The isolation and characterisation of the genes coding for the calcium channel affected by Lambert-Eaton myasthenic syndrome antibodies in NG108-15 cells

Lambert-Eaton myasthenic syndrome is a rare paraneoplastic and autoimmune disorder affecting the presynaptic voltage gated calcium channels at the neuromuscular junction. The aim of this project was to isolate the genes coding for the $\alpha 1$ and $\alpha 2$ subunits of the voltage-gated calcium channel from hybrid neuronal cell lines including NG108-15 whose potassium stimulated $^{45}Ca^{2+}$ flux is reduced by IgG from patients with Lambert-Eaton myasthenic syndrome.

The cDNA libraries were constructed in λgt10 from NG108-15 mRNA and screened with PCR products derived from the rabbit skeletal muscle $\alpha 1$ and $\alpha 2$-δ genes. A 2230 bp portion of the NG108-15 $\alpha 2$-δ gene homologous to bp 1000-3304 of the rabbit gene has been isolated and sequenced. This shows 48% homology to the amino acid sequence of the rabbit gene and 56% nucleotide homology with particular conservation of the C-terminal domains.

The NG108-15 $\alpha 2$ mRNA is 8.5 kb long and was found in the N18TG2 mouse neuroblastoma cell line from which NG108-15 is derived and in mouse brain. Rabbit $\alpha 2$-δ gene PCR primers were used to screen a rodent tissue cDNA panel to detect highly homologous sequences by PCR amplification. These products were detected only in mouse brain and lung and in rat skeletal muscle. The mouse brain and lung product amino acid sequences were 87% homologous to the rabbit gene while the rat skeletal muscle product was 81% homologous and both differed significantly from the homologous region of the NG108-15 gene.

This suggests that some of the diversity of voltage gated calcium channels arises from the expression of different $\alpha 2$-δ subunits within the 5 subunit VGCC complex as well as from diversity in the other subunits.

The isolation and characterisation of the genes coding for the calcium channel affected by Lambert-Eaton myasthenic syndrome antibodies in NG108-15 cells

A thesis presented for the partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Oxford

Anthony Stanislav Wierzbicki von Rosch

Wolfson College

1992

Hanc nigram niveamque mihi Iovis alitis alam,

Pro meritis caesar nobile stemma dedit,

Quod datur oc atavis clavum est, sed clavis omne,

Quod per se virtus propria ferre solet.

Jan Dantiscus (1485-1548)

The black and white spread of Jupiter's bird,
was given to me by the Emperor as a noble emblem for my services.
That which is inherited from one's ancestors is illustrious
but still more illustrious is that which a man achieves by his own merit.

This is to certify that all the work presented in this thesis
is my own (unless otherwise specified) and has not been submitted
for any other degree at this or any other university.

Acknowledgements

I would like to thank my supervisor David Beeson for his help and Beth Lang for her wholehearted cooperation and generosity with cells, clones, plasmids and reagents. I would also like to thank Professor George Elder and Dr. Paul Morgan at the Department of Medical Biochemistry, University College of Wales Hospital for their generosity with study leave. Lastly, I would like to thank all members of the Neurosciences group for their encouragement and advice about all aspects of this work.

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Abbreviations

General:

AA	amino acid
BP	base pair
LEMS	Lambert-Eaton myasthenic syndrome
NMJ	neuromuscular junction
RR	ryanodine receptor/calcium release channel
SCLC	small cell lung carcinoma
TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA buffer
VGCC	Voltage gated calcium channel
VGKC	Voltage gated potassium channel
VGSC	Voltage gated sodium channel

Amino acids:

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartate	P	proline
E	glutamate	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

Chapter 1

The structure and classification of calcium channels

1.1 General introduction

Neuronal transmission was first postulated as having an electrical component in 1872 by Hermann [Hermann, 1914]. Later Hodgkin & Huxley [1952] described axonal transmission in the squid giant axon in terms of countercurrent fluxes of sodium and potassium and as explicable by basic mathematical cable theory. Depolarisation of the membrane led to the opening of postulated channels through it and a flux of ions down their concentration gradients. This model served well to explain the propagation of the action potential. The history of the discovery of ion fluxes and their relation to the biophysics of axonal action potential transmission has been reviewed by Hille [1984].

Fatt and Katz [1959] discovered that in crustacean muscle fibres larger action potentials were obtained in the presence of choline than with sodium ions. They proceeded to show that sodium ions were not essential for action potentials in this tissue and postulated that instead calcium was responsible for the inward flux. Fatt & Ginsborg [1958] showed that calcium ions could be replaced by barium or strontium and the fibres would still conduct. Hagiwara & Naka [1964] extended this work using barnacle giant muscle fibres and showed that low internal calcium and calcium ion chelators induced spontaneous calcium spikes thereby showing a degree of control absent in sodium channels. They also used ^{45}calcium to show charge and ion movement across the membrane and therefore confirmed the existence of voltage sensitive calcium channels. These original experiments have since been extended to other phyla and it is now known that most cells express calcium channels and that calcium flux is involved in the process of exocytosis. Exocytosis includes secretion of

neurotransmitters in response to electrical depolarisation. However the debate as to whether calcium flux was essential for neurotransmitter release or whether an initial calcium flux preset the release apparatus for an electrical signal has only recently been resolved in favour of the calcium hypothesis [Mulley & Zucker, 1991].

The elucidation of the nature of calcium channels and their function has been greatly aided by the discovery of specific high affinity ligands [usually pharmacological] and by the occurrrence of antagonists in nature as toxins. The paradigm for these studies has been the use of α-bungarotoxin from the banded krait *Bungarus multicinctus* to study the nicotinic acetylcholine receptor [see Claudio, 1989].

More recently electrophysiological techniques and their attendant electronics and computer analysis have been developed. These techniques include single cell recording by attached micropipettes and then individual single channel recording from isolated membrane patches [Sakmann & Neher, 1974]. This has enabled the gating characteristics of individual channels to be elucidated and some attempts to be made to devise mathematical models for the structural transitions that occur during channel openning.

The molecular structure of the ion channels present in cells remained obscure until molecular biological techniques were developed and applied to the richest source of transmembrane channels - the electric organs of the rays *Torpedo californica* and *T. marmorata*. This specialised organ was used as a source of a high concentration of protein to enable partial peptide sequencing to be performed and used to isolate channels by oligonucleotide hybridisation. The first channel characterised by this technique was the α-subunit of the acetylcholine receptor [Noda *et al*, 1982] followed

by the sodium channel [Noda *et al*, 1984]. It has lately been applied to determine the nature of the chloride channel [Jentsch *et al*, 1990].

The T-tubule system of skeletal muscle was found to be an extremely rich source of dihydropyridine receptors. The calcium channel complex could be purified from this source by detergent extraction, functionally reconstituted in planar lipid bilayers and further purified for gel analysis. Using polyacrylamide gel electrophoresis [PAGE] it could be dissociated into its 4 component subunits on neutral PAGE gels- 165-170 kDa [α], 55 kDa [ß], and 32kDa [γ] [Curtis & Catterall, 1984]. Another subunit appeared on reduction [δ] and the α band was split into two components of 165kDa and 130kDa thereafter termed $\alpha 1$ and $\alpha 2$ respectively [fig 1 .1].

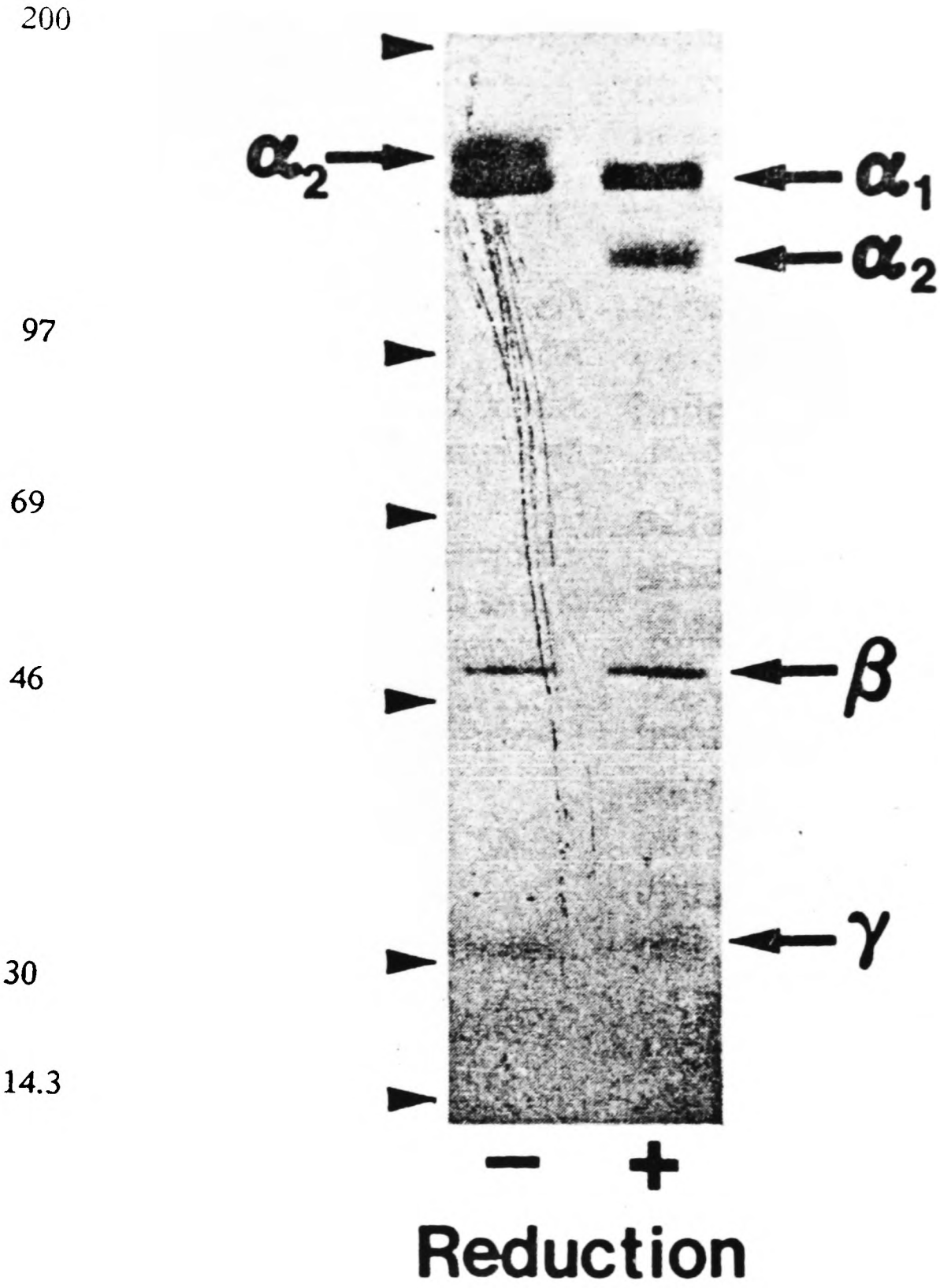


Fig 1.1

SDS-PAGE gel of purified skeletal muscle VGCC under nonreducing and reducing [SDS] conditions. The ^{3}H-nitrendipine labelled VGCC complex was isolated using 1% digitonin detergent from isolated T-tubule membranes and was purified by chromatography on wheat germ agglutinin sepharose, DEAE sepharose, and sucrose density gradient centrifugation. The complex was run down a 5-15% gradient PAGE gel with and without reduction with 20mM dithiothreitol.

Data from Curtis & Catterall [1984]

1.2 Calcium channels - general introduction

Calcium channels are a heterogeneous group of molecules. They can be classified into two major categories of intracellular and plasma membrane channels. The latter can be subdivided into 3 categories. Thus 4 types of calcium channel exist:

[i] receptor operated channels

[ii] mechanosensitive channels

[iii] voltage gated channels

[iv] intracellular channels

Members of each group have been electrophysiologically characterised. The excitatory amino acid receptors such as the glutamate or glycine receptors are examples of receptor operated channels which have been cloned [see Betz, 1990]. No mechanosensitive [calcium leak] channel has yet been cloned or fully characterised at the molecular level. Calcium release [ryanodine receptor] channels have been cloned and characterised and are known to be intimately related to voltage gated calcium channels [VGCCs] in the process of excitation-contraction [secretion] coupling. The VGCC has been partially cloned from a variety of tissues and is well characterised in skeletal muscle.

A large variety of review articles and books have addressed the subject of voltage gated calcium channels from a variety of perspectives. The books and reviews include Hille, [1984]; Numa, [1985]; Tsien *et al*,[1987]; Catterall, [1988]; Catterall *et al*, [1988]; Hosey & Lazdunski, [1988]; Wray *et al*, [1989]; Glover & Hames,[1990]; and Tsien *et al*, [1991].

1.3 Channel superfamilies

The concept of gene superfamilies is essentially the idea of evolution working at a molecular level. Thus, from a single fundamental starting structure encoded by an original ancestral gene, diversity is generated through the process of duplication and divergent evolution without significant alteration of the basic structure. A number of reviews exist that address the basics of channel design including Krueger, [1989]; Unwin, [1989]; and Montal, [1990].

These concepts have been used to group channels into families on the basis of amino acid homology and superfamilies on domain structure homology. The commonest feature used for homology grouping is the number of membrane spanning domains. The resulting classification is shown in table 1.1.

Receptor operated channels are generally pentameric in quaternary structure [Langosch *et al*, 1988] with 4 membrane spanning domains. G-protein coupled receptors generally have 7 membrane spanning regions and the paradigm is the rod photoreceptor channel [Kaupp *et al*, 1989; Nathans *et al*, 1986]. The voltage gated chloride channel has 13 membrane spanning regions [Jentsch *et al*, 1990]. Voltage-gated cation channels have 6 membrane spanning regions and are tetrameric in quaternary structure [Catterall *et al*, 1989].

Channel Family	Membrane spanning domains
Acetylcholine	4
Glutamate	4
GABA	4
cGMP gated	7
voltage gated anion	12/13
voltage gated cation	6
minimal channel	1x X [?]

Table 1.1

The superfamilies of ion channels as classified by their number of hydrophobic domains

The structure of certain domains in all these channel groups is very highly conserved. It is possible to formulate consensus structures for all the domains of these channels and other synaptic proteins such as synapsins and connexins with details of essential amino acids and essential characteristics at each point [see Betz, 1991].
This can be used as the basis for identifying further members of the families by molecular biological hybridisation techniques [see Montal, 1990 for further details].

1.4 Relation of VGCCs to VGSCs and VGKCs

VGCCs are structurally highly related to VGSCs and VGKCs and analysis of their structure may provide clues to the characteristics of each type of channel [fig 1.2]. Little is still known about the functional domains of VGCCs but two other members of the superfamily had been cloned far earlier and have been studied intensively. More information is available about genomic organisation, mutants, generation of molecular diversity, domain function, ion selection, gating and inactivation mechanisms for these channels and much of it seems to be relevant to VGCCs. The next sections will review briefly the structures of the various channels, their inactivation mechanisms, pores, genomic structures, generation of diversity and then the specific characteristics of VGCCs.

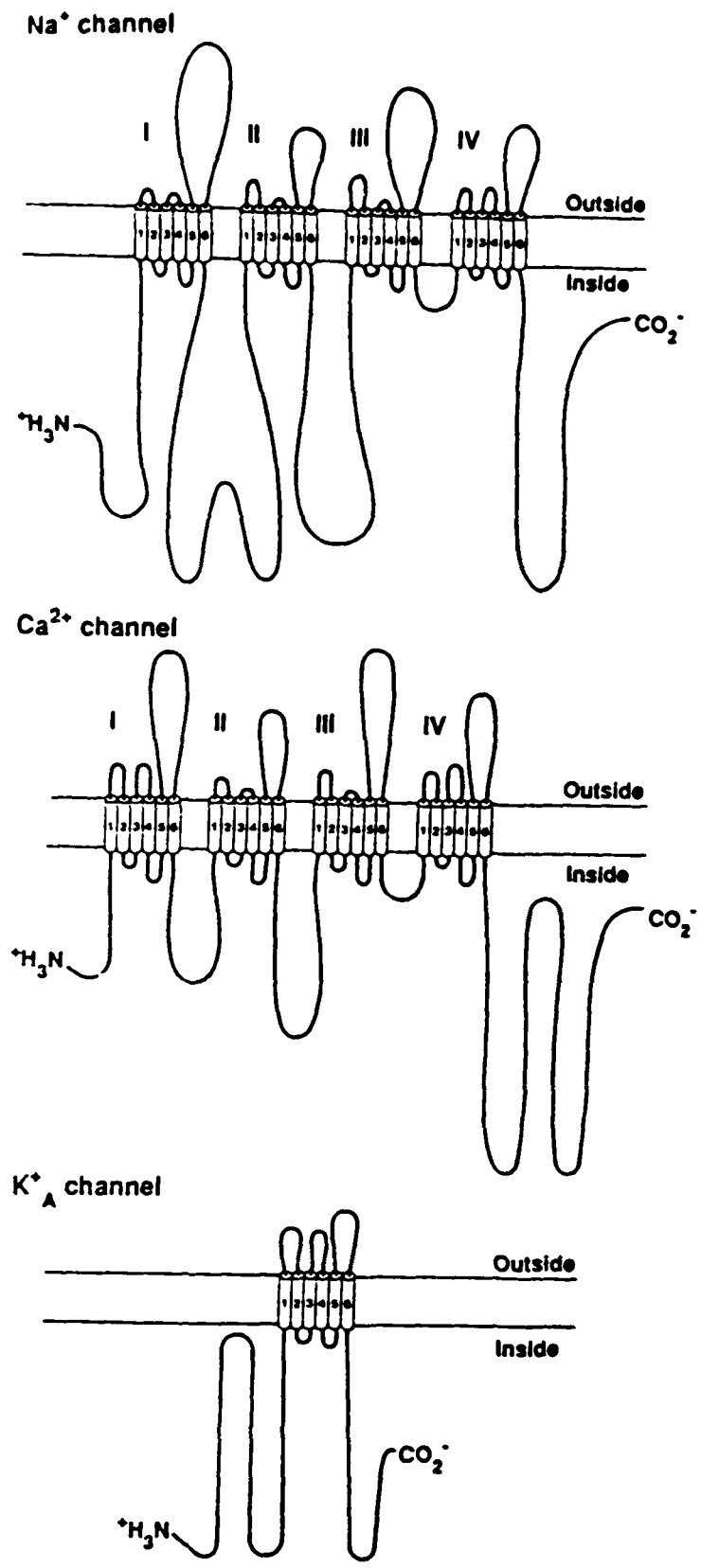


Fig 1.2

A comparison of the schematic structures of pore subunits of VGKCs, VGSCs and VGCCs. All these channels share a domain structure of 6 membrane spanning α-helices of which the fourth is a highly conserved voltage sensor. VGKCs exist as a hetero-multimer of 4 separate protein units whereas in VGSCs and VGCCs the 4 units are linked into a single large molecule. [After Catterall, 1989]

1.5 The structure of the voltage gated potassium channel

The potassium channel or VGKC is the simplest of the cation channel family in structure. It consists of one set of 6 membrane spanning regions which correspond to one repeat unit of the VGSC or VGCC. The basic structure of VGKCs has been reviewed by Catterall, [1988]. The basis of most research in potassium channels is *Drosophila melanogaster* mutants that have been bred for various characteristics. The genetics of ion channel mutants in *Drosophila* and in other systems has been reviewed by Salkoff & Tanouye, [1986,1987] and Ganetsky & Wu, [1989].

The *Shaker* product was 27% directly homologous and 47% conserved substitutions over 121 AAs [the S4 region] to the *Electroplax* sodium channel [Noda *et al*, 1982] and corresponded to one domain unit [H1- H6 membrane spanning domains]. Analysis of the *Shaker* locus revealed it to contain 21 exons over 113 kb which were alternatively spliced to produce VGKCs [Pongs *et al*, 1988]. Injection of two types of cDNA into oocytes resulted in the expression of multiple currents suggesting that the functional unit of the VGKC was a hetero-oligomer of probably 4 individual units [Pongs *et al*, 1988; Isacoff *et al*, 1988]. The diversity is such that at least 6 VGKCs are encoded by *Shaker* and *Shal* [another I_a channel], 2 by *Shab* [a delayed rectifier VGKC] and members of the *Shaw* [delayed rectifier] families are known. The number seems still to be increasing as different tissues are examined e.g. photoreceptors [Hardie *et al*, 1990].

1.6 Other relatives of VGKCs

More recent work in *Drosophila* has identified the *Slo* [Slowpoke] locus as encoding a calcium activated potassium channel of 1184 AAs encoded over an area of 40kb

[Atkinson *et al*, 1991]. The *Drosophila eag* [ether a gogo] gene is encoded over a region of 35 kb producing a transcript of 1174 amino acids. It is very similar to the VGKC group. It lacks the NEYFFDR motif of *Shaker* at the N-terminal which is known to be involved in inactivation [*vide infra*][Warmke *et al*, 1991]. Voltage clamp experiments with *eag* mutants [Zhong & Wu, 1991] showed that all 3 potassium currents [I_k, I_{cf} and I_{cs}] were affected in mutants unlike *Shaker* and its analogues [I_k] and *Slo* [I_{cf}]. This suggested that *eag* encoded a constant subunit of the full VGKC complex. The comparable region to *eag* in the VGCC seems to be the first domain repeat which is critical for gating [Tanabe *et al*, 1991]. The other domains of VGSCs and VGCCs seem to coincide with other variably expressed VGKC proteins. As yet these VGKC proteins which contribute to the 'tetrameric' VGKC have not been definitively identified with any functionally homologous domains in VGSCs or VGCCs. Other probable potassium channel mutants like *hyk* - hyperkinetic and *wei* remain to be characterised at the molecular and genomic level [Ganetsky & Wu, 1989].

1.7 The structure of the voltage gated sodium channels

The sodium channel was the first of the voltage gated channels to be described in detail. It was originally isolated from the the electric organ of the eel *Electroplax* [Noda *et al*, 1984] and since then others have been isolated e.g. rat brain [Noda *et al*, 1986], μ1 from rat skeletal muscle [Trimmer *et al*, 1989], and a tetrodotoxin insensitive channel characteristic of denervated rat muscle [Kallen *et al*, 1990]. The molecular structure of VGSCs discoveries has been reviewed by Agnew *et al*, [1988] and Catterall, [1991] and their tissue distribution by Grubman *et al*, [1988]. Guy, [1988]

reviewed the detailed modelling of VGSC structure and gating characteristics were examined by Guy [1988] and by Patlak [1991] in their reviews.

1.8 The structure of VGSCs

VGSCs purified from rat brain exist as a complex of 3 subunits- α [260 kDa], ß1 [36 kDa] and ß2 [33 kDa] of which the α and ß2 are linked by disulphide bridges [analogous to some elements of α2-δ interaction in VGCCs] while skeletal muscle VGSCs consist only of an α-subunit. VGSCs resemble VGCCs in their structure by the presence of these ancillary subunits but unlike in VGCCs these subunits are totally absent in skeletal muscle and partially [lung] or wholly [brain] present in other tissues. The VGCC however seems to comprise 5 subunits in all tissues.

A 200 AA stretch between domains I and II was noted in brain channels [Noda *et al*, 1986] which might be responsible for neuromodulation. Homology density plots showed that the 4 membrane spanning regions were 85% homologous and the III-IV segment was also conserved even in relation to VGSCs isolated from *Drosophila*. Neither of these areas are conserved in VGCCs and it has been suggetsed that the ion selectivitivity and special modulatory functions distinct to each channel reside in the inter-domain linkers as opposed to the membrane spanning domains [Catterall 1991a]. Mutations in the VGSC are responsible for the *para* [paralysed] mutant and affect membrane excitability [Ramaswami & Tanouye, 1989]. The *sei* and *tip*$^{\underline{E}}$ mutants of the sodium channel still remain to be characterised [Ganetsky & Wu, 1990].

1.9 The structure of the voltage-gated calcium channel

The L-channel of skeletal muscle is the dihydropyridine [DHP] binding , fast inactivating channel with a Ba^{2+} conductance of 15 - 25 pS [in 100 mM Ba^{2+}] found in the T tubule system. Catterall was able to purify the receptor from this source using the detergent CHAPS and determine that it comprised 5 subunits which he termed the $\alpha 1$, $\alpha 2$, ß , γ , and δ. These had apparent molecular weights of 165kDa, 130kDa, 55kDa, 32kDa and 22kDa respectively. The analysis of the characteristics of the subunits is shown in table 1.2 and a putative arrangement in fig 1.3.

Using a similar purification technique Tanabe *et al*, [1989] isolated the $\alpha 1$ protein and sequenced trypsin peptide digests. They used the sequence to design oligomer probes and screened a cDNA library from skeletal muscle. This enabled them to isolate a dihydropyridine binding protein of 1873 amino acids with a 29% homology to the rat brain R_{II} Na^+ channel [Noda *et al*, 1986]. The structure was a pseudo-tetramer of 6 hydrophobic membrane spanning unit domains which were more homologous [upto 60% nucleotide homology] to the VGSC than were the intervening sections. It contained 5 putative N-glycosylation sites and 7 putative cAMP dependent phosphorylation sites. $\alpha 1$ subunits were shown to vary between tissues [Ellis *et al*, 1989] with only partial homology between members of a postulated family of proteins.

The genomic structure of the $\alpha 1$ subunit of VGCCs is unknown but is probably analogous to the VGKC and VGSC. VGCCs share domain homologies with the other two channels. Notably they all contain a highly conserved S4 region which is thought to be the voltage sensor.

Subunit	α1	α2	ß	γ	δ
Structural:					
RMM [non]	155-200	165-220	52-65	30-33	-
[red]	155-200	130-160	52-65	30-33	24-33
Stoichiometry	1	1	1	1	vary
Glycoprotein	+/-	+++	-	+	+
Disulphide bridging	-	to δ	-	-	to α2
Membrane:					
MS domains	4x6	3	0	4	1
Cytoplasmic domains	++	+	+	+	+
Extracellular domains	++	+++	-	+	+/-
Function:					
DHP binding	+	-	-	-	-
Benzothiazepine binding	+	-	-	-	-
Arylalkylamine binding	+	-	-	-	-
ω-GVIA binding	-/+[?]	-/+[?]	-	-	-
Phosphorylation	+	+	++	+	-
Function in VGCC	Pore Protein, Voltage Sensor	Exp	Gating	Gating	Exp

Table 1.2
Structure, composition, properties and function of the subunits of the skeletal muscle VGCC
Non = nonreduced mass [kD], red = reduced mass [kD]
Exp = role in expression of α1 including DHP binding

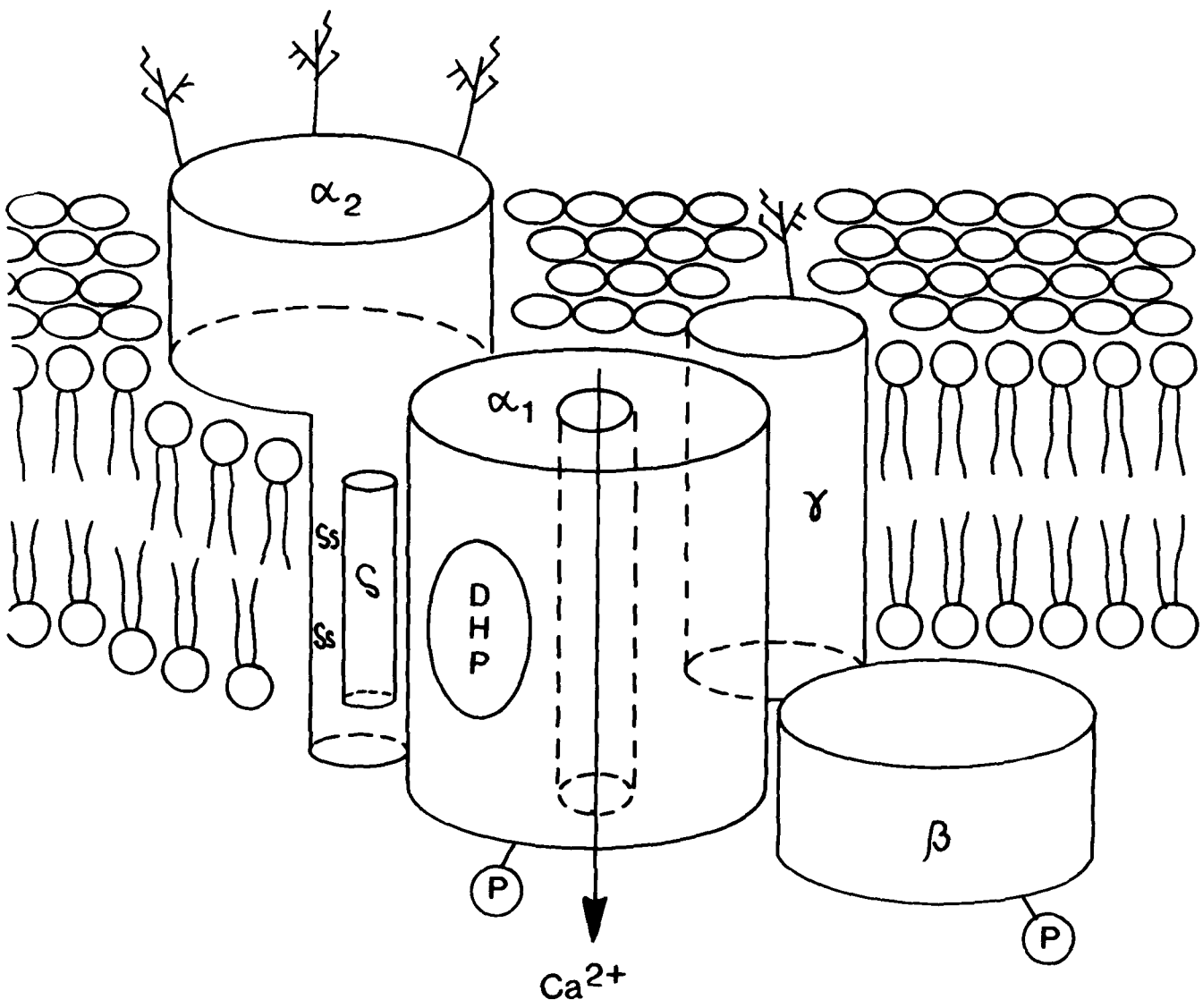


Fig 1.3

Putative arrangement of the subunits of the skeletal muscle VGCC in the plasma membrane. After Tsien, 1989.

The $\alpha 2$ subunit was isolated by screening expression libraries with antibodies raised to the purified subunit. The cDNA encoded a protein of 1106 amino acids. This was mostly extracellular at the amino terminus with 3 putative membrane spanning regions [MS 1 - 3][Ellis *et al*, 1989]. 16 possible glycosylation sites were identified in this molecule. Two of the membrane spanning regions were concentrated at the carboxy terminus [MS2 & MS3]. Hybridisation studies suggested that it was a universal component of VGCCs in a large variety of tissues.

The skeletal muscle ß subunit was cloned by protein purification, peptide sequencing and oligonucleotide hybridisation [Ruth *et al*, 1989]. The cDNA encoded a protein of 524 amino acids containing 4 α helical domains, no membrane spanning domains [19 AAs, average hydrophobicity index > 1.6] and multiple phosphorylation sites for cGMP kinase [1], protein kinase C [12], and cAMP dependent kinase [2]. Different related proteins were shown to be present in other tissues and the brain homologue has been sequenced [Pragnell *et al*, 1991]. The brain ß subunit [82% homologous] contains a 93 amino acid deletion at position 209 and a marked divergence in its C-terminal 135 residues. Its mRNA distribution seems to comap with that of ω-conotoxin binding VGCCs.

The γ subunit was cloned by a similar technique and shown to encode a protein of 222 amino acids lacking a signal sequence and containing 4 transmembrane domains [Bosse *et al*, 1990; Jay *et al*, 1990]. It showed 34% nucleotide homology in sections to the *mdr*2 drug resistance protein. No other specific hybridising analogues of γ were detected on northern blotting in different tissues implying that γ analogues show wide variation between tissues.

The δ subunit has not been formally cloned but tryptic digests of purified δ had identical sequences to the α2 subunit and the N-terminal of δ corresponded to AA_{935}, alanine 935 [A935] of the α2 sequence [De Jongh *et al*, 1990]. Antibodies to α2 sequence carboxy to this area identified two δ peptides of 24 kDa and 27 kDa which are linked by disulphide bridges to α2. Therefore δ is thought to be generated by post-translational processing of the α2-δ gene product by a peptidase with a di-alanine recognition site [fig 1.4]. The δ peptide shows variation in size with 3 δ peptides known all starting at the same point of RMM 25 kDa [δ1], 22 kDa [δ2], and 17 kDa [δ3]. Their stoichiometry of 1:0.31:0.47:0.08 with α2 suggests that δ usage is heterogeneous [Jay *et al*, 1991]. It is likely that the differences are due to glycosylation in a protein with one membrane spanning region. The large number of possible glycosylation sites in α2 makes this likely but it is not known which sites are glycosylated *in vivo*. The exact site[s] of disulphide bridging between the amino α2 cysteines [17] and carboxy δ cysteines [9] remains to be determined.

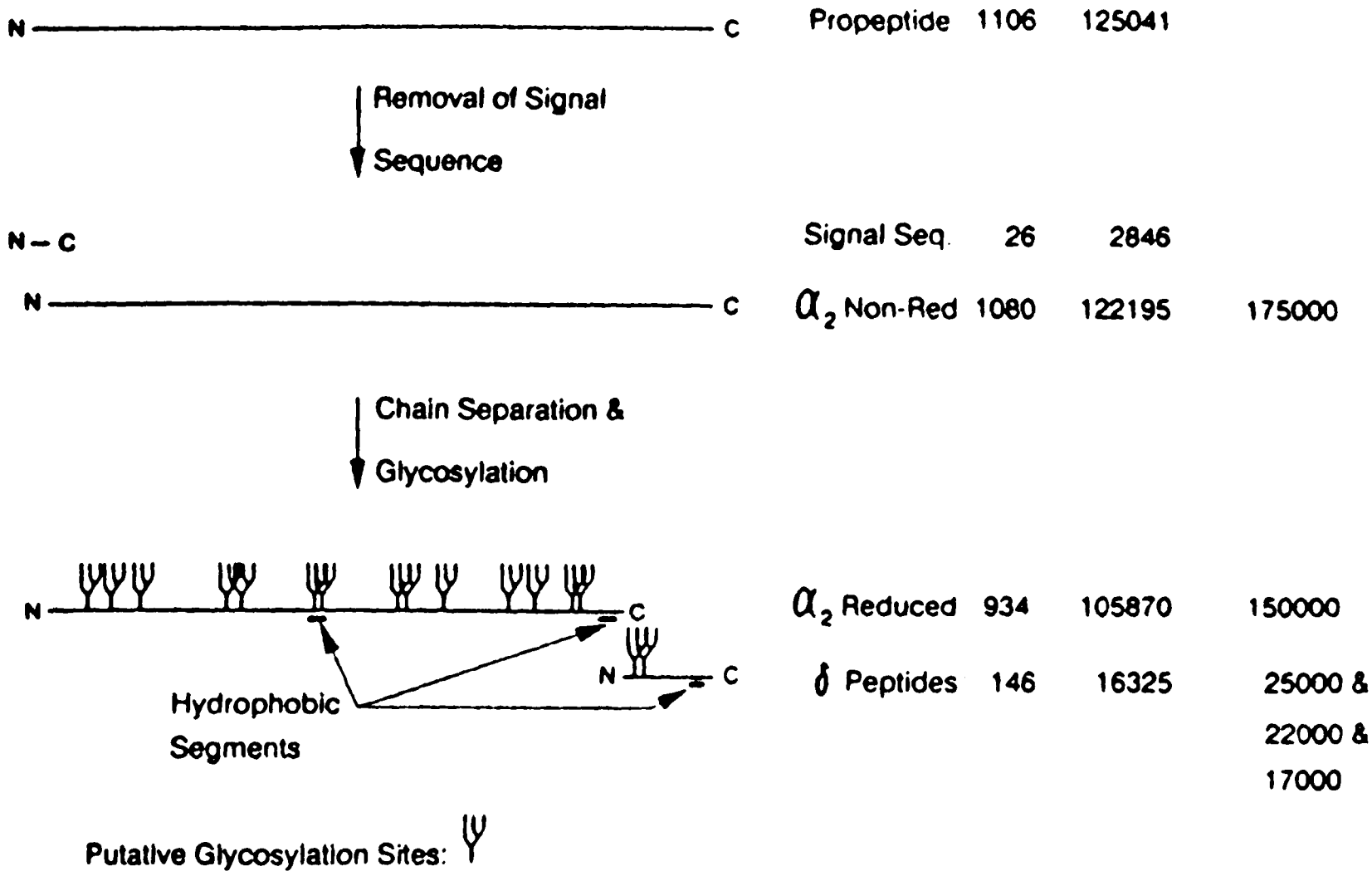


Fig 1.4

Processing of the $\alpha 2$-δ complex. Data from de Jongh *et al*, 1990

1.10 Kinetics and gating in voltage gated channels

The majority of data on the kinetics and inactivation of the voltage gated family comes from work on the smallest member - the VGKC though not all of it is applicable to the other members of the family. Most structural analysis has centred on the properties of the voltage sensor which was originally noted in the sodium channel. Two approaches have been used to study cloned channels: site directed mutagenesis and the construction of chimaeras between different channel cDNAs. However little work has correlated the two approaches in any particular channel cDNA.

1.11 The S4 domain

The S4 domain is thought to be the voltage sensor in the voltage gated channel family. Its structure consists of a positively charged amino acid every third residue embedded in a hydrophobic domain. The structure of the S4 domain in VGKCs, VGSCs and VGCCs has recently been noted to bear similarities to the coiled-coil structure of the 'leucine zipper'. This consists of a leucine every seventh amino acid and usually mediates dimerisation of DNA binding proteins [McCormack, 1989][fig 1.5] but can show homology to membrane spanning domains.

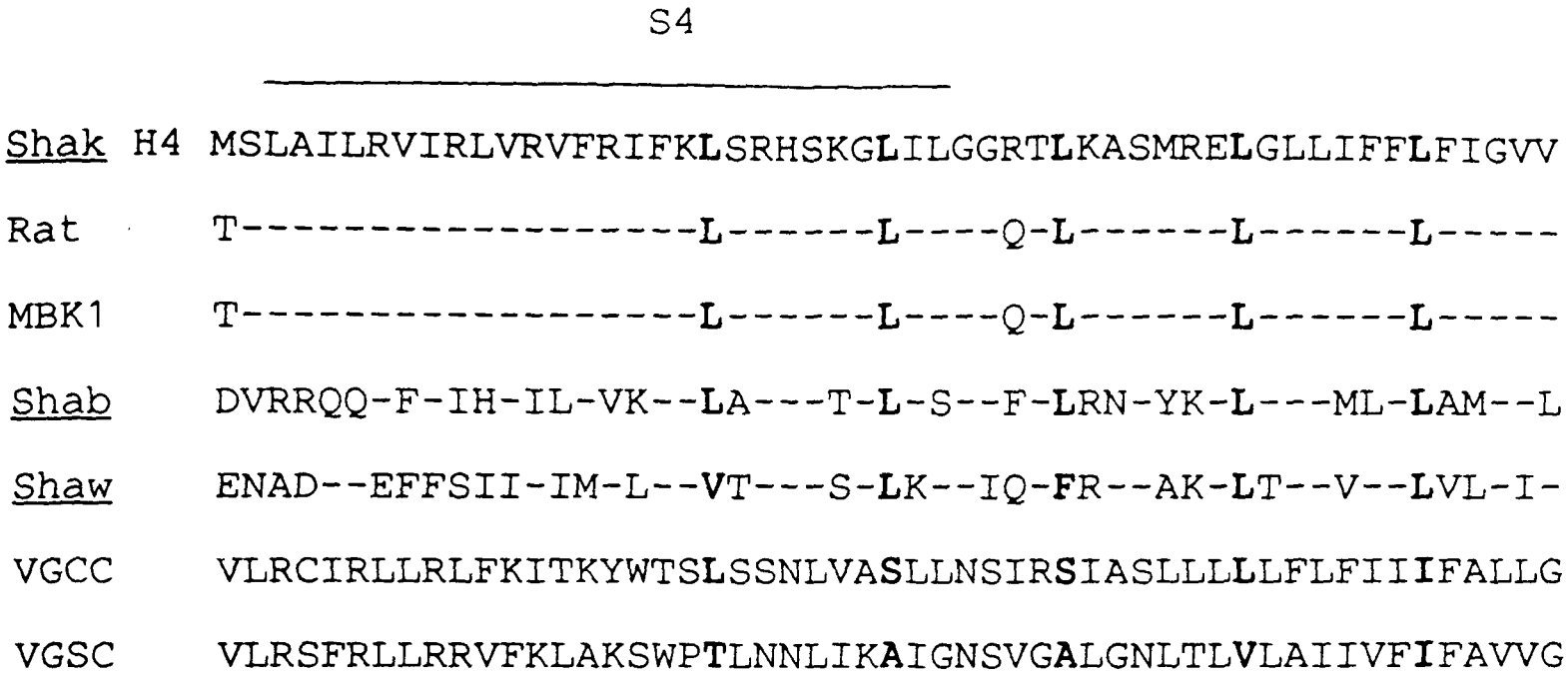


Figure 1.5
The preservation of domains resembling the leucine zipper in the S4 region of the voltage gated superfamily of genes. Preserved leucine residues or homologous hydrophobic amino acids serine, threonine or alanine are shown in bold type.

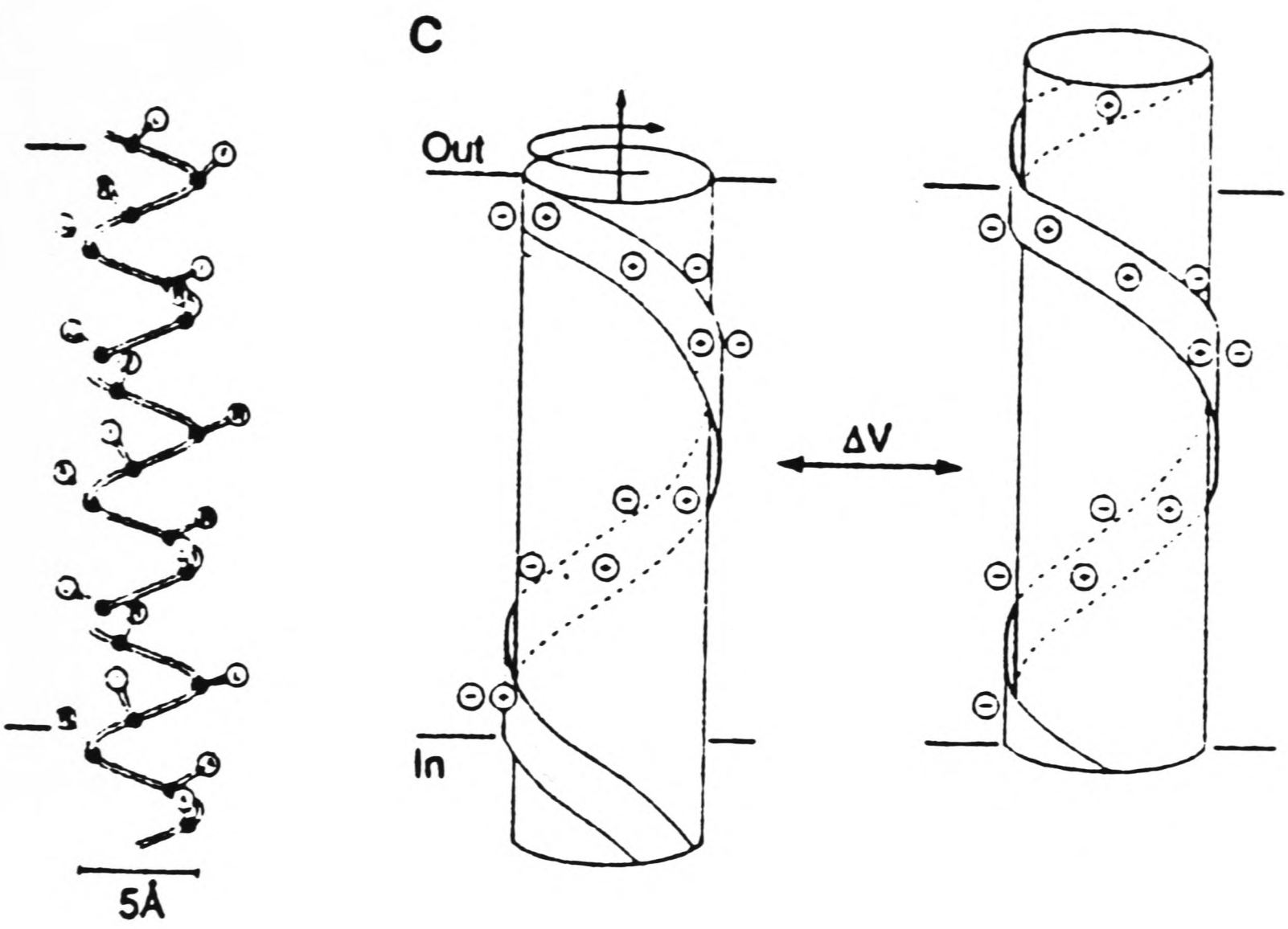


Fig 1.6

Structure of the S4 region of the VGKC and its possible change in conformation as caused a membrane voltage shift [ΔV]. After Aldrich, 1990.

The S4-H4 area with its heptad repeat is highly conserved in evolution as all but 3 AAs are invariant between members of the potassium channel gene family. Substitutions in the sodium channel S4 [Stuhmer *et al*, 1989; Liman *et al*, 1991][*vide infra*] have confirmed that the S4 is a voltage sensing domain and therefore by implication a definitive domain in the voltage gated superfamily of ion channels. Analogous work in *Shaker* type potassium channels have shown that conservative substitutions in the hydrophobic residues of the S4 region have as profound effects on voltage dependent gating as substitutions in the charged residues [McCormack *et al*, 1989; Lopez *et al*, 1991; Papazian *et al*, 1991]. Little work as yet has been performed in VGCCs.

The function of the S4 region is thought to be to change the shape of the channel in response to voltage shifts across the membrane by bending the α helix or sliding in the membrane and so the expected charge movement would be small due to steric constraints [Unwin, 1989; Zagotta & Aldritch, 1990]. It is noticeable however that the S4 regions of all the channels are only 85% homologous and differ between each domain or unit. This implies that voltage sensing may not be a simple process as uniformity of structure would be expected given the sensitivity of the S4 to mutations. Whether these domain differences have any functional role remains to be elucidated.

1.12 The model of VGKC inactivation

The S4 region is the only area involved in voltage dependence and seems to be relevant to inactivation [Bezanilla *et al*, 1991]. But, other areas of the protein are also necessary for inactivation to occur. Armstrong & Bezanilla, [1977] suggested a 'ball and chain' model for the inactivation process in sodium channels [fig 1.7]. The ball

and chain model was confirmed for the *Shaker* VGKC by mutagenesis of the N-terminal domain -with the first 19 AAs [10 hydrophobic] forming a ball and residues 20 - 83, the chain [Hoshi *et al*, 1990][see review by Barinaga, 1990]. Mutations in the ball slowed or removed fast inactivation. Deletions in the chain speed up inactivation whilst insertions slow it [Zagotta *et al*, 1990]. However the negatively charged acceptor on the core remains to be determined. Site directed mutagenesis and deletion variants identified the H1-H6 core as necessary for ion flux [Van Dongen *et al*, 1990]. Large N-terminal deletion alter kinetics but the addition of C-terminal mutants compensate for the N-terminal mutation [Van Dongen *et al*, 1990]. This is difficult to reconcile with the ball and chain model unless the C-terminal is involved in the acceptor ring. However other areas are involved in gating and inactivation e.g. the extracellular calcium binding site stabilising the closed state [squid; Armstrong & Miller, 1990].

No such ball and chain motifs are directly identifiable in VGSCs or VGCCS but it is known that VGSC and VGCC inactivation like that of VGKCs is susceptible to proteolysis suggesting a similar process involving an as yet unidentified cytoplasmic linker may occur. Candidate regions exist in the VGSC [III-IV inter-region] but no work has yet been carried out on VGCCs.

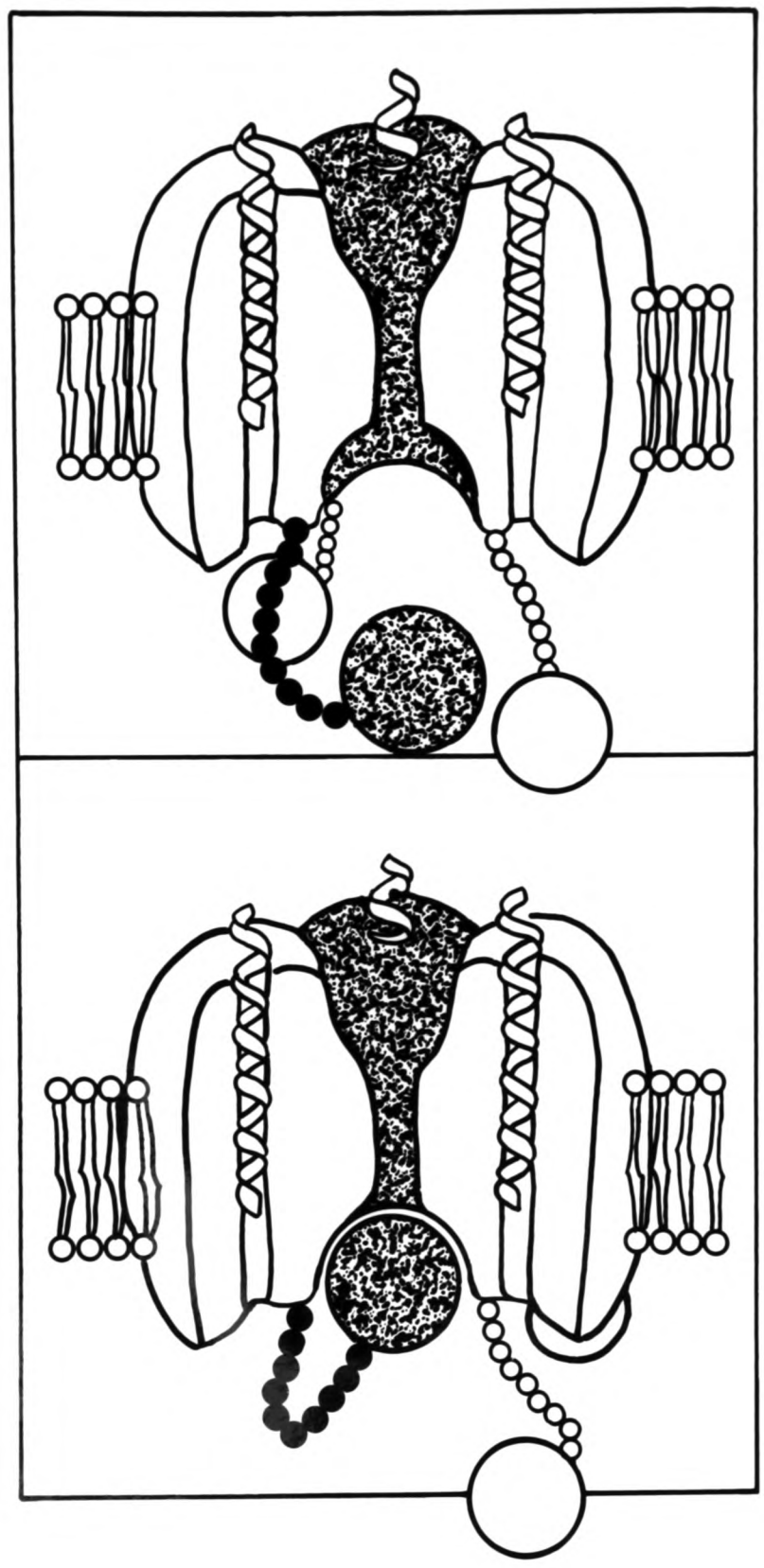


Fig 1.7

The 'ball and chain' model of inactivation of the VGKC as determined by Hoshi *et al*, 1990. The N-terminal ball containing the motif NEYFFDR of 8 predominantly hydrophobic amino acids is attached to a chain of 10 hydrophilic amino acids comprising the chain and interacts with an acceptor ring core on the membrane side of the VGKC.

1.13 Site directed mutagenesis of VGSCs

Stuhmer *et al*, [1989] expressed VGSCs altered by site directed mutagenesis in oocytes and provided evidence that mutations in the S4 region reducing net positive charge reduced the apparent gating charge and resulted in slower activation kinetics. The cloning of a rat VGSC II_a cDNA has enabled the exact residues affecting functioning channels to be determined. This clone differs in 7 AAs from the rat VGSC II clone [Numa *et al*, 1986] and a single change F860L changes VGSC II_a inactivation characteristics to VGSC II [Auld *et al*, 1990]. The critical region for inactivation was found to be the III-IV interregion [Moorman *et al*, 1990a]. Simply cutting the linker had an effect similar to mutagenesis. The III-IV linker might be a positively charged ball as occurs in the VGKC but site directed mutagenesis in this area in the rat brain type III VGSC - RSC3 [Moorman *et al*, 1990b] showed that inactivation was altered but not abolished. This implied that the mechanism involved the linker acting as an acceptor for another area in which E1438 and E1439 were essential. It has been suggested that the II-III inter-region may be a 'ball' for inactivation [Salkoff *et al*, 1990] but changes in this area do not significantly alter inactivation [Moorman *et al*, 1990b]. The exact roles of these regions in inactivation remain to be determined.

VGSCs can show lability in their inactivation kinetics such that the rat brain Na^+ III channel expressed from its cDNA shows both slow and fast inactivation in single channel patches [Moorman *et al*, 1990c]. This is postulated to be due to instability in the inactivation domain and it is interesting to note that calcium channels are also capable of showing two types on inactivation kinetics [*vide infra*]. It thus seems likely that the critical amino acids described by mutagenesis experiments determine only

short-term inactivation characteristics while the other gating modes may be due to alterations in different regions and possibly secondary structure [e.g. phosphorylation].

1.14 Structural requirements for gating

In contrast to VGKCs where no ancillary subunits are known, both VGSCs and VGCCs have auxiliary subunits. These are variable in tissue expression in VGSCs [ß1 and ß2]. The function of these subunits remains obscure as they are not necessary to generate recognisable gating in VGSCs in *Xenopus* oocytes. The degree of diversity in these subunits also remains to be determined.

1.15 The molecular biology of non-skeletal muscle VGCCs

The work done on the rabbit skeletal muscle calcium channel has now identified all the components of the VGCC in that tissue. However the structure of the VGCC in other tissues has not been characterised in such detail largely because the concentration of DHP binding sites is lower in these tissues. Arguments about their structure therefore rely on analogy with the skeletal muscle channel in assuming a structure of $\alpha 1$, $\alpha 2$-δ, ß and γ. Many of these appear to be present in other tissues in partially homologous isoforms unlike VGSCs where their presence at all is tissue specific [Catterall, 1991a & 1991b].

Northern blots from rabbit tissues and BC_3H1 cells [a mouse brain tumour line with muscle characteristics] identified 6.5kb transcripts for $\alpha 1$ in muscle and weaker 8.9 kb and 15.5 kb bands in other tissues. Two $\alpha 2$-δ transcripts of 7.0 kb [weak] and 8.0 kb were seen. 3 ß and 3 γ subunit transcripts of 1.6 - 3.0 kb were detected [Biel *et al*, 1991]. They suggested that $\alpha 1$, ß, and γ showed coordinate regulation with different

regulatory elements for $\alpha 2$-δ. However some of the larger transcripts seen in these tissues could be incompletely spliced RNA molecules.

Various experiments have been performed to assess the role of the subunits and domains within those subunits on VGCC gating. The $\alpha 1$ subunit cannnot be expressed on its own in *Xenopus* oocytes [Tanabe *et al*, 1989] but could be expressed in the presence of $\alpha 2$ [Mikami *et al*, 1990]. The gating characteristics of the channel were dependent on the $\alpha 1$ subunit present [Mikami *et al*, 1989] as was shown by the assembly of heteromultimer channels with $\alpha 1$ and $\alpha 2$ subunits from different sources- e.g. $\alpha 1_{sk}$ and $\alpha 1_{ca}$. The cardiac $\alpha 1$ was found to be capable of expression in *Xenopus* oocytes without the need for an $\alpha 2$. A better expression system for VGCCs was found to be transfection of mouse L cells [Perez-Reyes *et al*, 1989] where calcium fluxes could be generated by the transfection of $\alpha 1$ alone. However in these transfection experiments the $\alpha 1$ was not cleaved to the 175 kDa form. The absence of the induction of other subunits by the transfected protein was confirmed by the use of specific antisera. However the activation kinetics of $\alpha 1$ were 100 times slower than expected. $\alpha 2$-δ complexes were found to increase the amount of $\alpha 1$ installed in the membrane by a factor of 5 [Mikami *et al*, 1989] and therefore raise total flux but not flux per channel. Initially the minimum unit for expression of recognisable gating in mouse L cells was $\alpha 1$-ß-γ [Kim *et al*, 1990] but more recently stable L cell transfectants with $\alpha 1_{sk}$ and $ß_{sk}$ have been shown to generate recognisable gating characteristics and this implies that γ like $\alpha 2$-δ only increases expression and stabilises the $\alpha 1$-ß complex [Lacerda *et al*, 1991]. However more detailed studies on heteromultimeric channels in *Xenopus* oocytes have shown that $\alpha 2$-δ and γ accelerate activation and shift the VGCC voltage dependence to more negative potentials nearer

to the physiological level [Singer *et al*, 1991]. ß subunits raise the number of available DHP sites 10 fold and accelerate the kinetics of the $\alpha 1$ subunit in L cells [Varadi *et al*, 1991], but have only a slight effect on voltage dependence [Singer *et al*, 1991]. Whether more subtle effects are dependent on γ and $\alpha 2$-δ subunits remains to be determined. Also all the current work has been performed with skeletal and cardiac muscle type channels. Whether the similar effects occur with other VGCCs is not yet known but the functional VGCC seems to require all 5 subunits to generate channels with a fully normal time course and inactivation characteristics [Catterall, 1991b] at least in the case of the cardiac VGCC.

1.16 Mechanisms of generating diversity

A variety of mechanisms exist to generate diversity in a family of proteins. These include alternative genetic splicing and separate gene transcription. Most proteins are spliced from exons at the hnRNA level and the exons inserted can be very small as in acetylcholine receptors and dopamine receptors [Auld *et al*, 1990]. Alternatively in multimeric proteins diversity can be generated by hetero-oligomer formation. Both these mechanisms occur in the voltage-gated family.

1.17 Generation of calcium channel diversity

Two possible mechanisms exist to generate diversity in the VGCC. Either the individual subunits could vary between channels or the structure of the subunits could vary while conserving certain regions and be built up by alternative splicing. The VGKC mechanism of shuffling individual units of the tetrad after translation is

unavailable to VGCCs and VGSCs but pre-translational splice variation does occur in all three members of the family.

Very few VGCC subunits have been cloned in their entirity to date- 10 α1, 1 each of the rest - so the full nature of the diversity is still obscure but the data gathered so far suggests that both mechanisms operate.

The role of post-translational processing in generating diversity in different tissues is unknown in VGSCs and VGCCs except for carboxy terminal variants [*vide infra*] and the putative sensor only channel of the skeletal muscle T-tubule.

1.18 Generation of α1 subunit diversity

Screening of a rat brain cDNA library with a CaCh1 [rabbit skeletal muscle] probe led to the discovery of many nonidentical clones - 32 cDNAs on primary screening and a further 104 on rescreening with the original isolates [Snutch *et al*, 1990]. These cDNAs expressed VGCCs in oocytes and calcium currents were removed by the addition of antisense RNA [Lotan *et al*, 1989]. On this basis the genes were assigned to 4 families of transcripts and their frequency suggested that the main source of diversity in calcium channels resided in the α1 subunit. Initially 8 different VGCCs were identified in rat brain but later evidence seems to suggest that this is a gross underestimate.

Some α1 VGCC subunit genes have been completely cloned. These are shown in table 1.3. Most have been isolated by homology screening of cDNA libraries but others have relied on the conservation of S4 domains to isolate sequences [Huang *et al*, 1990; Perez-Reyes *et al*, 1991]. Application of S4 region PCR led to proposal that VGCCs were derived by alternative splicing from a selection of common precursors

[Snutch *et al*, 1990]. Perez-Reyes *et al*, [1990] showed that PCR generated VGCC sequences could be placed into 4 families - neural, neuroendocrine, cardiac and skeletal and that alternative splicing occurred in cardiac and neuroendocrine sequences. The alternatively spliced exons could be as short as 3 amino acids [Snutch *et al*, 1991]. Two alternative mechanisms could account for this splicing involving either skipped exons or alternate splice acceptor sites. The latter is thought to be more likely. However the family names that are proposed for these sequences do not necessarily reflect the tissue distribution of VGCC families as 'skeletal' type VGCCs are expressed in ovarian tissue and 'cardiac' type are found in ovarian tissue and in pancreatic ß cells [Perez-Reyes *et al*, 1991]. Therefore VGCC α1 genes should be classified in numbered rather than named groups [Tsien *et al*, 1991][fig 1.8].

Animal	Source	bp	% Exp	Electrophysiology			Pharmacology			
				V_p	Ω^{-1}		DHP	Cd^{2+}	ω-CgTx	FTX
				mV	pS					
Rabbit	Skm	6083	100	[+]	+20	?	[+]	[+]	[-]	[-]
Rabbit	Card	7036	76	[+]	+25	?	[+]	[+]	[-]	[-]
Rabbit	Lung	7374	65	[+]	+20	?	[+]	[+]	[-]	[-]
Rabbit	Brain 1	6819	45	[+]	+12	16	[-]	[+]	[-]	[+]
Rabbit	Brain 2	7272	40	[-]						
Carp	Skm	6103	65	[-]						
Rat	Aorta	6507	65	[-]						
Rat	Cbm	6636	33	[-]						
Rat	Brain	6975	71	[-]						
Rat	Brain	6423	78	[-]						
Rat	Brain	6431	77	[-]						

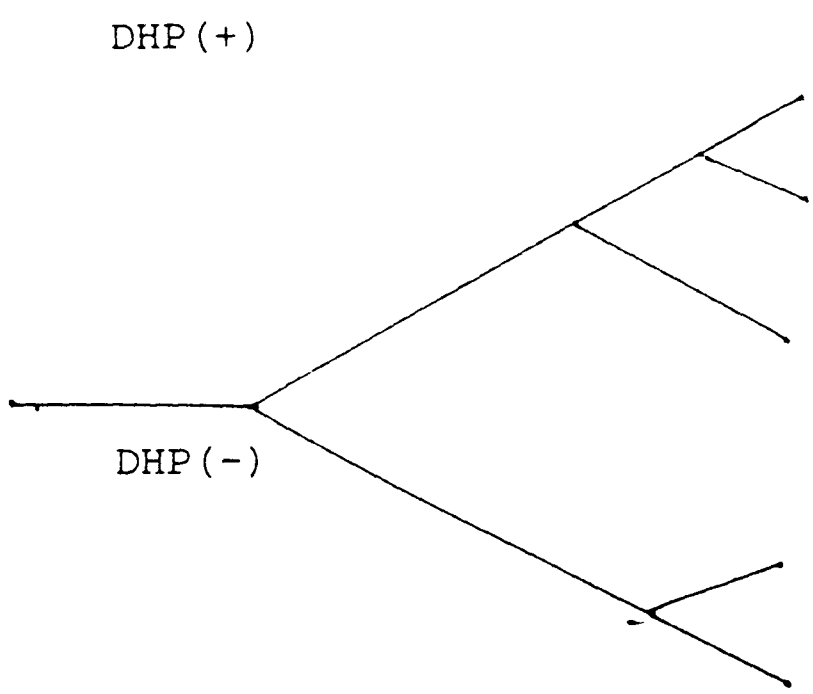
Table 1.3

α1-VGCC subunit sequences, expression and properties of VGCCs that have been fully cloned.

Skm = skeletal muscle, Card = cardiac, Cbm = cerebellum

Sources:

Tanabe	[1988]	Rabbit Skm	; Koch	[1989]	Rat Aorta
Mikami	[1989]	Rabbit card	; Starr	[1990]	Rat cbm
Biel	[1990]	Rabbit lung	; Snutch	[1991]	Rat brain
Mori	[1990]	Rabbit brain	; Hui	[1991]	Rat brain
Grabner	[1990]	Carp Skm	; Hui	[1991]	Rat brain



Molecular Divergence	Perez-Reyes Class	Snutch Class	Tissue Class	
DHP(+)	1	-	Skm	L
	2	C	Card	L
	3	D	N/End	L
DHP(-)	4	A	Neu	P?
	5	B	Neu	?

120 0

Figure 1.8

Relationship between PCR amplified segments of $\alpha 1$ subunits and the dendrogenetic tree. Data is derived from the classifications devised by Perez-Reyes and Snutch. Figure after Tsien et al, 1991.

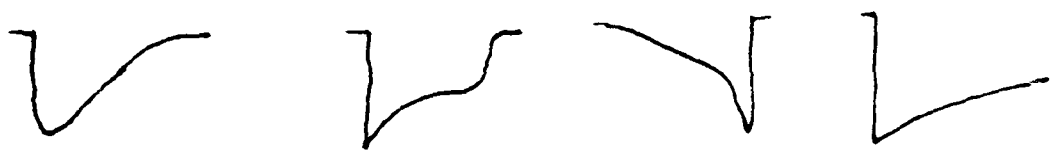
Class	T	N	L	P
Ba^{2+} flow [pS] 110mM	8	15	25	10-12
Single channel Kinetics	Late opening Brief burst	Long burst		
Inactivation	+	+	-	-
Conductance	$Ba^{2+}=Ca^{2+}$	$Ba^{2+}>Ca^{2+}$	$Ba^{2+}>Ca^{2+}$	$Ba^{2+}>Ca^{2+}$
I_p [mV]	- 20	+20	+10	-60
Waveform				
Inactivation 10mM Ca^{2+}	(+) to -70	(+) to -20	(+) to -20	(+) to +20
100mM Ca^{2+}	-100 to -60	-120 to -30	-60 to -10	-30 to +20
Rate of inactivation (τ) [ms]	20-50	50-80	>500	?
Pharmacology: Inorganic block	$Ni^{2+}>Cd^{2+}$	$Cd^{2+}>Ni^{2+}$	$Cd^{2+}>Ni^{2+}$	$Cd^{2+}>Ni^{2+}$
DHP	[-]	[-]	[+]	[-]
ωCgTx	[-]	[+]	[-]/[+]	[-]
Amiloride	[+]	[-]	[+]	[-]
ωAgaII	[-]	[-]	[-]	[+]
ωAgaIa	[+]	[+]	[-]	[-]
ωAgaIIIa	[-]	[+]	[+]	[-]

Table 1.4
Classification of Calcium Channels
Originally described by Fox et al [1985]
Source : chick sensory neuron

Differential expression of these genes has also been shown in rat brain for the isotypes rb-CI and rbCII in rat brain but did not correlate with the distribution of DHP binding sites in the brain as determined by labelling sections with ^{3}H-nifedipine [Cortes, 1984].

1.19 Structure of the ion pore and ion selection mechanism

Little data exists on the pore structure of the VGCC or VGSC. No large scale site directed mutagenesis has been performed in VGSCs or VGCCs in contrast to VGKCs. The VGSC is known to be partially permeable to calcium ions [Agnew, 1988] but ion selectivity can be reduced further by the action of hormones. The VGSC of rat myocytes can be converted to a VGCC by the action of atrio-naturetic peptide independent of G-protein effects [Sorbera & Morad, 1989]. Calcium ions may be required as a cofactor in VGSC gating as they can block the channel at high voltages and can alter channel gating directly via surface adsorption [Armstrong & Cota, 1991]. VGKCs have no requirement for calcium ions.

1.20 The structure of the VGSC and VGCC pore

Comparison of VGSC and VGCC structure showed that S3 domains are highly conserved and this sequence has been suggested to be pore lining just like the H4/H5 region of the VGKC. However the structure of the S3 domains bears little relation to the H loops found in VGKCs. Theoretically synthesising these regions and placing them in a planar membrane should result in the formation of a functional pore while areas not involved in pore formation should not be able to function as pore formimg units. IVS3 domains were synthesised and placed in lipid bilayers and the structure

analysed by computer modelling [Grove *et al*, 1991]. This structure had the right energy characteristics for a pore and was consistent with the presence of transmembrane α-helices. Structure and electrophysiological comparisons were made with the IVS5 domain which was not thought to be relevant to the pore. Electrophysiologically detectable pores were formed by $[IVS3]_4$ in the membrane which could be blocked by cadmium ions, nifedipine and verapamil and the structures were ion selective. They were also stereo selective for Bay K 8644(+) enantiomers. This is a fair approximation of the function of the channel pore and thus suggests that S3 domains line the pore in VGSCs and VGCCs.

1.21 Post-translational modification of voltage gated channels

Post-translational modification also plays a role as expression of the *Shaker* product in neurons produces a charybdotoxin sensitive current while expression in muscle produces an insensitive current [Zagotta *et al*, 1990]. There is no data yet on whether post-translational control occurs in VGSCs but it is known that the α1 subunit of the VGCC is cleaved specifically in muscle to generate a voltage sensor [de Jongh *et al*, 1989]. Two forms of the α1 subunit were identified with a monoclonal antibody αCP1 which recognises the C-terminal of the α1 subunit [de Jongh *et al*, 1989]. These two forms vary in size with the major component αCP1(-) of 175 kDa [90%] and a larger CP1(+) protein of 212 kDa comprising 10% of the isolated T tubule protein. A discrepancy existed between the calculated calcium flux from external sources through VGCCs and the amount of VGCCs present in the T tubule with 5% of the expected flux being seen [Schwartz *et al*, 1985; Catterall, 1986]. This lead to the suggestion that the $\alpha 1_{175}$ protein is generated by proteolysis of the $\alpha 1_{212}$ and is solely a voltage

sensor attached to the ryanodine receptor [Catterall, 1991a & 1991b]. The proteolysis removed 3 of the 6 cAMP dependent phosphorylation sites in α1. It has also been suggested that a tissue factor may downregulate channel ion flux possibly by a process involving phosphorylation of the α1 subunit [Lamb, 1991].

1.22 Developmental expression of VGKCs, VGSCs and VGCCs

The transcription of the rat RCK1-5 family shows that some of the diversity in VGKCs is generated by differential expression with age [Beckh & Pongs, 1990]. Similar differential expression is seen with VGSCs [Hallen *et al*, 1990] but there is little data yet on VGCCs. The only experiments that have been performed in the mouse myoblast line C2C12 and these showed that both α1 and α2 subunits are expressed in differentiating cells and that α1 induction occurs at the stage of myotube fusion [36 hours] while α2 only occurs in significant amounts after 36 hours. Thus α1 seems to be the initial regulator [Varadi *et al*, 1989]. α1 subunit genes contain regulatory domains for proteins such as myoD, steroids and neuropeptides though they seem relatively resistant to the effects of denervation [Varadi *et al*, 1990].

It is interseting to note that both VGSCs [*para* mutant] and VGCCs have been implicated in morphogenesis. The nap^{ts} locus in *Drosphila* which affects *para* gene expression also affects sex determination- the *mle* locus [male][Kernan *et al*, 1991]. Similarly DHPs have been shown to regulate *c-fos, fos-b* and *jun-B* oncogene expression in neurons and this may be relevant both to morphogenesis and to synaptic plasticity [Murphy *et al*, 1991].

1.23 The classification of voltage gated calcium channels

Calcium channels occur in a wide variety of cells and are found in the plasma membrane [see Hosey & Lazdunski, 1988]. They seem to be universal across phyla with VGCCs reported in bacteria [reviewed by Saimi *et al*, 1988] and protozoa e.g. the *Paramoecium pawn* and *dancer* mutants [reviewed by Kung & Saimi, 1985]. Algae and plants are known to have DHP receptors [Johannes *et al*, 1991] and to employ calcium signalling in response to mechanical stress. However, only the structure of animal VGCCs is known in detail.

Calcium channel currents are inward fluxes sensitive to calcium concentration evoked by membrane depolarisation. They are generally unaffected by sodium concentration or tetrodotoxin. Calcium fluxes are difficult to measure directly so channel conductance is measured in the presence of Mendeleev group II analogues e.g. barium and strontium. However magnesium ions block the channel as do divalent transition elements [cadmium, nickel and cobalt] and rare earth metals e.g. lanthanum. They are sensitive to simple organic compounds such as dihydropyridines [see Hagiwara & Byerley, 1981 for a review] and complex peptide toxins.

1.24 The pharmacology of calcium channels

The basic classification of VGCCs was evolved by Fox *et al* [1984, 1985] [see Miller, 1987 for a review] who characterised VGCCs in chick dorsal root ganglion into 3 groups based on their Ba^{2+} current conductance, inactivation characteristics and limited pharmacology in whole cell and single channel patch clamp. The terms T, L, and N originally stood for transient, long, and intermediate ['neither'] conductance on the basis of inactivation characteristics. The occurence of similar channels has been

noted in mammalian neurons and other neuronal cell types [Kostyuk *et al* 1987;1989]. The basic classification is shown in table 1.4. The classification has been extended to other cell types which have indicated that these properties define families of channels and not individual units [see McCleskey *et al*, 1986 for a review][table 1.5]. The classification has limitations in non-mammalian systems. In squid giant axon calcium channels are not related to any of the three basic types [see review by Charlton & Augustine, 1990] and are even cadmium insensitive. In *Drosophila* head tissue including brain the DHP and phenylalkylamine binding sites are independent and occur on different channels which are insensitive to the other antagonist [Pelzer *et al*, 1989]. Similar effects are seen with peptide toxins which vary in their efficacy depending on the species and tissue used [*vide infra*]. In mammals substantial differences are seen with conductivities and inactivation voltages for pharmacologically similar channels [Reuter & Porzig, 1988; Swandulla *et al*, 1991]. Therefore the original classification needs to be extended to include new families of channel and be based on molecular analysis of the channel proteins rather than cruder pharmacological or electrophysiological properties which may be inappropriate for defining characteristics in functional terms.

Table 1.5a
Regulation of amplitude of calcium channel flux in neurons by neurotransmitters, drugs and regulation mechanism

Neuron	Transmitter Receptor	Messenger	Current Potentiate	Inhibit
Chick Sensory DRG	NA (α2)	G PKC		L
	GABA-B	?		L T
	5-HT	?		L T
	Dopamine	?		L T
	?	PKC		L T
Rat Sensory	Adenosine 1	G		all
	GABA-B	G		all
	GABA-B	?		N
Mouse sensory	Dynorphin A	?		N
	Pentobarb	?		N L
	Adenosine	?		all
Rat Sympathetic	ACh [M1]	G		N
	NA	?		all
	Somatostatin	?		all
Rat Myenteric	NPY	G		N
	Dynorphin	?		N L
	NA	?		N L
Rat Hippocampal Pyramidal	Adenosine	?		N
	Muscarine	?		all
Granular	NA (β)	cAMP		N

Cell Line	Neurotransmitter Receptor	Mechanism	Current Potentiate	Current Inhibit
NG108-15 Neuroblastoma Glioma	Leu-Enkephalin	$G_{0\alpha}$		all
	Somatostatin	$G_{\alpha 1}$		all
	Noradrenaline	?		all
N x G	Enkephalin [D-Ala^2 D-Leu^5]	G		all
AtT-20	Somatostatin	G		all
	Isoproterenol (β)	cAMP		L
	?	PKC		L T
GH3 Hippocampal	?	PKC		L T

Table 1.5b

Regulation of calcium channel flux by neurotramnsmitters, drugs and regulatory mechanisms.

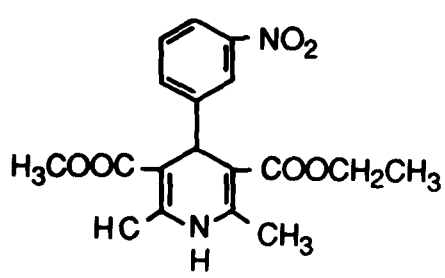
1.25 The electrophysiological properties of VGCCs

All 3 basic channel types activate from -80 mV [strong depolarisation] but small steps from the resting potential activate only T channels [-40 mV] while steps to +10 mV activate L & N [Dascal, 1990][see Hille, 1984 for a detailed review]. Holding the membrane potential at -40 mV inactivates T and N channels but depolarisation [to 0 mV] still gives L currents. Single channel studies show that T and N channels open preferentially giving transient currents while L channels open later and give a sustained current. In some neuronal cells a calcium current has been found that is sensitive to tetrodotoxin [von Sprechelsen *et al*, 1990] so some sodium channels may be reset to calcium selection as has been described in cardiac atria in response to cardio/neuropeptides [Sorbera & Morad, 1989].

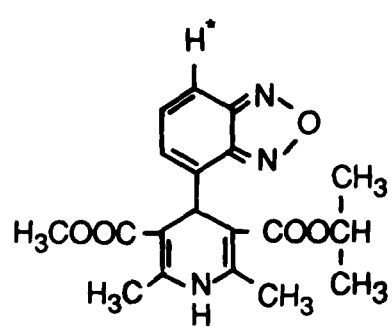
1.26 Pharmacological ligands of L-type VGCCs

L channels have been subject to the most study as they are the commonest type of VGCC and have a large number of ligands [fig 1.9]. The ligands commonly used for defining L-VGCCs are the dihydropyridines [DHPs]. Phenylalkylamines and other other compounds interact with DHPs in a non-competitive way suggesting that there are different but closely spaced sites on the L-channel for these compounds [fig 1.10]. The effects of these compounds on gating modes and their interactions with other ions, channel use and each other are complex and a detailed discussion can be found in Peers [1989] or Hille [1984].

1. 4 Dihydropyridines



Nitrendipine



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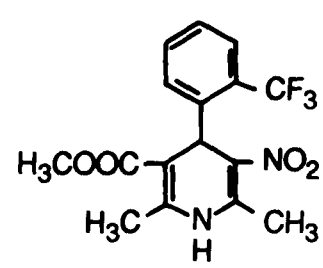
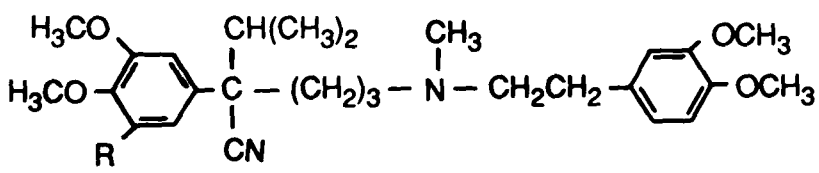


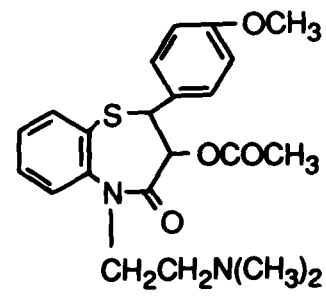
Bay K 8644

2. Phenylalkylamines



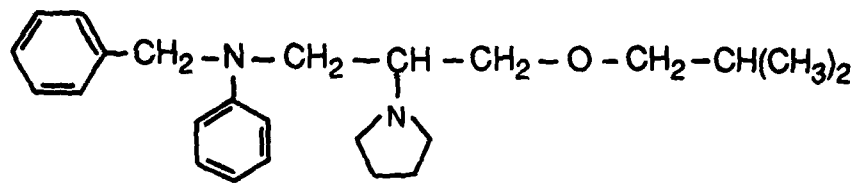
Verapamil R = H
D600 R = OCH_3

3. Benzothiazepines

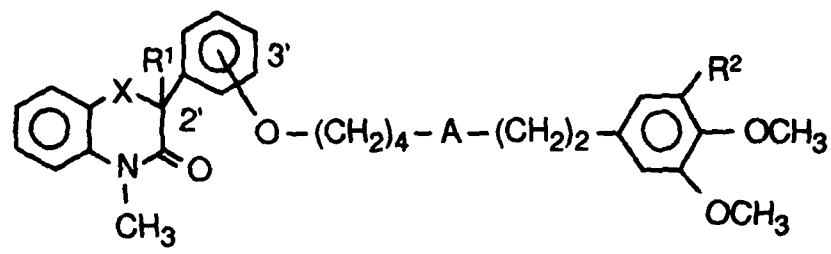


Diltiazem

4. **Bepridil**



6. **HOE 166**



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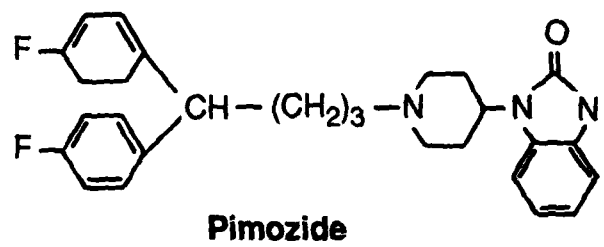

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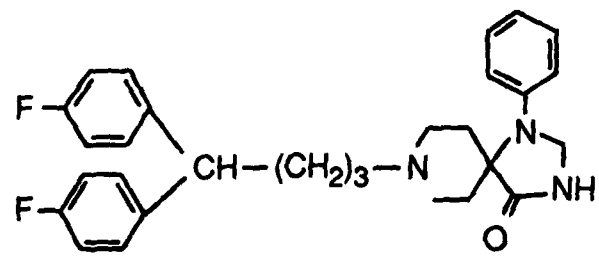

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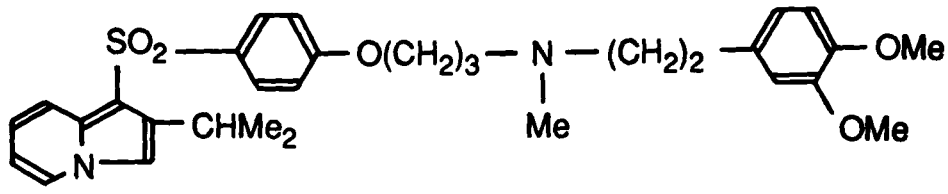

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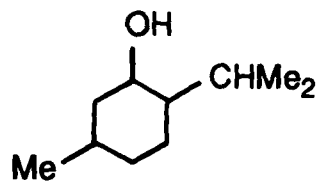

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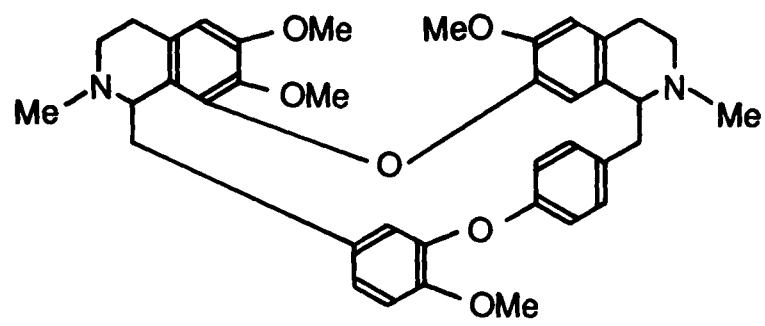

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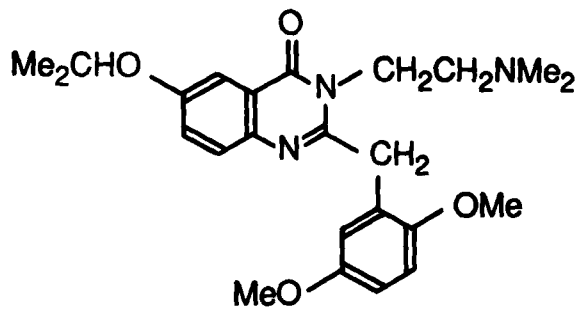

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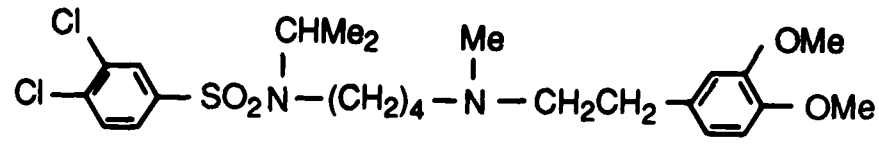

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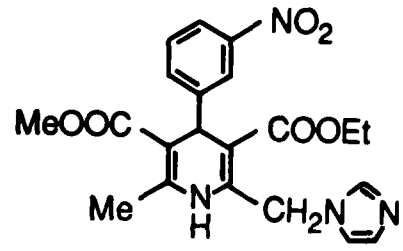

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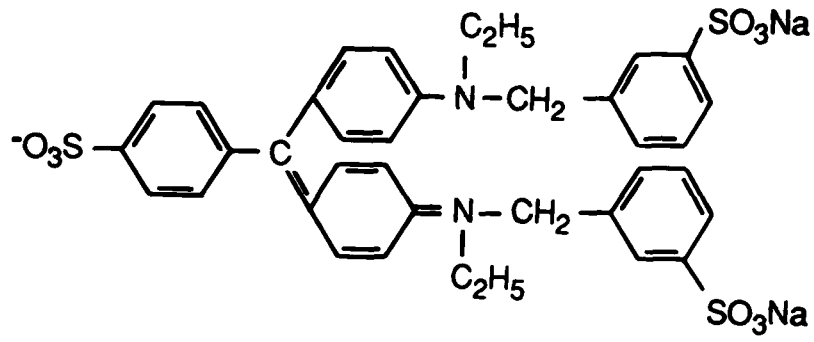
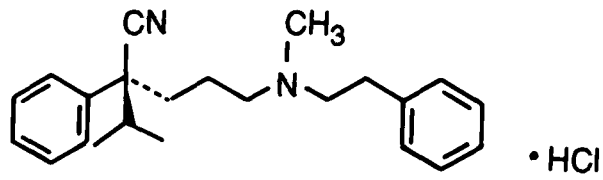


Lidoflazine



[s]-emopamil

Figure 1.9

The chemical structure of some commonly used ligands for L-type VGCCs.

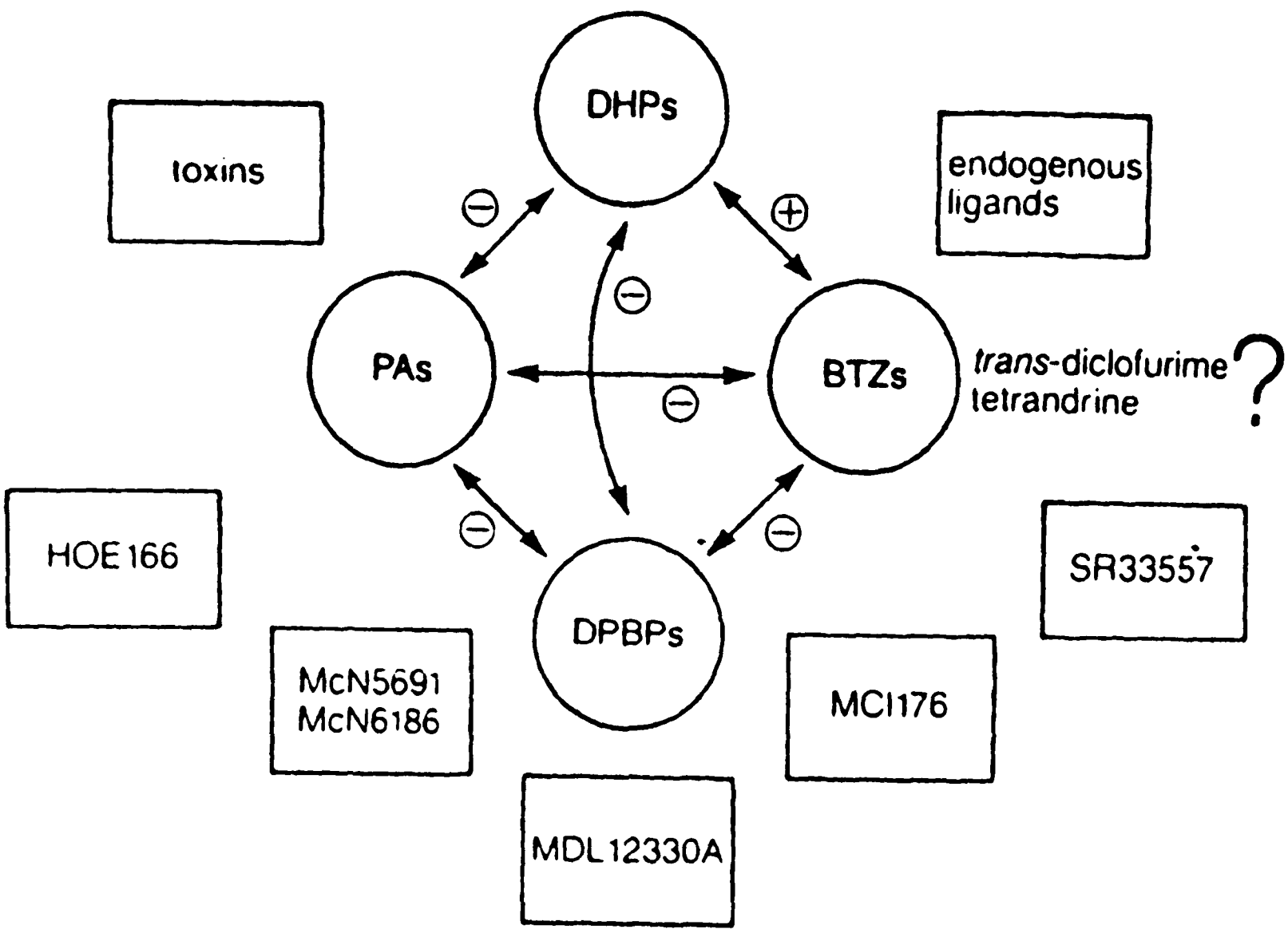


Fig 1.10

A schematic representation of the non-competitive pharmacological interactions that have been described in L-type VGCCs. The data was acquired using patch clamp electrophysiological techniques on a variety of cell lines in the presence of pharmacological ligands followed by mathematical analysis of the interaction between any two ligands. Ligands whose exact interaction with the well defined central group are unclear are shown nearest those to which they appear to interact. Endogenous ligands for the L-type VGCC have not yet been defined though they are postulated to exist.

Abbreviations: BTZ= benzothiazepine DHP= dihydropyridine

DPBP= diphenylbiperidine PA= phenylalkylamine

The phenylalkylamines e.g. verapamil also bind to the α1 subunit of the L-VGCC. Other ligands for L-VGCCs now exist [fig 1.9] and their pharmacology has been reviewed by Rampe and Triggle [1990]. The group of L-VGCCs has become heterogeneous with the discovery that some neuronal L-VGCCs also bind ω-conotoxin [L_N][Cruz *et al*, 1987] and the discovery that some L-VGCCs are latent and depend on previous depolarisation of the cell to show L-type characteristics [L_O][Bacskai & Friedman, 1990].

1.27 Identification of the DHP binding site

Molecular characterisation of the various homologous forms of L-type channel and specifically their α1 subunits [*vide infra*] has enabled photoaffinity labelling experiments and peptide sequencing to be correlated with the primary cDNA structure. Certain common motifs have emerged from these studies though only 2 direct homologues have been cloned - the α1 subunit from rabbit [Tanabe *et al*, 1989] and from the carp *Cyprinus carpio* [Grabner *et al*, 1991]. The latter encoded a protein of 1852 aminoacids and RMM 210,060 Da. In the non-membrane spanning sections one conserved domain was identified. The principal area of conservation was the 155 amino acid segment after IVS6 [figure 1.11]. This region contains the EF hand domain. The EF hand is two a helix loops separated by a calcium binding site over 29 amino acids and was noted to occur in VGCCs in contrast to VGKCs [Babitch, 1990]. This domain which has an amino acid consensus motif of:

EFKRIWAEYDPEAKGRIKHLDVVTLLRRI

in VGCCs is found in all calcium binding proteins after having been originally discovered in calmodulin [Handford *et al*, 1987]. The sequence after IVS6 was also

found to be identical in skeletal and cardiac VGCCs and has been identified independently as the DHP binding site on the basis of photoaffinty labelling. This was performed using tritiated azidopine and nitrendipine and led to the identification of 2 segments of binding: 1428 - 1437 [azidopine] and 1390 - 1399 [nitrendipine][Regulla *et al*, 1991]. This would place it intracellularly which would be consistent with the hydrophobic or at best amphipathic nature of the dihydropyridines [Rampe & Triggle, 1990]. The DHP binding site overlaps the EF hand domain which accounts for the dependence of DHP binding on the presence of calcium ions and the speed of blockade being dependent on the the net charge of the DHP. The region is shown in fig 1.12. However more recent work has implicated the III S5-S6 loop as a binding site suggesting that the DHP binding site may occur on the extracellular side of the VGCC [Nakayama *et al*, 1991].

The binding site for phenylalkylamines has been localised to the area just proximal to the DHP binding area by similar techniques and consists of the extracellular region from the amino acid leucine at position 1359 [L1359] to IVS6 [i.e IVS5-IVS6] which accounts for its partially competitive interaction with DHPs [Streissnig *et al*, 1990].

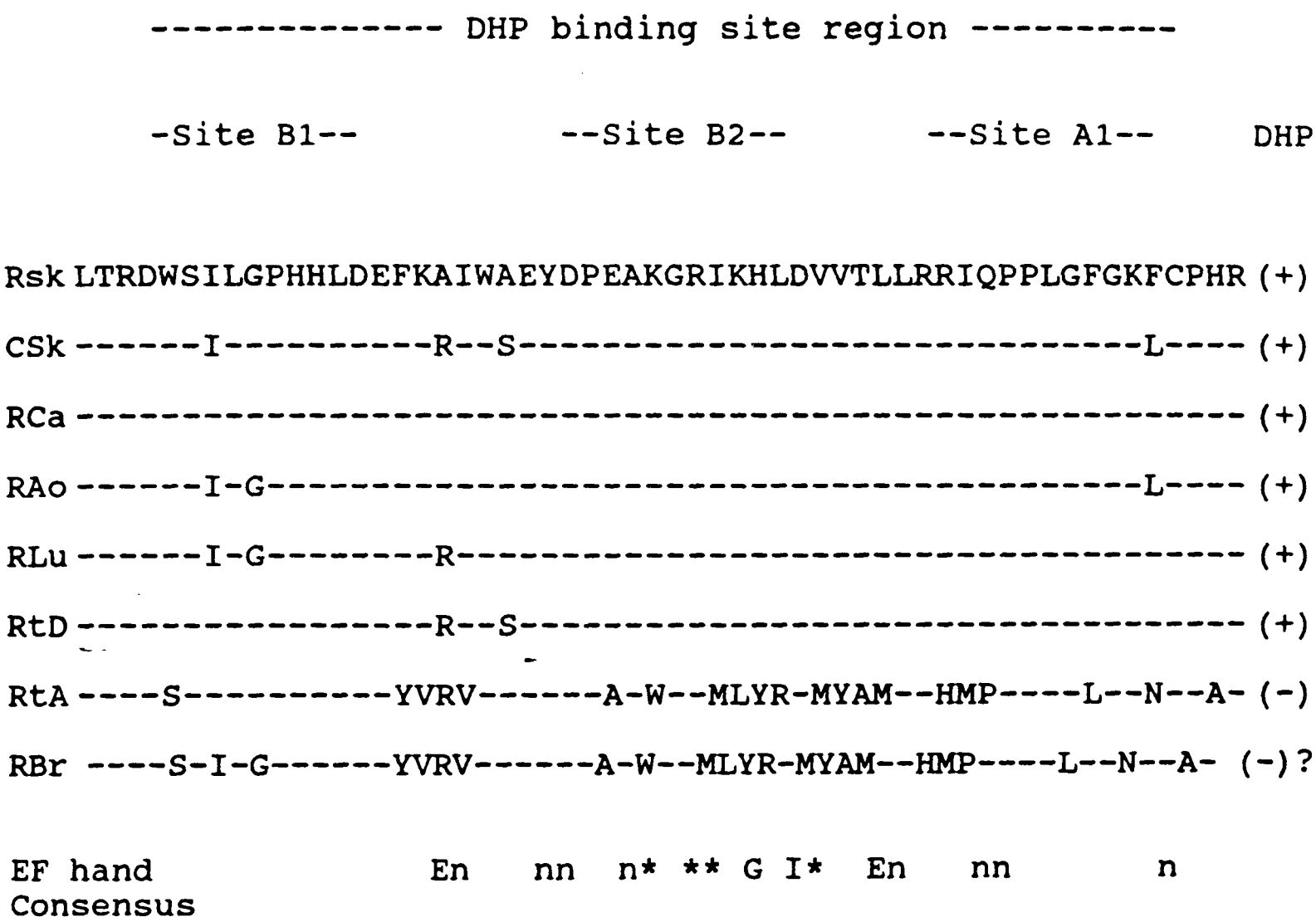


Figure 1.11

Comparison of EF hand and DHP binding site regions of the $\alpha 1$ subunits cloned in their entireity. The EF hand domain includes highly conserved residues and n = hydrophobic residues [L, I, V, F, M] and * = oxygen containing amino acids [D, N, E, Q, S, T]. Sites A1, B1 and B2 represent major labelling sites for nimodipine and azidopine respectively.
After Tsien *et al*, 1991

Animal	ω-CgTx	ω-CmTx
Elasmobranch	reversible	reversible
Frog: neuromuscular junction	irreversible	reversible
sympathetic ganglion	irreversible	reversible
Snake: Neuromuscular junction	irreversible	?
Chick: neuromuscular junction	?	irreversible
ciliary ganglion	irreversible	?
DRG currents: L & N:	irreversible	partial reversibility
: T	reversible	reversible
Human	reversible	reversible

Animal	preparation	effect
Aplysia	CNS neuron	none
Drosophila	bag cell	none
locust	muscle	none
elasmobranch	NMJ	none
frog	NMJ	blocks
	ciliary ganglion	blocks
	spinal cord	blocks
	cardiac muscle	blocks
snake	NMJ	blocks
chick	DRG	L&N irreversible T reversible
	myotube	none
	parasympathetics	blocks
mouse	NMJ	none
	sympathetic	none
rat	CA1 hippocampal cells	some currents blocked
guinea pig	cardiac muscle	none

Table 1.6

Effects of ω-conotoxins from *C.geographus* and *C. magnus* on different tissues and cell preparations (a) and whole animals (b). Data from Cruz *et al* [1987], Tsien *et al* [1991].

1.28 The pharmacology of N-type VGCCs

The conductance of N-channels is often difficult to distinguish from other high voltage activated channels and therefore they are defined by being blocked by the specific peptide toxin ω-conotoxin [ω-CgTx]. The *Conus* snails are piscivorous molluscs found in the Pacific which are known to be toxic to man [Hallen, 1914]. A few major reviews exist of conotoxins including Gray, [1988]; Thayer *et al*, [1988] and Cruz *et al*, [1989]. The naming of conotoxins arises from the arrangement of cysteine residues in the 27 amino acid peptide and is termed the 'framework' classification [Woodward *et al*,1990].

The most commonly used N-VGCC ligand is the ω-conotoxin derived from *C. geographus* [ω-GVIA][originally described by Olivera *et al*, 1985] but analogues exist in *C. magnus* [ω-MVIIA] and *C. textile* [Gray, 1988]. The structures of the analogues are shown in fig 1.13. The toxins have markedly different effects in various tissues implying that calcium channel diversity is significant between tissues as well as between species [Gray, 1988; Cruz *et al*, 1989][see table 1.6]. This is illustrated by the range of affinities [1-100 nM] depending on the toxin and target cell type.

Though ω-GVIA has been extensively used to study VGCCs the exact binding site of the iodinated toxin has remained obscure. The neuronal VGCC was identified as the target in 1985 [Cruz & Olivera, 1985]. Chemical crosslinking of ω-GVIA to chick brain VGCCs identified the binding site as a 140 + 30 kDa protein similar in size to the $\alpha 2$ subunit but another derivative bound to a 210 - 220 kDa protein consistent in size with the $\alpha 1$ subunit [Barhanin *et al*, 1990]. This suggested that the binding site may be in the $\alpha 1$-$\alpha 2$ gap. Marqueze *et al*, 1990 found that affinity labelled ω-conotoxin bound to a protein of 245 - 300 kDa in the rat foetus and 220 kDa in chick synaptosomes and suggested that this was a brain $\alpha 1$ subunit. The amount of glycosylation in neuronal

VGCCs is unknown and therefore binding to an $\alpha 1$ subunit of unglycosylated mass of 220 kDa with extra glycosylation accounting for the mass discrepancy is possible.

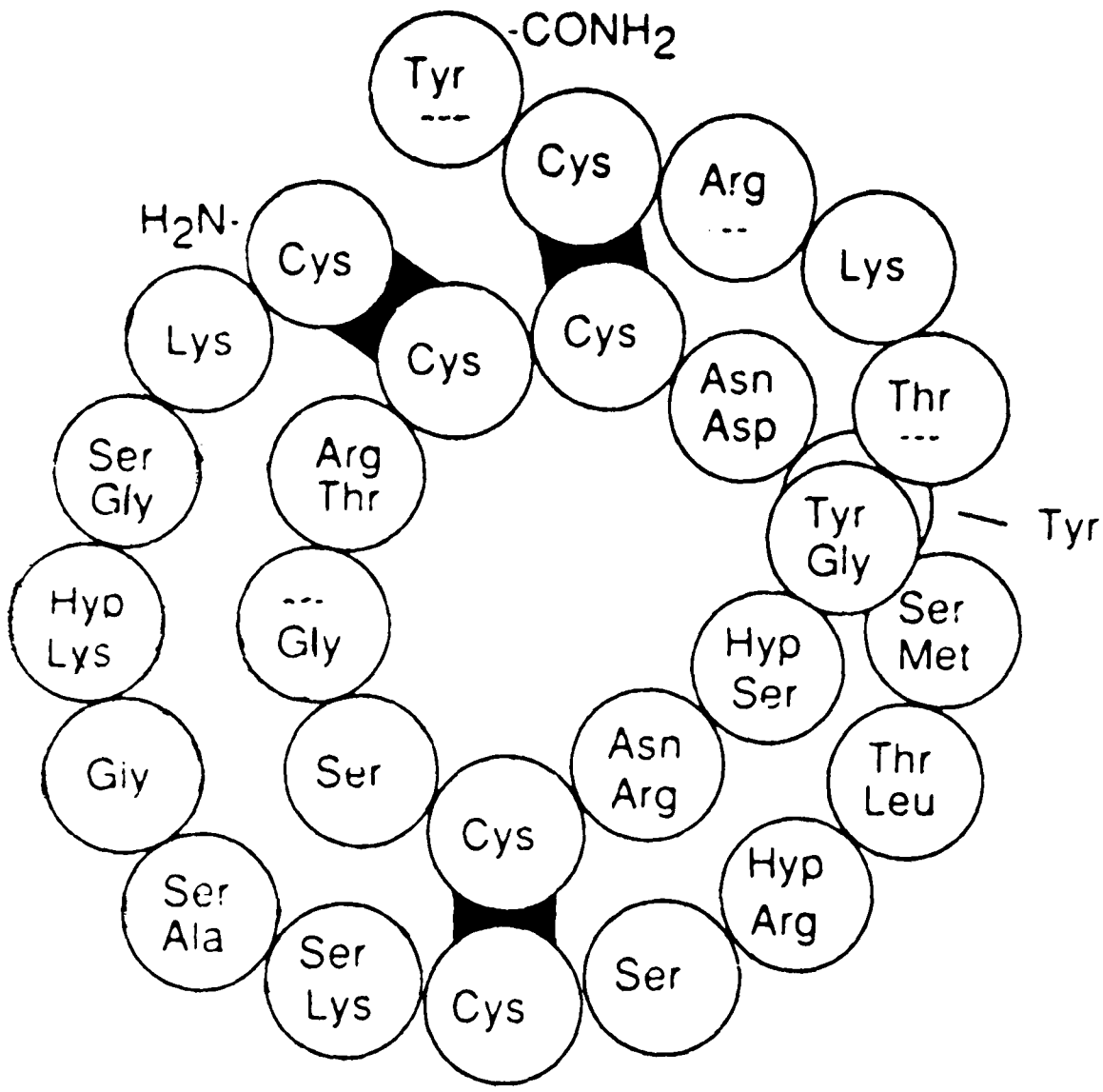


Fig 1.13

Structure of the ω-conotoxins and comparison of the structures of ω-GVIA [*Conus geographus*] and ω-MVIA [*Conus magnus*]. The amino acids are placed in positions on the ring with alternates marked at points of difference. The amino acids present in ω-GVIA are placed above those present in ω-MVIA. After Cruz *et al*, 1987

1.29 Ligands and T-type VGCCs

No utterly specific ligand exists for T-channels but amiloride is often used even though it binds to L-VGCCs as well [Garcia *et al*, 1989]. Other antagonists used to define T-channels include ethanol and nickel ions [Ni^{2+}][Tang *et al*, 1987]. However, usually these channels have to be defined by their inactivation characteristic.

1.30 P-type VGCCs and other toxin ligands

A new group of VGCC toxin ligands has been discovered in the the venom of the funnel web spider *Agelenopsis aperta* which blocked calcium spikes in guinea pig cerebellar Purkinje cells and in the squid giant axon [Llinas *et al*, 1989]. The fraction called FTX had a RMM of 0.2 - 0.4 kDa. The VGCCs defined by this crude toxin were not sensitive to either DHPs or ω-GVIA and had markedly different conductances to previously known channels. They have been termed P-channels. Similar effects were seen in oocytes injected with rat brain mRNA [Lin *et al*, 1990]. One P-type channel has been cloned from rabbit brain by Mori *et al*, [1991]. The toxin binding site has not yet been identified.

Further characterisation has isolated 4 large peptides from the venom of funnel web spiders: ω-AgaIA, ω-AgaIB, ω-AgaIIA [Adams *et al*, 1990] and ω-AgaIIIA [Mintz *et al*, 1991]. Type I toxins do not compete with ω-GVIA for binding sites on VGCCs unlike type IIA [Adams *et al*, 1989]. ω-AgaIA inhibits the DHP-(+) 202-791 sensitive VGCC [L-type] and binds partially reversibly to ω-GVIA sensitive channels [N] in rat dorsal root ganglion cells [Scott *et al*, 1990]. ω-AgaIIIA [8.5kDa] also binds to both L- and N- type channels [Mintz *et al*, 1991]. Further work will be needed to identify the agatoxins and clarify their spectrum of activity as compared to other toxins.

1.31 Reconciliation of the molecular and T,N,L,P classifications

There is not very much data to allow the structural classification of 4 main families of channels to be reconciled with the T,N,L,P. Only the sequence defining the DHP binding site on $\alpha 1$ subunits has been definitively identified and this overlaps two molecular families [1,2][Tsien *et al*, 1991]. The other toxin and ligand binding sites are unknown. It remains to be seen which classification has more functional relevance given the diversity of VGCCs. A completely new classification independent of pharmacological or evolutionary origins possibly on inter-domain linker structure may prove appropriate but this awaits the cloning and expression of more sequences.

1.32 Integrating currents with neuronal VGCCs

The location, type and control of VGCCs in neuronal tissue is less well understood than in skeletal muscle and has usually involved the study of synaptosomes or brain slices [Miller, 1987] by ion flux and later by patch clamp techniques. Four types of calcium channel have been identified in central neurons corresponding approximately to those found in the sensory spinal neurons [L, N, and T] but there is also a tetrodotoxin sensitive calcium current which could be a form of sodium channel [von Sprechelsen *et al*, 1990]. VGCCs are regulated by a variety of intracellular modulators coupled to presynaptic receptors and directly by electrical activity. Strong persistent depolarisations change the gating of L-VGCCs to a long opening high probability form on a voltage dependent basis [Pietrobon & Hess, 1990] and may thus regulate calcium influx. N-VGCCs are also capable of two inactivation characteristics dependent on slow switching [Plummer & Hess, 1991]. The interactions between depolarisation profiles, intracellular regulatory proteins, receptors and channels is complex and so

control of gating and currents will be complicated and probably tailored to each individual synapse. The use of action potential wave form patch clamping as opposed to continuous clamping has also caused some redefinition of channel inactivation characteristics in individual neurons [McCobb *et al*, 1991]. The effects of DHP agonists and antagonists on relative flux in cell lines has led to the model that N-channels exist in the synaptic plate while L-channels are outside it [McCleskey *et al*, 1985; Stanley & Atrakchi, 1990]. Studies of the neurohypophysis have confirmed the localisation [Lin N.F. *et al*, 1990]. A similar distribution of N-VGCCs subtype N_{pt} has been found at the endplates of cholinergic ciliary ganglion cells [Stanley, 1991]. Action potential waveform clamping has led to the concept that L-types regulate the facility and probability of longer term release while N-VGCCs regulate individual spike release [McCobb *et al*, 1991] while other data has suggested that L-VGCCs are responsible for low depolarisation transmission and N- for high [Lin N.F. *et al*, 1990]. However, analysis of calcium currents and their correlation with particular channel types does not necessarily coincide as a multiplicity of current types can be generated [Giffin *et al*, 1991; Schroeder *et al*, 1990] from a limited number of channels types [2] . To complicate matters even further isolated nerve terminals [from guinea pig hippocampus] are capable of differential release of amino acids, catecholamines and neuropeptides [Verhage *et al*, 1991]. The role of the various types of VGCC in selecting which transmitter is released in response to identical or different depolarising stimulus trains still remains to be determined.

1.33 Location of VGCCs on the neuronal cell body

While the location of axonal terminal channels is approximately defined the location of VGCCs on the rest of the neuronal cell body is far less clear. However some initial studies have begun to define areas that are particularly rich in VGCCs. Cultured rat hippocampal neurons express two types of VGCC and high voltage activated VGCCs [probably L/N-VGCCs] are found diffusely spaced over dendritic arborisations while low voltage activated channels [T-VGCCs] are localised to the the cell soma [Yaari *et al*, 1987][fig 1.14].

1.34 VGCCs in non-neuronal cells in the brain

Glial cells and their precursors contain a wide variety of ion channels [see Barres *et al*, 1990a & 1990b; Barres, 1991] as do some Schwann cells [Amedee *et al*, 1991] . Astrocytic glial cells express L-channels [Barres *et al*, 1990a] as do glial precursor cells [Barres *et al*, 1990b]. The activity and expression of these channels can be modulated by neurons in co-culture [Corvalan *et al*, 1990] but their functional role remains unclear.

1.35 Control of voltage gated calcium channels

A voluminous literature exists on the subject of control of VGCCs depending on the animal used, cell type examined and signalling mechanism [see Swandulla *et al*, 1991 for a review]. The most general review relating regulation of VGCCs and its relevance to disease is Ferrante & Triggle, [1990].

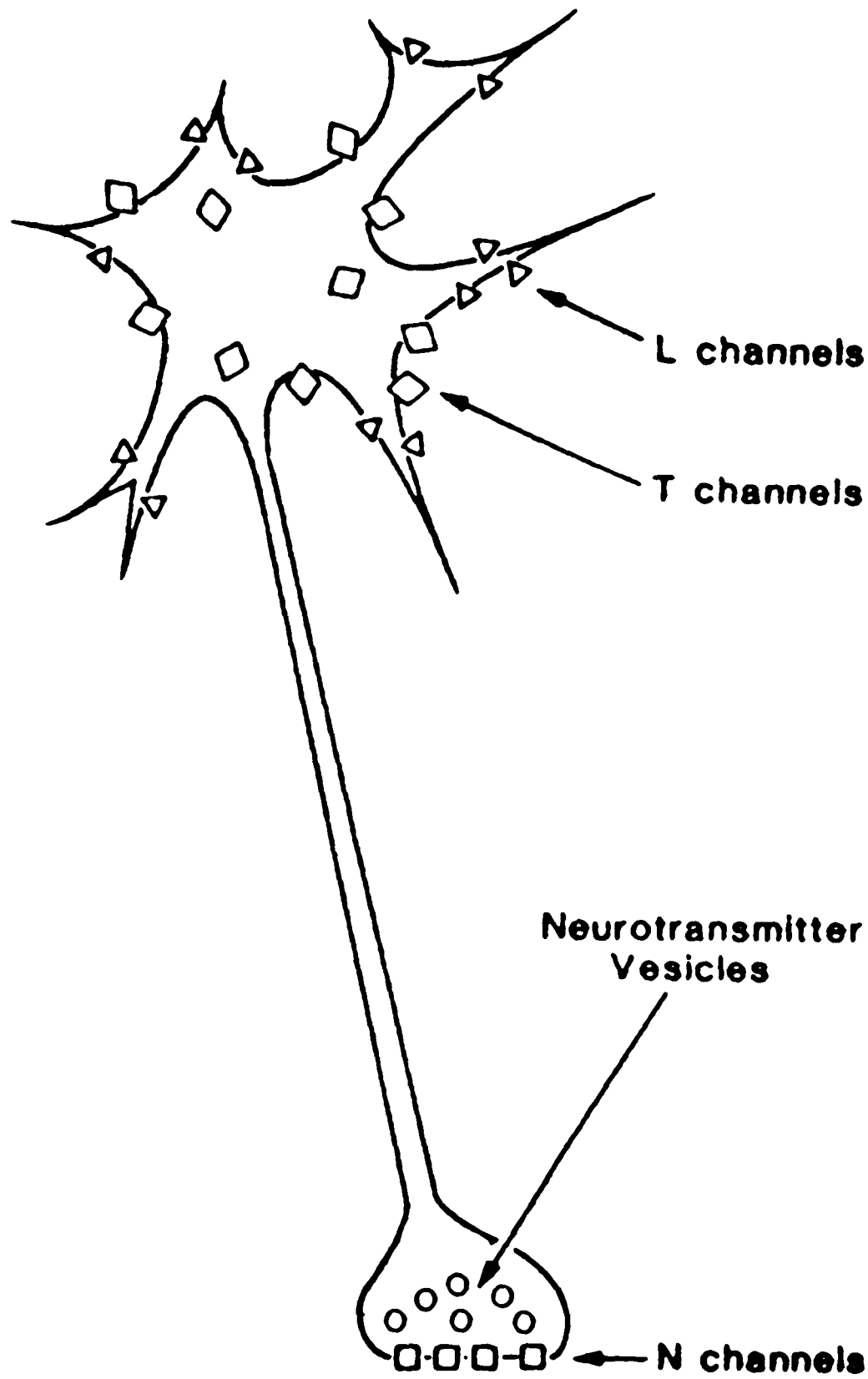


Figure 1.14

Schematic representation of the location of VGCC channel subtypes on a neuronal cell. This model is based on the chick dorsal root ganglion sensory neuron. After Miller, 1987

Organism	Channel: Potassium	Sodium	Calcium
Genetic: protozoa	?	?	*Pawn* *Dancer*
Drosophila	*Shaker* *Shaw* *Shal* *Shab* *Slo* *Eag*	*Para*	*?*
Mouse	?	?Deaf/waddler	*mdg*
Man Genetic Defect	?hereditary neuropathies	hyperkalemic periodic paralysis paramyotonia congenita Schwartz-Jampel[?]	cLEMS
Human disease:			
Autoimmune Paraneoplastic	?neuromyotonia	?	LEMS
Associated Protein Defect	-	?	malignant hyperthermia
Other	epilepsy[?]	epilepsy[?]	epilepsy[?]

Table 1.7
The voltage gated ion channel superfamily and its disease associations.
cLEMS = congenital LEMS

1.36 Diseases and the voltage gated superfamily

A comparative scheme of diseases and defects affecting the voltage-gated superfamily of genes is shown in table 1.7. In many cases the disease associations are not totally proven or the data is inadequate in some regard. Thus no genetic defects have yet been identified in *Drosophila* for calcium channels. No genetic defects are known in hereditary neuropathies and the basis for suggesting that these diseases might be caused by VGKC defects is the pattern of continuous muscle fibre activity which is consistent with defects in the potassium dependent repolarisation mechanism. The data for identifying neuromyotonia [Sinha *et al*, 1991] and Schwartz-Jampel syndrome [Spaans *et al*, 1991; Lehmann-Horn *et al*, 1991] as related to ion channels relies on gross pharmacological or electrophysiological findings and has to be treated as preliminary in the absence of single channel recordings. The data for paramyotonia congenita and hyperkalemic periodic paralysis is firmly established as both have been localised in affected kindreds to comap with the VGSC locus on chromosome 17 with LOD scores of greater than 5 in each kindred [total 20][Barchi, 1991; Ptacek *et al* 1991] and mutations have been identified in the VGSCs in these patients [Ptacek *et al*, 1991].

1.37 Diseases and voltage gated calcium channels

A variety of diseases have been associated with calcium channels. The NMDA receptor [receptor operated] has been associated with epilepsy [Barnard, 1990]. The calcium leak channel is known to show increased activity in myotubes of Duchenne muscular dystrophy [Fong *et al*, 1990]. A similar pattern is seen in the *mdx* mouse model of Duchenne muscular dystrophy. Mutations in the calcium release channel are

associated with malignant hyperthermia [MH]. These mutations are heterogeneous [Levitt *et al*, 1991] but one mutation does seem to occur in both humans and pigs - C614R [Gillard *et al*, 1991; Otsu *et al*, 1991]. No defect is known to occur in the voltage sensor in this disease though levels of DHP binding and α1 subunit expression are altered as a secondary phenomenon [Ervasti *et al*, 1989].

1.38 Muscular dysgenesis and the VGCC

Only one disease is characterised by mutations in the VGCC and this has been identified only in mice. Muscular dygenesis [*mdg*] is caused by an autosomal recessive mutation and characterised by abnormal myogenesis in the form of dilated myotubes at 13 days of gestation [see Powell, 1990; Adams & Beam, 1990 for reviews]. In culture the myotubes lack spontaneous contractions in culture. Potassium stimulated flux does however occur in diaphragmatic studies. Thus, the disease is due to a failure of E-C coupling.

Ultrastructural studies identified a defect in triad organisation which included a lack of SR feet. DHP binding was also significantly reduced. Immunoblots identifed most common muscle proteins except the DHP receptor [Knudson *et al*, 1989] and specifically the α1 subunit. The α2 is present in normal quantities. RFLP analysis of normal and *mdg* mice revealed an abnormal *Bgl I* and *Pst I* polymorphisms in *mdg* mice and northern blotting showed gross reduction in the levels of the α1 subunit. Microinjection of an expression plasmid containing the rabbit α1 gene restored E-C coupling and spontaneous contractions in *mdg* myotubes [Tanabe *et al*, 1989]. *Mdg* mice are now used as a model of myogenesis and their myotubes are used as cells for studying the expression of exogenous α1 subunits.

1.39 Diseases of the human VGCC

The situation with VGCCs in man is far more speculative. Only one disease, the autoimmune and paraneoplastic disease Lambert-Eaton myasthenic syndrome has been definitely proven to involve calcium channels. Other diseases have been associated with calcium channels on the basis of their response to calcium channel blockers and there is little hard evidence for a role of calcium channels in these conditions.

The majority of data comes from diseases in which dihydropyridines have been shown to confer a degree of benefit. However, the actions of the DHPs may occur at other sites besides the VGCC and seem to be more relevant in these studies to control of intracellular calcium pools rather than voltage gated calcium channels.

1.40 The Lambert-Eaton myasthenic syndrome

The only human disease in which VGCCs are known to be directly relevant is the paraneoplastic Lambert-Eaton myasthenic syndrome [LEMS]. This syndrome accounts for 8% of neurological complications of neoplastic disease [Elrington *et al*, 1991] and occurs in 3% of cases of small cell lung carcinoma [Sculier *et al*, 1987, Gomm *et al*, 1989]. No proven analogues exist for other voltage gated channels though neuromyotonia may involve antibodies to the voltage gated potassium channel [Sinha *et al*, 1991] and there is a report that IgG from patients with amyotrophic lateral sclerosis [motor neurone disease] can reduce charge movement in calcium channels in skeletal muscle T-tubules [Delbono *et al*, 1991]. There is also a report of a case of congenital LEMS on the basis of age and electrophysiological findings but no direct studies of the neuromuscular junction or of VGCCs were performed [Bady *et al*, 1987].

An analogous disease does affect receptor operated channels where myasthenia gravis is caused by antibodies to the acetylcholine receptor [see Engel, 1984; Lindstrom, 1985 for reviews].

A number of reviews have been written on LEMS including Engel, [1984]; Lindstrom, [1985]; Newsom-Davis, [1985]; O'Neill *et al*, [1988]; Vincent *et al*, [1989] and Lang *et al*, [1990].

1.41 History and clinical features of LEMS

LEMS was originally described by Eaton & Lambert in 1956 though an earlier case with comparable clinical features had been seen by Anderson *et al*, [1953]. It mostly occurs in adults and usually presents with fatigue, progressive proximal muscle weakness resulting in gait abnormalities. More severe cases tend to have ptosis and bulbar weakness. Tendon reflexes are usually reduced but are markedly increased after exercise. Autonomic effects include dry mouth, sexual impotence and occasional adrenergic dysfunction [Rubenstein *et al*, 1979]. No sensory symptoms are known to occur. Severe cases can show cerebellar degeneration and have histological type I muscle fibre atrophy [Squier *et al*, 1991].

Up to 60% of cases are associated with small cell lung carcinoma. LEMS usually presents before the tumour has presented but in certain cases the interval before tumour presentation can be upto 10 years. It has been noted in association with thymoma, renal carcinoma acute leukaemia, large cell lymphoma, breast adenocarcinoma, ovarian adenocarcinoma and squamous cell lung cancer.

Most other cases of LEMS [26%] are associated with autoimmune disorders including thyroiditis, pernicious anaemia, Sjogren's syndrome [O'Neill *et al*, 1988] and systemic

lupus erythematosus [Bromberg *et al*, 1989]. Many of these cases have relatives with autoimmune disease and 36% of the patients have raised autoantibody titres. Nonspecific autoimmune markers such as anti-IgG antibodies [rheumatoid factor] occur in 42% and anti-neuronal antibodies in 26% [Dropcho *et al*, 1989] of cases of LEMS. It is associated with HLA-B8 [62%], less with HLA-A1 and HLA-DR3, and with the immunoglobulin heavy chain marker GM2 [47%][Willcox *et al*, 1985]. Furthermore, plasmapheresis and immunosuppressive drug therapy [prednisolone and azathioprine] are often followed by clinical improvement [Newsom-Davis & Murray, 1984].

1.42 Physiology of LEMS

LEMS is characterised by distinctive neurophysiological findings. Electromyography shows a classical triad of low CMAP amplitude, decremental response at low rate of nerve stimulation and an incremental response at high rates of stimulation or following maximal voluntary contraction. This facilitation persists for a while to single nerve stimuli [Lambert, 1961]. Direct stimulation of the muscle shows normal contractility and motor nerve conduction is also normal [Elmqvist & Lambert, 1968]. The neurophysiological assessment of LEMS has been reviewed by Oh, [1989].

Intracellular recording from isolated muscle biopsy specimens has shown that the main abnormality is a reduction in quantal content, i.e. the number of acetylcholine molecules released by each nerve impulse, and in the resulting EPP amplitude [Lambert & Elmqvist, 1971] suggesting that the failure exists in the mechanism linking excitation and transmitter secretion. The frequency of miniature end-plate potentials [mEPPs] is unaffected [Lambert & Elmqvist, 1971; Cull-Candy *et al*, 1980] as is the

acetylcholine content of the secretory vesicles [Lindstrom & Lambert, 1978; Molenaar *et al*, 1984]. The electrophysiological findings in LEMS are exploited clinically by pharmacological therapy with 3,4 di-amino-pyridine which blocks potassium mediated repolarisation and increases calcium influx, thereby enhancing transmitter release, resulting in an increase in muscle strength [Lundh *et al*, 1977; Murray & Newsom-Davis, 1984].

1.43 Passive transfer studies in LEMS

Passive transfer studies in the mouse showed that diaphragmatic epp amplitudes showed facilitation by trains of impulses and that quantal content was significantly reduced by chronic treatment with LEMS IgG [Lang *et al*, 1981; Gwilt *et al*, 1981; Newsom-Davis *et al*, 1982; Kim, 1985; Lang *et al*, 1985]. Long term injection of LEMS sera or purified IgG into mice caused a reduction of quantal content [Lang *et al*, 1983]. The effect was independent of the action of presynaptic acting peptides such as *Clostridium botulinum* toxin [Dolly *et al*, 1986] or lautrotoxin [Lande *et al*, 1985]. The VGCC of the presynaptic nerve terminal was identified as the target of LEMS [Lang *et al*, 1981; 1984; 1985; 1987] but LEMS sera had no effect on the cardiac slow current which is verapamil sensitive [Lang *et al*, 1985; Lang *et al*, 1988]. This showed that only some calcium channels are the target of LEMS IgG and illustrates the range of calcium channel diversity. Purified LEMS IgG was capable of reducing prolactin release in rat pituitary GH_3 cells indicating that the VGCCs present at the neuromuscular junction show some similarity to the ones present in the brain [Login *et al*, 1987].

1.44 Aetiology of LEMS

LEMS IgG also reduced K^+ stimulated $^{45}Ca^{2+}$ influx into a human SCLC line [MAR][Roberts *et al*, 1985], an effect could be blocked with DHPs and verapamil. A variety of neuronal cell lines including PC-12 cells and NG108-15 cells had $^{45}Ca^{2+}$ flux reduced by LEMS IgG [Lang *et al*, 1988]. The IgG precipitated membrane fractions of small cell cancer cell xenografts labelled with PN-200-110 though the antibodies bound to other bands as well as the VGCC on western blots [Chester *et al*, 1987]. This suggested that the autoantibody response in LEMS in its paraneoplastic form might be triggered by tumour VGCCs that shared crossreactive determinants with nerve terminal VGCCs. The degree of inhibition of $^{45}Ca^{2+}$ influx into the cell lines correlated well with clinical and electrophysiological measures of disease severity. Electron microscopy of freeze fractured membranes from mouse diaphragm end plates showed that presynaptic active zones were disordered, and the parallel rows of active zone particles [that are believed to represent VGCCS] were disrupted. The number of particles was reduced and those that remained were clustered. This suggested that LEMS IgG crosslink and then decrease the number of VGCCs present in the active zones by encouraging internalisation [Fukuoka *et al*, 1987a]. Immunolocalisation of LEMS antibodies showed that they bound to the active zones [Fukuoka *et al*, 1987b]. Crosslinking is essential in the pathogenesis of LEMS as monovalent Fab fragments of the antibodies did not deplete active zone particles in contrast to divalent $F(ab)_2$ and intact IgG [Nagel *et al*, 1988].

1.45 Clinical Assays for LEMS

A variety of LEMS assay methodologies have been developed based on precipitation of solubilised VGCCs labelled with ^{125}I-ω-GVIA conotoxin by LEMS sera. Three assays have been reported by Sher *et al*, [1989,1990], Leys *et al,* [1989,1990,1991] and Lennon & Lambert, [1989]. They vary in the source of the antigen in that different cell lines are used - human SCLC line cells grown in nude mice [Lennon & Lambert, 1989], human neuroblastoma cells IMR-32 [Sher *et al*, 1989] and SKN-SH cells [Leys *et al*, 1989] respectively and in the method of immunoprecipitation used. Titres in the different assays range from .44 - 91% positive for EMG proven LEMS. Unlike the calcium flux studies the anti-VGCC antibody titres detected by the ^{125}I-ωconotoxin assays do not correlate with disease severity across individuals [Leys, 1990]. High titres are found in non-SCLC LEMS rather than tumour associated LEMS [Sher *et al*, 1989,1990; Leys *et al*, 1989,1990,1991] and the pathological role of the autoantibodies detected by these asssays is presently unclear.

1.46 The search for the LEMS antigen(s)

The LEMS antigen[s] has been identified electrophysiologically and morphologically as the VGCC or a structure closely associated with it, but it has not been defined in immunologically or molecularly. LEMS IgG is not monospecific in its determinants [Johnstone & Lang, unpublished] and this makes identification of the pathogenic antigen difficult. Therefore, most studies have used monoclonal antibodies to synaptosomes to isolate possible antigens which are then screened for binding of LEMS IgG. A monoclonal antibody raised to chick brain synaptosomes was used to immunoscreen a human brain expression library and identified a 58 kDa antigen which

on western blots binds to 10% of LEMS antisera and which may be the synaptic vesicle protein p65 [B. Marqueze, personal communication]. There is evidence to suggest that p65 is the synaptic protein - synaptotagmin. Synaptotagmin has been cloned [Perin *et al*, 1990] and exists in multiple isoforms especially at the N-terminal [Wendland *et al*, 1991]. The presence of antibodies reacting to this antigen could explain the discrepancies between ω-conotoxin binding assays and calcium flux LEMS assays as differences in synaptotagmin isoforms between cells could have significant effects on antibody binding to VGCC complexes.

1.47 Aims of this project

The previous sections have described the evidence that the LEMS antigen is the VGCC and that autoantibodies present in LEMS IgG can bind to VGCCs from different species and cell lines. The molecular structure of the skeletal muscle VGCC indicates that the extracellular portions of the VGCC are probably present only in the α1, α2, γ and δ subunits. These 4 subunits are encoded by 3 genes as the α2-δ gene encodes two subunits. The tertiary structure of the LEMS antibody binding site had to be conserved across species and some neuronal VGCCs for the the effects on calcium flux to occur. Therefore, the candidate antigen had to show a large degree of interspecies and inter-tissue homology. The two genes that best fitted these criteria were the α1 and α2-δ genes of which the latter seemed to show less tissue variability. The nucleotide sequences of the α1 and α2 subunits of the rabbit skeletal muscle VGCC were known [Tanabe *et al*, 1989; Ellis *et al*, 1989]. However LEMS IgG does not affect all VGCCs but only a small subpopulation and therefore the candidate gene had to show limited tissue variability and be present in both lung and neuromuscular

junction. It was hoped that the brain would contain similar VGCCs to the neuromuscular junction and could be used as a source of mRNA for a candidate gene to be cloned. Human brain tissue and explants were not available but the rat glioma x mouse neuroblastoma cell line NG108-15 was known to have VGCCs that were downregulated by LEMS sera [Peers, 1989] so it was chosen as the source of mRNA for finding the homologue of the human LEMS antigen. The F2.1D1 human dorsal root ganglion x mouse neuroblastoma line was derived from the N18TG2 mouse neuroblastoma line like the NG108-15 line and its $^{45}Ca^{2+}$ flux was also inhibited by LEMS IgG suggesting that a homologous antigen was present in both cell lines. It was hoped that similar genes could be cloned from both cell lines and that homologues would be present in human lung and neuromuscular junctions.

LEMS IgG was known to be multispecific and therefore it was thought that there was a low probability of identifying the target antigen by immunological screening so an approach based on molecular homology of candidate genes [crosshybridisation] would be more practical.

The isolation of long probes for the rabbit skeletal muscle $\alpha 1$ and $\alpha 2$-δ genes by polymerase chain reaction [PCR] and their use to try to identify homologues of the $\alpha 1$ and $\alpha 2$-δ genes in rodent and human DNA and RNA is described in chapter 3. The same PCR primers were used to assess the degree of cross species homology and tissue expression of these 2 genes to try to find which candidate gene had the degree of heterogeneity required in the LEMS antigen [chapter 4]. The probes were then used to try to isolate a candidate LEMS antigen gene from cDNA libraries made from the two cell lines and chapter 5 describes the cloning of an $\alpha 2$-δ gene from the NG108-15 cell line and the assessment of its tissue distribution.

Chapter 2

Materials and Methods

2.1 Cell lines used in neurobiology

Neuronal explants are difficult to culture and vary between batches. It is easier to use cell lines. Inhibition of K^+ induced Ca^{2+} flux by LEMS IgG and a variety of pharmacological agents have been assessed in a panel of 25 neuronal and neuroectodermal lines by Lang *et al*, [1990] and Leys [1991]. This project involved the use of two cell lines: F2.1D1 and NG108-15 cells. Both are neuronally derived tumour cell lines, capable of forming synapses and secreting acetylcholine and express LEMS sensitive VGCCs which could be highly homologous to presynaptic VGCCs.

2.2 The NG108-15 mouse neuroblastoma/rat glioma cell line

The NG108-15 cell line is derived from a fusion of the rat CU-B6-1 glioma line resistant to 6-thioguanine and the mouse neuroblastoma line N18TG2 resistant to 5-bromodeoxy uridine [Gleason *et al*, 1990]. The NG108-15 cell line is well characterised electrophysiologically and biochemically and shows a variety of properties including contact inhibition and limited dendritic differentiation in culture in the presence of 10% carbon dioxide, 50 μM prostaglandin E_1 and 10 μM isobutryl-methyl-xanthine [IBMX]. A fully differentiated phenotype is seen after 2 - 8 days growth under these conditions. It expresses acetylcholine, noradrenaline, opioid, adenosine, bradykinin and neurotensin receptors whose gating is affected by cyclic AMP, cyclic GMP, and inositol-1,3,5,-triphosphate [Brown *et al*, 1989]. The G-proteins G_o and G_i have also been found in NG108-15 cells [Brown *et al*, 1989]. It maintains electrical excitability and can generate calcium dependent action potentials [Reiser *et al*, 1977]. The cell line can release acetylcholine and can release it afterlong term exposure to $N^6O^{2'}$ di-butyryl-cAMP [McGee *et al*, 1978]. NG108-15 cells will form synapses with cultured

striated muscle cells. The synapses formed resemble those found in non-artificial systems in their behaviour and appearance [Nelson *et al*, 1976]. Work on synaptic development in NG108-15 cells transfected with synapsin IIb showed that transfection altered the processes that these cells put out. The transfectants had increased amounts of synapsin I and synapsin IIa, more dense core [transmitter containing] synaptic vesicles as opposed to clear vesicles and spontaneously formed synapses with other NG108 cells [Han H-Q *et al*, 1991]. The structures produced had the structural and electrophysiological characteristics of synapses as opposed to varicosities [Kelly, 1991].

2.3 Calcium channels in NG108-15 cells

NG108-15 cells contain 2 types of VGCC as determined by the effects of antagonists on flux e.g. nitrendipine, ω-conotoxin [ω-GVIA][Leys, 1991] or electrophysiological recording in the presence of gadolinium ions [Docherty, 1988]. LEMS IgG reduce the potassium stimulated calcium flux in this line [Roberts *et al*, 1985; Peers *et al*, 1989]. However it has to be noted that the maximal inhibition of calcium flux produced by blockade of putative L, N and T channels is 50% so there are other components to calcium channels in this cell line [Tsien *et al*, 1991]. NG108-15 cells have 45 fmoles/mg protein of ω-contoxin binding and <10 fmoles/mg protein of nitrendipine/ PN-200 binding which corresponds to approximately 10^4 receptors per cell [M.J. Seagar, personal communication]. Muscle T-tubules contain about 1000 fmoles/mg protein of dihydropyridine binding sites [Tanabe *et al*, 1987]. The origin and type of the VGCCs in the cell line is unknown: at the simplest it could consist of 6 channel types [one each of the L,N and T from each parent cell line] or at the other extreme

it could contain hybrid channels formed by the association of rat and mouse subunits in differing combinations.

2.4 The F2.1D1 human dorsal root ganglion/mouse neuroblastoma line

Less is known about the F2.1D1 line which was made by fusing human foetal dorsal root ganglion cells with the mouse neuroblastoma line N18TG2 [Dickson *et al*, 1983]. The line contained human chromosomes and was shown to differentiate in response to $N^6,O^{2'}$-dibutyryl-cAMP. In serum free medium extensive neurite out growth occurred. It contained neurofilaments and cell surface tetraganglioside on antibody staining. The cell line has been shown to contain 2 types of VGCC and have a calcium flux that can be significantly reduced by LEMs sera [Lang *et al*, 1989].

Both cell types were cultured in the presence of 10% carbon dioxide as recommended by the American tissue culture collection [ATCC], but the addition of PGE_1 and isobutyrlmethylxanthine [IBMX] or dibutyryl-cAMP were found to have little effect on calcium flux [Johnston & Lang, unpublished] so these 'differentiation inducers' were omitted from the cell culture protocol.

2.5 Cell Culture

Basic cell culture techniques used in this study are described in Freshney [1987].

An aliquot of each cell line [gift of Dr. B.Lang] was thawed from liquid nitrogen and thawed at 37^{0}C. It was then added to 1 ml of culture medium:

500 mls DMEM containing L-glutamine and

4500mg/l D-glucose [Gibco BRL]

50 mls foetal calf serum [Gibco BRL]

50 mls 50 x HAT medium [Gibco BRL]

1 ml 100mg/ml penicillin V, 100mg/ml streptomycin [Sigma]

and grown overnight at 37^{0}C under 10% CO_2 in a volume of 2 mls in a 25 cm^2 flask. The cells were then expanded into flasks with an area of 175 cm^2 containing 50mls medium and grown until 70% confluent [approximately 3 days].

Cells were suspended in the medium by agitation, pelleted at 3000g for 5 minutes and the pellet either resuspended in 100mls of medium for further growth or resuspended in 2 mls 4M guanidinium isothiocyanate, 50mM sodium acetate, 100mM EDTA and frozen in liquid nitrogen.

2.6 Preparation of Master Cell Stocks

One flask of cells at confluence was taken and the cells washed fresh in tissue culture medium and resuspended in 1ml 1 x culture medium containing 10% foetal calf serum at 0^{0}C and 1ml of ice cold medium containing 20% foetal calf serum and 50% dimethyl sulphoxide was added. The suspension was placed in a cryotube [Nunc Ltd] and frozen in a freezing box at the rate of 1^{0}C/minute to -70^{0}C and then transferred to liquid nitrogen.

2.7 Basic Molecular Biology Techniques

Molecular biological techniques are described in many standard texts and a variety were consulted including the following:

[i] Sambrook *et al*, [1990]

Molecular cloning: a laboratory manual

[ii] Perbal [1990]

A practical guide to molecular cloning

[iii] Berger and Kimmel [1990]

Molecular cloning techniques

[iv] Glover D.M., Hames B.D., [1989]

DNA cloning techniques; vols I - III

Many of the techniques used are described in great detail in these texts. Other techniques are described as they occur in the text.

A large variety of suppliers exist for molecular biology grade materials. Chemicals and solvents were obtained from BDH Ltd, Fluka Ltd and Sigma Ltd. Restriction enzymes and other molecular cloning enzymes, reagents and kits were obtained from Amersham Ltd, BCL Ltd, BRL Ltd, Pharmacia Ltd, Promega Corp, and Stratagene Ltd. Hybridisation membranes were obtained from Amersham Ltd and Schleicher & Schuell Ltd. Sequencing kits and reagents were obtained from U.S. Biochemical and Promega Corporations. Radioactive isotopes and radioactive molecular weight markers were obtained from Amersham Ltd.

2.8 Preparation of DNA

2 ml of cell suspension was defrozen and diluted in 48 mls of lysis solution:

50 mM sucrose

10 mM Tris-Cl pH 8.0

1 mM EDTA pH 8.0

The suspension was spun at 2000g for 5 minutes and the nuclear pellet resuspended in

4.5 mls 0.7M sodium chloride, 0.025 M EDTA pH 8.0

0.5 mls 5% sodium dodecyl sulphate

100 µl 10mg/ml proteinase K

The viscous suspension was incubated for 3 - 16 hours at 56^{o}C until the viscosity was visibly reduced.

The DNA solution was extracted with one volume of a 1:1 mix of equilibriated phenol and chloroform. The phenol had been melted, 8-hydroxy-quinoline added to 0.1%, and equilibriated with 1M Tris-Cl pH 7.5 as described in Sambrook *et al*, [1989]. The DNA solution was then extracted with an equal volume of chloroform and the aqueous [upper] layer containing the DNA was removed and 1/10 volume of 3M sodium acetate pH 5 added followed by 2.2 volumes of ethanol.

Strands of DNA were spooled out using a bent glass pipette and the DNA pellet washed in 70% ethanol, air dried and resuspended in 1.5 mls of 10mM Tris-Cl pH 7.5, 1mM EDTA [TE]. The DNA was allowed to dissolve overnight on a rotating wheel at 4^{o}C and the purity was assayed by measuring the absorbance at 260nm and 280nm. Yield was calculated on the basis that 1 $O.D._{260}$ unit contains 50 µg/ml of DNA.

Samples with an O.D. 260/280 ratio of >1.8 were used in futher experiments. Samples that did not meet this criterion were redigested with proteinase K and re-extracted.

2.9 Preparation of RNA

RNA is highly susceptible to degration by ribonucleases present on the skin and on glassware which are highly resistant to degration. All RNA work was performed using disposable sterilised plastic where possible but where glassware was used it was treated to reduce RNAse activity. Glassware was baked at 160^{o}C for 12 hours and then washed with a solution of 0.1% diethylpyrocarbonate [DEPC] for 30 minutes. The glassware was autoclaved on liquid cycle [40 minutes] to remove traces of the DEPC. All solutions for RNA work were similarly prepared by incubation with 0.1% DEPC. However it was not necessary to treat solutions of 10% SDS [which is itself a weak RNAse inhibitor], Tris [which reacts with DEPC] or sucrose. Solutions containing these chemicals were made up in RNAse free distilled water and autoclaved [except for sucrose solutions].

2.10 Caesium Chloride/guanidinium isothiocyanate RNA preparation

The caesium chloride [CsCl] method described is essentially that described by Chirgwin *et al*, [1987].

One tube of cells was defrozen and resuspended in 10 mls of chaotropic solution [solution D]:

4 M guanidinium isothiocyanate

5mM sodium citrate pH 7

0.1M ß-mercaptoethanol

The cells were vortexed into the solution and DNA was sheared by repeated passages through a 23 G needle. The solution was made upto 0.5% with L-sodium sarcosine [sarkosyl] and incubated at 65^{o}C for 2 minutes.

The solution was layered on top of a 3 ml cushion of 5.7M caesium chloride/ 100mM EDTA in a sterile polyallomer ultracentrifuge tube [Beckman]. The balanced tubes were spun for 18 hours at 35000rpm in a SW41 rotor in a Beckman LS392 series ultracentrifuge.

The layer of solution D was removed to within 1 cm of the cushion and the rest of the fluid poured out and the tubes allowed to drain. The bottom 1 cm of the tube was cut off with a scalpel blade and the RNA pellet dissolved in 1 ml of 10mM Tris-Cl pH 7.4/ 5mM EDTA / 1% SDS. The RNA was extracted with phenol-chloroform and chloroform and precipitated with 1/10 volume of 0.3M sodium acetate.

2.11 Preparation of RNA by the AGPC technique

The acid-guanidinium-phenol-chloroform [AGPC] technique was first described in 1987 as a fast single step preparative method for RNA by Chomczynski & Sacchi.

Cells were lysed and treated with sarkosyl as for the CsCl technique to give 10 mls of lysate. 1 ml of 0.2M sodium acetate pH 4 was added to the lysate and the mixture vortexed. Then 10 mls of phenol and 2 mls of chloroform were added and the suspension well shaken and incubated on ice for 30 minutes. The layers wre separated by centrifugation in Corex tubes [Dupont Ltd] in a Beckmann LS centrifuge [10000g

10 minutes at 4^{0}C]. The RNA containing upper layer was precipitated with an equal volume of isopropanol at -20^{0}C for 6-24 hours. The RNA was pelleted by centrifugation at 15000g for 15 minutes at 4^{0}C and resuspended in 0.75ml of solution D/ 0.5% sarkosyl and reprecipitated with isopropanol for 1 hour at -20^{0}C. The RNA was pelleted and the pellet washed with 70% ethanol [twice]. The pellet was resuspended in 400 µl of 10mM HEPES pH 7.

2.12 Oligo-dT cellulose chromatography

Messenger RNA was purified from the total RNA by oligo-dT chromatography. This was performed as described in Sambrook *et al* [1989] except that different salt solutions were used. All tubing and the column were pretreated by autoclaving, washing with 0.1 M sodium hydroxide and washed with 0.1% DEPC and then 0.5M KCl/ HEPES pH 7.0. The oligo-dT cellulose [Pharmacia Ltd] was hydrated in DEPC treated water and equilibriated with 10mM HEPES pH 7.0 / 0.5M potassium chloride. The RNA was dissolved in 400 µl of 10mM HEPES pH 7 and denatured at 65^{0}C for 2 minutes and cooled on ice. 4 volumes of 10mM HEPES pH 7.0/ 0.5 M KCl were added and the solution was passed down a 10 ml column containing a 1 ml bed of equilibriated oligo-dT cellulose. The eluate was monitored with a Uvicord B [Pharmacia/LKB] flow monitor set to 260 nm and pumped by a Pharmacia/LKB peristaltic pump. The column was washed with 20 volumes of 0.5M KCl / 10mM HEPES pH 7.0 and then 10 volumes of 0.1M KCl/ 10mM HEPES pH 7.0. The mRNA was eluted by adding 2 mls of warm [50^{0}C] 10mM HEPES pH 7.0. The column was then washed with 10 mls of 0.5M KCl/ 10mM HEPES pH 7.0 and the

RNA reapplied for a second purification. After 2 passages the mRNA was precipated by the addition of 1/20 volume of 4 M sodium chloride and 2.2 volumes of ethanol.

2.13 Preparation of plasmid probes

Probes were prepared by alkaline lysis plasmid preparation and caesium chloride centrifugation as described in Sambrook *et al*, [1989].

E. Coli RB791 cells tranfected with plasmids containing portions of the rabbit α1 and α2 subunit genes were supplied by Dr. B.Lang as 16% glycerol/ Luria broth stocks. The pGEM plasmids contained a 420bp PCR insert encoding part of the α1 subunit or a 618bp section of the α2 gene cloned into the polylinker at the *Sma* I site [D. Beeson & B. Lang].

A 50 µl aliquot of cells was added to 500 mls of Luria broth [LB] containing 50 µg/ml ampicillin and grown overnight in a shaking incubator [180 rpm] at 37^{o}c. The final cultures had an $O.D._{600}$ of >1. The cells were pelleted in 250 ml Beckmann bottles at 3000 g for 5 minutes and the plasmids prepared by the alkaline lysis method using an initial volume of 10 mls 50mM glucose/ 10mM Tris-Cl pH 7.5/ 1mM EDTA for resuspension. The supernatant after centrifugation to remove the protein was extracted with phenol-chloroform and chloroform and precipitated with 2.2 volumes of ethanol.

The plasmid pellet was dissolved in 10mls of TE pH 7.5 and 10g of caesium chloride were added. Ethidium bromide solution was added to a final concentration of 1mg/ml and the solution centrifuged briefly at 3000 g to remove the protein deposit. The supernatant was poured into a sealable ultraclear centrifuge tube [Beckmann] and the tubes heat sealed [Beckmann Heatsealer]. The tubes were spun to equilibrium in a

Beckmann 7-65 ultracentrifuge [24 hours at 60000 rpm in a TI70 rotor at 20^{o}C]. The tubes were removed from the centrifuge and the plasmid bands visualised in the tubes by daylight [or if faint by ultraviolet transillumination at 340 nm] and the lower band of supercoiled plasmid removed by puncturing the tube with a 19G syringe. The ethidium bromide was removed by 3 equivolume extractions with water saturated butan-1-ol and the cleared DNA solution dialysed against 5 dm^3 of TE pH 7.5 with 3 changes. The DNA was precipitated eventually with sodium acetate/ethanol and redissolved at 100 ng/µl in TE pH 7.5.

2.14 Purification of probe inserts

100 µg of plasmid was taken and digested to completion with 200 U of *EcoR1/HindIII* [Promega] in a volume of 1000 µl at 37^{o}C. 55 µl of sample buffer [Sambrook *et al*, 1989] was added to the digest solution and the digest separated on a 1% low melting point agarose gel. The appropriate band was cut out of the gel, melted at 65^{o}C and purified by the hot phenol technique [Sambrook *et al*, 1989]. The concentration of insert after extraction and precipitation was found by comparing an aliquot with PM2 markers of known concentration on an agarose gel. The probe was resuspended at a concentration of 50 ng/µl in TE.

2.15 Preparation of PM2 size markers

50 µg of PM2 phage DNA [Boehringer-Mannheim Ltd] were digested in a volume of 450 µl of TE with 100 U of *Hind* III until completion at 37^{o}C [approximately 4-6 hours]. Digestion was terminated with 5 ul of 0.5M EDTA. The digest was made upto 500 µl with sample buffer.

The sizes of bands produced by this digestion are:

5400

2350

1050

475

450

275

110

base pairs respectively.

2.16 Labelling of Probes

Probes were labelled by random priming or by end kinasing.

[a] Random primer labelling

50 ng of probe insert were taken denatured by boiling for 2 minutes and cooled on ice. Sequential additions were made of:

5 µl 10 x Klenow buffer [10mM Tris-Cl pH 7.6, 50mM $MgCl_2$, 1mM EDTA]

5 µl 100 mM dithiothreitol

10 µl 100 ng/µl random hexamers

4 µl 2.5 mM dNTPs

3 µl ^{32}P-α-dCTP [Amersham]

11 µl H_2O

2 µl Klenow polymerase [4 U/µl; Amersham]

The probe was incubated for 6 - 24 hours at 20^{0}C and then for 15 minutes at37^{0}C. When the Amersham 'Multiprime' kit became available it superseded this method of labelling.

[b] Kinase labelling

50 ng of unphosphorylated oligonucleotide were dissolved in 22 µl of TE. To this were added:

3 µl 10 x kinase buffer [10 mM Tris-Cl pH 7.6, 25mM $MgCl_2$,
10mM DTT, 2mM spermidine, 1mM EDTA]

3 µl ^{32}P-γ-ATP [Amersham]

2 µl T4 polynucleotide kinase [Pharmacia]

The labelling was incubated for 15 - 30 minutes at 37^{0}C.

2.17 Preparation of Labelled PM2 Markers

100ng of markers were prepared as described in section 2.15. The solution was extracted with phenol-chloroform, chloroform and precipitated with NaOAc/ ethanol. The precipitate was washed with 70% ethanol, air dried, and resuspended in 40ul of TE. 5 µl of 10 x Klenow buffer and 2.5 µl of a mixture of 100mM dGTP, dATP and dTTP [Pharmacia Ltd], 0.5 µl of ^{32}P-α-dCTP and 4 U of Klenow polymerase [Amersham Ltd] were added and the solution incubated for 15 minutes at 37^{0}C. The endfilled labelled markers were then separated from unincorporated nucleotides on a G-75 column.

2.18 Separation of unincorporated nucleotides

Labelled probes were passed down a 20cm column of 5 ml bed volume of G-75 sephadex equilibriated in 10mM Tris-Cl pH 7.5, 100mM NaCl, 1mM EDTA, 1% SDS. The first peak of radioactivity was collected and counted. This method was superseded by use of pre-packed 'NIP' columns [Pharmacia].

2.19 Preparation of competent cells

A glycerol stock of *E. coli* RB791 cells was purified by streaking out on a LB agar plate and a single colony grown up overnight at 37^{0}C. This was used to inoculate a 100ml culture and grown until the O.D.$_{600}$ was 0.5. The culture was cooled in ice, spun down at 3000 g for 5 minutes and resuspended in 15 mls of ice cold solution RF1:

10mM Morpholino-ethane sulfonic acid pH 6.2

100mM Rubidium chloride

50mM Manganese chloride

10mM Calcium chloride

and incubated for 15 minutes at 0^{0}C.

The cells were spun down again and gently resuspended in 8 mls of ice cold RF2:

1M 3-(N-Morpholino) propane sulphonic acid pH 6.5

75mM Calcium chloride

10mM Rubidium chloride

15% glycerol

The cells were snap frozen in 200 µl aliquots in liquid nitrogen and stored at -70^{0}c until used.

2.20 Transformation of *E.Coli* RB791s

The ligation reaction [typically 10 µl] was diluted in sterile H_2O to a volume of 50 µl. 10 - 15 µl were used per transformation. Competent cells were defrozen on ice and an aliquot of the ligation reaction was added. The cells were incubated at 0^oC or 4^oc for 1 hour and were then heat shocked at 42^oC for 1.5 minutes before being returned to ice for another 2 minutes. The cells were diluted in 4 volumes of L-broth and incubated for 1 hour at 37^oC.

1.5% agar plates containing 200 µg/ml ampicillin were prepared and just before use were spread with 2 mls of L-broth containing 10 µl of 1M isopropylthiogalactoside [IPTG] and 20 µl 2% X-galactoside in N'N' dimethylformamide [X-Gal/DMF]. The plates were dried at 65^oC for 2 minutes and kept at 37^oC until use [< 1 hour].

The cell suspension was spread over the indicator plates and incubated overnight at 37^oC.

2.21 Preparation of competent JM101 Cells

E. coli JM 101 cells were streaked on a minimal media plate containing 30 µM vitamin B_1. Individual colonies were used to grow up initial culture stocks in L-broth. 100 µl of the initial culture was taken and added to 10mls of fresh L-broth and incubated until the $O.D._{600}$ was about 0.5. The cells were then cooled on ice, pelleted and resuspended in 5mls of 50mM calcium chloride. They were incubated on ice for 15 minutes, repelleted and resuspended in 2mls of 50mM calcium chloride. The cells were then kept on ice for a further one hour before use.

2.22 Transformation of *E.Coli* JM 101s

5 µl of a diluted M13 ligation reation was added to a 200 µl aliquot of cells and incubated for 15 minutes on ice. Cells were heat shocked for 2 minutes at 42^{0}C for 2 minutes, replaced on ice for 2 minutes and added to 4 mls of warm 0.8% top agar containing 10 ul of 1M IPTG and 20ul of 2% X-Gal/DMF. The plates were grown overnight at 37^{0}C.

2.23 Preparation of M13 single strands

Individual clear plaques were picked witha sterile toothpick and used to inoculate 2ml of L-broth containing 20 µl of competent JM101s. The cells were grown for 6 hours at 37^{0}C and pelleted. 0.75mls of supernatant was removed and added to 0.75mls of 2M NaCl/20% PEG and left to precipitate for 15 minutes. The precipitate was collected by centrifugation in a microfuge [5 minutes], all the PEG/NaCl solution was removed, and the precipitate was resuspended in 200 µl of TE. The protein was removed by the addition of 100 µl of phenol and incubation at room temperature for 15 minutes. The phases were separated by the addition of 100 µl of chloroform and centrifugation. The aqueous layer was re-extracted with phenol-chloroform, chloroform and precipitated with ethanol and sodium acetate. The single strands were spun down, washed with 70% ethanol and resuspended in 24 µl of 0.1 x TE and used for sequencing.

2.24 Sequencing of Single Strands

Single strand sequencing was performed by the Sanger dideoxy method [Sanger, 1975] as described in the Sequenase^{tm} kit [United States Biochemical Corporation]. All annealing, labelling, extension and termination reactions were usually performed as described by the manufacturer.

When low concentrations of single strands were obtained or when sequence close to the primer was required the following technique was employed in preference to the use of manganese ions.

The method of Tsang and Bentley [1988] uses 60 - 600 ng of template and this is annealed with 0.2 pM primer before performing a dCTP minus labelling at 37^{0}C for 5 minutes in the presence of 1 μCi of ^{35}S-α-dATP, dGTP and dTTP at at 0.2 μM, 6mM DTT and 2U of Sequenase. 2 μl of this reaction were added to 2 μl of extension mixes [80 μM dNTPs, 8 μM ddNTP] and the reaction incubated for 5 minutes at 37^{0}C. Termination was carried out as usual.

2.25 Sequencing Gels

Sequencing gels containing 8M urea and 6% acrylamide were poured using preprepared solutions supplied by Sequagel [Flowgen]. For 100mls of gel solution the following volumes were required:

10 mls buffer

24 mls concentrate

66 mls diluent

Amberlite beads were added [1g/20mls] and the solution deionised on a stirring plate for 15 minutes. The gel solution was filtered just before use.

The gel solution was degassed and cooled on ice for 10 minutes. 150 µl of 10% ammonium persulphate [AMPS] and 50 µl of TEMED were added to the gel solution and the gel was cast between prepared plates.

40 x 30 cm sequencing plates [BRL Ltd] were cleaned in 10% hydrochloric acid and washed with detergent [Lipsol] and 70% ethanol. The plates were finally wiped over with chloroform. The top plate was siliconised by being wiped over with 4 mls dimethyldichlorosilane solution and the residual acid removed 10 minutes later with 70% ethanol. The plates were assembled with spacers and 75 mls of gel solution was poured in between the plates. Reversed combs were used to form gels slots. Once polymerised the combs were inverted, the gel slots washed out with 1 x TBE and the gel pre-run at 60W for 15 minutes.

A 0.5 µl volume of sample was added per well in the order ATGC. Gels were run at 60W until the bromophenol blue ran off the end or longer with a change of buffer. In short runs the lower reservoir was filled with TBE to which 1/20 volume of sodium phosphate pH 8.4 had been added. The gels were prerun as usual. This provided a countercurrent of ions and formed a continuous salt gradient in the gel enabling easier reading of short and medium length runs [].

Once fully run the gels were fixed in 2 dm^3 10% acetic acid/10% methanol which also eluted the urea and were dried under vacuum at 80^{o}C for 15-30 minutes.

2.26 Plasmid Sequencing

Plasmids were grown up as usual and alkaline lysis plasmid preparation performed. Previous experience had shown that RNAse A digestion alone or sodium hydroxide denaturation/degradation alone [Jones & Schofield, 1991] did not give sufficiently high

quality plasmid for sequencing. Therefore the method of Saunders and Burke [1990] was used to prepare purified plasmid.

The plasmid pellet was resuspended in 100 µl of TE and 0.1 g of caesium chloride was added. The solution was shaken until the salt dissolved and then 10 µl of 10% ethidium bromide were added. The protein was pelleted by centrifugation and the ethidium bromide extracted from the supernatant with 3 separate extractions with 50 µl of isopropanol. The aqueous layer was precipitated at room temperature by the addition of 400 µl of TE, 45 µl of sodium acetate and 720 µl of isopropanol. The precipitate was washed with 70% ethanol and resuspended in 40 µl of 0.1 x TE.

The plasmid was denatured by adding 3 µl of 2M NaOH to 7 µl of plasmid solution, incubating at room temperature for 10 minutes and then neutralised by adding 3 µl of 3M NaOAc, 7 µl of H_2O and precipitated with 60 µl of ethanol. The plasmid was spun down, washed with 70% ethanol and resuspended in 0.1 x TE. An annealing reaction carried out under standard conditions.

Chapter 3

Initial experiments

3.1 Introduction : choice of probe

Voltage gated channels for potassium, sodium and calcium are members of a gene superfamily. It was not known whether this family included all the members of the voltage gated channels or whether other families existed. Peers, [1988] had shown that in NG108-15 cells the target of LEMS antibodies was a calcium channel. A representative of a class of L-channel α1-subunits had been characterised at the molecular level by Tanabe *et al*, [1987] from rabbit skeletal muscle. It ought to be posssible to screen libraries with a region of this gene likely to have been conserved in evolution. The membrane spanning domains of voltage gated channels show a minimum 30% homology at amino acid level across the superfamily and 60% in individual ion channels but the S4 membrane spanning region shows a higher degree of homology across the family [50%] and in individual ion channels [80%] due to its structure of $[Arg\text{-}X\text{-}Y]_{4\text{-}7}$ and its possible role as a voltage sensor. A possible problem was that probes to the S4 region would pick up the other members of the superfamily by cross-hybridisation but it was hoped the stringency of screening would eliminate this.

The α2 gene cloned by Ellis *et al*, [1989] had no known homologues, so choice of a probe region could be arbitrary. However as membrane spanning regions are usually conserved across members of gene superfamilies and in individual families of proteins [Betz, 1991], it was decided to use a membrane spanning region probe for the α2 gene as well as for the α1.

3.2 Strategy

The initial idea was to screen cDNA libraries with oligomer probes derived from the α1 and α2 sequences. The second S4 region of the α1 subunit was chosen for a candidate probe as this area encodes the highly conserved region of the voltage sensor [S4] in voltage gated channel superfamily. This particular region seemed to be slightly different from the consensus motif found in sodium channels and potassium channels as opposed to the other S4 regions which were highly similar [table 3.1]. However the genetic code is degenerate and so there are still multiple possibilites for the coding sequence.

A variety of approaches were therefore available :

[1] a multiple set of degenerate oligonucleotides derived from the amino acid sequence of the region,

[2] a single oligomer containing many inosine residues,

[3] a single long oligomer to the known sequence.

A single long probe of 100bp was chosen to ensure reasonable specificity, avoid technical difficulties in screening with mixtures of oligonucleotides, lower expense and allow crosshybridisation if total amino acid homology was not present. This would have a reasonable probability of detecting a 65% homologous gene [Kajimura *et al*, 1991].

Less data was available on α2 sequences so the 75bp probe was chosen from an area that on theoretical grounds was likely to show a degree of conservation. In the paper describing the α2 sequence Ellis *et al*, [1989] used cloned sequences derived from the α1 and α2 sequences to probe human and rabbit genomic DNA and to assess the expression of mRNA in a variety of human and rabbit tissues. These blots showed that

crosshybridising sequences were present in human tissues when probed with rabbit sequences and that different tissues gave positive signals that may have been due to either crosshybridisation to a closely related gene or a gene with the same product. Therefore oligomer probes to these sequences ought to be able to hybridise to related sequences in the F2.1D1 and NG108-15 cell lines. Therefore the probe sequence is derived from the second membrane spanning region [ms2] of the $\alpha 2$ subunit [fig 3.2]. Oligonucleotide probes were obtained from British Biotechnology Ltd.

3.3 Parallel Work

Dr. D. Beeson and Dr. B.Lang used the same oligonucleotide probes to screen oligo-dT primed cDNA libraries from rabbit and human skeletal muscle with no result. Therefore they made cDNA from rabbit skeletal muscle using specific primers [$\alpha 1$: 1656 - 1677; 2055 - 2076][$\alpha 2$: 2532 -2554; 3132 - 3155] and used PCR to amplify segments of the $\alpha 1$ and $\alpha 2$-δ genes. These were subcloned into pUC8 or pGEM-5Z and the probes were made available to me.

IIS2 region:

Rabbit	SkM	ANRVLLSLFTIEMLLKMYSL
Carp	SkM	--VI--A------VM---A-
Eel	VGSC	G-L-FTGI--A--F--IIAM
Rabbit	Lung	A-KA--A---A---------
Rabbit	aorta	--KA--A---A---------
Rabbit	cardiac	--KA--A----------G-
Rat	brain	--KA--A---A---------
Rat	brain	--K---A---C---V-----
Rat	brain	-EFIF-G--MS--FI---G-
Rabbit	brain	-EFIF-G--MS--FI---G-

IIS5 region:

Rabbit	SkM	LLLLLFIFIIIFALLGMQLF
Carp	SkM	----------V-
Eel	VGSC	-T-V-A-IVF---VV-----
Rabbit	lung	------L-----S-------
Rabbit	aorta	------L-----S-------
Rabbit	cardiac	----------S-------
Rat	brain	------L-----S-------
Rat	brain	------L-----S-------
Rat	brain	--F---L--VV---------
Rabbit	brain	------L--VV---------

IIS4 region:

Rabbit	SkM	ISVLRCIRLLRRLFKITKYW
Carp	SkM	---M----------
Eel	VGSC	L----SF-----V--LA-S-
Rabbit	lung	------V-----I----R--
Rabbit	aorta	------V-----I----R--
Rabbit	cardiac	------V-----I----R--
Rat	brain	------V-----I----R--
Rat	brain	V--F--V-----I--V-RH-
Rat	brain	-----AL-----I--V----
Rabbit	brain	-----AL-----I--V----

IVS4 region:

Rabbit	SkM	SAFFRLFRVMRRLIKLLSRA
Carp	SkM	IT-------L-------M-S
Eel	VGSC	-RLA-IG-IL-----GAKGI
Rabbit	lung	IT----------V-----G
Rabbit	aorta	IT----------V-----G
Rabbit	cardiac	IT----------V-----G
Rat	brain	----------
Rat	brain	----------
Rat	brain	LSIL----AA-------RQ-
Rabbit	brain	----------RQ-

Figure 3.1
Comparison of amino acid sequences of conserved membrane spanning regions of the VGCC and VGSC. PCR primers were chosen from the 5' end of the IIS2 and the 3' end of the IIS5 and an oligonucleotide probe was obtained overlapping the IIS4 region

Rabbit skeletal muscle α1 VGCC subunit:

```
1550        GCCATGACGCCGCTGGGCATCTCCGTGTTGCGCTGCATCCGCCTCCTGAGG
             A  M  T  P  L  G  I  S  V  L  R  C  I  R  L  L  R

Rat brain AT----T----C-----G---------C--A-------G--G--G--C---
[ Snutch ]

Rat brain CT----T-T--C------G----T-----C--G--TG-A--T---------
[ Hui ]



            CTCTTCAAGATCACCAAGTACTGGACGTCGCTCAGCAACCTGGTGGCC  1650
             L  F  K  I  T  K  Y  W  T  S  L  S  N  L  V  A

            A---------------G--------AC--C--G---------G----G

            A-------AG-G----G-C-------T--C--G------T-------G
```

Figure 3.2a
Comparison of two rabbit brain sequences overlapping the region of the IIS4 membrane spanning domain and adjacent areas from the rabbit skeletal muscle α1-VGCC subunit gene used as an oligonucleotide probe

Rabbit VGCC α2:

```
     2760 TCGGCTTATGTGCCATCAATAGCAGACATACTGCAGATT
           S  A  Y  V  P  S  I  A  D  I  L  Q  I

NG108-15  ------------------T-C-A-A-----C------

          GGATGGTGGGCCACTGCTGCTGCTGCCTGGTCTATTCTT 2835
           G  W  W  A  T  T  A  A  A  W  S  I  L

          --C----------------------------C-----C
```

Figure 3.2b
Comparison of the rabbit skeltal muscle α2 sequence for the second membrane spanning region and the homologous sequence in the α2 gene isolated from NG108-15 cells.

3.4 Manufacture of probes

Plasmid containing bacteria were taken and grown up in 500 ml cultures and plasmid was purified from the culture by the use of alkaline lysis and caesium chloride centrifugation. Yields for the 2 cultures are shown in table 3.1a.

50 μg of purified plasmid were digested with 50 U of *EcoR1* and *HindIII* in a volume of 500ul for 4 hours at 37^{0}C and the digest run out on a 1% LMP agarose gel [fig 3.3]. The relevant bands were cut out, purified by the hot phenol technique of extraction, precipitated and resuspended in a volume of 100 μl of TE. The amount of DNA present in each tube was assessed by the use of UV spectrophotometry and the probe diluted to 10 ng/μl [table 3.1b]. 50 ng of probe were used in random primed labelling reactions and the probes were Cherenkov counted in a Beckmann LS1701 series counter set for ^{32}P counting. The yields of probe are shown in table 3.1c.

[a]

Plasmid	O.D.$_{260}$	O.D.$_{280}$	Yield	Total	Purity
			µg/µl	mg	
pGEM-5Z	98	46	1.90	3800	2.9
pUC8-α1	80	30	1.60	3200	2.6
pUC8-α2	86	44	1.72	3400	2.0

[b]

Plasmid	Probe	Total
	µg/ml	µg
pUC8-α1	750	75
pUC8-α2	1130	113

[c]

Probe	Counts	Activity
	cpm/µl	cpm/ng
α1	1057294	4.2×10^7
α2	2662819	1.1×10^8

Table 3.1
Yields and purity of DNA from caesium chloride density preparations of plasmid cultures [a]. Yield of 418mer α1 and 620mer α2 probes from 500µg of plasmid [b]. Yield and activity of radioactive probe labelled by random priming [c]. Labelling used 25ng of probe and produced 400µl of column eluate/sample.

3.5 Initial experiments

The labelled probes were used to screen the southern blots of digests of F2.1.D1 and NG108-15 genomic DNA.

20 μg of genomic DNA derived from the NG108-15, F2.1D1 cell lines and human and rabbit peripheral blood lymphocytes were digested with *EcoR1* at 37^{0}C for 16 hours in the presence of 2mM spermidine. The restriction fragments were run on a 0.8% agarose-TBE gel [50V, 16 hours], blotted onto nylon membranes and baked. The filters were prehybridised for 30 minutes. Hybridisation was carried out in 6 x SSCE hybridisation solution containing 50% formamide, 10 x Denhardt's solution, 100 μg/ml of boiled sonicated herring sperm DNA and 1 x 10^{6} cpm/ml of rabbit α1 or α2 probes at 42^{0}C for 16 hours. The filters were washed initially at low stringency - 2 x SSC at 20^{0}C. Both probes bound to the filters in a nonspecific manner so the hybridisations were repeated at higher stringencies of washing [2 x SSC at 20, 40, 50, 60 ^{0}C]. 2 x SSC at 40^{0}C was chosen as the temperature and stringency for screening as it this the lowest stringency that blots with definable bands and reasonably low background [figs 3.4, 3.5].

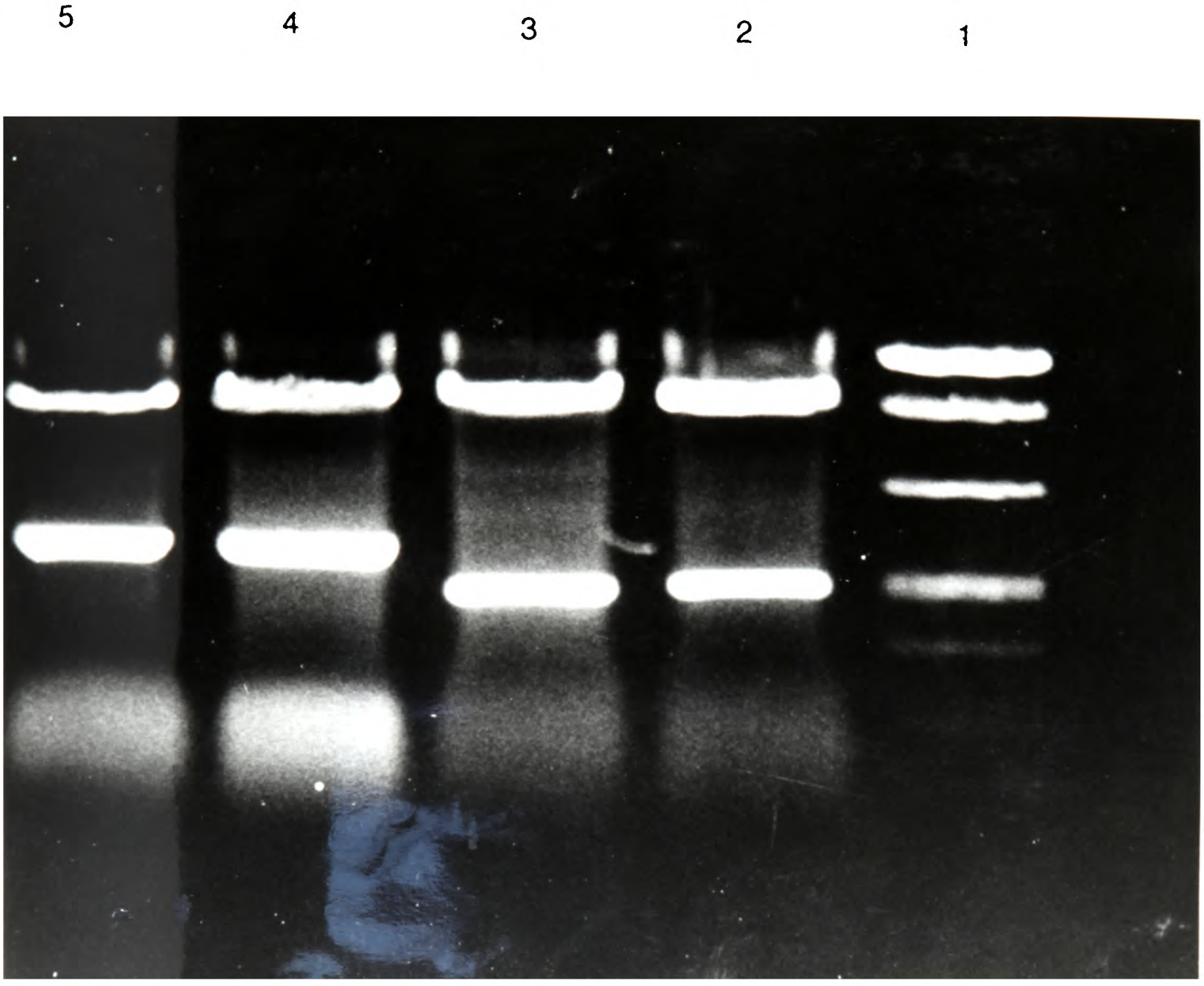


Figure 3.3

An *EcoR1* and *HindIII* digest of plasmids containing rabbit skeletal muscle α1 [lanes 2,3] and α2-δ [lanes 4,5] as compared with PM2 markers [lane 1]. The α1 insert has a size of 420bp and the α2-δ insert 618bp.

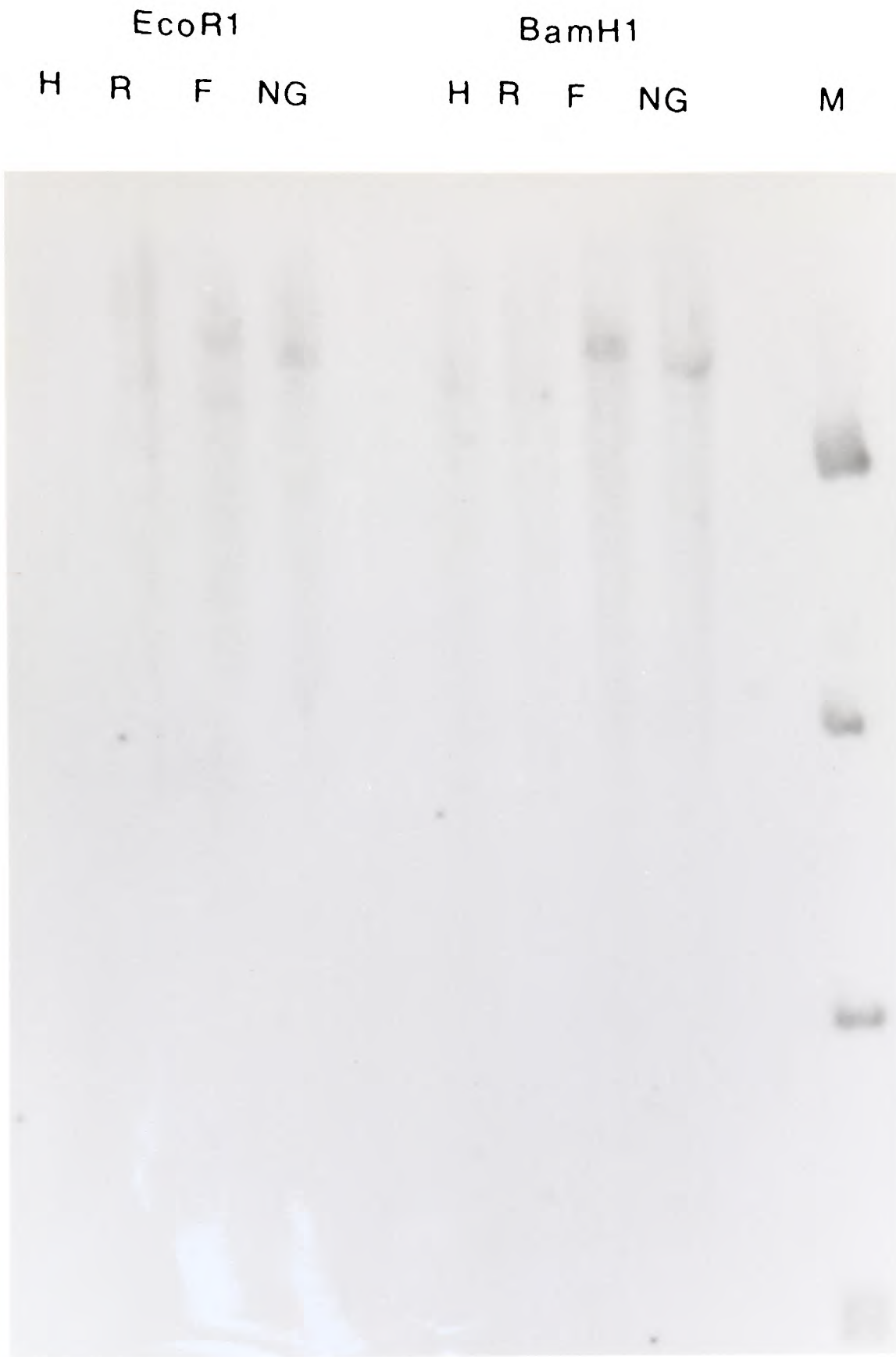


Figure 3.4

Southern blot of 20μg of human [H], rabbit [R], F2.1D1 [F], and NG108-15 [NG] genomic DNA digested with *EcoR1* or *BamH1* and hybridised with with $0.5x10^6$ cpm/ml of rabbit skeletal muscle α1 probe [418bp]. The filter was washed with 2xSSC at 40^0C. Autoradiography was for 5 days. Markers [M] are a labelled *HindIII* digest of PM2.

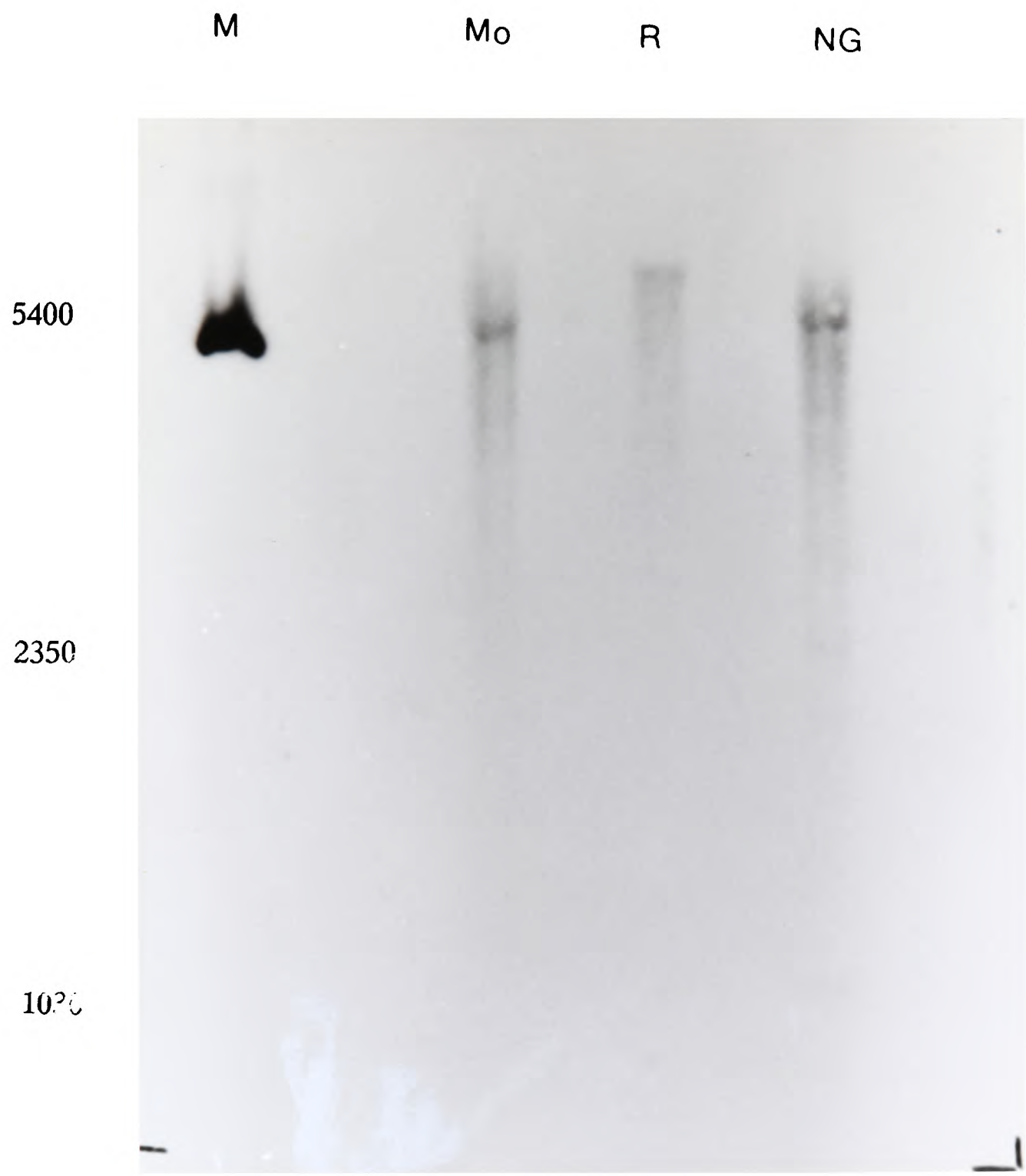


Figure 3.5

Southern blot of 20μg of *HindIII* digested genomic DNA from rat blood [R], mouse blood [M] and NG108-15 cells. Markers [M] are restriction fragments off *HindIII* digested PM2 DNA. The blot was hybridised with 0.5×10^6 cpm/ml of NG108-15 $\alpha 2$ probe [NG1.242]. The filters was washed with 0.2 x SSC at 40^{o}C. Autoradiography was for 5 days. The blot shows the similarity in size of the genomic restriction fragments of the mouse and NG108-15 cells as compared to rat genomic DNA.

3.6 Confirmation of α1 probe sequence

The opportunity was taken during the optimisation of screening conditions to check the sequence of the α1 probe. 10 ng of *EcoR1/HindIII* cut purified probe was ligated to *EcoR1 /HindIII* digested M13 vector. Two control ligations were also set up containing [a] no insert and [b] no vector. 5 µl of the diluted ligation mix [50 µl total] used to transform competent JM101 cells. A blank transformation containing no DNA was also performed. The results are shown in table 3.2. Twelve clear plaques were picked from the α1 containing plate and used to grow up single strands. These were then sequenced by the Sanger dideoxy technique [Sequenase].

The resulting sequence is shown in fig 3.7. Both strands were sequenced and the results were compared with those obtained by Dr B. Lang for the same insert and the original sequence as published by Tanabe *et al* [1989]. A similar confirmation was performed by Dr. B. Lang on the α2 sequence.

Ligation	Yield	
	White	Blue
JM101 cells	0	0
Insert + ligase	0	0
Vector[EcoR1/HindIII] no ligase	0	0
Vector[EcoR1/HindIII] + ligase	1	6
Vector [EcoR1] + ligase	2	>500
Vector + insert MB	24	10
Vector + insert MLu	29	13
Vector + insert RM	13	14

Table 3.2

Yields of transformants from ligations of *EcoR1/HindIII* digested M13 vector and purified *EcoR1/HindIII* digested $\alpha 1$ and $\alpha 2$ probes.

```
                          15                  30                  45
CTC AAC ACC CTG TCC ATC GCC TCG GAG CAC CAC AAC CAG CCG CTC TGG
L   N   T   L   S   I   A   S   E   H   H   N   Q   P   L   W
                  60                  75                  90
CTG CTG ACC CAC TTG CAA GAC ATC GCC AAT CGA GTG CTG CTG TCA CTC
L   L   T   H   L   Q   D   I   A   N   R   V   L   L   S   L
            105                 120                 135
TTC ACC ATC GAG ATG CTG CTG AAG ATG TAC GGG CTG GGC CTG CGC CAC
F   T   I   E   M   L   L   K   M   Y   G   L   G   L   R   H
        150                 165                 180
TAC TTC ATG TCC ATC TTC AAC CGG TTC GAC TGC TTC GTG GTG TGC AGC
Y   F   M   S   I   F   N   R   F   D   C   F   V   V   C   S
  195                 210                 225                 240
GGC ATC CTG GAG CTG CTG GTG GAG TCG GGC GCC ATG ACG CCG CTG GGG
G   I   L   E   L   L   V   E   S   G   A   M   T   P   L   G
                  255                 270                 285
ATC TCC GTG TTG CGC TGC ATC CGC CTC CTG AGG CTC TTC AAG ATC AGC
I   S   V   L   R   C   I   R   L   L   R   L   F   K   I   S
                  300                 315                 330
AAG TAC TGG ACG TCG CTC AGC AAC CTG GTG GCC TCC CTC CTC AAC TAC
K   Y   W   T   S   L   S   N   L   V   A   S   L   L   N   Y
            345                 360                 375
ATC GGC TCC ATC CGC TCC ATC GCC TCG CTG CTG CTG CTG CTG TTC CTC
 I   G   S   I   R   S   I   A   S   L   L   L   L   L   F   L
        390                 405                 415
TTC ATC ATC ATC TTC GCC CTG CTG GGC ATG CAG CTC TTC GGG GGG
F   I   I   I   F   A   L   L   G   M   Q   L   F   G   G
```

Figure 3.7

Sequence of α1 subunit PCR product in an M13mp18 sequencing vextor derived from 4 overlapping clones on both strands showing the insertion YIG at 335-343.

3.8 Discussion

The sequence of the $\alpha 2$ PCR product was identical with that described by Ellis *et al*, [1989]. The $\alpha 1$ sequence shows 4 changes relative to the consensus sequence of $\alpha 1$ as described by Tanabe *et al*, [1988] and confirmed by Ellis *et al*, [1989]. The major change in the $\alpha 1$ sequence comprises an 8 nucleotide or 3 amino acid insertion causing a change of threonine [T] to tyrosine-isoleucine-glycine [YIG] at amino acid position 113. It was possible that this was a spliced mini-exon [Perez-Reyes *et al*, 1990] but subsequent resequencing of the same clone after repeating the ligation showed the absence of this insertion. Therefore the insertion was assumed to be artefactual as a result of sequencing only one product molecule of a PCR reaction subcloned in pUC. Both the rabbit skeletal muscle $\alpha 1$ sequences published to date also agree at that site [Tanabe *et al*, 1988; Ellis *et al*, 1989]. The insertion could be due to errors in the amplification of the gene by Taq polymerase or reduplication in that area caused by secondary structure and multiple reading by the polymerases of Taq or *E. coli* while replicating the M13 single strand sequence. Such a large insertion error was thought to be due to the replication error of the M13 single strand rather than Taq polymerase as the latter usually substitutes nucleotides at any one point rather than causing insertions [Ehrlich, 1990]. It illustrated the difficulty of using just one plasmid clone derived from a PCR amplification of a gene sequence for sequence information. Thereafter all PCR amplified sequences were determined by the use of three separate clones to minimise this kind of incorporation or reduplication error. The alternative method to reduce incorporation errors is the use of direct sequencing of the PCR product. This uses stringent amplification at the lowest possible magnesium concentration and the sequencing of PCR products directly without subcloning to give

a population average sequence thereby minimising individual incorporation errors. The problems involved in this approach are the need for a single product to be produced by the PCR reaction and the availability of bead coupled purified sequencing PCR primers to remove extraneous complementary strand primer [Ehrlich 1990].

The use of ordinary unpurified primers was found to give unreliable results [data not shown] for sequencing of PCR products. The greatest problem was the difficulty of obtaining sequence information from near the primer. The Sequenase^{tm} [USB] T7 polymerase sequencing kit relies on a ratio of primer to template to determine the number of nucleotides added in the labelling reaction and this proved difficult to standardise for direct sequencing. It was also difficult to remove the complementary primer from the reaction and experiments with asymetric PCR [Ehrlich, 1989] [data not shown] were unsucessful. Biotin coupled oligonucleotides have become available which allow easy purification of the PCR product for sequencing and are intensively labelled enough to provide a good radioactive signal for sequence near the primer [e.g. Dynabead^{tm}, Dynalabs Ltd]. This technique is now used as the basis for fluorescent labelled automated sequencing and is the preferred technique for direct sequencing of PCR products. However, satisfactory results were obtained with primers for plasmids or M13 single stranded DNA so this new technique was not employed in this project.

Chapter 4

Tissue expression of the VGCC-$\alpha 2$ subunit gene

4.1 Introduction

Many proteins have tissue specific forms and this is one of the methods of ensuring diversity of control of gene products. In the case of ion channels tissue specific forms are known in voltage gated sodium channels where the α subunit is constant but in the brain both a ß1 and a ß2 subunit occur [Catterall, 1987]. Voltage gated potasssium channels also vary between tissues with the variation being generated by alternative splicing of gene constructs [man] or in having large numbers of separate gene products encoding one exon [*Drosophila*]. Alternative splicing is also known to generate diversity in receptor operated channels such as the glycine or GABA receptors.

In the case of VGCCs, tissue specific expression exists for the α1 subunit [brain, lung, cardiac, vascular, skeletal muscle to date]. The ß and γ subunits also show tissue specific expression whose origin remains to be determined but it seems likely that different genes exist as no cross-hybridisation is seen between tissues e.g. brain and skeletal muscle on northern blots [Biel *et al*, 1991].

The α2 subunit showed far more limited tissue diversity. A variety of δ products of different sizes exist and it was not known whether this variation was tissue specific or simply an artefact generated by the isolation procedure.

Generally tissue variation in genes is examined by the use of northern blotting which will show the presence or absence of messenger RNA under high stringency conditions. This method is susceptible to errors induced by highly homologous products which crosshybridise to the probe. The problem is more significant with longer probes. The more sensitive method of cRNA hybridisation to the mRNA of interest followed by RNAse digestion overcomes this disadvantage but has not been applied to the α2 gene. However the α2 gene mRNA is rare so a more sensitive

method capable of detecting differences would be preferable. Polymerase chain reaction is both highly specific and very sensitive and easily allows products to be sequenced to confirm any differences.

The aim of this part of the project was to use polymerase chain reaction amplification under stringent conditions to test whether the $\alpha 2$ subunit was tissue invariant and to analyse any differences detected if it was not.

4.2 Polymerase Chain Reaction

Polymerase chain reaction [PCR] was initially described by Mullis *et al*, [1987] and later improved by the discovery of heat stable polymerases [Saiki *et al*, 1988]. The technique relies on the double complementary strands of DNA and directional synthesis to amplify regions by sequentially denaturing the strands, annealing oligonucleotide primers complementary to each template and extending those primers in an iterative process [Ehrlich, 1989].

In 1989 the technique was applied to mRNA using first strand cDNA as the template for PCR amplification in order to isolate tyrosine kinase homologues from zebra fish [Kamb *et al*, 1989]. It was decided to design PCR primers to the $\alpha 1$ and $\alpha 2$ sequences and to amplify segments of these genes to which probes existed. These amplification products should be the same in all tissues if the genes are highly homologous or should be absent if significant differences in sequence occurs between tissues.

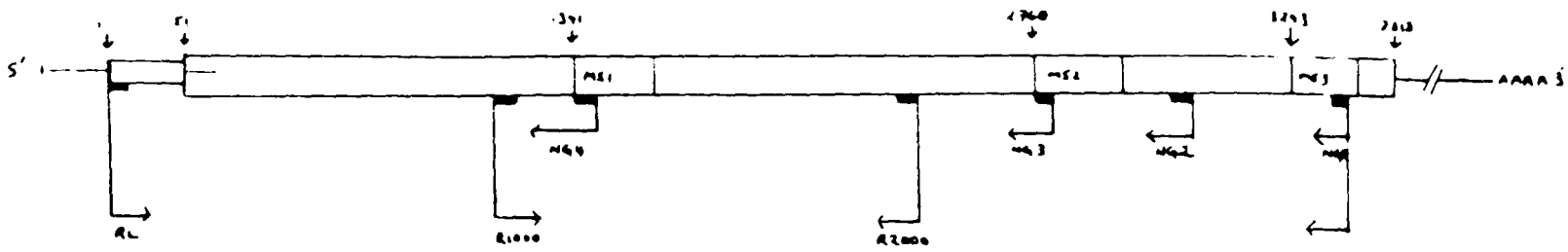
4.3 Methods

The basic technique of polymerase chain reaction is described in Ehrlich [1990]. Oligonucleotide primers to the conserved 2S1 and 2S5 regions of the α1 subunit- basepairs 1326 - 1350 and complementary to 1720 - 1744 and 2 arbitrary sequences [2532 - 2554 and complementary to 3132 - 3155] present in the α2 subunit were obtained [British Biotechnology Ltd] [fig 4.1]. These would result in amplification of regions of 420bp and 618bp respectively.

The annealing temperature of the primers was calculated by the following formula:

$$T_a = 4 (G/C) + 2 (A/T) \ ^{o}C$$

where G/C is the number of purines in the primer and A/T the number of pyrimidines.



PCR primer	5'	3'	bp
Rabbit leader	ATGGCTGCGGGCCGCCCGCTG		1-20
Rabbit 1000	CGGATC-GATTATAAGAAGGGCTTTTAG		999-1019
NG4	AACAGCTATGACATG		1326-1341
Rabbit 2000	CGGATC-GTAATCCTCTTGGTGCTAGGA		1980-2000
NG3	GCTATTGATGGCACA		2754-2769
NG2	GTCATTATCGAAGAA		2952-2967
NG1/rabbit MS3	TGCCAGAAACCAGCCA		2987-3303
NG reverse	GAGGCAGTTGAAATG		2874-2889
α2 forward [sense]	AAACGAAACAGTGATGTAATGGA		2532-2554
α2 reverse	CTTAACGATTACACAAGGATCTG		3132-3155
α1 forward [sense]	CTCAACACCCTGTCCATCGCC		1326-1350
α1 reverse	CCCCCGAAGAGCTGCATGCCC		1720-1744

Figure 4.1
Map of the rabbit α2-δ gene showing positions of PCR primers. Sequences of primers to both rabbit α1 and α2 genes and for the NG108-15 gene are shown below the map.

4.4 Precautions

PCR is well known to be very sensitive to small quantities of contaminants. A variety of precautions were taken to mininmise the chance of this:

[1] A separate set of Gilson pipettes was used where possible and positive displacement pipettes reserved for PCR were used for the addition of small volumes.

[2] All PCR reactions were set up in a different room.

[3] Solutions were specifically reserved for PCR and not used for any other purpose.

[4] All solutions and pipette ends were treated with UV irradiation [302 nm] for 2 minutes before use [Sarkar & Sommer, 1990].

4.4 Methods

cDNA was synthesised from mRNA prepared by the method of Chomcynski & Sacchi, 1987. 10 µg of mRNA in 20 µl of DEPC treated water was denatured at $65^{o}C$ for 3 minutes and cooled on ice. cDNA synthesis was performed in an eppendorf tube containing:

5 µl 10 x S1 buffer [100mM Tris-Cl pH8.3, 50mM NaCl, 25mM $MgCl_2$]

5 µl 0.1M DTT

1 µl 25mM dNTPs [Pharmacia Ltd]

1 µl RNasin [5U/µl][BRL Ltd]

0.5 µl oligo dT [100 ng/µl]

0.5 µl random hexamers [200 ng/µl][Amersham Ltd]

25 µl H_2O

The contents were equilibriated at $37^{o}C$ for 2 minutes.

The mRNA was added followed by 20 U of Moloney leukaemia virus reverse transciptase [Superscript^{tm}][BRL Ltd] and then incubated at 37^{o}C for 2 hours. The reaction was terminated by the addition of 2.5 µl of 0.5M EDTA.

A parallel reaction was performed with the substitution of 100 ng/µl of 2 specific short primers for the α1 and α2 subunits. These were complementary to base pairs 2859 - 2874 and 4650 - 4665 in the α1 subunit and 3215 - 3301 in the α2.

The first strand cDNA was phenol-chloroform extracted and precipitated with 1/10 volume of 3M NaOAc, 2.2 volumes of ethanol [-70^{o}C, 15 minutes], washed with 70% ethanol and resuspended in 100 µl of 0.1 x TE.

4.6 The PCR reaction

The PCR reaction was carried out as follows:

10 µl 10 x Taq buffer: 100mM Tris-Cl pH 8.3, 15mM $MgCl_2$,

0.1% tween

1 µl 25mM dNTPs

1 µl primers [100 ng/µl]

86 µl H_2O

1 µl template DNA

0.5 µl Taq polymerase [5 U/µl]

Amplification was performed on a programmable temperature cycling machine [MJ Research Ltd] initially under the following conditions:

Denature	[T_d]	94^{o}C 1 minute	
Anneal	[T_a]	45^{o}C 1 minute	
Extend	[T_e]	72^{o}C 2 minutes	Cycles 40

10 µl of reaction products were run out on a 1% agarose gel and stained with ethidium bromide.

Control reactions lacking template [negative control] were included in all PCR runs. A positive control was used only in initial optimisation reactions to reduce the possibility of contamination in test amplifications.

4.7 Optimisation of PCR conditions

A variety of parameters were optimised in the PCR reaction:

[a] annealing temperature

Repeat episodes of PCR were carried out raising the annealing temperature of the reaction towards the T_m of the primers in steps of 5^oC. An optimal temperature was chosen as close as possible to the T_m that still gave amplifying products and with clearly defined bands.

[b] magnesium concentration

A magnesium ion titration was performed by the addition of 5 µl amounts of a various of magnesium chloride solutions [20 - 100mM in steps of 20mM] to the reaction and repeating the amplification at the optimal temperature thereby giving a range of magnesium ion concentrations from 1.5mM to 6.5mM.

[c] primer concentration

The concentration of the primers in the amplification reaction was varied in three serial 10 fold dilutions from 1 µg/µl at the optimal annealing temperature and magnesium concentration.

4.8 Checking the quality of cDNA synthesised

Adequate cDNA synthesis was checked by the use of PCR amplification to analyse quality and purity of synthesis by the method of Corrochano [1991]. This involves PCR amplification of a segment of the human histidyl-tRNA synthetase enzyme which generates a fragment of 128bp from cDNA and 400bp when genomic DNA contamination is present. The segment to be amplified occurs 1.8kb from the oligo-dT tail and therefore allows some check of the quality of cDNA synthesis. The primers can also be used to hybridise across species as the molecule is highly conserved. The primer sequences are to the regions nucleotides 37-56 [1F] and complementary to 127-146 [1R].

1F	5'	CTTCAGGGAGAGCGCGTGCG 3'
1R	5'	TCATCAGGACCCAGCTGTGC 3'

Primers were used as directed at a magnesium concentration of 1.5mM and amplification was performed in the presence of 5% DMSO to improve specificity.

T_d	94°C	0.5 minutes	
T_a	55°C	0.5 minutes	
T_e	72°C	1.0 minutes	40 cycles

10 µl of the reaction were run out on a 2% agarose gel and visualised under UV transillumination.

4.9 Southern blotting of PCR products

10 µl samples of PCR amplifications were run on 1% agarose gels in the presence of labelled PM2 markers. The gel was blotted onto a nylon membrane and hybridised with 0.2×10^6 cpm/ml of end kinased α1 or α2 probes, washed with 2 x SSC at 40^0c for 15 minutes and exposed for 3 days at -70^0C.

4.10 Subcloning PCR products

The products were subcloned into pUC8. Briefly, 20 µl of the PCR reaction was run on a 1.5% agarose gel containing 10 µg/ml ethidium bromide and the band visualised, cut out and then extracted from the agarose, and precipitated with NaOAc/EtOH. The DNA was resuspended in 25 µl of H_2O.

Kinasing Reaction: 24 µl DNA

3 µl 10x kinase buffer [50mM Tris-Cl,

10 mM DTT, 20mM $MgCl_2$]

3 µl 10 mM ATP

2 µl T4 DNA kinase [20 U]

The reaction was incubated at 37^0C for 30 minutes.

The ends were made flush with the Klenow fragment of *E.coli* DNA polymerase I as follows:

Flush ending: 30 µl kinase reaction

3 µl 2.5 mM dNTPs

1 µl Klenow DNA polymerase I [10 U] [Amersham Ltd]

The reaction was incubated at 37^0C for 15 minutes. The DNA was extracted with

phenol-chloroform, chloroform and precipitated with the addition of 1/10 volume 0.3M NaOAc and 2.2 volumes of ethanol. After spinning down and washing with 70% ethanol the DNA was resuspended in 20 µl to give an approximate concentration of 100 ng/µl.

The vectors used for subcloning were pGEM-5Z and pUC8. Vector plasmid was prepared by the method in Perbal [1989].

Preparation of vector:

Preprepared plasmid was digested with the *HincII* restriction enzyme to produce blunt ended linearised plasmid.

1 µg of plasmid [10 µl]

34 µl TE

4 µl 10 x *HincII* restriction buffer

1 µl *HincII* [10 U]

The plasmid was digested for 1 hour at 37^0C and completeness of digestion checked on an agarose minigel.

The vector was dephosphorylated to reduce background ligations by the addition of:

5 µl 10 x CIAP buffer [10mM Tris pH 7.4, 10mM $ZnCl_2$]

1 µl of calf intestinal alkaline phosphatase [1 U/µl][BRL Ltd]

and incubated at 37^0C for 15 minutes and 56^0C for 15 minutes. The plasmid was then phenol-chloroform extracted and precipitated as before. The vector was resuspended at 50 ng/µl in TE.

The vector was ligated to the PCR insert as follows:

Ligation: Vector 50 ng [1 µl]

Insert 5 ng [1 µl of diluted insert]

2 µl 5 x ligase buffer

5 µl H_2O

1 µl T4 ligase

The ligation was incubated at 16^{0}C for 16 hours. The ligation reaction was diluted to 50 µl in TE and 10 µl used to transform a 150 µl aliquot of competent RB791 cells. White colonies were picked and transferred with a sterile toothpick to a gridded nylon membrane on a LB agar plate containing 200 µg/ml ampicillin and grown overnight. A duplicate nylon filter was overlaid on the first, marked out by punching through both with 19 G needle and then separted off. This filter was placed on Whatman 3MM paper soaked in 0.5M NaOH / 1.5 M NaCl for 5 minutes then onto another soaked in 0.5 M Tris-Cl pH 8/ 1.5M NaCl [2 minutes] and then onto paper containing 2 x SSC. The filter was pressed hard between 2 sheets of dry Whatman paper and then baked for 2 hours at 85^{0}C.

Filters were prehybridised in perspex chambers as for southern blots and probed with kinased oligonucleotide probes [0.1 x 10^6 cpm/ml]. The filters were washed in 2 x SSC at 50^{0}C for 15 minutes before autoradiography for 12 hours.

However if there were less than 10 white colonies per transformation inserts were analysed by individual 1.5 ml alkaline lysis plasmid preparation and a restriction digest with *EcoR1* and *HindIII* to release the insert. The digested DNA was then run on an ethidium bromide containing 1% agarose gel and visualised by UV transillumination. Inserts were isolated by running some digest on an LMP agarose gel and hot phenol

extraction and ligated to M13mp18 or M13mp19 for sequencing. Sequencing was performed by the dideoxy chain termination method of Sanger *et al*, [1975] on both strands from either plasmid or M13.

4.11 Results

The approximate yield of cDNA was 3-5 μg and transcripts of upto 2.4kb were visible on a ethidium bromide containing agarose gel.

Results of the check amplification are shown in fig 4.2. All cDNA was of good quality but did contain some genomic DNA. Multiple additional bands were seen in both rodent cDNA preparations whose origin is unknown but suggests that other partially homologous genomic sequences exist in rodent as opposed to lagomorph cDNA or human genomic DNA.

The optimal conditions were determined experimentally for the primer pairs. Results of magnesium ion titrations are shown for $\alpha 1$ and $\alpha 2$ primers in fig 4.3. It was found that primer concentration was not a significant factor in optimisation and so a primer concentration of 0.5 μg/ml was used routinely.

PCR amplification of rabbit cDNA resulted in bands of the expected size [fig 4.3] which hybridised to kinased oligomer probes [fig 4.4]. Blots were later screened with rabbit PCR products that had been subcloned into pUC8, digested by restriction enzymes, purified by low melting point agarose gel separation and random primer labelled. This enabled exposure times to be reduced to 1 - 3 hours.

cDNA from NG108-15 and F2.1D1 cell lines did not amplify at all.

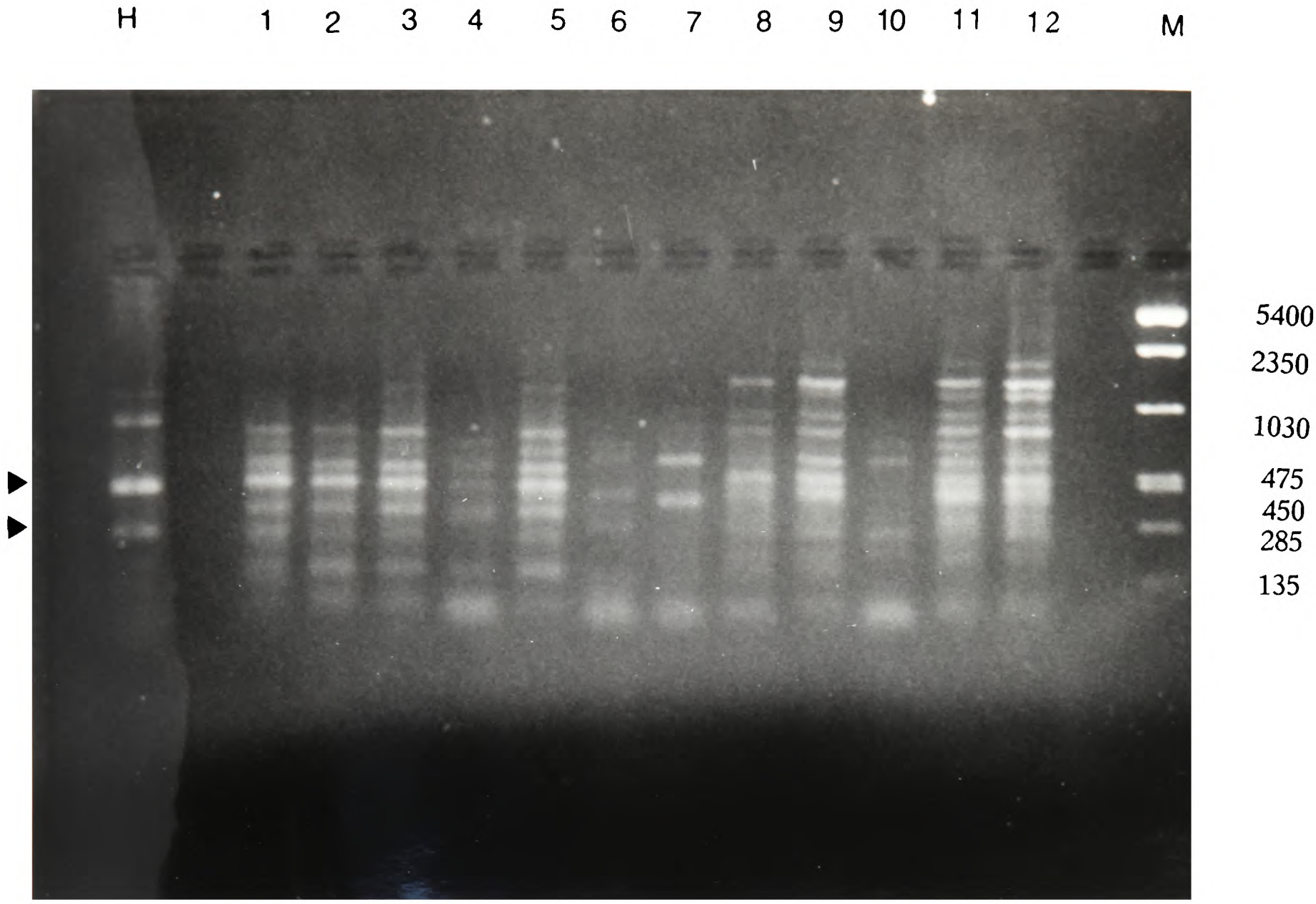


Figure 4.2

Check PCR of cDNA derived from rodent tissues with primers to human histidyl tRNA synthase gene with cycling conditions of 94^{o}C/0.5 min 55^{o}C/0.5 min 72^{o}C/1 min. Lane H: human skeletal muscle cDNA Lanes 1-6:mouse ; lanes 7-12 rat Tissues lanes 1-6; 7-12: brain, lung, skeletal muscle, liver, kidney, heart muscle. Markers PM2/*HindIII* digest [M]. All cDNA was made with both oligo-dT, random hexamers and specific primers. The human transcript [135 bp] and genomic [420 bp] amplified products are marked. Similar products are present in the other lanes. The multiple bands present in rodent tissues are probably slightly homologous genpomic sequences present in the animals.

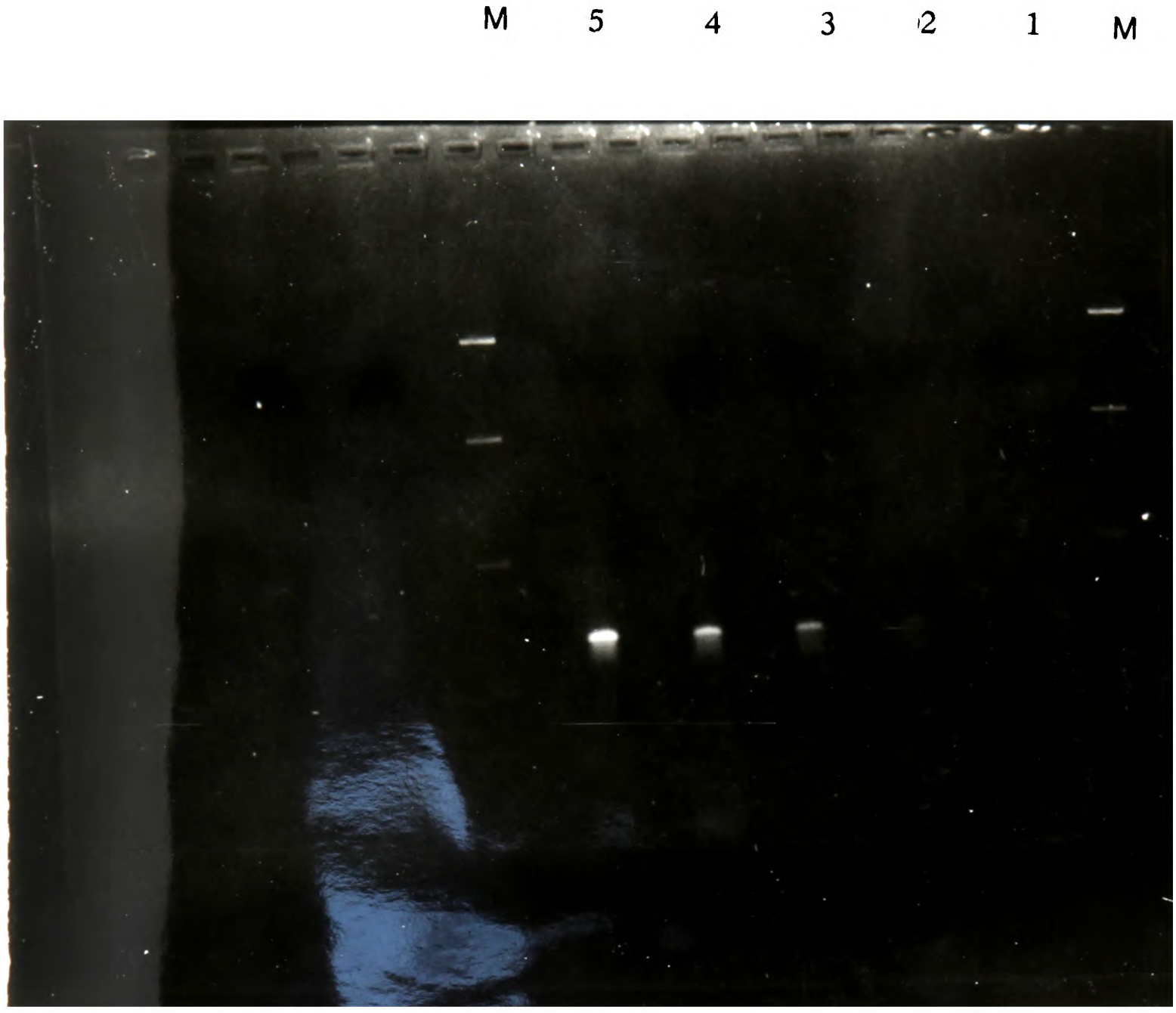
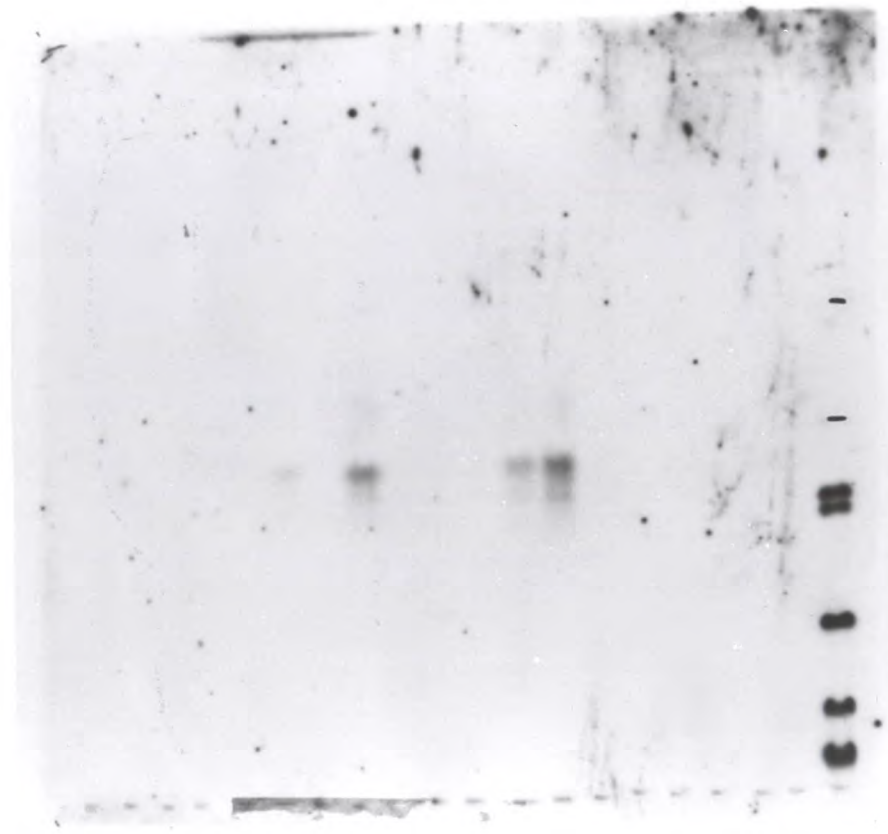


Figure 4.3

Titration of magnesium concentrations for $\alpha 2$-δ VGCC PCR primers using rabbit skeletal muscle oligo-dT and random hexamer primed cDNA. Cycling conditions 94°C/1min; 55°C/1min; 72°C/2mins. Magnesium concentration 1.5mM [1], 2.5mM [2], 3.5mM [3], 4.5mM [5], 5.5mM [6]. Markers PM2/ *HindIII* digest [M].



B

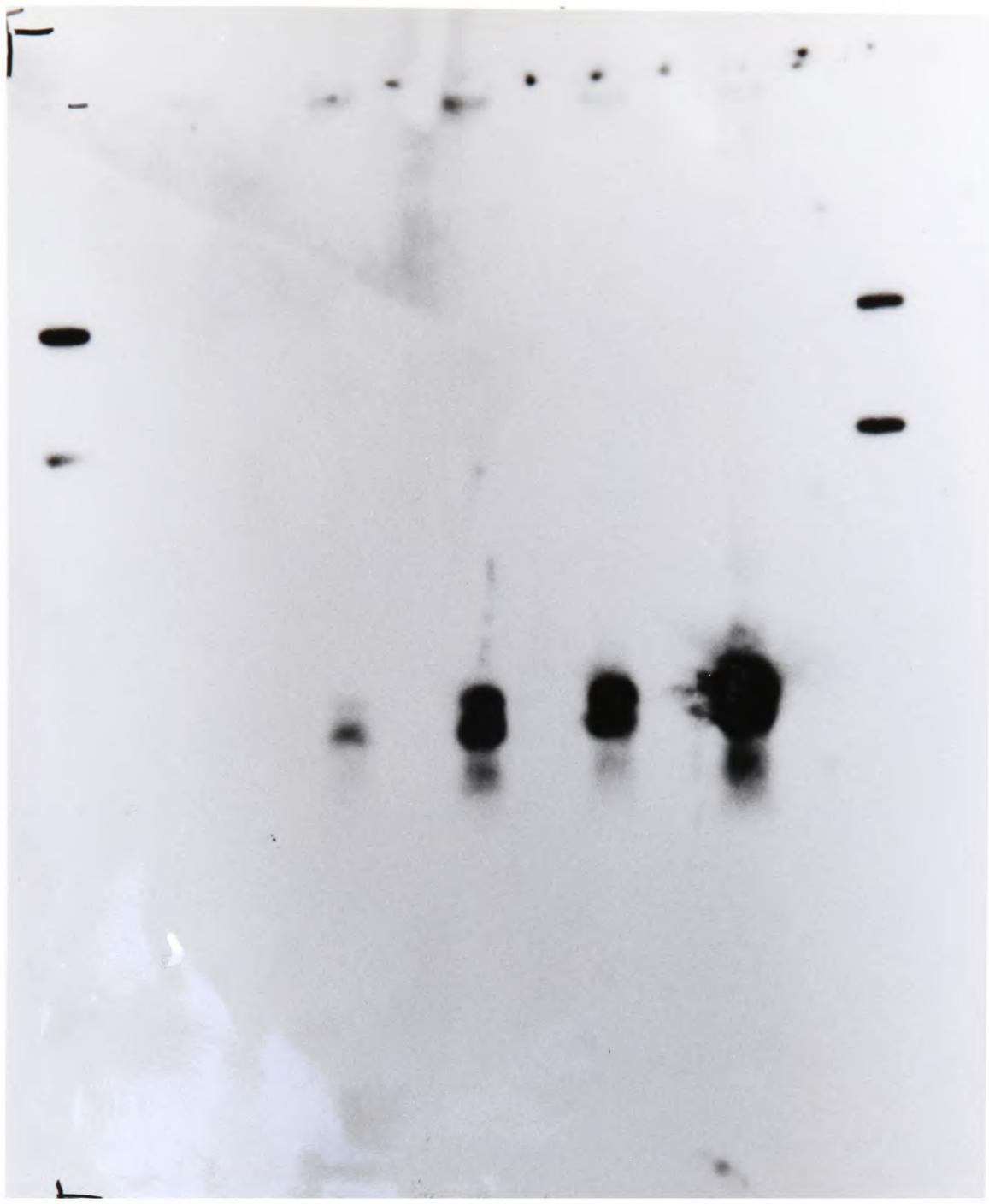


Figure 4.4

Southern blots of rabbit skeletal muscle α1 [a] and α2-δ [b] products hybridised to their respective oligomer probes. Hybridisation was performed at 42^{o}C in 5mls of 6xSSCE, 10xDenhardt's, 100µg/ml herring sperm DNA, 50% formamide with 0.5x10^{6} cpm/ml of probe. Filters were washed in 2xSSC at 60^{o}C and autoradiographed for 5 days at -70^{o}C.

4.8 Application of PCR to Rodent Tissues

Methods and results

The PCR was repeated with cDNA derived from rodent tissues - brain, lung, skeletal muscle, cardiac muscle, liver and kidney from rat and mouse. In each case 1 - 10g of tissue were removed from freshly killed animals and frozen in liquid nitrogen. The organs were crushed with a hammer and the powder weighed and resuspended in guanidinium isothiocyanate solution. An equal amount of tissue [approximately 5g] was used to prepare total RNA by the method previously described. Yields are shown in table 4.1. The mRNA was purified by oligo-dT cellulose chromatography and 1 μg used for first strand cDNA synthesis. RNA quality was checked by running 1 μg mRNA on a formaldehyde-MOPS-agarose miningel.

Primers for first strand cDNA synthesis comprised a mixture of 2 α1 primers [bp 2859 - 2874 and 4650 - 4665], α2-δ [bp 3285 - 3301], and a separate tube containing 100 ng of random hexamers and 100 ng of poly-dT. The contents of the first strand reactions were mixed after cDNA synthesis and an aliquot used for the PCR reaction. All cDNA syntheses were conducted in duplicate. 100 ng of the cDNA were used as template for PCR amplification under the conditions previously described. DMSO was added as a specificity enhancer to a concentration of 5% in all PCR reactions. 10 μl of the PCR reaction were run out on a 1.2% agarose gel and products of the correct size [618 bp] were observed with mouse liver α1 and with mouse brain and lung and rat skeletal muscle for α2 [fig 4.5].

The gels were denatured and blotted by standard techniques and the resulting Southern blots probed with 0.1×10^6 cpm/ml of random primer labelled rabbit α1 or α2. The filters were washed with 2 x SSC at 70^0C and were autoradiographed for 2

hours [$\alpha 2$] or 2 days [$\alpha 1$]. Hybridising bands were observed with the $\alpha 1$ probe in mouse liver [fig 4.6] and in rat muscle, mouse brain, lung with $\alpha 2$ [fig 4.7]. This confirmed that the amplified products did show homology to the $\alpha 1$ or $\alpha 2$ sequences. The remainder of the PCR product was phenol-chloroform extracted and precipitated. The DNA was resuspended in 20 µl of TE and run out on a 1.2% LMP-agarose gel and the product band extracted with hot phenol and re-precipitated. The products ends were kinased, polished with Klenow polymerase in the same tube for 30 minutes at 37^{0}C, extracted with phenol-chloroform and precipitated. One tenth of the resuspended DNA [2 µl] was run out on an agarose gel and compared with PM2 markers to obtain an approximate idea of the concentration of the insert. The products were ligated to pUC8 and the plasmid used to transform competent RB791 cells. The results of the transformation are shown in table 4.2. The transformants were transferred to hybond-N filters and positives identified by screening with rabbit probes under the conditions described for Southern blotting. Screening of subcloned inserts gave results as shown in fig 4.8 and the positive colonies were picked, grown up in L-broth containing 50 µg/ml ampicillin and then sequenced either from plasmid or from M13. The size of the inserts present in these positive clones was checked by digesting an aliquot of the plasmid preparation with *EcoR1/HindIII* [fig 4.9].

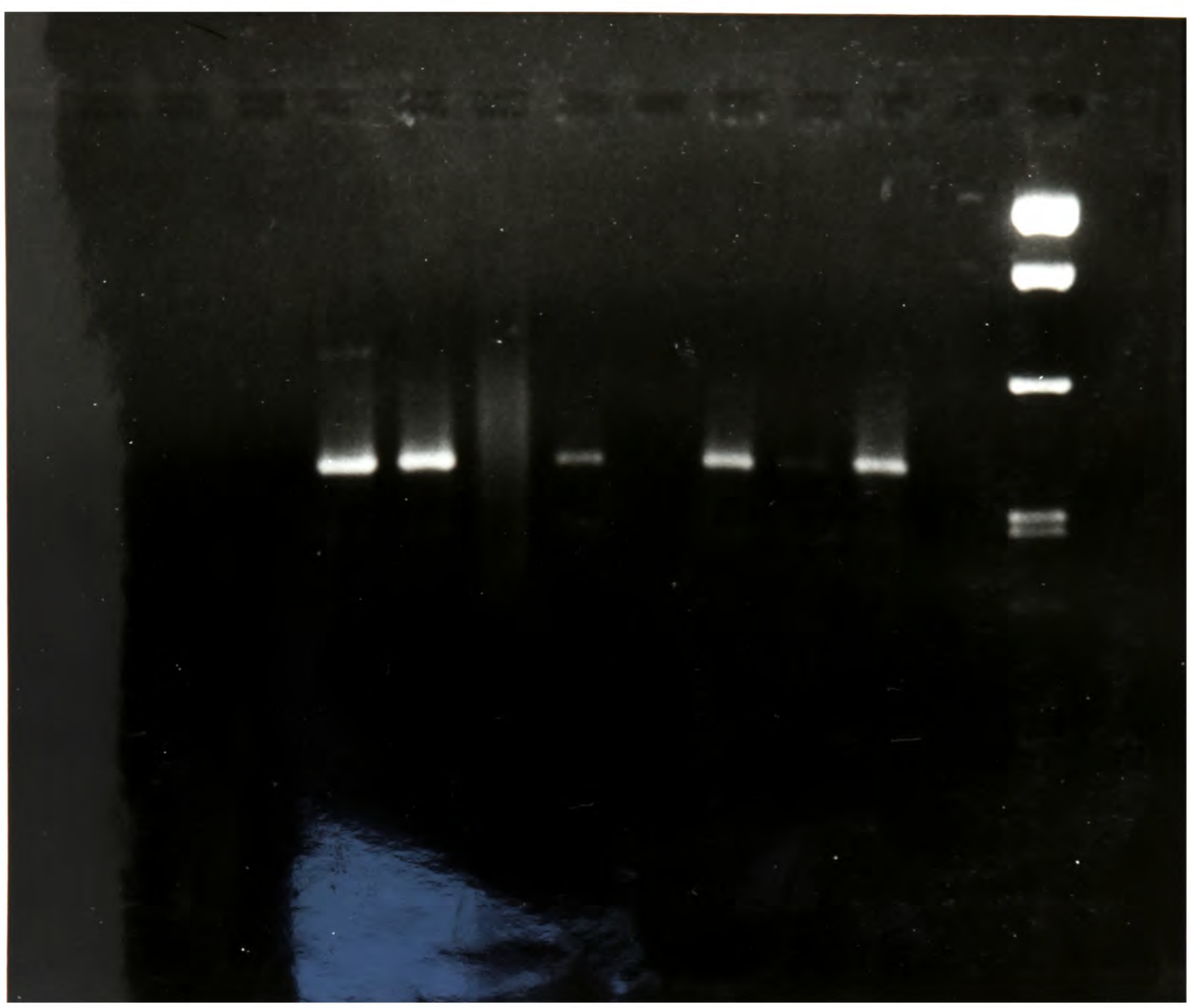


Figure 4.5

PCR amplification using primers to the $\alpha 2$-δ subunit of the rabbit skeletal muscle VGCC and specifically and randomly primed rodent cDNA from rat brain [1,2], rat lung [3], rabbit skeletal muscle [4,5], rat skeletal muscle [6,7], mouse brain [8,9], mouse lung [10], mouse skeletal muscle [11]. Conditions for cycling 94^{0}C/1 min; 45^{0}C/1 min; 72^{0}C/2 mins; 3.5mM $MgSO_4$. Markers PM2/*HindIII* digest [M]. The cDNA used is the same as used in the check amplification [fig 4.2].

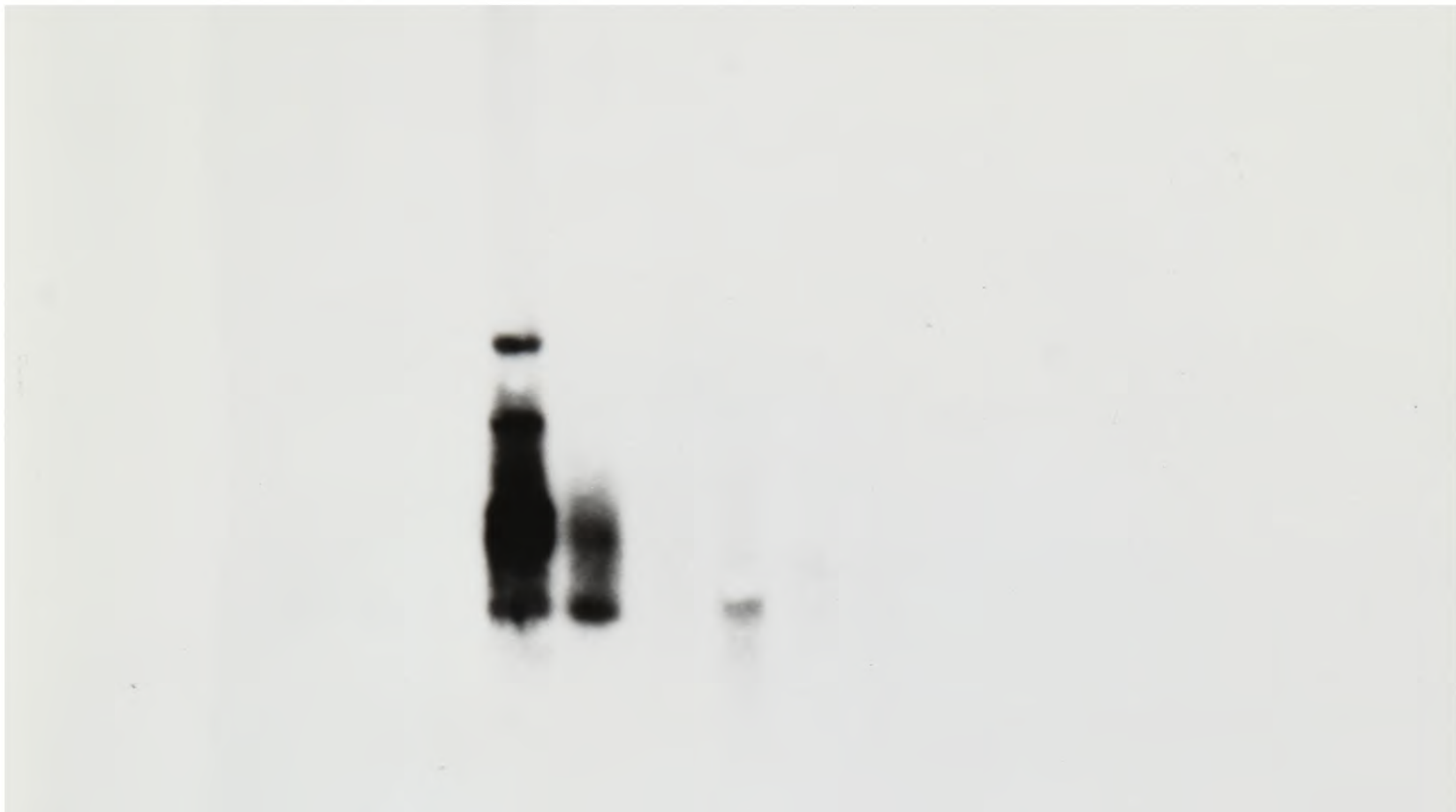


Figure 4.6

Southern blot of PCR amplified products using primers for the α1 subunit from rodent tissues with a rabbit α1 subunit probe [420bp]. Mouse lanes 1-6. Rat lanes 7-12. Brain [1,7]; lung [2,8]; skeletal muscle [3,9], liver [4,10], kidney [5,11] heart [6,12]. Markers PM2/*HindIII* digest [M]. Hybridisation was performed at 42^{o}C in 5 mls 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 50% formamide and $0.1x10^{6}$cpm/ml probe. The filter was washed at 2xSSC at 50^{o}C and autoradiographed for 2 days.

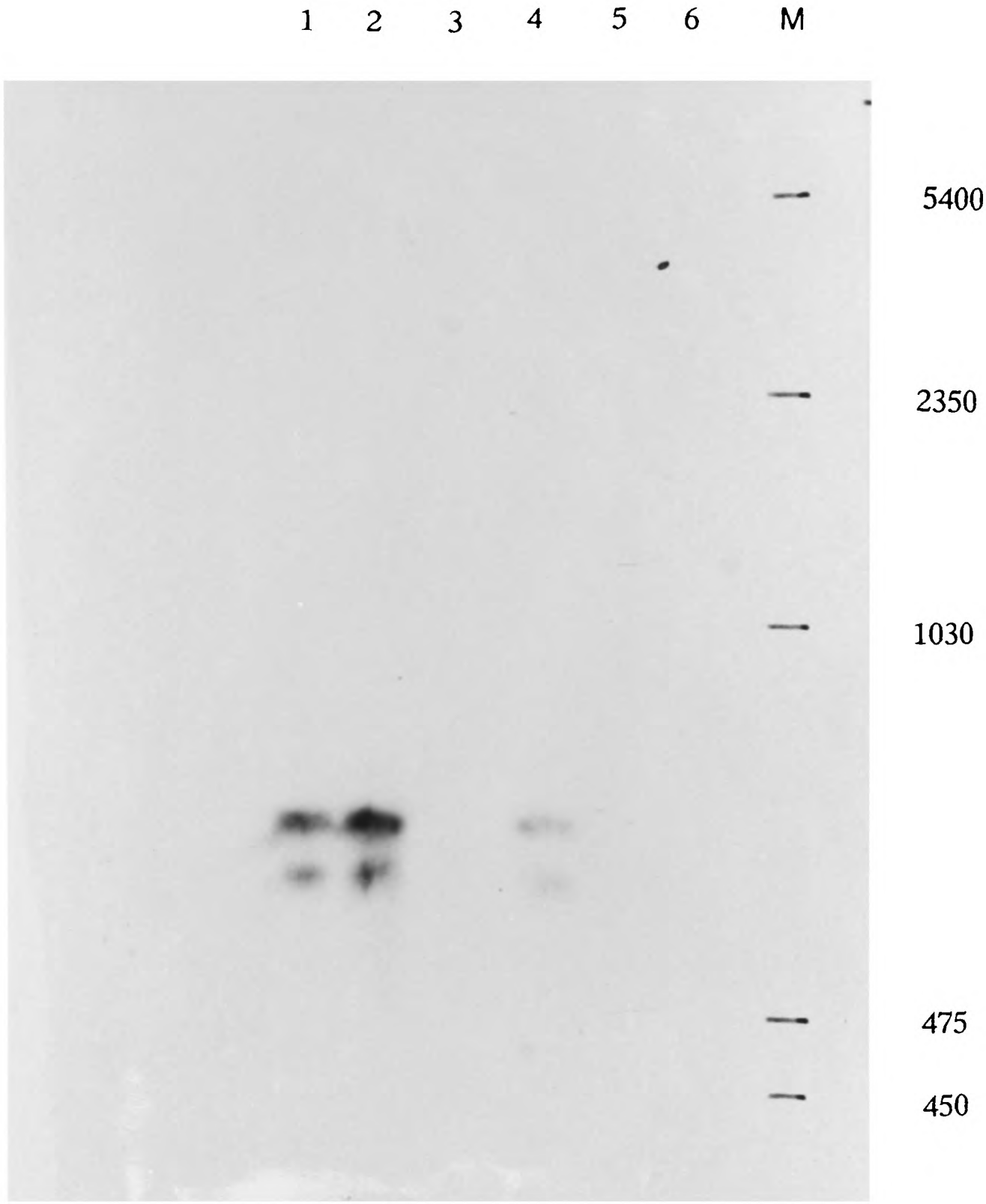


Figure 4.7

Southern blot of $\alpha 2$-δ PCR amplified products from rodent tissues with rabbit skeletal muscle $\alpha 2$-δ probe [618bp]. Lanes 1-3 mouse; lanes 4-6 rat. Brain [1,6]; lung [2,5]; muscle [3,4]. Markers PM2/*HindIII* digest. Hybridisation was carried out in 5mls 6xSSCE, 10xDenhardt's, 100µg/ml herring sperm DNA, 50% formamide and 0.1×10^6 cpm/ml probe at 42^0C. The filter was washed in 2xSSC at 70^0C and autoradiographed for 2 hours.

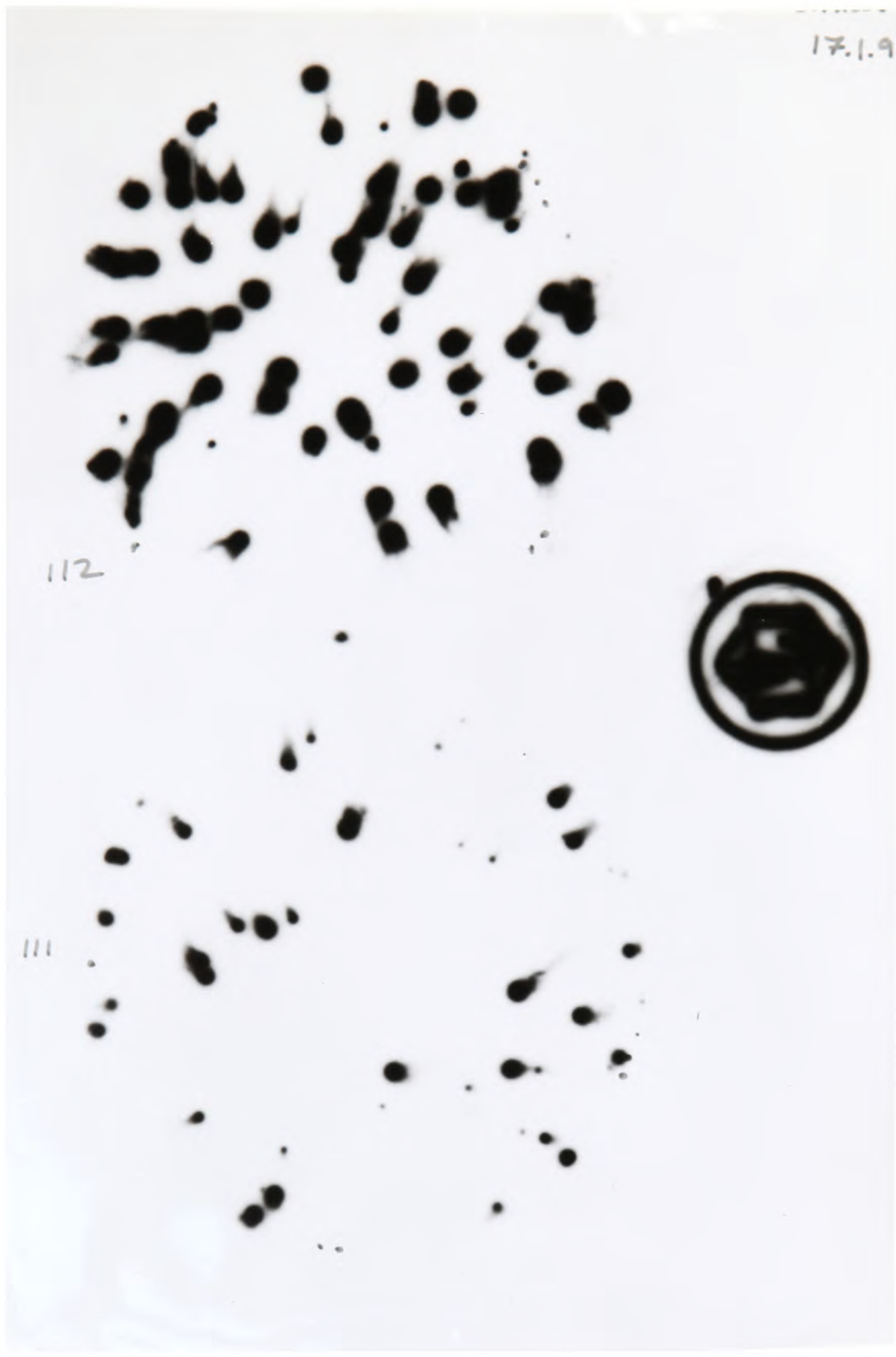


Figure 4.8

Filter hybridisation of pUC8 transformants containing inserts of mouse brain α2-δ PCR products. Hybridisation was carried out in 20mls 6xSSCE, 10x Denhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8 plasmid and 0.1×10^6cpm/ml rabbit α2 probe at 42^{o}C. Filters were washed in 2xSSC at 70^{o}C and autoradiographed for 2 hours.

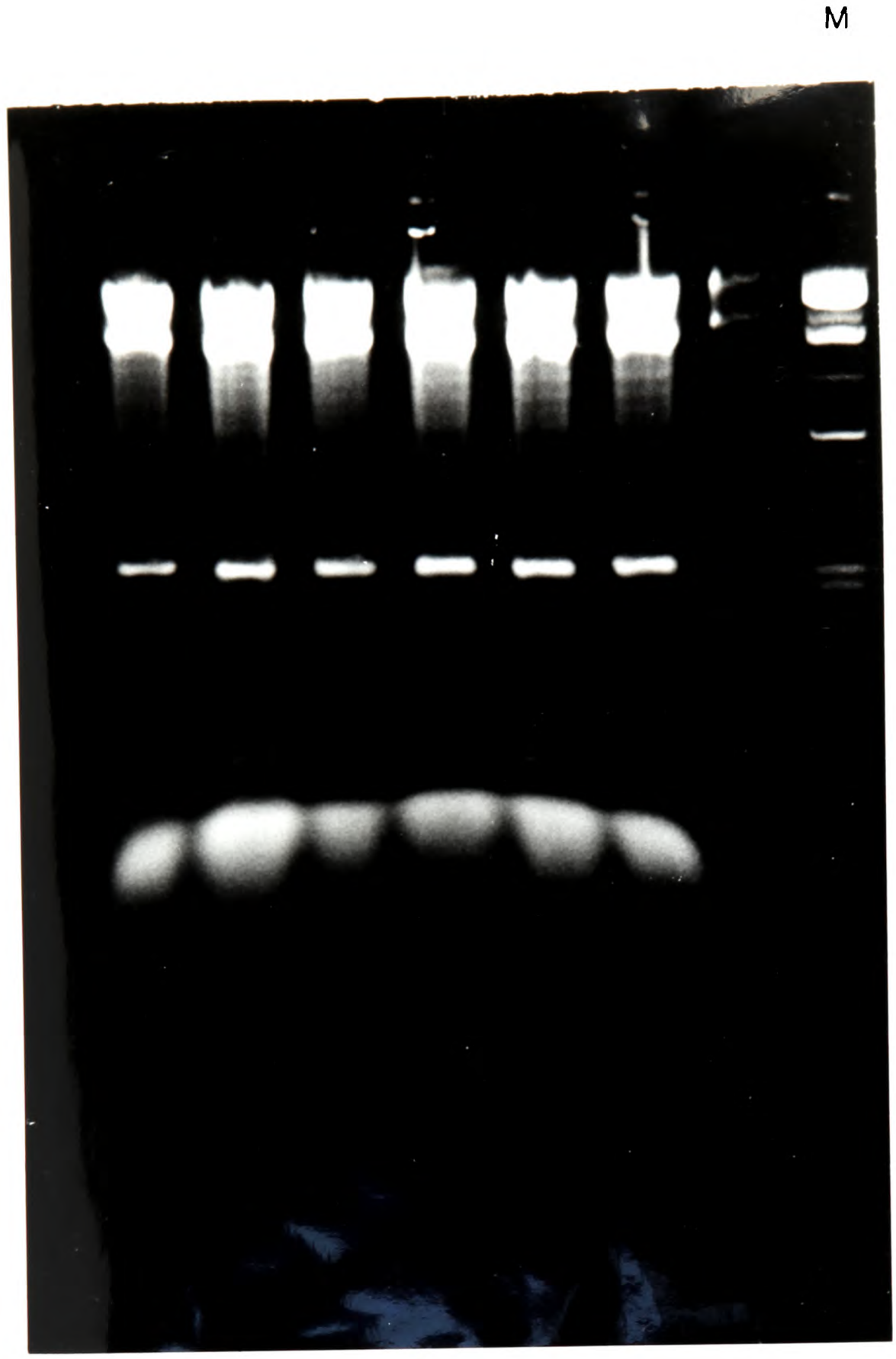


Figure 4.9

Alkaline lysis plasmid preparations of colonies containing plasmids with $\alpha 2$-δ PCR inserts. The plasmids were digested with *EcoR1* and *HindIII*. The digests were run out on a 1% agarose gel. The digest product is shorter than 618bp due to the presence of a *HindIII* restriction site in the mouse PCR product. The smaller fragment is too faint to be clearly visible in all lanes.

Figure 4.10

A sequencing gel of the mouse brain $\alpha 2$-δ PCR product. pUC8/$\alpha 2$-δ ligated plasmids were sequenced from alkali denatured caesium chloride/ethidium bromide purified double stranded plasmid by the Sequenase^{tm} dideoxy terminator method.

Lanes are loaded in the order ATGC.

The $\alpha 1$ product was not sequenced as the yield of the product was low even with optimisation and it was not possible to subclone it.

Three $\alpha 2$ inserts were subcloned and sequenced. A typical sequencing gel for plasmid sequencing is shown in fig 4.10. The inserts were short and were sequenced in both directions and from away from a convenient *HindIII* site present in the inserts. The complete $\alpha 2$ sequences are shown in figs 4.11 - 4.13 and a comparison of the amino acid sequences in fig 4.14. Gel sequences were analysed on a Viglen II computer using the program RTDNA [Elsevier Biosciences]. This enabled the sequence to be translated in all possible frames and also allowed restriction sites to be determined. The best possible match was used as the basis for the final sequence.

Further experiments have been performed using primers to 1000-1021 and 1979-2000 bp of the $\alpha 2$ gene and some of these have been shown to hybridise to a homologous portion of the NG108 $\alpha 2$ gene [mouse lung, mouse brain] while a product in rat muscle hybridises to a 21bp oligomer in MS1 of the NG108 and rabbit gene [figs 4.15, 4.16]. These products have not yet been subcloned and sequenced owing to the highly heterogeneous nature of the amplification products produced in these reactions. Further work is needed to subclone and define these products by hybridisation screening and then sequencing.

```
                                                                  30
TGT GTG ATT CTA GAT GAT GGT GGG TTT CTT TTG ATG GCC AAC CAT GAT
C   V   I   L   D   D   G   G   F   L   L   M   A   N   H   D
                60                                            90
GAT TAT ACC AAT TAC ATT GGA AGC TTC TTT GGA GAG ATT GAT CCA AGC
D   Y   T   N   Y   I   G   S   F   F   G   E   I   D   P   S
                                        120
TTG ATG AGA CAC CTG GTC AAT ATA TCT GTT TAT GCC TTT AAC AAA TCT
L   M   R   H   L   V   S   V   Y   A   F   N   K   S   Y   D
      150                                           180
TAT GAT TAT CAG TCG GTG TGC GAA CCT GGT CCA AAG CAG GGA GCA GGG
Y   Q   S   V   C   E   P   G   A   A   P   K   Q   G   A   G
                        210                                   240
CAC CGC TCG GCT TAT GTG CCC TCA ATA GCA GAC ATA ATT CAG ATT GGA
H   R   S   A   Y   V   P   S   I   A   D   I   L   Q   I   G
                                        270
TGG TGG GCC ACT GCT GCT GCC TGG TCT ATT CTT CAG CAG TTT CTG CTG
W   W   A   T   A   A   A   W   S   I   L   Q   Q   F   L   L
                300                                           330
CAG TTT TTG AGC TTT TCT CGG CTC CTT GAG GCA GTC GAG ATG GAG GAG
Q   F   L   S   F   S   R   L   L   E   A   V   E   M   E   E
                                        360
GAT GCA GCA TTC ACT ACG AGT ATG TCA AGC CAG AGC TGC ATC ACG CAG
D   A   A   F   T   T   S   M   S   S   Q   S   C   I   T   Q
      390                                           420
CAT ACC CAT TAT TAT TTC TTC GAC AAT GAC TCG ATA TCG TTC TCC GGG
H   T   H   Y   Y   F   F   D   N   D   S   I   S   F   S   G
                        450                                   480
GTA TTA GAC TGT GGG AAC TGT CAG AGA ATC TTC ATG GTA GAA AAG CTC
V   L   D   C   G   N   C   Q   R   I   F   M   V   E   K   L
                                        510
ATG TGC TCC GGG TTA ATA TTC ATA ATG GTA GAG TCA AAG GGC ACA TGT
M   C   S   G   L   I   F   I   M   V   E   S   K   G   T   C
                540                                           570
CCC TGT GAT ACA CGG ATA CTC ATA CAA GCA GAG CAA ACT TCT TCT GAT
P   C   D   T   R   I   L   I   Q   A   E   Q   T   S   S   D

GGC
G
```

Figure 4.11

The nucleotide and amino acid sequence of the mouse brain $\alpha 2$-δ PCR product excluding primers. Nucleotide and amino acid changes compared to the rabbit skeletal muscle $\alpha 2$-δ sequence are shown in bold.

```
                                                  30
TGT GTG ATT CTA GAT GAT GGT GGG TTT CTT TTG ATG GCC AAC CAT GAT
C   V   I   L   D   D   G   G   F   L   L   M   A   N   H   D
                60                                        90
GAT TAT ACC AAT TAC ATT GGA AGC TTC TTT GGA GAG ATT GAT CCA AGC
D   Y   T   N   Y   I   G   S   F   F   G   E   I   D   P   S
                                  120
TTG ATG AGA CAC CTG GTC AAT ATA TCT GTT TAT GCC TTT AAC AAA TCT
L   M   R   H   L   V   N   I   S   V   Y   A   F   N   K   S
      150                                       180
TAT GAT TAT CAG TCG GTG TGC GAA CCT GGT GCC GCG CCA AAG CAG GGA
Y   D   Y   Q   S   V   C   E   P   G   A   A   P   K   Q   G
                          210                             240
GCA GGG CAC CGC TCG GCT TAT GTG CCC TCA ATA GCA GAC ATA CTG CAG
A   G   H   R   S   A   Y   V   P   S   I   A   D   I   L   Q
                                  270
ATT GGA TGG TGG GCC ACT GCT GCT GCC TGG TCT ATT CTT CAG CAG TTT
I   G   W   W   A   T   A   A   A   W   S   I   L   Q   Q   F
                300                               330
CTG TTG CAG TTT AGC TTT TCT CGG CTC CTT GAG GCA GTC GAG ATG GAG
L   L   Q   F   S   F   S   R   L   L   E   A   V   E   M   E
                                  360
GAG GAT GCA GCA TTC ACT ACG AGT ATA TCA AGC CAG AGC TGC ATC ACG
E   D   A   A   F   T   A   S   I   S   S   Q   S   C   I   T
      390                                       420
CAG CAT ACC CAT TAT TTC TTC GAC AAT GAC AGC ATA TCG TTC TCC GGG
Q   H   T   H   Y   F   F   D   N   D   S   I   S   F   S   G
                          450                             480
GTA TTA GAC TGT GGG AAC TGT CAG AGA ATC TTC ATG GTA GAA AAG CTC
V   L   D   C   G   N   C   Q   R   I   F   M   V   E   K   L
                                  510
ATG TGC TCC GGG TTA ATA TTC ATA ATG GTA GAG TCA AAG GGC ACA TGT
M   C   S   G   L   I   F   I   M   V   E   S   K   G   T   C
                540                               570
CCC TGT GAT ACT CGG ATA CTC ATA CAA GCA GAG CAA ACT TCT TCT GAT
P   C   D   T   R   I   L   I   Q   A   E   Q   T   S   S   D

GGC
G
```

Figure 4.12

The nucleotide and amino acid sequences of the mouse lung α2-δ PCR product. The nucleotide and amino acid changes from the rabbit α2 sequence are shown in bold.

```
                                                                30
TGT GTG ATA CTA GAT GAC GGT GGG TTT CTT TTG ATG GCA ATA CAT GAT
C   V   I   L   D   D   G   G   F   L   L   M   A   I   H   D
                60                                          90
GAT TAT ACC AAC CAG ATT GGA AGA TTC TTT GGA GAA ATT GAT CCA AGC
D   Y   T   N   Q   I   G   R   F   F   G   E   I   D   P   S
                                        120
TTG ATG AAC CAC CTG GTC AAT ATT TCG GTA TAT GCC TTC AAC AAA TCT
L   M   N   H   L   V   N   I   S   V   Y   A   F   N   K   S
      150                                       180
TAT GAT TAT CAG TCG GTC TGT GAA CCT GGT GCC GCC CCC AAG CAG GGA
Y   D   Y   Q   S   V   C   E   P   G   A   A   P   K   Q   G
                        210                                 240
GCA GGG CAC CGG TCT GCA TAT GTC CCA TCA ATT ATA GAC ATA CTC CAG
A   G   H   R   S   A   Y   V   P   S   I   I   D   I   L   Q
                                        270
ATT GGA TGG TGG GCT ACT GCA GCA GCC TGG TCT ATT TTG CAG CAG AGC
I   G   W   W   A   T   A   A   A   W   S   I   L   Q   Q   S
                300                                         330
CTA CTA AGC CTT CTA CTA CCA CGT CTT CTT GAG GCA GCA GAC ATG GAG
L   L   S   L   L   L   P   R   L   L   E   A   A   D   M   E
                                        360
GAG GAC GAC TTC ACT GCT TCA ATG TCA AAG CAG AGC TGC ATC ACG GAG
E   D   D   F   T   A   S   M   S   K   Q   S   C   I   T   E
      390                                       420
CAG ACC CAG TAT TTC TTC GAT AAG GAC ACG GCA ACG TTC AGC ATG GGC
Q   T   Q   Y   F   F   D   K   D   T   A   T   F   S   M   G
                        450                                 480
GTA CTG GAA CGT GGT AAG TGC AGT CGT ATA TTT CAG TTA GAG AAG CGC
V   L   E   R   G   K   C   S   R   I   F   Q   L   E   K   R
                                        510
ATG AGC ACT AAT TTC ATA TTC ATA ATG GTA GAG CGC ATA GGG ACA TGT
M   S   T   N   F   I   F   I   M   V   E   R   I   G   T   C
                540                                         570
CCC TGT GAC ACG ATA CGG CTG CTC ATA CAA GCA GGA CAA ACT TCT GAT
P   C   D   T   I   R   L   L   I   Q   A   G   Q   T   S   D

GGG
G
```

Figure 4.13

The nucleotide and amino acid sequence of the rat skeletal muscle $\alpha 2$-δ PCR product.

Changes from the rabbit sequence are shown in bold.

```
Rabbit muscle   KRNSDVMDCVILDDGGFLLMANHDDYTNQIGRFFGEIDPSLMRHLV
Mouse brain     ------------------------------Y--S--------------
Mouse lung      ------------------------------Y--S--------------
Rat muscle      ----------------------I---------T--------------
NG108-15        FHTWIMF-...----------SIY---RLHVRA--TV-GHY-VERPL

NISVYDFNKSYDYQSVCEPGAAPKQGAGHRSAYVPSIADILQIGWWATAAAWSILQQFLLSL
-------------------------------------T-----------------------QF
-------------------------------------T-----------------------QF
-------------------------------------T-------------------------
RL---------------D-----R-------------A--------------------L-CD-

TFPRLLEAADMEDNDFTASMSLQSCITQQTQYFFDNDSLSFSGVLDCGNCSRIFMVEKLMNT
S-S-----VE--EDA------S-------------------------------------R
S-S-----VE--EDA------S-------------------------------------R
LL----------ED-------K-----E----------A-------R-K-----HL--R-S-
Q-------VE--EDA----L--------------K-----I--I-A-----.QDL---T----

NLIFIMVESLGTCPCDTRLLIQAEQTSDGPDPCDMVK
Q-------RK-H--------------------------
Q-------RK-H--------------------------
--------R--------------G-------------
T-GS-------D--------M-----A-------S---
```

Figure 4.14
Comparison of partial amino acid sequences of α2 VGCC subunits amplified at high stringency from rodent tissues with the rabbit skeletal muscle α2 subunit.
PCR primers in bold
2nd membrane spanning region underlined

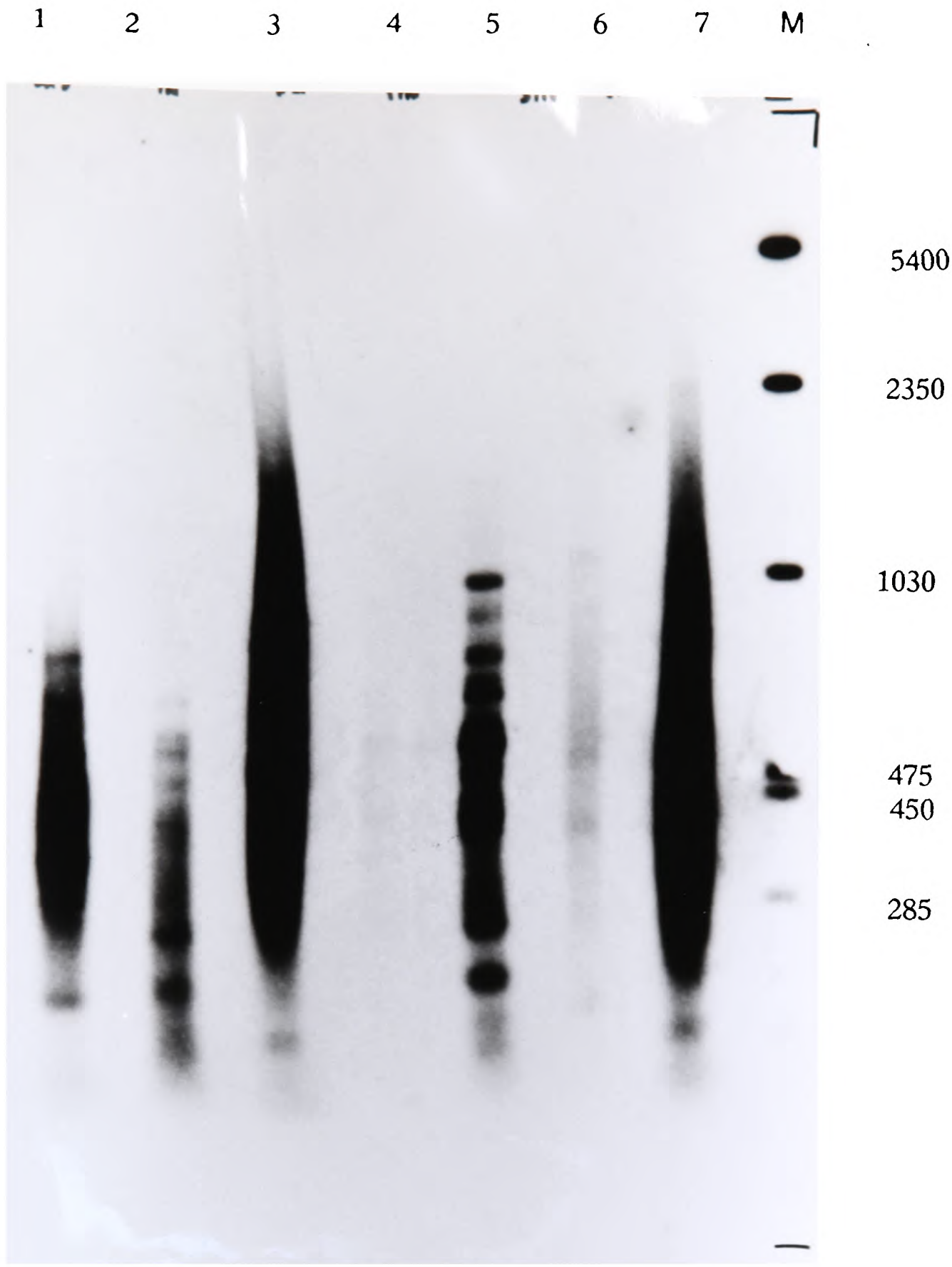


Figure 4.15

Southern blot of mouse brain, lung and rat muscle PCR products amplified with primers to bp 999-1020 and 1977-1998 of the rabbit skeletal muscle $\alpha 2$-δ sequence. Mouse brain [1,2], mouse lung [3,4] and rat muscle [5,6] and human skeletal muscle [7] at 3.5mM and 4.5mM $MgSO_4$ [even lanes]. Markers PM2/*HindIII* digest [M]. Blots were hybridised at 42^{o}C in 5mls of 6xSSCE, 10x Denhardt's, 100µg/ml herring sperm DNA, 50% formamide with $0.1x10^{6}$cpm/ml rabbit 1000-2000 probe. Filters were washed with 0.2xSSC at 70^{o}C and autoradiographed overnight.

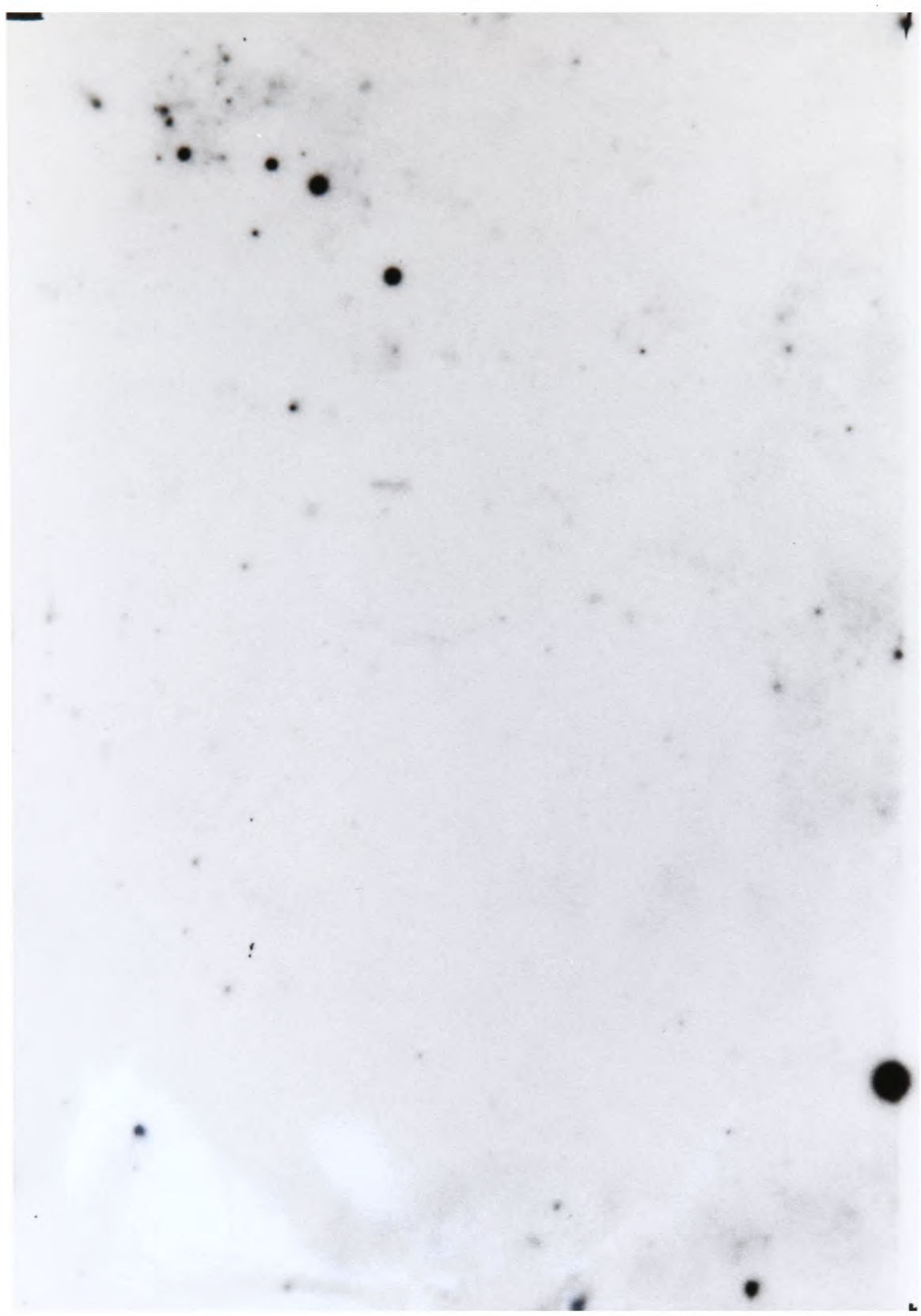


Figure 4.16

Southern blot of PCR amplified DNA homologous to rabbit skeletal muscle $\alpha 2$-δ bp 999-1998 from mouse brain, lung and rat muscle hybridised to a 21bp oligomer to the rabbit $\alpha 2$ first membrane spanning region. The filter was hybridised in 5mls of 6xSSCE, 10xDenhardt's, 100µg/ml herring sperm DNA, and 0.5×10^6cpm/ml probe at 65^0C. The filter was washed with 2xSSC at 40^0C and autoradiographed for 5 days.

4.10 Confirmation of the Sequences

The 3 $\alpha 2$ sequences were analysed by use of the RTDNA to determine the restiction sites present in the fragments. The products were then reamplified with the dNTP cocktail containing 0.25 µl of ^{32}P-α-dCTP per reaction, purified and aliquots were digested with a selection of restriction enzymes used according to manufacturer's instructions. The enzymes chosen were

[1] *HindIII*

[2] *PstI*

[3] *TaqI*

[4] *MspI*

[5] *ScaI*

[6] *HinfI*

Results are shown in table 4.3. Products of the digestion were run out on 2% agarose gels.

However, if the sequences were very similar and this technique would not differentiate subtle changes especially with small DNA fragment size differences. Therefore the technique of polymerase chain reaction - single strand conformational polymorphism [PCR-SSCP] mapping was used as this can detect at least 50% of single base changes [Orita *et al*, 1990a].

Source	Restriction Enzyme						
	AluI	MspI	TaqI	HindIII	HinfI	PstI	ScaI
Mouse brain	-	3	3	+	-	+	-
Mouse lung	-	3	3	+	-	+	-
Rat muscle	2	3	3	+	+	+	-
NG108-15	n/a	n/a	n/a	+	-	-	+

Table 4.3

Restriction enzyme analysis of $\alpha 2$ PCR products from various sources

Data on the NG108-15 cDNA predicts restriction sites for the 4 cutters [AluI, MspI, and TaqI] but these have not been formally checked and are therefore labelled not available [n/a].

4.11 PCR-SSCP Mapping

The technique of PCR-SSCP was developed by Orita *et al*, [1990a,b,1991] and relies on the differences in mobility of partially denatured single strand DNA in non-denaturing polyacrylamide gels. It has been used to detect single base pair differences in short PCR fragments in *Alu* repeats [Orita *et al*, 1989] and in a variety of genes [Suzuki *et al*, 1991].

4.12 PCR-SSCP Method

PCR amplification was performed under stringent conditions as stated previously with labelling of both strands by the addition of 0.5 µl of ^{32}P-α-dCTP on the final cycle of the PCR.

Product quality was checked on a minigel and the radiolabelled fragments precipitated after phenol-chloroform extraction. The yield was determined by counting the pellets in a Beckmann series 376 counter set for Cherenkov radiation ^{32}P counting. The yields are shown below:

Rabbit	$\alpha 2$	1.16×10^7/µl
Mouse brain	$\alpha 2$	0.44×10^7/µl
Mouse lung	$\alpha 2$	0.62×10^7/µl
Rat Muscle	$\alpha 2$	0.87×10^7/µl

The pellets were resuspended in TE to give 10^4 cpm/µl.

The non-denaturing gel was poured according to the method of Orita *et al*, [1989]. A 6% polyacylamide-TBE containing 10% glycerol was poured between 40 x 20 cm plates with a spacer thickness of 0.1 cm. The gel was allowed to set and the apparatus was moved to a cold room [4^{0}C]. Two aluminium plates were clamped to both faces

of the gel plates and the apparatus was allowed to equilibriate to ambient temperature.

4 µl of sample was added to 4 µl of sequence gel loading buffer containing 50% formamide [USB, Sequenase kit] and heated to 85^{0}C for 3 minutes to fully denature the sample. 2 µl aliquots were loaded onto the gel from a hot plate so as not to allow them to renature before running into the gel.

The gel was run at 30W for 6 hours with a thermocouple to measure the plate temperature. At no point did this exceed 35^{0}C. The gel was then removed from the apparatus, fixed in 10% methanol-10% acetic acid for 30 minutes and dried under vacuum for 30 minutes at 85^{0}C. The gel was autoradiographed overnight.

4.13 Results

Though the original method recommends a 0.2mm thickness gel early experiments showed that a thicker gel suffered fewer problems from heating and that a 40cm length allowed good resolution of differences between 620mers at 6 hours.

The results of the PCR-SSCP analysis of the 4 $\alpha 2$ PCR fragments is shown in fig 4.17.

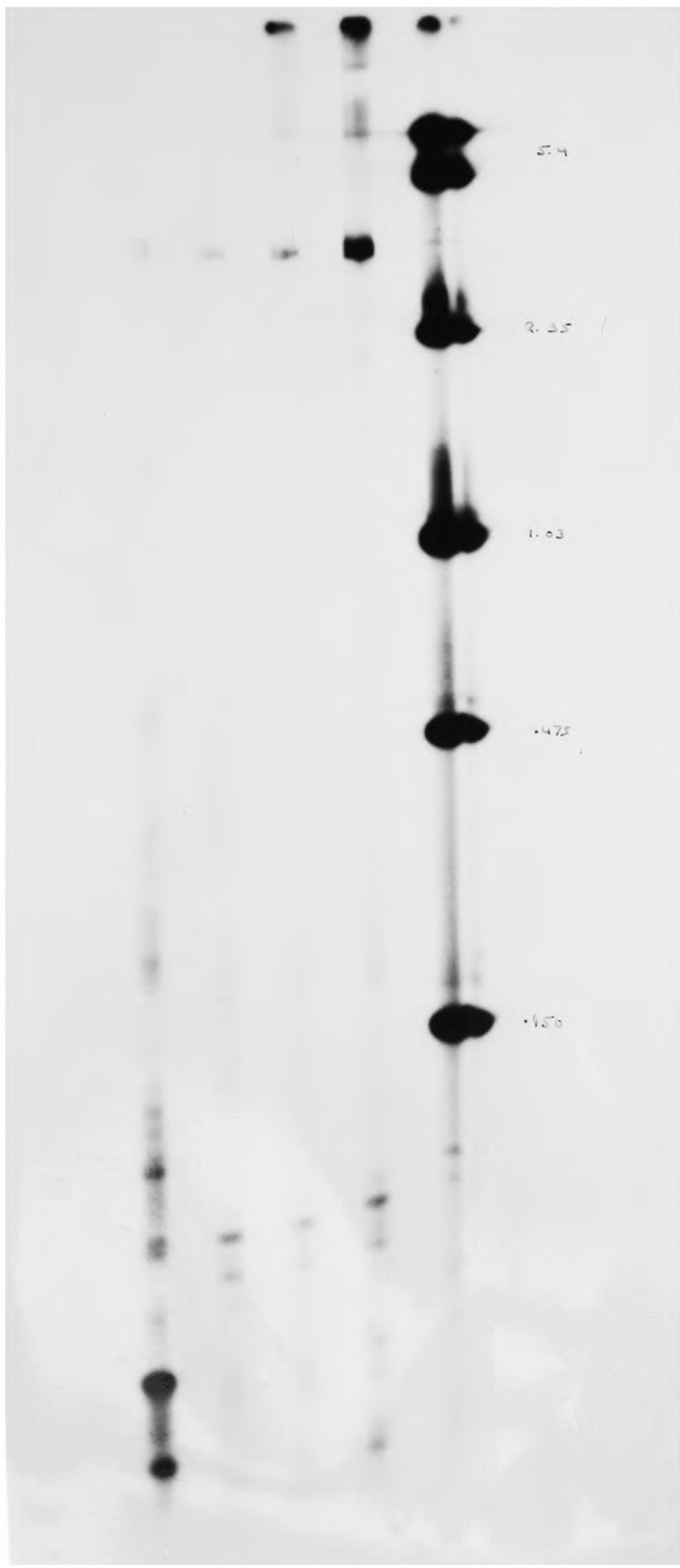


Figure 4.17

PCR-SSCP analysis of $\alpha 2$ PCR products [$4x10^4$ cpm/lane] run a 6% PAGE-TBE/10% glycerol gel run at 15W for 6 hours at4^{0}C. The gel was fixed in 10% acetic acid/10% methanol, dried and autoradiographed for 6 hours. The whole gel is shown as better resolution of differences is visible with the smaller satellite products of PCR amplification.

4.14 Discussion

The original paper describing the sequence of the $\alpha 2$ subunit of the VGCC showed Southern and northern blots that implied that the $\alpha 2$ subunit was universal between tissues [Ellis *et al*, 1989]. However others have noted differences in immunoreactivity and been unable to show the presence of $\alpha 2$ subunits in some tissues that contain VGCCs [Catterall, 1989; Biel *et al*, 1991]. If $\alpha 2$ subunits were identical between tissues it would be expected that PCR amplification under stringent conditions would produce identical bands in all tissues. A similar cross-species PCR using a single set of primers had been used by Arpagaus *et al*, [1991] to probe sequence homologies in tissue butylcholinesterases. A possible problem was that nonfidelity of Taq polymerase relative to *E. coli* DNA polymerase would result in sequence differences being due to Taq errors alone. However, the error rates quoted for Taq polymerase by Arpagaus suggested that this was not significant [12 errors/15000bp over 30 cycles]. Another possible problem was the lack of prior knowledge about sequences complementary to the PCR primers in the template DNA as stringent annealing conditions would be required. However reasonable interspecies homology especially at the 3' end of the primer would still allow amplification to proceed. However some method of reducing nonspecific priming would be desirable. Two approaches were available: calculating an optimised T_m or adding specificity enhancers to the reaction. Most studies rely on an approximation to calculate the annealing temperature for PCR primers. The most commonly used formula is:

$$T_a = 4 (G/C) + 2 (A/T) \ ^oC$$

This formula is approximate and tends to produce non-specific products. However more accurate formulae depend on having a computer program e.g. OLIGO to

determine the annealing temperature - usually by nearest neighbour analysis of the less stable primer pair utilising the Gibbs free energy of helix formation corrected by potassium concentration and for the size and nature of the product [Rychlik *et al*, 1990]. In practice the approximate formula was found to give good results for products under 1 kb.

Other approaches exist to reduce nonspecific priming by the addition of a variety of different molecules to the amplification reaction. These include deionised formamide [Sarkar *et al*, 1990], tetramethylammonium chloride [Hung *et al*, 1990], 7-deaza-2'-deoxyguanosine 5'-triphosphate [McConlogue *et al*, 1988], dimethylsulphoxide [Winship *et al*, 1989] and a variety of patented products. It was decided to use one of the cheaper reagents. Preliminary experiments showed little difference between formamide and DMSO so the latter was used at a concentration of 5% as no deionisation was required.

The results presented here show that $\alpha 2$ products show some variation between tissues though the exact nature of the origin of that variation remains to be determined. This agrees with recent findings by Biel *et al*, [1991] who showed a lack of a hybridising $\alpha 2$ subunit in the BIN52 insulinoma line on northern blots. Other tissues had multiple $\alpha 2$ subunit mRNAs of size 8.5kb and 7.0kb. The full extent of $\alpha 2$ subunit diversity remains to be determined.

Partial sequences have been determined for 2 $\alpha 2$ subunits from mouse [brain and lung] and rat skeletal muscle. These show 88% and 84% homology to the rabbit sequence at nucleotide level and 83% and 79% homology at amino acid level. It is notable that the majority of the changes in the amino acid sequence are conservative. In contrast $\alpha 2$ products obtained by Dr. B. Lang showed greater than 95% nucleotide

homology between rabbit skeletal muscle and human skeletal muscle, [Lang, unpublished]. This is not incompatible with other homology data which show for instance 92.4% homology between rabbit and human butrylcholinesterase but only 86.3% between human and mouse [Arpagaus *et al*, 1990] as species vary in their codon preference for any particular amino acid.

Two methods were used to check the sequences determined given that PCR amplification with Taq polymerase generates errors at 1 in 1000 to 1 in 3000bp [Ehrlich *et al*, 1989]. All sequences were determined from 3 separate pUC8 clones and in both directions to minimise errors. Published sequences may contain an error rate of upto 1- 5 % [Krawetz, 1989] so efforts were made to check sequence predictions by restriction enzyme analysis. Work with restriction fragment length polymorphisms [RFLPs] in genomic digests has shown that only 1 - 5 % of changes are resolved by the deletion or creation of a restriction site and in highly conserved sequences such changes are rare [Cotton, 1989]. In the case of the $\alpha 2$ PCR products changes were rare and often produced only a small change in fragment size and were therefore difficult to resolve. Therefore PCR-SSCP was applied to the PCR products as this method can allow the separation of single base pair changes in short PCR products and can detect upto 10% of sequence alterations in longer fragments [Orita, 1990]. This confirmed that the mouse, rat and rabbit products were different.

Future work in this area would determine the differences over the full length of the $\alpha 2$ gene and this could be used to assess whether alternative splicing did occur or whether there were different gene products and what relevance these differences have to generation of VGCC diversity.

Chapter 5

Cloning the $\alpha 2$-VGCC subunit gene from NG108-15 cells

5.1 Introduction

The characterisation of protein structure by molecular cloning involves the construction of complementary DNA libraries and their screening by a variety of techniques.

The method chosen depends on the initial available experimental data. In the case of cloning VGCCs the possibilities were the use of expression cloning [Frech *et al*, 1989], G-protein homology cloning [Matsuda *et al*, 1987], antibody screening of expression vector libraries [Glover & Hames, 1990] and DNA homology cloning [Glover & Hames, 1990].

An example of expression cloning has been its use in characterising potassium channel isoforms and involves the fractionation of mRNA and injection of individual fractions into *Xenopus* oocytes. The channel containing fraction is detected electrophysiologically, the channel protein is purified, and N-terminal sequenced to provide a probe for screening of cDNA libraries [Jentsch *et al*, 1990]. Although the electrophysiological data were available for VGCCs the techniques were considered too time consuming and also might not identify the relevant subunit. G-protein homology cloning involves screening cDNA libraries with motifs expressing G-protein recognition domains [Kaupp *et al*, 1989] but this would not necessarily result in the cloning of a VGCC. Antibody screening of expression libraries constructed in vectors such as λgt11 depends on unitary recognition by polyclonal or monoclonal sera. The antibodies present in LEMS are probably heterologous in their target specificities and the only monoclonals available are directed against the skeletal muscle form of the VGCC whose amino acid sequemnce and conformation might not be homologous to the neuronal VGCC. Such screening would have involved the use of large amounts of

LEMS sera of definite reproducible specificity but the low patient numbers, low frequency of plasmapheresis, effects of immunosuppression and interpatient variability all suggested that this was not a practical method. However if sequence data is available then screening of cDNA libraries for nucleotide homology is the most efficient method. Two nucleotide sequences of VGCC subunits had been published - the $\alpha 1$ subunit [Tanabe *et al*, 1988] and the $\alpha 2$ subunit [Ellis *et al*, 1989]. Therefore, it was decided to use homology screening of cDNA libraries derived from cell lines whose VGCCs are partially sensitive to LEMS sera antibodies.

5.2 Strategy

The initial idea had been to screen cDNA libraries with oligomer probes derived from the $\alpha 1$ and $\alpha 2$ sequences. These had proved to be unable to bind to southern blots or to rabbit skeletal muscle libraries [Dr. D. Beeson]. Also no specific probes had been isolated by the use of single primer pair PCR on cDNA derived from the NG108-15 or F2.1D1 cell lines. Therefore it was decided to make cDNA libraries from NG108-15 cells and from F2.1D1 cells and to screen these with PCR product probes derived from the rabbit skeletal muscle $\alpha 1$ and $\alpha 2$ genes.

5.3 Methods and results - introduction

cDNA libraries are constructed by reverse transcribing purified mRNA, converting the single strand to double strands by the use of DNA polymerase and ligating the insert cDNA in to λgt10 [for hybridisation screening] or λgt11 [antibody and hybridisation screening] by the use of linker or adaptor molecules. The latter serve to increase the efficiency of cloning to around 20% . More recently newer phagemid vectors have become available e.g. λ-ZAP that include origin sequences for plasmids e.g. pBluescript and M13 single strands in the 'stuffer' section flanking the polylinker and obviate the need for subcloning.

The construction of λgt10 libraries was the technique used in the laboratory so this was chosen as the vector of choice for the initial cDNA library.

5.4 Isolation of mRNA

RNA was prepared from 2 flasks of NG108-15 cells or F2.1D1 cells by the guanidium isothiocyanate/caesium chloride technique and by the acid guanidinium/phenol/chloroform technique. The quality of the total RNA was assessed by quantitating the yield and visually examining the quality on a gel. The mRNA was purified by the use of 2 rounds of oligo-dT chromatography and the quality of the resultant mRNA assessed visually on a gel, by northern blots and by checking its purity in *in vitro* expression studies using rabbit reticulocyte lysate.

5.5 Comparison of RNA isolation techniques

Beeson [1987] had previously compared 4 RNA isolation techniques - hot phenol, proteinase k, lithium chloride, guanidinium isothiocyanate/ caesium chloride and found that the caesium chloride cushion technique gave the best results. The comparison was repeated using the best previous method and the new AGPC technique.

2 flasks of cells were taken and total RNA was prepared by both techniques as described in chapter 2. The yield and quality were assessed spectrophotometrically [table 5.1] and then 50μg aliquots of total RNA were run out on a 1% MOPS-formaldehyde-agarose gel.

5.6 Electrophoresis of RNA and northern blotting

All solutions and glassware were treated to reduce or remove RNAase activity as described in chapter 2. The protocol used for RNA electrophoresis used is that of Lehrach *et al* [1989] as modified by Fourney *et al* [1990].

Agarose gel solution was prepared in a DEPC/baked glass flask:

agarose 3g

DEPC treated H_2O 260mls

The agarose was dissolved by boiling the solution in a microwave oven. The agarose solution was allowed to cool to 60^0c and 30mls of 10xMOPs [200mM MOPS pH 7.0, 50mM sodium acetate, 10mM EDTA] buffer was added followed by 5 mls of formaldehyde [37% by volume, BDH]. The solution was mixed and poured into a BRL electrophoresis tray that had been cleaned with 1% SDS and was reserved for RNA electrophoresis. The gel was allowed to set for one hour.

Samples were prepared by the following technique:

5µl of RNA solution was added to 25µl of fresh RNA sample buffer:

RNA sample buffer:

750 µl deionised formamide

240 µl formaldehyde

150 µl 10 x MOPS pH 7.0

80 µl 10% bromophenol blue

100 µl dH_2O

100 µl 80% glycerol [analar grade]

All aqueous solutions had been DEPC treated and autoclaved as had the glycerol which which was made up as a 80% solution.

The RNA sample was heated at 65^{0}C for 5 minutes and then cooled on ice. Before loading 1ul of 1mg/ml EtBr solution was added to each sample.

Gels were run unsubmerged at 100V for 3 hours or 30V overnight.

Fully run gels were soaked in 50mM NaOH/50mM NaCl for 5 minutes, followed by 10 x SSC for 10 minutes before being blotted onto nylon filters with 10 x SSC. Blotted filters were fixed by UV irradiation at 302nm for 2 minutes followed by baking for 2 hours at 85^{0}C. Filters were hybridised under the same conditions as Southern blots except for the addition of 1/10 volume of 10% dextran sulphate to the prehybridisation and hybridisation solutions.

The quality comparison between the two methods of RNA preparation is shown in fig 5.1. Initial experiments did not show any binding of α1 or α2 probes to the filters.

Method	Yield	Purity [O.D. 260/280 ratio]
GTC/CsCl	3530	1.9
AGPC	4200	1.8

Table 5.1

Comparison of the amount and purity of total RNA isolated by the guanidinium isothiocyanate/caesium chloride and AGPC techniques from one $75cm^2$ flask of frozen cells.

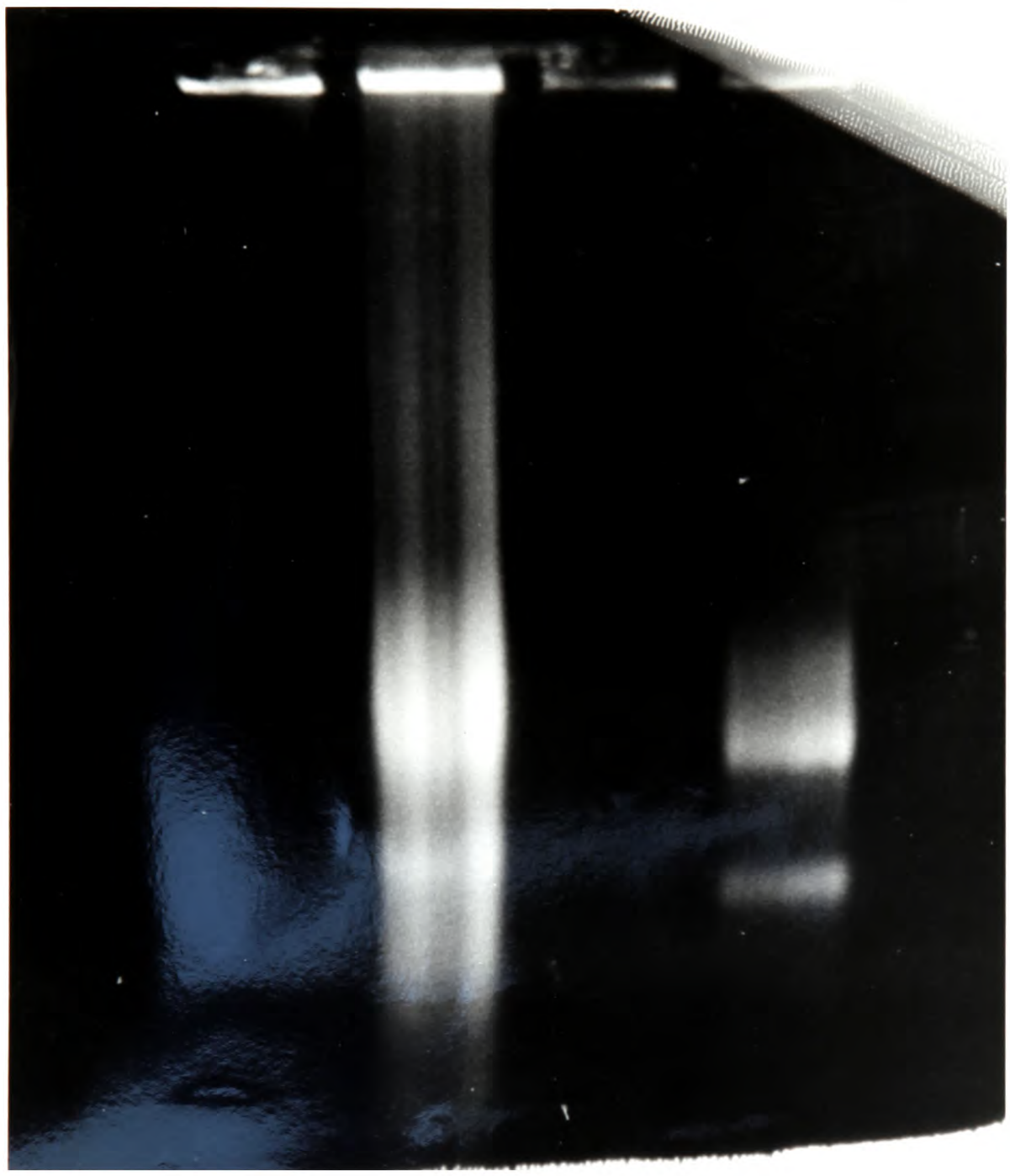


Figure 5.1

Comparison of RNA prepared by the guanidinium isothiocyanate/caesium chloride and AGPC techniques. 10µg of total RNA were electrophoresed on a 1% agarose/MOPS/formaldehyde gel. The RNA was stained with ethidium bromide present in the sample buffer [1mg/ml]

5.7 Translation of mRNA

A further quality control on the mRNA produced by these methods was its effectiveness in the rabbit reticulocyte lysate system as a substrate for *in vitro* translation.

The method used was that recommended by the manufacturer's of the BRL translation system:

mRNA was dissolved in 10mM HEPES pH 7.0 at a concentration of 100ng/µl. The solution was heat denatured at 65^{0}C for 2 minutes and cooled on ice.

25 µl aliquots of rabbit reticulocyte lysate were thawed on ice from -70^{0}C. These contained the lysate, hemin, phosphocreatine, phosphocreatine kinase, tRNAs and other modulating factors, 5mM $MgCl_2$, and 50mM KCl.

2.5 µl of amino acids minus methionine

1.0 µl ^{35}S-methionine

1.0 µl of 30mM cAMP [optional]

were added to the lysate followed by the denatured RNA [1µl]. The lysate was mixed gently and incubated at 30^{0}C for 30 minutes.

The ^{35}S-methionine was stored in individual aliquots at -70^{0}C from the time of its arrival as oxidation of the amino acid has been found to inhibit lysate translation. cAMP was added under the manufacturer's recommendations [Promega] to deinhibit initiation factor II [eIF-2] which may be inhibited by the presence of double stranded RNA [Berger & Kimmel, 1990].

The reaction was stopped by the addition of 12 µl of 3 x FSB.

Sample buffer [3xFSB]:

10 ml H_2O

3 ml ß-mercaptoethanol [6%]

20 mls 10% SDS [4%]

6 mls 1M Tris-Cl pH 6.8 [120mM]

1 ml Bromophenol blue

10 mls glycerol

The protein was denatured by boiling the tubes for 1 minute and removing the pellet by centrifugation. 1µl of supernatant was run on a 12.5% denaturing SDS-PAGE gel [as in the method of Laemmli *et al*, 1970].

Gel plates and 1 mm spacers were cleaned as described for sequencing plates. The gel solution was made up as follows:

8 mls 30% acrylamide, 0.2% bis-acrylamide [Sequagel]

7.5 mls Tris pH 8.8

200 µl 10% SDS

150 µl 10% ammonium persulphate [AMPS]

10 µl N,N,N',N'-tetramethylethylenediamine [TEMED]

The gel was poured and allowed to set under cover of water saturated butan-1-ol. After the running gel had set a stacking gel was poured on top and allowed to set:

Stacking gel: 550 µl acrylamide solution

625 µl Tris-Cl pH 6.8

3775 µl H_2O

50 µl 10% SDS

38 µl 10% AMPS

5 µl TEMED

The comb was inserted and the gel allowed to set. The gel was set up, wells were washed out and the gel was run in 50mM Tris-Cl pH 8.8/ 50mM glycine buffer at 250V for 2-3 hours.

The gel plates were separated and the gel was fixed in 10% acetic acid/10% methanol for 30 minutes. The gel was then soaked in fluorography reagent [Amplify^{tm} , Amersham Ltd] for 15 minutes and dried under vacuum at 80^{o}C for 1 hour. The gel was exposed directly to film with intensifying screens for 3 days.

Controls used in every lysate experiment were:

[a] no RNA

[b] globin mRNA- expected size 16kDa

[c] BMV virus RNA- expected size 109kDa, 94kDa, 35kDa, 20kDa, and 15kDa

[d] ^{14}C-labelled protein markers [Amersham Ltd]

Results with the lysate are shown in fig 5.2.

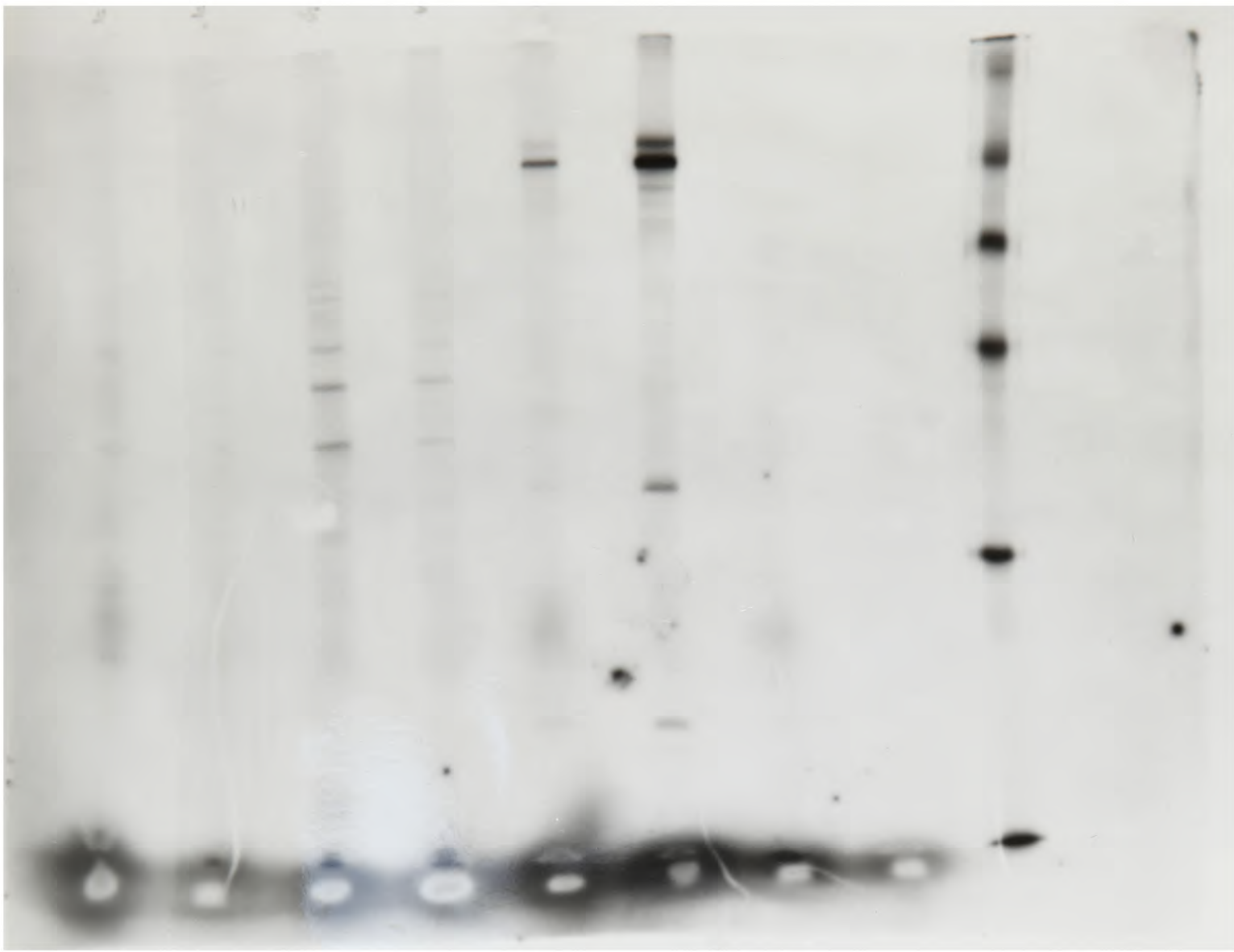


Figure 5.2

Reticulocyte lysate translation of mRNA prepared by the AGPC technique over a 30 minute period at 30°C. The reaction contained 100ng of mRNA, 25µl of rabbit reticulocyte lysate [BRL Ltd], 2.5µl of amino acids minus methionine, 1µl of ^{35}S-methionine and DEPC treated water to a total volume of 30µl. 1µl of 150mM cAMP was added to some tubes.

Sample: No RNA [1]; globin mRNA [2]; BMV RNA [3,4]; NG108-15 mRNA [5,6]; F2.1D1 mRNA [7,8]. All even lanes except lane 2 contain samples incubated in the presence of 5mM cAMP

Both NG108-15 and F2.1D1 mRNA were capable of translation and the presence of cAMP enhanced the translation of all RNAs. Previous work [detailed in Berger & Kimmel, 1989] had shown the importance of the use of fresh thio-amino acid and variation in the magnesium ion and potassium ion concentrations in achieving satisfactory translation. Magnesium titration [1- 5 mM] and potassium titration [30-60 mM] were not found to have a significant effect on the translation of cell line RNA in this study [data not shown] and the use of 5mM $MgCl_2$ and 50mM KCl was adequate. The effect of cAMP in increasing the translation of BMV virus RNA was unexpected as eIF-2 is only supposed to be inhibited by double stranded 5-methyl guanosine capped mRNAs while BMV viral RNA has no cap. Globin mRNA gave a translation product of 16 kD which was difficult to resolve on 12.5% PAGE gels as it ran very close to the front of unincorporated ^{35}S-methionine.

mRNAs produced by both methods was adequate under the criteria of yield, purity, and translation efficiency. It was decided to use the AGPC technique to prepare the RNA as it was the quickest and involved least handling of purified RNA. No antisera were available to the VGCC or its subunits so it was not possible to use this technique combined with western blotting to detect expression of VGCC subunits. It was also thought that the amount of VGCC protein synthesised in this system would be below the detection threshold.

5.8 Size purification of mRNA

The mRNAs for the rabbit α1 and α2 skeletal muscle genes are large [α1 6.5kb, α2 8.8 kb] and previous northern blots had shown them to be relatively rare. Therefore it was decided to enrich the rare mRNAs of interest by selecting only large RNAs [> 5.4 kb] for reverse transcription. Two methods were available to size separate mRNA in quantity and of acceptable quality:

[a] oligo-dT coupled DBT paper

[b] sucrose density gradient centrifugation.

These methods were compared for their efficiency in separating mRNA by size and yielding large quantities of purified mRNA.

[a] a standard 1% MOPS-formaldhyde-agarose gel was run containing EtBr adducted RNA. The gel region containing RNA greater than 28S rRNA [> 5.4 kb] was cut out and blotted onto oligo-dT paper [Amersham] using 0.5M KCl/ 10mM HEPES pH 7.0 overnight. The mRNA was eluted by immersing the strip of oligo-dT paper in 10mM HEPES at 56^{0}C. The mRNA was precipitated with NaOAc/ ethanol and the yield quantitated using the $O.D._{260}$. The gel fragment used for blotting was illuminated under UV light and compared with a series of RNA standards mixed with 1mg/ml ethidium bromide.

[b] a continuous 5 - 30% sucrose gradient was poured in a polyallomer ultracentrifuge tube as follows:

10 ml aliquots were made of sucrose steps by mixing in a sterile tube:

1 ml 10 x gradient buffer:

[100mM HEPES pH 7.0, 0.1M EDTA pH 8.0,

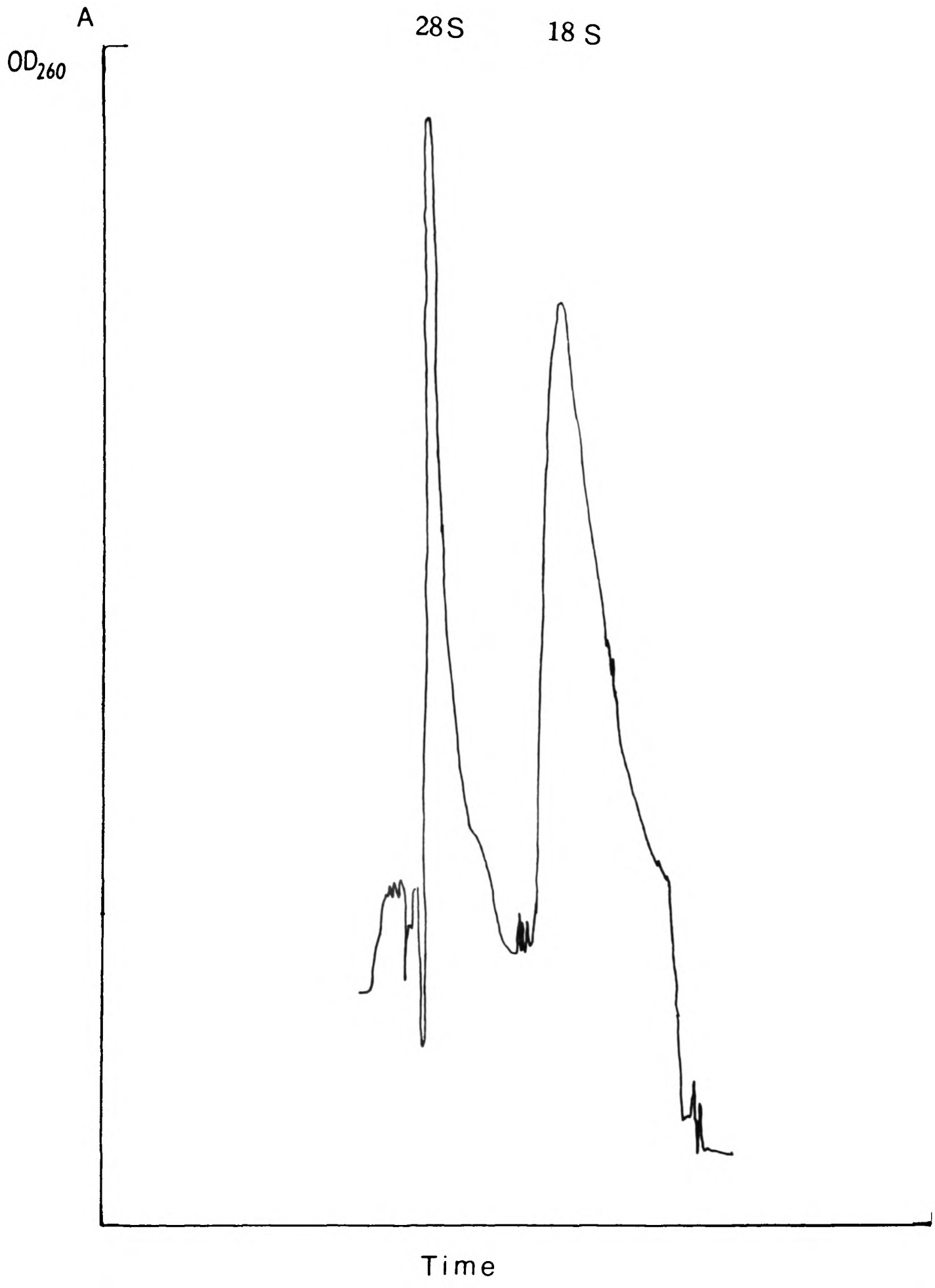
1% lithium dodecyl sulphate]

1-6 mls 50 % sucrose [molecular biology grade] solution [nonautoclaved]

7-3 mls H_2O

1.5 ml steps were layered into the polyallomer tube [Beckman] in decreasing order of density. The tube and and an identical balance tube were allowed to partially equilibriate overnight at 4^{o}C. mRNA was denatured before loading by heating to 65^{0}c for 3 minutes and cooling on ice. The mRNA was added to 2 mls of 5% sucrose gradient solution and layered on the top of the gradient. The gradient was centrifuged for 16 hours at 40000rpm at 4^{o}C in a Beckmann SW 41 rotor. The centrifuge was allowed to spin down without any brake and the gradient pumped off using an LKB-Pharmacia peristaltic pump at a rate of 0.1mls per minute using a 18G needle inserted at the base of the tube. The RNA flow was monitored using a Uvicord spectrophotometer set to 260nm [LKB-Pharmacia] in series with the pump [fig 5.3]. 0.2 ml aliquots were collected and precipitated with 3M NaOAc/ethanol.

The oligo-dT paper showed a significant residue of mRNA was left in the gel [fig 5.4] and the yield of RNA eluted from the paper was 4.8 μg. The sucrose density gradient gave a far greater yield of 10 μg from the original 200 μg of mRNA. It was decided to use the gradient fractionation method for library preparation.


A
OD_{260}
28 S
18 S
Time

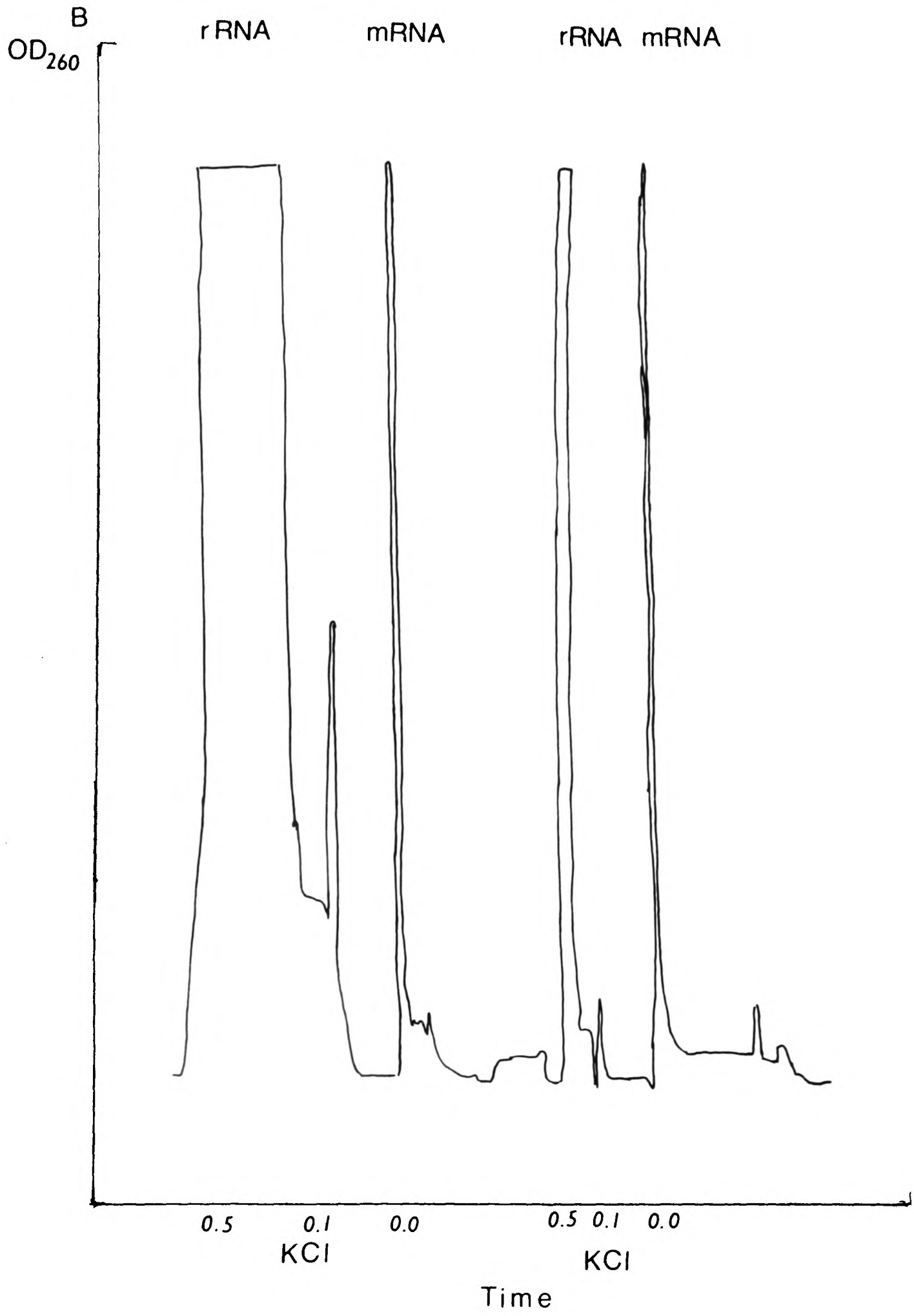

B
OD_{260}
rRNA
mRNA
rRNA
mRNA
0.5
0.1
0.0
KCl
0.5
0.1
0.0
KCl
Time

Figure 5.3

Size fractionation of mRNA by sucrose gradient centrifugation. 200μg of mRNA was loaded on 5-30% continuous sucrose gradient and centrifuged at 40000rpm for 24 hours. RNA was pumped off from the bottom of the gradient and detected by its absorbance at 260nm [a]. The elution profile of total RNA during 2 rounds of oligo-dT chromatography is shown for comparison [b]. 2mg of denatured total RNA in $1cm^3$ of 10mM HEPES pH 7.0, 0.5M KCl was loaded onto a 10cm oligo-dT column [bed volume $3cm^3$] equlilibriated in 10mM HEPES pH 7.0, 0.5 M KCl. Unbound RNA eluted with 10mM HEPES pH 7.0, 0.5M KCl [10 volumes] and then 10mM HEPES pH 7.0, 0.1M KCl [10 volumes]. The poly(A)$^+$ RNA was eluted from the column with 10mM HEPES pH 7.0 [1volume] at 37^0C.

rRNA= ribosomal RNA mRNA= messenger RNA

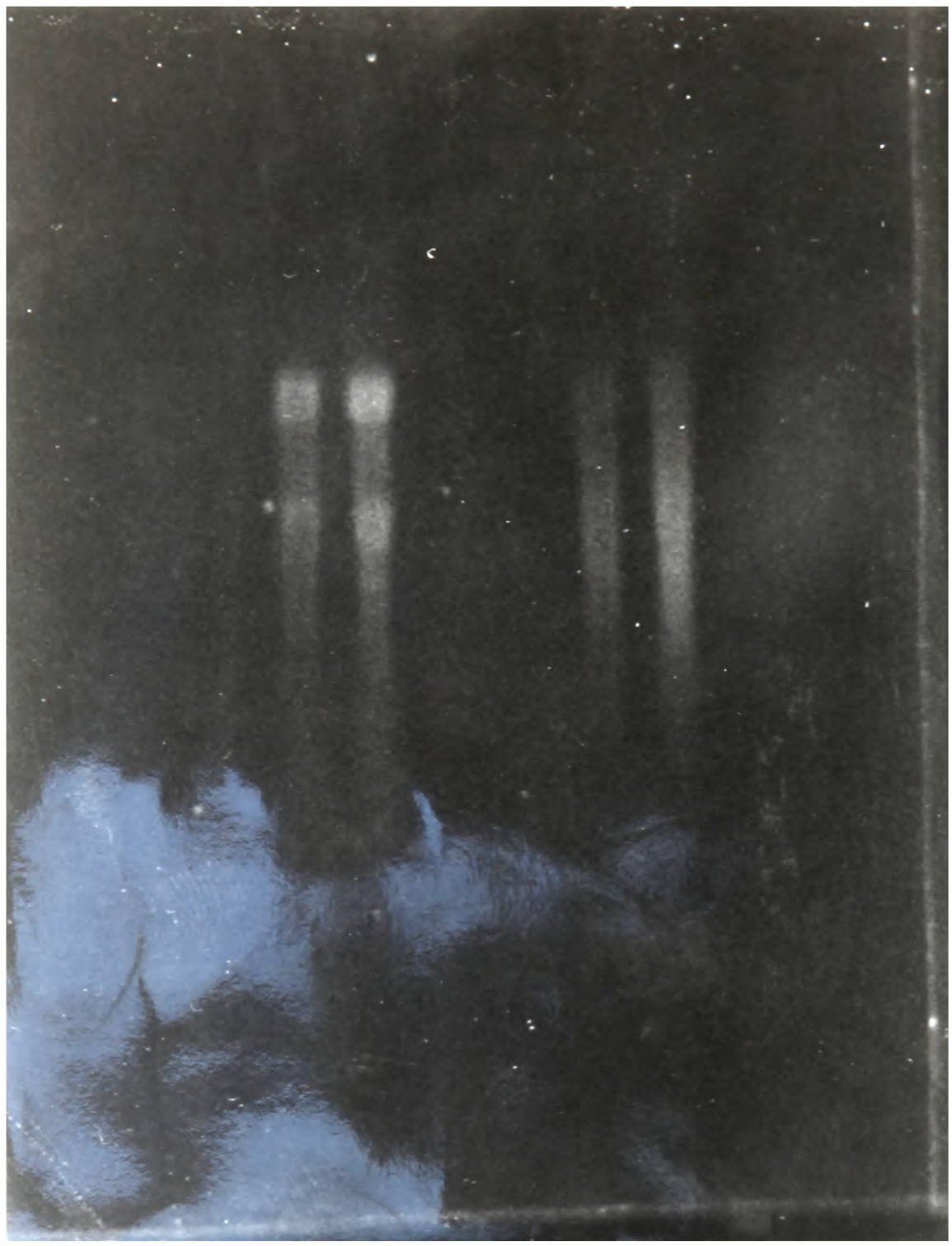


Figure 5.4

Oligo-dT coupled paper purification of 200μg of RNA was attempted on a 1% agarose/MOPS/formaldehyde gel. The gel was blotted overnight to transfer the RNA and the RNA residue on the gel was stained with 0.1 mg/ml ethidium bromide.

5.10 Identification of the $\alpha 2$ fraction

The RNA aliquots from the gradient were resuspended in 50 µl of 10mM HEPES pH 7.0 and 10 µl were used for RNA dot blotting to identify the fraction for library construction.

10 µl duplicate samples of mRNA were mixed with 20 µl of 10 x SSC and 15 µl of formaldehyde and were dot blotted using a vacuum in a BRL dot blotting apparatus onto wetted hybond-N [Amersham] lying on 2 sheets of dry Whatmann 3MM paper. The filter was washed with 3 rinses of 10 x SSC and then baked at 65^{0}C for 2 hours. The filters were hybridised with 10^{6} cpm/ml of rabbit $\alpha 1$ and $\alpha 2$ probes respectively and washed with 5 x SSC at room temperature until there was no detectable background. The filters were exposed for autoradiography with intensifying screens for 5 days. The other 10 µl was treated similarly but was probed with labelled 28S RNA and autoradiographed overnight.

No hybridisation was observed with the $\alpha 1$ probe at all and only the NG108-15 filter bound the $\alpha 2$ probe [fig 5.5]. The fraction with the most intense binding [fraction 6] was used to make cDNA. The remaining fractions greater than 5.4 kb in size were pooled for each cell line and used to make auxiliary libraries on the assumption that a rare mRNA might be cloned but not be present in sufficient quantity to produce a signal on a dot blot in 24 hours.

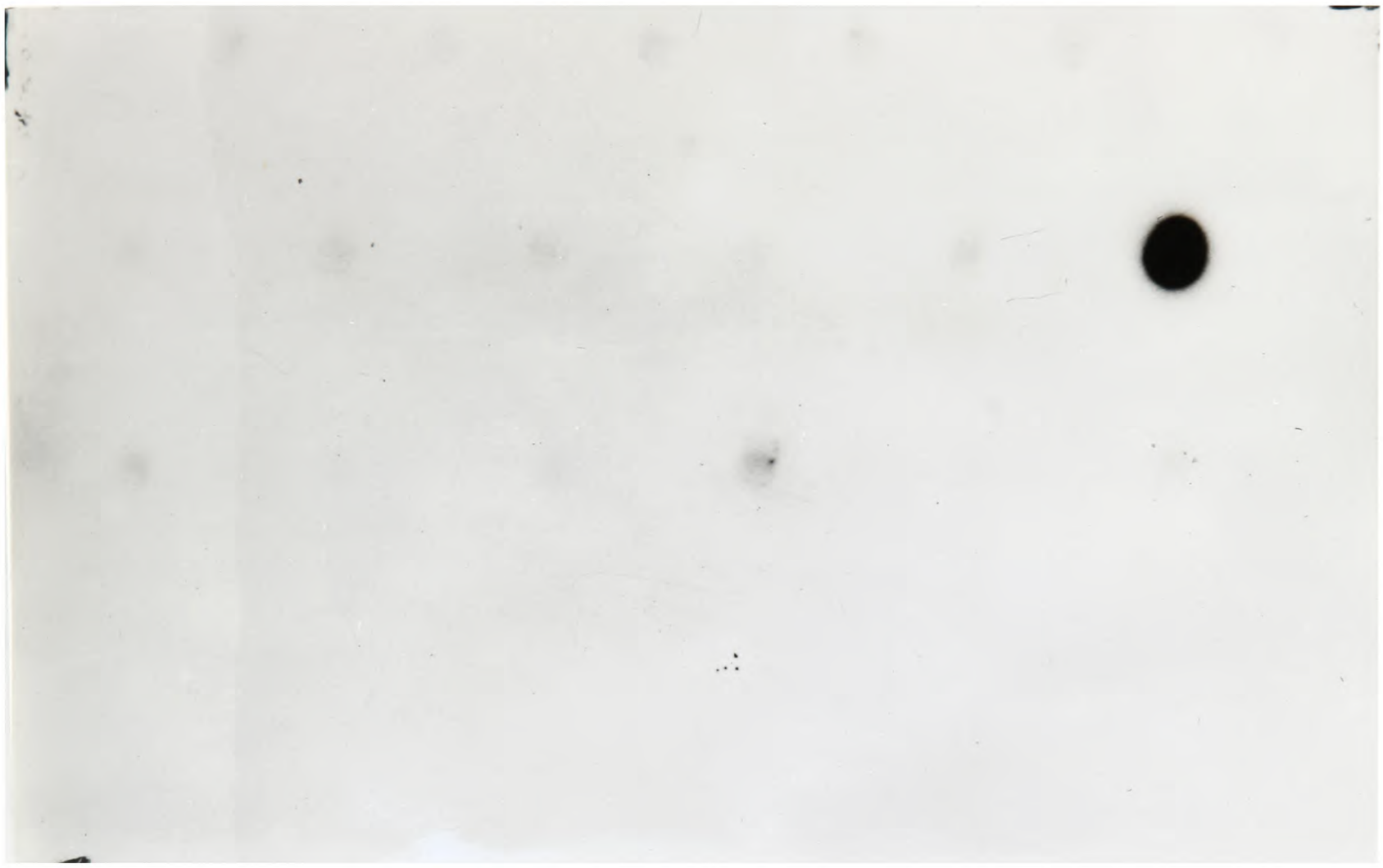


Figure 5.5

Northern dot blot of mRNA fractions from a sucrose gradient. 1/20 of the sample was added to 1 volume 15xSSC, 30% formaldehyde and vacuum blotted on to hybond-N. The filter was baked and hybridised in 5mls 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8, 50% formamide at 42^{o}C with $0.5x10^{6}$ cpm/ml rabbit α2-δ probe. The filter was washed with 2xSSC at 50^{o}C and autoradiographed for 5 days. A positive control duplicate filter was hybridised with 28S and 18S RNA probes. All dots contained varying quantities of ribosomal RNA [data not shown].

5.10 cDNA synthesis

cDNA synthesis was performed by standard techniques [chapter 4]. mRNA was denatured by heating at 65^{o}C for 3 minutes then cooled on ice. The reaction mix was made up as follows:

5 µl 10x cDNA(1) buffer [500mM Tris-Cl pH 8.3 at 22^{o}C,

750mM KCl, 1M DTT, 30mM $MgCl_2$]

5 µl 5mM dNTPs [Pharmacia]

1 µl primer [200 ng/µl]

RNA was added at a concentration of 100 ng/µl [upto 2.5 µg] and the volume made upto 48 µl with H_2O. 2 µl [20 U] of RNase H (-) moloney leukaemia virus reverse transcriptase [Superscript^{tm}] was added. 10 µl of the reaction mix was immediately removed and added to a tube containing 0.5 µl of ^{32}P-α- dCTP. Both reactions were incubated in parallel at 37^{o}C for 1 hour. 1 µl of the labelled reaction was kept for alkaline agarose electrophoresis.

Second strand synthesis was performed in the same tube by the addition of:

32 µl of cDNA (2) buffer [250mM Tris-Cl pH 8.3, 1M KCl,

50mM $MgCl_2$, 50mM DTT]

16 µl of 5mM dNTPs

1 µl of ^{32}P-α-dCTP

10 µl [250 U] *E. coli* DNA polymerase I [BRL Ltd]

1 µl [8.5 U] RNase H [BRL Ltd]

The reaction was incubated for 2 hours at 16^{o}C.

The ends of the cDNA were polished by the addition of 2 µl [20U] of T4 DNA polymerase and incubation at 37^{o}C for 15 minutes. The reaction was stopped by the

addition of 10 µl of 500mM EDTA pH 8.0. The cDNA was purified by phenol-chloroform, chloroform extraction and precipitated with NaOAc/ethanol. The pellet was spun down, washed with 70% ethanol and resuspended in TE at a concentration of 0.5 µg/µl.

5.11 Calculation of cDNA yield

The yield of cDNA was calculated by calculating the percentage of ^{32}P-α-dCTP incorporated as an analogue of nonradioactive incorporation in to the cDNA and then working out the yield in nanomoles of cDNA and converting this to nanogrammes.

cDNA = { % ^{32}P-α-dCTP/ 100 } x moles of dCTP x RMM of dsDNA/base

Results of cDNA syntheses are shown in table 5.2 along with the calculated yields. The yield of cDNA synthesised by this technique was typically 30% of the original amount of mRNA and generally 1 µg of cDNA was obtained from each reaction. This was approximately the amount expected [Amersham cDNA synthesis kit: manufacturer's protocol].

Cell Source	RNA amount	cDNA yield	% yield
	µg	ng	
NG/hexamer	10	5282	52.5
NG/α2 primer	5	651	13.0
NG/oligo dT	5	421	8.5
Mouse brain hexamer	10	6646	65.0
Mouse lung hexamer	5	1077	21.7
Rat muscle hexamer	5	990	19.8

Table 5.2

Yields of second strand cDNA from a variety of synthesis reactions

5.12 Alkaline gel electrophoresis

1% agarose gels were poured in 50mM Tris-Cl pH 7.5, 50mM NaCl, 1mM EDTA and soaked in 50mM NaOH, 50mM NaCl, 1mM EDTA for 30 minutes to form the alkaline gel.

The sample [1 µl] for running in the gel was dissolved in alkaline elctrophoresis sample buffer:

10 x sample buffer: 500mM NaOH

500mM NaCl

1% bromophenol blue

The alkaline agarose gel was run in 50mM NaCl, 50mM NaOH at 30V for 4 hours. The gel was dried under vacuum for 30 minutes at 85^{0}C onto hybond-N and the gel exposed for autoradiography for 4 hours.

The results of a typical cDNA synthesis are shown in fig 5.6. cDNA synthesis under these conditions showing single strand upto 7kb in size was considered acceptable.

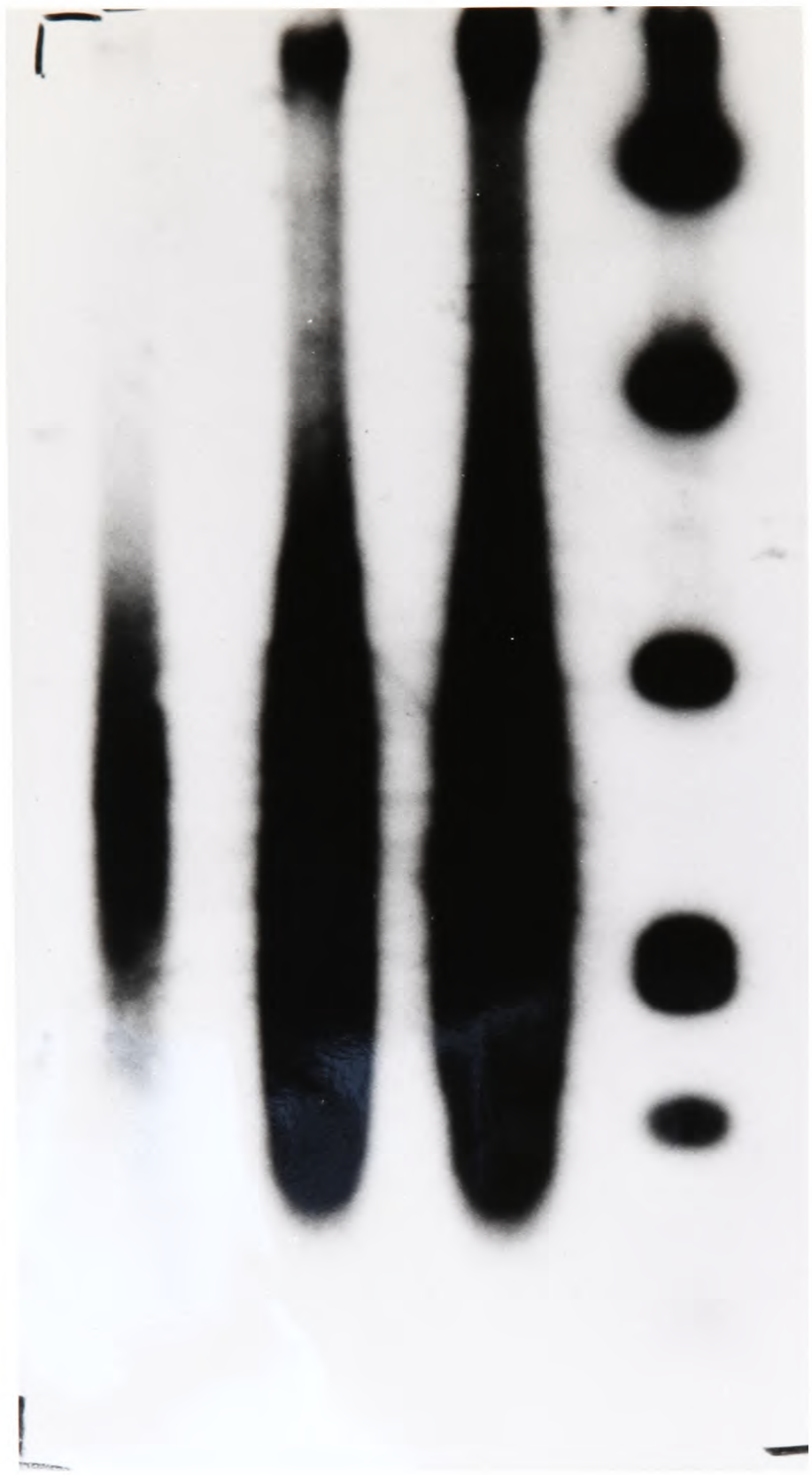


Figure 5.6

Alkaline gel electrophoresis of labelled 2nd strand cDNA. cDNA was labelled on 2nd strand synthesis and 0.1µl were electrophoresed on a 1% alkaline agarose gel, dried and autoradiographed overnight.

5.13 Methylation of cDNA

The preferred method for cDNA ligation into the λgt10 vector was the use of kinased ecoR1 linkers. This is thought slightly preferable to the use of phosphorylated adaptors [Berger & Kimmel 1989] though in practice little difference was found. The inserts were methylated using *ecoR1* methylase to prevent digestion of internal *EcoR1* sites in the cDNA after linker ligation [*vide infra*].

The cDNA was resuspended at a concentration of 100 ng/μl in TE.

A 50 μl methylation reaction was set up as follows:

45 μl	cDNA in TE
2.5 μl	S-adenosyl methionine
2.5 μl	*E. coli EcoR1* methylase [25 U]

The reaction was incubated at 37^{0}C for 30 minutes and the cDNA purified by phenol-chloroform, chloroform extraction and NaOAc/ethanol precipitation.

The success of the reaction was checked by taking an aliquot of cDNA resuspended in TE and digesting it with *EcoR1*:

44 μl	cDNA in TE [0.25 μg]
5 μl	*EcoR1* buffer
1 μl	*EcoR1* [20 U]

The cDNA was digested for 4 hours at 37^{0}C and compared with a digest of an equal amount of unmethylated cDNA on a 1% agarose gel. Satisfactory methylation prevented digestion of the cDNA giving rise to cDNA of greater size in comparison to the unmethylated cDNA as shown in fig 5.7.

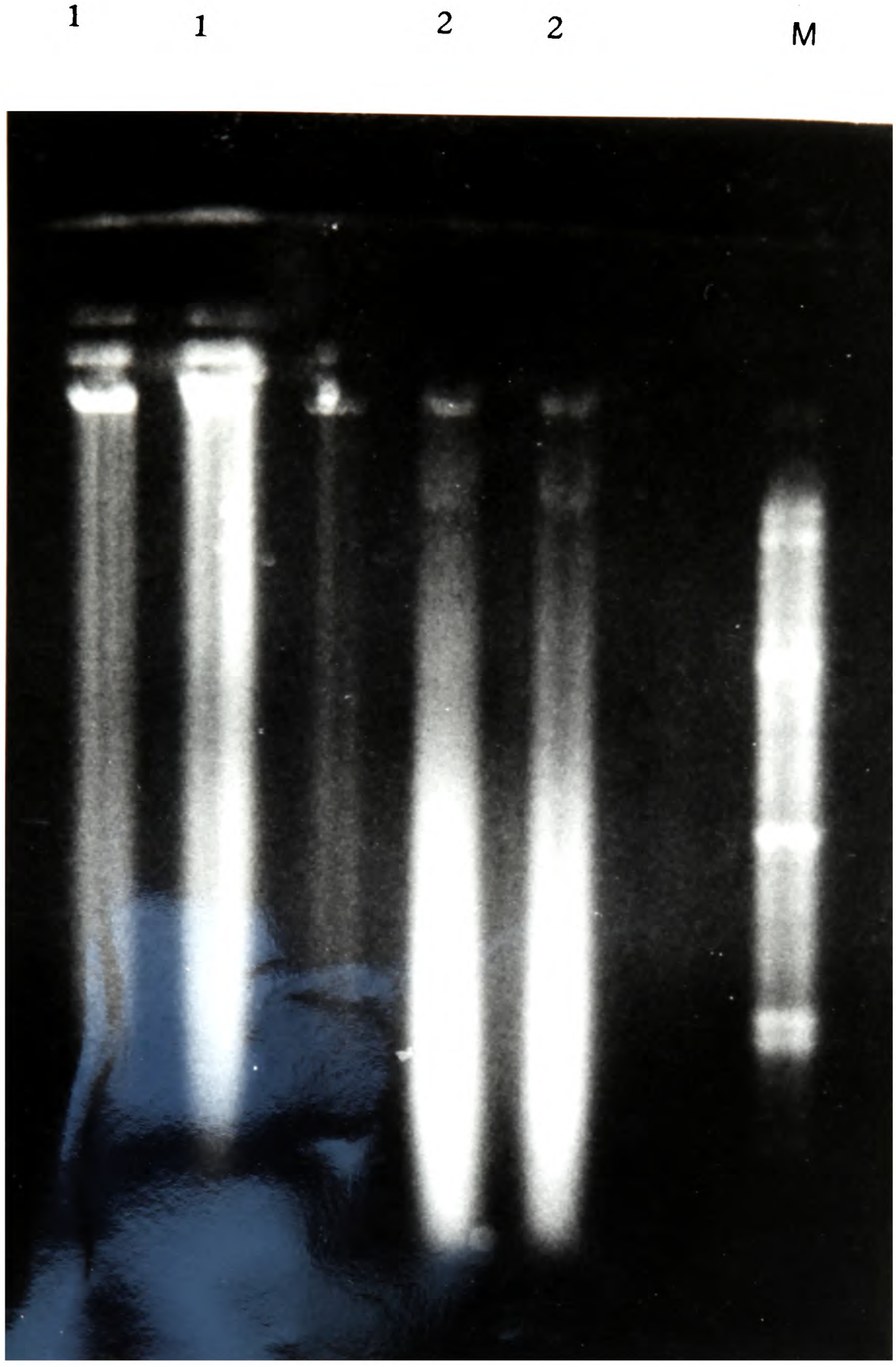


Figure 5.7

Comparison of methylated [lane 1] and unmethylated [lane 2] cDNA digested with 50U *EcoR1* for 2 hours and run out on a 1% agarose gel. Markers [M] = PM2/*HindIII* digest.

5.14 Synthesis of kinased linkers

Linkers are short oligomers of single stranded DNA that when ligated to cDNA generate ecoR1 at the ends of the cDNA molecules. The linkers were prepared as follows:

1 µg *EcoR1* linkers [Pharmacia] were dissolved in 42 µl of 0.1 x TE. The linkers were kinased in the presence of 4 µl of 10mM ATP, 1ul of ^{32}P-α-ATP and 20 U of polynucleotide kinase. The linkers were purified from the protein, precipitated and resuspended in 100 ng aliquots at 100 ng/µl in 0.1 x TE.

The quality of the linkers was assessed by the use of ligation and digestion:

2 aliquots of linkers were self-ligated:

100 ng linkers [1 µl]

7 µl H_2O

2 µl 5x ligase buffer [Amersham]

1 µl T4 ligase

The linkers were self ligated by incubation at 16^{0}C for 16 hours. The ligase was inactivated by heating at 65^{0}C for 10 minutes. One aliquot was digested with 10 U *EcoR1* in a volume of 50 µl for 4 hours while the other aliquot was retained for gel analysis.

A 5% PAGE gel was poured in 1 x TBE buffer between 20 x 15 cm plates with 1mm spacers:

37 mls H_2O

5 mls 10 x TBE

8 mls 30% acrylamide, 0.8% bis-acrylamide

150 µl 10% AMPS

30 µl TEMED

A stacking gel was poured on top of the running gel and the comb inserted:

3.995 mls H_2O

0.5 mls 10 x TBE

0.625 ml 30% acrylamide/ 0.8% bis-acrylamide

38 µl 10% AMPS

5 µl TEMED

The samples of undigested linkers, digested linkers and an unligated aliquot of linkers were dissolved in DNA sample buffer. The 3 samples were loaded onto the gel and the gel was run at 150V for 2 hours. The gel was fixed in 10% acetic acid/ 10% methanol and dried on to hybond-N under vacuum at 85^{0}C for 1 hour. The dried gel was exposed for autoradiography overnight.

The result of a linker quality assay is shown in fig 5.8. Successfully kinased linkers should ligate to form concatemers and be cut to a product of different size on digestion as shown in fig 5.8.

Figure 5.8

A 5% PAGE gel showing unligated linkers [12 bp] [lane 1], concatemers formed on self-ligation [lane 2], and *EcoR1* digested linkers [15bp for *EcoR1* linkers].

5.15 Ligation of cDNA to linkers

The ligation reaction was set up as follows:

200 ng cDNA in 6 µl 0.1 x TE

100 ng kinased ecoR1 linkers [1 µl]

2 µl 5 x ligase buffer [BRL]

1 µl T4 ligase

The reaction was incubated for 16 hours at 16^{0}C. The ligase was inactivated by incubation at 65^{0}C for 10 minutes. Surplus added linkers were digested in a volume of 500 µl with 50 U of *EcoR1* at 37^{0}C for 4 hours. The digest was extracted with phenol-chloroform, chloroform and precipitated with an equal volume of 4M NH_4OAc and precipitated with 2.2 volumes of ethanol. The precipitate was resuspended in 75 µl of 0.1 x TE and linkers and small cDNA were separated by passage down a spun column.

Two forms of spun columns were used: Select 5L [Pharmacia] with an exclusion limit of 297bp and Chromaspin-1000 [Clontech] selecting at 1 kb. The columns were used according to manufacturer's instructions. The column was shaken, the top bung removed and the sepharose allowed to settle before the bottom bung was removed. The column was spun twice at 1800 rpm [1000 g] in a Beckmann LS 32 centrifuge and the eluate discarded. The sample was loaded on to the column and allowed to equilibriate for 1 minute before the columns were spun for 5 minutes at 1000g. The eluate was collected and precipitated. The final precipitate was counted and the amount of remaining cDNA estimated.

The size selected cDNA was resuspended in 0.1 x TE at a concentration of 25 ng/µl.

5.16 Ligation of cDNA to λgt10

Early experiments had shown that λgt10 produced in the laboratory had a lower packagable titre than commercially available λgt10. Difficulties had also been experienced in making dephosphorylated λgt10 arms [D.Beeson, personal communication]. Commercial λgt10 arms were employed in the ligation:

2 µl	λgt10 arms [1 µg] [BRL]
2 µl	cDNA [50 ng]
5 µl	H_2O
2 µl	5 x ligase buffer
1 µl	T4 ligase

The ligation was incubated at 16^0C for 16 hours. The ligation was diluted to 50ul in TE. 4 µl of the ligation reaction was packaged using a commercial packaging reaction kit according to manufacturer's instructions [Amersham Ltd].

5.17 Preparation of competent cells

Two types of competent cells were employed for titration and growing of λgt10 phage. They were C_{600} and C_{600} Hfla. These were prepared by the protocol in Sambrook *et al*, [1990].

5.18 Titration of λgt10

Packaged phage was diluted to a volume of 500 µl in 10mM Tris-Cl pH 7.0 / 10mM $MgSO_4$ after packaging for 2 hours at 20^0C. Serial 10 fold dilutions were made from a 5 µl [titre: 10^{-2}] aliquot to 10^{-6} in 10mM Tris pH 8.0, 10 mM $MgCl_2$. These gave final dilutions of 10^2 to 10^6. Whole dilutions were added to 150 µl of either C_{600} or

C_{600} Hfla cells and incubated at 20°C for 10 minutes. The phage and cells were then mixed with 4 mls of 0.8% top agar and poured onto agar plates. The plaques were grown overnight and titres for each cell type calculated.

No significant difference was found between C_{600} and C_{600} Hfla cells indicating a trivial background due to self-ligated phage [table 5.6]. In all subsequent experiments only C_{600} Hfla cells were used.

The cloning efficiency was calculated by the formula:

$$\text{Cloning efficiency [/}\mu\text{g cDNA]} = \frac{\text{recombinants x 1000}}{\text{ng cDNA used}}$$

Efficiencies were expected to be greater than 10^6 pfu/µg cDNA.

Details of the libraries constructed and the efficiencies of cloning are given in table 5.4.

5.19 Initial library plating and amplification

The whole of the packaged phage was added to C_{600} Hfla cells so that every 50,000 pfu of phage were added to 0.5 mls of cells. The phages were allowed to attach as before and the library was plated using top agarose on agarose 137mm plates and grown for 6-8 hours [partial confluence].

The library was used for direct screening or the phage eluted with 10 mls elution buffer [50mM Tris pH 7.5, 10mM $MgSO_4$, 50mM $Mg[\ OAc\ ]_2$] overnight at 4°C. The library was stored at 4°C in the presence of 1% chloroform.

Library	Titre		Yield	Insert size
	NM514	L87		
	pfu/ml $x10^5$	pfu/ml $x10^5$	/µg cDNA $X10^7$	bp
NG/oligo	180	-		300
NG/hexamer	150	-		350
NG1	78	-	10.4	550
NG2	256	-		1000
NG3	365	-		650
F2.1D1/oligo	120	-		500
F2.1D1/hexamer	320	-		500
F2.1	54	-		500
Mouse brain	780	-		350
Mouse lung	1260	-		350
Rat brain	1350	-		350
λgt10 arms [k]	>800	>800	-	-
λgt10 arms[cip]	3	5	-	-
λgt10 arms/self	50	60	-	-
λgt10/ΦX174	176	183	1.76	-
No DNA	4	5	-	-
No T4 ligase	0	0	-	-

Table 5.3
Yields of cDNA libraries constructed from cell lines and tissues. ΦX174 EcoR1 linked cDNA [2.3 kb] used as control insert. Commercial λgt10 arms used were kinased [k] or used as supplied - treated with calf alkaline phosphatase [cip]. Self made λgt10 arms were made by the plate lysis/ EcoR1 digestion technique. All libraries were made with CIP treated arms.

5.20 Preparation of screening filters

Nylon filters were placed on the surface of the bacterial lawn and lifted off after 30 seconds. The filters were denatured, neutralised and baked under standard conditions [Sambrook *et al*, 1990]. Screening was conducted by standard Southern hybridisation.

5.21 Isolation of positive plaques

Individual positive signals on autoradiographs were aligned with the phage master plates and the plaques picked by punching out plugs of agarose with a pasteur pipette. The phage was eluted from the plugs by incubation with 1 ml of elution buffer [10mM Tris-Cl pH 8.0, 50mM $MgSO_4$, 10mM $Mg(OAC)_2$, 1mM EDTA] overnight at 4^0C. For secondary and tertiary screening 1 µl of the phage solution was diluted in 1ml of elution buffer and 1 µl of that used to infect 150 µl of C_{600} Hfla cells. The secondary and tertiary screens were carried out on 87mm agar plates. This dilution protocol gave approximately 150 - 300 plaques per plate. It was expected that positives should comprise 5 - 10% of plaques on secondary screening. Tertiary screens were expected to be > 50% positives.

5.22 Isolation of λgt10 phage inserts

λgt10 phage preparation was used to assess the average insert size in the library before screening and then to isolate the inserts from positive clones. Two methods were used:

[a] Plate isolation technique

3 agarose plates of phage were grown up with a concentration of phage to give almost confluent lysis. This was determined empirically for each set of phage but 5 - 15 µl of diluted eluate from an individual plaque was usually necessary. The protocol suggested by Amersham Ltd was found to be the best:

The phage was eluted overnight from the plates using a volume of 4 mls elution buffer per 87mm plate. The eluate was precipitated with an equal volume of 2M NaCl / 20% PEG for 1 hour on ice. The phage was spun down at 3000 rpm for 15 minutes and all the supernatant carefully removed. The phage was redissolved in 0.75 mls of TE and an equal volume of a 1:1 volume mix of DE52 cellulose [Whatmann] equilibriated in TE was added. The mix was shaken for 10 minutes and the cellulose spun down. The supernatant [volume about 1ml] was retained and the following were added:

42 µl 10% SDS

2 µl 10 mg/ml proteinase K

The solution was incubated for 15 minutes at room temperature and then 104 µl of 5M KOAc pH 5.0 were added. The solution was heated to 90^{0}C for 20 minutes and cooled on ice. The precipitate was removed by centrifugation [microfuge] and the supernatant extracted with phenol-chloroform, chloroform and precipitated at room temperature with 2.2 volumes of ethanol. The λgt10 DNA was air dried and resuspended in 50 µl TE.

25 µl of phage was digested in a a volume of 50 µl with 10U of *EcoR1* in the presence of 2mM spermidine. The reaction product was run out on a 1% agarose gel to assess insert size [fig 5.9].

If the inserts were small, the ends of the phage were labelled by filling in the digested ends with 2.5mM dNTPs minus dATP and 0.5 µl of ^{32}P-α-dATP. The phage was run out on a gel as before, the gel dried onto a nylon filter and exposed for autoradiography overnight [fig 5.10].

[b] later the PCR based method of Rasmussen *et al* [1989] was used to assess insert size:

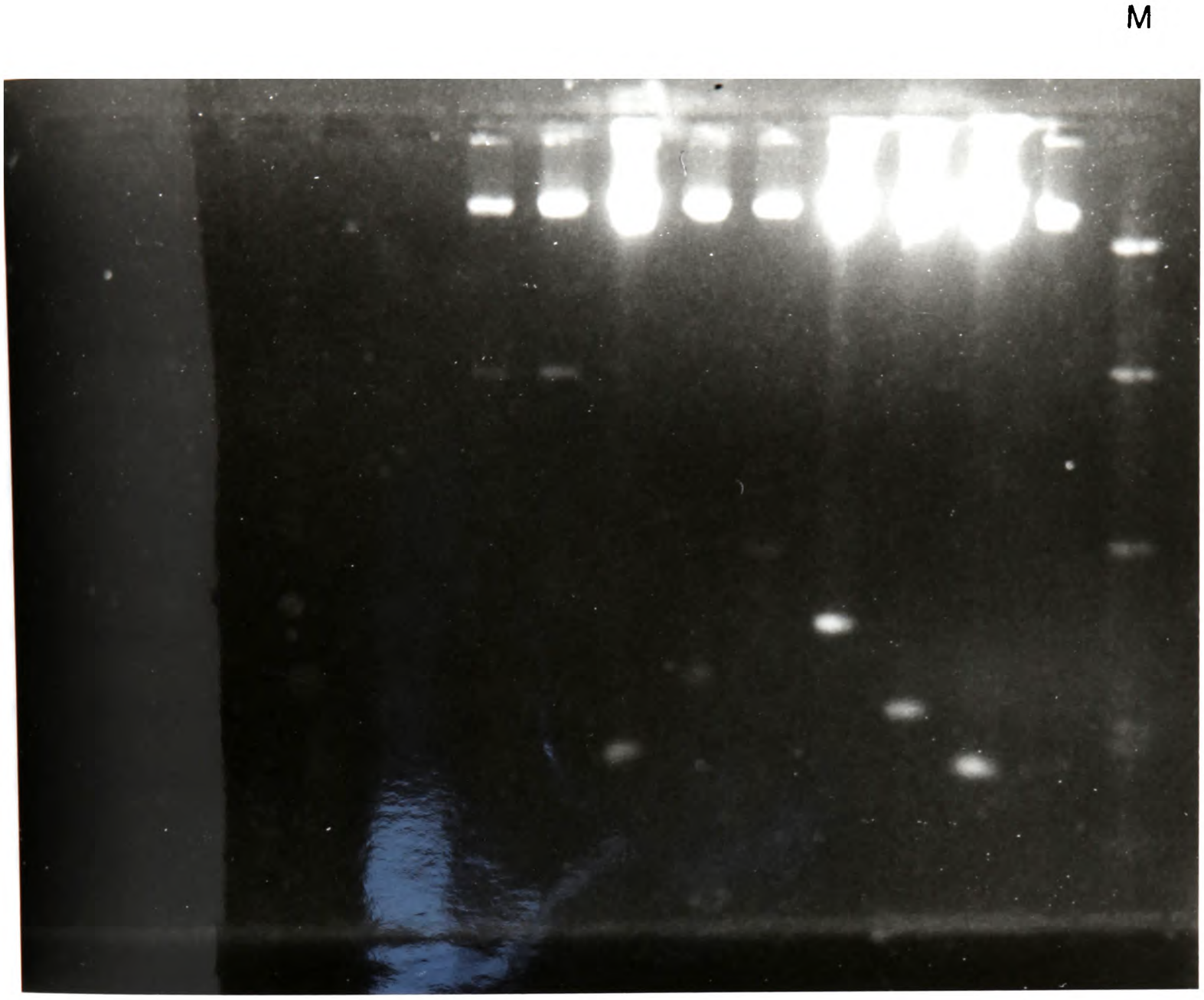
λgt10 phage plaques were picked with a sterile toothpick and the toothpick washed in 10 µl of H_2O. 10 µl of 2 x PCR buffer [20mM Tris-CL pH 8.3, 22mM KCl, 3.2mM $MgCl_2$, 2mM DTT] was added followed by 100 ng of proteinase K and 2 µl 1% SDS. The mix was incubated at 37^{o}C for 1 hour followed by 85^{o}C for 10 minutes. Debris was removed by microcentrifugation for 5 minutes and 4 µl of the supernatant were used in a PCR reaction of 50 µl containing 50 µl 1xPCR buffer, 250 µM dNTPs and 0.5 µg of λgt10 arm primers [New England Biolabs]. The sample was denatured for 2 minutes at 95^{o}C before addition of 0.25 µl [2.5 U] of Taq polymerase. Cycling conditions were:

T_d = 94^{o}C 1 minute

T_a = 55^{o}C 1 minute

T_e = 72^{o}C 3 minutes 25 cycles

Insert size was determined by running 10 µl of reaction mix out on a agarose minigel [fig 5.11].


M
5400
2350
1030
475
450
285

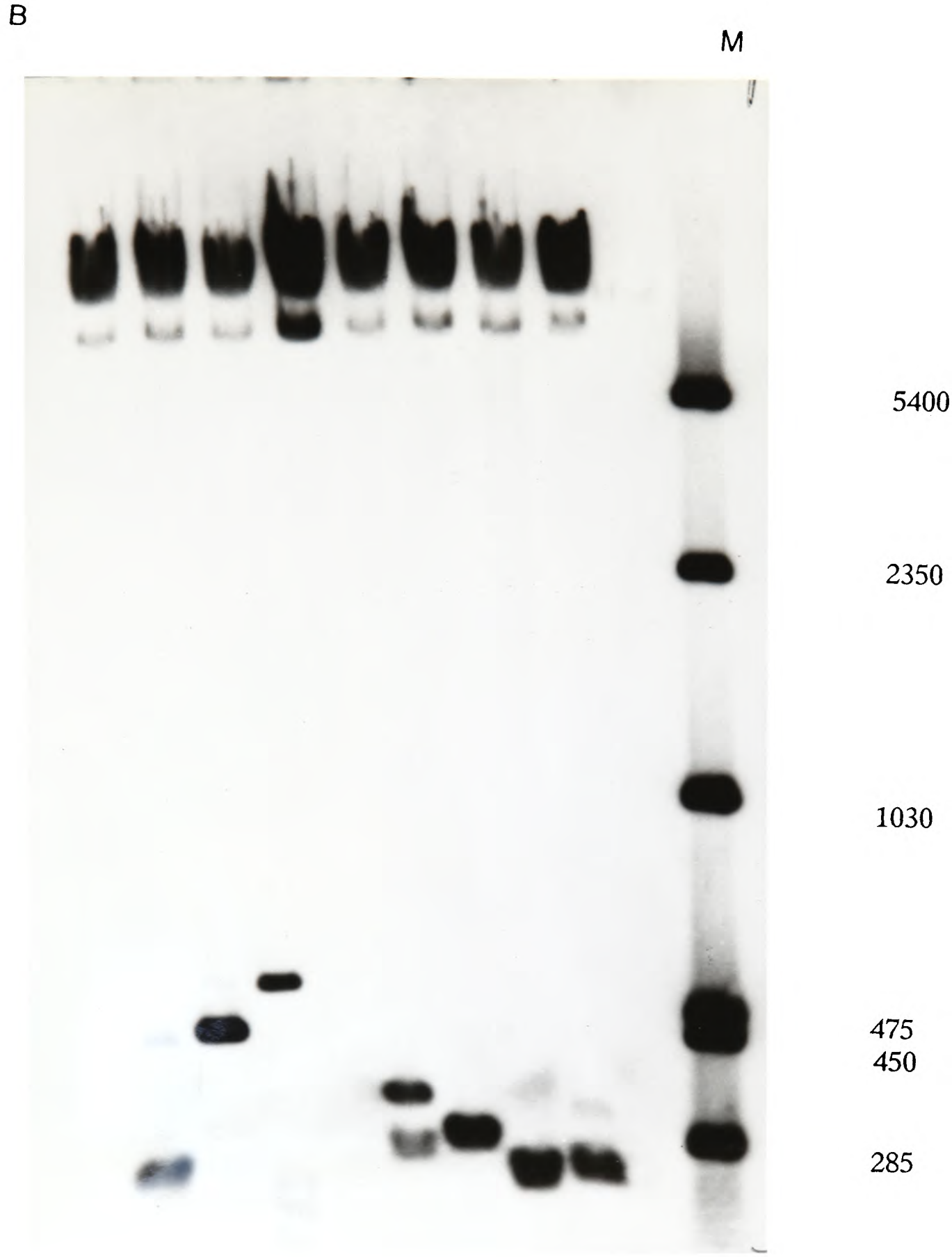


Fig 5.10

λgt10 library clone DNA prepared by DE52 cellulose chromatography, proteinase K digestion and sodium acetate precipitation [Amersham protocol] digested with *EcoR1* and run on a 0.8% agarose gel [a] or labelled with ^{32}P-α-dATP end-filling by Klenow polymerase and run on a 1% agarose gel, vacuum dried onto hybond-N and autoradiographed overnight [b].

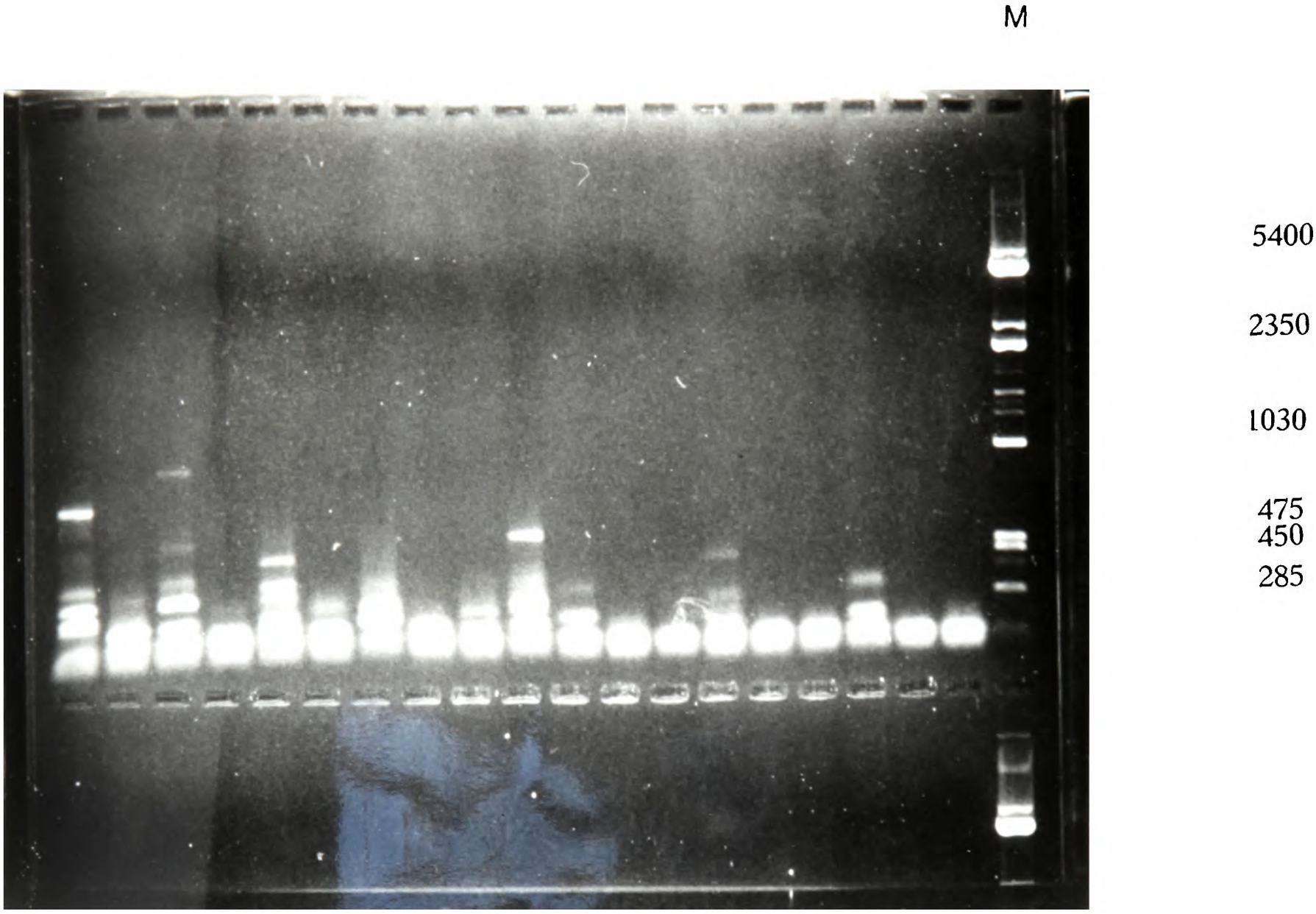


Figure 5.11

Inserts from multiple λgt10 clones from a specifically primed mouse brain α1 and α2 cDNA library selected for inserts of size >300bp amplified by PCR using λ specific primers. The DNA from a dense colony punch was prepared by the method of Rasmussen *et al*, 1987 and amplified with λ specific primers. Cycling conditions were 94^{0}C/1min; 55^{0}C/1min; 72^{0}C/3mins; 25 cycles.

5.23 Initial Library Construction

The size selected F2.1D1 and NG108-15 mRNA was used to construct libraries using a mix of 2 primers specific for the α1 and α2 rabbit skeletal muscle genes. Antisense primers to the rabbit sequences were obtained from British Biotechnology:

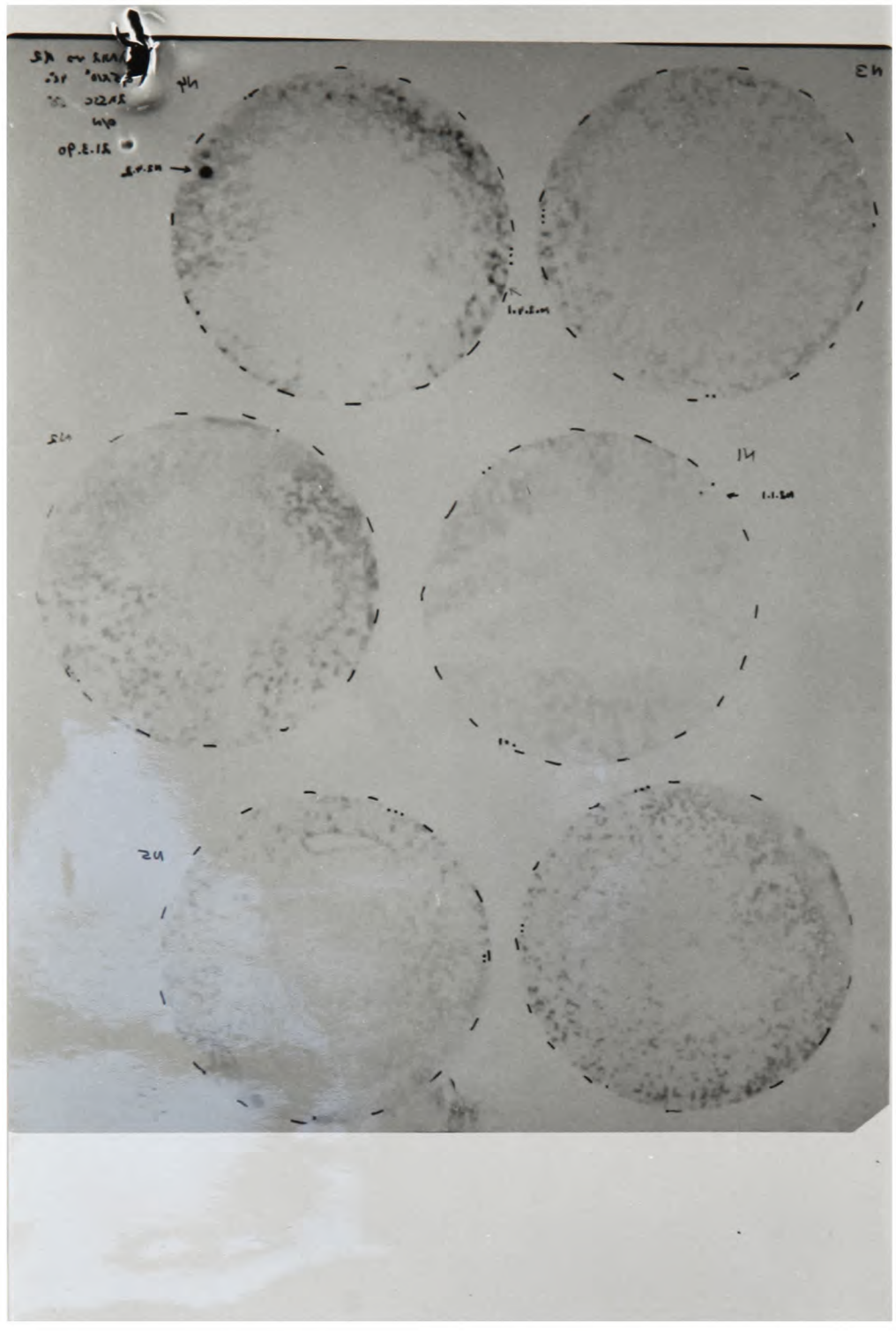
α1 primer 5' GCTTCATGAATTCGGAAG 3' 4598-4616 T_m= 52^{0}C

α2 primer 5' TGCCAGAAACCAGCCA 3' 3287-3303 T_m= 50^{0}C

Additional aliquots of mRNA were used for synthesis of random hexamer primed libraries from each cell line.

Yields of cDNA, and library titres are shown in table 5.3. The resulting unamplified specifically primed libraries were screened with the rabbit PCR product probes at a concentration of 10^5 cpm/ml in perspex hybridisation chambers containing a volume of 20 mls of hybridisation solution. False positive background was reduced by the addition of sonicated salmon sperm DNA at a concentration of 100 µg/ml and heat denatured pUC8 plasmid at 1 µg/ml. Low stringency screening was performed and the filters were washed in 2 x SSC at 25^{0}C and autoradiographed initially for 1 day and then put down for a further 5 days to look for fainter signals.

The results of screening are shown in table 5.4. Initially a large number of putative clones were obtained but only one clone found on screening with the α2 probe gave a strong signal - NG1-242. This clone was purified and rescreened subsequently on two occasions [figs 5.12 a-b].



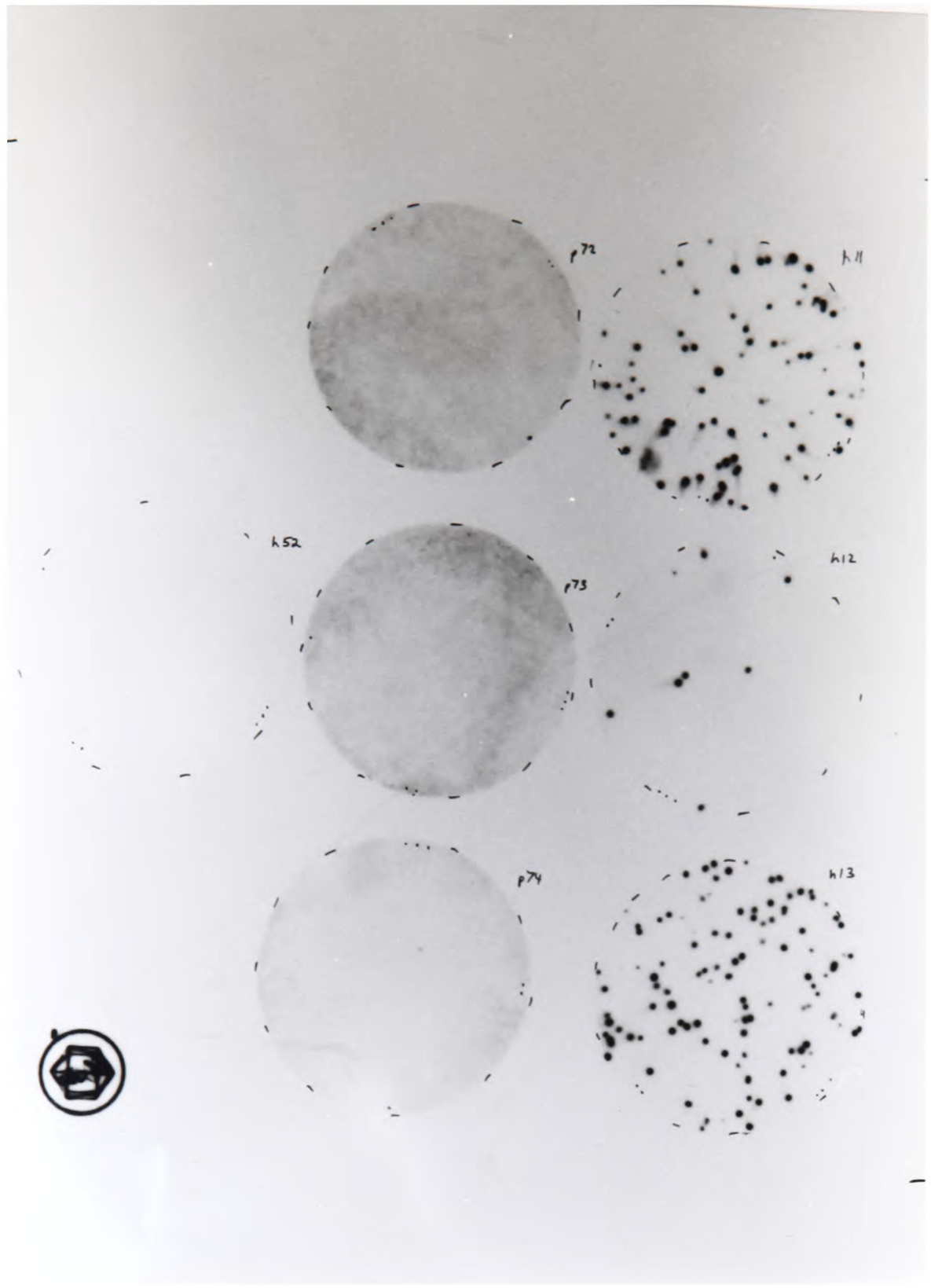


Figure 5.12

Screening of λgt10 library constructed from NG108-15 cells with primers specific to $\alpha 1$ and $\alpha 2$ VGCC subunit sequences. Primary screen [a] Secondary screen [b] Tertiary screen [b] as indicated. Colony filters were screened at a density of $5x10^4$ pfu/plate. Filters were hybridised with 40mls 6xSSCE, 10x Denhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8, 50% formamide and $0.1x10^6$cpm/ml rabbit $\alpha 2$ probe at 42^oC and washed with 2xSSC at 50^oC. Autoradiography was for 2 days.

The tertiary screen filter also retained signal when washed with 2 x SSC at 50^{o}C - the same conditions as used on the dot blot. Phage was purified from the final screen and the insert isolated by *EcoR1* digestion and subcloned into *EcoR1* cut pUC8 [fig 5.13a]. The pUC8-insert ligation was plated on RB791 cells. As there were few individual white colonies containing insert the individual colonies were grown up and the plasmids prepared and *EcoR1* digested. The results of this digest are shown in fig 5.13b. A plasmid containing the same 450mer insert as the phage was picked and the insert was subcloned into M13mp18 and sequenced. 12 single strands were sequenced to obtain both directions of reading and the sequence of NG1-242 is shown in fig 5.14. The sequence was translated using the program RTDNA [Elsevier] and restriction sites predicted.

This sequence predicted the presence of a *Sca1* restriction site which is not present in the rabbit sequence at all. The plasmid was grown up in 500 ml culture and purified plasmid prepared by the caesium chloride centrifugation technique. The insert was isolated by ecoR1 digestion and purified from plasmid sequences on a LMP agarose gel. This would be used to rescreen the libraries for further positives. An aliquot [5 µg] of the prepared insert was digested with *ScaI* and the results of this are shown in fig 5.15. This confirmed the sequence as determined by dideoxy sequencing as correct and proved that no rabbit plasmid contaminant had been isolated.

No other confirmed positives were isolated from the primed libraries for either gene. Similar results were obtained when the random hexamer primed libraries were screened with the 2 rabbit probes.

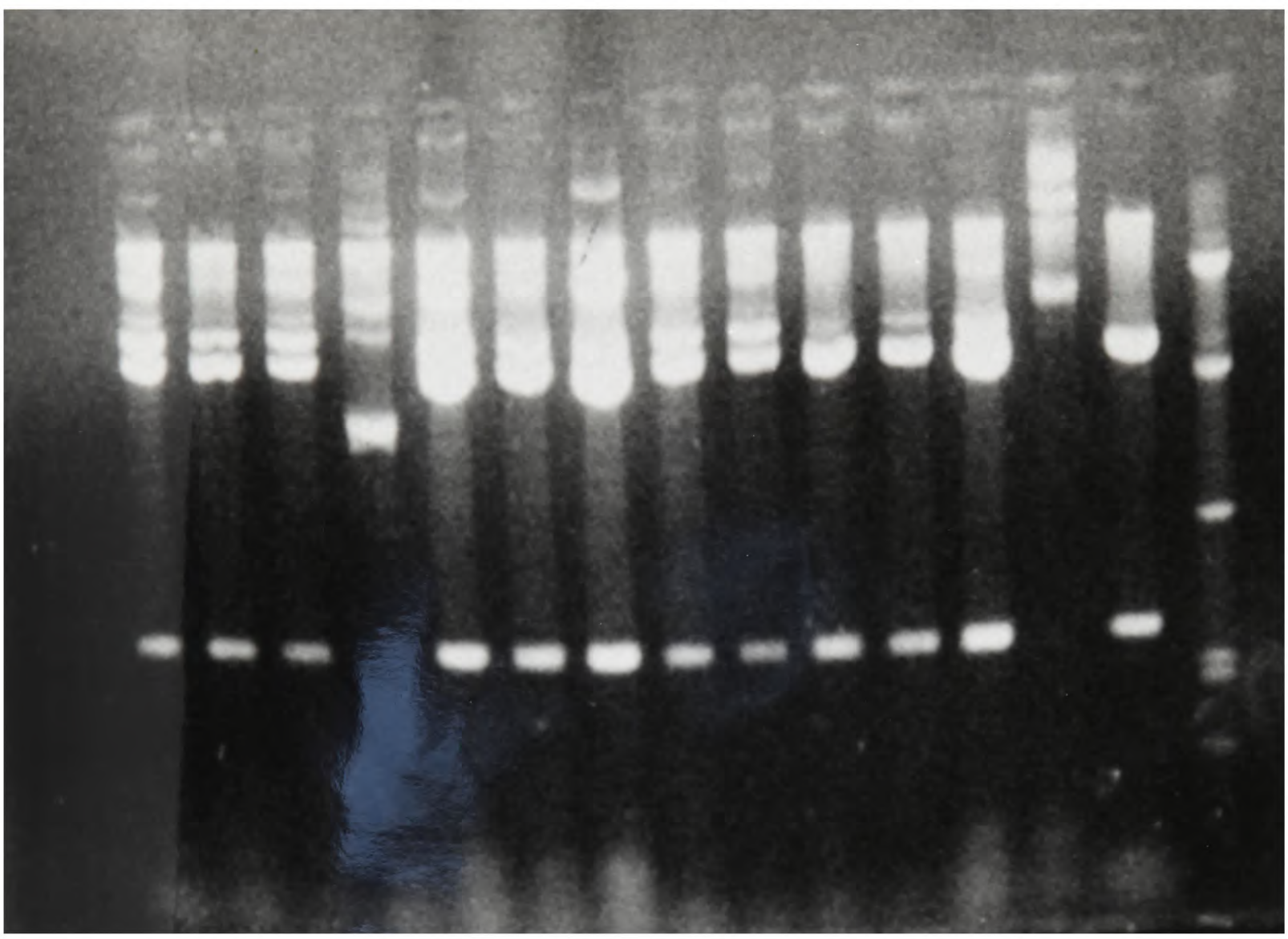


Figure 5.13b

The *EcoR1/HindIII* digested insert from the λgt10 phage clone NG1.242 was isolated from a low melting point gel [as in 5.13a] and 20ng were ligated to 100ng of *EcoR1/HindIII* digested pUC8. 10ng plasmid was transfected into 200μl of *E.coli* RB791 cells and white [lacZ operon disrupted] colonies were picked and the presence of inserts assessed by alkaline lysis plasmid preparation and *EcoR1/HindIII* restriction digestion. The digests were run out on a 1% agarose gel.

```
                                                        30
TAT GTG CCA TGA ATT ACA ACA ATA CTC CAG ATT GGC TGG TGG GCC ACT
Y   V   P   S   I   A   A   I   L   Q   I   G   W   W   A   T
                60                                          90
GCT GCC GCC TGG TCC ATT CTC CAG CAG CTA CTC TGT GAT TTG CAA TTT
A   A   A   W   S   I   L   Q   Q   L   L   C   D   L   Q   F
                                    120
CCA CGG CTC CTT GAG GCA GTT GAA ATG GAA GAG GAT GCT TTC ACA GCT
P   R   L   L   E   A   V   E   M   E   E   D   A   F   T   A
      150                                   180
TCC CTG TCT AAG CAG TGC TGC ATC ACA GAA CAA ACT CAG TAC TTC TTC
S   L   S   K   Q   S   C   I   T   E   Q   T   Q   Y   F   F
                        210                             240
AAG AAC GAT TCA ATA TCA TTC AGT GGC ATT CTG GCA TGT GGA AAC TGT
K   N   D   S   L   S   F   S   G   L   L   A   C   G   N   C
                                        270
CAG GAT CTT CAT GTG GAG GAG CTT ATG AAC ACT AAT CTA GGC TCT ATA
Q   D   L   H   V   E   E   L   M   N   T   N   L   G   S   I
                300                                 330
ATG GTG GAG TCT AAG GGG CAG TGT TGT GAC ACT CGC CTG GTC ATG CAA
M   V   E   S   K   G   H   C   C   D   T   R   L   L   M   Q
                                    360
GCA GAG CAG GCA TCA GAC GGA CCT GAT CCT TGC TCG ATG GTA AAG CAG
A   E   Q   A   S   D   G   P   D   P   C   S   M   V   K   Q
      390                                   420
CCG CGT TAT AGG AAA GGA CCT GAC GTG TGC TTC GAC AAT AAC GTT CTT
P   R   Y   R   K   G   P   D   V   C   F   D   N   N   V   L
                        450                             480
GAA GAC TAT ACT GAC TGT GGC GGC GTA TAT GGA TTA AAT CCA TCT CTA
E   D   Y   T   D   C   G   G   V   Y   G   L   N   P   S   L
                                        510
TGG TCC ATT ATA GCT ATT CAA TTT GGC CTC CTA TGG CTG GTT TCT GGC
W   S   I   I   A   I   Q   F   G   L   L   W   L   V   S   G
```

Figure 5.14

Nucleotide and amino acid sequence of the clone NG1.242. The predicted unique *ScaI* restriction site is arrowed.

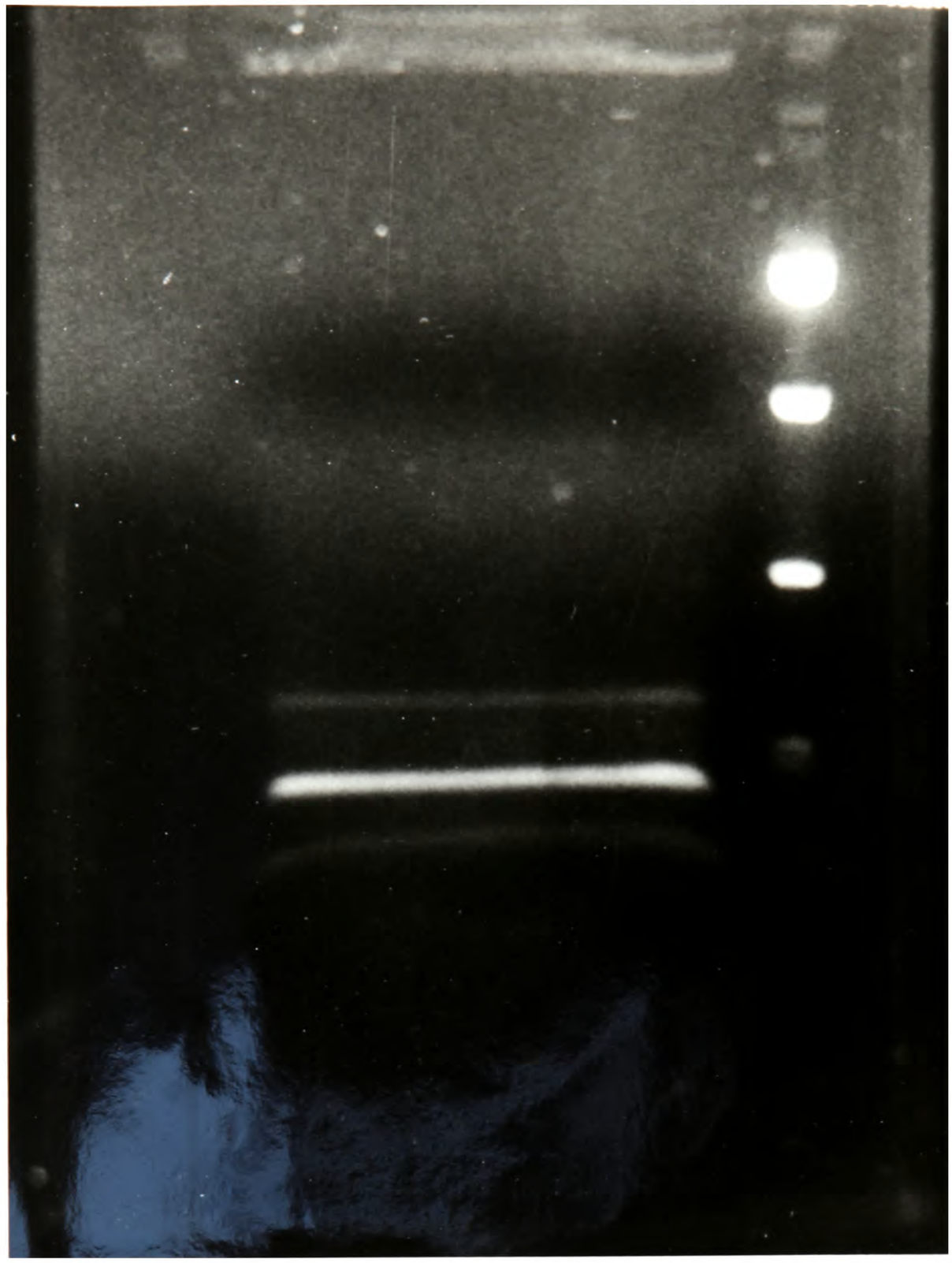


Figure 5.15

ScaI digest of the purified insert of the clone NG1.242. The λgt10 clone NG1.242 was purified and the insert isolated by *EcoR1/HindIII* digestion and subcloned into *EcoR1/HindIII* digested pUC8. Plasmid was prepared from a 1ml LB culture by the alkaline lysis technique, digested with *EcoR1/HindIII* and the insert isolated on a LMP gel. The isolated insert was purified by the hot phenol technique and 0.5μg were digested with 10U of *ScaI* at 37^{o}C for 1 hour. The digest was run on a 2% agarose gel.

5.24 The clone NG1-242

This clone encoded 158 amino acids [475bp] segment of the $\alpha 2$ VGCC subunit gene starting at the primer in MS3 and encoding all but 4 amino acids of MS2. The sequence showed 85% homology at nucleotide level and 75% [135/158] amino acid homology to the rabbit $\alpha 2$ gene. The changes were principally conservative [13] especially in the membrane spanning domains where only 3 out of 37 amino acids were changed - none of which would alter the hydrophobicity of these regions. Comparison of this sequence with the EMBL database using the Staden [Staden, 1987] and Wisconsin GCG7 packages [Devereux *et al*, 1984] on the Oxford Vax series II computer confirmed the identification of homology to the $\alpha 2$ gene and no significant homology to any other protein in the dtabase.

The region corresponding to the 3' PCR primer showed 4 base pair changes with 2/4 occurring at the 3' end and therefore likely to reduce the possibliity of annealing of the PCR primer [Ehrlich, 1991]. The clone did not extend far enough for the 5' primer site to be analysed. Two possible disulphide bridge and glycosylation sites exist in the putative extracellular domain. The cysteine residues were preserved as were two glycosylation sites though the position of the latter differed from the rabbit $\alpha 2$ sequence.

5.26 Extension of the cloned sequence

The library made in the previous section was rescreened with NG1-242 and no further positive clones were isolated. Similar results were obtained when the random hexamer [4×10^5 pfu primary clones] and oligo-dT primed [3.5×10^5 pfu primary

clones] libraries were screened. The gene that was wanted was obviously rare and therefore more sensitive techniques would have to be used.

A new primer was obtained from British Biotechnology with the sequence:

5' GTCATTATCGAAGAA 3' T_m = 40^oC

corresponding to the middle of the newly isolated extracellular domain. This was used to construct a library in λgt10 from unfractionated mRNA as previously described.

A primary library of 2.87×10^5 pfu was obtained. The library contained 95% inserts with an average size of 1.3 kb. It was screened with the NG1.242 probe at high stringency [0.1 x SSC, 50^oC]. The results of primary secondary and tertiary screens are shown in table 5.4. A solitary positive clone ws obtained and sequenced. This was termed NG2.h41. This clone encoded 360 bp of sequence from the primer completing the second membrane spanning domain and extending partially in to the intracellular portion of the gene.

The sequence of NG2.h41 is shown in fig 5.16.

As this strategy had been successful to date it was decided to continue using this approach to extend the sequence. A new primer NG3 was obtained to the beginning of MS2 and used to construct a new library on the same basis.

Primer NG3: 5' GCTATTGATGGCACA 3' T_m = 44^oC

A further 1.5×10^5 pfu were obtained and another small section of gene isolated as NG3.354 [270bp] [fig 5.17].

Library		Results of screening			Clone
		Primary	Secondary	Tertiary	
NG oligo	α1	7	1	0	
	α2	6	1	0	
NG hexamer	α1	16	8	0	
	α2	23	5	0	
NG1p	α1	9	4	0	
	α2	19	2	1	NG1.242
F2.1D1 oligo	α1	3	0	0	
	α2	5	2	0	
F2.1D1 hexamer	α1	7	2	0	
	α2	9	1	0	
F2.1D1p	α1	21	4	0	
	α2	17	3	0	
NG2	α2	15	3	1	NG2.h41
NG3	α2	45	18	3	NG3.354
Rat brain	α1	23	4	2	α1.RB2.1
	α2	13	5	0	α1.RB9.1
Mouse brain	α1	64	13	1	α1.MB2.15
	α2	9	2	0	
Mouse lung	α1	46	22	1	α1.ML2.13
	α2	23	6	0	

Table 5.4

Colony screening counts for primary, secondary and tertiary screens for NG108-15 primer libraries

```
                                                        30
CCT TTG AGA CTT AGC TAC GCG TTC AAT AAA TCT TAT GAC TAT CAG TCG
P   L   R   L   S   Y   A   F   N   K   S   Y   D   Y   Q   S
                60                                          90
GTG TGT GAC CCT GGT GCT GCG CCC AAG CGC GGC GCA GGG CAC CGC TCG
V   C   E   P   G   A   A   P   K   R   G   A   G   H   R   S
                                    120
GCT TAT GTG CCA TGA ATT GCA GCA ATA CTC CAG ATT GGC TGG TGG GCC
A   Y   V   P   S   I   A   A   I   L   Q   I   G   W   W   A
      150                                       180
ACT GCT GCC GCC TGG TCC ATT CTC CAG CAG CTA CTC TGT GAT TTG CAA
T   A   A   A   W   S   I   L   Q   Q   L   L   C   D   L   Q
                    210                                     240
TTT CCA CGG CTC CTT GAG GCA GTT GAA ATG GAA GAG GAT GCT TTC ACA
F   P   R   L   L   E   A   V   E   M   E   E   D   A   F   T
                                        270
GCT TCC CTG TCT AAG CAG AGC TGC ATC ACA GAA CAA ACT CAG TAC TTC
A   S   L   S   K   Q   S   C   I   T   E   Q   T   Q   Y   F
              300                                       330
TTC AAG AAC GAT TCA ATA TCA TTC AGT GGC ATT CTG GCA TGT GGA AAC
F   K   N   D   S   L   S   F   S   G   L   L   A   C   G   N
                                    360
TGT CAG GAT CTT CAT GTG GAG AAG
C   Q   D   L   H   V   E   K
```

Figure 5.16

Sequence of the second clone- insert NG2.h41

```
                                                        30
GCC GAA AAT GTT GAG AAG GTT GTT AGA CCG CCA CAA AAT CCG GTG GTG
A   E   N   V   E   K   V   V   R   P   P   Q   N   P   V   V
                60                                          90
ATT AAT ACT TTC CAC ACT TGG ATC ATG TTC GTC ATA CTG TTG ATG CGG
I   N   T   F   H   T   W   I   M   F   V   I   L   L   M   R
                                    120
TCA ATC TAT GAT GAT GCC TAT CGG TTG CAT GTA CGC GCT TTC ACT GTA
S   I   Y   D   D   A   Y   R   L   H   V   R   A   F   T   V
      150                                       180
ATA GGT CAT TAT CTT GTG GAA CGT CCT TTG AGA CTT AGC TAC GCG TTC
I   G   H   Y   L   V   E   R   P   L   R   L   S   Y   A   F
                        210                             240
AAT AAA TCT TAT GAC TAT CAG TCG GTG TGT GAC CCT GGT GCT GCG CCC
N   K   S   Y   D   Y   Q   S   V   C   E   P   G   A   A   P
                                    270
AAG CGC GGC GCA GGG CAC CGC TCG GCT TAT
K   R   G   A   G   H   R   S   A   Y
```

Figure 5.17

The sequence of the insert from the clone NG3.354

This method was thought to be to slow to isolate the full length gene so a variety of different methods were explored to try to isolate the full length gene using the primers already available.

Since the original description of cloning methodology, a variety of new more sensitive techniques had been described. Two of these were applied to the problem of the NG108-15 $\alpha 2$ gene.

5.27 Anchor PCR library construction

The original technique of using PCR to isolate genes if the sequence for only one primer is available had been described by Frohland *et al* [1989]. The technique relies on reverse transcribing a first strand under normal conditions with a specific primer and then tailing an aliquot with adenosine residues using the enzyme terminal transferase under conditions such that only 20 - 25 adenosine residues will be added. An aliquot of this reaction was then amplified by PCR between the original 5' primer and a poly-dT primer, subcloned into a vector [e.g. plasmid] and then screened with a separate probe for hybridising sequences. This technique had been shown to isolate 1500 bp segments of genes [Frohland *et al*, 1989] and to be usable starting with 50 ng of total RNA [Domec *et al*, 1990]. It was decided to use this method with a few minor variations - poly dG tailing replaced poly dA as dG tails are supposed to be self limiting in length [Berger & Kimmel, 1990] and attempts were made to use Vent^{tm} polymerase [*Thermus littoralis*] instead of Taq [*Thermus aquaticus*] to reduce errors in incorporation due to the 5'-3' exonuclease proof-reading capacity available in Vent^{tm}. The primer used for amplification was:

$$5'\ GGAATTC_{17}\ \ 3'\ T_m = > 70^0C$$

The restriction site was added to facilitate orientation of the primer on the dG tail and to help in subcloning the final products. Both reactions gave visible yields of product upto 7kb in size. Both sets of reactions were later optimised for magnesium concentration. For Taq polymerase this was performed for the range 1.5 - 6.5mM $MgCl_2$ by the addition of 5 µl of 20 - 100mM $MgCl_2$ and for Vent polymerase 2 - 8mM $MgSO_4$ by the addition of 1 - 6 µl of 100mM $MgSO_4$.

25 µl aliquots of the PCR reaction were run out on LMP agarose gels and the region between 1.5 and 3.0 kb excised and purified. The purified cDNA was kinased, filled with DNA polymerase I and 100 ng ligated to *EcoR1* linkers. The reaction was digested with excess *EcoR1* for 4 hours and cDNA separated from the linkers by spun column chromatography. 25 ng of cDNA were used in the final ligation to λgt10. A fifth of the reaction was packaged and analysed for number of plaques and insert size.

A further aliquot of 100 ng of cDNA was size selected by column chromatography to remove small products. The cDNA was kinased and filled with DNA polymerase I before being digested with 20 U of *EcoR1* for 2 hours. The cDNA was purified from protein, precipitated and 25 ng used in a ligation reaction with 100 ng of *EcoR1/HincII* digested phosphatased pGEM-3Z vector.

Both sets of reaction products were run out on agarose gels and optimum magnesium concentrations selected on the basis of the greatest yield of products greater than 3 kb in size. For these primers this was found to be 3 mM for Taq polymerase and 2 mM for Vent polymerase. The presence of hybridising products in the mixture was checked by southern blotting the gel and hybridising NG2.h41 probe to the products.

It was noticeable that both produced large PCR products but more are produced with the use of Taq polymerase. Hybridisation revealed a non-specific smear of products

implying the existence of many individual tailed products of differing sizes. However it was noticeable that some products were very much larger than the largest possible size for the gene [2.7 kb]. This could have been due to primer polymerisation at the end of the products or the presence of very long dG tails.

Yields of the product were calculated using the counted radioactivity incorporation and the products purified. Libraries were constructed from both amplification reactions with titres of 7.4×10^4 pfu and 1.05×10^5 pfu respectively. However analysis of the inserts showed that none of them contained any insert of any significant size. No ligation products were obtained with pGEM-3Z.

A variety of improvements could be made to the technique in this method. The initial amount of starting cDNA seemed to be too large and so allowed the generation of significant amounts of non-specific products in the amplification. The problem of non-specific products has been noted to be a significant limitation in the use of anchor cloning with particular products accounting for upto 50% of the yield even using optimised conditions [Kajimura *et al*, 1990]. Reduction in the amount of cDNA to an amount representing about 1000 molecules of mRNA would allow far more stringent amplification. The second problem encountered was the inability to use the restriction site in the dC primer on attempting to subclone products directly into plasmids [*EcoR1* / *HincII* digested]. This has been noted to occur close to the polymer section of the primer [Frohmann *et al*, 1989] and so the use of a longer primer might be neccessary with for instance the following sequence:

$$5' \textit{EcoR1} - \textit{BamH1} - \textit{HindIII} - C_{17} \text{ or } A_{17} \quad 3'$$

However the price of using this kind of primer would be the lower fidelity of Taq polymerase. This would have to be used as the amplifying polymerase as the

preference of proof-reading polymerases is to digest primers to 15 - 18 bp from the 3' end thus leaving only restriction sites in such 5' extended primers.

It has been stated [Frohmann, 1990] that a poly-A primer suffers from fewer ratchetting artefacts than a poly-C primer and so it might be better to substitute dT tailing as in the original paper. Others [Domec *et al*, 1990] seem to feel that the choice of tailing nucleotide makes little difference. It also remains to be seen whether the presence of polymeric sequences makes the cDNA more difficult to ligate into vectors such as λgt10.

The failure of this technique was not critical as other PCR methodology had been used to amplify further portions of the gene.

5.30 PCR amplification of the NG108-15 α2 gene

Additional primers had been obtained to continue the tissue specificity work described in chapter 4. These were to the following sequences:

Rabbit 5'-3' 1-20

BamH1-ATGGCTGCGGGCCGCCCGCTG T_m= 76^{0}C

Rabbit 5'-3 1000-1020

BamH1-GATTATAAGAAGGGCTTTTAG T_m = 54^{0}C

Rabbit 3'-5' 1980-2000

BamH1-GTAATCCTCTTGGTGCTAGGA T_m = 58^{0}C

These primers were tried in PCR amplifications with NG108-15 cDNA made with the primer NG1. The following combinations were tried:

5' primer	3' primer
Rabbit 1-20,	NG1
Rabbit 1-20,	NG3
Rabbit 1000-1020,	rabbit 1980-2000
Rabbit 1000-1020,	NG1
Rabbit 1000-1020,	NG3

Amplification conditions and results are shown in table 5.5.

All the products were heterogeneous and were detected by the use of NG2.h41 probe at high stringency. A band of the expected size was detected with the combination rabbit 1000-1020 and NG1. This gave a product of 3.2 kb [fig 5.18]. When a $1/10^6$ dilution of the PCR reaction was reamplified in the presence of the primer NG3 the band shifted in size to 2.4kb and still hybridised to the probe [fig 5.19]. No hybridising products were obtained when the rabbit $\alpha 2$ probe to the region 1000-2000 was used. The shorter product named NG3 was subcloned into pUC8. No convenient restriction sites within it were found on digestion with a panel of restriction enzymes:

AccI, *BamH1*, *BglII*, *EcoR1*, *HindIII*, *KpnI*, and *PvuII*

Therefore, the PCR product was reamplified from an aliquot of the original PCR and aliquots were purified with Geneclean I [according to manufacturer's instructions] and digested with 4 cutter enzymes to allow subcloning and sequencing of the shotgun digests in M13 vectors.

Three digests were performed:

[i] *MspI* [BRL Ltd]

[ii] *BstIIIa* [a *SauIIIa* isoschizomer][Pharmacia]

[iii] *AluI* [BRL Ltd]

Small aliqouts of the digests were checked and the remainder ligated to the respective cohesive M13 vectors:

[i] *AccI* cut M13/mp18

[ii] *BamH1* cut M13/mp18

[iii] *HincII* cut M13/mp18

The single stranded vector was plated onto competent JM101 cells and 36 individual plaques were analysed. A further 48 were subsequently sequenced

to confirm and overlap the original sequences in both directions. The possibility that close restriction sites could lead to the loss of small clones was minimised by performing digestions with 3 enzymes and assembling an overlapping sequence from all 3 digests.

The sequencing map of NG3 is shown in fig 5.20. The sequence of NG3 comprises of bp 1 - 1742 of the full sequence of the $\alpha 2$-δ gene.

PCR conditions					Results
Forward	Reverse	T_a	Extension	cDNA	Band size [bp]
RLead	R2000	50	5 mins	Rab/hex	2000
R1000	R2000	50	3 mins	Rab/hex	1000
R1000	R2000	50	5 mins	MLu/NG1 RM/NG1 MB/NG1	1000 1000 1000
R1000	NG1	50	5 mins	NG/hex	2300
R1000	NG3	50	3 mins	NG/NG1	1700
R1000	NG3	50	3 mins	NG/hex	1700

Table 5.5

PCR cycling conditions and results with primers to the rabbit and NG108-15 sequences using cDNA from NG108-15, F2.1D1, and brain, lung and skeletal muscle from rat and mouse.

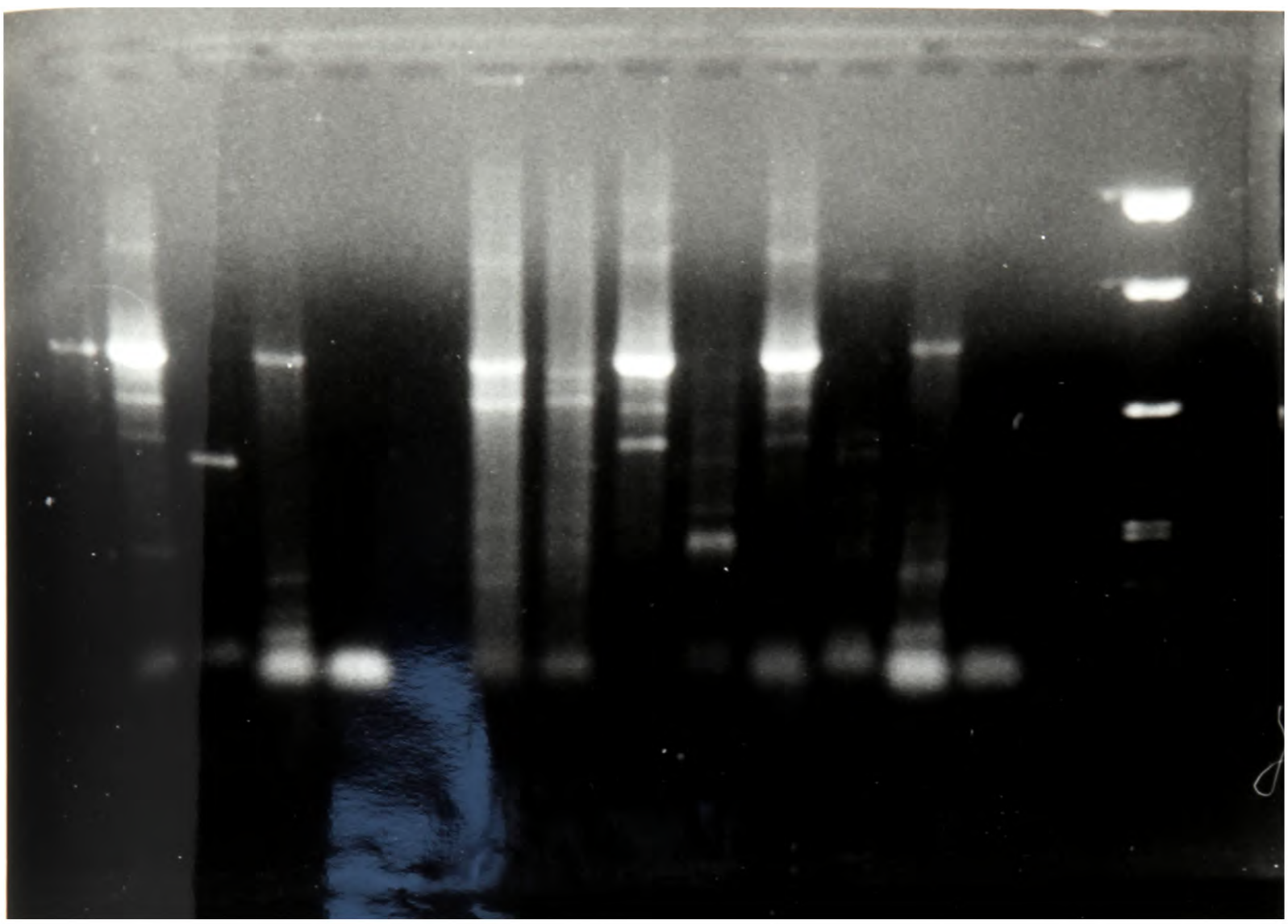


Figure 5.18

PCR amplification of rabbit skeletal muscle, NG108-15 or rodent tissue [brain, lung, and muscle] random hex $\alpha 2$-δ specific [primer NG1][NG1] or oligo-dT primed dT] cDNA [100ng] using the primers NG1, NG3, rabbit leader [-21-0][RL] and rabbit 999-1020 [RI][0.1µg].

Cycling conditions 94^{o}C/ 1min; 50^{o}C/1min; 72^{o}C/3mins; 30 cycles; 3mM $MgSO_4$

The cDNA source and the primer(s) used to produce it are shown in the top line above the relevant lane with the primer pairs shown in line 2 [5' primer] and line 3 [3' primer]. M= PM2 *HindIII* digest markers.

PCR primer pair

R1000 NG1

R1000 NG3

M

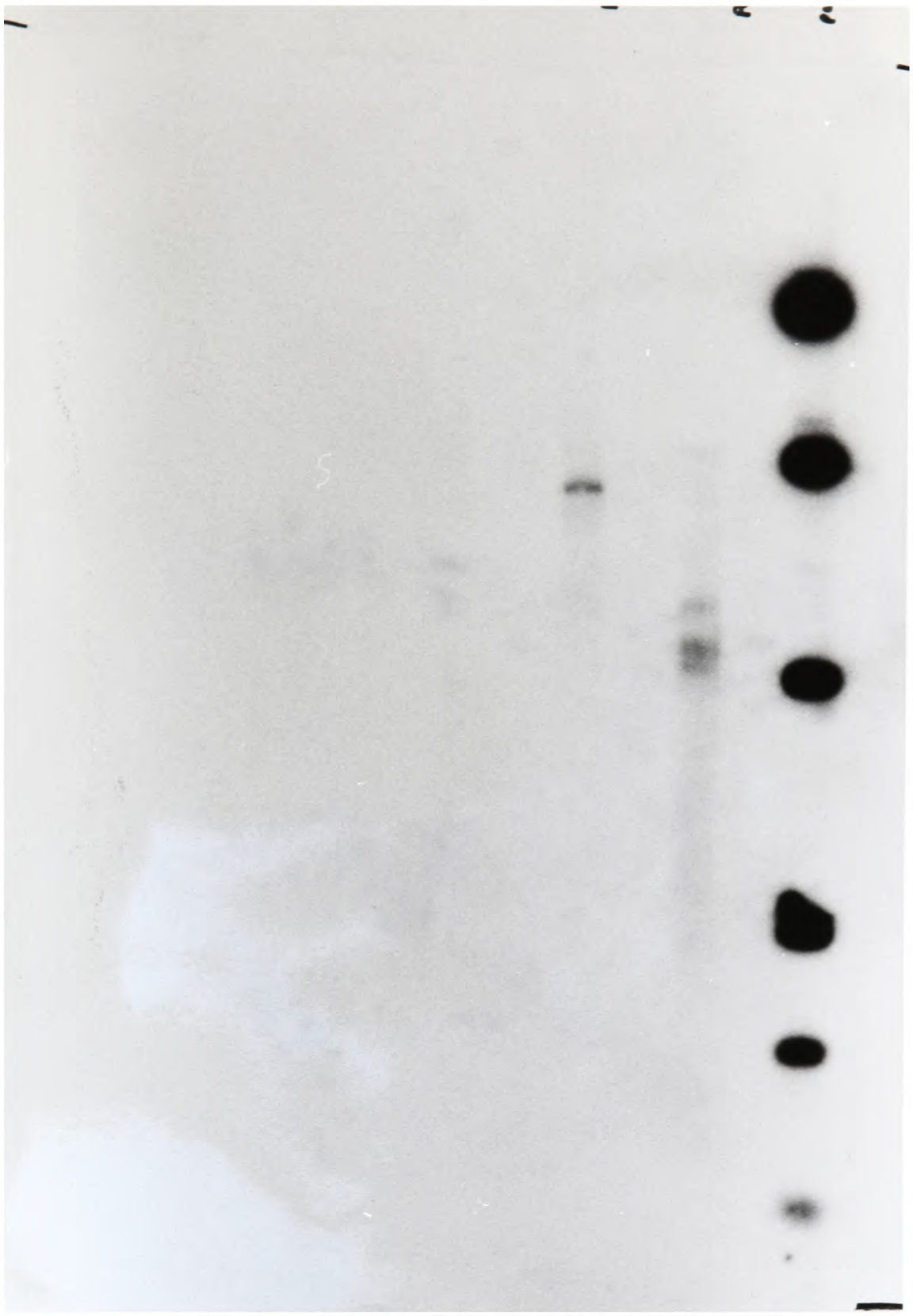
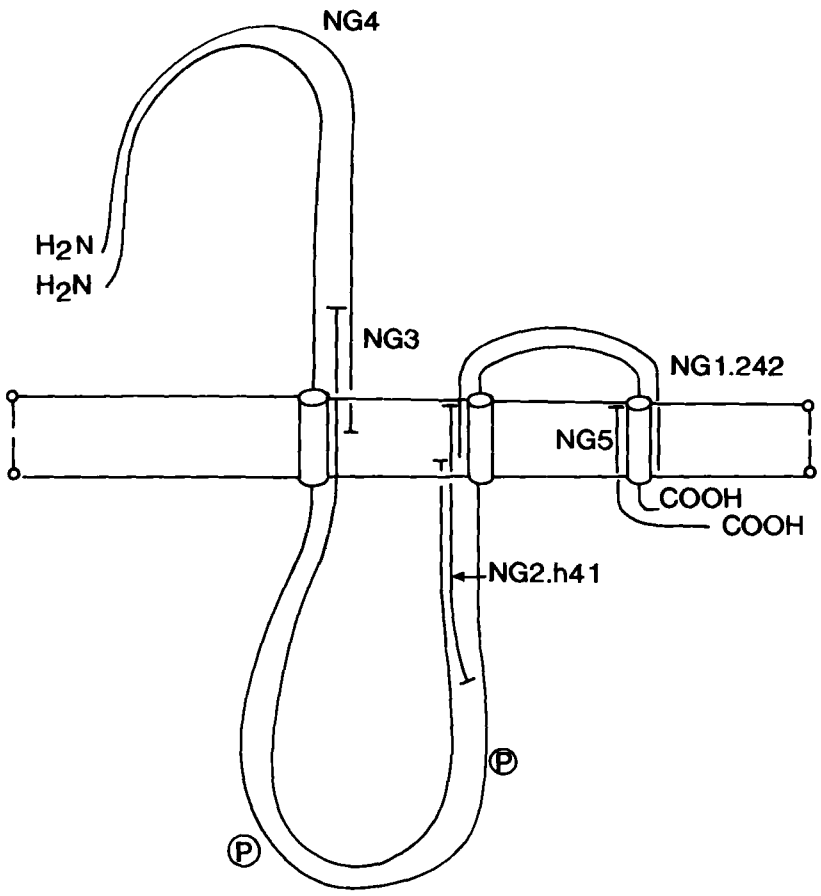


Figure 5.19

Southern blot of 100ng NG108-15 random primed cDNA amplified with the primer pair NG1, rabbit 999-1020 [0.1µg each] in a volume 0f 100µl and 1µl of a 10^6 dilution of that PCR reaction reamplified in a volume of 100µl with 0.1µg of primers NG3 and rabbit 999-1020. Cycling conditions 94^0C/1min; 50^0C/1min; 72^0C/3min; 35 cycles, 3mM $MgSO_4$. The blot was hybridised in 5mls of 6xSSCE, 10xDenhardt's, 100µg/ml herring sperm DNA, 50% formamide at 42^0C with $0.1x10^6$cpm/ml NG2.h41. The blot was washed with 0.2xSSC at 60^0C and autoradiographed for 3 days. The insert NG2.h41 contains sequence present in both PCR products and therefore hybridises to both products unlike NG1.242 which only hybridises to the 2.3kb rabbit 999-1020/NG1 PCR product [data not shown].


NG4
H_2N
H_2N
NG3
NG1.242
NG5
COOH
COOH
NG2.h41
Ⓟ
Ⓟ

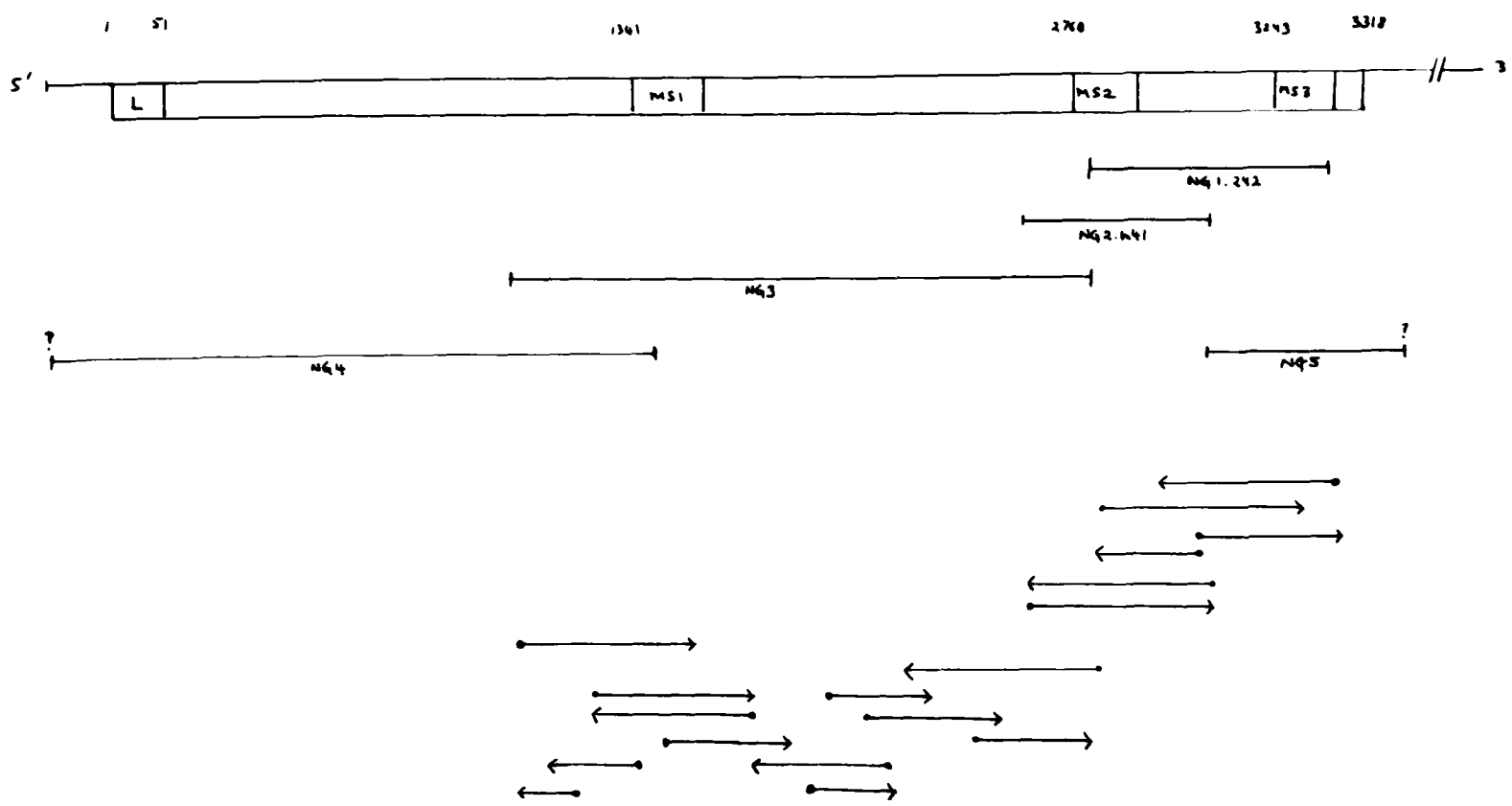


Figure 5.20

The sequencing map of the $\alpha 2$-δ gene product of NG108-15 cells.

The PCR product NG3 was sequenced by triple shotgun digestion using *MspI*, *AluI* and *BstIIIa* [a *SauIIIa* isoschizomer] and subcloning into compatibly digested M13 vectors [*AccI*, *HincII*, and *BamH1*]. The clones NG2.h41 and NG1.242 were sequenced from both ends using M13/pUC primers. NG1.242 was also sequenced using an internal *ScaI* site followed by Klenow infilling and the fragments were ligated into a blunt end M13 vector [*HincII* digest]. NG2.h41 was sequenced using an internal ***indIII*** site and the fragments ligated into *HindIII* digested M13.

The sequence encodes the area homologous to that found in the rabbit gene from the initial extracellullar portion through the first membrane spanning region and then up to the second membrane spanning region. It is markedly different from the rabbit sequence having only 45% amino acid homology. This confirms the result of the PCR using the primer pair r1000-1020 and r1980-2000. Notably both cAMP phosphorylation sites present in the rabbit gene are absent in the NG108-15 product.

5.31 Isolation of the remainder of the NG108-15 $\alpha 2$ gene

It was not possible to use the rabbit leader sequence to amplify a homologue from NG108-15 cells. However a method had recently been described in which the primer to the uncharacterised end of the gene is provided by a vector specific sequence [Wilson-Shaw *et al*, 1991; Gibbons *et al*, 1991]. These methods use a λ specific primer to amplify the unknown end of a gene whilst a specific primer is used to amplify the known end and had been applied to sequence the γ subunit of a $GABA_A$ receptor from mouse brain [Wilson-Shaw *et al*, 1991] and sea urchin dynein [Gibbons *et al*, 1991]. It could therefore be used to amplify either end of a gene depending on the orientation chosen for the primer pair. Packaging a library is known to be an inefficient reaction in which many molecules are not packaged because they have only one λ arm attached or are too short or too long [> 7.0 kb]. The likely yield of packaging is about 10% of cDNA molecules present in the ligation reaction [Berger & Kimmel, 1989]. Therefore full-length gene products might well be present in the ligation reaction with at least one λ arm attached. It was decided to attempt PCR amplication from original unpackaged aliquots of the libraries NG1 and NG/h using

each λ primer in parallel and NG3 and a primer to the first membrane spanning region [NG4]:

5' AACAGCTATGACATG 3' T_m = 42°C

The results of the amplification reactions are shown in fig 5.21. Products of 3.3 kb were obtained with both NG1 and NG/h source DNA and either of the λ primers and NG4. These products hybridised to NG3 and NG1.242 at high stringency [fig 5.21b]. A similar experiment was performed with a reverse primer NG/rev to obtain the remainder of the 3' coding region and a product of 1.0 kb obtained. The products were subcloned into pUC 8 by standard tecniques and the product analysed. Digestion of the plasmids with *EcoR1* and *PstI* showed that the NG4 amplified product was 2.0 kb in length and that the NG/rev product was 0.6 kb in length.

The initial products were very large compared to expectation. The size of the 5' product was expected to be 1.8 kb if full-length and the excess size was ascribed to polymerised *EcoR1* linkers which had not been initially digested as neither of the two enzymes generated a large restriction fragment. The final digested products of 2.0 kb and 0.6 kb would be expected to yield the remaining portions of the coding segment of the gene. The sequence from these products will yield the 5' end of the NG108-15 α2 gene [approx 1.1 kb] and confirm the sequencing of NG3 while the 3' product NG/rev would give 200 bp of extra sequence at the 3' end where 5 amino acids present in the rabbit homologue have not been isolated. These products will be subcloned and sequenced in the future.

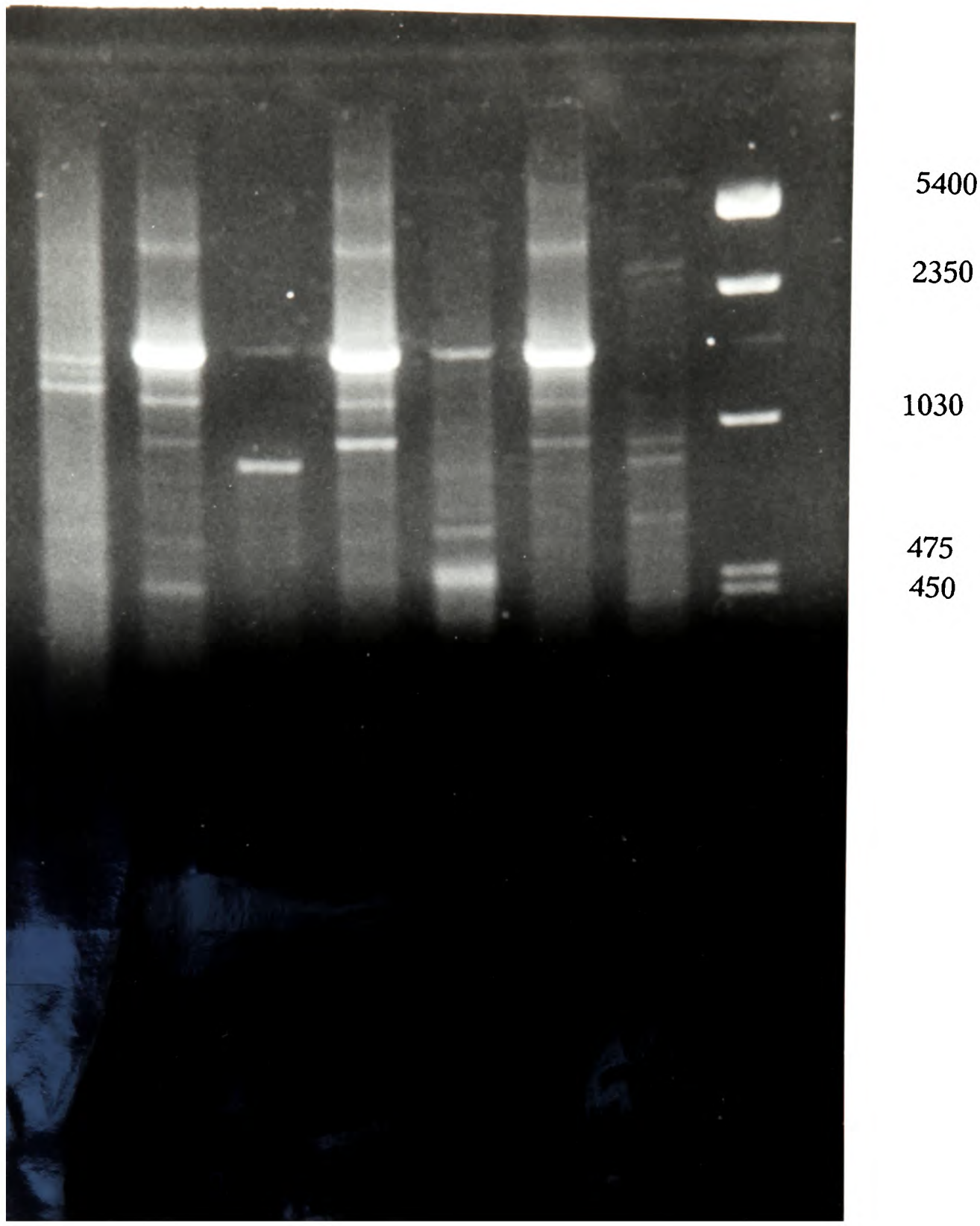
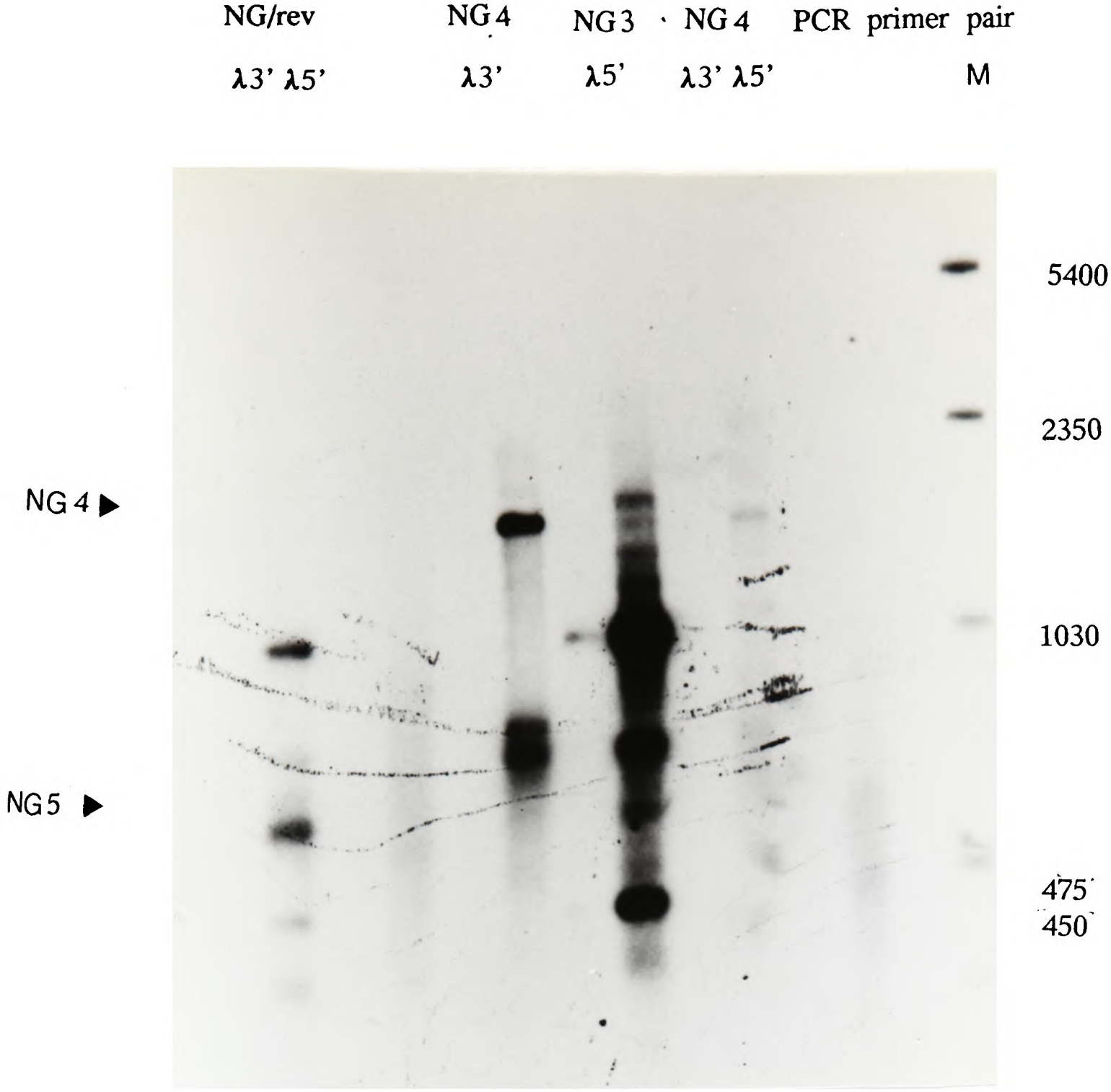


Figure 5.21

[a] PCR amplification of NG108-15 library cDNA [approx 100ng aliquot of ligated but unpackaged phage] with λgt10 specific primers- forward [F] and reverse [R] as one oligomer and primers NG4 or NG/Rev as the other in a volume of 100µl. The heterogeneous amplified products comprised phage sequences that contained an insert with homology to the $\alpha 2$-δ gene primers. Conditions 94^{0}C/1min; 56^{0}C/1min; 72^{0}C/3mins; 35 cycles, 2.5mM $MgSO_4$.



[b] The possibility of the presence of products partially homologous to the primers only being amplified in the PCR reaction led to the need to identify those that were homologous to the rest of the gene. The $\alpha 2$-δ products were identified by high stringency Southern blotting. The PCR products were blotted onto hybond-N and the filters hybridised at 42^{0}C in 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 10μg/ml pUC8 and 50% formamide containing $0.1x10^{6}$ cpm/ml of purified NG3 and NG1.242 inserts as probes. The blot was washed with 0.1xSSC at 70^{0}C and autoradiographed for 6 hours. The hybridising bands were subsequently isolated on LMP gels and subcloned into pGEM-5Z.

5.32 Tissue distribution of the NG108-15 $\alpha 2$ gene product

Total RNA was prepared from rodent tissues by the AGPC technique. The tissues used were brain, lung, skeletal muscle, cardiac muscle, liver and kidney from both rats and mice. RNA was also prepared from NG108-15, N18/TG2 cells and F2.1D1 cells. Northern blots were performed with 50 µg of tissue total RNA or 10 µg of cell line total RNA and were run on a 0.8% agarose/MOPS/formaldehyde gels and blotted onto hybond-N as described by Fourney *et al* [1987]. Blots were hybridised under standard conditions with $0.1x10^6$ cpm/ml of purified probes derived from the clones NG1.242 [δ region of gene] and NG3 [$\alpha 2$ region of gene] in the presence of 5% dextran sulphate. The blots were washed with 0.1 x SSC at 60^oC and autoradiographed for 7-14 days.

The results of these blots are shown in figs 5.22 - 5.24. A strongly hybridising product of 8.5 kb was detected in NG108-15 cells and at a lower level in N18/TG2 cells from which all three cell lines are derived. No hybridising band was detected in the F2.1D1 cell line. This suggests that the $\alpha 2$ gene expressed in NG108-15 cells is derived from the mouse neuroblastoma line N18/TG2 but this has been lost in the construction of the F2.1D1 cell line. This probably occurred while the F2.1D1 hybridoma was stabilising and losing chromosomes after fusion.

Northern blots with the rodent tissues showed hybridisation of the NG1.242 insert to a 8.5 kb band present in all mouse tissues except kidney. No hybridising bands were seen in rat tissues. Northern blots with the NG3 probe however showed hybridisation only to an 8.5 kb product in mouse brain. This suggested that the δ portion of the gene is conserved tissues but that the $\alpha 2$ portion of the gene varies between different tissues. The mechanism for this variation remains obscure but the findings are

compatible with alternative splicing of $\alpha 2$ and δ exons to form a large $\alpha 2$-δ transcript which is translated and then cleaved post-translation to separate $\alpha 2$ and δ peptides. The gene splicing site probably does not correspond to the protein cleavage site as the sequences are very similar up to the second membrane spanning region 12 amino acids N-terminal to the di-alanine cleavage site.

Figure 5.22

Northern blot of 10 μg of total RNA run on a 0.8% agarose/MOPS/formaldehyde gel. Lane 2 NG108-15, lane 1 N18/TG2, lane 3 F2.1D1. Markers PM2/*HindIII* digest [M]. The blot was hybridised in 5mls 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8, 50% formamide at 42^{o}C and $0.5x10^{6}$ cpm/ml NG1.242 probe. The blot was washed in 0.1xSSC at 60^{o}C and autoradiographed for 5 days. This blot shows the presence of an 8.5kb mRNA homologous to the probe in NG108-15 cells and more faintly in the mouse neuroblastoma ancestral line N18TG2. However, the mRNA is not present in the F2.1D1 line which is also derived from the N18TG2 line.

Figure 5.23

Northern blot of 50 μg of total RNA from rat [lanes 1-6] and mouse [lanes 7-12] tissues. Tissues: brain [1,7],lung [2,8], muscle [3,9], liver [4,10], heart [5,11] and kidney [6,12]. Markers PM2/*HindIII* digest. RNA was run on a 0.8% gel and the blot was hydridised in 5 mls of 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8, 5% dextran sulphate and 50% formamide with $0.1x10^6$ cpm/ml of probe NG1.242 at 42^oC. The blot was washed in 0.1xSSC at 60^oC and autoradiographed for 10 days.

The NG108-15 line is derived from a fusion of a rat glioma cell line and a mouse neuroblastoma. Homologous sequences ought to be present in rodents from which the cell line is ultimately derived. The NG108-15 α2-δ gene is present in mouse lines [fig 5.22]. Here the δ region of the NG108-15 α2-δ gene shows the presence of homologous sequences in mouse of size 8.5kb.

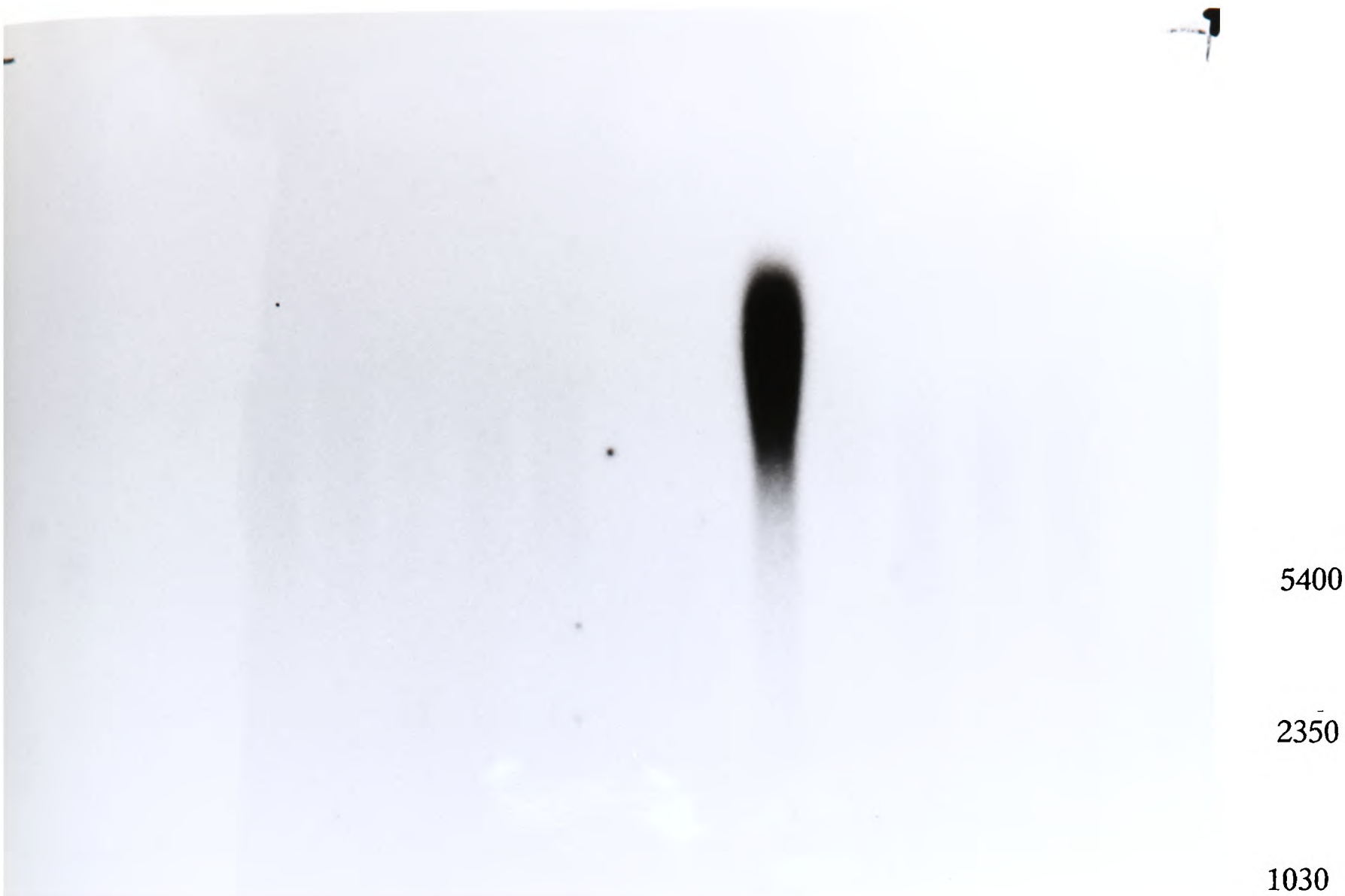


Figure 5.24

Northern blot of 50 μg of total RNA from rat [lanes 1-6] and mouse tissues [lanes 7-12]. Tissues: brain [1,7], lung [2,8], muscle [3,9], liver [4,10], heart [5,11] and kidney [6,12]. Markers PM2/*HindIII* digest. The RNA was run on a 0.8% gel and the blot was hybridised in 5mls of 6xSSCE, 10xDenhardt's, 100μg/ml herring sperm DNA, 1μg/ml pUC8, 5% dextran sulphate and 50% formamide with $0.1x10^6$ cpm/ml of NG3 probe at 42^oC. The blot was washed in 0.1xSSC at 60^oC and autoradiographed for 7 days.

Homologous regions to the δ region of the NG108-15 $\alpha 2$-δ gene are present in mouse tissues [fig 5.23]. This blot examines the presence of sequences homologous to the $\alpha 2$ region in both rat and mouse tissues. A 8.5kb band is seen only in mouse brain.

5.33 Computer analysis of NG108-15 and rabbit skeletal muscle $\alpha 2$ genes

To date, 2238 nucleotides of the sequence of the NG108-15 $\alpha 2$ gene have been isolated and these encode 746 amino acids corresponding to amino acids 327 -1103 of the rabbit $\alpha 2$ gene as originally described by Ellis *et al* [1989]. All the analyses detailed in the following section were performed using the Wisconsin GCG7 series of programs running on the Vax computer at Oxford. The sequence of the NG108-15 $\alpha 2$ is shown in fig 5.25 with its restriction map. The rabbit skeletal muscle $\alpha 2$ gene is shown in fig 5.26 for comparison. Some of the changes in restriction sites have been confirmed: the unique *Sca1* site at 1883 in the NG gene and the lack of *AccI*, *BglII*, *EcoR1*, *HindIII*, *KpnI*, and *PvuII* sites in the region 1-1760. The sequences are 48% identical at amino acid level and 62% comparable as opposed to 62% homology at nucleotide level. The major regions of smilarity are the 3' end including both membrane spanning regions and the MS2 - MS3 interrregion [78% nucleotide homology over 742 bp]. Comparison of the NG108-15 $\alpha 2$ gene with the EMBL and GenBank databases revealed significant similarity to only the rabbit $\alpha 2$ gene and the rat connexin gene [see fig 5.27a] both in the 3' end of the gene. Connexin genes are known to be distantly related to the receptor gated ion channel proteins and are found in the nerve terminal [Betz, 1990]. Amino acid comparison with the SwissProtein database again revealed homology to the rabbit $\alpha 2$ gene and a 17 amino acid section at 492 - 508 showed 47% homology to the 1248 - 1263 region of the rabbit cardiac $\alpha 1$ gene [fig 5.27b]. The significance of the latter finding is unclear.

[a]

```
                                                  30
ATT ATC GCG AAG GGT ATT ACC GAC TAT AAC AAC GGC TTA TCT CAG CTG
I   T   A   K   G   I   T   D   Y   K   K   G   F   S   Q   L
                60                                          90
CTC AAT GCT AAT TTA TCT AGC GCC AGC CCC GAC ACC ATC ATC CCG ATA
L   N   A   N   L   S   R   A   S   P   D   T   I   I   P   L
                                        120
ACC GGC CTC TCC GGC ATC CGT TAC AGA CAA GCT GTT ACA GTG TCC GGG
T   G   L   S   G   I   R   Y   R   Q   A   V   T   V   S   G
      150                                       180
GTC GCT TGT GTG GAG GTG TTT ACC GTA TCC GTG GGA CAA GCT TGC AGG
V   A   C   V   E   V   F   T   V   S   V   G   Q   A   S   R
                            210                             240
GGT GGC CAG AGC TGG AGG ATG GCG TGC GAG GCA TAC ATC GGT ACG GAT
G   G   Q   S   W   R   M   A   C   E   A   Y   I   G   T   D
                                            270
GAG CTT AGT TCC ATC ACC TCT TTG AGA GCG CGT ACA CAT ATG TAC CCA
E   L   S   S   I   T   S   L   R   A   R   T   H   M   Y   P
                300                                         330
GAC ACA CAA GGA GAA AAG CCC GTA ATG GCC CTG AGG GGT AAA GCA ATG
D   T   Q   G   E   K   P   V   M   A   L   R   G   K   A   M
                                        360
TTT CAA GTG CAA TGG ACA GAC GTG TAC CTA AAC GCG CTT CTT ATA ATA
F   Q   V   Q   W   T   N   V   Y   L   N   A   L   L   I   I
      390                                       420
TCT GTG GAT TCC TCT AGA GTC CCC GCC TTC AAC CTC ACC GGT CAA TTC
S   V   D   S   S   R   V   P   A   F   N   L   T   G   Q   F
                            450                             480
GAA AAT AAG ACT CAA AAC ATC AAT AAC GCC CTG CAG CTC ATC CTT GGC
E   N   K   T   Q   N   L   K   N   A   L   Q   L   I   L   G
                                            510
GTC ATG GGA GTC TGT CCG TCT CTG TGT GTC ATC CTG CAT GTA ACA CCT
V   M   G   V   C   P   S   L   C   V   I   L   H   V   T   P
                540                                         570
CGA GTA ACC GGA TCT CTA GAG TCG GTG GTG GTA CAG TTT GGC GCA TTG
R   V   T   G   S   L   E   S   V   A   V   Q   F   G   A   L
                                        600
CTC CCC GTG CTT CTT GCG ATG CTT CCG TTC CCG CTC ATT GCG GTA GGC
L   P   V   L   L   A   M   L   P   F   P   L   L   A   V   G
      630                                       660
TAC ATG CAG GAC GAC CAG CAT AGA CGG ACA AAT AAA TGG GCT AAG TTA
Y   M   Q   D   D   Q   H   R   R   T   N   K   W   A   K   L
                            690                             720
TCT AGT CAG GAA CCT GTG ATC CTG GAC GGA GTG GAC GAA GAT GAG CTG
S   S   Q   E   P   V   T   L   D   G   V   D   E   D   E   L
                                            750
CAG GGC CCA GAG CGG ATG TTG GAG ATA GAT GCA GAA GAA GTT GTC ATA
Q   G   P   E   R   M   L   E   I   D   G   E   E   V   V   I
                780                                         810
AGC ACA CGT GTA CAG AGC GAC CCA GAG CTG GTG GAT GGA GGC CGT CTT
S   T   R   V   Q   S   D   P   E   L   V   D   G   G   R   L
                                        840
TAC ACA TGG TCA GGA TGC ACA AGT CTC TAC AGC CCC CTT GTG CTG CTC
Y   T   W   S   G   C   T   S   L   Y   S   P   L   V   L   L
```

```
      870                                                   900
CTC CCC ACC TAC CTT TTC TAT TAT ATC AAG CTA ATT TGT TTA GTA GAC
L   P   T   Y   L   F   Y   Y   I   K   L   I   C   L   V   D
                        930                                   960
ATT ACG TAC GTA ATT GTT TAC TCC ACC ACC TTA AAG CCA GAT TAT TTA
I   T   Y   V   I   V   Y   S   T   T   L   K   P   D   Y   F
                                        990
TCA GCG TCA AAG GGC TAT ACA GCA TTG GCA CCC AGT GAC TAT TGC AGC
S   A   S   K   G   Y   T   A   L   A   P   R   D   Y   C   S
              1020                                    1050
TGC GAC ATA AAG GTC TCC CAC CCC GAC CTG CAG TTT CTC CTT GTG TTC
C   D   L   K   V   S   H   P   D   L   Q   F   L   L   V   L
                                1080
CTT ATA GTG ATT GGT CAT TTG ACT CCT ATA TTT CCT GCT TGC CTT CTG
L   I   V   I   G   H   L   T   P   I   F   P   A   C   L   L
      1110                                          1140
AGC TCG TAT TTA AGG TCG GTC CTG CTG GAT TCG GAT CTG GAG ACA GGC
S   S   Y   L   R   S   V   L   L   D   S   D   L   E   T   G
                        1170                                  1200
GAG GCA GAC CAG TTC TGG GCA CTT CAG GGC AAT ATT GAT GTA AAA CTG
E   A   D   Q   F   W   A   L   Q   G   N   I   D   V   K   L
                                        1230
AGG TTT ACA GGT GGC CTC TGG CGA AAA GTC CCA TCG GAG GCA CTC GAG
R   F   T   G   G   L   W   R   K   V   P   S   E   A   L   E
              1260                                    1290
CTC ACC ATG GAG CAC CCG TAC ATA ACC GAG CTC AGC CTA GAC GGA CGT
L   T   M   E   H   P   Y   I   T   E   L   S   L   D   G   R
                                1320
CCA CAA GAT AAT GAC CTA TAC AGT GTC TTC AGT GTG CGG TTC CCG AAG
P   Q   D   N   D   L   Y   S   V   F   S   V   R   F   P   K
      1350                                          1380
GCC GAA GTG CCA GGC GTC ATG GCA AAA GAC CCA GAG TTG AGC AAA ACG
A   E   V   P   G   V   M   A   K   D   P   E   L   S   K   T
                        1410                                  1440
TTC AGT AAT GCC CAG AAG CCA AAG CGT TCC CTC TTG AAA AAC ACC ACC
F   S   N   A   Q   K   P   K   R   S   L   L   K   N   T   T
                                        1470
TCT GCC GAA AAT GTT GAG AAG GTT GTT AGA CCG CCA CAA AAT CCG GTG
S   A   E   N   V   E   K   V   V   R   P   P   Q   N   P   V
              1500                                    1530
GTG ATT AAT ACT TTC CAC ACT TGG ATC ATG TTC GTC ATA CTG TTG ATG
V   I   N   T   F   H   T   W   I   M   F   V   I   L   L   M
                                1560
CGG TCA ATC TAT GAT GAT GCC TAT CGG TTG CAT GTA CGC GCT TTC ACT
R   S   I   Y   D   D   A   Y   R   L   H   V   R   A   F   T
      1590                                          1620
GTA ATA GGT CAT TAT CTT GTG GAA CGT CCT TTG AGA CTT AGC TAC GCG
V   I   G   H   Y   L   V   E   R   P   L   R   L   S   Y   A
                        1650                                  1680
TTC AAT AAA TCT TAT GAC TAT CAG TCG GTG TGT GAC CCT GGT GCT GCG
F   N   K   S   Y   D   Y   Q   S   V   C   E   P   G   A   A
                                        1710
CCC AAG CGC GGC GCA GGG CAC CGC TCG GCT TAT GTG CCA TCA ATT GCA
P   K   R   G   A   G   H   R   S   A   Y   V   P   S   I   A
```

```
                1740                                                1770
GCA ATA CTC CAG ATT GGC TGG TGG GCC ACT GCT GCC GCC TGG TCC ATT
A   I   L   Q   I   G   W   W   A   T   A   A   A   W   S   I
                                1800
CTC CAG CAG CTA CTC TGT GAT TTG CAA TTT CCA CGG CTC CTT GAG GCA
L   Q   Q   L   L   C   D   L   Q   F   P   R   L   L   E   A
      1830                                              1860
GTT GAA ATG GAA GAG GAT GCT TTC ACA GCT TCC CTG TCT AAG CAG AGC
V   E   M   E   E   D   A   F   T   A   S   L   S   K   Q   S
                            1890                                1920
TGC ATC ACA GAA CAA ACT CAG TAC TTC TTC AAG AAC GAT TCA TTA TCA
C   I   T   E   Q   T   Q   Y   F   F   K   N   D   S   L   S
                                    1950
TTC AGT GGC TTA CTG GCA TGT GGA AAC TGT CAG GAT CTT CAT GTG GAG
F   S   G   L   L   A   C   G   N   C   Q   D   L   H   V   E
                1980                                        2010
AAG CTT ATG AAC ACT AAT CTA GGC TCT ATA ATG GTG GAG TCT AAG GGG
T   L   M   N   T   N   L   G   S   I   M   V   E   S   K   G
                                2040
CAG TGT TGT GAC ACT CGC CTG CTC ATG CAA GCA GAG CAG GCA TCA GAC
H   C   C   D   T   R   L   L   M   Q   A   E   Q   A   S   D
      2070                                              2100
GGA CCT GAT CCT TGC TCG ATG GTA AAG CAG CCG CGT TAT AGG AAA GGA
G   P   D   P   C   S   M   V   K   Q   P   R   Y   R   K   G
                            2130                                2160
CCT GAC GTG TGC TTC GAC AAT AAC GTT CTT GAA GAC TAT ACT GAC TGT
P   D   V   C   F   D   N   N   V   L   E   D   Y   T   D   C
                                    2190
GGC GGC GTA TAT GGA TTA AAT CCA TCT CTA TGG TCC ATT ATA GCT ATT
G   G   V   Y   G   L   N   P   S   L   W   S   I   I   A   I
                2220
CAA TTT GGC CTC CTA TGG CTG GTT TCT GGC
Q   F   G   L   L   W   L   V   S   G
```

Figure 5.25a

The nucleotide and amino acid sequence of the NG108-15 $\alpha 2$ gene

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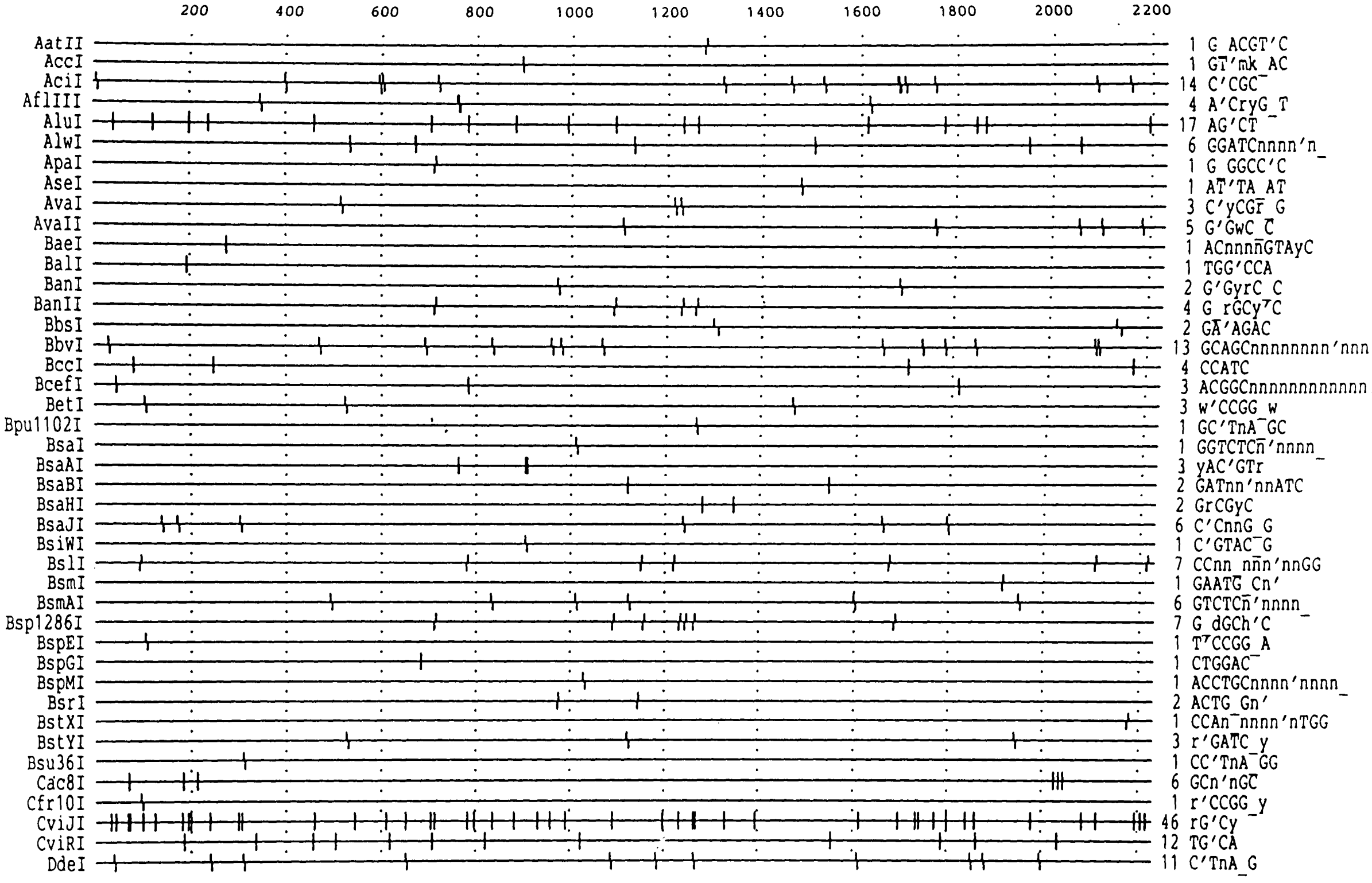

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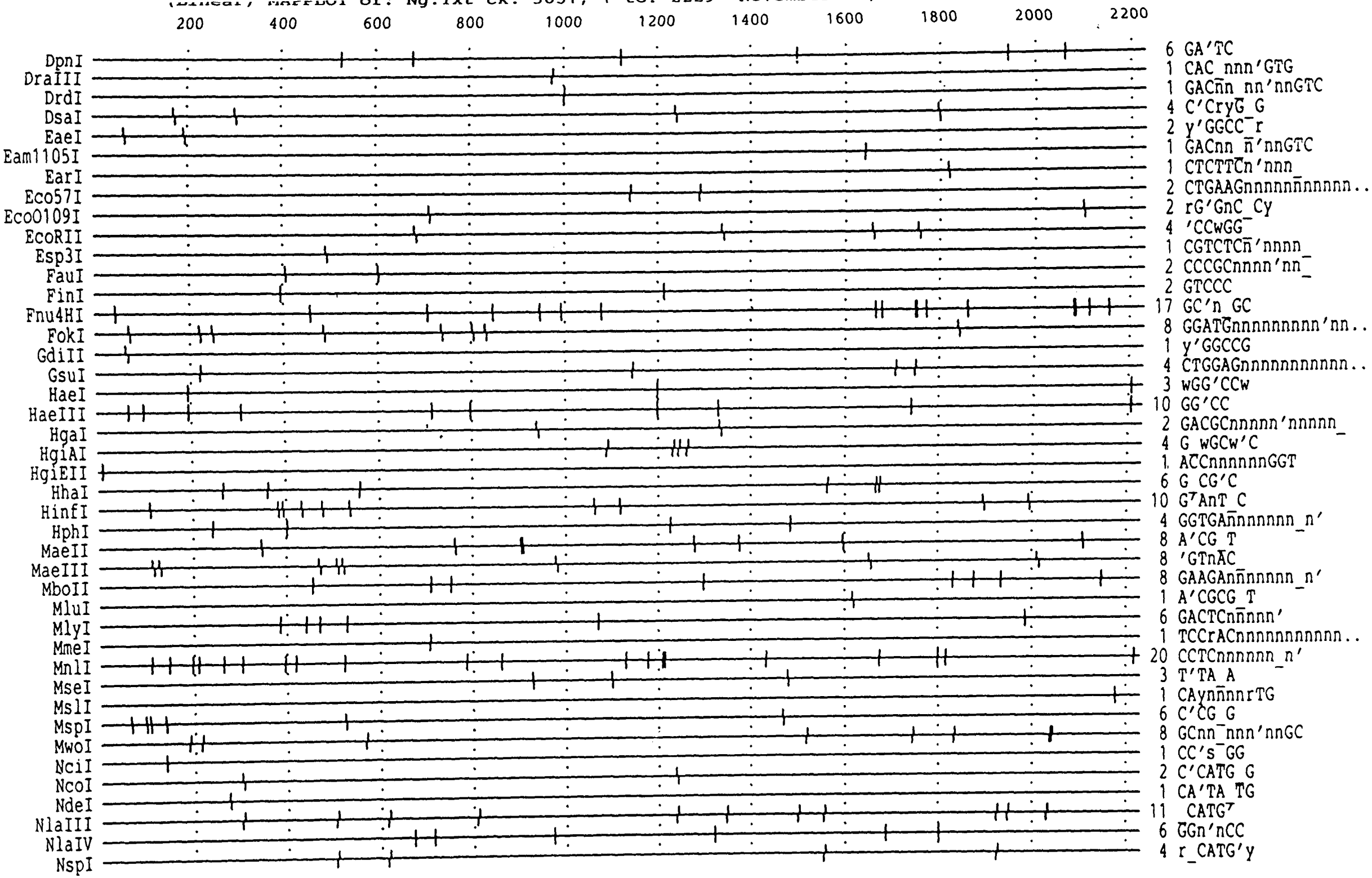

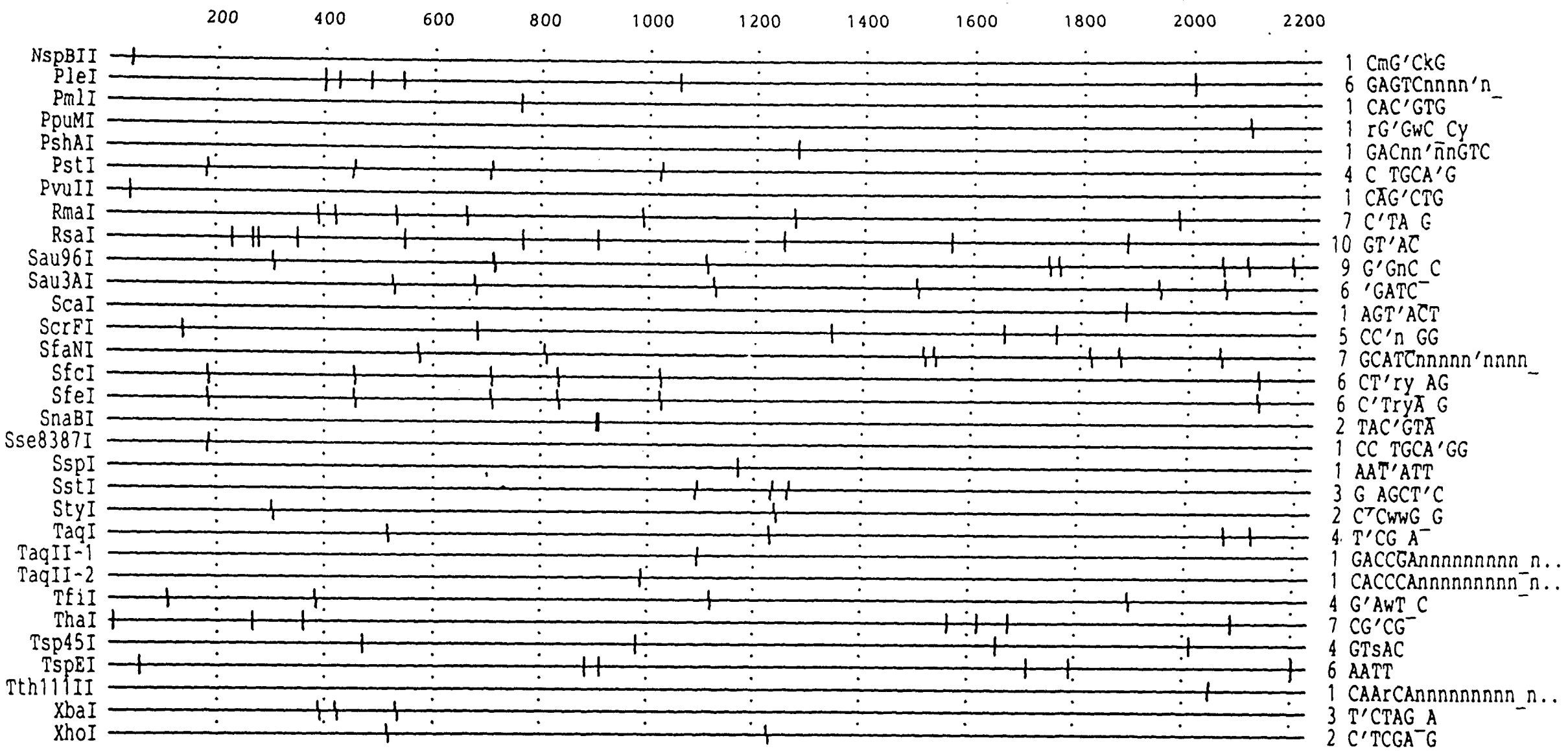

Enzymes that do not cut:

AflII	AgeI	AlwNI	ApaBI	ApaLI	AscI	AvrII	BamHI	BcgI	BcgI	BclI	BglI
BglII	Bpu10I	BsgI	BsiI	BsiEI	BspHI	BssHII	Bst1107I	BstEII	ClaI	DraI	DrdII
EciI	Eco47III	EcoNI	EcoRI	EcoRV	FseI	FsiI	FspI	HaeII	HincII	HindIII	HpaI
KpnI	MunI	NaeI	NarI	NheI	NotI	NruI	NsiI	NspV	PacI	Pfl1108I	PflMI
PmeI	PvuI	RleAI	RsrII	SacII	SalI	SapI	SfiI	SgrAI	SmaI	SpeI	SphI
			StuI	SwaI	Tth111I	XcmI	XmaIII	XmnI			

Enzymes excluded; MinCuts: 1 MaxCuts: 350000

NONE

Figure 5.25b

The resrtiction map of the isolated section of the NG108-15 α2-δ gene as generated using the Wisconsin GCG7 program [Devereux *et al*, 1984].

```
  1 ..........................ITAKGITDYN.....GLSQLLNAN 19
                              |||||||||.     ::.|||| |
301 QDVSCFQHLVQANVRNKKVLKDAVNNITAKGITDYKKGFSFAFEQLLNYN 350

 20 LSPASPDTIIPITGLSGIRYRQAV..TVSGVACVEVFTVSVGQ.ACRGGQ 66
    :|.|..:.|| : . :|    |.:  . .    | |||.|||| .:  |.
351 VSRANCNKIIMLFTDGGEERAQEIFAKYNKDKKVRVFTFSVGQHNYDRGP 400

 67 SWRMACEAYIGTDELSSITSLRARTHMYPDTQGKPMALRGKAMFQVQWTD 116
      :||||.     |:.||..:| .|: | |. |:||.| |.   |||||:
401 IQWMACENKGYYYEIPSIGAIRINTQEYLDVLGRPMVLAGDKAKQVQWTN 450

117 VYLNALLIISVDSSRVPAFNLTPALENKTQNINNALQLIFVTGVCPSLCV 166
    |||:|| :  | .: :|.||:|..:|||| |:.|.| :: | || .||
451 VYLDALELGLVITGTLPVFNITGQFENKT.NLKNQL.ILGVMGVDVSLED 498

167 ILHVTPRVTGSLESVAVQFGALLPVLL.AMLPFPLIAVGYMQDDQHRRTN 215
    | ::|||.| : ::  ..:::   ||| : |. . |:||.   : ::|
499 IKRLTPRFTLCPNGYYFAIDPNGYVLLHPNLQPKPIGVGIPTINLRKRRP 548

216 KWAKLSSQEPVILDGVDED.....ELQGPERMLEIDGEEVVISTRVQSDP 260
    . .. .|||||.|| :|.:     .:: .::|:: :::| .:.| |.|:.
549 NVQNPKSQEPVTLDFLDAELENDIKVEIRNKMIDGESGEKTFRTLVKSQD 598

261 E..LVDGGRLYTWSGCTSL.YSPLVLLLPTYLFYYIKLICLVDITYVIVY 307
    |  : .|.| |||....:  ||.|.|:|||| |||||  .  .|| .
599 ERYIDKGNRTYTWTPVNGTDYSSLALVLPTYSFYYIKAKIEETITQARYS 648

308 STLKPDYLAASKGYTALAPSDYPSCDIKVSHPDLQFLLVFLIVIGHLTPI 357
    .||||| :..| ||| |||.||.| |:|.|. : :||| |  .|:: ||
649 ETLKPDNFEES.GYTFLAPRDYCS.DLKPSDNNTEFLLNFNEFIDRKTPN 696

358 FPAALLSSYLRSVLLDSDLETGEADQFWALQGNI.DVKLRF...TGGLWR 403
     |..  ...:..||||.::..: .:.:|. | || :|| ||   .||: |
697 NPSC.NTDLINRVLLDAGFTNELVQNYWSKQKNIKGVKARFVVTDGGITR 745

404 KVPSEALELTMEHPYITELSLDGRPQDNDLYSVFSVRFPKAEVPGVMAKD 453
      |.|| |   |:| . | |:  |. ||| | ||...: . ..||. ..:
746 VYPKEAGENWQENPETYEDSFYKRSLDNDNY.VFTAPYFNKSGPGAYESG 794

454 PELSKTFSNAQKPKRSFLKNTTSAENVE.........KVVRPPQNPVVI 493
      :||...   ..|  :|| .. : .::          . :|.| ...|.
795 IMVSKAVEIYIQGK..LLKPAVVGIKIDVNSWIENFTKTSIRDPCAGPVC 842

494 NTFHTWIMF..VIL....LLMRSIYDDAYRLHVRAFTVIGHYLVERPLRL 537
    :. :.. ::  |||    :|:.. .||  .  .| |. |:. |: : :.:
843 DCKRNSDVMDCVILDDGGFLLMANHDDYTNQIGRFFGEIDPSLMRHLVNI 892

538 S.YAFNKSYDYQSVCDPGAAPKRGGGHRSAYVPSITDILQIGWWATAAAW 586
    | |||||||||||||:||||||.|:||||||||||.||||||||||||||
893 SVYAFNKSYDYQSVCEPGAAPKQGAGHRSAYVPSIADILQIGWWATAAAW 942

587 SILQQLLCDLQFPRLLEAVEMEEDAFTASLSKQSCITEQTQYFFKNDSIS 636
    |||||:| .| |||||||.:||:|.||||:|||||||||||||.||| |
943 SILQQFLLSLTFPRLLEAADMEDDDFTASMSKQSCITEQTQYFFDNDSKS 992

637 FSGILACGNC.QDLHVETLMNTTLGSIMVESKGDCCDDTRLLMQAEQASD 685
    |||:|.|||| . :|||.||||.|  |||||||.|. |||||:||||.||
993 FSGVLDCGNCSRIFHVEKLMNTNLIFIMVESKGTCPCDTRLLIQAEQTSD 1042

686 GPDPCSMVKQPRYRKGPDVGCFDTTVLEDYTDCGGVYG.NPSLWSIIAIQ 734
    |||||.|||||||||||| |||..|||||||||||  | |||||||:||
1043 GPDPCDMVKQPRYRKGPDV.CFDNNVLEDYTDCGGVSGLNPSLWSIIGIQ 1091

735 FGLLWLVSG.......  743
    |.|||||||
1092 FVLLWLVSGSRHCLL* 1107
```

Figure 5.26

Comparison of the portion of NG108-15 $\alpha 2$ gene and the homologous portion of the rabbit $\alpha 2$ gene [amino acids 327-1103] using the Wisconsin GCG7 program [Devereux *et al,* 1984]

	Sequence	%	Number
Nucleotide:	Rabbit alpha 2 VGCC subunit	63	1116
	"	72	729
	Rat connexin-31	72	192
	Rat connexin-31	69	102
	E. coli Rts plasmid	58	135
	S. cerevisiae TRP1 & AMPr	80	67
	plasmid pAA3.7X	71	102
	plasmid R100 repA	71	104
	plasmid cole1 rop	71	104
	Chicken retinoate ß-BP	87	30
Protein:	Rabbit alpha 2 VGCC subunit	49	484
	Rat acylaminoreleasing enzyme	37	19
	Pig acylaminoreleasing enzyme	37	19
	Pseudomonas NOSD protein	25	43
	EBV latent membrane protein	34	35
	Rabbit α1 VGCC subunit	47	17
	Human DNF1552 protein	37	19
	Tobacco NADH oxoreductase	41	34
	Spinach 250kDa protein	38	21
	Serratia iron transporter	24	139

Figure 5.27

Comparison of the nucleotide and amino acid sequences of the NG108-15 α2 with other sequences in the GenBank, EMBL and Swiss protein databases.

Hydropathy analysis of both sequences was performed using the Goldman and Klyte & Doolittle algorithms [figs 5.28 and 5.29]. The rabbit $\alpha 2$ gene is difficult to model using this protocol as only 2 membrane spanning regions [bp 1344 - 1413; AA 438 - 471] and [bp 3243 - 3303; AA 1081 - 1101] are easy to identify. The second membrane spanning region [bp 2760- 2835; AA 920 - 945] has a lower hydrophobicity [-1.2 over 20 amino acids] than is usually required for a membrane spanning region [-1.6 over 19 amino acids]. The structure and folding of the rabbit $\alpha 2$ gene is still controversial [Catterall, 1991] and is principally related to placing the cAMP phosphorylation sites and asparagine glycosylation sites correctly. Motif analysis of both genes is shown in fig 5.30 and 5.31. The current model places 6 glycosylation sites internally due to the hydrophilic nature of the MS1- MS2 interregion. In the NG108-15 gene this region is more hydrophobic and the two possible rabbit cAMP phosphorylation sites [AA 500, 845] are lost. The hydropathy profile is consistent with 5 membrane spanning domains if hydrophobic domains containing modification sites are not counted. A model with 7 membrane spanning domains is needed if all hydrophobic domains are considered membrane spanning. The 5 membrane spanning unit model comprises the 3 regions recognised in the rabbit gene plus two extra membrane spanning regions at AA 160-180 [rabbit AA 487 - 507] and AA 340 - 360 [rabbit AA 627 - 647]. The extra two regions change the folding of the rabbit gene to place more glycosylation sites extracellularly but do not affect the positioning of the cAMP phosphorylation sites. The changes do not affect the siting of the N-terminal which is assumed to be extracellular due to the presence of a signal sequence in the protein. The difficulty with this model is that these regions are amphipathic in the rabbit gene and not hydrophobic. There are multiple possible protein kinase C sites in both genes and it

is difficult to assess which are significant in the abscence of site directed mutagenesis. The tyrosine phosphorylation sites are reduced to a possible two from three but it is doubtful that 3 exist as 2 sites overlap in the rabbit gene. It is difficult to come to any conclusion about the relevance of the phosphorylation sites as no evidence is available about which modulatory systems affect the $\alpha 2$ subunit.

PEPPLOT of: Ng.Pep ck: 3457, 1 to 743 November 29, 1991 14:36
TRANSLATE of: ng check: 3051 from: 1 to: 2229

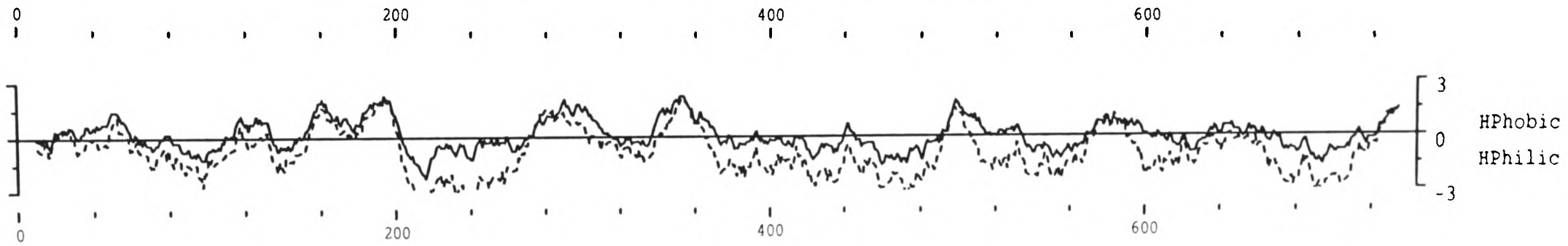

Figure 5.28

Hydropathy analysis of the NG108-15 $\alpha 2$ gene product by the Goldman and Klyte & Doolittle algorithms.

PEPPLOT of: Raba2n.Pep ck: 6626, 1 to 1107 November 29, 1991 14:30
TRANSLATE of: raba2n.txt check: 871 from: 309 to: 3629

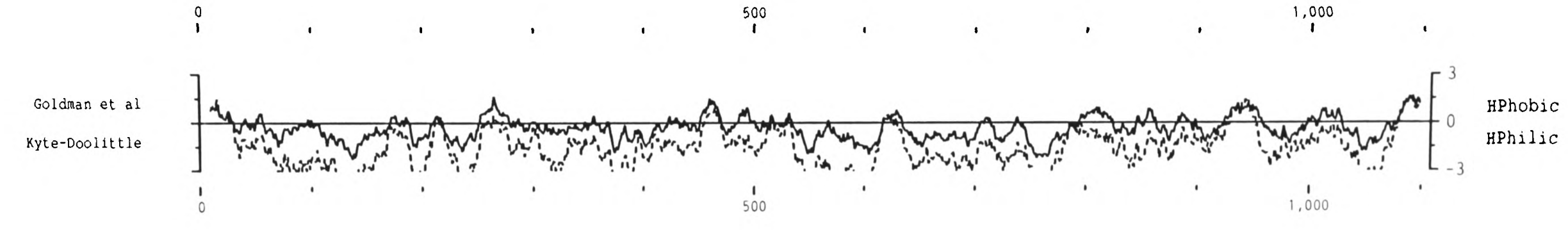

Figure 5.29

Hydropathy analysis of the rabbit $\alpha 2$ gene [amino acids 327-1103] by the Goldman and Klyte & Doolittle algorithms.

s from: ng.pep

tches: 0 November 19, 1991 15:35 ..

Ng.Pep Check: 3457 Length: 743 ! TRANSLATE of: ng check: 305

ycosylation N~(P)(S,T)~(P)

		N~P(T)~P	
143:	TPALE	NKTQ	NINNA
		N~P(T)~P	
473:	RSFLK	NTTS	AENVE
		N~P(S)~P	
542:	LSYAF	NKSY	DYQSV
		N~P(S)~P	
632:	QYFFK	NDSI	SFSGI
		N~P(T)~P	
656:	VETLM	NTTL	GSIMV

osphо_Site (S,T)x2(D,E)

		(S)x{2}(E)	
221:	KWAKL	SSQE	PVILD
		(S)x{2}(E)	
258:	STRVQ	SDPE	LVDGG
		(S)x{2}(D)	
337:	CDIKV	SHPD	LQFLL
		(S)x{2}(E)	
374:	SVLLD	SDLE	TGEAD
		(T)x{2}(E)	
475:	FLKNT	TSAE	NVEKV
		(S)x{2}(D)	
510:	LLLMR	SIYD	DAYRL
		(S)x{2}(D)	
549:	SYDYQ	SVCD	PGAAP
		(S)x{2}(D)	
570:	SAYVP	SITD	ILQIG
		(S)x{2}(D)	
666:	SIMVE	SKGD	CCDDT
		(T)x{2}(E)	
710:	GCFDT	TVLE	DYTDC

tyl G~(E,D,R,K,H,P,F,Y,W)x2(S,T,A,G,C,N)~(P)

		G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P	
159:	LIFVT	GVCPSL	CVILH
		G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P	
176:	TPRVT	GSLESV	AVQFG
		G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P	

561:	APKRG	GGHRSA	YVPSI
		G~(E,D,R,K,H,P,F,Y,W)x{2}(C)~P	
639:	SISFS	GILACG	NCQDL
		G~(E,D,R,K,H,P,F,Y,W)x{2}(T)~P	
705:	KGPDV	GCFDTT	VLEDY
		G~(E,D,R,K,H,P,F,Y,W)x{2}(G)~P	
719:	DYTDC	GGVYGN	PSLWS

hospho_Site (S,T)x(R,K)

		(T)x(K)	
2:	I	TAK	GITDY
		(S)x(R)	
67:	CRGGQ	SWR	MACEA
		(S)x(R)	
86:	LSSIT	SLR	ARTHM
		(S)x(R)	
129:	IISVD	SSR	VPAFN
		(T)x(R)	
171:	VILHV	TPR	VTGSL
		(T)x(K)	
214:	DQHRR	TNK	WAKLS
		(S)x(R)	
253:	EEVVI	STR	VQSDP
		(T)x(K)	
309:	VIVYS	TLK	PDYLA
		(S)x(R)	
438:	LYSVF	SVR	FPKAE

hospho_Site (R,K)x{2,3}(D,E)x{2,3}Y

		(R)x{2}(D)x{3}Y	
427:	LSLDG	RPQDNDLY	SVFSV
		(R)x{3}(D)x{2}Y	
509:	ILLLM	RSIYDDAY	RLHVR

Figure 5.30

The glycosylation, phosphorylation and other motif sites present in the isolated portion of the NG108-15 $\alpha 2$ gene.

s from: raba2n.pep

tches: 0 November 23, 1991 20:57 ..

Raba2n.Pep Check: 6626 Length: 1,107 ! TRANSLATE of: raba2n.txt ch

ycosylation N~(P)(S,T)~(P)

		N~P(S)~P	
94:	EKLLS	NRSK	ALVRL
		N~P(S)~P	
138:	LDPEK	NDSE	PGSQR
		N~P(T)~P	
186:	VLNEL	NWTS	ALDDV
		N~P(T)~P	
326:	KDAVN	NITA	KGITD
		N~P(S)~P	
350:	QLLNY	NVSR	ANCNK
		N~P(T)~P	
470:	TLPVF	NITG	QFENK
		N~P(T)~P	
477:	TGQFE	NKTN	LKNQL
		N~P(T)~P	
606:	YIDKG	NRTY	TWTPV
		N~P(T)~P	
615:	TWTPV	NGTD	YSSLA
		N~P(T)~P	
678:	LKPSD	NNTE	FLLNF
		N~P(S)~P	
784:	TAPYF	NKSG	PGAYE
		N~P(T)~P	
827:	NSWIE	NFTK	TSIRD
		N~P(S)~P	
891:	MRHLV	NISV	YAFNK
		N~P(S)~P	
898:	SVYAF	NKSY	DYQSV
		N~P(S)~P	
988:	QYFFD	NDSK	SFSGV
		N~P(S)~P	
1,001:	VLDCG	NCSR	IFHVE

Phospho_Site (R,K)2x(S,T)
(R,K){2}x(T)

500:	SLEDI	KRLT	PRFTL
		(R,K){2}x(S)	
845:	PVCDC	KRNS	DVMDC

spho_Site (S,T)x2(D,E)

```
                (S)x{2}(E)
  24: ILIGP        SSEE      PFPSA

                (S)x{2}(D)
  37: AVTIK        SWVD      KMQED

                (S)x{2}(D)
 189: ELNWT        SALD      DVFKK

                (S)x{2}(D)
 252: IQGAA        SPKD      MLILV

                (S)x{2}(E)
 277: KLIRT        SVSE      MLETL

                (T)x{2}(D)
 284: SEMLE        TLSD      DDFVN

                (S)x{2}(D)
 286: MLETL        SDDD      FVNVA

                (S)x{2}(D)
 495: MGVDV        SLED      IKRLT

                (S)x{2}(E)
 596: RTLVK        SQDE      RYIDK

                (T)x{2}(D)
 761: QENPE        TYED      SFYKR

                (S)x{2}(E)
 823: KIDVN        SWIE      NFTKT

                (S)x{2}(D)
 832: NFTKT        SIRD      PCAGP

                (S)x{2}(E)
 905: SYDYQ        SVCE      PGAAP

                (S)x{2}(D)
 926: SAYVP        SIAD      ILQIG
```

lation C~(D,E,N,Q)(L,I,V,M)x>

```
                C~(D,E,N,Q)(L)x
1,104: SGSRH          CLL*
```

inoglycan SGxG

```
 786: PYFNK SGPG AYESG
```

l G~(E,D,R,K,H,P,F,Y,W)x2(S,T,A,G,C,N)~(P)

```
                G~(E,D,R,K,H,P,F,Y,W)x{2}(G)~P
 211: LWQVF                GSATGL                    ARYYP

                G~(E,D,R,K,H,P,F,Y,W)x{2}(G)~P
 264: LVDVS                GSVSGL                    TLKLI

                G~(E,D,R,K,H,P,F,Y,W)x{2}(T)~P
 459: DALEL                GLVITG                    TLPVF

                G~(E,D,R,K,H,P,F,Y,W)x{2}(N)~P
```

473:	VFNIT	GQFENK	TNLKN
491:	ILGVM	G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P GVDVSL	EDIKR
616:	WTPVN	G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P GTDYSS	LALVL
789:	NKSGP	G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P GAYESG	IMVSK
794:	GAYES	G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P GIMVSK	AVEIY
995:	SKSFS	G~(E,D,R,K,H,P,F,Y,W)x{2}(C)~P GVLDCG	NCSRI
1,025:	MVESK	G~(E,D,R,K,H,P,F,Y,W)x{2}(C)~P GTCPCD	TRLLI
1,075:	DYTDC	G~(E,D,R,K,H,P,F,Y,W)x{2}(G)~P GGVSGL	NPSLW
1,079:	CGGVS	G~(E,D,R,K,H,P,F,Y,W)x{2}(S)~P GLNPSL	WSIIG
1,100:	LWLVS	G~(E,D,R,K,H,P,F,Y,W)x{2}(C)~P GSRHCL	L*

ospho_Site		(S,T)x(R,K) (T)x(K)	
34:	FPSAV	TIK	SWVDK
93:	IEKLL	(S)x(R) SNR	SKALV
144:	DSEPG	(S)x(R) SQR	IKPVF
252:	IQGAA	(S)x(K) SPK	DMLIL
270:	SVSGL	(T)x(K) TLK	LIRTS
328:	AVNNI	(T)x(K) TAK	GITDY
503:	DIKRL	(T)x(R) TPR	FTLCP
589:	ESGEK	(T)x(R) TFR	TLVKS
650:	ARYSE	(T)x(K) TLK	PDNFE
832:	NFTKT	(S)x(R) SIR	DPCAG

Pho_Site		(R,K)x{2,3}(D,E)x{2,3}Y (K)x{2}(D)x{2}Y

595:	FRTLV	KSQDERY	IDKGN
768:	EDSFY	(K)x{3}(D)x{3}Y KRSLDNDNY	VFTAP
769:	DSFYK	(R)x{2}(D)x{3}Y RSLDNDNY	VFTAP

Figure 5.31

The glycosylation, phosphorylation and other modification sites present in the rabbit $\alpha 2$ gene [amino acids 327-1103].

It is known that the $\alpha 2$ gene is glycosylated up to 30% of its mass [de Jongh *et al*, 1990] but the sugar sequences have not been characterised. Therefore the question as to whether a few complex glycosylation sites exist or many sites are lightly glycosylated remains open. The presence of large numbers of possible sites in both genes [5 in the NG108-15 gene, 12 in the relevant region of the rabbit] makes it difficult to decide which are relevant. The other 4 sites in the rabbit gene occur 5' to the region of determined sequence. However, in both genes there seem to be 2 glycosylation sites in the region of the δ peptide but these are not identical in their sites. The two putative structures are shown in fig 5.32 and the modification site analyses are superimposed on the 2 models according to the 3 membrane spanning region and 5 membrane spanning region models.

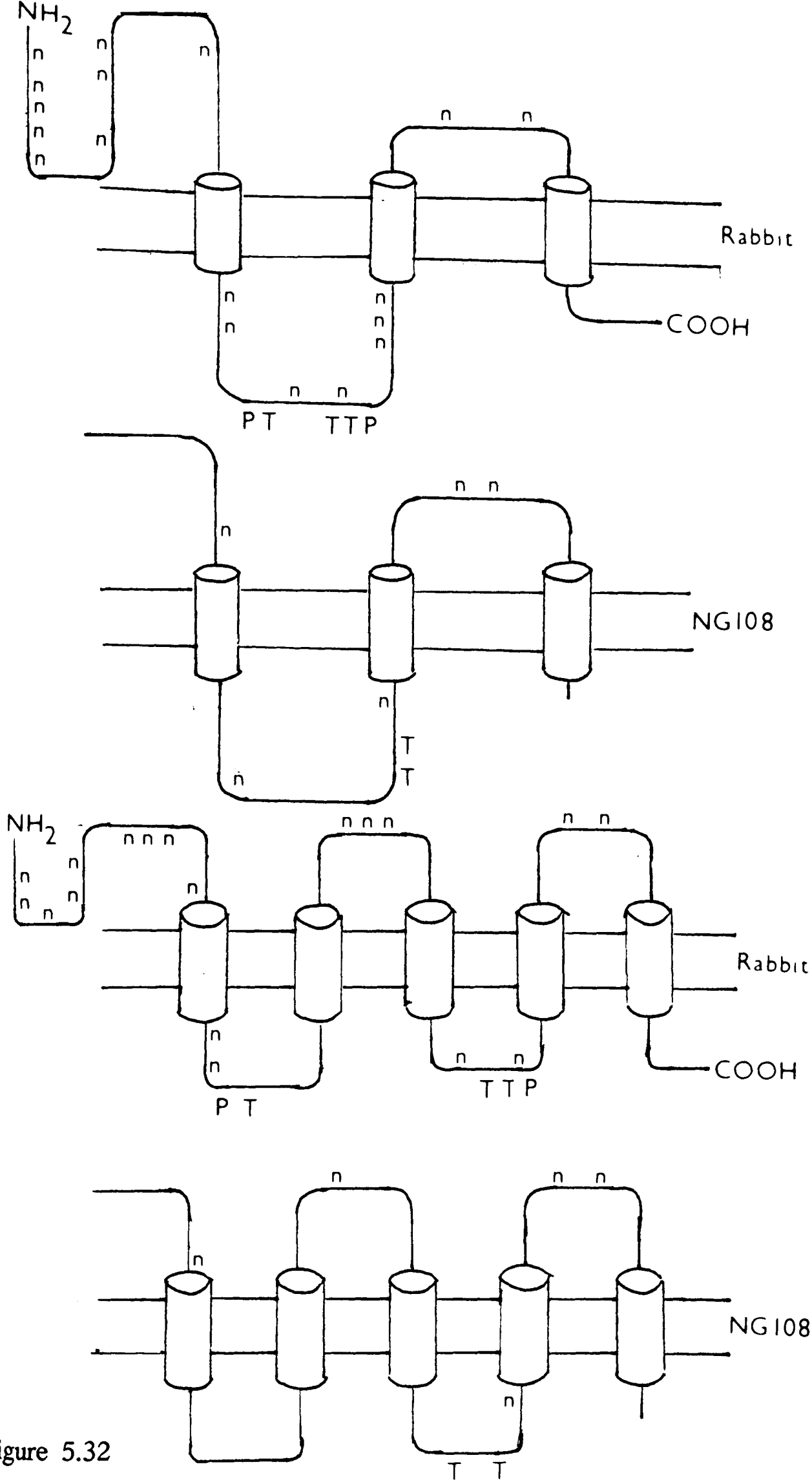

Figure 5.32

Alternative models of the NG108-15 and rabbit skeletal muscle α2-δ gene products with 3 or 5 membrane spanning regions showing the position of the modification sites. n=glycosylation site; P=cAMP phosphorylation site; T=tyrosine phosphorylation site

The resemblance of the 3' ends of the two genes corresponding to the δ peptides suggests an explanation for the tissue invariability of the α2-δ product described by Ellis *et al*, [1989]. The close similarity of these regions in contrast to the 5' ends suggests that this area was detected on both northern and Southern blots. Northern blots using a 3' [δ region] probe [NG1-242] support the the universal hypothesis indicating a mouse origin for the NG108-15 α2-δ gene. The δ regions of the NG108-15 α2 gene and the mouse brain α2 PCR product differ significantly implying the expression of different α2-δ genes in different cells in the brain with the NG108-15 α2-δ representing a neuronal lineage protein. The mouse brain PCR product probably represents another cell type's α2-δ gene. The 5' probe [α2 region] is brain specific on northern blots suggesting that α2 segments vary much more significantly between tissues than δ segements. The two α2 segments are only 34% homologous over the region up to the second membrane spanning domain of the rabbit gene or the putative α2-δ cleavage site. This suggests that α2-δ gene diversity arises by a complex mechanism involving the splicing of alternative but similar δ segement exons on to highly variable α2 exons at the level of transcription followed by peptide cleavage after translation to generate the final protein. Whether the variation in α2-δ genes is due to splicing variation or due to separate genes will only be determined once the genomic structure of the genes has been analysed.

It is known that α1 genes are alternatively spliced from genomic and PCR analysis but whether the separate families of genes are encoded as separate gene clusters remains to be determined. Little is known about the ß subunit but the cloning of the brain ß allows some conclusions to be drawn. The brain ß subunit which has a 96 amino acid deletion in the middle of the gene compared with the skeletal muscle gene

and a different 3' end but the other residues are identical. This structure suggests splicing variants. Little is known about variation in the γ gene.

5.33 Conclusions

The α2-δ gene shows a greater degree of variation in its general structure than has been thought previously. The variation is more complex in origin than in the ß and γ subunits but is less complex than the α1 gene. It seems to consist of a highly conserved section [δ] and a variable 5' end [α2]. The broad distribution of α2-δ gene on northern blots allied with the sequence information suggests that δ subunits are highly conserved. These may associate with alternative α2 regions which are highly tissue variable. This association may occur by splicing variation as generally happens in ion channels but alternative genes are not excluded. The elucidation of the generation of calcium channel diversity awaits the cloning of more α2-δ subunit genes from other tissues and the analysis of their genomic structure.

Chapter 6

Future Experiments

6.1 The NG108-15 $\alpha 2$ gene

Currently the remaining portions of the gene as subcloned in pUC8 are being sequenced and once this is completed and the full sequence analysed the full gene would be assembled in a plasmid.

The NG108-15 $\alpha 2$ gene could be used to isolate the human brain homologue from brain cDNA libraries. Also the sequences could be compared to find any conserved regions that might act as antigenic determinants for LEMS antibodies in both systems. The $\alpha 2$ gene has not been mapped in either mouse or man so its chromosomal position could be determined by somatic cell PCR and *in situ* hybridisation and its relevance to other nearby genes determined. Screening genomic libraries with the full length cDNA would also allow the intron/exon structure of the gene to be determined and it would be interesting to compare $\alpha 2$ genes from different organs to assess whether alternative splicing exists for exons in this gene.

The full length cDNA would be expressed in a prokaryotic system using a plasmid such as pREX or a modern fusion-protein vector and the protein product purified and used to screen LEMS sera for antibodies. This would determine whether any antibodies in LEMS sera react with the $\alpha 2$ subunit. The gene could be expressed either in the baculovirus-insect cell system or tranfected into a cell line lacking $\alpha 2$ subunits or VGCCs and the antibody screening repeated.

Short expression constructs would be used in studies to map the T cell epitopes present in LEMS in analagous experiments to those already being carried out with constructs derived from the various subunits of the acetylcholine recptor.

Once a stably expressed system had been constructed it would be possible to examine whether the $\alpha 2$ gene comprises part of the the conotoxin binding site or whether it is

the binding site for other toxins. Co-expression of either of the two $\alpha 2$ subunits with different $\alpha 1$ subunits would allow the control and pharmacology of partially reconstructed VGCCs to be studied by electrophysiological techniques and allow better assessment of the role of the $\alpha 2$ subunit in the VGCC.

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ar) MAP of: Ocalp1b check: 2593 from: 1 to: 3802

CALP1B standard; RNA; MAM; 3802 BP.
21948;
6-JUL-1989 (Rel. 20, Last updated, Version 1)
2-APR-1989 (Rel. 19, Created)
abbit dihydropyridine-sensitive calcium channel alpha-2 subunit
RNA, complete cds.
alcium channel protein; DHP-sensitive channel alpha-2 subunit;
ihydropryridine-sensitive calcium channel alpha-2 subunit;
-type calcium channel.
ryctolagus cuniculus (rabbit)
ukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia;
heria; Eutheria; Lagomorpha; Leporidae.
1]
-3802
llis S.B., Williams M.E., Ways N.R., Brenner R., Sharp A.H., . . .

181 enzymes: *

November 19, 1991 15:09 ..

```
                    B           C    F
          C         s C        BfH C n       NBNS
          a         s aHHAT   BNsraAaHuBMMMANlslg
          c         H chhvh   aaa1ecch4asnwcaaaar
          8         I 8aaaa   nrH0Ii8aHnploieIJIA
          I         I IIIII   IIIIIIIIIIIIIIVIVI
                      /  /        //  / / // //
  AGAAGGGAGGGCGAGCGTGGTGTGTGCGCGCTCGGGCGCCGGCGGCACCGCCGAGGTCTG
1 --------+---------+---------+---------+---------+---------+---------+ 60
  TCTTCCCTCCCGCTCGCACCACACACGCGCGAGCCCGCGGCCGCCGTGGCGGCTCCAGAC

  R  R  E  G  E  R  G  V  C  A  L  G  R  R  R  H  R  R  G  L   -
   E  G  R  A  S  V  V  C  A  R  S  G  A  G  G  T  A  E  V  C  -
    K  G  G  R  A  W  C  V  R  A  R  A  P  A  A  P  P  R  S  V -

                        F           F     F F        N
                        n           n C B n nB  H  NsSC
          M             AM Au M M.  u vAsDuBusHBaMNlpavT
          w             cn c4 n w   4 icas4a4ahbewaaBcih
          o             il iH l o   H JiJaHnHHavIorIIIJa
          I             II II I I   I IIIIIIIIIIIIVIIII
                          /             /// /   ////// /
  TTGGCAAAAGTCGCCCTTGATGGCGGCGGAGGCGAGGCAGCCGCGGCGCCGAACAGCCGA
61 --------+---------+---------+---------+---------+---------+---------+ 120
  AACCGTTTTCAGCGGGAACTACCGCCGCCTCCGCTCCGTCGGCGCCGCGGCTTGTCGGCT

  L  A  K  V  A  L  D  G  G  G  G  E  A  A  A  A  P  N  S  R   -
   W  Q  K  S  P  L  M  A  A  E  A  R  Q  P  R  R  R  T  A  D  -
    G  K  S  R  P  *  W  R  R  R  R  G  S  R  G  A  E  Q  P  T -

                                                B
                                                s
  B                     S                       p     F         F  F
  s C    C     AN     a  C           B   C B1     nH        n  n
  sFaHHNAaHRTTvlMAEu Aa     F     c   v a2   HAua       Au AuM
  Hachhhccgmhhaawcc9 cc     a     e   i n8   hc4e       c4 c4w
  Iu8aaei8aaaaIIoii6 i8     u     f   J I6   aiHI       iH iHo
  IIIIIIIIIIIIIVIIII II     I     I   I II   IIII       II III
     / / /////     /  /                  /     //         /  /
  CGCGCGCTAGCGGGGTCCGCCCGCCCCTTTCCCAGAGCCCAGCGCCGCCGTTCGCCGCCG
21 --------+---------+---------+---------+---------+---------+---------+ 180
  GCGCGCGATCGCCCCAGGCGGGCGGGGAAAGGGTCTCGGGTCGCGGCGGCAAGCGGCGGC
```

```
 R   A   L   A   G   S   A   R   P   F   P   R   A   Q   R   R   R   S   P   P   -
  A   R   *   R   G   P   P   A   P   F   P   E   P   S   A   A   V   R   R   R  -
   R   A   S   G   V   R   P   P   L   S   Q   S   P   A   P   P   F   A   A   A -

                                                                 T
 F    F          B                F   F   F   F          N   aF      F
 n   Bn   C    Cs C               n   n   n   n    B C   s  Sqn      nH    A
AuMAcu Aa   AasFaHHFTTM      Au  AuMAuFAuM  AsBaDBpFaIuT  HAua    v
c4wce4 cc   ccHachhahhw      c4  c4wc4ac4w  cascssBacI4h  hc4e    a
iHoifH i8   i8Iu8aauaao      iH  iHoiHuiHo  iJl8alIuI-Ha  aiHI    I
IIIIII II   IIIIIIIIIII      II  IIIIIIIII  IIIIIIIII1II  IIII    I
 /  //  /    /   /  //        /   /   /  //  / //  /  /   /    //
CCGCCGCCCGCCCGCGCCGTTCGCCGCCGCCGCCCGCGGGTGGCAGCGCCGCTCG
181 ---------+---------+---------+---------+---------+---------+ 240
GGCGGCGGGCGGGCGCGCGGCAAGCGGCGGCGGCGGGCGCCCACCGTCGCGGCGAGC

 P   P   P   A   R   A   P   F   A   A   A   A   A   R   G   W   Q   R   R   S   -
  R   R   P   P   A   R   R   S   P   P   P   P   P   A   G   G   S   A   A   R  -
   A   A   R   P   R   A   V   R   R   R   R   R   P   R   V   A   A   P   L   G -

       S        H C    H          S S
  BN   aCB BB   a fCCCa         NNaBa S SS           C C
BFslMNuvsAssBBeBravveMBMNANNllusuMcMccS             v a           AB
biaasc9iavaassIs1ciiIsssacccaa9i9wrnrrm             i c           lb
vnJIpi6JJaJJlII08JJIplpeiiiII6E6oFlFFa              J 8           uv
IIIVIIIIIIIIIIIIIIIIIIIIIIIIIVVIIIIIIIII            I I           II
/  / //   ///// / / // // ///// / / ///                           /
GTCCCCGGCCCCGGGGCCGGCTGGGGGGCGGTCGGGGCGTGTGAGGGGCTTGCTCCCAGC
241 ---------+---------+---------+---------+---------+---------+ 300
CAGGGGCCGGGGCCCCGGCCGACCCCCCGCCAGCCCCGCACACTCCCCGAACGAGGGTCG

 V   P   G   P   G   A   G   W   G   A   V   G   A   C   E   G   L   A   P   S   -
  S   P   A   P   G   P   A   G   G   R   S   G   R   V   R   G   L   L   P   A  -
   P   R   P   R   G   R   L   G   G   G   R   G   V   *   G   A   C   S   Q   L -

                F       FHS    N   E   H
CC             C n    CMC naa C sC Cc   BaS              C        C
avNT    FM    vAu    abvAueuAaBpa voFHsec      H         a    AM v
cirh    aw    ic4    coic4I9ccsBc iRaapIr      g         c    lw i
8Jua    uo    JiH    8IJiHI6i8lI8 JIueGIF      a         8    wo J
IIII    II    III    IIIIIIIIIIII IIIIIII      I         I    II I
 / /            /    /  /// / /    /// //                         /
TCGCGAAGATGGCTGCGGGCCGCCCGCTGGCCTGGACGCTGACACTTTGGCAGGCGTGGC
301 ---------+---------+---------+---------+---------+---------+ 360
AGCGCTTCTACCGACGCCCGGCGGGCGACCGGACCTGCGACTGTGAAACCGTCCGCACCG

 S   R   R   W   L   R   A   A   R   W   P   G   R   *   H   F   G   R   R   G   -
  R   E   D   G   C   G   P   P   A   G   L   D   A   D   T   L   A   G   V   A  -
   A   K  |M   A   A   G   R   P   L   A   W   T   L   T   L   W   Q   A   W   L -

              E    B
              c    s                                      T
 S    S      So   pH  ES                       M T        a
 a    Ba     aOCB B1a Nca       BCN          C a s        q
MuD   su D   u1vcAa2eMlou    M  cvl          v e p        I
m3p   a3 p   90iepn8Ina59    n  eia          i I 4        I
eAn   BA n   69JfaI6IlI76    l  fJI          J I 5        -
III   II I   IIIIIIIIIVII    I  IIV          I I I        2
 /                /////  /       /
TGATCCTGATCGGGCCCTCGTCGGAGGAGCCGTTCCCTTCAGCCGTCACTATCAAGTCAT
361 ---------+---------+---------+---------+---------+---------+ 420
ACTAGGACTAGCCCGGGAGCAGCCTCCTCGGCAAGGGAAGTCGGCAGTGATAGTTCAGTA
```

```
*  S  *  S  G  P  R  R  R  S  R  S  L  Q  P  S  L  S  S  H  -
 D  P  D  R  A  L  V  G  G  A  V  P  F  S  R  H  Y  Q  V  M -
  I  L  I  G  P  S  S  E  E  P  F  P  S  A  V  T  I  K  S  W -

                        T
N                    E  tM T
1S           C       c Sha s M                     H
af           v    B  o c1e p b      B    M   M     i    B  P
Ia           i    b  R r1I 4 o      s    w   l     n    c  l
IN           R    s  I F1I 5 I      r    o   y     f    c  e
II           I    I  I III I I      I    I   I     I    I  I
 /.                     /
GGGTGGATAAGATGCAAGAAGACCTGGTCACACTGGCAAAAACAGCAAGTGGAGTCCATC
21 ---------+---------+---------+---------+---------+---------+ 480
CCCACCTATTCTACGTTCTTCTGGACCAGTGTGACCGTTTTTGTCGTTCACCTCAGGTAG

G  W  I  R  C  K  K  T  W  S  H  W  Q  K  Q  Q  V  E  S  I  -
 G  G  *  D  A  R  R  P  G  H  T  G  K  N  S  K  W  S  P  S  -
  V  D  K  M  Q  E  D  L  V  T  L  A  K  T  A  S  G  V  H  Q -

                                    B
                                    s
                                    t
  C                                 1        ND         C M
 Av                                A1        lr         v a
 li                                c0        ad         i e
 uJ                                c7        II         R I
 II                                II        VI         I I
  /
AGCTTGTTGATATTTATGAGAAATATCAAGATTTGTATACTGTGGAACCAAATAATGCAC
181 ---------+---------+---------+---------+---------+---------+ 540
TCGAACAACTATAAATACTCTTTATAGTTCTAAACATATGACACCTTGGTTTATTACGTG

S  L  L  I  F  M  R  N  I  K  I  C  I  L  W  N  Q  I  M  H  -
 A  C  *  Y  L  *  E  I  S  R  F  V  Y  C  G  T  K  *  C  T  -
  L  V  D  I  Y  E  K  Y  Q  D  L  Y  T  V  E  P  N  N  A  R -

                                   H
      N            F               i                  S
     CsP      T   CBnC       .     n  C             BB a     C
    Avpv      s   vsuv       B     d Av  D          gsDu     v
    liBu      p   im4i       b     I li  d          ltp3     i
    uJII      E   RAHJ       v     I uJ  e          IYnA     J
    IIII      I   IIII       I     I II  I          IIII     I
     ///          /                  /               / /
GTCAGCTGGTGGAAATTGCAGCCAGAGACATTGAGAAGCTTCTCAGCAACAGATCTAAAG
41 ---------+---------+---------+---------+---------+---------+ 600
CAGTCGACCACCTTTAACGTCGGTCTCTGTAACTCTTCGAAGAGTCGTTGTCTAGATTTC

V  S  W  W  K  L  Q  P  E  T  L  R  S  F  S  A  T  D  L  K  -
 S  A  G  G  N  C  S  Q  R  H  *  E  A  S  Q  Q  Q  I  *  S  -
  Q  L  V  E  I  A  A  R  D  I  E  K  L  L  S  N  R  S  K  A -

                                                    T
                                                    t
 E       E                             F            h
BcS     c S  C                         n C          1
soc     oHc  v B    M                  u v    M  B B1B
aRr     Rhr  i s    w                  4 i    n  s b1s
JIF     IaF  J l    o                  H J    l  l vIl
III     III  I I    I                  I I    I  I III
 /       /
CCCTGGTGCGCCTGGCTTTGGAAGCAGAGAAAGTTCAAGCAGCCCACCAATGGAGGGAAG
01 ---------+---------+---------+---------+---------+---------+ 660
```

```
GGGACCACGCGGACCGAAACCTTCGTCTCTTTCAAGTTCGTCGGGTGGTTACCTCCCTTC

P  W  C  A  W  L  W  K  Q  R  K  F  K  Q  P  T  N  G  G  K   -
 P  G  A  P  G  F  G  S  R  E  S  S  S  S  P  P  M  E  G  R  -
  L  V  R  L  A  L  E  A  E  K  V  Q  A  A  H  Q  W  R  E  D -

                        T
                        t
                        h          .     S     S
     C CM               1                a     Ba
     v ab               A1          T    uAD   su D F
     i co               c1          h    3lp   a3 p o
     R 8I               cI          a    Awn   BA n k
     I II               II          I    III   II I I
ATTTTGCAAGCAATGAAGTTGTCTACTATAACGCGAAGGATGATCTTGATCCTGAAAAAA
661 ---------+---------+---------+---------+---------+---------+---------+ 720
TAAAACGTTCGTTACTTCAACAGATGATATTGCGCTTCCTACTAGAACTAGGACTTTTTT

I  L  Q  A  M  K  L  S  T  I  T  R  R  M  I  L  I  L  K  K   -
 F  C  K  Q  *  S  C  L  L  *  R  E  G  *  S  *  S  *  K  K  -
  F  A  S  N  E  V  V  Y  Y  N  A  K  D  D  L  D  P  E  K  N -

           E    F       S
          DcS   n C     a              S
          roc  Mu v     u DB    A      f
          dRr  n4 i     3 pb    l      a
          IIF  lH J     A nv    w      N
          III  II I     I II    I      I
           /
ATGACAGTGAACCAGGCAGCCAGAGGATCAAACCTGTTTTCATTGACGATGCTAACTTTA
721 ---------+---------+---------+---------+---------+---------+---------+ 780
TACTGTCACTTGGTCCGTCGGTCTCCTAGTTTGGACAAAAGTAACTGCTACGATTGAAAT

M  T  V  N  Q  A  A  R  G  S  N  L  F  S  L  T  M  L  T  L   -
 *  Q  *  T  R  Q  P  E  D  Q  T  C  F  H  *  R  C  *  L  *  -
  D  S  E  P  G  S  Q  R  I  K  P  V  F  I  D  D  A  N  F  R -

                       F   N                                S
              M        C   n  CsP                           a
 B            b        a   u Avpv        B                  u DT
 b            o        c   4 liBu        b                  3 pa
 s            I        8   H uJII        v                  A nq
 I            I        I   I IIII        I                  I II
                           ///
GAAGACAAGTATCCTATCAGCACGCAGCTGTCCATATCCCCACTGACATCTATGAAGGAT
781 ---------+---------+---------+---------+---------+---------+---------+ 840
CTTCTGTTCATAGGATAGTCGTGCGTCGACAGGTATAGGGGTGACTGTAGATACTTCCTA

E  D  K  Y  P  I  S  T  Q  L  S  I  S  P  L  T  S  M  K  D   -
 K  T  S  I  L  S  A  R  S  C  P  Y  P  H  *  H  L  *  R  I  -
  R  Q  V  S  Y  Q  H  A  A  V  H  I  P  T  D  I  Y  E  G  S -

                          B                   M
    A        M            s B       D        a          M
    l        s            p s       d        e          n
    w        e            G r       e        I          l
    I        I            I I       I        I          I
CGACAATCGTGTTAAACGAACTCAACTGGACAAGTGCCTTAGATGACGTTTTCAAAAAAA
841 ---------+---------+---------+---------+---------+---------+---------+ 900
GCTGTTAGCACAATTTGCTTGAGTTGACCTGTTCACGGAATCTACTGCAAAAGTTTTTTT

R  Q  S  C  *  T  N  S  T  G  Q  V  P  *  M  T  F  S  K  K   -
 D  N  R  V  K  R  T  Q  L  D  K  C  L  R  *  R  F  Q  K  K  -
  T  I  V  L  N  E  L  N  W  T  S  A  L  D  D  V  F  K  K  N -
```

```
                          T
                          t
                          h                             E  HH
                    BM    1                           C Bc Caa
 T       B          sb  M1                            vBsoHveeM
 a       b          po  s1                            istRaiIIs
 q       s          MI  lI                            JrXIeJIIp
 I       I          II  II                            IIIIIIIII
                                                        ///  ///
 ATCGAGAGGAAGACCCTTCACTGTTGTGGCAGGTGTTTGGCAGTGCCACTGGCCTGGCCC
01 ---------+---------+---------+---------+---------+---------+ 960
 TAGCTCTCCTTCTGGGAAGTGACAACACCGTCCACAAACCGTCACGGTGACCGGACCGGG

 I  E  R  K  T  L  H  C  C  G  R  C  L  A  V  P  L  A  W  P  -
  S  R  G  R  P  F  T  V  V  A  G  V  W  Q  C  H  W  P  G  P  -
   R  E  E  D  P  S  L  L  W  Q  V  F  G  S  A  T  G  L  A  R -

                                                            T
                                                            t
 S                    N                                 S   h
 aSS         C     B  l              C                  a   1
 Nucc        Av  X sDNSa             v                  u D 1
 c9rr        li  c asctI             i                  3 p 1
 i6FF        uJ  m JaoyI             J                  A n I
 IIII        II  I IIIII             I                  I I I
 ////         /     ///
 GGTATTACCCAGCTTCTCCATGGGTTGATAATAGCCGAACCCCAAACAAGATTGATCTTT
61 ---------+---------+---------+---------+---------+---------+ 1020
 CCATAATGGGTCGAAGAGGTACCCAACTATTATCGGCTTGGGGTTTGTTCTAACTAGAAA

 G  I  T  Q  L  L  H  G  L  I  I  A  E  P  Q  T  R  L  I  F  -
  V  L  P  S  F  S  M  G  *  *  *  P  N  P  K  Q  D  *  S  L  -
   Y  Y  P  A  S  P  W  V  D  N  S  R  T  P  N  K  I  D  L  Y -

                       N                 F
                  B    l  M    B         n C          S
      R     F B   sDNSaBRbF    sS        u v          f
      s     o b   asctIbsoo    at        4 i          a
      a     k s   JaoyIvaIk    Jy        H R          N
      I     I I   IIIIIIIII    II        I I          I
                   ///          /
 ATGATGTACGCAGAAGACCATGGTACATCCAAGGTGCTGCATCCCCTAAAGATATGCTTA
21 ---------+---------+---------+---------+---------+---------+ 1080
 TACTACATGCGTCTTCTGGTACCATGTAGGTTCCACGACGTAGGGGATTTCTATACGAAT

 M  M  Y  A  E  D  H  G  T  S  K  V  L  H  P  L  K  I  C  L  -
  *  C  T  Q  K  T  M  V  H  P  R  C  C  I  P  *  R  Y  A  Y  -
   D  V  R  R  R  P  W  Y  I  Q  G  A  A  S  P  K  D  M  L  I -

                                                   B      P
                      F          F       F       BsMM     s
                      o          o       o       epsm     h
                      k          k       k       tEpe     A
                      I          I       I       IIII     I
                                                  //
 TTCTGGTGGATGTGAGTGGAAGCGTTAGTGGACTGACACTCAAACTCATCCGGACATCCG
81 ---------+---------+---------+---------+---------+---------+ 1140
 AAGACCACCTACACTCACCTTCGCAATCACCTGACTGTGAGTTTGAGTAGGCCTGTAGGC

 F  W  W  M  *  V  E  A  L  V  D  *  H  S  N  S  S  G  H  P  -
  S  G  G  C  E  W  K  R  *  W  T  D  T  Q  T  H  P  D  I  R  -
   L  V  D  V  S  G  S  V  S  G  L  T  L  K  L  I  R  T  S  V -
```

```
      BE
      ss
      mp                      D      M            M    C     M
      A3                      d      n            a    v     s
      II                      e      l            e    i     e
       /                      I      I            I    J     I
                                                  I    I
1141  TCTCCGAAATGTTGGAAACCCTCTCAGATGATGATTTTGTGAACGTGGCTTCATTTAACA
      ---------+---------+---------+---------+---------+---------+  1200
      AGAGGCTTTACAACCTTTGGGAGAGTCTACTACTAAAACACTTGCACCGAAGTAAATTGT

      S  P  K  C  W  K  P  S  Q  M  M  I  L  *  T  W  L  H  L  T  -
       L  R  N  V  G  N  P  L  R  *  *  F  C  E  R  G  F  I  *  Q  -
        S  E  M  L  E  T  L  S  D  D  D  F  V  N  V  A  S  F  N  S -

                                                             T
                                                             t
       B                     F                               h
       p                     Cn                              1
       u   BD               Avu   F                          1
       1   bd               li4   o                          1
       0   ve               uJH   k                          I
       I   II               III   I                          I
                             //
1201  GCAATGCTCAGGATGTAAGCTGCTTTCAGCACCTTGTCCAAGCAAATGTAAGAAATAAGA
      ---------+---------+---------+---------+---------+---------+  1260
      CGTTACGAGTCCTACATTCGACGAAAGTCGTGGAACAGGTTCGTTTACATTCTTTATTCT

      A  M  L  R  M  *  A  A  F  S  T  L  S  K  Q  M  *  E  I  R  -
       Q  C  S  G  C  K  L  L  S  A  P  C  P  S  K  C  K  K  *  E  -
        N  A  Q  D  V  S  C  F  Q  H  L  V  Q  A  N  V  R  N  K  K -

       S            C                        H
       f            v                        iT
       a            i                        nf
       N            R                        fi
       I            I                        II
                                              /
1261  AAGTGTTGAAAGATGCAGTGAATAATATCACAGCAAAAGGAATCACAGATTATAAGAAGG
      ---------+---------+---------+---------+---------+---------+  1320
      TTCACAACTTTCTACGTCACTTATTATAGTGTCGTTTTCCTTAGTGTCTAATATTCTTCC

      K  C  *  K  M  Q  *  I  I  S  Q  Q  K  E  S  Q  I  I  R  R  -
       S  V  E  R  C  S  E  *  Y  H  S  K  R  N  H  R  L  *  E  G  -
        V  L  K  D  A  V  N  N  I  T  A  K  G  I  T  D  Y  K  K  G -

                            F   FN
      C                     n  CnsP   T                 C      C
      v         B           u AvupvM  sB                v      v
      i         b           4 li4Bus  pb                i      i
      J         v           H uJHIIe  Ev                J      R
      I         I           I IIIIII  II                I      I
                                ////
1321  GCTTTAGTTTTGCTTTTGAGCAGCTGCTTAATTATAATGTATCCAGAGCCAACTGCAATA
      ---------+---------+---------+---------+---------+---------+  1380
      CGAAATCAAAACGAAAACTCGTCGACGAATTAATATTACATAGGTCTCGGTTGACGTTAT

      A  L  V  L  L  L  S  S  C  L  I  I  M  Y  P  E  P  T  A  I  -
       L  *  F  C  F  *  A  A  A  *  L  *  C  I  Q  S  Q  L  Q  *  -
        F  S  F  A  F  E  Q  L  L  N  Y  N  V  S  R  A  N  C  N  K -

                                                 B
                                                 s
                  N                              pE
                  l                           CBB1cMS
```

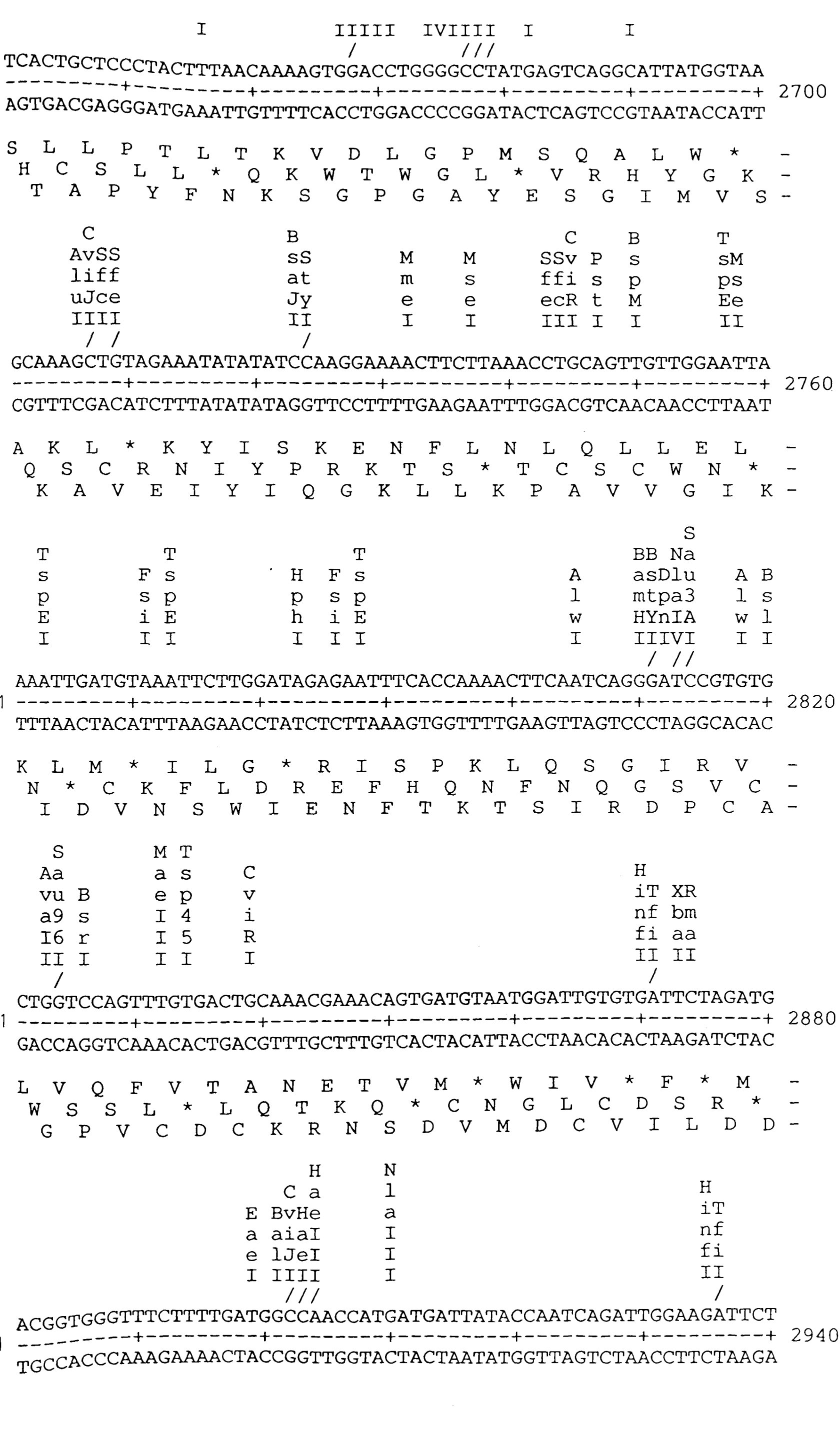
```
           a        M        E                   vsa2obc
           I        n        a                   ian8Ror
           I        l        r                   JJI6IIF
           I        I        I                   IIIIIII
                                                     ////
  AGATTATCATGTTGTTCACGGACGGAGGAGAAGAGAGAGCCCAGGAGATATTTGCCAAAT
381 ---------+---------+---------+---------+---------+---------+ 1440
  TCTAATAGTACAACAAGTGCCTGCCTCCTCTTCTCTCGGGTCCTCTATAAACGGTTTA

  R  L  S  C  C  S  R  T  E  E  K  R  E  P  R  R  Y  L  P  N   -
   D  Y  H  V  V  H  G  R  R  R  R  E  S  P  G  D  I  C  Q  I  -
    I  I  M  L  F  T  D  G  G  E  E  R  A  Q  E  I  F  A  K  Y -

                    A                          H
                    fBM                      C a       T
                   Rlsa            D       E BvHe      s M
                   sIae            d       a aiaI      p n
                   aIAI            e       e lJeI      E l
                   IIII            I       I IIII      I I
                      /                      ///
  ACAATAAAGACAAGAAAGTACGTGTATTCACATTCTCAGTTGGCCAACATAATTACGACA
441 ---------+---------+---------+---------+---------+---------+ 1500
  TGTTATTTCTGTTCTTTCATGCACATAAGTGTAAGAGTCAACCGGTTGTATTAATGCTGT

  T  I  K  T  R  K  Y  V  Y  S  H  S  Q  L  A  N  I  I  T  T   -
   Q  *  R  Q  E  S  T  C  I  H  I  L  S  W  P  T  *  L  R  Q  -
    N  K  D  K  K  V  R  V  F  T  F  S  V  G  Q  H  N  Y  D  R -

     E
     c
     o S
    AOPa                                               T
    v1pu               C C                             
    a0u9               v a     F           F       F s    B    B
    I9M6               i c     o           o       s p    c    s
    IIII               J 8     k           k       i E    c    l
     ///               I I     I           I       I I    I    I
  GAGGACCTATTCAGTGGATGGCTTGCGAAAATAAAGGTTATTATTATGAAATTCCATCCA
501 ---------+---------+---------+---------+---------+---------+ 1560
  CTCCTGGATAAGTCACCTACCGAACGCTTTTATTTCCAATAATAATACTTTAAGGTAGGT

  E  D  L  F  S  G  W  L  A  K  I  K  V  I  I  M  K  F  H  P   -
   R  T  Y  S  V  D  G  L  R  K  *  R  L  L  L  *  N  S  I  H  -
    G  P  I  Q  W  M  A  C  E  N  K  G  Y  Y  Y  E  I  P  S  I -

                                                           M
  P  NC        T                                           b
  f  lv        sAM    D         R             B            o
  l  ai        pss    d         m             b            I
  M  IJ        Eee    e         a             s            I
  I  VI        III    I         I             I
  /               /
  TTGGAGCCATAAGAATTAATACTCAGGAATACCTAGATGTTCTGGGAAGACCGATGGTTT
1561 ---------+---------+---------+---------+---------+---------+ 1620
  AACCTCGGTATTCTTAATTATGAGTCCTTATGGATCTACAAGACCCTTCTGGCTACCAAA

  L  E  P  *  E  L  I  L  R  N  T  *  M  F  W  E  D  R  W  F   -
   W  S  H  K  N  *  Y  S  G  I  P  R  C  S  G  K  T  D  G  F  -
    G  A  I  R  I  N  T  Q  E  Y  L  D  V  L  G  R  P  M  V  L -

               B
     T         p
     a         u                        E
     q         1C                  S    c S      C
  B  I        A1vD                 f   Ro c      v       B F
  s
```

```
m    I          l0id                  a    sR r      i        s o
A    -          u2Je                  N    aI F      R        r k
I    1          IIII                  I    II I      I        I I
                 //
TAGCAGGAGACAAAGCTAAGCAAGTCCAATGGACAAATGTGTACCTGGATGCACTGGAAC
1621 ---------+---------+---------+---------+---------+---------+ 1680
ATCGTCCTCTGTTTCGATTCGTTCAGGTTACCTGTTTACACATGGACCTACGTGACCTTG

 *  Q  E  T  K  L  S  K  S  N  G  Q  M  C  T  W  M  H  W  N  -
  S  R  R  Q  S  *  A  S  P  M  D  K  C  V  P  G  C  T  G  T  -
   A  G  D  K  A  K  Q  V  Q  W  T  N  V  Y  L  D  A  L  E  L -

                                                   H
            M        M                           C aT
  B         b    B   Bb BME                    E BBvHes
  s         o    s   bo esa                    a asiaIp
  r         I    r   sI tpr                    e lrJeIE
  I         I    I   II III                    I IIIIII
                     /                             ////
TGGGACTTGTCATTACTGGAACTCTTCCGGTCTTCAACATAACTGGCCAATTTGAAAATA
1681 ---------+---------+---------+---------+---------+---------+ 1740
ACCCTGAACAGTAATGACCTTGAGAAGGCCAGAAGTTGTATTGACCGGTTAAACTTTTAT

W  D  L  S  L  L  E  L  F  R  S  S  T  *  L  A  N  L  K  I   -
 G  T  C  H  Y  W  N  S  S  G  L  Q  H  N  W  P  I  *  K  *  -
  G  L  V  I  T  G  T  L  P  V  F  N  I  T  G  Q  F  E  N  K -

                     N
            D       CsHP
      M     r       AvpivTX
      s     d       liBnufc
      e     I       uJIfIim
      I     I       IIIIIII
                     // ///
AGACAAACTTAAAGAACCAGCTGATTCTTGGAGTGATGGGAGTTGATGTGTCTTTGGAAG
1741 ---------+---------+---------+---------+---------+---------+ 1800
TCTGTTTGAATTTCTTGGTCGACTAAGAACCTCACTACCCTCAACTACACAGAAACCTTC

R  Q  T  *  R  T  S  *  F  L  E  *  W  E  L  M  C  L  W  K   -
 D  K  L  K  E  P  A  D  S  W  S  D  G  S  *  C  V  F  G  R  -
  T  N  L  K  N  Q  L  I  L  G  V  M  G  V  D  V  S  L  E  D -

                                                             S
    M                                       C           C T a
M   b           M                       BM  v           AvMs u
s   o           a                       gw  i           liup 3
e   I           e                       lo  J           wRnE A
I   I           I                       II  I           IIII I
                                         /               //
ATATTAAAAGACTGACACCACGTTTTACACTCTGCCCCAATGGCTACTATTTTGCAATTG
1801 ---------+---------+---------+---------+---------+---------+ 1860
TATAATTTTCTGACTGTGGTGCAAAATGTGAGACGGGGTTACCGATGATAAAACGTTAAC

I  L  K  D  *  H  H  V  L  H  S  A  P  M  A  T  I  L  Q  L   -
 Y  *  K  T  D  T  T  F  Y  T  L  P  Q  W  L  L  F  C  N  *  -
  I  K  R  L  T  P  R  F  T  L  C  P  N  G  Y  Y  F  A  I  D -

                                                            B
                                                            s
                                                            t
                E                                           1
                c     M                 C      C            A1
D       F       o     b                 v      v            c0
p       o       5     o                 i      i            c7
n       k       7     I                 J      J
```

```
I        I       I    I           I     I             II
ATCCTAATGGTTATGTGTTATTACATCCAAATCTTCAGCCAAAGCCTATTGGTGTAGGTA
861 ---------+---------+---------+---------+---------+---------+ 1920
TAGGATTACCAATACACAATAATGTAGGTTTAGAAGTCGGTTTCGGATAACCACATCCAT

I  L  M  V  M  C  Y  Y  I  Q  I  F  S  Q  S  L  L  V  *  V   -
 S  *  W  L  C  V  I  T  S  K  S  S  A  K  A  Y  W  C  R  Y  -
  P  N  G  Y  V  L  L  H  P  N  L  Q  P  K  P  I  G  V  G  I -

               T   T       B                                  NC
               sAM s       Bs                           D     lvB
               pss p       sm                           d     ais
               Eee E       aA                           e     IJr
               III I       II                           I     VII
                 /          /
TACCAACAATTAATTTGAGAAAAAGGAGACCCAATGTTCAGAACCCCAAATCTCAGGAGC
921 ---------+---------+---------+---------+---------+---------+ 1980
ATGGTTGTTAATTAAACTCTTTTTCCTCTGGGTTACAAGTCTTGGGGTTTAGAGTCCTCG

Y  Q  Q  L  I  *  E  K  G  D  P  M  F  R  T  P  N  L  R  S   -
 T  N  N  *  F  E  K  K  E  T  Q  C  S  E  P  Q  I  S  G  A  -
  P  T  I  N  L  R  K  R  R  P  N  V  Q  N  P  K  S  Q  E  P -

 M T
 a s      S              C                                 H
 e p      Mf        T    v  M                    M M       iT NT
 I 4      ma        a    i  n                    s s       nf sa
 I 5      eN        q    R  l                    e l       fi pq
 I I      II        I    I  I                    I I       II VI
                                                             /  /
CAGTGACATTGGATTTCCTCGATGCAGAGTTGGAGAATGACATTAAAGTGGAGATTCGAA
981 ---------+---------+---------+---------+---------+---------+ 2040
GTCACTGTAACCTAAAGGAGCTACGTCTCAACCTCTTACTGTAATTTCACCTCTAAGCTT

Q  *  H  W  I  S  S  M  Q  S  W  R  M  T  L  K  W  R  F  E   -
 S  D  I  G  F  P  R  C  R  V  G  E  *  H  *  S  G  D  S  K  -
  V  T  L  D  F  L  D  A  E  L  E  N  D  I  K  V  E  I  R  N -

      S
      a
      u DCT                                         M
      3 pla                                         s
      A naq                                         e
      I III                                         I
         /
ATAAAATGATCGATGGAGAAAGTGGAGAAAAAACATTCAGAACTCTGGTTAAATCTCAAG
041 ---------+---------+---------+---------+---------+---------+ 2100
TATTTTACTAGCTACCTCTTTCACCTCTTTTTTGTAAGTCTTGAGACCAATTTAGAGTTC

I  K  *  S  M  E  K  V  E  K  K  H  S  E  L  W  L  N  L  K   -
 *  N  D  R  W  R  K  W  R  K  N  I  Q  N  S  G  *  I  S  R  -
  K  M  I  D  G  E  S  G  E  K  T  F  R  T  L  V  K  S  Q  D -

                                   A                 H
                                   f MB   H   P      i
                                   lMasPP i   s      nBM
                                   Isealm n   h      csl
                                   IlIAel f   A      Ily
                                   IIIIII I   I      III
                                       //             /
ATGAGAGATATATTGACAAAGGAAACAGGACATACACGTGGACTCCTGTCAACGGCACAG
101 ---------+---------+---------+---------+---------+---------+ 2160
TACTCTCTATATAACTGTTTCCTTTGTCCTGTATGTGCACCTGAGGACAGTTGCCGTGTC
```

```
 M  R  D  I  L  T  K  E  T  G  H  T  R  G  L  L  S  T  A  Q   -
  *  E  I  Y  *  Q  R  K  Q  D  I  H  V  D  S  C  Q  R  H  R  -
 E  R  Y  I  D  K  G  N  R  T  Y  T  W  T  P  V  N  G  T  D -

                H
     B       CB a                                      C   B
     c       vsHeS          SS                         v  E s
     e       iaaIt          ff                         i  a m
     f       JJeIy          ec                         J  r A
     I       IIIII          II                         I  I I
              ///
ATTATAGCAGTTTGGCCTTGGTATTACCAACCTACAGTTTTTACTATATAAAAGCCAAAA
161 ---------+---------+---------+---------+---------+---------+ 2220
TAATATCGTCAAACCGGAACCATAATGGTTGGATGTCAAAAATGATATATTTTCGGTTTT

 I  I  A  V  W  P  W  Y  Y  Q  P  T  V  F  T  I  *  K  P  K   -
  L  *  Q  F  G  L  G  I  T  N  L  Q  F  L  L  Y  K  S  Q  N  -
   Y  S  S  L  A  L  V  L  P  T  Y  S  F  Y  Y  I  K  A  K  I -

                    H
          M     C a                            T     H
          bD    vHe                  BM        s     i
          od    iaI                  es        p     n
          Ie    JeI                  tp        E     f
          II    III                  II        I     I
                 //
TAGAAGAGACAATAACTCAGGCCAGATATTCAGAAACACTGAAACCGGATAATTTTGAAG
221 ---------+---------+---------+---------+---------+---------+ 2280
ATCTTCTCTGTTATTGAGTCCGGTCTATAAGTCTTTGTGACTTTGGCCTATTAAAACTTC

 *  K  R  Q  *  L  R  P  D  I  Q  K  H  *  N  R  I  I  L  K   -
  R  R  D  N  N  S  G  Q  I  F  R  N  T  E  T  G  *  F  *  R  -
   E  E  T  I  T  Q  A  R  Y  S  E  T  L  K  P  D  N  F  E  E -

                              EM T
    C M                      Cca s
T   v b        R            SSvoePp    M
f   i o        m            ffi5Is4    s
i   J I        a            ecR7It5    e
I   I I        I            IIIIIII    I
/                              / /
AATCTGGCTACACATTCCTAGCACCAAGAGATTACTGCAGTGACCTTAAACCTTCAGATA
281 ---------+---------+---------+---------+---------+---------+ 2340
TTAGACCGATGTGTAAGGATCGTGGTTCTCTAATGACGTCACTGGAATTTGGAAGTCTAT

 N  L  A  T  H  S  *  H  Q  E  I  T  A  V  T  L  N  L  Q  I   -
  I  W  L  H  I  P  S  T  K  R  L  L  Q  *  P  *  T  F  R  *  -
   S  G  Y  T  F  L  A  P  R  D  Y  C  S  D  L  K  P  S  D  N -

        T         T
      F s      MDF s                  F
      s p      srs p                  o
      i E      eai E                  k
      I I      III I                  I
ATAACACTGAATTTCTTTTAAATTTCAATGAGTTTATTGATAGAAAAACTCCAAACAACC
341 ---------+---------+---------+---------+---------+---------+ 2400
TATTGTGACTTAAAGAAAATTTAAAGTTACTCAAATAACTATCTTTTTGAGGTTTGTTGG

 I  T  L  N  F  F  *  I  S  M  S  L  L  I  E  K  L  Q  T  T   -
  *  H  *  I  S  F  K  F  Q  *  V  Y  *  *  K  N  S  K  Q  P  -
   N  T  E  F  L  L  N  F  N  E  F  I  D  R  K  T  P  N  N  P -

      T
      t
```

```
       h
       1                   HS              C C C
B      1             AMM   if      P     v a v    F
c      1             sls   na      l     i c i    o
c      I             eye   fN      e     R 8 J    k
I      I             III   II      I     I I I    I
                     //
    CATCCTGTAATACAGACTTGATTAATAGAGTCTTGCTGGATGCAGGCTTTACAAATGAAC
401 ---------+---------+---------+---------+---------+---------+ 2460
    GTAGGACATTATGTCTGAACTAATTATCTCAGAACGACCTACGTCCGAAATGTTTACTTG

    H  P  V  I  Q  T  *  L  I  E  S  C  W  M  Q  A  L  Q  M  N  -
     I  L  *  Y  R  L  D  *  *  S  L  A  G  C  R  L  Y  K  *  T -
      S  C  N  T  D  L  I  N  R  V  L  L  D  A  G  F  T  N  E  L-

                                                              M
             T                           M                    a
    X        s       B                G  b                    e
    m        p       s                s  o                    I
    n        E       r                u  I                    I
    I        I       I                I  I                    I
    TTGTTCAAAATTACTGGAGTAAGCAGAAGAATATCAAGGGAGTGAAAGCACGGTTTGTTG
461 ---------+---------+---------+---------+---------+---------+ 2520
    AACAAGTTTTAATGACCTCATTCGTCTTCTTATAGTTCCCTCACTTTCGTGCCAAACAAC

    L  F  K  I  T  G  V  S  R  R  I  S  R  E  *  K  H  G  L  L  -
     C  S  K  L  L  E  *  A  E  E  Y  Q  G  S  E  S  T  V  C  C -
      V  Q  N  Y  W  S  K  Q  K  N  I  K  G  V  K  A  R  F  V  V-

    T
    s                                     C         T          B
    p                           M         v         s          s
    4                           n         i         p          m
    5                           l         J         E          A
    I                           I         I         I          I
    TGACTGATGGTGGGATTACCAGAGTTTATCCCAAAGAGGCTGGAGAAAATTGGCAGGAAA
521 ---------+---------+---------+---------+---------+---------+ 2580
    ACTGACTACCACCCTAATGGTCTCAAATAGGGTTTCTCCGACCTCTTTTAACCGTCCTTT

    *  L  M  V  G  L  P  E  F  I  P  K  R  L  E  K  I  G  R  K  -
     D  *  W  W  D  Y  Q  S  L  S  Q  R  G  W  R  K  L  A  G  K -
      T  D  G  G  I  T  R  V  Y  P  K  E  A  G  E  N  W  Q  E  N-

                          C   M        NC                 M
    G         N    B     Av   b        lv  T       M      a
    s         d    b     li   o        ai  a       n      e
    u         e    s     uJ   I        IJ  q       l      I
    I         I    I     II   I        VI  I       I      I
                          /
    ACCCAGAGACATATGAAGACAGCTTCTATAAAAGGAGCCTCGATAATGATAACTACGTTT
581 ---------+---------+---------+---------+---------+---------+ 2640
    TGGGTCTCTGTATACTTCTGTCGAAGATATTTTCCTCGGAGCTATTACTATTGATGCAAA

    T  Q  R  H  M  K  T  A  S  I  K  G  A  S  I  M  I  T  T  F  -
     P  R  D  I  *  R  Q  L  L  *  K  E  P  R  *  *  *  L  R  F -
      P  E  T  Y  E  D  S  F  Y  K  R  S  L  D  N  D  N  Y  V  F-

                                     E
                                     c
                           SE        o  H S
                          AacBS      ONCa a  H
                   M      vuosc      1lveMu  i       P
                   s      a9Rar      0aiI19  n       l
                   e      I6IJF      9IJIy6  f       e
```


TCACTGCTCCCTACTTTAACAAAAGTGGACCTGGGGCCTATGAGTCAGGCATTATGGTAA
2641 2700
AGTGACGAGGGATGAAATTGTTTTCACCTGGACCCCGGATACTCAGTCCGTAATACCATT
S L L P T L T K V D L G P M S Q A L W *
H C S L L * Q K W T W G L * V R H Y G K
T A P Y F N K S G P G A Y E S G I M V S
GCAAAGCTGTAGAAATATATATCCAAGGAAAACTTCTTAAACCTGCAGTTGTTGGAATTA
2701 2760
CGTTTCGACATCTTTATATATAGGTTCCTTTTGAAGAATTTGGACGTCAACAACCTTAAT
A K L * K Y I S K E N F L N L Q L L E L
Q S C R N I Y P R K T S * T C S C W N *
K A V E I Y I Q G K L L K P A V V G I K
AAATTGATGTAAATTCTTGGATAGAGAATTTCACCAAAACTTCAATCAGGGATCCGTGTG
2761 2820
TTTAACTACATTTAAGAACCTATCTCTTAAAGTGGTTTTGAAGTTAGTCCCTAGGCACAC
K L M * I L G * R I S P K L Q S G I R V
N * C K F L D R E F H Q N F N Q G S V C
I D V N S W I E N F T K T S I R D P C A
CTGGTCCAGTTTGTGACTGCAAACGAAACAGTGATGTAATGGATTGTGTGATTCTAGATG
2821 2880
GACCAGGTCAAACACTGACGTTTGCTTTGTCACTACATTACCTAACACACTAAGATCTAC
L V Q F V T A N E T V M * W I V * F * M
W S S L * L Q T K Q * C N G L C D S R *
G P V C D C K R N S D V M D C V I L D D
ACGGTGGGTTTCTTTTGATGGCCAACCATGATGATTATACCAATCAGATTGGAAGATTCT
2881 2940
TGCCACCCAAAGAAAACTACCGGTTGGTACTACTAATATGGTTAGTCTAACCTTCTAAGA

```
T  V  G  F  F  *  W  P  T  M  M  I  I  P  I  R  L  E  D  S  -
 R  W  V  S  F  D  G  Q  P  *  *  L  Y  Q  S  D  W  K  I  L  -
  G  G  F  L  L  M  A  N  H  D  D  Y  T  N  Q  I  G  R  F  F -

                                   E
          H                        a
       S  i                        m
  M    a  n  BC                 E  1S                         M
  b A  u D d Asv                c  1c                         s
  o l  3 p I lmi                o  0r                         e
  I w  A n I uAJ                R  5F                         I
  I I  I I I III                I  II
               /                    /
TTGGAGAGATTGATCCAAGCTTGATGAGACACCTGGTCAATATATCAGTTTATGCCTTTA
941 ---------+---------+---------+---------+---------+---------+ 3000
AACCTCTCTAACTAGGTTCGAACTACTCTGTGGACCAGTTATATAGTCAAATACGGAAAT

L  E  R  L  I  Q  A  *  *  D  T  W  S  I  Y  Q  F  M  P  L  -
 W  R  D  *  S  K  L  D  E  T  P  G  Q  Y  I  S  L  C  L  *  -
  G  E  I  D  P  S  L  M  R  H  L  V  N  I  S  V  Y  A  F  N -

                                  E     F
                                  c S   n
                           B      o c   u   H     B
                           b      R r   4   h     s
                           v      I F   H   a     l
                           I      I I   I   I     I
ACAAATCTTATGATTATCAGTCGGTGTGTGAACCTGGTGCTGCGCCAAAGCAGGGAGCAG
001 ---------+---------+---------+---------+---------+---------+ 3060
TGTTTAGAATACTAATAGTCAGCCACACACTTGGACCACGACGCGGTTTCGTCCCTCGTC

T  N  L  M  I  I  S  R  C  V  N  L  V  L  R  Q  S  R  E  Q  -
 Q  I  L  *  L  S  V  G  V  *  T  W  C  C  A  K  A  G  S  R  -
  K  S  Y  D  Y  Q  S  V  C  E  P  G  A  A  P  K  Q  G  A  G -

   B
   s
   p                                                     S  H
  N1        C                        C                   a Ca
B 12A       v         B             SSv P          B  B  u ve
a a8c       i         c             ffi s          b  b  9 iI
n I6i       J         c             ecR t          v  v  6 JI
I VII       I         I             III I          I  I  I II
                                                             /
GGCACCGCTCGGCTTATGTGCCATCAATAGCAGACATACTGCAGATTGGATGGTGGGCCA
061 ---------+---------+---------+---------+---------+---------+ 3120
CCGTGGCGAGCCGAATACACGGTAGTTATCGTCTGTATGACGTCTAACCTACCACCCGGT

G  T  A  R  L  M  C  H  Q  *  Q  T  Y  C  R  L  D  G  G  P  -
 A  P  L  G  L  C  A  I  N  S  R  H  T  A  D  W  M  V  G  H  -
  H  R  S  A  Y  V  P  S  I  A  D  I  L  Q  I  G  W  W  A  T -

  F EF E
  n cn c SM                       A                  B    C N
F Mu ou o cb                      l                  sD   vMl
o w4 54 R ro                      w                  as   ina
k oH 7H I FI                      N                  Ja   JlI
I II II I II                      I                  II   IIV
                                                      /    //
CTGCTGCTGCCTGGTCTATTCTTCAGCAGTTTCTGTTGAGTTTGACTTTTCCACGGCTCC
121 ---------+---------+---------+---------+---------+---------+ 3180
GACGACGACGGACCAGATAAGAAGTCGTCAAAGACAACTCAAACTGAAAAGGTGCCGAGG
```

```
 L  L  L  P  G  L  F  F  S  S  F  C  *  V  *  L  F  H  G  S   -
  C  C  C  L  V  Y  S  S  A  V  S  V  E  F  D  F  S  T  A  P  -
   A  A  A  W  S  I  L  Q  Q  F  L  L  S  L  T  F  P  R  L  L -

     F    N                               N         F
     n  BC sP                             l        CnC
     u  AcvMpvB  B              F      B  a  M B   Avuv
     4  leinBuc  b              o      b  I  n c   li4i
     H  ufJlIIg  v              k      v  I  l g   uJHR
     I  IIIIIII  I              I      I  I  I I   IIII
         /////                                      //
 TTGAGGCAGCTGATATGGAGGATGACGACTTCACTGCCTCCATGTCAAAGCAGAGCTGCA
81 ---------+---------+---------+---------+---------+---------+ 3240
 AACTCCGTCGACTATACCTCCTACTGCTGAAGTGACGGAGGTACAGTTTCGTCTCGACGT

 L  R  Q  L  I  W  R  M  T  T  S  L  P  P  C  Q  S  R  A  A   -
  *  G  S  *  Y  G  G  *  R  L  H  C  L  H  V  K  A  E  L  H  -
   E  A  A  D  M  E  D  D  D  F  T  A  S  M  S  K  Q  S  C  I -

      S     M                                          R
   D  f     Bb         T                               l
   d  a     so         a                               e
   e  N     rI         q                               A
   I  I     II         I                               I
             /
 TCACTGAGCAAACCCAGTATTTCTTCGATAATGACAGCAAATCGTTCAGTGGGGTATTAG
241 ---------+---------+---------+---------+---------+---------+ 3300
 AGTGACTCGTTTGGGTCATAAAGAAGCTATTACTGTCGTTTAGCAAGTCACCCCATAATC

 S  L  S  K  P  S  I  S  S  I  M  T  A  N  R  S  V  G  Y  *   -
  H  *  A  N  P  V  F  L  R  *  *  Q  Q  I  V  Q  W  G  I  R  -
   T  E  Q  T  Q  Y  F  F  D  N  D  S  K  S  F  S  G  V  L  D -

                             N            N
       T      H              l      CB    l       T
       s      iT             a     Avs    a       s M   S
       p      nf             I     lip    I       p s   s
       E      fi             I     uJH    I       E e   p
       I      II             I     III    I       I I   I
              /                    /
 ACTGTGGGAATTGTTCCAGAATCTTTCATGTAGAAAAGCTCATGAACACCAATTTAATAT
301 ---------+---------+---------+---------+---------+---------+ 3360
 TGACACCCTTAACAAGGTCTTAGAAAGTACATCTTTTCGAGTACTTGTGGTTAAATTATA

 T  V  G  I  V  P  E  S  F  M  *  K  S  S  *  T  P  I  *  Y   -
  L  W  E  L  F  Q  N  L  S  C  R  K  A  H  E  H  Q  F  N  I  -
   C  G  N  C  S  R  I  F  H  V  E  K  L  M  N  T  N  L  I  F -

                        A   N    M T        F
                        f   l    a s       Cn           B
                        l   aFNB e p       vu           c
                        I   Iisb I 4       i4           e
                        I   Inpv I 5       JH           f
                        I   IIII I I       II           I
                              /
 TCATAATGGTAGAGAGCAAGGGGACATGTCCCTGTGACACACGGCTGCTCATACAAGCAG
361 ---------+---------+---------+---------+---------+---------+ 3420
 AGTATTACCATCTCTCGTTCCCCTGTACAGGGACACTGTGTGCCGACGAGTATGTTCGTC

 S  *  W  *  R  A  R  G  H  V  P  V  T  H  G  C  S  Y  K  Q   -
  H  N  G  R  E  Q  G  D  M  S  L  *  H  T  A  A  H  T  S  R  -
   I  M  V  E  S  K  G  T  C  P  C  D  T  R  L  L  I  Q  A  E -

          T
```

```
     t
     h        S   S                                               S
     1       Aa  Ba                                   E           a
     1      Avu  suD                    M             c T         u
     1      la9  t3p                    s             o a         9
     I      wI6  YAn                    e             R q         6
     I      III  III                    I             V I         I
            //   /
AGCAAACTTCTGATGGACCAGATCCTTGTGATATGGTTAAGCAACCCAGATATCGAAAAG
421 ---------+---------+---------+---------+---------+---------+ 3480
TCGTTTGAAGACTACCTGGTCTAGGAACACTATACCAATTCGTTGGGTCTATAGCTTTTC

S  K  L  L  M  D  Q  I  L  V  I  W  L  S  N  P  D  I  E  K   -
 A  N  F  *  W  T  R  S  L  *  Y  G  *  A  T  Q  I  S  K  R  -
  Q  T  S  D  G  P  D  P  C  D  M  V  K  Q  P  R  Y  R  K  G -

  H                        E
 Ca                        c S
 ve                      D M o c                A   G
 iI                      r n R r                c   s
 JI                      d l I F                i   u
 II                      I I I I                I   I
  /
GGCCAGATGTCTGCTTTGACAACAATGTCCTGGAGGATTATACTGACTGCGGTGGGGTCT
481 ---------+---------+---------+---------+---------+---------+ 3540
CCGGTCTACAGACGAAACTGTTGTTACAGGACCTCCTAATATGACTGACGCCACCCCAGA

G  Q  M  S  A  L  T  T  M  S  W  R  I  I  L  T  A  V  G  S   -
 A  R  C  L  L  *  Q  Q  C  P  G  G  L  Y  *  L  R  W  G  L  -
  P  D  V  C  F  D  N  N  V  L  E  D  Y  T  D  C  G  G  V  S -

                         S
  B                      Aa                                C
 Bs  M                   vu  B    B              R         v
 sm  s                   a9  c    s              s         i
 aA  e                   I6  c    l              a         J
 II  I                   II  I    I              I         I
  /                      /
CTGGATTAAATCCTTCCCTGTGGTCCATCATCGGGATACAGTTTGTACTGCTTTGGCTGG
541 ---------+---------+---------+---------+---------+---------+ 3600
GACCTAATTTAGGAAGGGACACCAGGTAGTAGCCCTATGTCAAACATGACGAAACCGACC

L  D  *  I  L  P  C  G  P  S  S  G  Y  S  L  Y  C  F  G  W   -
 W  I  K  S  F  P  V  V  H  H  R  D  T  V  C  T  A  L  A  G  -
  G  L  N  P  S  L  W  S  I  I  G  I  Q  F  V  L  L  W  L  V -

     F
     n      AA                                   T
     u      1pMB                        G       sM
     4      wawb                        s       ps
     H      NBov                        u       Ee
     I      IIII                        I       II
             /                 Stop
TTTCTGGCAGCAGACACTGCCTGTTA|TGA|CCTTCTAAAACCAAATCTCCATAATTAAACT
601 ---------+---------+---------+---------+---------+---------+ 3660
AAAGACCGTCGTCTGTGACGGACAATACTGGAAGATTTTGGTTTAGAGGTATTAATTTGA

F  L  A  A  D  T  A  C  Y  D  L  L  K  P  N  L  H  N  *  T   -
 F  W  Q  Q  T  L  P  V  M  T  F  *  N  Q  I  S  I  I  K  L  -
  S  G  S  R  H  C  L  L  * [P  S  K  T  K  S  P  *  L  N  S -
                             del ->

                 SN                         H
   A             al                         i
   l     A       uaD             MM         n       M
```

```
      w       l     3Ip              ns            c      s
      N       w     AIn              le            I      e
      I       I     III              II            I      I
CCAGACCCTGCCACAACATGATCCCTCCGTTATGTTAAAGTAGGGTCAACTGTTAAATCA
---------+---------+---------+---------+---------+---------+---------+ 3720
GGTCTGGGACGGTGTTGTACTAGGGAGGCAATACAATTTCATCCCAGTTGACAATTTAGT

P  D  P  A  T  T  *  S  L  R  Y  V  K  V  G  S  T  V  K  S  -
 Q  T  L  P  Q  H  D  P  S  V  M  L  K  *  G  Q  L  L  N  Q  -
  R  P  C  H  N  M  I  P  P  L  C  *  S  R  V  N  C  *  I  R -

                                      B
                                      s
          S  H           N          pB
        C a  Ca      B    l     C B1s          H          N
       Av u  ve      sDNSMa     v a2uD P  H  i       B BMl
       li 9  iI      asctnI     i n83d l  h  n       b ala
       uJ 6  JI      JaoylI     J I66e e  a  f       v nyI
       II I  II      IIIIII     I IIII I  I  I       I IIV
        /    /        ///  /       ///                  /
GAACATTAGCTGGGCCTCTGCCATGGCAGAGCCCTAAGGCGCAGACTCATCAGGCACCCA
---------+---------+---------+---------+---------+---------+---------+ 3780
CTTGTAATCGACCCGGAGACGGTACCGTCTCGGGATTCCGCGTCTGAGTAGTCCGTGGGT

E  H  *  L  G  L  C  H  G  R  A  L  R  R  R  L  I  R  H  P  -
 N  I  S  W  A  S  A  M  A  E  P  *  G  A  D  S  S  G  T  H  -
  T  L  A  G  P  L  P  W  Q  S  P  K  A  Q  T  H  Q  A  P  T -

          T
    F     N a
  C nC    l q
  vBuv    aNI
  is4i    IsI
    JrHR  Ip-
    IIII  II2
      /     //
CTGGCTGCATGTCAGGGTGTCC
---------+---------+-- 3802
GACCGACGTACAGTCCCACAGG

L  A  A  C  Q  G  V    -
 W  L  H  V  R  V  S   -
  G  C  M  S  G  C     -
```

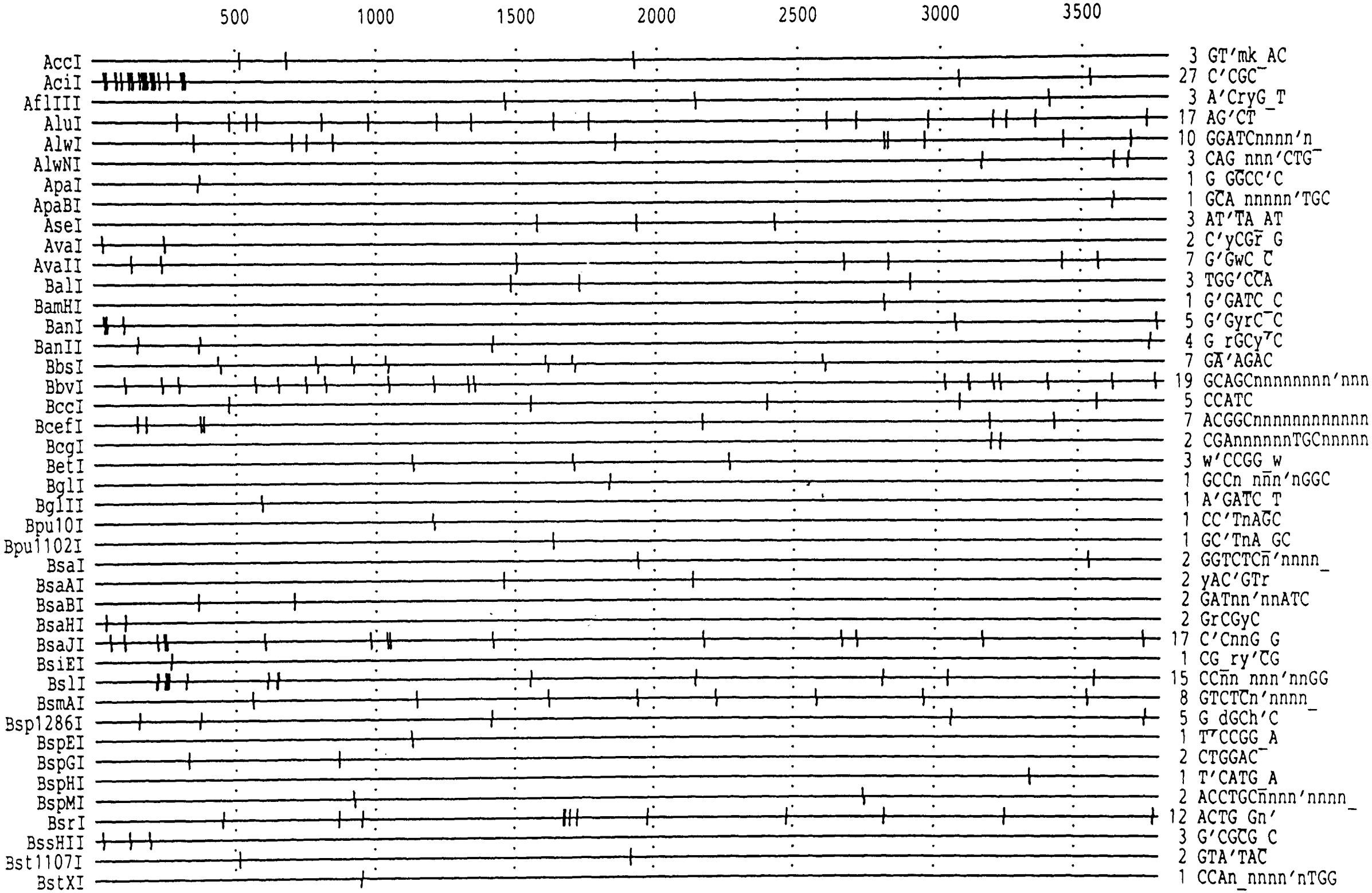
es that do cut:

ccI	AciI	AflIII	AluI	AlwI	AlwNI	ApaI	ApaBI
seI	AvaI	AvaII	BalI	BamHI	BanI	BanII	BbsI
bvI	BccI	BcefI	BcgI	BetI	BglI	BglII	Bpu10I
02I	BsaI	BsaAI	BsaBI	BsaHI	BsaJI	BsiEI	BslI
mAI	Bsp1286I	BspEI	BspGI	BspHI	BspMI	BsrI	BssHII
07I	BstXI	BstYI	Bsu36I	Cac8I	Cfr10I	ClaI	CviJI
iRI	DdeI	DpnI	DraI	DrdI	DrdII	DsaI	EaeI
05I	EarI	EciI	Eco57I	EcoO109I	EcoRII	EcoRV	Esp3I
auI	FinI	Fnu4HI	FokI	FsiI	GsuI	HaeI	HaeII
III	HgaI	HhaI	HincII	HindIII	HinfI	HphI	MaeII
III	MboII	MlyI	MmeI	MnlI	MseI	MslI	MspI
unI	MwoI	NaeI	NarI	NciI	NcoI	NdeI	NheI
III	NlaIV	NruI	NspI	NspV	NspBII	PflMI	PleI
mlI	PpuMI	PshAI	PstI	PvuII	RleAI	RmaI	RsaI
cII	Sau96I	Sau3AI	ScrFI	SfaNI	SfcI	SfeI	SgrAI
maI	SspI	StyI	TaqI	TaqII-1	TaqII-2	TfiI	ThaI
45I	TspEI	Tth111I	Tth111II	XbaI	XcmI	XmnI	

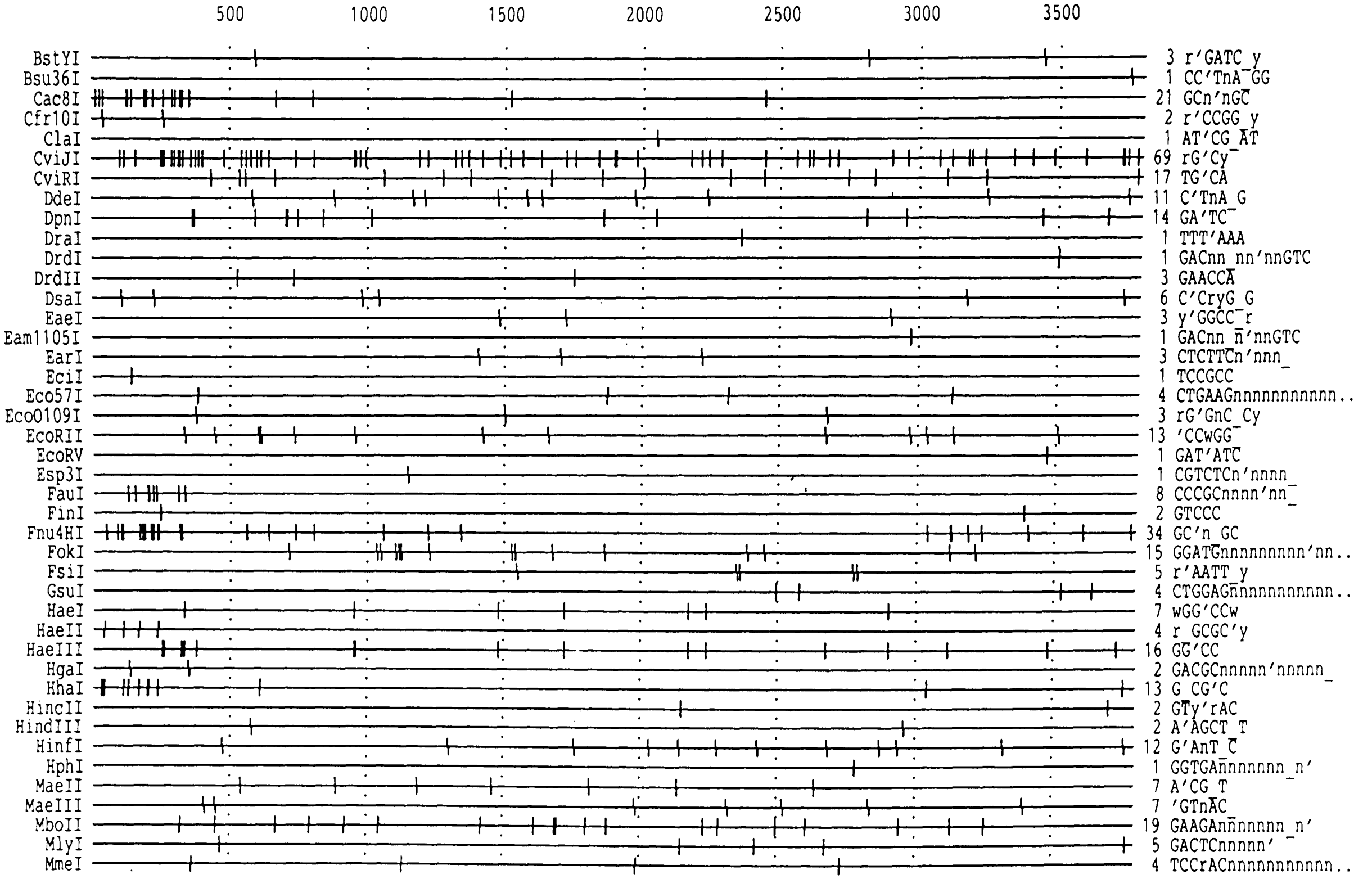
es that do not cut:

tII	AflII	AgeI	ApaLI	AscI	AvrII	BaeI	BclI
sgI	BsiI	BsiWI	BsmI	BstEII	DraIII	Eco47III	EcoNI
oRI	FseI	FspI	GdiII	HgiAI	HgiEII	HpaI	KpnI
luI	NotI	NsiI	PacI	Pfl1108I	PmeI	PvuI	RsrII
alI	SapI	ScaI	SfiI	SnaBI	SpeI	SphI	Sse8387I
stI	StuI	SwaI	XhoI	XmaIII			

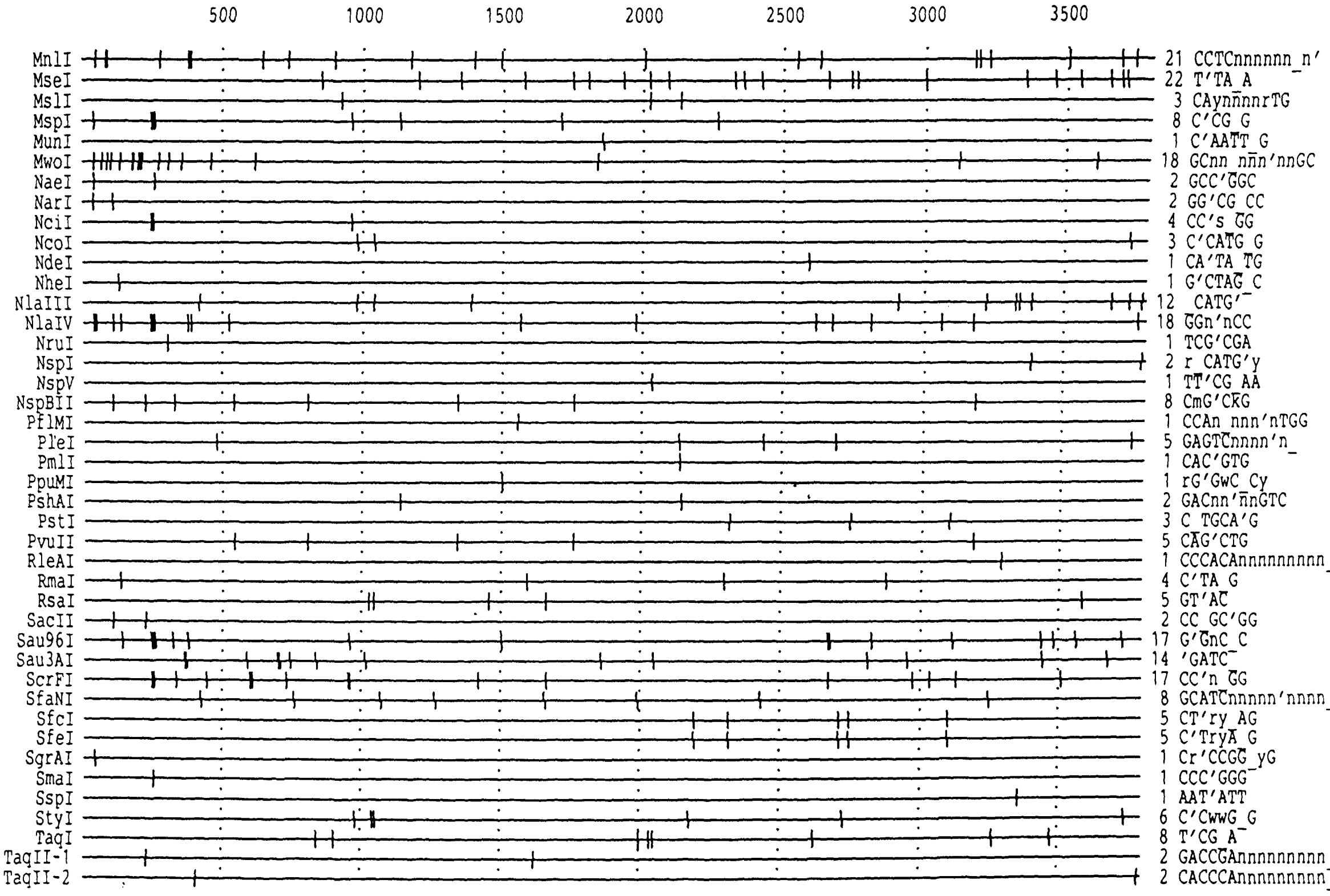
(Linear) MAPPLOT of: Raba2n.Txt ck: 871, 1 to: 3802 November 24, 1991 18:16.



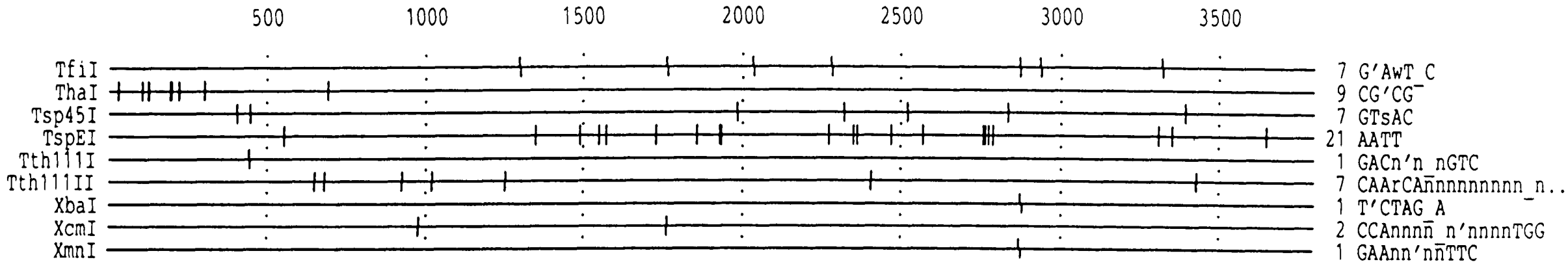
(Linear) MAPPLOT of: Raba2n.Txt ck: 871, 1 to: 3802 November 24, 1991 18:17.



(Linear) MAPPLOT of: Raba2n.Txt ck: 871, 1 to: 3802 November 24, 1991 18:18.



(Linear) MAPPLOT of: Raba2n.Txt ck: 871, 1 to: 3802 November 24, 1991 18:19.



Enzymes that do not cut:

AatII	AflII	AgeI	ApaLI	AscI	AvrII	BaeI	BclI	BsgI	BsiI	BsiWI	BsmI
BstEII	DraIII	Eco47III	EcoNI	EcoRI	FseI	FspI	GdiII	HgiAI	HgiEII	HpaI	KpnI
MluI	NotI	NsiI	PacI	Pfl1108I	PmeI	PvuI	RsrII	SalI	SapI	ScaI	SfiI
	SnaBI	SpeI	SphI	Sse8387I	SstI	StuI	SwaI	XhoI	XmaIII		

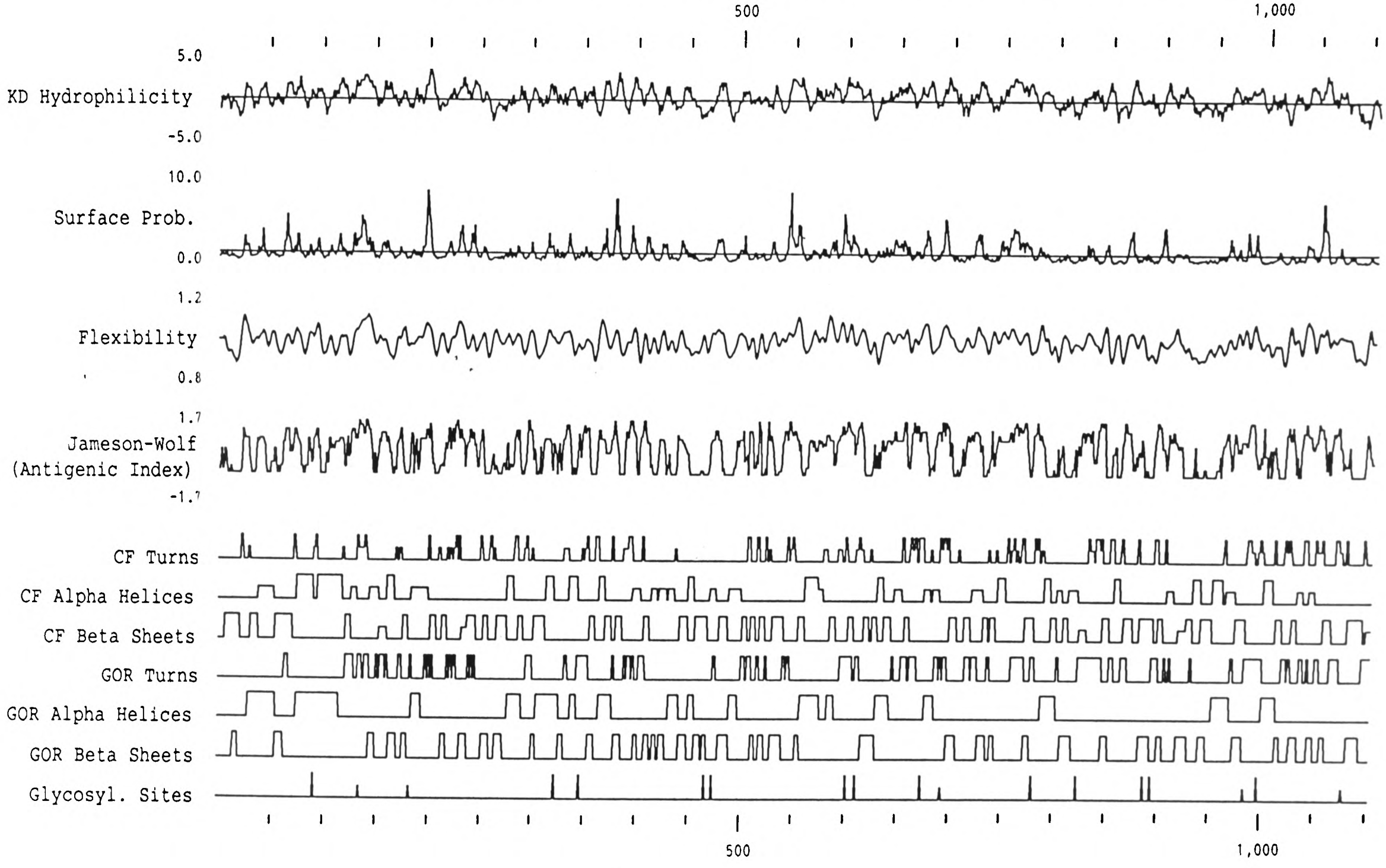
Enzymes excluded; MinCuts: 1 MaxCuts: 350000

NONE

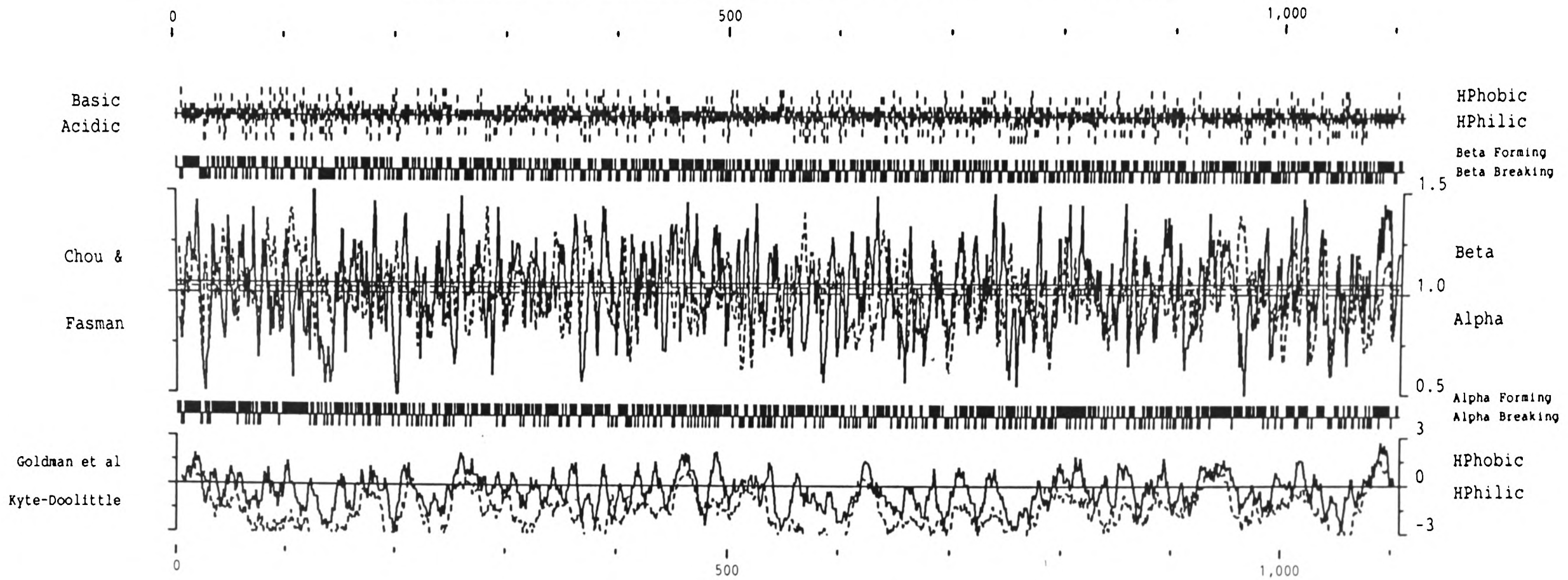
PLOTSTRUCTURE of: raba2n.p2s November 24, 1991 18:22

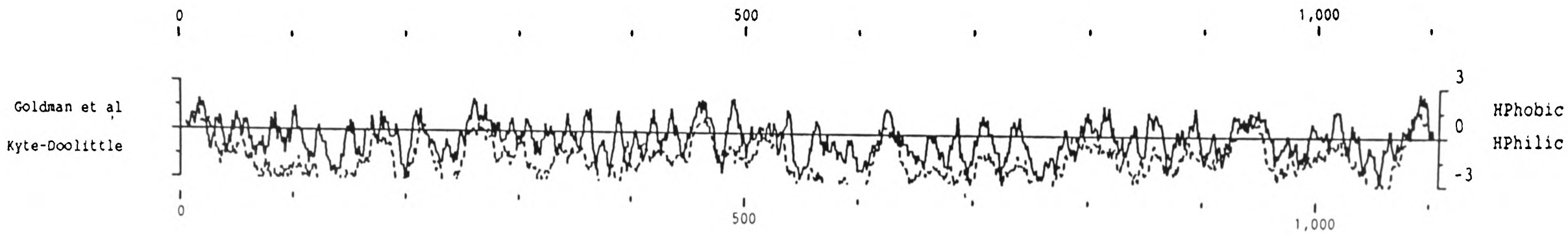
PEPTIDESTRUCTURE of: raba2n.pep Ck: 6626, 1 to: 1106

TRANSLATE of: raba2n.txt check: 871 from: 309 to: 3629



PEPPLOT of: Raba2n.Pep ck: 6626, 1 to 1107 November 24, 1991 17:37
TRANSLATE of: raba2n.txt check: 871 from: 309 to: 3629

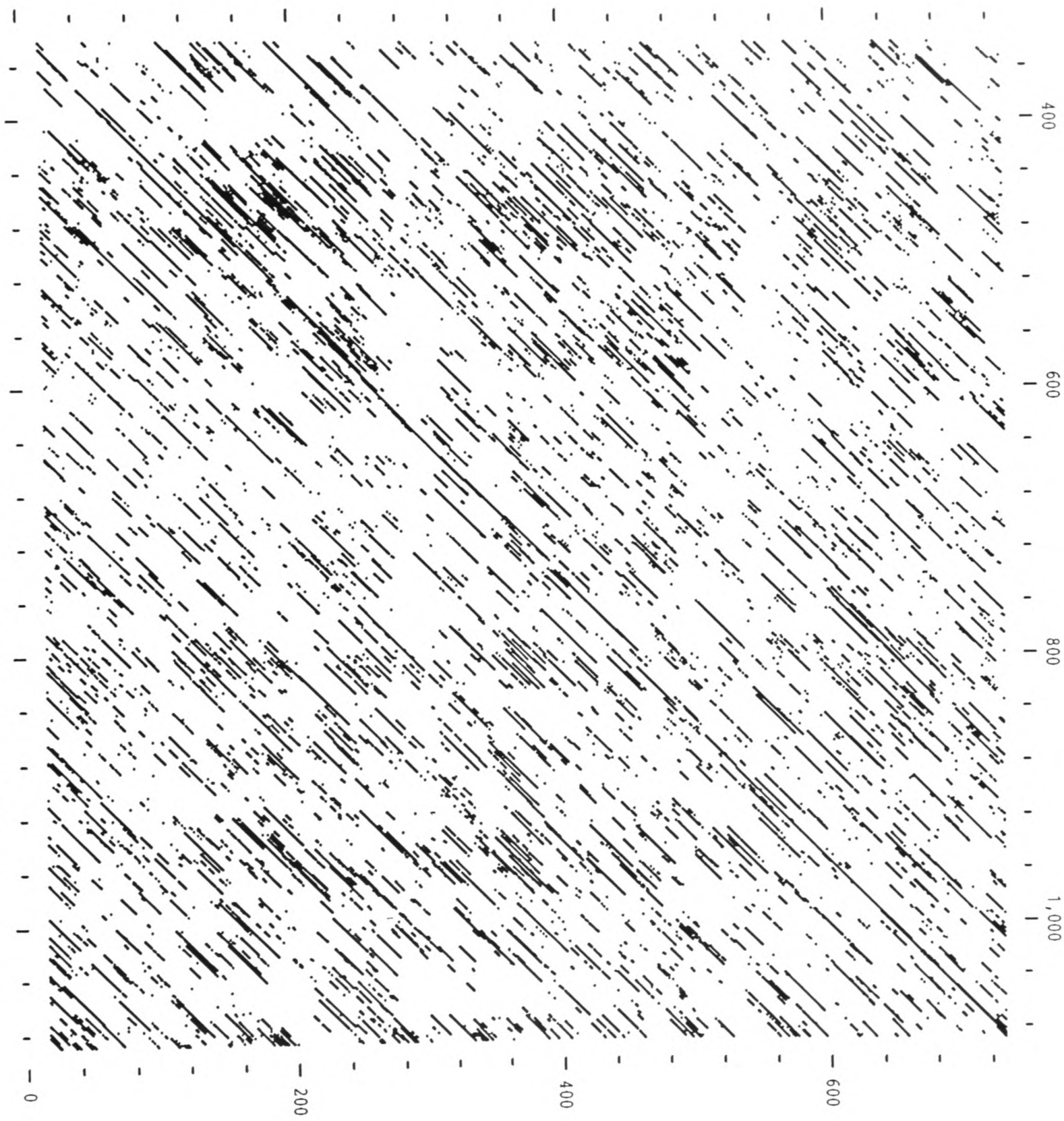



PEPPLOT of: Raba2n.Pep ck: 6626, 1 to 1107 November 24, 1991 17:45
TRANSLATE of: raba2n.txt check: 871 from: 309 to: 3629
0
500
1,000
Goldman et al
Kyte-Doolittle
3
0
-3
HPhobic
HPhilic
0
500
1,000

DOTPLOT of: raba2n.pnt Density: 846.59 November 24, 1991 17:54

COMPARE Window: 30 Stringency: 9.0 Points: 25,091

Ng.Pep ck: 3,457, 1 to 743



Raba2n.Pep ck: 6,626, 327 to 1,102

DOTPLOT of: raba2n.pnt Density: 924.17 November 24, 1991 17:17

COMPARE Window: 30 Stringency: 9.0 Points: 38,218

Ng.Pep ck: 3,457, 1 to 743

Raba2n.Pep ck: 6,626, 1 to 1,107