTGCCAGGGC CTGACGGGAA GGAGTGTGTG		
ntelpep	..G..L..L. .E..L..C.. L..P..G..P ..D..G..K. .E..C..V..		
	851		900
a28HOL4	GTGAAGGAAG TGGTTCCTGG GGACAGCGTC AACAGCCTTC TCAGCATCCT		
ntelpep	V..K..E..V ..V..P..G. .D..S..V.. N..S..L..L ..S..I..L.		
	901		950
a28HOL4	GGATGTCATC ACCGGTCACC AGCATCCCCA GCGGACCGTG TCTGCCCCGG		
ntelpep	.D..V..I.. T..G..H..Q ..H..P..Q. .R..T..V.. S..A..R..A		
	951		1000
a28HOL4	CGGCCCGGGA CTCCACGGTG CTGCGCCTGC CGGTGGAAGC ATTCTCCGCG		
ntelpep	..A..R..D. .S..T..V.. L..R..L..P ..V..E..A. .F..S..A..		
	1001		1050
a28HOL4	GTCTTCACCA AGTACCCGGA GAGCTTGGTG CGGGTCGTGC AGATCATCAT		
ntelpep	V..F..T..K ..Y..P..E. .S..L..V.. R..V..V..Q ..I..I..M.		
	1051		1100
a28HOL4	GGTGCGGCTG CAGCGAGTCA CCTTACTGGC ACTGCACAAC TACCTGGGTC		
ntelpep	.V..R..L.. Q..R..V..T ..L..L..A. .L..H..N.. Y..L..G..L		
	1101		1150
a28HOL4	TGACCAATGA GCTCTTCAGC CACGAGATCC AGCCCCTGCG TCTGTTCCcC		
ntelpep	..T..N..E. .L..F..S.. H..E..I..Q ..P..L..R. .L..F..P..		
	1151		1200
a28HOL4	AGCCCCGGCC TCCCAActcg CACCAGCCct GtGCGGGGCT CCAAGAGAAT		
ntelpep	S..P..G..L ..P..T..R. .T..S..P.. V..R..G..S ..K..R..M.		
	1201		1250
a28HOL4	GGTCAGCACC TCAGCTACAG ACGAGCCCAG GGAGACCCCA GGGCGGCCAC		
ntelpep	.V..S..T.. S..A..T..D ..E..P..R. .E..T..P.. G..R..P..P		
	1251		1300
a28HOL4	CCGATCCCAC CGGGGCCCCG CTGCCTGGAC CTACAGGGGA CCCTGTGAAG		
ntelpep	..D..P..T. .G..A..P.. L..P..G..P ..T..G..D. .P..V..K..		
	1301		1350
a28HOL4	CCCACATCCC TGGAAACCCC CTCGGCCCCT CTGCTGAGCC GCTGCGTCTC		
ntelpep	P..T..S..L ..E..T..P. .S..A..P.. L..L..S..R ..C..V..S.		
	1351		1400
a28HOL4	CATGCCAGGG GACATCTCAG GCTTGCAGGG TGGCCCCCGC TCCGACTTCG		
ntelpep	.M..P..G.. D..I..S..G ..L..Q..G. .G..P..R.. S..D..F..D		
	1401		1450
a28HOL4	ACATGGCCTA TGAGCGTGGC CGGATCTCCG TGTCCCTGCA GGAAGAGGCC		
ntelpep	..M..A..Y. .E..R..G.. R..I..S..V ..S..L..Q. .E..E..A..		
	1451		1500
a28HOL4	TCCGGGGGGT CCCTGGCAGC CCCCCTCGG ACCCCCACTC AGGAGCCTCG		
ntelpep	S..G..G..S ..L..A..A. .P..A..R.. T..P..T..Q ..E..P..R.		
	1501		1550
a28HOL4	TGAGCAGCCG GCAGGCGCCT GTGAATACAG CTACTGTGAG GATGAGTCGG		
ntelpep	.E..Q..P.. A..G..A..C ..E..Y..S. .Y..C..E.. D..E..S..A		

	1551		1600
a28HOL4	CCACTGGTGG CTGCCcTTTC GGGCCCTACC AGGGCCGCCA GACCAGCAGC		
ntelpep	..T..G..G. .C..P..F.. G..P..Y..Q ..G..R..Q. .T..S..S..		
	1601		1650
a28HOL4	ATCTTCGAGG CAGCAAAGCA GGAGCTGGCC AAGCTGATGC GGATTGAGGA		
ntelpep	I..F..E..A ..A..K..Q. .E..L..A.. K..L..M..R ..I..E..D.		
	1651		1700
a28HOL4	CCCCTCCCTC CTGAACAGCA GAGTCTTGCT GCACCcCGCC AAAGCTGGCA		
ntelpep	.P..S..L.. L..N..S..R ..V..L..L. .H..H..A.. K..A..G..T		
	1701		1750
a28HOL4	CCATCATTCG CCGCCAGGGA GACCAGGACG TGAGCCTGCA CTTCGTGCTC		
ntelpep	..I..I..A. .R..Q..G.. D..Q..D..V ..S..L..H. .F..V..L..		
	1751		1800
a28HOL4	TGGGGCTGCC TGCACGTGTA CCAGCGCATG ATCGACAAGG CGGAGGACGT		
ntelpep	W..G..C..L ..H..V..Y. .Q..R..M.. I..D..K..A ..E..D..V.		
	1801		1850
a28HOL4	GTGCCTGTTC GTAGCGCAGC CCGGGGAACT GGTGGGGCAG CTGGCGGTGC		
ntelpep	.C..L..F.. V..A..Q..P ..G..E..L. .V..G..Q.. L..A..V..L		
	1851		1900
a28HOL4	TCACTGGCGA ACCTCTCATC TTCACACTGC GAGCCCAACG CGACTGCACC		
ntelpep	..T..G..E. .P..L..I.. F..T..L..R ..A..Q..R. .D..C..T..		
	1901		1950
a28HOL4	TTCCTGCGGA TCTCCAAGTC CGACTTCTAT GAGATCATGC GCGCACAGCC		
ntelpep	F..L..R..I ..S..K..S. .D..F..Y.. E..I..M..R ..A..Q..P.		
	1951		2000
a28HOL4	CAGTGTGGTG CTGAGTGCGG CGCACACGGT GGCAGCCAGG ATGTGCGcCCT		
ntelpep	.S..V..V.. L..S..A..A ..H..T..V. .A..A..R.. M..S..P..F		
	2001		2050
a28HOL4	TCGTGCGCCA GATGGACTTc GCCATCgaCT GGACTIONCAGT GGAGGCGGGA		
ntelpep	..V..R..Q. .M..D..F.. A..I..D..W ..T..A..V. .E..A..G..		
	2051		2100
a28HOL4	CGCGCGCTGT ACAGGCAGGG CGACCGCTCC GACTGCACTT ACATCGTGCT		
ntelpep	R..A..L..Y ..R..Q..G. .D..R..S.. D..C..T..Y ..I..V..L.		
	2101		2150
a28HOL4	CAATGGGCGG CTGCGTAGCG TGATCCAGCG AGGCAGTGGC AAGAAGGAGC		
ntelpep	.N..G..R.. L..R..S..V ..I..Q..R. .G..S..G.. K..K..E..L		
	2151		2200
a28HOL4	TGGTGGGCGA GTACGGCCGC GGCGACCTCA TCGGCGTGGT GGAGGCACTG		
ntelpep	..V..G..E. .Y..G..R.. G..D..L..I ..G..V..V. .E..A..L..		
	2201		2250
a28HOL4	ACCCGGCAGC CGCGAGCCAC GACGGTGCAC GCGGTGCGCG ACACGGAGCT		
ntelpep	T..R..Q..P ..R..A..T. .T..V..H.. A..V..R..D ..T..E..L.		
	2251		2300
a28HOL4	GGCCAAGCTT CCCGAGGGCA CCTTGGGTCA CATCAAACGC CGGTACCCGC		
ntelpep	.A..K..L.. P..E..G..T ..L..G..H. .I..K..R.. R..Y..P..Q		
	2301		2350
a28HOL4	AGGTCGTGAC CCGCCTTATC CACCTACTGA GCCAGAAAAT TCTAGGGAAT		
ntelpep	..V..V..T. .R..L..I.. H..L..L..S ..Q..K..I. .L..G..N..		

	2351		2400
a28HOL4	TTGCAGCAGC TGCAAGGACC CTTCCCAGCA GGCTCTGGGT TGGGTGTGCC		
ntelpep	L..Q..Q..L ..Q..G..P. .F..P..A.. G..S..G..L ..G..V..P.		
	2401		2450
a28HOL4	CCCACACTCG GAACTCACCA ACCCAGCCAG CAACCTGGCA ACTGTGGCAA		
ntelpep	.P..H..S.. E..L..T..N ..P..A..S. .N..L..A.. T..V..A..I		
	2451		2500
a28HOL4	TCCTGCCTGT GTGTGCTGAG GTCCCCATGG TGGCCTTCAC GCTGGAGCTG		
ntelpep	..L..P..V. .C..A..E.. V..P..M..V ..A..F..T. .L..E..L..		
	2501		2550
a28HOL4	CAGCACGCCC TGCAGGCCAT CGGTCCGACG CTACTCCTTA ACAGTGACAT		
ntelpep	Q..H..A..L ..Q..A..I. .G..P..T.. L..L..L..N ..S..D..I.		
	2551		2600
a28HOL4	CATCCGGGCA CGCCTGGGGG CCTCCGCACT GGATAGCATC CAAGAGTTCC		
ntelpep	.I..R..A.. R..L..G..A ..S..A..L. .D..S..I.. Q..E..F..R		
	2601		2650
a28HOL4	GGCTGTCAGG GTGGCTGGCC CAGCAGGAGG ATGCACACCG TATCGTACTC		
ntelpep	..L..S..G. .W..L..A.. Q..Q..E..D ..A..H..R. .I..V..L..		
	2651		2700
a28HOL4	TACCAGACGG ACGCCTCGCT GACgCCCTGG ACCGTGCGCT GCCTGCGACA		
ntelpep	Y..Q..T..D ..A..S..L. .T..P..W.. T..V..R..C ..L..R..Q.		
	2701		2750
a28HOL4	GGCCGACTGC ATCCTCaTTG TGGGCCTGGG GGACCAGGAG CCTACCCTCG		
ntelpep	.A..D..C.. I..L..I..V ..G..L..G. .D..Q..E.. P..T..L..G		
	2751		2800
a28HOL4	GCCAgCTGGA GCAGATGCTG GAGAACACGG CTGTGCGCGC CCTTAAGCAg		
ntelpep	..Q..L..E. .Q..M..L.. E..N..T..A ..V..R..A. .L..K..Q..		
	2801		2850
a28HOL4	CTAgTCCTGC TCCACCgAgA gGAGGGCGCG GGCCCCACGC GCACCGTGGA		
ntelpep	L..V..L..L ..H..R..E. .E..G..A.. G..P..T..R ..T..V..E.		
	2851		2900
a28HOL4	GTGGCTAAAT ATGCGCAGCT GGTgCTCGGG GCACCTGCAC CTGCgCTGTC		
ntelpep	.W..L..N.. M..R..S..W ..C..S..G. .H..L..H.. L..R..C..P		
	2901		2950
a28HOL4	CGCGCCGCTT CTTTTCGCGC CGCAGCCCTG CCAAGCTGCA TGAGCTCTTC		
ntelpep	..R..R..L. .F..S..R.. R..S..P..A ..K..L..H. .E..L..F..		
	2951		3000
a28HOL4	GACAAGGTTT TCTCCAGGCG CGCGGACCGG CACAGcgacT TCTCCCGCTT		
ntelpep	D..K..V..F ..S..R..R. .A..D..R.. H..S..D..F ..S..R..L.		
	3001		3050
a28HOL4	GGCGAGGGTG CTCACGGGGA ACACCATTCG CCTTGTGCTA GGCGGGGGCG		
ntelpep	.A..R..V.. L..T..G..N ..T..I..A. .L..V..L.. G..G..G..G		
	3051		3100
a28HOL4	GGGCCAGGGG CTGCTCGCAC ATCGGAGTAC TAAAGGCATT AGAGGAGGCG		
ntelpep	..A..R..G. .C..S..H.. I..G..V..L ..K..A..L. .E..E..A..		
	3101		3150
a28HOL4	GGGGTCCCCG TGGACCTGGT GGGCGGCACG TCCATTGGCT CTTTCATCGG		
ntelpep	G..V..P..V ..D..L..V. .G..G..T.. S..I..G..S ..F..I..G.		

	3151		3200
a28HOL4	AGCGTTGTAC GCGGAGGAGC GCAGCGCCAG CCGCACGAAG CAGCGGGGCC		
ntelpep	.A..L..Y.. A..E...E..R ..S..A..S. .R..T..K.. Q..R..A..R		
	3201		3250
a28HOL4	GGGAGTGGGC CAAGAGCATG ACTTCGGtGC TGGAACTGT GTTGGACCTC		
ntelpep	..E..W..A. .K..S..M.. T..S..V..L ..E..P..V. .L..D..L..		
	3251		3300
a28HOL4	ACGTACCCAG TCACCTcCAT GTTCACTGGG TCTGcCTTTA ACCGCAGCAT		
ntelpep	T..Y..P..V ..T..S..M. .F..T..G.. S..A..F..N ..R..S..I.		
	3301		3350
a28HOL4	TCATCGGGTC TTCCAGGATA AGCAGATTGA GGACCTGTGG CTGCCTTACT		
ntelpep	.H..R..V.. F..Q..D..K ..Q..I..E. .D..L..W.. L..P..Y..F		
	3351		3400
a28HOL4	TcAACGTGAC CACAGATATC ACCGCcTCaG CCATGCgAGT cCACAAAGAT		
ntelpep	..N..V..T. .T..D..I.. T..A..S..A ..M..R..V. .H..K..D..		
	3401		3450
a28HOL4	GGCTCCCTGT GGCGGTACct GCGCGCCAGC ATGACgCTGT CGGGCTACCT		
ntelpep	G..S..L..W ..R..Y..L. .R..A..S.. M..T..L..S ..G..Y..L.		
	3451		3500
a28HOL4	GCCCCGCTG TGCGAcCCCA AgGACGGGCA CCTACTCATG GATGGCGGCT		
ntelpep	.P..P..L.. C..D..P..K ..D..G..H. .L..L..M.. D..G..G..Y		
	3501		3550
a28HOL4	ACATCAACAA TCTGCCAGCG GACATCGCCC GCAGCATGGG TGCCAAAACG		
ntelpep	..I..N..N. .L..P..A.. D..I..A..R ..S..M..G. .A..K..T..		
	3551		3600
a28HOL4	GTCATCGCCA TTgACGTGGG GAGCCAGGAT gAGaCGGACc TCAGCACCTA		
ntelpep	V..I..A..I ..D..V..G. .S..Q..D.. E..T..D..L ..S..T..Y.		
	3601		3650
a28HOL4	CGGGGACAGC CTGTCCGGCT GGTGgCTgCT GTGGAAgCGG CTgAATCCCT		
ntelpep	.G..D..S.. L..S..G..W ..W..L..L. .W..K..R.. L..N..P..W		
	3651		3700
a28HOL4	GGGCTGACAA GGTAAAGGTT CCAGACATGG CTGAAATCCA GTCCCGCcTG		
ntelpep	..A..D..K. .V..K..V.. P..D..M..A ..E..I..Q. .S..R..L..		
	3701		3750
a28HOL4	GCCTACGTGT CCTGTGTgCG GCAGCTAGAG GTTGTCAAGT CCAGCTCcTA		
ntelpep	A..Y..V..S ..C..V..R. .Q..L..E.. V..V..K..S ..S..S..Y.		
	3751		3800
a28HOL4	CTGCGAGTAC CTGCGCCCCG CCATCGACTG CTTCAAGACC ATGGACTTTG		
ntelpep	.C..E..Y.. L..R..P..P ..I..D..C. .F..K..T.. M..D..F..G		
	3801		3850
a28HOL4	GGAAGTTCGA CCAGATCTAT GATGTGGGCT ACCAGTACGG GAAGGCGGTG		
ntelpep	..K..F..D. .Q..I..Y.. D..V..G..Y ..Q..Y..G. .K..A..V..		
	3851		3900
a28HOL4	TTTGAGAGCT GGAGCCGTGG CAACGTCATT GAGAAAATGC TCACAGACCG		
ntelpep	F..G..G..W ..S..R..G. .N..V..I.. E..K..M..L ..T..D..R.		
	3901		3950
a28HOL4	GCGGTCTACA GACCTTAATG AGAGCCGCCG TGCAGACGTG CTTGCCTTCC		
ntelpep	.R..S..T.. D..L..N..E ..S..R..R. .A..D..V.. L..A..F..P		

	3951		4000
a28HOL4	CAAGCTCTGG CTTCACTGAC TTGGCAGAGA TTGTGTCCCG GATTGAGCCC		
ntelpep	..S..S..G. .F..T..D.. L..A..E..I ..V..S..R. .I..E..P..		
	4001		4050
a28HOL4	CCCACGAGCT ATGTCTCTGA TGGCTGTGCT GACGGAGAGG AGTCAGATTG		
ntelpep	P..T..S..Y ..V..S..D. .G..C..A.. D..G..E..E ..S..D..C.		
	4051		4100
a28HOL4	TCTGACAGAG TATGAGGaGG ACGCCcGACC CGACTGCTCG AgGGATGAAG		
ntelpep	.L..T..E.. Y..E..E..D ..A..G..P. .D..C..S.. R..D..E..G		
	4101		4150
a28HOL4	GGGGGTCCCC CGAGGGCGCA AGCCCCAGCA CTGCCTCCGA GATGGAGGAG		
ntelpep	..G..S..P. .E..G..A.. S..P..S..T ..A..S..E. .M..E..E..		
	4151		4200
a28HOL4	GAgAAGTCGA TTCTCCGGCA ACGACgCTGT CTGcCCCAGG AgCCGCCCCG		
ntelpep	E..K..S..I ..L..R..Q. .R..R..C.. L..P..Q..E ..P..P..G.		
	4201		4250
a28HOL4	cTCAGCCACA GATGCCTGAG GACCTCGaCA GGGGTCACCC CCTCCCTCCC		
ntelpep	.S..A..T.. D..A..*..G ..P..R..Q. .G..S..P.. P..P..S..H		
	4251		4300
a28HOL4	ACCCCTGGAC TGGGCTGGGG GTGGCCCCGT GGGGGTAGCT CACTCCCCCT		
ntelpep	..P..W..T. .G..L..G.. V..A..P..W ..G..*..L. .T..P..P..		
	4301		4350
a28HOL4	CCTGCTGCTA TGCCTGTGAC CCCCgCGGCC CACACACTGG ACTGACCTGC		
ntelpep	P..A..A..M ..P..V..T. .P..A..A.. H..T..L..D ..*..P..A.		
	4351		4400
a28HOL4	CCTGAGCGGG GATGCAGTGT TGCACTGATG ACTTGACCAG CCCCTCCCCC		
ntelpep	.L..S..G.. D..A..V..L ..H..*..*.. .L..D..Q.. P..L..P..Q		
	4401		4440
a28HOL4	AATAAACTCG CCTCTTGGA AAAAAAAAAa aaAAAAAAAA		
ntelpep	..*..T..R. .L..L..E.. K..K..K..K ..K..K..		

References

- Abou-Donia M. B. (1981) Organophosphorus ester induced delayed neurotoxicity. *Ann. Rev. Pharmacol. Toxicol.* 21, 511-548
- Aldridge W. N., (1954) Tricresylphosphate and cholinesterase. *Biochem. J.* 56 pp185-189
- Altschul S. F., Madden T. L., Alejandro A., Schaumlffer A. A., Zhang J., Zhang Z., Miller W, and Lipman D. J., (1997) Gapped BLAST and PSI-BLAST: a New Generation of Protein Database Search Programs. *Nucleic Acids Res.* 25 pp3389-3402.
- Argos P., (1989) Predictions of Protein Structure from Gene and Amino Acid Sequences. IRL press. *Protein Structure a Practical Approach.* Edited by Creighton T. E. pp169-190
- Auld V. J., Fetter R. D., Broadie K. B. and Goodman C. S., (1995) Gliotactin, a Novel Transmembrane Protein on Peripheral Glia, Is Required to Form the Blood-Nerve Barrier in *Drosophila*. *Cell* 81 pp757-767
- Ayyub C., Paranjape J., Rodrigues V. and Siddiqi O., (1990) Genetics of Olfactory Behavior in *Drosophila Melanogaster*. *J. Neurogenetics* 6 pp243-262
- Bitstrup P. L., Bonnell J. A. and Beckett A. G., (1953) Paralysis Following Poisoning by a New Organic phosphorus insecticide (mipafox) *Brit. Med. j.* 1 pp1068-69
- Bouldin T. W. and Cavanagh J. B., Organophosphate Neuropathy: I. A Teased-Fiber Study of the Spatio-Temporal Spread of Axonal Degeneration. *Am. Ass. Pathologists* 94(2) pp241-251
- Brendel V., Bucher P., Nourbakhsh I., Blaisdell B. E. and Karlin S. (1992) Methods and Algorithms for Statistical Analysis of Protein Sequences. *Proc. Natl. Acad. Sci. USA* 89 pp2002-2006
- Brenner S., (1988) The Molecular Evolution of Genes and Proteins: a Tale of Two Serines. *Nature* 334 pp528-530
- Carlson F. R., (1996) Olfaction in *Drosophila* from Odor to Behavior. *TIG* 12 (5) pp175-180
- Carrington C. D. and Abou-Donia M. B., (1985) Axoplasmic Transport and Turnaround of Neurotoxic Esterase in Hen Sciatic Nerve. *J. Neurochem.* 44 pp616-621
- Casida J. E., Eto M., and Baron R. L., (1961) Biological Activity of Tri o-cresyl Phosphate metabolite *Nature* 191 pp1396-1397

Creighton T. E., (1993) *Proteins Structure and Molecular Properties*. W. H. Freeman and Company pp261-325

Chatel J.-M., Grassi J., Frobert Y., Massoulié J. and Vallette F.-M., (1993) Existence of an Inactive Pool of Acetylcholinesterase in Chicken Brain. *Neurobiology* 90 pp2476-2480

Clothier B. and Johnson M. K., (1974) Rapid Ageing of Neurotoxic Esterase after Inhibition by Di-isopropyl Phosphorofluoridate. *Biochem. J.* 177 pp549-558

Clark M., Lehrach H., Appel B., Eisen J., Johnson S., Marra M., Eddy S., Hillier L., Allen M., Bowles L., Dubuque T., Geisel G., Jost S., Kucaba T., Lacy, M., Le N., Lennon G., Martin J., Moore B., Schellenberg K., Steptoe M., Tan F., Theising B., White Y., Wylie T., Waterston R., Wilson R. (1997) WashU Zebrafish EST Project. Unpublished AC: AA494858

Clontech (1997) *Marathon-Ready cDNA User manual* (PT1156-1 version PR64097)

Chemnitus J. M., Haselmeyer K. H. and Zech R., (1984) Neurotoxic Esterase: Gel Filtration and Isoelectric Focusing of Carboxylesterases Solubilized from Hen Brain. *Life Sci.* 34 pp1119-1125

Dang C. V., Barrett J., Villagarcia M., Resar L. M. S., Kato G. J. and Fearon E. R., (1991) Intracellular Leucine Zipper Interactions Suggest C-Myc Hetero-Oligomerization. *Molecular and Cellular Biology* 11(2) pp954-962

Davies K. E. and Read A. P., (1988) *Locating Genes. Molecular Basis of Inherited Disease Second Edition* IRL press. Edited by D. Rickwood pp15-36

Davis C. S. and Richardson R., (1987) Neurotoxic Esterase: Characterisation of the Solubilized Enzyme and the Conditions for its Solubilization from Chicken Brain Microsomal Membranes with Ionic, Zwitterionic, or non-ionic detergents. *Biochem. Pharmacol* 36 pp1393-1399

Devlin K., Churcher C., Barrell B. G. and Rajandream M. A., unpublished em:Z46729, sw: Q04958

Drapeau G. R. (1976) [38] *Protease from Staphylococcus aureus. Methods in Enzymology.* 45, pp469-475

Dry I. B. and Robinson S. P., (1994) Molecular cloning and characterisation of grape berry polyphenol oxidase *Plant Molecular Biology* 26 pp495-502

Edwards J. B. D. M., Ravassard P., Icard-Liepkalns C. and Mallet J. (1995) *cDNA Cloning by RT-PCR. IRL press. PCR 2: A Practical Approach.* Edited by McPherson M. J., Hames B. D. and Taylor G. R. pp89-118

Escalera S. de la., Bockamp E.-O., Moya F., Piovant M. and Jiménez F., (1990) Characterization and gene cloning of neurotactin, a *Drosophila* transmembrane protein related to cholinesterases. *EMBO Journal* 9 (11) pp3593-3601

Eto M., Casida J. E. and Eto T., (1962) Hydroxylation and Cyclization Reactions Involved in the Metabolism of Tri *o*-cresyl Phosphate. *Biochem. Pharmacol.* 11 pp337-352

Frohman M. A., (1990) RACE: Rapid Amplification of cDNA Ends. *PCR Protocols: A guide to Methods and Applications.* Academic Press, Inc. Edited by Innis M.A., Gelfand D. H., Sninsky J. J. and White T. J. pp28-38

Glynn P., Rüffer-Turner M. E., Read D. J., Wylie S. and Johnson M. K (1993) Molecular Characterisation of Neuropathy Target Esterase: Proteolysis of the [3H]DFP-Labelled Polypeptide. *Chem.-Biol. Interactions* 87, 361-367

Glynn P., Read D. J., Guo R., Wylie S., and Johnson M. K., (1994) Synthesis and characterization of a biotinylated organophosphorus ester for detection and affinity purification of a brain serine esterase: neuropathy target esterase. *Biochem. J.* 301, 551-556

Glynn P., Holton J. L., Nolan C. C., Read D. J., Brown L., Hubbard A. and Cavanagh J. B., (1998) Neuropathy Target Esterase: Immunolocalization to Neuronal Cell Bodies and Axons. *Neuroscience* 83(1) pp295-302

Gnagey A. L., Forte M., and Rosenberg T. L., (1997) Isolation and Characterization of Acetylcholinesterase from *Drosophila*. *J. Biol. Chem.* 262(27) pp13290-13298

Goldenring J. R., Shen K. R., Vaughan H. D., and Modlin I. M., (1993) Identification of a Small GTP-binding Protein, Rab25, Expressed in the Gastrointestinal Mucosa, Kidney, and Lung. *J. Biol. Chem.* 268(25) pp18419-18422

Heisenberg and Böhl (1979) Isolation of Brain Mutants of *Drosophila* by Histological Means. *Z. Naturforsch* 34 pp143-147

Hasan G., (1990) Molecular cloning of an Olfactory Gene from *Drosophila Melanogaster*. *Proc. Natl. Acad. Sci. USA.* 87 pp9037--9041

Hofmann K. and Stoffel W., (1993) TMbase- A Database of Membrane Spanning Protein Segments *Biol. Chem. Hoppe-Seyler* 347 pp166

Holmstedt B., (1963) Structure-activity Relationships of the Organophosphorus Anticholinesterase Agents. *Cholinesterases and Anticholinesterase Agents.* Berlin, Heidelberg: Springer. Edited by Koelle G. B. pp428-485

Henikoff and Henikoff, (1992) Amino Acid Substitution Matrices from Protein Blocks. *Proc. Natl. Acad. Sci. USA* 89; 10915-10919

Hope I. A., Albertson D. G., Martinelli S. D., Lynch A. S., Sonnhammer E. and Durbin R., (1996) The *C. elegans* Expression Pattern Database: a Beginning TIG 12(9) pp370-371. URL:http://eatworms.swmed.edu/Worm_labs/Hope

Ichtchenko K., Hata Y., Nguyen T., Ullrich B., Messler M., Moomaw C. and Südhof T. C., (1995) Neuroligin 1: A Splice Site-Specific Ligand for b-Neurexins. *Cell* 81 pp435-443

Ichtchenko K., Nguyen T. and Südhof T. C., (1996) Structure, Alternative Splicing, and Neurexin Binding of Multiple Neuroligins. *J. Biological Chemistry* 271 (5) pp2676-2682

Inoue N., Fujishiro K., Mori K. and Matsuoka M., (1988) Triorthocresyl Phosphate Poisoning - A Review of Human Cases. *J. UOEH* 10 (4) pp433-442

Iwabuchi K., Li B., Bartel P. and Fields S. (1993) Use of the 2-Hybrid System to Identify the Domain of P53 Involved in Oligomerization. *Oncogene* 8(6), pp1693-1696

Johnson M. K., (1969a) A Phosphorylation Site in Brain and the Delayed Neurotoxic Effect of Some Organophosphorus Compounds. *Biochem. J.* 111 pp487-495

Johnson M. K., (1969b) The Delayed Neurotoxic Effect of Some Organophosphorus Compounds. Identification of the Phosphorylation Site as an Esterase. *Biochem. J.* 144 pp711-717

Johnson M. K., (1970) Organophosphorus and Other Inhibitors of Brain 'Neurotoxic esterase' and the Development of Delayed Neurotoxicity in Hens. *Biochem. J.* 120 pp523-531

Johnson M. K., (1975) Organophosphorus Esters Causing Delayed Neurotoxic Effects: Mechanism of Action and Structure/Activity Studies. *Arch.Toxicol.* 34 pp259-288

Johnson M. K. (1975b) Structure-Activity Relationships for Substrates and Inhibitors of Hen Brain Neurotoxic Esterase. *Biochem. Pharmacol.* 24 pp797-805

Johnson M. K., (1978) Improved Assay Of Neurotoxic Esterase for Screening Organophosphates for Delayed Neurotoxic Potential. *Arch. Toxicol.* 37 pp113-115

Johnson M. K., (1982) The Target for Initiation of Delayed Neurotoxicity by Organophosphates Esters: Biochemical Studies and Toxicological Applications. *Rev. Biochem. Toxicol* Vol 4 New York: Elsevier. Edited by Hodgson E., Bend J. R. and Philpot R. M. pp141-212

Johnson M. K. and Read D. J., (1987) The Influence of Chirality on the Delayed neuropathic potential of some organophosphate esters: delayed neuropathic and protective respectively are caused by the L(-) and D(+) isomers of EPN and its oxon. *Toxicol. Appl. Pharmacol.* 90 pp103-115

Johnson M. K., (1988) Sensitivity and Selectivity of Compounds Interacting with Neuropathy Target Esterase: Further Structure/Activity Studies. *Biochem. Pharmacol.* 37 pp4094-4104

Johnson M. K., (1992) Molecular Events in Delayed Neuropathy: Experimental Aspects of Neuropathy Target Esterase. *Clinical and Experimental Toxicology of Organophosphates and Carbamates*. Butterworth Heinemann. Edited by Ballantyne B. and Marrs T. C. pp90-113

Johnson W. G., Murphy M., Murphy W. I. and Bloom A. D. (1987) Recessive congenital cerebellar disorder in a genetic isolate: CPD type VII *Neurology*(Abstract) 28 pp352-353.

Kidd K. K. and Ruano G. (1995) Optimizing PCR. *PCR 2: A Practical Approach*. Edited by McPherson M. J., Hames B. D. and Taylor G. R. pp1-22

Kozak M., (1986) Point mutations Define a Sequence Flanking the AUG Initiator Codon that Modulates Translation by Eukaryotic Ribosomes. *Cell* 44 pp283-292

Krejci E., Duval N., Chatonnet A., Vincens P., and Massoulie J., (1991) Cholinesterase-like domains in enzymes and structural proteins: Functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proc. Natl. Acad. Sci. USA* 88, pp6647-6651

Kretschmar D., Hasan G., Sharma S., Heisenberg M. and Benzer S., (1997) The swiss cheese mutant causes glial hyperwrapping and brain degeneration in *Drosophila*. *J. Neurosci.* 17 (19) pp7425-7432

Kwok S., Kellogg D. E., McKinney N., Spasic D., Goda L., Levenson C. and Sninsky J. J., (1990) Effects of Primer-Template Mismatches on the Polymerase Chain Reaction: Human Immunodeficiency Virus Type 1 Model Studies. *Nucleic Acids Research* 18 (4) pp999-1005

Laemmli U. K., (1971) Cleavage of Structural Proteins During Assembly of Bacteriophage T4. *Nature* 227 pp680-685

Lamballe F., Klein R., and Barbacid M. (1991) trkC, a New Member of the trk Family of Tyrosine Protein Kinases Is a Receptor of Neurotrophin-3. *Cell* 66 pp967-979

Layer P. G., Weikert T. and Alber R., (1993) Cholinesterases Regulate Neurite Growth of Chick Nerve Cells In Vitro by Means of a Non-enzymic Mechanism. *Cell Tissue Res* 273 pp219-226

Lee C. C. and Caskey C. T., (1990) cDNA Cloning using Degenerate Primers. PCR Protocols: A guide to Methods and Applications. Academic Press, Inc. Edited by Innis M.A, Gelfand D. H., Sninsky J. J. and White T. J. pp46-53

Lennon G. G., Auffray C., Polymeropoulos M. and Soares M. B., (1996) The I.M.A.G.E. Consortium: An Integrated Molecular Analysis of Genomes and their Expression. Genomics 33 pp151-152

Lotti M., (1992) The Pathogenesis of Organophosphate Polyneuropathy. Crit. Rev. Toxicol. 21(6) pp465-487

Lundberg K. S., Shoemaker D. D., Adams M. W. W., Short J. M., Sorge J. A., and Mathur E. J. (1991) High-fidelity Amplification Using a Thermostable DNA Polymerase Isolated from *Pyrococcus furiosus*. Gene 108 pp1-6

Lush, M. J., Li, Y., Read, D., Whillis, A. C. and Glynn, P. (1998) Neuropathy Target Esterase (NTE) and a homologous *Drosophila* neurodegenerative-associated mutant protein contains a novel domain conserved from bacteria to man. Biochem. J. 332 pp1-4

Luque E. A., Lemanski L. F., and Dube D. K., (1994) Molecular Cloning, Sequencing and Expression of an Isoform of Cardiac Alpha-tropomyosin from the Mexican Axolotl (*Ambystoma Mexicanum*). Biochem. and Biophys Research Communications 203(1) pp319-325

Mackay C. E., Hammock B. D. and Wilson B. W. (1996) Identification and Isolation of a 155kDa Protein with Neuropathy Target Esterase Activity. Fundamental and Applied Toxicology 30 pp23-30

McPherson M. J., Jones K. M. and Gurr S. J. (1991) PCR with highly degenerate primers. PCR: A Practical Approach. IRL press. Edited by McPherson M. J., Quirke P., and Taylor G. R. pp171-186

Meredith C. and Johnson M. K., (1989) Species Distribution of Paraoxon-Resistant Brain Polypeptides Radiolabelled with Diisopropyl Phosphorofluoridate ($[^3\text{H}]\text{DiPF}$): Electrophoretic Assay for the Aged Polypeptide of $[^3\text{H}]\text{DiPF}$ -Labelled Neuropathy Target Esterase. J. Neurochem, 52, 1248-1252

Meredith C. and Johnson M. K., (1988) Neuropathy Target Esterase: Rates of Turnover In Vivo Following Covalent Inhibition with Phenyl Di-n-Pentylphosphinate. J. Neurochem. 51(4) pp1097-1101

Hirokawa T., Boon-Chieng S. and Mitaku S., (1998) SOSUI: Classification and Secondary Structure Prediction System for Membrane Proteins. in press, Bioinformatics, formerly known as CABIOS

Morgan J. P., and Penovich P., (1978) Jamaica Ginger paralysis. Archives Neurology 35 pp530-532

Moretto A., Lotti M., Sabri M. I. and Spencer P. S., (1987) Progressive Deficit of Retrograde Axonal Transport is Associated with the Pathogenesis of di-n-butyl dichlorvos axonopathy. *J. Neurochem* 49 pp1515-1522

Nystuen A., Benke P. J., Merren J., Stone E., and Sheffield V. C., (1996) A Cerebellar Ataxia Locus Identified by DNA pooling to Search for Linkage Disequilibrium in an Isolated Population from the Cayman Islands. *Human Mol. Genet.* 5 (4) pp525-531

Ochman H., Medhora M. M., Garza D., and Hartl D. L. (1990) Amplification of Flanking Sequences by Inverse PCR. *PCR Protocols: A guide to Methods and Applications.* Academic Press, Inc. Edited by Innis M.A, Gelfand D. H., Sninsky J. J. and White T. J. pp219-227

Olender E. H. and Simoni R. D., (1992) The intracellular Targeting and membrane Topology of 3-Hydroxy-3-methyl Glutaryl-CoA Reductase. *J. Biol. Chem.* 267 pp4233-4235

Olson P. F., Fessler L. I., Nelson R. E., Sterne R. E., Campbell A. G. and Fessler J. H., (1990) Glutactin, a Novel *Drosophila* Basement Membrane-related Glycoprotein with Sequence Similarity to Serine Esterases. *EMBO J.* 9(4) pp1219-1227

OMIM (1997), Online Mendelian Inheritance in Man. Center for Medical Genetics, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 1997. World Wide Web URL: <http://www.ncbi.nlm.nih.gov/omim/>

Paterson J. and O'Hare K., (1991) Structure and Transcription of the Singed Locus of *Drosophila Melanogaster*. *Genetics* 129 pp1073-1084

Poulsen E. and Aldridge W. N., (1964) Studies On Esterases In The Chicken Central Nervous System. *Biochem. J.* 90 pp182-189

Pope C. N. and Padillia S. S., (1989) Chromatographic characterization of neurotoxic esterase. *Biochem. Pharmacol.* 38 pp181-188

Promega (1996) Promega Protocols and Application Guide 3rd Edition Promega, pp49-51

QIAGEN (1997) QIAquick Spin Handbook. QIAGEN

Rice G. C., McCabe S., Pennica D. P., Woronicz J., Borree J. and Goeddel D. V. (1990) Expression Cloning by Transient Expression Utilising Fluorescence Activated Cell Sorting. *DNA and Protein Engineering Techniques* 2 (1) pp18-22

Rüffer-Turner M. E., Read D. J., and Johnson M. K., (1992) Purification of Neuropathy Target Esterase from Avian Brain After Prelavelling with [3H]Disopropyl Phosphorfluoridate. *J. Neurochem.* 58 (1) pp135-141

Sambrook J., Fritsch E. F. and Maniatis T., (1989a) *Molecular Cloning, A Laboratory Manual* (2nd edition) Cold Spring Harbor Laboratory Press

Sambrook J., Fritsch E. F. and Maniatis T., (1989b) *Molecular Cloning, A Laboratory Manual* (2nd edition) Cold Spring Harbor Laboratory Press pp12.4

Schmidt H., Dresselhaus T., Buck F. and Heinz E., (1994) Purification and PCR-based cDNA Cloning of a Plastidial n-5 Desaturase. *Plant Molecular Biology* 26 pp631-642

Schmidt J. V., Carver L. A., and Bradfield C. A. (1993) Molecular characterization of the Murind Ahr Gene. *J. Biol. Chem.* 268, 22203-22209

Schuler G.D., Boguski M. S., Stewart E. A., Stein L. D., Gyapay G., Rice K., White R. E., Rodriguez-Tomé P., Aggarwal A., Bajorek E., Bentolila S., Birren B. B., Butler A., Castle A. B., Chiannikulchai N., Chu A., Clee C., Cowles S., Day P. J. R., Dibling T., Drouot N., Dunham I., Duprat S., East C., Edwards C., Fan J.-B., Fang N., Fizames C., Garrett C., Green L., Hadley D., Ma J., MacGilvery A., Mader C., Maratukulam A., Matisse T. C., Mckusick K. B., Morissette J., Mungall A., Muselet D., Nusbaum H. C., Page D. C., Peck S., Perkins S., Piercy M., Qin F., Quackenbush J., Ranby S., Reif T., Rozen S., Sanders C., She X., Silva J., Slonim D. K., Soderlund C., Sun W.-L., Tabar P., Thangarajh T., Vega-Czarny N., Vollrath D., Voyticky S., Wilmer T., Wu X., Adams M. D., Auffray C., Walter N. A. R., Brandon R., Dehejia A., Goodfellow P. N., Houlgatte R., Hudson Jr J. R., Ide S. E., Iorio K. R., Lee W. Y., Seki N., Nagase T., Ishikawa K., Nomura N., Phillips C., Polymeropoulos M. H., Sandusky M., Schmitt K., Berry R., Swanson K., Torres R., Venter J. C., Sikela J. M., Beckmann J. S., Weissenbach J., Myers R. M., Cox D. R., James M. R., Bentley D., Deloukas P., Lander E. S. and Hudson T. J. (1996) A Gene Map of the Human Genome. *Science* 274 pp540-546

Schwartz, R. M. and Dayhoff, M. O. [1979] *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Washington D.C. Edited by Dayhoff, M. O. pp353-358

Silver J., (1991) *Inverse Polymerase Chain Reaction. PCR: A Practical Approach*. IRL press. Edited by McPherson M. J., Quirke P., and Taylor G. R. pp137-146

Simmonds R. J., 1992 *Chemistry of Biomolecules An Introduction*. Royal Society of Chemistry. pp94-95

Smith M.I., Elvove E. and Frazier W. H., (1930) The pharmacological action of certain phenol esters with special reference to the etiology of so-called ginger paralysis *Public Health Reports*, 45 (42) pp2509-2524

Stratagene, (1997) *pCR-Script Amp SK(+) Cloning Kit. Instruction Manual* (Revision #076004)

Taylor G. R., (1991) Polymerase Chain Reaction: Basic Principles and Automation. PCR: A Practical Approach. IRL press. Edited by McPherson M. J., Quirke P., and Taylor G. R. pp1-14

Thomas T. C., Székács A., Hammock B. D., Wilson B. W. and McNamee M. G. (1990) Characterization of Neuropathy Target Esterase using Trifluoromethylketones. *Biochem. Pharmacol.* 257 pp106-116

Thomas T. C., Székács A., Hammock B. D., Wilson B. W. and McNamee M. G. (1993) Affinity Chromatography of Neuropathy Target Esterase. *Chemico-Biological Interactions* 87 No. 1-3 pp347-360

Tindall, K. R., and Kunkel T. A. (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* 27; 6008-6013

Wicking C. and Williamson B. (1991) From Linked Marker to Gene. *TIG* 7 (9) pp288-293

Williams D. G. and Johnson M. K. (1981) Gel Electrophoretic Identification of Hen Brain Neurotoxic Esterase, Labelled with Tritiated Diisopropyl Phosphorofluoridate. *Biochem J.* 199 pp323-333

Wilson R., Ainscough R., Anderson K., Baynes C., Berks M., Bonfield J., Burton J., Connell M., Copsey T., Cooper J., Coulson A., Craxton M., Dear S., Du Z., Durbin R., Favello A., Fraser A., Fulton L., Gardner A., Green P., Hawkins T., Hillier L., Jier M., Johnston L., Jones M., Kershaw J., Kirsten J., Laisster N., Latreille P., Lightning J., Lloyd C., Mortimore B., O'callaghan M., Parsons J., Percy C., Rifken L., Roopra A., Saunders D., Shownkeen R., Sims M., Smaldon N., Smith A., Smith M., Sonnhammer E., Staden R., Sulston J., Thierry-Mieg J., Thomas K., Vaudin M., Vaughan K., Waterson R., Watson A., Weinstock L., Wilkinson-Sproat J., Wohldman P. (1994). 2.2 Mb of Contiguous Nucleotide Sequence from Chromosome III of *C. elegans*. *Nature* 368 32-38

Yoshida M., Tomizawa M., Wu S.-Y., Quistad G. B. and Casida (1995) Neuropathy Target Esterase of Hen Brain: Active Site Reactions with 2-[octyl-³H]Octyl-4H-1,3,2-Benzodioxaphosphorin 2-Oxide and 2-Octyl-4H-1,3,2-[aryl-³H]Benzodioxaphosphorin 2-Oxide. *J. Neurochem.* 64(4) pp 1680-1687