THE $GABA_A$ RECEPTOR δ SUBUNIT GENE PROMOTER : CHARACTERISATION AND USE

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A dissertation submitted for the degree of Doctor of Philosophy of the University of Glasgow

February 1998

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For my husband Stuart

and for my children Ross and Ailie

with love

Also to JR (Grandad) in loving memory

Preface

The work presented in this thesis was performed entirely by the author, except where acknowledged. I declare that my thesis contains unique work and will not be submitted for any other degree, diploma or qualification at any other University.

A. Brett Roberts
December 1997

Acknowledgements

I would like to thank Professor R.W. Davies for giving me the opportunity to study for this degree and for his help throughout the last three years. I would also like to thank my colleagues for their help and advice. Particular thanks go to the technical staff - - Lynn Loughlin (Supertech) for her help and friendship, Irene Houghton and Gareth Westrop, and to Team Scotland : Nicola Craig (for keeping me sane and making me laugh), Vicki Laird and Thora Glencorse.

I am especially indebted to Dr Thora Glencorse for her help, advice and encouragement.

I would also like to thank Prof. B. Clements and Dr A. MacLean (Dept. of Virology, Glasgow) for help with the virus work. Thanks also to Dr Jackie Campbell and Neil Bennett (Dept. of Anatomy, Glasgow University) for performing the stereotactic injections.

I acknowledge the Medical Research Council for supporting this work, and Glasgow University.

Special thanks go to my husband Stuart for all his support and encouragement throughout the last 3 years and my children for letting me be an absent Mum for so long.

Contents

List of Figures

Chapter 5

Chapter 6

List of Tables

Abbreviations

A	antisense
Amp	ampicillin
BAP	bacterial alkaline phosphatase
b-HLH	basic helix loop helix
b-HLH-Zip	basic helix - loop - helix - leucine zipper.
bZIP	basic leucine zipper
BDNF	brain-derived neurotropic factor
bp	basepairs
BSF1	brain specific factor 1
BZ	benzodiazepine
CAT	chloramphenicol acetyl transferase
ChAT	choline acetyl transferase
CMV	cytomegalovirus
CNS	central nervous system
c.p.e.	cytopathic effect
Cys	cystine
DBH	dopamine β-hydroxylase
DG	dentate gyrus
DMCM	6,7-dimethyl-4-ethylcarboline-3-carboxylate methyl ester
E	early
ES	embryonic stem cells
EST	expressed sequence tag
G	cerebellar granule cell
GABA	γ-aminobutyric acid
gc	granule cell
gD	glycoprotein D
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
gl	glial cell
GlyR	glycine receptor
HMBA	N,N hexamethylene bis-acrylamide
HSV	herpes simplex virus
IE	immediate early
IR	internal repeat
I_R	inverted repeat
Kan	kanamycin
Kb	kilobase pairs

L	late
lacZ	β-galactosidase
LAT	latency associated transcript
LINES	long interspersed elements
LTR	long term repeat
Luc	luciferase
M	cerebellar molecular layer
mcs	multiple cloning site
misc	miscellaneous
nAChR	nicotinic acetylcholine receptor
neo^r	neomycin resistance
NRSE	neural specific silencer element
NSE	neuron specific enolase
ori	origin of replication
P	Purkinje cell layer
PBS	phosphate buffered saline
pfu	plaque forming unit
PGK	phosphoglycerate kinase
PoDg	posterior area of the dentate gyrus
Poly(A)	polyadenylation site
PPE	preproenkephalin
S	sense
SINES	short interspersed elements
SNc	substantia nigra pars compacta
SP	signal peptide
TAP	tobacco acid phosphatase
TBPS	tert-butylbicyclophosphorothionate
TF	transcription factor
TH	tyrosine hydroxylase
tk	thymidine kinase
TM1-TM4	transmembrane domains
TR	terminal repeat
U_L	unique long
U_S	unique short
V	ventricles
ZF	zinc finger

Summary

Viral vectors represent powerful tools in the rapidly developing field of gene therapy of the mammalian central nervous system. Interest in HSV - 1 in particular as a vector relates primarily to its ability to establish long - term latent infections in neuronal cells. A goal of this research was the development of a safe HSV - 1 vector that would deliver a reporter gene to target cells in the CNS and restrict its expression to a specific cell - type by the use of a neuronal promoter. A second goal was to add to the present limited understanding of neuron - specific promoter structure.

The murine $GABA_A$ receptor δ subunit gene promoter was selected for use as a prototype neuronal promoter as it is moderately expressed and has a well characterised restricted expression pattern in the CNS. Characterisation of the 5' - promoter region of the $GABA_A$ receptor δ subunit gene was achieved by sequencing 10.5 kb of DNA from the 5' - upstream region. Analysis of this sequence revealed the presence of several putative recognition sites for transcription factors. Identification of the transcription start points showed two main clusters of start sites located 58 and 108 bases upstream from the translational start point. Comparison of about 3.5 kb of DNA sequence of the promoters of the rat and mouse genes showed a high degree of conservation of noncoding sequence between the species. The recognition site for a putative regulatory factor BSF 1 identified in the 5' - upstream sequence of the rat δ gene was demonstrated to be absent in the mouse 5' - flanking sequence. Analysis of a range of promoter - reporter constructs in the NB4 1A3 cell line suggested the presence of a silencing element located between 4.5 kb and 6.3 kb upstream of the translational start point.

As a preliminary step to evaluating *in vivo* mutagenesis investigations of $GABA_A$ receptor δ subunit gene promoter function, targeted disruption of the $GABA_A$ receptor δ subunit gene in embryonic stem cells was achieved. Two genomic DNA fragments from the $GABA_A$ receptor δ subunit gene were selected and inserted into a simple replacement vector (pNT) containing the neomycin gene and the HSV thymidine kinase gene. A successful targeting event would result in the removal of the first exon, 4.5 kb of

promoter sequence and 2 kb of DNA from the first intron. Two targeted embryonic stem cell lines were isolated and can be used to make mice homozygous for the mutated gene. The $GABA_A$ receptor δ subunit gene had previously been assigned to human Chromosome 1p. Genetic mapping of the gene to rat chromosome 5 and mouse chromosome 4 was performed. These results agree with expected regions of synteny between human, mouse and rat.

A range of recombinant HSV - 1 viruses were produced which contained the *E coli lacZ* gene driven by different promoters. Two loci within the HSV - 1 genome were chosen as sites of insertion, LAT and U_L43 in two HSV - 1 viral variants, 1716 and 1764. Infection of primary cerebellar granule cell cultures with a range of these recombinant viruses revealed that the cerebellar granule cells were not readily infected by this viral variant. Stereotactic injection of the viruses into the cerebellum of adult rats failed to show specific β - galactosidase expression.

Nevertheless, the $GABA_A$ receptor δ subunit gene promoter - *lacZ* fusion constructs can now be transferred to new HSV - 1 vectors with better growth characteristics thus furthering the original goal of this work.

Chapter 1

General Introduction

For many tissues, for example liver, muscle and pancreas, DNA elements and their cognate transcription factors that mediate regulation of particular genes have been identified. The corresponding elements and transcription factors responsible for expression in the nervous system are still relatively unknown. The mammalian brain is the most complex vertebrate organ, comprising as many as 1000 different neuronal cell types (Jessell and Kandal, 1993). Understanding neuronal gene regulation is essential when investigating diseases of the central nervous system as abnormal regulation of gene expression is thought to underlie certain neurological conditions. In addition, changes in gene expression are thought to underlie some aspects of the plasticity of the nervous system, such as learning and memory (Black *et al.*, 1987 ; Goelet *et al.*, 1986).

1.1 GABA RECEPTORS

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and has been shown to occur in about 30% of all central synapses (Bloom and Iversen, 1971). The actions of GABA are mediated by at least 3 different receptor classes that have been defined pharmacologically : $GABA_A$, $GABA_B$ (Bowery, 1993) and $GABA_C$ receptors. $GABA_A$ receptors form chloride - ion selective channels (Bormann, 1988; Silvilotti and Nistri, 1991) and are stimulated by GABA, muscimol and isoguvacine and inhibited by bicuculline and picrotoxin. $GABA_B$ receptors are 7 - transmembrane domain G protein - linked receptors that appear to be coupled to Ca^{2+} and/or K^+ channels (Bormann, 1988; Bowery, 1993). They are stimulated by GABA and baclofen and are inhibited by phaclofen. A third class of GABA receptor, the $GABA_C$ receptors are stimulated by GABA, but are insensitive to both bicuculline and baclofen (Quian and Dowling, 1993; Feigenspan *et al.*, 1993). These receptors are directly associated with Cl^- ion channels. A subunit family recently identified contains 2 members, ρ1 and ρ2 (Cutting *et al.*, 1991; Cutting *et al.*, 1992) and is found mainly in the retina. Although the ρ subunits display 30% - 38% sequence homology to the $GABA_A$ receptor subunits, pharmacological studies show that these subunits differ from the $GABA_A$ receptor subunits in that they are insensitive to baclofen (Cutting *et al.*, 1991, 1992; Shimada *et al.*, 1992) and as such have been classified as $GABA_C$ receptors.

1.1.1 The $GABA_A$ receptor

$GABA_A$ receptors belong to a superfamily of ligand - gated ion channels which includes nicotinic acetylcholine receptors (nAChR), glycine receptors (GlyR) and the $5\text{-}HT_3$ receptor. It has been proposed that these receptors have evolved from a common ancestor by gene duplication and sequence mutation (Schofield *et al.*, 1987). It is thought that the $GABA_A$ receptor and GlyR receptor families diverged from the original acetylcholine - gated ion channel (Arbas *et al.*, 1991). Subsequently, the $GABA_A$ receptor itself diverged from an original single gene to give the present array of genes encoding subunit families and subunit isoforms within the families. The $GABA_A$ receptor has received attention as it is the site of action of a number of important centrally acting drugs. It forms a chloride - ion selective channel and contains specific binding sites for GABA, picrotoxin, barbiturates, benzodiazepines and anaesthetic steroids.

Due to the ubiquitous presence of $GABA_A$ receptors in the CNS, they have been implicated in the involvement of several neurological diseases which are thought to involve defects in inhibitory transmission e.g. epilepsy, panic disorder, anxiety and depression (Matsumoto, 1989). Several naturally occurring mutations of $GABA_A$ receptor subunit genes have been found in animals and these may be involved in some human diseases. P^{cp} mice with a 95% penetrant, recessive, neonatally - lethal cleft plate have been found to have a deletion of the cleft palate locus (cp1) on chromosome 7. This deletion has been found to include the genes encoding the $GABA_A$ receptor subunits $\alpha 5$, $\gamma 3$ and $\beta 3$. (Culiat *et al.*, 1993; Nakatsu *et al.*, 1993). Mice containing a deletion which included the $\alpha 5$ and $\gamma 3$ subunit genes, but not the $\beta 3$ subunit gene, developed a normal palate (Culiat *et al.*, 1994). Furthermore, the cleft palate phenotype was rescued by introduction of a $\beta 3$ subunit transgene, under the control of a β - actin promoter, into mice homozygous for the cleft palate deletion (Culiat *et al.*, 1995). Thus it has been proposed that deletion of the $\beta 3$ subunit gene contributes to the clefting defect in P^{cp} mice. The human counterpart of the region deleted in P^{cp} mice is Chromosome 15q11-q13. It is possible that mutations occurring at this locus, especially those involving the $\beta 3$ subunit gene, might contribute to familial or sporadic craniofacial abnormalities in man.

Another abnormality linked to human Chromosome 15q11-q13 is Angelman Syndrome (AS) which is characterised by severe mental retardation, microencephaly, seizures, ataxia and craniofacial abnormalities (Angelmann, 1965; Magenis *et al.*, 1990). It has been shown in some patients that deletion of the β3 subunit gene, but not the α5 subunit gene, results in AS (Sinnett *et al.*, 1993). However, a single patient containing a translocation was found with an intact β3 gene (Reis *et al.*, 1993). Also, an AS - like paternal imprinting has not been detected for the corresponding region of the mouse chromosome 7 (Nicholls *et al.*, 1993). Thus the involvement of $GABA_A$ receptor subunit genes in the aetiology of AS remains to be determined.

The receptor is comprised of 5 protein subunits arranged in a pentameric array around a central core which forms the ion channel (Nayeem *et al.*, 1994). The exact identity of the 5 subunits which form this array are unknown for any $GABA_A$ receptor *in vivo.* There are at least 7 distinct subunit classes and molecular cloning studies have revealed many subunit isoforms within these classes. Thus there exists a large family of closely related receptor subunits (α1-6, β1-4, γ1-4, δ, ρ 1-2, ε and π) with properties that are similar but distinct; thus the possible number of receptor subtypes is immense.

1.1.2 Pharmacology of the $GABA_A$ receptor

Much of our understanding of the pharmacology of the $GABA_A$ receptor has been obtained from the study of neurones of the vertebrate CNS. As well as the GABA binding site, for which a number of different agonists and antagonists are known, there are several other ligand binding sites. Barbiturates, neurosteroids, ethanol and general anaesthetics all act through their independent modulatory sites on the receptor to allosterically enhance the effect of GABA. Compounds acting at the benzodiazepine (BZ) binding site of the $GABA_A$ receptor can allosterically enhance the effect of GABA (BZ agonists), inhibit the effect of GABA (BZ inverse agonists) or simply inhibit the effects of agonists and inverse agonists (BZ antagonists). Also, picrotoxin and TBPS (t-butylbicyclophosphorothionate) act at a unique site at or close to the channel pore to inhibit the effect of GABA.

1.1.2.1 The GABA binding site

$GABA_A$ receptors are activated by GABA and its structural analogues e.g. muscimol and THIP (4,5,6,7-tetrahydroisoxazolopyridin-3-ol). When GABA binds to the $GABA_A$ receptor it results in an increased chloride conductance of the supporting neuronal membrane (Curtis *et al.*, 1968; Kelly *et al.*, 1969). This increase is due to the opening of the ion channel of the $GABA_A$ receptor resulting in the passage of chloride ions into the cell. The agonist induced current decreases on continued exposure to high agonist concentrations as a result of receptor desensitisation (Mathers, 1987). The gating properties of the channel have been investigated using single channel current recording techniques (Hamill *et al.*, 1981). The single channel currents, recorded from mouse spinal cord neurons in culture, show that for the main conductance state of the $GABA_A$ receptor, channels open either singly or in bursts of several openings. Increasing the concentration of GABA affects the receptor in several ways. It increases the frequency with which the channels open as well as increasing the time for which the channels stay open. It also results in an increased frequency of occurrence of bursts of channel openings (MacDonald *et al.*, 1989a; Twyman *et al.*, 1990).

Experimental evidence suggests that at least 2 molecules of GABA must bind to the receptor for full activation of the channel (Sakmann *et al.*, 1983). Initially the properties of agonist binding sites of the $GABA_A$ receptor were studied using GABA, however the recognition site and the agonist recognition process were investigated using [3H] - GABA (Burch *et al.*, 1983), and a number of different GABA analogues e.g. muscimol (Krogsgaard-Larson *et al.*, 1979) and N - methyl GABA (Krogsgaard-Larson and Johnston, 1978). Bicuculline acts as a competitive antagonist at the $GABA_A$ receptor (Curtis *et al.*, 1970; Barker *et al.*, 1983). The phenylaminopyridazine, SR95531, also shows competitive inhibition of $GABA_A$ receptor mediated responses and is about 20 times more potent than bicuculline (Mienville and Vicini, 1987).

1.1.2.2 The benzodiazepine site

The benzodiazepines (BZ) are the most frequently prescribed psychoactive drugs and are used largely for the treatment of anxiety and insomnia. They are also potent anticonvulsants and muscle relaxants (Martin, 1990). Studies have shown that the BZs

produce a facilitation of $GABA_A$ receptor mediated transmission at both pre - and postsynaptic sites in the mammalian CNS (Haefely and Polc, 1983). They appear to increase the frequency of channel opening with little effect on the channel open time or channel conductance (Study and Barker, 1981). Single channel analysis has indicated that the increase in channel opening frequency is due to an increased occurrence of bursting activity, though the average duration of the bursts did not increase in the presence of the BZs (Twyman *et al.*, 1989). Biochemical experiments demonstrated the existence of specific high affinity binding sites for BZs on brain membranes that are closely associated with $GABA_A$ receptors (Braestrup and Squires, 1977). Thus binding of [^{3}H] - flunitrazepam to brain membranes was chloride - dependent and stimulated by GABA, muscimol (Tallman *et al.*, 1978; Olsen, 1982) and a large selection of GABA analogues (Braestrup and Nielsen, 1983). This stimulation was blocked by bicuculline and other $GABA_A$ receptor antagonists.

Richards *et al.* (1983) have mapped the distribution of the BZ binding sites in the mammalian CNS in considerable detail, and have found that they have a similar distribution to the $GABA_A$ receptor binding sites. However, the match is not exact suggesting that not all $GABA_A$ receptors have an associated BZ binding site, although there appears to be a close association between the two recognition sites. Further studies have shown that purification of the BZ recognition site results in co - purification of the $GABA_A$ receptor indicating that the two recognition sites are located on a common protein structure.

Prior to cloning of the subunit cDNAs, GABA/BZ receptors were subdivided into two pharmacological classes BZI and BZII, which could be differentiated by their different binding affinities for the triazolopyridazine CL 218 872 and zolpidem (Squires *et al.*, 1979). BZI type receptors have a higher affinity for these compounds and constitute the predominant $GABA_A$ receptor class in the CNS. The low affinity type II receptors are found principally in the cortex, hippocampus and spinal cord (Olsen and Tobin, 1990). There are also known to be a large population of receptors in the cerebellum which bind the BZ inverse agonist Ro15-4513 with high affinity but are insensitive to classical BZs e.g. diazepam (Sieghart *et al.*, 1987).

Photoaffinity labelling experiments have shown that the pharmacology of the BZ site is defined by the type of α and γ subunit present in the receptor (Stephenson *et al.*, 1990).

1.1.2.3 The barbiturate site

Anticonvulsant and anaesthetic barbiturates potentiate the electrophysiological response to GABA but their mechanism of action differs from that of the BZs. At low concentrations they seem to facilitate GABA - mediated transmission by increasing the channel opening time, while having no effect on channel conductance or opening frequency (Study and Barker, 1981). Channel - gating studies have revealed that the barbiturates increase the channel burst duration but have no effect on bursting frequency (Twyman *et al.*, 1989; Macdonald *et al.*, 1989b). High concentrations of the barbiturates can directly activate $GABA_A$ receptors (Higashi and Nishi, 1982).

1.1.2.4 The steroid site

Steroids have been found to enhance $GABA_A$ receptor function e.g. 5α-pregnan-3α-ol-20-one (3α-OH-DHP) and 5α-pregnan-3α,21-diol-20-one allosterically enhance the response to GABA (Puia *et al.*, 1990; Hadingham *et al.*, 1993) and this has been shown to be due to both an increase in the duration of channel opening and an increase in the frequency of openings (Twyman and MacDonald, 1992). Other steroids (e.g. pregnanolone sulphate) inhibit the response to GABA (Majewska *et al.*, 1990).

1.1.2.5 Picrotoxin/TBPS site

Picrotoxin is a noncompetitive antagonist of the $GABA_A$ receptor and appears to act at an allosteric site located close to the channel pore. It competitively displaces the cage convulsant tert-butylbicyclophosphorothionate (TBPS) from its specific high affinity binding sites on brain membranes (Squires *et al.*, 1983). Pribilla *et al.* (1992) showed that residues in the TM2 region of the glycine receptor β subunit were implicated in conferring picrotoxin resistance to heteromeric α/β glycine receptors and so it is possible that corresponding amino acids in the $GABA_A$ receptor TM2 region provide the binding site for picrotoxin. All recombinant forms tested so far are blocked by picrotoxin in a similar manner, and thus the contribution of different subunits to this site remains to be determined.

1.1.2.6 Other sites

In addition to the major sites already listed, the $GABA_A$ receptor also appears to contain binding sites for a number of other modulators including Zn^{2+}, avermectin and alcohols (Sieghart, 1992). **Figure 1.1** (Stephenson, 1995) illustrates the complex pharmacology of the mammalian $GABA_A$ receptors, displaying a variety of allosteric interactions which lead to increases or decreases in GABA mediated transmission.

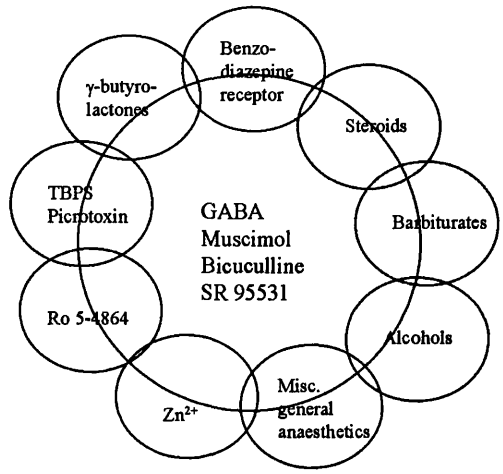


Figure 1.1 Schematic presentation of the various binding sites associated with $GABA_A$ receptors

Abbreviations used : misc. = miscellaneous. (Adapted from Stephenson, 1995).

1.1.3 Structure of the $GABA_A$ receptor

Purification of the $GABA_A$ receptor from bovine brain led to the isolation of cDNAs encoding the α1 and β1 subunits (Schofield *et al.*, 1987). The deduced amino acid sequences of the encoded polypeptides were found to share approximately 35% homology. Comparison of these amino acid sequences to those of the nAChR and glycine receptors, revealed structural similarities, leading to the proposal that these receptors are members of a ligand - gated ion channel superfamily (Barnard *et al.*, 1987; Schofield *et al.*, 1987).

Figure 1.2 illustrates the basic structure of the $GABA_A$ receptor. The N and C terminal domains are thought to be extracellular. The N terminal region contains a number of putative N -linked glycosylation sites (Barnard *et al.*, 1987; Schofield *et al.*, 1987) and a Cys - Cys loop which has been proposed to play a role in agonist binding (Cockcroft *et al.*, 1990). There are 4 putative membrane spanning domains (TM1 - TM4), the second of which is proposed to form the inner lining of the ion channel. Between the third and fourth transmembrane domains is a hydrophilic cytoplasmic region of highly variable sequence containing phosphorylation sites that are in intracellular regulatory mechanisms (Schofield *et al.*, 1987; Olsen and Tobin, 1990).

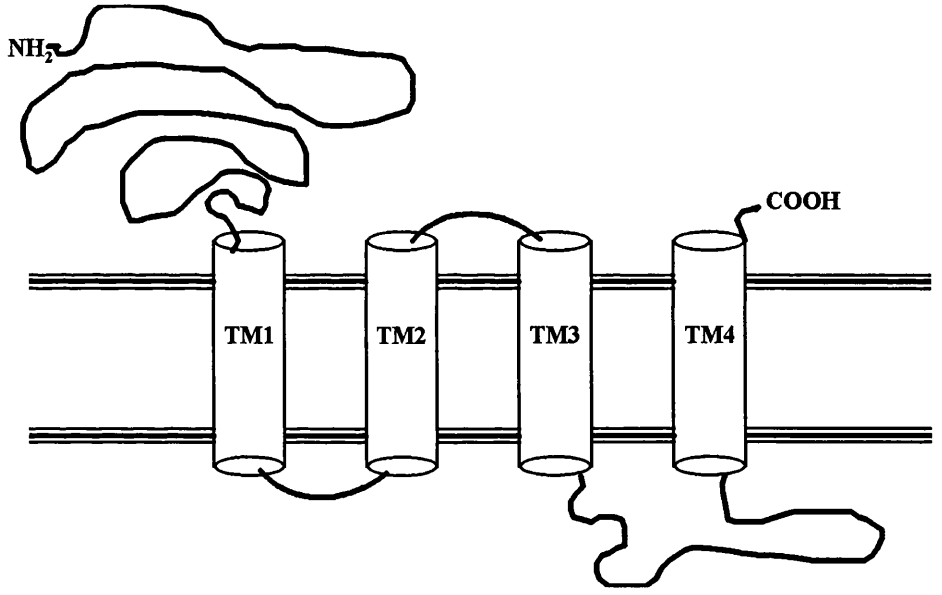


Figure 1.2 Basic structure of the $GABA_A$ receptor subunits

A schematic diagram showing the basic structural features common to all the $GABA_A$ receptor subunit genes. The N and C terminal domains are thought to be extracellular. The transmembrane domains are depicted as cylinders within the cell membrane. Abbreviations used are : NH_2 = N terminal domain, TM1 - TM4 = transmembrane domains, COOH = C terminal domain.

$GABA_A$ receptors have been solubilised from mammalian brain membranes using a variety of non-denaturing detergents. This solubilisation was followed by purification by BZ affinity chromatography (Stephenson, 1988). Analysis of purified $GABA_A$ receptors by SDS - PAGE revealed a multi-subunit structure. Initially it was thought that the purified receptor contained 2 major subunits, α (53 kDa) and β (57 kDa) (Stephenson, 1988; Sieghart, 1991). However, based on an overall complex molecular weight of 220000 - 240000 determined by radiation inactivation (Chang and Barnard, 1982) or by gel filtration chromatography (Sigel *et al.*, 1983; Martini *et al.*, 1982), it was proposed that the purified receptor was a hetero - oligomeric complex with a stoichiometry of α2β2 (Casalotti *et al.*, 1986; Mamalaki *et al.*, 1987). Microheterogeneity of both bands was demonstrated by protein staining, photoaffinity labelling and immunoblotting (Bureau and Olsen, 1990; Fuchs *et al.*, 1990; Park and deBlas, 1991).

Subsequent studies revealed the existence of 2 other α subunits (Levitan *et al.*, 1988). They also showed that by combining one of the α subunits (α1, α2, α3) with the β1 subunit in *Xenopus oocytes*, functional receptors could be produced. Levitan *et al.* (1988) were unable to demonstrate a robust benzodiazepine effect with these receptors, suggesting that an additional subunit, or factor, was required to produce $GABA_A$ receptors that had the full range of characteristics found *in vivo*.

cDNA clones encoding further $GABA_A$ receptor subunits were identified by using a degenerate pool of 23 - base oligonucleotides. One such clone encoded a polypeptide (γ2) that shares about 40% sequence identity with the α1 and β1 subunits. When γ2 was co - expressed with the α1 and β1 subunits in heterologous expression systems, the resultant receptor had a high - affinity binding site for benzodiazepines and showed potentiation of the GABA mediated response by BZ (Pritchett *et al.*, 1989a; Ymer *et al.*, 1989a, b).

Initially, 4 different $GABA_A$ receptor subunit families have been identified on the basis of sequence similarity and have been named α, β, γ and δ. More recently a further two putative subunit families have been isolated - ε and π (Davies *et al.*, 1997: Hedblom and

Kirkness, 1997).There is 30% - 40% sequence identity among the subunit families and greater than 70% sequence homology between members of the same family.

1.1.4 Molecular biology of the $GABA_A$ receptor

1.1.4.1 Receptor heterogeneity

A large number of $GABA_A$ receptor subunit cDNAs have been isolated. Initially, in the rat, 13 different subunits were identified, and these were divided into 4 families according to sequence homology i.e. α (1-6), β (1-3), γ (1-3) and δ (Wisden and Seeburg, 1992). More recently, two novel $GABA_A$ receptor subunit classes have been identified. After screening an EST (expressed sequence tag) database with a peptide consensus sequence from known family members, a new class of subunit - ε was identified (Davies *et al.*, 1997). The sequence of this EST was used to isolate a cDNA clone from a hippocampal library. This contained a large open reading frame which encoded a 506 amino acid polypeptide showing 38% - 43% amino acid identity to the γ - subunits and 28% - 30% identity to the α - subunits.

The other $GABA_A$ receptor subunit class recently identified has been named π (Hedblom and Kirkness, 1997). This was also identified by searching an EST database. The sequence from this EST was used isolate a cDNA clone from a pancreatic carcinoma library. The cDNA contains an open reading frame encoding a 440 amino acid polypeptide which is most closely related to the β - subunits (37% amino acid identity), the δ subunit (35%).

Experimental work on $GABA_A$ receptors from non - mammalian species has shown a high degree of conservation through evolution. One example of this is the chicken α1 subunit which shows 98% homology to any mammalian α1 subunit (Bateson *et al.*, 1991). Two novel subunits have been identified in the chick, β4 (Lasham *et al.*, 1991) and γ4 (Harvey *et al.*, 1993). As yet these have not been identified for other vertebrate species.

The identification of alternate splice variants of some $GABA_A$ receptor subunits has further increased the pool of possible receptor subunit combinations. The γ2 subunit exists in 2 forms which differ by the presence ($\gamma2_L$) or absence ($\gamma2_S$) of 8 amino acids in the

region that lies between the 3rd and 4th transmembrane (TM) domains. This has been shown to be present in bovine, human (Whiting *et al.*, 1990), mouse (Kofuji *et al.*, 1991) and chicken brains (Glencorse *et al.*, 1990, 1992) and the 8 amino acid insertion present in the $\gamma 2_L$ variant is absolutely conserved between these species. Alternative splicing of the human β3 (Kirkness and Fraser, 1993) and rat α6 (Korpi *et al.*, 1994) genes have also been identified. Splice variants of the chick β2 and β4 subunits have been shown (Bateson *et al.*, 1991; Harvey *et al.*, 1994). The insertion of 17 amino acids between TM3 and TM4 distinguishes the β2L variant from the shorter β2S form (Harvey *et al.*, 1994). The β4 forms differ by the presence (β4') or absence (β4) of 4 amino acids in the region that lies between the 3rd and 4th TN domain (Bateson *et al.*, 1991).

1.1.4.2 Receptor subunit families

Heterologous expression studies of recombinant receptors have identified specific roles for individual subunit classes. However, there are also many characteristics of the receptors that are specified by interactions between subunits of different classes.

1.1.4.2.1 α - subunits

This is the largest subunit class comprising 6 members. It is believed that they specify the heterogeneity of the BZ binding site. Two types of BZ receptor were originally proposed on the basis of pharmacology and distribution. The BZI subtype is found throughout the brain and displays a high affinity for Cl218-872 (a triazolopyridazine) and β - carbolines. The BZII subtype is found principally in the cortex, hippocampus and spinal cord. (Doble and Martin, 1992; Wisden and Seeburg, 1992). In heterologous expression systems, varying the α subunit in combination with the β and γ subunits suggested differences in BZ pharmacology, GABA - BZ interaction and steroid modulation of GABA responses, but not barbiturate, picrotoxin or bicuculline sensitivity. Ligand - binding studies, using αx βxγ2 combinations, revealed a correlation between the presence of an α1 subunit with BZI pharmacology and α2 or α3 with BZII pharmacology (Pritchett *et al.*, 1989b). Altering the particular β subunit present appeared to have no effect. It has also been shown that the α5 subunit can confer a BZII pharmacology different to that seen for the α2 or α3 subunits, demonstrating heterogeneity within the BZII receptor type. Receptors

containing α5 show a lower affinity for zolpidem than those containing α2 or α3, and a higher affinity for Cl218-872 (Pritchett and Seeburg, 1990). Receptors containing an α4 or α6 subunit appear to constitute a subfamily of $GABA_A$ receptors with a distinct type of BZ pharmacology. α6 - containing receptors exhibit high affinity binding of muscimol, Ro 15-4513 and the inverse agonist DMCM (6,7-dimethyl-4-ethylcarboline-3-carboxylate methyl ester), but do not bind other BZs or β - carbolines (Lüddens *et al.*, 1990). The α6 subunit is only expressed in the cerebellum and is selective for Ro 15-4513, an antagonist of alcohol induced motor incoordination and ataxia. Receptors containing the α4 subunit display pharmacological characteristics similar to those observed for subunit combinations containing the α6 subunit. Thus muscimol and Ro 15-4513 bind with high affinity, while diazepam, flunitrazepam or CL 218,872 do not bind (Wisden *et al.*, 1991).

Using site-directed mutagenesis, a single amino acid change between α1 and α3 subunits has been shown to confer BZI or BZII pharmacology. Substitution of a single glutamate residue (Glu-201) in the α3 subunit by a glycine residue (Gly-210, which appears in the corresponding position of the α1 subunit), confers on the hybrid receptor ($\alpha3_{G225E}\beta2\gamma2$) high affinity for Cl218-872, effectively changing the pharmacology of the receptor from BZII to BZI (Pritchett and Seeburg, 1991). Similarly, a specific histidine residue (His-100) present in some α subunits (α2, 3 and 5, His 102 in α1) has been shown to be necessary for high affinity BZ agonist binding (Wieland *et al.*, 1992; Korpi *et al.*, 1993).

GABA sensitivity appears to be altered depending on which α subunit is combined with β1. The α4β1 combination is less sensitive to GABA than α1β1 (Khrestchatisky *et al.*, 1989), while the α5β1 combination is more sensitive than α1β1 or α3β1 (Malherbe *et al.*, 1990; Sigel *et al.*, 1990). Addition of γ2 seems to maintain or even increase the effects of the α subunit present on GABA sensitivity (Sigel *et al.*, 1990). The α subunit also has an effect on the steroid action. Combinations of α1β1 or α3β1 display greater potentiation than α2β1 (Shingai *et al.*, 1991).

A major role in determining the nature of binding sites for GABA and some of the allosteric effectors has therefore been assigned to the α subunit.

1.1.4.2.2 β - subunits

The β subunits are highly sequence - conserved, do not have a hydrophilic C terminus and contain a larger intracellular domain than other subunits (Levitan *et al.*, 1988). While all of the β subunits tested form GABA - responsive chloride channels when co - expressed with the α1 subunit, the β2 subunit has been shown to be the most prevalent (Benke *et al.*, 1994). The markedly different mRNA distributions of the 3 β subunit isoforms in the mammalian CNS appear to suggest a functional significance for their existence (Wisden *et al.*, 1992: Laurie *et al.*, 1992a; Laurie *et al.*, 1992b). They are known to play a role in the formation of part of the neurotransmitter binding site, as well as influencing receptor assembly and mediating some of the affects of protein kinase phosphorylation of $GABA_A$ receptors (Connolly *et al.*, 1996; Moss and Smart, 1996). *In vitro* and *in vivo* phosphorylation studies suggested that β subunits all contain highly conserved phosphorylation sites for protein kinase A (PKA) within their major intracellular domains (Kano and Konnerth, 1992; Moss and Smart, 1996). However, McDonald *et al.* (1998) demonstrated that only the β1 and β3 subunit – containing receptors (but not β2) can be phosphorylated by PKA. They showed that the phosphorylation of either the β1 or β3 subunits resulted in either reduced or enhanced GABA - activated currents Thus the differential behaviour of neuronal $GABA_A$ receptors .following phosphorylation by PKA can be accounted for by the presence of different β subunits in the receptor complex.

The initial pharmacological studies of $GABA_A$ receptors focused on the role of the α and γ subunits. In 1993, Hadingham *et al.* demonstrated that neither the affinity nor efficacy of a number of benzodiazepine binding site compounds, a barbiturate and several neurosteroids were influenced by the type of β subunit present in the receptor molecule. Thus they proposed that the β subunit did not significantly influence the benzodiazepine, barbiturate or steroid site pharmacologies of $GABA_A$ receptor subtypes. However, Hill - Venning *et al.* (1997) demonstrated that the general anaesthetic etomidate enhanced GABA - activated currents of recombinant $GABA_A$ receptors in a manner that was dependent upon the identity of both the α and β subunit isoforms. They concluded that the subtype of β subunit influences the potency with which etomidate potentiates GABA-

evoked currents. Thus while initial studies led to the belief that the β subunits played mainly a structural role in the $GABA_A$ receptor, while also forming with the α and γ subunits the GABA agonist binding sites, it now appears that they play more complex role in the $GABA_A$ receptor pharmacology.

1.1.4.2.3 γ - subunits

There are clear differences in both the binding and the functional properties of recombinant receptors containing different γ subunits. All 3 γ subunits, when combined with α or β subunits, can confer different BZ binding on recombinant receptor complexes (Knoflach *et al.*, 1991; Pritchett *et al.*, 1989b; Ymer *et al.*, 1990). Some BZ effects, however, may be attributed to differential co-operative interactions between γ and α subunits.

In recombinant receptors containing an α and β subunit, the γ1 subunit produces a marked decrease in affinity for the antagonist Ro 15-1788 and inverse agonist DMCM in comparison to γ2 - containing receptors (Ymer *et al.*, 1990). In contrast, γ3 - containing receptors show a marked decrease in BZ agonist affinity compared to γ2 receptors, while both γ2 and γ3 have similar affinities for antagonists and inverse agonists (Herb *et al.*, 1992). Exchanging the γ2 subunit for a γ1 subunit in αβγ combinations changes the action of DMCM from that of agonist to antagonist (Puia *et al.*, 1991). In general, the higher affinities of BZ ligands for γ2 - containing receptors is reflected in increased modulation of GABA - gated currents (Herb *et al.*, 1992; Knoflach *et al.*, 1991; Puia *et al.*, 1992).

The $\gamma 2_L$ subunit alternative splice variant has been shown to be responsible for conferring ethanol enhancement in receptors expressed in *Xenopus oocytes* from recombinant clones and brain mRNA (Wafford *et al.*, 1991). The previously proposed phosphorylation site within the 8 amino acid insert of the $\gamma 2_L$ subunit (Whiting *et al.*, 1990) appears to be essential for this effect.

1.1.4.2.4 δ subunit

The δ subunit is discussed in detail in **Section 1.3**.

1.1.4.2.5 ε - subunit

The pharmacological properties of this subunit have been investigated by *in vitro* expression studies (Davies *et al.*, 1997). They showed that the ε subunit behaves differently from the other $GABA_A$ receptor subunits. It was demonstrated that the ε subunit could assemble with other subunits and it appeared to confer an insensitivity to the potentiating effects of intravenous anaesthetic agents. It also abolishes the normal outward rectification of recombinant receptors in which it assembles. It is thus possible that the ε subunit can confer additional pharmacological properties to $GABA_A$ receptors.

1.1.4.2.6 π - subunit

While $GABA_A$ receptors have been detected in non - neuronal cells, their precise function in these cells has not been determined. The π subunit has been detected in a number of different human tissues, but is particularly abundant in the uterus. The pharmacological properties conferred by the π subunit were examined by *in vitro* expression studies (Hedblom and Kirkness, 1997). Like the ε subunit, the π subunit behaved differently from the other $GABA_A$ receptor subunits. It assembled with other subunits to form recombinant receptors with unique ligand - binding properties. In particular, the π subunit reduced the sensitivity of the receptors to the endogenous steroid pregnanolone, which has been proposed to regulate uterine motility by inhibiting contractions (Majewska and Vaupel, 1991). The physiological significance of this effect has yet to be determined.

1.1.5 $GABA_A$ receptor expression patterns

1.1.5.1 Cell - specific expression

The existence of 19 $GABA_A$ receptor subunit genes (to date) has led to much speculation about the molecular structure of the possible $GABA_A$ receptor types. It is probable that different combinations of subunits are present in different neuronal populations. Several approaches have been used to attempt to identify the structure of *in vivo* receptors, e.g. *in situ* hybridisation to determine the sites of gene expression, immunocytochemistry to identify the location of the receptor polypeptides and immunoprecipitation using subunit - specific antibodies.

Wisden *et al.* (1992) studied the expression pattern of 13 $GABA_A$ receptor subunit - encoding genes in the adult rat brain by *in situ* hybridisation. Each mRNA displayed a unique distribution ranging from ubiquitous (α1 mRNA) to narrowly confined (α6 mRNA was present only in cerebellum granule cells). This work allows for some plausible deductions of possible receptor subunit combinations. The α1 and β2 mRNAs are most widely co - distributed in the brain. Also the γ2 mRNA often co-localises with this pair. The α1β2γ2 receptor which exhibits BZI pharmacology (Pritchett *et al.*, 1989b), is proposed to be the major $GABA_A$ receptor subtype (Laurie *et al.*, 1992a ; Shivers *et al.*, 1989 ; Wisden *et al.*, 1992). Similarly, the major receptors comprising the BZII subtypes are proposed to be α2β3γ2, α3βxγ2 and α5β1γ2 combinations (Wisden *et al.*, 1992). Another frequently occurring combination is that of α2 and β3 mRNAs, together with various γ subtype mRNAs. The distribution and intensity of the α5 and β1 probe hybridisation signals appear to be identical throughout most of the brain, suggesting that they are co - expressed and may co assemble into receptors. In a large number of regions (principally the thalamic nuclei) the δ subunit mRNA co - localises with the α1, α4 and β 2 mRNAs, although its distribution is more restricted. The δ subunit expression pattern is discussed more fully in **Section 1.3** of this chapter.

The expression pattern of the ε subunit was determined by Northern blot analysis (Davies *et al.*, 1997). While little expression was evident in whole brain samples, there were higher levels of transcripts detected in amygdala, the thalamus and especially in the subthalamic

nuclei. The latter is of particular interest as the transcripts of the γ and δ subunit are detected only at relatively low levels in the subthalamic nuclei which may suggest the possible involvement of alternative receptor subunits in this region.

1.1.5.2 Developmental expression

The role of the perinatal $GABA_A$ system appears to differ greatly from that in the adult CNS. In the foetal and neonatal hippocampus, GABA - activated chloride channels lead to marked membrane depolarisation (Ben-Ari *et al.*, 1989). Furthermore, activation of neonatal $GABA_A$ receptors induces a rise in intracellular calcium concentration in both cerebellar and cortical neurones (Connor *et al.*, 1987; Yuste and Katz, 1991). Increased intracellular calcium is an important factor in neuronal growth and differentiation (Kater and Guthrie, 1990; Spitzer, 1991). Consistent with the different roles of GABA in the neonate and adult, are the observed changes in the pharmacological properties of $GABA_A$ receptors during rat and primate brain development. For instance, the proportions of $GABA_A$ and BZ receptor subtypes alter, with low - affinity $GABA_A$ receptors appearing later than those of high affinity, and type II and I BZ receptors predominating in the neonate and adult respectively (Chisholm *et al.*, 1983: Reichelt *et al.*, 1991).

Laurie *et al.* (1992b) performed gene expression studies of $GABA_A$ receptor subunits during both embryonic and postnatal development in the rat brain. They observed that the expression of each $GABA_A$ receptor subunit gene changes during early development, and these changes appear to coincide with the alteration of GABAs role from excitatory, neurotrophic factor to inhibitory neurotransmitter.

Thus, each subunit appears to display a specific developmental expression pattern. The levels of the α2, α3, α4 and α5 subunit transcripts (Laurie *et al.*, 1992b) are much higher in the brains of developing rats than in those of adult animals, whereas the α1 and α6 subunit transcripts (Laurie *et al.*, 1992b) are undetectable before birth and reach peak expression in the adult rat brain. The β2 and δ subunit transcripts are also present predominantly in the adult rat brain (Laurie *et al.*, 1992b). The alternative splicing of the γ 2 subunit transcript in the rat is also developmentally regulated, with levels of $\gamma 2_S$ - subunit transcripts remaining fairly constant from birth to adult while the $\gamma 2_L$ - subunit

transcripts rise from being almost undetectable at birth to peak expression levels in the adult rat brain (Bovolin *et al.*, 1992b).

Laurie *et al.* (1992b) have shown that in regions of the brain that express only a subset of $GABA_A$ receptor subunit genes, a developmental switch from one subunit combination to another can occur. For example, in the globus pallidus an $\alpha2/\alpha3\beta2\gamma1$ combination changes to that of an $\alpha1\beta2\gamma1/\gamma2$ combination (Laurie *et al.*, 1992b). Such switches in expression are not universal however, with some brain regions and cell types displaying differential developmental profiles. Thus, in the cerebellum, the levels of $\alpha1$, $\beta2$, $\beta3$ and γ 2 subunit transcripts in Purkinje cells did not alter from post - natal day 6 to adulthood (Laurie *et al.*, 1992b). In contrast, the large number of transcript types detected in post - migratory cerebellar granule cells could be classified into those that showed little change in levels from post - natal day 6 to adulthood ($\alpha2$, $\alpha3$, $\beta1$, $\gamma1$ and $\gamma3$), and those ($\alpha1$, $\alpha6$, $\beta2$, $\beta3$, $\gamma2$ and δ) that showed a significant increase in their levels over the same time period (Laurie *et al.*, 1992b). Thus in 2 different cell types of this brain region, the $\alpha1$, $\beta2$, $\beta3$ and $\gamma2$ subunit transcripts display differential developmental regulation.

1.2 NEURON - SPECIFIC GENE EXPRESSION

By comparison with other tissues (Johnston and McKnight, 1989; Struhl, 1991), neuron - specific gene expression probably occurs at the transcriptional level through the interaction of *cis* - acting regulatory sequences with gene regulatory proteins within the nervous system. Transcription of eukaryotic protein - coding genes is performed by RNA polymerase II (pol II), which combines with numerous auxiliary factors to form a "core transcription complex" (Gill, 1994). In many genes this transcription complex binds to a TATA box which is a consensus sequence that has been shown to be present in the promoter region of a large number of genes and lies about 30 bases upstream of the transcription start site. However, many genes lack TATA boxes and so in these genes, the pol II transcription complex must bind to, and initiate transcription from less well defined initiator elements (Gill, 1994).

All these initiator complexes appear to require additional factors to achieve *in vivo* gene transcription patterns. Thus numerous *cis* - acting regulatory DNA sequences which can

mediate transcriptional activation (enhancers) or transcriptional repression (silencers) have been identified. These sequence - specific regions bind transcription factor proteins (TFs) and it is through these TFs binding to the DNA (protein - DNA interactions) and to each other (protein - protein interactions) that constitutive, inducible and tissue - specific regulation of gene expression can be achieved (**Figure 1.3**; Struhl,1991; Tjian and Maniatis, 1994).

Figure 1.3 ***Schematic diagram of regulatory elements in genomic regions of a prototypical gene.***

Organisation of regulatory elements in the genomic region of a typical eukaryotic gene. Multiple positive and negative regulatory elements can be located in the 5' - upstream, intronic and 3' - regions. (Adapted from Grant and Wisden, 1997).

Structure - function studies of various cloned eukaryotic transcription factors have revealed a modular structure consisting of DNA binding and transcriptional activation domains combined, in some cases with specific homo - or heterodimer interfaces (Mitchell and Tjian, 1989). A comparison of amino acid sequences from several cloned genes has led to the identification of distinct structural motifs responsible for transcriptional activation, DNA binding and dimerization. Transcriptional activation domains appear to include various structural features for example, acidic helices and glutamine - rich, proline - rich and serine/threonine domains (Ptashne, 1988; Courey *et al.*, 1989; Mermod *et al.*, 1989; Tanaka and Herr, 1990). These are however as yet poorly defined.

Characterisation of DNA binding domains has revealed more clearly defined structural features. These include zinc fingers, helix - turn - helix/homeo domains, basic leucine

zipper (b-ZIP) proteins and a variety of other "basic" motifs (Miller *et al.*, 1985; Kadonaga *et al.*, 1987; Clerc *et al.*, 1988; Hoey and Devine, 1988; Landschulz *et al.*, 1988; Müller *et al.*, 1988; Sturm *et al.*, 1988).

In addition to structural domains that form interactions with DNA, some regulatory proteins also contain protein - protein binding domains. In particular some classes of DNA - binding proteins rely on the formation of specific homo - or heterodimers to create a functional DNA recognition site. One dimerisation motif identified is the so - called "leucine repeat" of the b-ZIP class of DNA - binding proteins (Landschulz *et al.*, 1988). This amphipathic α - helix motif is characterised by the presence of a leucine residue at every seventh position. Two leucine repeats aligned in a parallel fashion are thought to form a coiled - coil structure resulting in either homodimers or heterodimers. Examples of this include the CCAAT/enhancer binding protein, Jun - Fos and GCN4 (Hope and Struhl, 1987; Kouzarrides and Ziff, 1988; Landschulz *et al.*, 1988 and 1989; O'Shea *et al.*, 1989; Turner and Tjian, 1989).

Another class of DNA - binding and dimerisation domain identified is the helix-loop-helix (HLH) motif (Murre *et al.*, 1989b). This motif has been found in a variety of enhancer binding proteins and putative transcription factors involved in the control of cellular proliferation and differentiation (Edmonson and Olsen, 1989; Mellentin *et al.*, 1989; Murre *et al.*, 1989b; Beckmann *et al.*, 1990; Henthron *et al.*, 1990). A distinct feature of HLH proteins is the ability to form heteromeric complexes with many different members of this large family. An example of this is with muscle - specific factors MyoD and myogenin which can form heterodimeric complexes with each other as well as homodimers (Murre *et al.*, 1989b).

Another family of transcription factors identified is the basic helix-loop-helix-leucine zipper (b-HLH-Zip) family (or c-myc family). This includes many nuclear eukaryotic proteins e.g. Myc, Max, Mad, AP-4, TFEB, MiTF, USF and ADD1 (Amati and Land, 1994; Baxevanis and Vinson, 1993; Tassabehji *et al.*, 1994; Tontonoz *et al.*, 1993; Henrion *et al.*, 1996). Through binding to the target DNA as homo - or heterodimers, all these factors regulate a variety of genes involved in important cellular processes including

differentiation, cell cycle regulation and apoptosis. Thus the combined action of different HLH proteins, leucine zippers and b-HLH-Zip proteins may provide an intricate network for directing eukaryotic gene transcription.

Many neuronal genes are expressed in more than one, but still a limited number of cell types. This restricted expression can be controlled by the presence of cell - specific positive enhancers (**Figure 1.4**). These bind transcription factors which are specific for each cell type in which the gene is expressed.

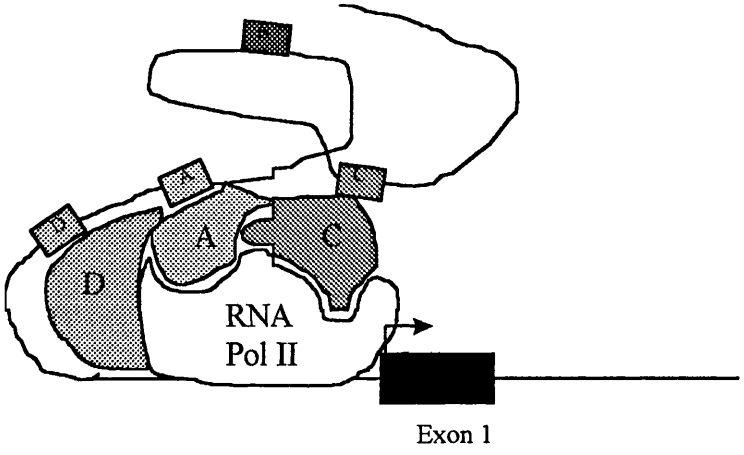


Figure 1.4 Activation of gene expression by enhancer elements

The binding of TFs A, C and D to their respective DNA recognition sequences, and their intramolecular interactions with each other and the RNA pol II - protein complex results in activation of gene transcription. (Adapted from Grant and Wisden, 1997).

An example of this is found in the *Drosophila FMRFamide* neuropeptide gene promoter region (Schneider *et al.*, 1993). This contains multiple discrete enhancer regions which are required for expression in different neuronal cell types in the fly brain. Another example of restricted expression is found in the olfactory system where genes encoding proteins involved in olfactory transduction are expressed exclusively in the sensory neurons of the olfactory system (Wang *et al.*, 1993). Analysis of the regulatory regions of six of these

genes identified a recognition sequence for a putative transcription factor binding site (Kudrycki *et al.*, 1993; Wang *et al.*, 1993). Kudrycki *et al.* (1993) showed that this region was sufficient to restrict reporter gene expression to the olfactory epithelium. The TF that binds to this motif (Olf-1) was isolated using a yeast one - hybrid system and belongs to the HLH class of TFs (Wang and Reed, 1993).

Unlike other tissue - specific genes, the majority of neuron - specific genes that have been studied to date appear to use negative regulation as a major mechanism for neuron - specific regulation (Mandel and McKinnon, 1993). The reasons for this are unclear, however it could be due to the fact that, to a much greater extent than other tissues, the nervous system contains thousands of distinct cell types within a single "tissue". Thus, in addition to mechanisms specifying "brain - specific" gene expression, there must also be mechanisms in place to restrict expression to a particular subset of neurons. These two forms of negative regulation can be achieved with the use of silencers (**Figure 1.5**). Global silencing involves repression of neuronal genes in nonneuronal cells and restricted silencing results in the restriction of expression of a neuron - specific gene to only a subset of neurons (Grant and Wisden, 1997).

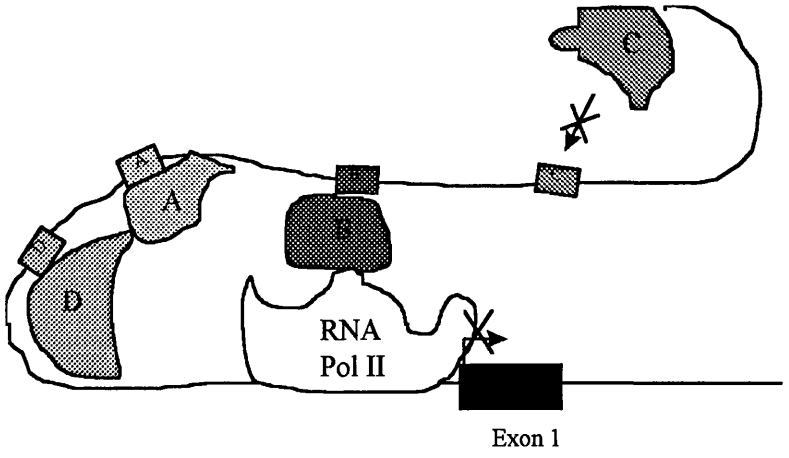


Figure 1.5 Repression of gene expression from silencing elements

The binding of TF B to its recognition site sterically blocks the binding of TF C to its binding site. In addition, the presence of the TF B complex disrupts the RNA pol II - protein complex. Thus the D-A-C protein complex seen in **Figure 1.4** is not formed and gene transcription is repressed (Adapted from Grant and Wisden, 1997).

Several regions identified in the promoters of neuron - specific genes have been shown to act as silencers. One of these is the neural restrictive silencer element (NRSE). NRSEs from various genes (**Table 1.1**) have been shown to have some functional role in silencing the expression of neuronal genes in nonneuronal cells by binding a sequence - specific protein found in nonneuronal cells, but absent in neuronal cells (Kraner *et al.*, 1992; Mori *et al.*, 1992; Maui *et al.*, 1990; Li *et al.*, 1993).

Table 1.1 NRSE consensus sequences

Gene	NRSE sequence	Reference
SCG10	TTCAGAACCACGGACAGCACC	Mori *et al.*, 1992
Na II	TTCAGAACCACGGACAGCACC	Maue *et al.*, 1990
DBH	AGCAGCTCCTCGGACCTCA-G	Ishiguro *et al.*, 1993
Synapsin	TTCAGCACCGCGGACAGTGCC	Li *et al.*, 1993
α3 NKA	CTTAGCTTCTCGGTGG-CGCC	Pathak *et al.*, 1994

DNA consensus sequences for the neural restrictive silencer element for which a functional activity in nonneuronal cells has been demonstrated by deletion analysis and protein binding studies. (Table adapted from Grant and Wisden, 1997)
Abbreviations used : NRSE = neural restrictive silencer element, Na II = sodium channel type II, DBH = dopamine β - hydroxylase, α3 NKA = Na,K - ATPase α3 subunit.

Sequence analysis has identified putative NRSE sequences in other neuron - specific genes including the brain - derived neurotrophic factor (BDNF), synaptotagmin, glycine receptor subunit and the β2 neuronal nicotinic receptor subunit genes (Bessis *et al.*, 1995; Schoenherr and Anderson, 1995; Schoenherr *et al.*, 1996).

A cDNA encoding a zinc finger TF has been isolated by two different groups (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). The protein encoded by this DNA, neural restrictive silencer factor (NRSF) has been shown to bind NRSE sequences from SCG10, Na II channel, synapsin I and BDNF genes (Schoenherr and Anderson, 1995). It has also been shown to repress transcription, in PC12 cells, from a reporter plasmid which contains

an NRSE (Chong *et al.*, 1995; Schoenherr and Anderson, 1995). NRSF is expressed in a wide range of nonneuronal tissues, and while it has been found in neuronal progenitor cells, it is absent in fully differentiated neurons (Schoenherr and Anderson, 1995). Thus it is probable that NRSF plays a role in the silencing of some NSRE containing neuronal genes in nonneuronal cells.

Other regions identified as putative silencers are E boxes which are DNA binding sites for the basic helix - loop - helix (bHLH) class of transcription factors. The promoter region of the gene encoding the tyrosine hydroxylase (TH) enzyme has been demonstrated to contain an E box consensus site. A 23 bp segment of the upstream promoter region, important for tissue - specific expression, contains an AP-1 motif, which is the binding site for leucine zipper TFs such as c-fos and c-jun. This motif overlaps with a dyad symmetry element centred around an E - box consensus site (Yoon and Chikaraishi, 1992). They demonstrated that while reporter constructs containing only the AP-1 site were expressed in both neuronal and nonneuronal cells, constructs containing the AP-1 site and the region of dyad symmetry were expressed only in the neuronal cells. This data points to the E - box having some role in silencing expression of neuronal cells in nonneuronal tissue.

An example of restricted silencing is shown for the ChAT (choline acetyl transferase) gene, which is expressed only in the cholinergic neurons in the nervous system. Li *et al.* (1993) demonstrated that the promoter region of the human ChAT gene contains two major silencing regions which act together to silence ChAT expression in noncholinergic cells. The proximal silencer element contains two E - box sequences both of which must be present to repress transcription in noncholinergic cells (Li *et al.*, 1993).

Some genes utilise both forms of silencing. The glial fibrillary acidic protein (GFAP) is found in astrocytes and Schwann glial cells in the nervous system. Two silencing elements (GDR1 and GDR2) are present outwith the 5' - region. GDR1 acts as a global silencer and is responsible for brain - specific expression, while GDR1 and GDR2 act together to restrict expression to glial cells (Kaneko and Sueoka, 1993).

While the examples given above have described using either activation or repression, many neuron - specific genes are regulated using both positive and negative regulatory regions (e.g. synapsin I gene, DBH gene, L7/pcp-2 gene). For example, a positive enhancer may be present in a gene that needs a complementary silencer to bring about the required expression pattern. However a different gene with the same positive regulatory element but different silencer elements can generate a different expression pattern. Thus a network may exist whereby the same regulatory elements and transcription factors can control different genes in different ways within the same cell.

Not all regulatory elements reside in the 5' - regions of genes. In many cases, regulatory enhancer/silencer regions are found in introns or 3' - to the polyadenylation site of the gene (**Figure 1.6**) (Echelard *et al.*, 1994; Marshall *et al.*, 1994; Zimmerman *et al.*, 1994). For these regions to form interactions with the basal promoter complexes, some structural rearrangement of the gene (looping of DNA) must occur.

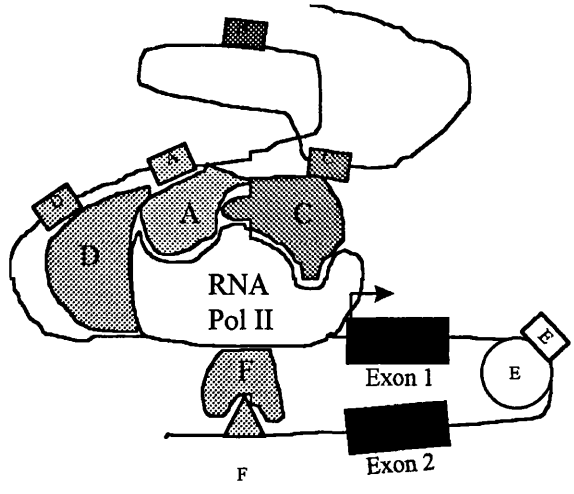


Figure 1.6 Activation of gene expression from intronic and 3' - DNA elements

The binding of TF E to its DNA recognition site causes a conformational change that brings the positive activating TF F which is bound to its 3' - DNA element, into contact with the RNA pol II complex and further activates transcription. (Adapted from Grant and Wisden)

One example of this is the nestin gene. Zimmerman *et al.* (1994), demonstrated that sequences present within the 5' - region of the nestin gene were insufficient to correctly direct expression of the β - galactosidase gene. However, on inclusion of intronic regions, expression similar to that shown for the endogenous gene was observed. Thus the 5' - region appears to act only as a basal promoter while the regulatory elements that govern the cell - specific expression of the nestin gene reside in the intronic regions.

Expression of genes which exist as clusters in the genome (e.g. the globins, olfactory genes) may be subject to co - ordinate control. Two possible regulatory mechanisms have been proposed. (**Figure 1.7a** and **b**). Each gene in the array may possess a unique binding site for a specific TF. In a cell type that expresses only that particular TF, the gene in the cluster that contains the binding site for that TF will be the only one transcribed. This system has been proposed for the selection of sensory olfactory neuron - specific genes from a background of non - olfactory expressed genes by the positive olfactory - specific TF Olf-1 (Wang and Reed, 1993).

An alternative mechanism is demonstrated in **Figure 1.7b**. There may be a single control region which mediates transcriptional activation of all the genes in the cluster. These "locus control regions" (LCRs) have been demonstrated to be important in regulating the expression of specific genes from the human β - globin gene cluster (Orkin, 1995).

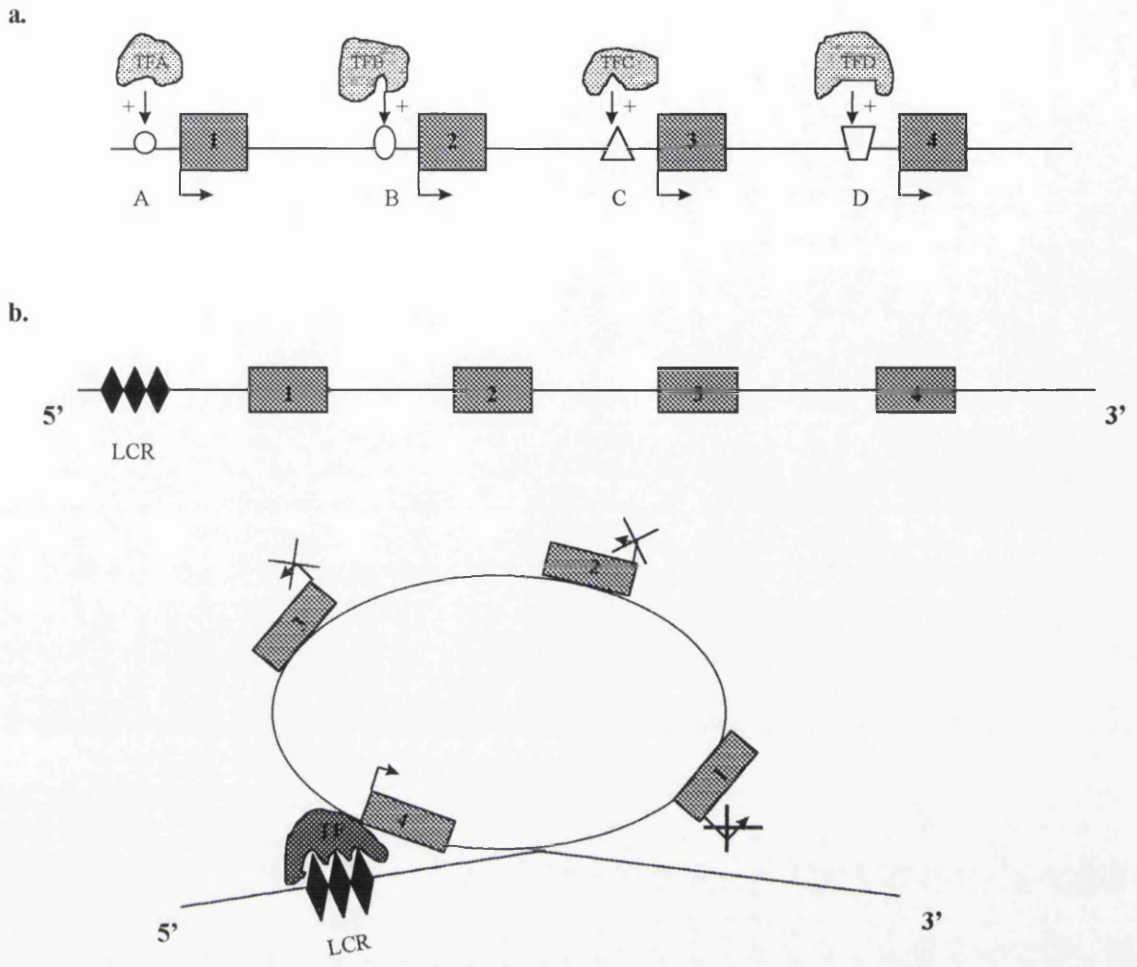


Figure 1.7 ***Schematic presentation of possible methods of regulation of expression of genes arranged in a cluster on a chromosome.***

a. Each gene is transcriptionally activated by its own unique cell - specific TF. 1 - 4 are the genes arranged in a cluster from which only one gene is expressed at any one time. Gene 1 will only be expressed in cells that express TF A, as this is the only gene present in the cluster that contains the TF A binding site. Similarly, gene 2 will only be expressed in cells that express TF B and so on.

b. The genes 1 - 4 are under the co - ordinate regulation of an LCR residing 5' - to the cluster. The LCR is a site at which specific TFs bind. Looping of the DNA can bring a proximal promoter element of one of the 4 genes into contact with the TF - bound LCR and thus cause activation of that gene from the cluster. (Adapted from Grant and Wisden, 1997).

Other mechanisms such as chromatin structure and differential methylation of CpG sites are also important in regulating differential gene expression (Bird and Tweedie, 1995; Grosschedl, 1995; McKnight and Schibler, 1993; Paranjape *et al.*, 1994).

While the regulatory regions of a number of neuronal genes have been identified, the transcription factors and the associated proteins which act on them remain largely unknown. In fact, there are likely to be thousands of transcription factors needed for regulating gene expression in the brain (He and Rosenfeld, 1991; Twyman and Jones, 1995).

1.3 $GABA_A$ RECEPTOR δ SUBUNIT GENE

1.3.1 Gene structure

The cDNA encoding the $GABA_A$ receptor δ subunit gene was isolated by screening a rat cDNA library with a degenerate oligonucleotide probe designed to a conserved region in the second transmembrane domain of $GABA_A$ receptors (Shivers *et al.*, 1989). It was shown to belong to the $GABA_A$ receptor family by its structural similarity to other members. The cDNA encodes a 450 residue peptide whose primary sequence predicts an N - terminal signal peptide, a disulphide - bonded loop, a β - structural loop, two adjacent N - linked glycosylation sites in the putative extracellular domain and four transmembrane segments (**Figure 1.8**).

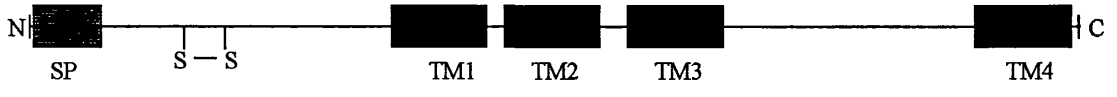


Figure 1.8 Primary structure of the $GABA_A$ receptor δ subunit gene

The figure shows the main structural features found in all $GABA_A$ receptors. Abbreviations used : SP = signal peptide, TM = transmembrane domains, S-S = disulphide bonded loop, N = N - terminal, C = C - terminal.

There is a high degree of sequence conservation within the transmembrane regions between the δ subunit, other $GABA_A$ receptors and the glycine receptor. There is a particularly low sequence conservation in the putative intracellular loop (between TM3 and 4) of all subunits.

Shivers *et al.* (1989) looked at the functional expression of the δ subunit. The δ subunit, when transfected into human embryonic kidney 293 cells, was shown to co - assemble and form homomeric GABA - gated chloride channels, which were bicuculline and picrotoxin sensitive, but showed no BZ - mediated potentiation. Thus, the δ subunit alone, is unable to form native receptors but is a constituent of heteromeric $GABA_A$ receptors.

The murine $GABA_A$ receptor δ subunit gene was isolated from two mouse genomic libraries, and its structure determined (Sommer *et al.*, 1990; **Figure 1.9**). The gene is contained within 13 kb of genomic DNA, and has 9 exons. The largest intron (8kb) is found between exons 1 and 2, while the smallest (70bp) lies between exons 3 and 4. An intron interrupts the coding sequence of the second transmembrane domain (TM2).

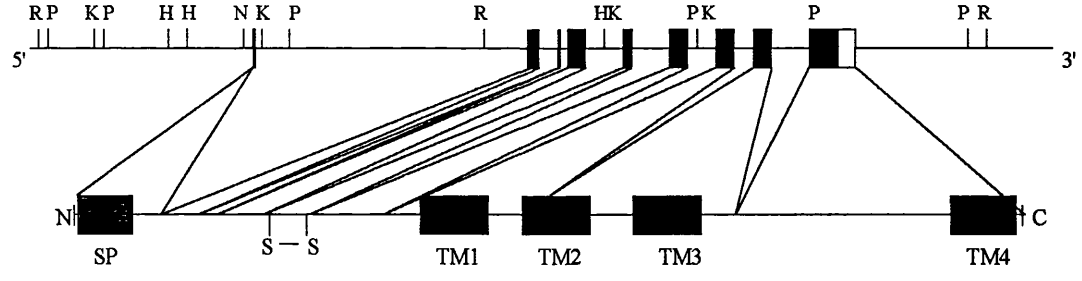


Figure 1.9 Structure of the murine $GABA_A$ receptor δ subunit gene

Exon assignment (upper line, 5' - 3') to the coding region (lower line, N - C) of the δ subunit cDNA. Abbreviations used : N = N - terminal, C = C - terminal, TM = transmembrane domain, S - S = disulphide bonded loop. Restriction enzymes : R = *Eco* RI, P = *Pst* I, H = *Hin*d III, K = *Kpn* I, N = *Not* I. (Adapted from Sommer *et al.*, 1990).

The transcription initiation region was mapped to a region -131 to - 95 (where +1 is the position of translational initiation), by PCR analysis of the 5' - flanking region using murine cDNA as a template (Sommer *et al.*, 1990). Sequence analysis of 800 bp of the immediate 5' - upstream region was performed. This revealed that the δ promoter region lacks TATA and CCAAT boxes, and is rich in CpG dinucleotides. Although CpG islands (Bird, 1987) and promoters lacking TATA sequences are typical for so called "housekeeping" genes, they have been shown to be present in genes that are expressed in a tissue - specific manner as with the δ subunit gene. Putative binding sites for the Sp1 and AP-4 transcription factors were also demonstrated. Other mammalian $GABA_A$ receptor subunit gene promoters identified to date, including the human β3 (Kirkness and Fraser, 1993) and the human α5 (Kim *et al.*, 1997) have also been shown to lack TATA and CCAAT box sequences and to display a high CpG content and Py - rich initiator elements.

Partial gene characterisation of the rat $GABA_A$ receptor δ subunit gene was performed (Motejlek *et al.*, 1994). The positions of the exons were found to be well conserved with the exon positions of the murine δ subunit gene (Sommer *et al.*, 1990). The rat δ promoter region was also analysed and showed similarities to the murine gene promoter region, i.e. it lacks TATA and CCAAT boxes, is CpG rich and contains putative binding sites for the Sp1 and AP-4 transcription factors. The transcription initiation sites were mapped by RNase protection and primer extension. There appears to be a cluster of start sites over a 30 bp region about 78 - 107 bp upstream from the translational start site. This coincides with the area of transcriptional initiation identified for the murine $GABA_A$ receptor δ subunit gene (Sommer *et al.*, 1990).

The 5' - flanking region of the rat δ subunit gene shows two extended purine nucleotide stretches, one of which includes binding sites for a novel brain - specific factor, BSF1 (Motejlek *et al.*, 1994). BSF1 was identified by bandshift assays using nuclear extracts from brain and liver, as a control. The binding site is represented seven times as tandemly repeated and partially overlapping 22 bp elements. A consensus sequence for the purine element is shown in **Figure 1.10**.

GAGAGGGGAGAGGGGGGAGGGG

Figure 1.10 Consensus sequence for the binding site of BSF1 in the rat $GABA_A$ receptor δ subunit gene promoter

Consensus sequence for the purine 22 bp repeat element identified in the 5' - flanking region of the rat $GABA_A$ receptor δ subunit gene.

These repeat elements appear to be distantly related to several purine elements present in the promoter region of other genes e.g the Purkinje cell - specific Pcp-2 gene and the glial fibrillary acidic protein (Vandaele *et al.*, 1991; Miura *et al.*, 1990).

1.3.2 δ expression patterns

As previously mentioned, the expression pattern of 13 $GABA_A$ receptor subunit genes was determined in adult rat brain by *in situ* hybridisation (Wisden *et al.*, 1992). **Table 1.2** lists the distribution of the δ mRNA in the CNS. As can be seen, the δ subunit is expressed fairly selectively, and is most abundant in cerebellar granule cells, dentate gyrus and in the neurones of some thalamic nuclei.

Table 1.2 Distribution of δ mRNA in CNS

Tissue	δ mRNA
Olfactory bulb	+
Neocortex	+
Hippocampus	++
Basal nuclei	+
Amygdala	0
Septum	0
Thalamus	++
Hypothalamus	0

Tissue	δ mRNA
Midbrain	0
Inferior colliculi	0
Substantia nigra	0
Cerebellum	+++

Summary of *in situ* hybridisation signals obtained with ^{35}S - labelled oligonucleotide probes on serial sections. +++ = intense, ++ = strongly positive, + = positive, 0 = not detectable.

Laurie *et al.* (1992b) investigated the developmental expression patterns of various $GABA_A$ receptor subunit genes as described in **Section 1.1.5.2**. Each subunit transcript showed specific temporal expression patterns, and in many neurons, showed specific regional expression patterns. The δ mRNA appeared postnatally and increased in expression with age. In the cortex, the δ mRNA appeared around P0 and peaked in expression at P12, whereas in the dentate gyrus, it was detectable at P12 with a gradual increase to peak expression in the adult. In the postmigratory granule and periglomerular cells of the olfactory bulb the lateral thalamic nuclei and the cerebellar granule cells, the δ mRNA was detected around P6 and reached adult levels by P12.

1.3.3 Pharmacology

While $GABA_A$ receptor subtypes composed of α, β and γ subunits have been studied, relatively little is known about the electrophysiological and pharmacological properties of $GABA_A$ receptor subtypes containing the δ subunit. *In situ* hybridisation studies in rat brain suggest that the distribution of the δ subunit in the brain resembles that of the high affinity $GABA_A$ receptor sites labelled with [^3H] - muscimol and lacking BZ binding (Benke *et al.*, 1991; Laurie *et al.*, 1992a). Immunohistochemical mapping of $GABA_A$ receptor subtypes containing the δ subunit in the rat brain have shown a similar distribution pattern as seen for the δ subunit (Benke *et al.*, 1991). In the hippocampal dentate gyrus granule cell, δ subunit mRNA co - localises with α1, β1 and $\gamma 2_L$ subunit

mRNAs while in the cerebellum it co - localises with α1, α6, β and γ2 subunit mRNAs (Laurie *et al.*, 1992b).

The electrophysiological and pharmacological properties of functional recombinant $GABA_A$ receptors, expressed in L929 cells co - transfected with different combinations of α1, β1, $\gamma2_L$ and δ subunits, were examined (Saxena and MacDonald, 1994). $\alpha1\beta1\gamma2_L$ and $\alpha1\beta1\gamma2_L\delta$ subunit combinations were functionally expressed with high efficiency, while α1 β1δ showed low but more detectable levels of expression of functional $GABA_A$ receptors than $\alpha1\delta\gamma2_L$ and $\delta\beta1\gamma2_L$. The presence of the δ subunit slowed the rate of acute desensitisation of GABA - evoked current during GABA application, and the rate of recovery of GABA - evoked current following GABA application.

These 3 different $GABA_A$ receptor subtypes also showed distinct pharmacological profiles with differential sensitivity to block by zinc. Zinc was found to be a potent blocker of α1β 1δ $GABA_A$ receptor channels, a moderate blocker of $\alpha1\beta1\gamma2_L\delta$ $GABA_A$ receptor channels, and did not block the $\alpha1\beta1\gamma2_L$ $GABA_A$ receptor channels. This suggests that the presence of the δ subunit creates a site to which Zn^{2+} can bind, despite the presence of the γ subunit, which has been shown previously to render αβγ $GABA_A$ receptor complexes insensitive to Zn^{2+} (Draguhn *et al.*, 1990). Thus the δ subunit could cause a change in the structure of the channel, which is consistent with the finding that the open channel duration of $\alpha1\beta1\gamma2_L\delta$ subtypes is much longer than that of the $\alpha1\beta1\gamma2_L$ subtypes.

1.3.4 Chromosomal assignment of human $GABA_A$ receptor δ subunit gene

The $GABA_A$ receptor δ subunit gene has been localised to the short arm of human chromosome 1 (Sommer *et al.*, 1990), by screening human - rat somatic cell hybrid cell lines. This demonstrates that the δ gene does not form part of a cluster which has been seen for some of the other $GABA_A$ receptor subunits (**Chapter 9**).

1.3.5 Recent research developments

Jones *et al.* (1997) have produced an α6 knockout mouse that appears to affect the production of the δ subunit protein in cerebellar granule cells. They disrupted exon 8 of the mouse α6 subunit gene by homologous recombination, and investigated the levels of other subunits in α6 -/- cerebellar granule cells by immunoprecipitation, immunocytochemistry and immunoblot analysis. The δ subunit was the only one affected. While the δ subunit mRNA was shown to be present at wild - type levels in the mutant granule cells, the protein appeared to be absent. As the knockout would result in a truncated α6 subunit protein being produced, it is possible that .this sequesters the δ subunit protein in some way. Whether this binding would happen with the endogenous α6 protein is unknown. Thus, Jones *et al.* (1997) have demonstrated a potential association between the α6 and δ subunits in cerebellar granule cells. It should also be noted that these mice showed no abnormalities in motor behaviour.

Another group, Mihalek *et al.* (1997) have produced a targeted disruption of the δ subunit gene. They have inactivated the gene by disrupting exon 8, in a similar manner to that described by Jones *et al.* (1997) for α6 gene disruption. They performed Western blot analysis to confirm the absence of the δ protein. After further studies, they concluded that the mutant mice were viable, fertile and healthy and that the δ subunit was not required for normal brain development.

1.4 RESEARCH PROJECT

The long - term goal of this project was the development of a safe HSV - 1 gene therapy vector for neuron - specific delivery. A second goal was to add to the present limited knowledge of neuronal promoter structure. Thus the two main requirements were the production of an HSV - 1 viral vector that could deliver DNA to neurons with minimal amount of cell killing, and the characterisation and incorporation of a neuron - specific promoter which could be used to drive cell - type restricted expression in neurons. The $GABA_A$ receptor δ subunit gene promoter was selected as a protoype neuronal promoter as it has a well characterised restricted cell - type specific expression pattern in the CNS.

The experimental goals necessary to achieve the above aims are described below. The characterisation of the 5' - upstream region of the $GABA_A$ receptor δ subunit gene involved identification of transcription star sites and the sequence characterisation of an upstream region large enough to be likely to encompass the majority of *cis* - acting regulatory elements. Experimental localisation of putative cis - acting regulatory regions was performed by deletion analysis in transfected neuronal cell lines. This involved the assembly of a series of promoter fragments linked to a marker gene (*lacZ*) and their subsequent transfection into NB4 1A3 cell cultures.

Using the knowledge gained about regulatory elements from the localisation studies, *in vivo* mutation analysis could be performed. As an initial experiment preparatory *to in vivo* analysis, a simple gene knockout experiment to create a null mutation of the $GABA_A$ receptor δ subunit gene was performed using a basic replacement vector system.

The development of an HSV - 1 vector system involved the incorporation of the δ promoter - marker gene constructs into HSV - 1 vectors. Testing of the δ - *lacZ* HSV - 1 vectors for β - galactosidase activity in primary cell culture and *in vivo* was performed.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Bacterial growth

2.1.1.1 Bacterial strains

Table 2.1 Escherichia coli strain information

Strain	Description	Genotype	Reference
TG-1	a restriction and recombination - deficient host for plasmids	*supE*, *hsd*5Δ, *thi*, Δ(*lac-proAB*), F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Gibson,1984
TG-2	a recombination - deficient derivative of TG-1	*supE*, *hsd*5Δ, *thi*, Δ(*lac-proAB*), Δ(*srl-recA*) 306::Tn*10*(*tet*^{r}), F'[*traD*36, *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]	Sambrook, *et al.*, 1989
DH5α	a recombination - deficient host for pBluescript plasmids	*supE*44, Δ*lacU*169(ϕ 80, *lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1	Hanahan, 1983
TOP10F'	recommended host strain for plasmid pZErO-2.1 ™	F'[*lacI*^{q}, Tn*10*(*tet*^{r})], *mcrA*, Δ(*mrr-hsdRMS*-mcrBC), ϕ 80, *lacZ*ΔM15, Δ*lacX*74, *deoR*, *recA*1, *araD*139, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*, (*str*^{r}), *endA*1, *nupG*	Grant, 1990 Invitrogen
INV αF'	does not express the *lacI* repressor	F' *endA*1, *recA*1, *hsdR*17(r_k^-, m_k+), *supE*44, *thi*-1, *gyrA*96, *relA*1, ϕ 80, *lacZ*ΔM15, Δ(*lacZYA-argF*), *U*169, λ$^-$	Grant, 1990 Invitrogen

Bacterial strains used in the work are indicated by name, description, genotype and are referenced. Media for growth of bacteria were made as in Sambrook *et al.* (1989).

2.1.1.2 Bacterial media requirements

For all bacterial strains, either L Broth (16 g/l bactotryptone; 10 g/l bacto-yeast extract; 5 g/l NaCl) or 2 x YT (10 g/l bactotryptone; 5 g/l bacto-yeast extract; 10 g/l NaCl) were used. These were obtained from the Molecular Genetics prep. room. The antibiotics

required by the plasmids were : Ampicillin (100 μg/ml), Kanamycin (50 μg /ml) or Tetracyclin (50 μg /ml).

2.1.2 Restriction and modifying enzymes

All restriction endonucleases and modifying enzymes were obtained from the following commercial sources: Gibco BRL, Promega, New England Biolabs, Boehringer Mannheim, Pharmacia, and Cambio. Restriction endonucleases were used according to the manufacturers' instructions or were buffered in One For All (Pharmacia). Modifying enzymes were used according to the manufacturers' instructions.

2.1.3 Biochemicals and Chemicals

All biochemical, chemical and molecular biological reagents used were of Analar or equivalent grade and were obtained from the following commercial sources: BDH, Sigma, Pharmacia, Fisons, Fischer and Gibco BRL.

2.1.4 Molecular size standards

DNA Standards (sizes in base pairs)

1Kb Ladder	12216 11198 10180 9162 8144 7126 6108 5090 4072
(Gibco BRL)	3054 2036 1636 1018 506/517 396 344 298 220
	201 154 134 75
100bp ladder	2072 1500 1400 1300 1200 1100 1000 900 800 700
(Gibco BRL)	600 500 400 300 200 100

2.1.5 Radioactive deoxyribonucleotides

Radioactively - labelled deoxyribonucleotides [α-32P]dCTP (3000Ci/mmol), [γ-32P]ddATP (3000Ci/mmol) and [35S]dATP (1250Ci/mmol) were supplied by DuPont NEN™

2.1.6. Large scale DNA purification

Large scale preparation of DNA plasmids was achieved using Qiagen DNA purification kits supplied by Qiagen. DNA purification from agarose gels was achieved using a Qiaex

kit supplied by Qiagen. RNAse A (used in the small scale preparation of DNA) was supplied by Sigma.

2.1.7 Vectors

Table 2.2 Plasmid vectors

Name	Description	Use	Reference/ Company
pBluescript II SK/KS(+)	Phagemids derived from pUC19. Differ only in orientation of mcs. Amp^r.	Cloning and sequencing	Messing,1983; Yanish-Perron *et al.*, 1985. Stratagene
pGL3-Basic	Modified from pGL2-basic vector for increased expression. Contains firefly luciferase gene, also mcs. Amp^r	Construction of series of promoter - reporter plasmids.	Groskreutz *et al.*, 1995. Promega
pGL3-Control	As above only contains SV40 promoter and enhancer sequences upstream of the luc. gene. Amp^r	Control plasmid for promoter constructs	Groskreutz *et al.*, 1995. Promega
pNSSA-β	Promoter -less vector, contains β -galactosidase gene Amp^r	Source of *lacZ* gene for promoter constructs	MacGregor and Caskey, 1989 Clontech
pZErO™-2.1	Contains the lethal E. coli gene, ccdB, fused to *lacZα*. Insertion of DNA disrupts *lacZα* -ccdB gene fusion permitting growth only of positive recombinants. Kan^r	Inserted HSV - 1 flanking sequences to enable production of recombinant HSV-1 viruses.	Bernard *et al.*, 1994 Invitrogen
pLitmus 29	Plasmid cloning vector, contains ColE1 and M13 origins, contains mcs. Amp^r	Cloning vector for constructs	Evans, *et al.*, 1995 NEB

Name	Description	Use	Reference/ Company
pSCC9	Contains neo^r gene driven by tk promoter, 2 tk genes driven by own promoters. Amp^r	Gene Targeting	Chauhan and Gottesman, 1992
pNT	Contains neo^r gene driven by PGK promoter, tk gene driven by PGK promoter. Amp^r	Gene Targeting	W. Skarnes (personal comm.)
pCR2.1	Has single 3' deoxythymidine(T) residue. Contains mcs. Amp^r and Kan^r.	For cloning PCR products	Clark, 1988; Mead *et al.*, 1991 Invitrogen
p35(PacI)	pGem vector backbone. Contains HSV-1 flanking sequences (91610-96751). Amp^r	For recombination into UL43 region of mutant HSV-1.	MacLean *et al.*, 1991a
pBL(PacI)	pGem vector backbone. Contains HSV-1 flanking sequences (118,774-119,675 and 121327-122023). Amp^r	For recombination into LAT region of mutant HSV-1.	Coffin *et al.*, 1996

Vector name, description, use and source are listed.

Abbreviations used are : mcs = multiple cloning site, *lacZ* = β-galactosidase gene, Amp^r = ampicillin resistance gene, Kan^r = kanamycin resistance gene, tk = thymidine kinase, neo^r = neomycin resistance, PGK = phosphoglycerate kinase.

2.1.8 Oligonucleotide primers

All oligonucleotide linkers (**Table 2.3**), PCR primers (**Table 2.4**) and sequencing primers (**Table 2.5**) were synthesized by Cruachem or Gibco BRL.

Table 2.3 Oligonucleotide primers for linkers

Primer Name	Oligonucleotide Sequence	Use	Source
BRNK1	5'-CTAGCTGGACCTGTCCCGCGCAGGTAACCG GTAC-3' [S]	Construct Design	BRL
BRNK2	5'-CGGTTACCTGCGCGGGACAGGTCCAG-3' [A]	Construct Design	BRL
BRKPK	5'-CTTAATTAAGGTAC-3'	Construct Design	BRL
BROBPB	5'-GATCTTCCCTTAATTAGGGAA-3'	Construct Design	BRL
TAG117/8	5'-GATCTCCTTAATTAAGGA-3'	Construct Design	BRL
TAG138	5'-CTTAATTAACTCGAGTCTAGAGAATTCAAGC TTGGATCCGCGATAAGAATGCGGCCGCGGT GACCTGTAC-3'	Construct Design	BRL
TAG139	5'-AGGTCACCGCGGCCGCATTCTTATCGCGGAT CCAAGCTTGAATTCTCTAGACTCGAGTTAAT TAAGGTAC-3'	Construct Design	BRL
TAG 119	5'-CTCCCCCGGGGGAATAAGAATGCGGCCGC TAAACTATGCTAGCTAGGAGCT-3'	Construct Design	BRL
TAG120	5'-CCTAGCTAGCATAGTTTAGCGGCCGCATTCT TATTCCCCCGGGGGAGGTA-3'	Construct Design	BRL
TAG126	5'-CCCCAAGCTTCCC-3'	Construct Design	BRL
TAG127	5'-GGGAAGCTTGGGGGTAC-3'	Construct Design	BRL
BRXSE1	5'-TCGAGGACTAGTCGG-3' [S]	Construct Design	BRL
BRXSE2	5'-AATTCCGACTAGTCC-3' [S]	Construct Design	BRL
NSF	5'-TTTAATTAAACTAGTCTCGAGAGATCTTTAA TTAAATGCA-3'	Shuttle Vector	In House
NSB	5'-AAATTAATTTCTAGAGAGCTCTGATCAAATT AATTTACGT-3'	Shuttle Vector	In House
RNA Oligo	5'-GGAUCCUAAACAAUUAACCCUCAAA-3' [S]	Oligo-capping	Cruachem

Oligonucleotide name, sequence, procedural use and source are indicated. All probes are written 5' to 3'. Primers BRNK1/2, TAG138/139, TAG119/120, TAG126/127 and BRXSE1/2 are paired. Abbreviations used are: [S] = sense and [A] = antisense.

Table 2.4 Oligonucleotide primers for PCR amplification

Primer Name	Oligonucleotide Sequence	Use	Source
TAG135	5'-GAGACGGATCCTAAACAATTAACCCTCAAA-3' [S]	Oligo capping	Cruachem
BRδ01	5'-CCAGGATATTTCCAGGTTGGAGCCCACG-3' [A]	Oligo capping	Cruachem
BR006	5'-CATGGTGCGGCTGCGTGCAGAGCAGGAGCGCA GCAG-3' [A]	Oligo capping	BRL
BROCI	5'-CTGAGATATGGTCAATGCTGGCCACCTC-3' [A]	Oligo capping	BRL
BROCII	5'-CACGTAGTCCCCAATGTCATTCATTGCC-3' [A]	Oligo capping	BRL
BRBSF3	5'-TATGGGTCTGTCTTTACCTC-3' [S]	Rat mapping	BRL
BRBSF4	5'-CAGAGCAAGAATAGGAGTG-3' [A]	Rat mapping	BRL
D5mgh9	5'-GTGGCAGCAGCACACAAG-3' [S]	Rat mapping	BRL
D5Mgh9	5'-GGCCAAGCTGGAGAATTACA-3' [A]	Rat mapping	BRL
D5Mit7	5'-CCCCCACTGTTTTTGTCTGT-3' [S]	Rat mapping	Research genetics
D5Mit7	5'-TCTGTTATGGGATCTGATGCC-3' [A]	Rat mapping	Research genetics
D5Mit9	5'- CTACTGGCCGTAGTGTTTGC-3' [S]	Rat mapping	Research genetics
D5Mit9	5'-CCACTGTGGTTGCTGTTCAG-3' [A]	Rat mapping	Research genetics

Oligonucleotide name, sequence, procedural use and source are indicated. All listed probes are written 5' to 3'. TAG135 was paired with BRδ01 and BR006 (which are nested primers), and BROCI and BROCII (nested primers). BRORAT1 and BRORAT2 are paired primers. Abbreviations used are: [S] = sense and [A] = antisense.

Table 2.5 Oligonucleotide primers for sequencing

Primer Name	Oligonucleotide Sequence	Use	Source
M13 Forward	5'-TGTAAAACGACGGCCAGT-3'	Sequencing	BRL
M13 Reverse	5'-AGCGGATAACAATTTCACACAGGA-3'	Sequencing	BRL
BRO13FI	5'-GAGATAGCCAATAATAG-3' [S]	Sequencing	Cruachem
BRO13RI	5'-CAACTTCCCTCATAAGA-3' [A]	Sequencing	Cruachem
BRO13F2	5'-GAGATAGTCAATAATAGT-3' [S]	Sequencing	BRL
BRO13R2	5'-CACCTTCCCTCATAAGAG-3' [A]	Sequencing	BRL
BRO16F2	5'-GAGAAGATCAGAAGAAAGCT-3' [S]	Sequencing	BRL
BRO17F2	5'-TACAGTGTTCTTGTACTA-3' [A]	Sequencing	BRL
BRO16F3	5'-GGGTGTTCTTTAAGTATTTG-3' [S]	Sequencing	BRL
BRO17F3	5'-GTATGGGTTAGGGGACCTGC-3' [A]	Sequencing	BRL
BRO17F4	5'-CCTGGTCCCACTGTTCTCAC-3' [A]	Sequencing	BRL
BRO18F2	5'-GCATGGATAACAAAGCCC-3' [A]	Sequencing	BRL
BR018F3	5'-GGGAAATGCACTAAATACA-3' [A]	Sequencing	BRL
BRO18F4	5'-CTAACCAAAGAGCTGTCAACCA-3' [A]	Sequencing	BRL
BR018F5	5'-TATGTGTGCTAATGTCTAAC-3' [A]	Sequencing	BRL
BRO18F6a	5'-GAGTACTTCATACTCATCGT-3' [A]	Sequencing	BRL
BR018F7	5'-ACAGGTGACTGCAGAGCT-3' [A]	Sequencing	BRL
BRO18F7a	5'-GGAACGACTGCTTAATTT-3' [A]	Sequencing	BRL
BRO18F7b	5'-CAAACTTCTACAGGCCCA-3' [A]	Sequencing	BRL
BRO18F8	5'-CAGGATGGCCTCGAACTCAG-3' [A]	Sequencing	BRL
BRO18RI	5'-TCATTTCTCTGATGAAATAC-3' [S]	Sequencing	BRL
BRO18RIa	5'-AAACCTGCAGGCCAGTCATG-3' [S]	Sequencing	BRL
BRO18R2	5'-ACTCTATCTGAAAATCACAAGA-3' [S]	Sequencing	BRL
BRO18R3	5'-GTATAGGATCTCCTTAGCGC-3' [S]	Sequencing	BRL
BRO18R4	5'-TGGCTAGCAAACCCTCACTG-3' [S]	Sequencing	BRL
BRO18R5a	5'-CAGGACTTAAGCCCATGGGT-3' [S]	Sequencing	BRL
BRO18X1	5'-CCACGGACTCTGGGCCTGTA-3' [S]	Sequencing	BRL
BRO18X2	5'-GGATGGTGCAGCCACTTTGG-3' [S]	Sequencing	BRL
BRO18X3a	5'-TGTGGATGCATGCAGCCA -3' [S]	Sequencing	BRL
BRO18X3b	5'-AGCTCTGCAGTCACCTGT -3' [S]	Sequencing	BRL
BRO30F1	5'-CACAGCAGTGTTTAGCATCC-3' [A]	Sequencing	BRL
BRO30R1	5'-CTCTTTCACGTTGCCTCA-3' [S]	Sequencing	BRL

Primer Name	Oligonucleotide Sequence	Use	Source
BRO31F1	5'-CATGCTTGCCTGGATGCTGC-3' [S]	Sequencing	BRL
BRO31R1	5'-GACTCAGGTTACCTGCAGAG-3' [A]	Sequencing	BRL
BRO30F2a	5'- GTCGTGGGATAGAACTAGGT-3' [A]	Sequencing	BRL
BRO30R2	5'-GGAATCAAGAACCAAAGGAT-3' [S]	Sequencing	BRL
BRO30R2a	5'-TATCTCAATATGCCAGTTGG-3' [S]	Sequencing	BRL
BRO31F2	5'-CCTGGGAAGCTGACTGTT-3' [S]	Sequencing	BRL
BRO31R2	5'-ATAGGAGGAAAGTAACAG-3' [A]	Sequencing	BRL
BR030F3	5'-GAAATCCTTTGGTTCTTGAT-3' [A]	Sequencing	BRL
BRO30R3	5'-GATGACTGCAGGGGATTTGG-3' [S]	Sequencing	BRL
BRO31F3	5'-GCCAGCCTGGTCTACAAGTG-3' [S]	Sequencing	BRL
BRO31R3	5'-ACGTATGTACGTACAGGCAA-3' [A]	Sequencing	BRL
BRO31F3a	5'-TATCCCAGCACTTAGGAGGC-3' [S]	Sequencing	BRL
BRO30F4	5'-TACTATAGTGGGGATTATAC-3' [A]	Sequencing	BRL
BRO30R4	5'-TGTGCGGTGGGCACTCACTG-3' [S]	Sequencing	BRL
BRO31R4	5'-GCACCAGGCATGCATGTTAG-3' [A]	Sequencing	BRL
BRO31R4b	5'-AGAAGAAGGCATCAGATCTCC-3' [A]	Sequencing	BRL
BRO31F5	5'-AAGATGGTATCAGATCGCCT-3' [S]	Sequencing	BRL
BRO31R5	5'-CGCCACCATGCCCAGCACAC-3' [A]	Sequencing	BRL
BRO31F6	5'-CTTTCCTCCTATCTGGAACA-3' [S]	Sequencing	BRL
BRO31R6	5'-TGTTCTCCTCGGTGTTTTCG-3' [A]	Sequencing	BRL
BRO31F7	5'-CACAAGGATGAGTCAGAGAA-3' [S]	Sequencing	BRL
BRO31R7	5'-CGATGAAAGTTCTACATCTT-3' [A]	Sequencing	BRL

Oligonucleotide name, sequence, procedural use and source are indicated. All listed probes are written 5' to 3'. Abbreviations used are: [S] = sense and [A] = antisense.

2.1.9 DNA sequencing

DNA sequencing was performed using an ABI 373 Stretch automated sequencer (Applied Biosystems). Sequencing reactions were performed on a Perkin Elmer 9600 PCR machine using ABI Terminator Ready Reaction Mix (A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dITP, dATP, dCTP, dTTP, Tris -Hcl (pH 9.0), $MgCl_2$, Thermal stable pyrophosphatase and AmpliTaq DNA Polymerase, FS).

Acrylamide/bis/urea gel mix (6%) was supplied by Anachem. Ammonium persulphate was supplied by IBI and Temed was supplied by Sigma.

2.1.10 RNA isolation

2.1.10.1 Total RNA isolation

Total RNA was isolated from a variety of fresh and frozen tissues using Tri Reagent™ (Sigma). For the preparation of RNA and mRNA, all solutions (except those containing Tris) and plasticware (e.g. Eppendorf tubes, tips, polypropylene tubes) were treated with a solution of 0.1% DEPC (diethylpyrocarbonate) overnight and then autoclaved to destroy the DEPC. Gloves were worn at all times to protect against residual RNases.

2.1.10.2 mRNA isolation

mRNA was purified from total RNA using oligo - dT cellulose supplied by Sigma. The following solutions were required :

Binding buffer	10 mM Hepes, 1 mM EDTA, 0.1% SDS, 0.4 M NaCl	pH 7.4
Elution buffer	10 mM Hepes, 1 mM EDTA, 0.1% SDS	pH 7.4
Wash buffer	10 mM Hepes, 1 mM EDTA, 0.1% SDS, 0.1 M NaCl	pH 7.4

2.1.11 Cell culture

2.1.11.1 Cell lines

Table 2.6 Cell lines

Cell line	Description	Use	Reference
BHK21/C13	Baby hamster kidney cells	HSV-1 growth and manipulation	MacPherson and Stoker,1962
NB4 1A3	Mouse C-1300 Neuroblastoma	*In vitro* analysis of promoter constructs	Buonassisi *et al.*, 1962
CGR8.8	Embryonic Stem Cell	Gene targeting	W. Skarnes, Personal Communication

Cell lines used in this work are indicated by name and description. There experimental use is also noted and they are referenced.

All the cell lines were grown in a humidified atmosphere at 37°C with 5% CO_2, unless otherwise stated.

2.1.11.2 Medium for cell lines

Unless otherwise stated, all medium and components were supplied by Gibco BRL.

2.1.11.2.1 BHK21/C13

ETC_{10} medium (Glasgow modified eagle's medium, 10% newborn calf serum, 100 u/ml Penicillin/100 µg/ml Streptomycin, 10% Tryptose phosphate broth) was used for all cell culture work involving BHK21/C13 cells.

$ETMC_{10}$ medium (Glasgow modified eagle's medium, 10% newborn calf serum, 100 u/ml Penicillin/100 µg/ml Streptomycin, 10% Tryptose phosphate broth, 1.5% carboxylmethyl cellulose sodium salt) was used as a semi - solid overlay to prevent virus spreading through the plate.

For the viral variant 1764 to plaque N,N - hexamethylene bis - acetamide (HMBA) was essential. It was added to the medium at a concentration of 3 mM.

2.1.11.2.2 NB4 1A3

Nut Mix F-10 with glutamax (2 mM), 15% horse serum, 5% calf serum, 100 u/ml Penicillin/100 µg/ml Streptomycin.

2.1.11.2.3 CGR8.8

1 x Glasgow medium, 0.2475% sodium bicarbonate, 1 x non-essential amino acids, 10 mM sodium pyruvate, 10 x Glutamax, 1 mM β-mercaptoethanol (Sigma), 10% heat-inactivated foetal calf serum (Sigma), 44 µls of LIF (10^7 stock), 100 u/ml Penicillin/100 µg/ml Streptomycin.

For selective medium, the following was added : 0.02% G418 (made as a 20 mg/ml stock and filter sterilised, Boehringer-Mannheim) and 2 x 10^6 M gancyclovir (Syntex).

2.1.11.3 Solutions for gene targeting

2.1.11.3.1 Trypsin solution

The trypsin solution used for passing the cells contained : 0.25% trypsin (Difco), 372 mgs EDTA, 1% chicken serum (Flow labs). This was filter-sterilised, aliquoted and stored at -20°C.

A 0.1% solution of gelatin (Sigma) was used to coat the tissue culture plates .

2.1.11.3.2 Freezing medium

DMSO (10%) in the standard medium was used to freeze down cells for storage.

2.1.11.4 Solutions for cell transfections

The transfection experiments were carried out in Opitem 1 with glutamax-1 (Gibco BRL), which is a serum free medium.

The transfection reagent used was DOTAP (Boehringer Mannheim).

The DNA for transfections was prepared using Qiagen tips (**Section 2.2.1.3.2**).

The DNA and DOTAP were diluted to working concentrations in 20 mM HEPES buffer (pH 7.4) - filter sterilised.

2.1.11.5 β - galactosidase assays

Cell extracts were assayed using the Luminescent β - galactosidase Genetic Reporter System II detection kit from Clontech.

Cells lysis buffer 100 mM potassium phosphate (pH 7.8)
0.2% Triton - X - 100
1 mM DTT

2.1.11.6 Luciferase assays

Cell extracts were assayed using the Luciferase Assay System detection kit from Promega.

The β - galactosidase and luciferase reactions were measured with a Turner TD-20e luminometer.

2.1.12 Cerebellar granule cell cultures

2.1.12.1 Medium for granule cells

EBSS (Earle's balanced salt solution, minus Ca^{2+}, minus Mg^{2+}) (Gibco BRL)

BME (Eagle;s basal medium, plus 200 mM L-Glutamine), 10% fetal calf serum (heat inactivated), 25 mM KCl, 100 u/ml Penicillin/100 μg/ml Streptomycin, 50 μg/ml Gentamicin.

2.1.12.2 Solutions for granule cell cultures

The following stock solutions were prepared in EBSS (w/out Ca^{2+} and Mg^{2+}) and filter - sterilised. Enzymes were prepared fresh on day of cell preparation.

Trypsin	100 mg/20 ml
Soyabean trypsin inhibitor	26.6 mg/4 ml
Dnase I	3.52 mg/4 ml (This should be dissolved in 3.56 ml of EBSS and 0.44 ml of 1 M $MgCl_2$ and filter - sterilised. Final concentration of $MgCl_2$ is 5 MM after dilution with enzymes and cells).
Cytosine-β-arabinofuranoside	100 mM
Poly-D-lysine (Sigma)	100 μg/ml (Tissue culture flasks and trays were treated with a 10 μg/ml solution, left at room temperature for 15 min, washed once with sterile water and allowed to dry).

2.1.12.3 Solutions for granule cell transfections

Medium	BME (**Section 2.1.12.1**)
Glutamate receptor inhibitor (GRI)	1 mM Kynurenic acid, 10 mM $MgCl_2$, 5 mM Hepes (pH 7.5) Filter sterilise
Calcium chloride	250 mM
HBS	137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 15 mM D-glucose, 42 mM Hepes, (pH 7.07)
Glycerol	5 %
DMSO	2 %

2.1.12.4 Solutions for granule cells - histochemistry

Wash solution	TBS (20 mM Tris (pH 7.4), 150 mM NaCl, 0.004 % SDS)
Fix solution	0.2 % gluteraldehyde, 20 mM Tris (pH 7.4), 150 mM NaCl
X - gal stain	0.2 M Sodium Phosphate buffer, 1 mM MgCl2, 0.04 % SDS, 0.3 % X - gal (in DMF), 0.005 M potassium ferrocyanide, 0.005 % potassium ferricyanide

2.1.13 Histochemistry of rat brains by X - gal staining

Wash Buffer is used as the basis for most of the solutions.

1 x Wash Buffer	0.1 M disodium hydrogen phosphate (anhydrous), 0.1 M sodium dihydrogen phosphate.1 H_2O, 0.02 M $MgCl_2$.
Fix solution	0.2% gluteraldehyde, 0.005 M EGTA (pH 8.0) in 1 x Wash Buffer. This should be stored at 4°C.
X - gal stain	0.005 M potassium ferrocyanide, 0.005 M potassium ferricyanide, 0.1% X - gal (in DMF) in 1 x Wash Buffer. This should be filtered (0.3 μm) and stored in foil at 4°C.
Neutral red	0.05 M sodium phosphate buffer (pH 3.3) with 1% (w/v) neutral red dye.

TESPA solution is used to coat the slides used for sectioning the brains. It contains acetone with 2% TESPA (Sigma).

Mounting of Slides Histoclear (National Diagnostics) and DPX (BDH) were used to mount coverslips on the sectioned slides.

2.2 METHODS

2.2.1 Basic molecular biology techniques

Standard molecular biology techniques such as bacterial growth, restriction enzyme digestion, small scale plasmid DNA isolation, ligation, transformation, phenol extraction, ethanol precipitation, agarose gel electrophoresis, radioactive labelling of probes and Southern blotting were performed as modifications of those described by Sambrook *et al.* (1989).

2.2.1.1 Bacterial growth

2.2.1.1.1 Bacterial transformation

Overnight bacterial cultures were set up in glass universals, by innoculating 5 ml of medium (either L Broth or 2 x YT medium) with a single bacterial colony and then incubating in a 37°C shaker for 16 hours. 0.5 ml of this culture was then used to innoculate 50 ml of fresh medium and this was incubated at 37°C, with shaking, until the culture OD reached 0.4 - 0.6. The bacterial cells were harvested by centrifugation at 4000 r. p.m. for 10 min in a Beckman J2-21.

The cell pellet was gently resuspended in 2 ml Transformation Buffer [10 mM MES buffer (pH 6.5), 100 mM rubidium chloride, 45 mM manganese chloride, 10 mM Calcium chloride, 3 mM hexaminecobalt chloride] and centrifuged as before. The cell pellet was resuspended in 1 ml of Transformation Buffer. To this was added 68 µl dimethyl formamide and 34 µl β - mercaptoethanol. The cells were incubated on ice for 15 min, and 100 µl of cells were mixed with the DNA sample and incubated on ice for 30 min. After heat - shocking at 42°C for 90 sec, 800 µl of medium was added and the sample incubated at 37°C for 30 min. After centrifugation at 6000 r.p.m. for 5 sec, most of the supernatant was removed, the cell pellet was resuspended in the residual liquid and then plated out on L Broth plates containing the appropriate antibiotic.

2.2.1.1.2 Bacterial plasmid growth - large scale.

A flask containing 100 ml of the appropriate medium plus antibiotic was innoculated with a single bacterial culture and then incubated, with shaking, at 37°C overnight. The bacterial cells were harvested as described in **Section 2.2.1.1.1** above.

2.2.1.2 Restriction enzyme digestion

Digestion of DNA was performed in either One For All buffer, or in the buffer provided with the enzyme. Unless otherwise stated, all DNA digestion was performed at 37°C.

2.2.1.3 DNA isolation

2.2.1.3.1. Small scale plasmid DNA isolation

DNA was purified using a modified alkaline lysis method (Birnboim and Doly, 1979). 1.5 ml of bacterial culture was harvested in a microfuge at 14000 r.p.m. for 1 min. The supernatant was discarded and the cell pellet was resuspended in 200 µl of GTE (Glucose/Tris/EDTA), 300 µl of solution P2 (2M NaOH/0.1% SDS) was added and the sample mixed by inverting gently. 300 µl of P3 (3M K Acetate pH 4.8) was then added and the sample mixed by invertion until a white precipitate formed. After centrifugation at 14000 r.p.m, the supernatant was transferred to a new tube containing 200 µg RNAse A, and incubated at 37°C for 20 min. 500 µl of chloroform : isoamyl alcohol (24:1 v/v) was added and, after mixing, the sample was centrifuged at 14000 r.p.m. for 3 min.

The upper aqueous phase was taken into a new tube containing 500 µl of propan - 2 -ol, mixed and the DNA precipitated by centrifugation at 14000 r.p.m. for 10 min. The DNA pellet was washed in 250 µl of 70 % ethanol and then, after centrifugation for 3 min and removal of the ethanol, the pellet was dried at 37°C for 10 min. The DNA was resuspended in 30 µl of sterile water and stored at -20°C.

2.2.1.3.2. Large scale plasmid DNA isolation

Large scale plasmid DNA isolations were performed using Qiagen 100 tips or 500 tips as described in the manufacturers protocol manual.

2.2.1.4 Ligation

All ligations were performed in the same way. In general, 10 ng of vector DNA was used in a molar ratio of 1:3 with the insert DNA. The ligations were performed in a total volume of 10 µl with 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM dATP and 4 units of T4 DNA ligase, and incubated at 16°C overnight.

2.2.1.5 Klenow reactions

Digested DNA that was to be end - filled was phenol extracted, ethanol precipitated and resuspended in 16 µl of water. 2 µl of 10 x Klenow buffer, 2µl of 10 mM dNTPs, 10 units of Klenow enzyme and 10 units of T4 DNA Polymerase were added and the sample incubated at 37°C for 30 min. The enzymes were heat inactivated at 65°C for 10 min and placed on ice. The DNA was then ready for further manipulations.

2.2.1.6 Phenol extraction

Phenol extractions were performed using a solution of phenol : chloroform : isoamyl alcohol (25:24:1 v/v) ($P/CHCl_3$). An equal volume of $P/CHCl_3$ was added to the sample. After vortexing, the sample was left at room temperature for 5 min and re - vortexed before centrifugation at 14000 r.p.m. for 5 min. The upper aqueous phase was transferred to a new tube and the DNA was subsequently precipitated with ethanol (see **Section 2.2.1.7**).

2.2.1.7 Ethanol precipitation

Unless stated otherwise, all ethanol precipitations were performed in the following manner. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M with 2.5 volumes of absolute ethanol. The sample was placed at -20°C for 30 min and the DNA harvested by centrifugation at 14000 r.p.m. for 10 min. The DNA pellet was washed and dried as described in **Section 2.2.1.3.1**. The DNA pellet was resuspended in the appropriate volume of sterile water and stored at -20°C.

2.2.1.8 Agarose gel electrophoresis

DNA was visualised on 1 x TBE agarose gels ranging from 0.5% for fragments of > 4 kb in length, to 2% for fragments of < 500 bp in length. 20 μg of ethidium bromide was added per 100 ml of gel solution.

2.2.1.9 Radioactive labelling of probes

2.2.1.9.1. Oligonucleotide probes

Oligonucleotide probes were end - labelled using T4 Polynucleotide Kinase (PNK). 50 ng of oligonucleotide, 10 μC of [γ–^{32}P] ddATP, 3 μl of 10 x PNK buffer and 4 units of PNK were mixed with water in a final volume of 30 μl and incubated at 37°C for 30 min. The oligonucleotide probe was purified from the unincorporated radioactivity by separation on a G25 nick column (Pharmacia) according to the manufacturers' instructions.

2.2.1.9.2. Random - primed probes

Linearised DNA fragments (50 ng) were randomly primed with 30 μC of [α–^{32}P] dCTP using a Ready - to - Go kit (Pharmacia) according to the manufacturers' instructions. The radioactively labelled probe was purified from the unincorporated radioactivity by separation on a G50 nick column (Pharmacia) according to the manufacturers' instructions.

2.2.1.10 Southern blotting

All incubations were performed at room temperature. Gels containing DNA samples for Southern blotting were denatured (1.5 M NaCl/0.5 M NaOH) for 20 min for medium - sized gels and 30 min for large gels. The gels were neutralised (1.5 M NaCl/0.5 M Tris pH 8) for the same time as the denaturing step and then soaked in 6 x SSC for 10 min. The gel was then inverted and the blot set up as shown in Sambrook *et al.* (1989). After blotting overnight, the nylon membrane (Micron) was washed in 6 x SSC to remove traces of agarose, and heat - baked at 80°C for 2 hours. The membrane was then pre - hybridised in 2 x Dextran hybridisation buffer (10% dextran sulphate, 3.34% Sarkosyl NL30, 33.4% 20x SCP [2 M NaCl, 0.6 M Na_2HPO_4, 0.02 M EDTA pH 6.2]) for 2 hours

at 65°C before the purified DNA probe was added. The hybridisation was left at 65°C overnight. The membrane was washed in :

2 x SSC/0.1% SDS for 15 min at room temperature

2 x SSC/0.1% SDS for 15 min at 65°C

0.1 x SSC/0.1% SDS for 30 min at 65°C.

The DNA bands were visualised by autoradiography with Fuji X - ray film at -70°C for the required time.

2.2.2 Subcloning of DNA from lambda clones

Lambda clones containing the whole of the $GABA_A$ receptor δ subunit gene were isolated by Dr D. Livingstone. Subclones of the 5' - upstream sequence were required for DNA sequence analysis and also for the generation of a series of promoter - deletion constructs. Subcloning of the lambda DNA into pBluescript was performed using standard molecular biological techniques.

2.2.3 Sequencing of pBluescript double - stranded templates

2.2.3.1 Template DNA preparation

DNA from pBluescript subclones was prepared as described in **Section 2.2.1.3.1.**. The DNA pellet was re - precipitated by adding NaCl (0.8M) and PEG (6.5%). The sample was incubated on ice for 20 min, centrifuged at 14000 r.p.m. for 20 min at 4°C and, after washing in 70% ethanol and drying, was resuspended in sterile water. The DNA was visualized by agarose gel electrophoresis to estimate the concentration of the DNA.

2.2.3.2 DNA sequencing of double - stranded templates

Sequencing was performed using an ABI automated sequencer. Template DNA was mixed, in a thin walled PCR microtube (Costar), with 3.2 pmoles of the relevant sequencing primer and 8 µl of sequencing mix in a total volume of 20 µl. Amplification was achieved using 25 cycles, each cycle comprising : 10 sec denaturation at 96°C, 10 sec annealing at 50°C and 5 min extension at 60°C.

The sequencing reactions were precipitated by adding sodium acetate [0.2 M (pH 4.5)] and 2.5 volumes of ethanol. The samples were incubated on ice for 20 min, centrifuged for 20 min at 14000 r.p.m. and after washing and drying, the pellets were resuspended in 4 µl of formamide loading buffer. The DNA samples were denatured at 80°C for 2 min before being loaded onto the acrylamide gel. The sequencing gel apparatus was assembled and electrophoresis carried out according to the manufacturers' instructions (ABI 373 users manual, Applied Biosystems).

2.2.3.3 Analysis of DNA sequence data

The sequence data was analysed using the sequence editor program (Seqed v 1.0.3, ABI) and using GeneJockey II (Biosoft). Further analysis of the sequence was performed using various computer programs :

TFSEARCH (http://pdapl.trc.rwcp.or.jp/research/db/TFSEARCH.html)

TESS (http://agave.humgen.upenn.edu/tess/index.htmml)

RepeatMasker 2 (www@ftp.genome.washington.edu) and the EMBL database.

2.2.4 The Polymerase Chain Reaction

The polymerase chain reaction (Saiki *et al.*,1988), was performed for a number of different procedures. Two different PCR machines were used, either a Perkin Elmer GeneAmp System 9600 or a Perkin Elmer Cetus DNA Thermal Cycler. In all cases, Taq DNA polymerase (Promega) was used. The reaction conditions varied depending on the primers used, but all included a hot start whereby the samples are heated to 99°C for 10 min before the addition of the Taq, and a 3 step amplification process involving a denaturation step, an elongation step and an extension step. The number of cycles of amplification varied according to the procedure involved.

2.2.5 RNA purification

2.2.5.1 Total RNA preparation

The tissue sample was homogenised in Tri Reagent (1 ml/100 mg of tissue) in a Polytron, and incubated at room temperature for 5 min. Chloroform (0.2 ml/100 mg of tissue) was added and the sample shaken vigorously for 15 sec before being incubated at room

temperature for 2 - 15 min. The sample was centrifuged at 12000 x g for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube containing Propan - 2 ol (0.5 ml/100 mg of tissue). The sample was incubated at room temperature for 10 min, and then centrifuged at 12000 x g for 10 min at 4°C. The supernatant was removed and the pellet washed in 1 ml of 70% ethanol. After centrifugation at 12000 x g for 10 min at 4°C, the RNA pellet was dried briefly at room temperature before being resuspended in DEPC - treated water. If the RNA is to be stored, this should be performed in ethanol at -70°C. The expected yield of RNA from brain tissue using this method is 1 - 1.5 μg/mg of tissue.

2.2.5.2 mRNA preparation

The solutions used are described in **Section 2.1.10.2.** This method was adapted from Sambrook *et al.* (1989). The total RNA pellet (**Section 2.2.5.1**) was resuspended in elution buffer to a final concentration of 1 mg/ml. 0.4 M NaCl was added and the sample heated to 65°C for 5 min prior to being applied to the oligo - dT cellulose column.

The column was prepared by resuspending 0.2 g of oligo - dT cellulose in 10 ml of binding buffer. This is then poured into a Disposocolumn (Biorad) and allowed to settle. The column is washed with :

0.1 M NaOH (10 ml)
DEPC - treated water (10 ml)
Binding buffer (25 ml)

The RNA is heated to 65°C for 5 min and then applied to the column. The eluent is collected and then re - applied. This is repeated twice. The column is washed with :

Binding buffer (25 ml)
Wash buffer (10 ml)

The mRNA was eluted with 6 x 0.5 ml aliquots of elution buffer which had been heated to 65°C. Each fraction was collected separately and the OD_{260} determined. The samples containing the mRNA were combined (generally fractions 2 to 5) and precipitated with

100 mM NaCl and 2.5 vol. of ethanol at -70°C for 30 min. After centrifugation at 12000 x g for 20 min, the mRNA was washed with 70% ethanol, dried briefly at room temperature and resuspended in 10 mM HEPES/ 0.005 M EDTA (pH 7.5) to give a final concentration of 0.5 mg mRNA/ml. At this stage, the sample was transferred to an Eppendorf tube. The mRNA was precipitated with 0.1 vol. of 3 M potassium acetate (pH5.2), 2.5 vol ethanol and stored at -70°C until required.

When required, the mRNA is centrifuged for 10 min, washed in 70% ethanol, dried briefly at room temperature and resuspended in DEPC - treated water.

2.2.6 Oligo - capping

This method has been modified from that used by Maruyama and Sugano (1994), and involves replacing the 5' - mRNA cap structure with a ribonucleotide oligomer (r-oligo), amplifying the 5' - end of interest using RT - PCR and then sequencing the resultant product(s). The first few steps of this procedure were performed under RNAse - free conditions and in the presence of RNasin, an RNase inhibitor. Each purification step involved at least one phenol/chloroform extraction, with glycogen (20 μg/μl) added as a carrier, and then precipitation with 0.3 M sodium acetate (pH 5.5) and 2.5 volumes of ethanol.

2.2.6.1 Removal of 5' - phosphate

mRNA was prepared as described in **Section 2.2.5.**. Approximately 5 μg of mRNA was treated with bacterial alkaline phosphatase (BAP) to remove the 5' - phosphate of any uncapped RNA molecules. 10 μl of 10 x dephosphorylation buffer, 150 u of BAP and 40 u of Rnasin were added to the mRNA in total volume of 100 μl with DEPC - treated water. This was incubated at 42°C for 60 min. The BAP - RNA was phenol/chloroform extracted 3 times before being ethanol precipitated and resuspended in DEPC - treated water.

2.2.6.2 Removal of 5' - cap

The 5' - cap structure was removed by incubation with tobacco alkaline phosphatase [(TAP) Cambio]. 20 µl of 10 x TAP buffer, 5 u of TAP and 40 u of RNasin were added to the BAP - RNA in a total volume of 200 µl and incubated at 37°C for 60 min. This was followed by a single phenol/chloroform extraction, ethanol precipitation and then resuspension in DEPC - treated water.

2.2.6.3 Ligation of ribo - oligonucleotide

A ribo - oligonucleotide was then ligated onto the 5' - end of any molecules that had a 5' - phosphate group, i.e. only those RNA molecules that had their cap structure removed. 1.5 µg of TAP - RNA was incubated overnight, at 16°C, with 80 u RNasin, 1.5 µg of ribo - oligonucleotide, 10 µl of T4 RNA ligase buffer, 200 u of T4 RNA ligase and 50 µl PEG (50%) in a total volume of 100 µl. The r - oligo - RNA was phenol/chloroform extracted once, ethanol precipitated and then re - precipitated with 0.3 vol. of 7.5 M ammonium acetate (pH 7) and 2.5 volumes of ethanol.

2.2.6.4 First strand cDNA synthesis

This ligated RNA was used as a template for first strand synthesis. One third of the r - oligo - RNA (0.5 µg) was denatured at 95°C for 5 min, snap cooled on ice and incubated with 9 µg of random hexamers (Gibco BRL), 2.5 µl of 5 mM dNTP's (Gibco BRL), 40 u of RNasin, 2 µl of 10 x Reverse Transcriptase buffer and 20 u of Superscript II Reverse Transcriptase (Gibco BRL), in a total volume of 20 µl, at 23°C for 10 min, 42°C for 45 min and at 95°C for 10 min.

2.2.6.5 Amplification of 5' - end of cDNA

The sequence of the 5' - end of the mRNA was amplified by RT - PCR using a 5' - oligonucleotide primer (TAG 135) whose sequence is complementary to that of the ribo - oligonucleotide and a 3' - oligonucleotide primer (BROδ1 or BROCI) specific for the δ GABAR subunit gene. A second round PCR was performed using varying concentrations of the first round product as template (1 µl of undiluted, 1 in 10 dilution and 1 in 100 dilution). The same 5' - oligonucleotide primer but different (nested) 3' - oligonucleotide

primers (BR004 or BROCII) were used in the second round PCR reaaction. The conditions for both rounds of PCR were as follows : denaturation of the template and primer at 94°C before the addition of Taq polymerase followed by 25 cycles, each cycle comprising 15 sec denaturation at 95°C, 30 sec annealing of primer to template at 60°C and 30 sec extension at 72°C. The final cycle included an elongation step of 2 min.

The second round products were visualised on 2% TBE agarose gels. A plug of gel containing each DNA band was removed with a yellow tip, placed in an Eppendorf tube containing 200 µl of water and heated to 95°C for 5 min. This was then used as a template for further PCR amplification of the products. These amplified DNA bands were then subcloned into a TA vector. This was performed using a TA cloning kit (Invitrogen). Blue/white selection was used to identify transformants with inserts and the DNA from these was prepared for sequencing (**Section 2.2.3.1**)

2.2.7 Production of recombinant viruses

2.2.7.1 Insertion of promoter - reporter gene cassettes into an HSV - 1 shuttle vector

Insertion of the promoter - reporter gene cassettes (**Chapter 4**) into the HSV shuttle vectors was achieved using standard molecular biological techniques. The promoter constructs were designed to allow for the excision of the promoter - reporter cassette when cut with *Pac* I enzyme (NEB) and thus the HSV shuttle vectors were modified by insertion of a *Pac* I linker (**Table 2.3**).

2.2.7.2 Recombination of shuttle vectors with an HSV - 1 host

All cell growth and medium requirements are described in **Section 2.1.11**.

The standard method for introducing HSV DNA into cells is the calcium phosphate precipitation/DMSO boost method (Graham and van der Eb, 1973; Stow and Wilkie, 1976). Plasmid DNA was linearised (outwith the HSV sequences) and then purified by phenol extraction and ethanol precipitation. Two different molar ratios of plasmid DNA to viral DNA were used in the procedure : 5x and 20x. The following were mixed in an Eppendorf : HSV DNA (0.5 µg), 400 µl HEBS (130 mM NaCl, 4.9 mM KCl, 1.6 mM

Na_2HPO_4, 5.5 mM D - glucose, 21 mM Hepes, pH 7.05), carrier DNA (5 μg of calf thymus DNA) and the appropriate quantity of linearised plasmid. Finally $CaCl_2$ (130 mM final concentration) was added to the mixture. The sample was gently mixed and incubated at room temperature for 5 min, to allow a calcium phosphate precipitate to form.

BHK21/C13 cells (3 x 10^6 cells) were grown at 37°C to 70% confluence. After removing the medium, the DNA mixture was gently added to the cells and incubated at 37°C for 40 min. The plates were overlaid with medium and incubated at 37°C for 4 hours. The medium was removed and the plates washed gently with medium. 1 ml of a HEBS solution containing DMSO (25%) was added to the plates and left for precisely 4 min after which it was removed and the cells washed immediately with medium. The cells were overlaid with medium and incubated at 37°C for 4 to 5 days until viral plaques formed.

The infected cells were scraped off the plate into the medium, transferred into a 15ml Falcon tube and centrifuged for 10 min at 2000 r.p.m. in a Beckman GPR centrifuge. The supernatant was removed and the pellet was resuspended in medium, transferred into a bijou and sonicated to disrupt the cells. This recombinant viral stock was stored at -70°C.

The 1764 viral variant requires a chemical - HMBA (N,N - hexamethylene bis - acetamide) for growth *in vitro*.

2.2.7.3 Isolation of single plaques from the recombinant virus stock

60 mM plastic Petri dishes were seeded with 3 x 10^6 BHK21/C13 cells and incubated at 37°C until the cells reached 85% confluence. Ten fold serial dilutions of the recombinant viral stock (typically 10^{-2} to 10^{-4}) were prepared in medium or PBS + 5% calf serum. The medium was removed from the plates and 100 μl of each dilution was added to the cells. The plates were incubated at 37°C for 45 min, overlaid with $ETMC_{10}$ and incubated at 37 °C until plaques formed.

Plaques were picked (using a microscope at 10 x magnification) into screw - capped storage tubes containing 200 µl of medium. This viral stock was then stored at -70°C.

2.2.7.4 Screening for recombinant virus

Recombinant viruses were identified by preparing viral DNA from the above stocks. The DNA was then analysed by dot blots followed by Southern blotting analysis.

Twentyfour well plates were seeded with 5 x 10^5 BHK21/C13 cells and incubated at 37°C until the cells reached 85% confluence. Each well was infected with 100 µl virus (from the single plaque stock) directly into the medium and incubated at 37°C until plaques had formed (approximately 3 day). The medium was discarded and 250 µl of cell lysis buffer (0.6% SDS,10 mM EDTA,10 mM Tris pH 7.5) + protease (500 µg/ml) was added to each well. The cells were transferred to an Eppendorf tube and lysed by incubation at 37°C overnight. The DNA was then extracted with the addition of sodium perchlorate (100 mM), as described in **Section 2.2.8.4.3**.

The solutions for the dot blot and Southern blot analysis are the same and are described in **Section 2.2.1.10**. For the first round purification, one third of each DNA sample was loaded onto a dot blot apparatus. The membrane was then probed at high stringency (65°C) overnight (using radioactively labelled cDNA that had been randomly primed, **Section 2.2.1.9.2**), washed at high stringency and then the DNA bands visualised by autoradiography with Fuji X - ray film at -70°C for the required time. Any positive viruses were then verified by Southern blot analysis and further purified. Several rounds of purification are required to isolate a pure recombinant virus.

2.2.7.5 Large-scale viral DNA purification

BHK21/C13 cells were grown at 37°C to 90% confluence in roller bottles which hold 1 x 10^8 cells. The cells were infected at a multiplicity of infection (moi) of 0.003 plaque forming units (pfu)/cell in a minimal volume of medium (20 ml). Each roller bottle will typically give a yield of 100 µg DNA. The incubation was then continued for 5 days at 31°C until cpe (cytopathic effect) was complete. The cells were shaken into the medium

and then transferred into 250 ml centrifuge tubes. The cells were pelleted by centrifugation at 2000 rpm for 15 min at 4°C in a Beckman GPR centrifuge. The supernatant was carefully removed and stored at 4°C.

Cytoplasmic virus was extracted from the cells by lysing the plasma membrane but keeping the nuclear membrane intact. This was achieved by resuspending the cell pellet in RSB (10 mM Tris-HCl, (pH7.5), 10 mM KCl, 1.5 mM $MgCl_2$) + NP40 (0.5 % v/v) and incubating at 4°C for 10 min. The nuclei were pelleted at 2000 rpm for 10 min at 4°C and the supernatant combined with the cell supernatant. The pellet was then discarded. The virus was harvested from the supernatant, by centrifugation at 12000 rpm for 2 hours at 4°C in a GSA rotor in a Sorvall RC5C centrifuge. The supernatant was disposed of and the viral pellet resuspended in NTE (10 mM Tris-HCl (pH7.5), 10 mM NaCl, 1 mM EDTA), transferred to a glass universal bottle and sonicated until the virus was completely resuspended.. Lysis of the virus was achieved by the addition of 2.5% SDS/10 mM EDTA and incubation at 37°C for 5 min.

The sample was then phenol extracted 4 times, chloroform/isoamyl alcohol extracted once and the DNA precipitated by the addition of 2 volumes of ethanol. After washing, the DNA pellet was dried briefly at room temperature before being resuspended in sterile water plus RNase A (10 μg/ml) and incubated overnight at 37°C. The viral DNA was then aliquoted and stored at -70°C until required.

2.2.7.6 Preparation of high titre viral stock

For this purpose, 10 roller bottles were seeded with BHK21/C13 cells and grown at 37°C until the cells reached 80% confluence. The medium was removed from the bottles and replaced with 20 ml of medium containing 10^6 pfu of virus. The incubation was continued for 5 days at 31°C until cell lysis was complete. The cells were shaken into the medium and then transferred into 250 ml centrifuge tubes. The cells were pelleted by centrifugation at 2000 rpm for 15 min at 4°C in a Beckman GPR centrifuge. The supernatant was transferred into a fresh centrifuge tube, and the virus harvested from the

supernatant by centrifugation at 12000 rpm for 2 hours at 4°C in a GSA rotor in a Sorvall RC5C centrifuge.

The supernatant was discarded, and the viral pellet resuspended in 5 ml of residual supernatant. The viral stock was sonicated, in a glass container, for 5 - 10 min until completely resuspended. The stock was then aliquoted and stored at -70°C.

2.2.7.7 Titration of viral stocks

60 mM plastic petri dishes were seeded with 3 x 10^6 BHK21/C13 cells and incubated at 37°C until the cells reached 85% confluence. Ten fold serial dilutions of the viral stock (typically 10^{-1} - 10^{-7}) were prepared in medium or PBS + 5% calf serum. When preparing the dilutions, it is necessary to use fresh tips as the virus will stick to the tips and introduce inaccuracies in the titration.

The medium was removed from the plates and 100 µl of the serially diluted virus stock was added gently to the cells. After swirling gently to ensure even coverage of virus, the plates were incubated at 37°C for 1 hour to allow adsorption of the virus onto the cells.

The cells were overlaid with $ETMC_{10}$ and incubated at 37°C until plaques formed (usually 2-3 days). The overlay medium was removed and the cells stained for a minimum of 2 hours at room temperature with Giemsa. The stain was removed by washing, the plates drained and the plaques counted using a plate microscope.

It is best to count the dilutions with 20 - 200 plaques/plate, and to count duplicate plates. The titre can be calculated as follows :

10^{-7} plate = 20 plaques

10^{-6} plate = 200 plaques

= 2 x 10^8 pfu in 100 µl inoculum.

Therefore the titre is 2 x 10^9 pfu/ml.

2.2.8 Gene targeting

2.2.8.1 Construction of vector

DNA fragments from the lambda subclones were introduced into the targeting vector using standard molecular biological techniques (**Sections 2.2.1.1 - 2.2.1.4**)

2.2.8.2 Growth of cells

All cells were grown in flasks or plates treated with 0.1% gelatin for a minimum of 1 hour at 4°C. The amount of medium required depended on the size of tissue culture vessel used. The cells were grown at 37°C with 5% CO_2.

Initially, cells were grown from a stock frozen in liquid nitrogen. The vial was thawed quickly at 37°C. 1 ml of medium was added and the cells were transferred to a gelatinised 5 cm^2 flask with 6 ml of medium. The cells were grown to about 80% confluence before being split into a larger flask. The medium was removed and the cells washed twice in 1x PBS to remove traces of medium which would inhibit the trypsin. 1ml of trypsin was added and left for 2 - 4 min until the cells were observed coming off the flask. 5ml of medium was added to stop the trypsin, and the cells were pipetted into a 75 cm^2 gelatinised flask with 22 ml of medium.

The cells were grown to 80% confluence before again being split , this time into 2 x 150 cm^2 flasks. 50 ml of medium is required for growth of cells in these flasks. The cells were then ready for the transfection procedure.

2.2.8.3 Transfection of ES cells

The DNA requires to be linear for transfection, therefore 50 µg of DNA was linearised with the appropriate enzyme. This was confirmed by agarose gel electrophoresis. The digested DNA was then precipitated with 0.3 M sodium acetate (pH 5.2) and 2.5 vol. of ethanol as described previously (**Section 2.2.1.6**). The removal of the 70% ethanol wash and the drying stage were performed in the hood for sterility purposes. The DNA was resuspended in 50 µl of sterile water and heated to 65°C.

2 x 150 cm^2 flasks of cells at 85% confluence were used for the transfection. The medium was removed and the cells were washed twice with 1x PBS. The cells were treated with trypsin and transferred in 10 ml of medium, to 15 ml Falcon tubes. The cells were harvested at 1000 rpm for 1 min in a Jouan CR312 benchtop centrifuge. The medium was carefully removed and the cells were gently resuspended in 1x PBS. The cells were again harvested at 1000 rpm for 1 min. This washing stage was repeated twice more. The cells were then combined in 1x PBS to give a final volume of 0.8 ml.

The cells were transferred to the Eppendorf tube containing the denatured DNA and mixed gently being careful not to introduce air bubbles. This mix was then transferred to a 0.2 ml cuvette and the cells electroporated with a single pulse of 0.8 Kv, 250 μFd, 600 ohms. The cell/DNA mix was then incubated at room temperature for 20 min. The cells were transferred to a 50 ml Falcon tube containing 30 ml of medium and 2 ml of this were added to 15 gelatinised petri dishes. The volume was made up to 10 ml with medium and the cells were incubated at 37°C. After 24 hours, the cells were grown in medium containing G418 and gancyclovir. The medium was changed every day.

2.2.8.4 Screening for targeted cell lines

2.2.8.4.1 Selection of resistant colonies

Initially, the cell growth was normal. After a few days a large amount of cell death was observed. It is critical to change the medium regularly as the presence of dead cells has a deleterious effect on cell growth. After about 10 - 12 days, resistant colonies could be seen and these were allowed to grow for several days before being picked.

The medium was removed and the cells washed twice in 1x PBS. 7.5 ml of PBS was then added to the plate. The colonies of cells were picked using yellow tips and transferred to gelatinised 24 well plates. Each well of cells was treated with trypsin before adding 2 ml of medium and incubating at 37°C. The medium was changed after 2 days initially and then daily after that. When the cells were confluent, they were harvested. One third of the cells were frozen down and stored in liquid nitrogen. The rest were used to prepare DNA.

2.2.8.4.2 Freezing of selected colonies

The medium was removed from the wells, the cells washed twice in 1x PBS and then treated with trypsin. 1ml of freezing medium was added to the wells and the cells were scraped off using a 1 ml pipette. 0.3 ml of cells were transferred to storage tubes, while the remainder of the cells were transferred to Eppendorf tubes. Only 20 wells were processed at one time as the DMSO in the freezing medium is toxic to the cells. The cells in the storage tubes were frozen slowly at -70°C before being transferred to liquid nitrogen. The cells in the Eppendorf tubes were harvested by centrifugation and the medium removed. The cell pellets were stored at -70°C.

2.2.8.4.3 Preparation of genomic DNA

This method has been modified from the Nucleon genomic DNA isolation kit from Scotlab. The cell pellets were resuspended in 340 µl Solution B (400 mM Tris (pH 8), 60 mM EDTA, 150 mM NaCL, 1% SDS). Sodium perchlorate (0.88 M) was added and the samples were incubated at 65°C for 10 min. 580 µl of chloroform : iso amyl alcohol (24:1) was added and the samples were incubated on a roller for 20 min at room temperature. The samples were centifuged for 5 min at 14000 rpm and the upper aqueous layer (400 µl) transferred to an Eppendorf tube using a cut blue tip. Two volumes of ethanol were added and the tubes gently inverted until the DNA precipitate was visible. After a 5 min centrifugation and wash with 70% ethanol, the DNA was dried briefly at room temperature and resuspended in 60 µl sterile water overnight at 4°C.

2.2.8.4.4 Screening of genomic DNA

The DNA samples were screened by Southern blot analysis (**Section 2.2.1.10**). For each clone, one sixth of the DNA was digested with the appropriate enzyme in a volume of 100 µl. The samples were then ethanol precipitated (**Section 2.2.1.7**) and resuspended in 20 µl of water plus loading dye. The digested DNA samples were separated by electrophoresis on 0.6% TBE agarose gels overnight at 25 volts. The gels were denatured, neutralised and blotted overnight. The blots were probed with random primed DNA fragments (**Section 2.2.1.9.2**) and visualised by autoradiography with Fuji X - ray film at -70°C for the required time.

2.2.9 *In vitro* analysis of promoter -reporter constructs

2.2.9.1 Neuronal cell cultures

2.2.9.1.1 Preparation of cells

The day prior to transfection, the cells were plated out in 6 well (35 mm) trays to yield a density of approximately 90% confluence at the time of transfection. Immediately before transfection, the cells were washed twice with 2 ml of Optimem 1. Finally, the cells were overlaid with 1 ml of Optimem 1.

2.2.9.1.2 Transfection of cells

Each plasmid was transfected in triplicate. In total, 3 μg of DNA was transfected per well. This consisted of 2 μg of the *lacZ* constructs and 1 μg of the internal control plasmid. The ratio of DNA to DOTAP used was 1 μg of DNA per 6 μl of DOTAP.

3 μg of DNA was diluted to 0.1 μg/μl in Hepes buffer (30 μl). In a separate tube, the DOTAP (18 μl) was diluted to 60 μl with Hepes buffer. The DOTAP solution was added to the DNA and incubated at room temperature for 15 min. The DNA : DOTAP mix was added to the cells dropwise, and the plates swirled gently to ensure proper mixing. The cells were incubated at 37°C for 6 hours. The medium was removed and the cells were overlaid with 2 ml of medium (with serum). The cells were incubated for 42 hours post - transfection before being harvested.

2.2.9.1.3 Harvesting of transfected cells

The medium was removed and the cells were washed 3 times with 1 x PBS. After removal of residual liquid, 80 μl of cell lysis buffer was added to each well. The cells were incubated at room temperature for 5 min and then scraped off and transferred into an Eppendorf tube. The cells were centrifuged for 1 min to pellet the cell debris and the supernatant was transferred to a fresh tube. The cell extract could be stored at -70°C at this stage.

2.2.9.1.4 β - galactosidase assays of cell extracts

30 µl of cell extract was mixed, in an Eppendorf tube, with 200 µl of β - galactosidase assay reagent. This was incubated, in the dark, at room temperature for 1 hour before being transferred to a tube for the luminometer.

The luminometer was set with a delay of 10 sec and to read for an integrate of 15 sec. A reading was taken for each sample.

2.2.9.1.5 Luciferase assays of cell extracts

10 µl of cell extract was mixed, in a luminometer tube, with 100 µl of luciferase assay reagent and a reading taken immediately. The luminometer was set with a delay of 3 sec and to read for an integrate of 10 sec.

2.2.9.2 Cerebellar granule cell cultures

2.2.9.2.1 Preparation of cells

Cerebella were removed from 7 - 9 day postnatal rats (Sprague - Dawley) and placed in a petri dish with EBSS (maximum of 10 per procedure). The cerebella were placed in a row in the lid of a petri dish, and triple chopped using a sterile double - edged scalpel. The cerebella were transferred to a 50 ml Falcon tube with 10 ml of supplemented BME medium. 10 ml of trypsin (0.25% w/v, final concentration, **Section 2.1.12.2**) was added to the cells and mixed 6 times by tituration. The cells were incubated at 37°C for 15 min.

1 ml of soyabean inhibitor (0.03% w/v, final concentration) was added and mixed for 1 min by tituration. 1 ml of DNase I (0.004%, final concentration) was added and mixed for 1 min by tituration. The cells were incubated at 37°C for 10 min.

5 ml of supplemented BME medium was added and the cells were mixed for 2 min by tituration. The cells were centrifuged at 1000 rpm for 90 sec, and the supernatant collected in a fresh Falcon tube and maintained at 37°C. The cell pellet was washed and titurated in 10 ml of supplemented BME medium before being re - centrifuged at 1000 rpm for 1 min. This washing step was repeated 2 more times. The supernatants were

pooled and maintained at 37°C. A 20 µl aliquot of cells was mixed 1:1 with trypan blue (0.4% stock) and counted using a Fuchs - Rosenthal counting chamber (depth 0.2 mm, 1/16 mm^2).

Cells were seeded at a density of : 1.5 x 10^6 cells/12 well plate in 1 ml of supplemented BME medium and maintained in an incubator at 37°C with 5% CO_2.
This is day 1 of the granule cell cultures.

The medium was replaced after 48 hours with fresh supplemented BME medium containing 10 µM cytosine-β-arabinofuranoside to prevent division of non - neuronal cells.

The medium was supplemented with Glucose solution (5 mM) every 3 days.

2.2.9.2.2 Transfection of granule cell cultures

Each plasmid was transfected in triplicate. 2 µg of DNA was transfected per well. This consisted of reporter constructs and pUC18 control DNA (added to standardise the DNA concentrations). This calcium phosphate precipitation method has be modified from that of Xia *et al.* (1996).The solutions for this are listed in **Section 2.1.12.3**.

The medium was removed from the wells and combined. Glucose (5 mM) was added to the medium and it was stored at 37°C until required (conditioned medium). 1 ml of BME + GRI was added to the wells and the cultures incubated at 37°C for 1 hour.

The DNA was mixed with the $CaCl_2$ and then this was slowly added dropwise to an equal volume of HBS. The DNA precipitate was allowed to form by incubation at room temperature for 25 min. The DNA mix was added to the cells dropwise and swirled gently to ensure proper mixing. The cells were incubated at 37°C for 1 hour. The cells were treated with a GRI/HBS supplemented with either 5 % Glycerol or 2 % DMSO for 2 min before being washed 3 times with BME. The cells were overlaid with 1.5 ml of conditioned medium and incubated at 37°C for 48 hours.

2.2.9.2.3 X - gal staining of granule cell cultures

The medium was removed and the cells washed 2 times with 1 x PBS. The cells were fixed by incubation with fix solution for 5 min at room temperature. The cells were washed 3 times with TBS and then stained with X - gal solution by incubating at 37°C overnight.

2.2.10 Analysis of HSV - 1 promoter constructs

2.2.10.1 In vitro analysis in cerebellar granule cells

2.2.10.1.1 Infection of granule cells

The viruses were diluted in BME to 1 x 10^8 pfu/ml. The cells were infected with 1 x 10^6 pfu/well which gave a MOI of 1. Each virus was infected in duplicate wells.

The medium was removed and stored as described in **Section 2.2.9.2.2** above (conditioned medium). The virus was added to the wells in 0.5 ml of medium and the cells incubated at 37°C for 1 hour. The medium was removed and replaced with conditioned medium. The infected cells were incubated at 37°C for the required time.

2.2.10.1.2 X - gal staining of infected cells

The medium was removed and the cells washed 2 times with BME (minus serum). The cells were fixed with 0.2 % gluteraldehyde in BME (minus serum) by incubating at room temperature for 5 min. The cells were washed 3 times with BME (minus serum) and stained by incubation at 37°C overnight with X - gal solution **(Section 2.1.12.3)**

2.2.10.2 In vivo analysis by stereotactic injection into rats

2.2.10.2.1 Intracerebellar inoculation of animals

Approximately 1 x 10^5 pfu of each virus was stereotactically injected under Rompun/ Vetalar (1:2) anaesthesia into the cerebellum of 200 g - 300 g, adult male AO rats. The virus was injected over a period of 1 min in a volume of 1 µl. At day 5 post - injection, animals were sacrificed by decapitation. The brains were removed and frozen immediately on dry ice. The brains were stored at -70°C.

2.2.10.2.2 Histochemistry of extracted brains

Glass slides were treated with TESPA solution, washed twice in acetone and stored at 4°C with dessicant.

Brains were removed from -70°C and mounted ready for sectioning. Brains were sectioned horizontally at 30 μm on a cryostat onto treated glass slides. The sections for histochemical staining were fixed with a gluteraldehyde solution for 30 min at room temperature. After washing 3 times in Wash Buffer, the sections were stained in X - gal solution overnight at 37°C. The slides were rinsed once in Wash Buffer, re - fixed for 30 min, rinsed again in Wash Buffer and then counter - stained with neutral red for 20 min. Sections were dehydrated in steps : 70% ethanol, 95% ethanol, 100% ethanol and finally Propan - 2 - ol. The sections were treated with Histoclear, cover - slips were applied with DPX and then dried at room temperature overnight in the dark.

Chapter 3

Characterisation of the $GABA_A$ Receptor δ Subunit Gene Promoter

3.1 INTRODUCTION

Early studies using a combination of *in vitro* mutagenesis and DNA - mediated gene transfer identified two distinct types of *cis* - acting regulatory sequences. Basal promoter regions are located close to the initiation site and act in a position - dependent manner, while enhancers and silencers, which can be located far from the initiation site, act in a position - and orientation - independent manner. Basal promoters can be subdivided into proximal elements, e.g. the cap site and the TATA box, and distal elements which can be spread over hundreds of base pairs. These *cis* - acting elements operate by interacting with protein factors. A combination of different interacting elements can generate various types of transcriptional control.

The $GABA_A$ receptor δ subunit gene has been partially characterised for both the mouse and rat, and some analysis of the putative promoter regions for both animals has been performed. This information has been discussed in detail in **Section 1.3.1**. The $GABA_A$ receptor δ subunit gene promoter lacks a TATA box and is rich in CpG dinucleotides. DNA recognition sequences have been identified for the Sp1 and AP-4 transcription factors. An area of transcription initiation has been identified in the mouse 5' - upstream region (Sommer *et al.*, 1990), and this coincides with transcription initiation sites that have been identified for the rat (Motejlek *et al.*, 1994). Also in the rat, a region containing tandemly repeated purine elements has been identified as binding a novel element - BSF1 (brain specific factor 1).

Recently, Dr D. Livingstone (Prof. R.W. Davies laboratory) performed analysis of short δ promoter fragments (close to the translational start site) driving luciferase gene expression in two cell lines, NB4 1A3 and GT1 - 7. The results showed that the region containing the putative Sp1 binding sites is critical for expression.

Lüscher reported in an abstract in 1993, the use of transgenic mice lines carrying a *lacZ* gene driven by a 6.5 kb fragment from the rat δ subunit gene 5' - flanking region to identify neuron - type specific regulatory elements. They reported faithful neuron - specific expression in most regions of the CNS but not in the cerebellum, thus they were unable to show the correct developmental profile of the native gene. They concluded

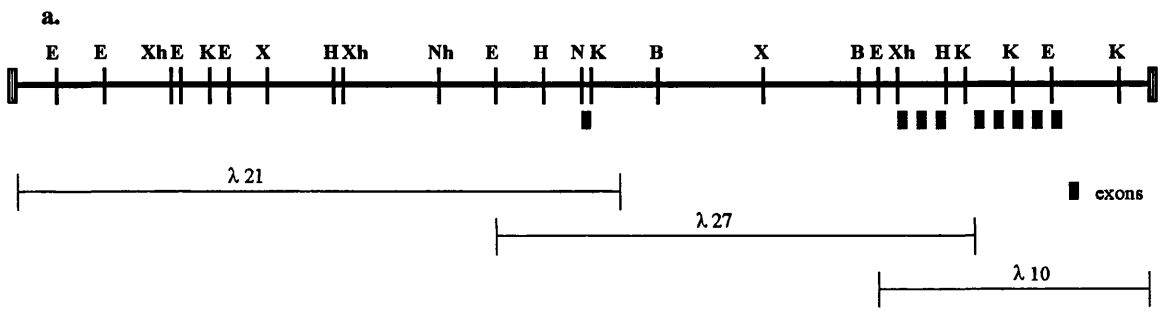
therefore that the construct lacked sequences essential for the inherent developmental expression profile of the $GABA_A$ receptor δ subunit gene.

In this chapter, a large region (10.6 kb) of the 5' - flanking region of the murine $GABA_A$ receptor δ subunit gene has been sequenced and analysed. The transcription initiation points have been identified, as have several regions that could be involved in the regulation of the gene.

3.2 RESULTS

3.2.1 Subcloning of the promoter region for sequence analysis

The $GABA_A$ receptor δ subunit gene was isolated from a murine 129 genomic DNA library (Dr D. Livingstone). Three overlapping lambda clones contained the whole gene (**Figure 3.1a**). Dr Livingstone mapped these clones by restriction analysis and found differences from the published data. In order to sequence the promoter region, 10.6 kb of 5' - flanking DNA was subcloned into Bluescript SKII (+) and KSII (+) (**Figure 3.1b**).



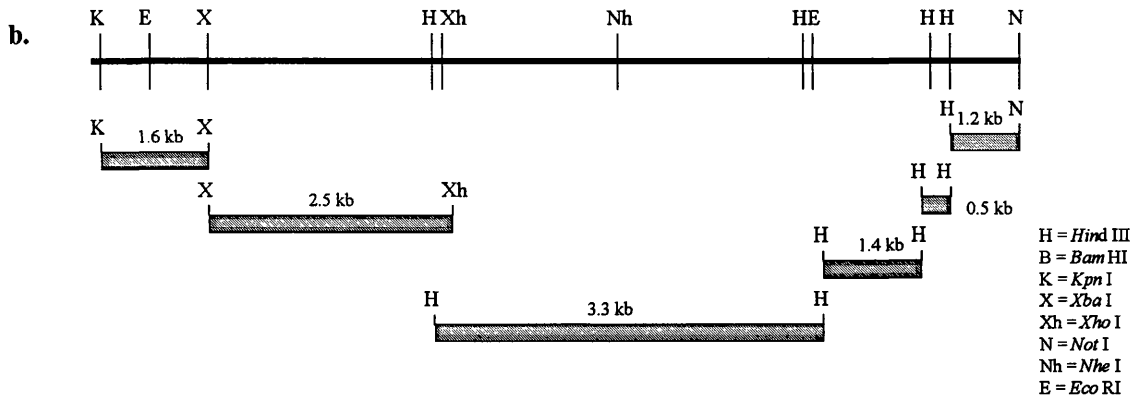


Figure 3.1 Genomic restriction map

a. Restriction map of $GABA_A$ receptor δ subunit gene genomic DNA, and overlapping lambda clones which contain the whole gene.

b. Promoter fragments (approximate sizes in base pairs) subcloned into pBluescript for sequence analysis.

The figure is not drawn to scale.

3.2.2 Identification of transcription start sites

Transcription start sites were identified using the oligo - capping technique (**Section 2.2.6**, Maruyama *et al.*, 1994). The PCR reactions were performed (using the same conditions) 3 times with different oligonucleotide primers. The products of the oligo - capping were visualised on a 2% TBE agarose gels, an example of which is shown in **Figure 3.2**. Initially, primers were designed to exons 2 and 1 (BRδ01, BR006, **Table 2.4**). 2 major products were obtained. These were subcloned into a TA vector (Promega) and 20 clones were analysed by sequencing **(Section 2.2.2.3.2)**. These results have been presented in a histogram **(Figure 3.3a)**. There appear to be several start sites, but 2 major clusters.

In the second experiment, the 3' - primers were designed to exons 3 and 2 (BROCI, BROCII, **Table 2.4**). 20 TA clones were analysed by sequencing and these results corroborated those of the first experiment i.e. several start sites with 2 main clusters **(Figure 3.3b)**. The experiment was repeated a third time with a newly prepared 5' - oligonucleotide primer from Cruachem, and the BROCI and BROCII 3' - primers. Again 20 clones were analysed by sequencing **(Figure 3.3c)**. **Figure 3.4** shows the results of the 3 experiments combined. The overall positions of the transcription start sites and the primers used are shown in **Figure 3.5**.

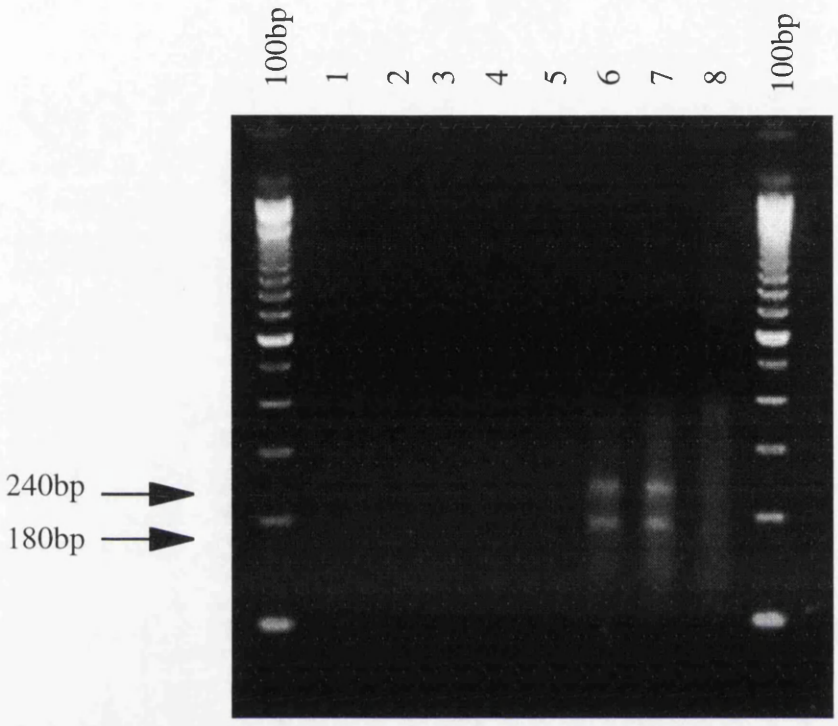


Figure 3.2 PCR products produced by Oligo - capping

2% TBE agarose gel showing second round PCR products from oligo-capping experiment. Lane 1 = no DNA control, Lanes 2 - 5 = single primer controls, with and without DNA, Lanes 6 - 8 = reactions using varying concentrations of DNA template. Primers used were TAG 135 and BROCII (**Table 2.4**). The 2 major products (and their sizes) are indicated with arrows.

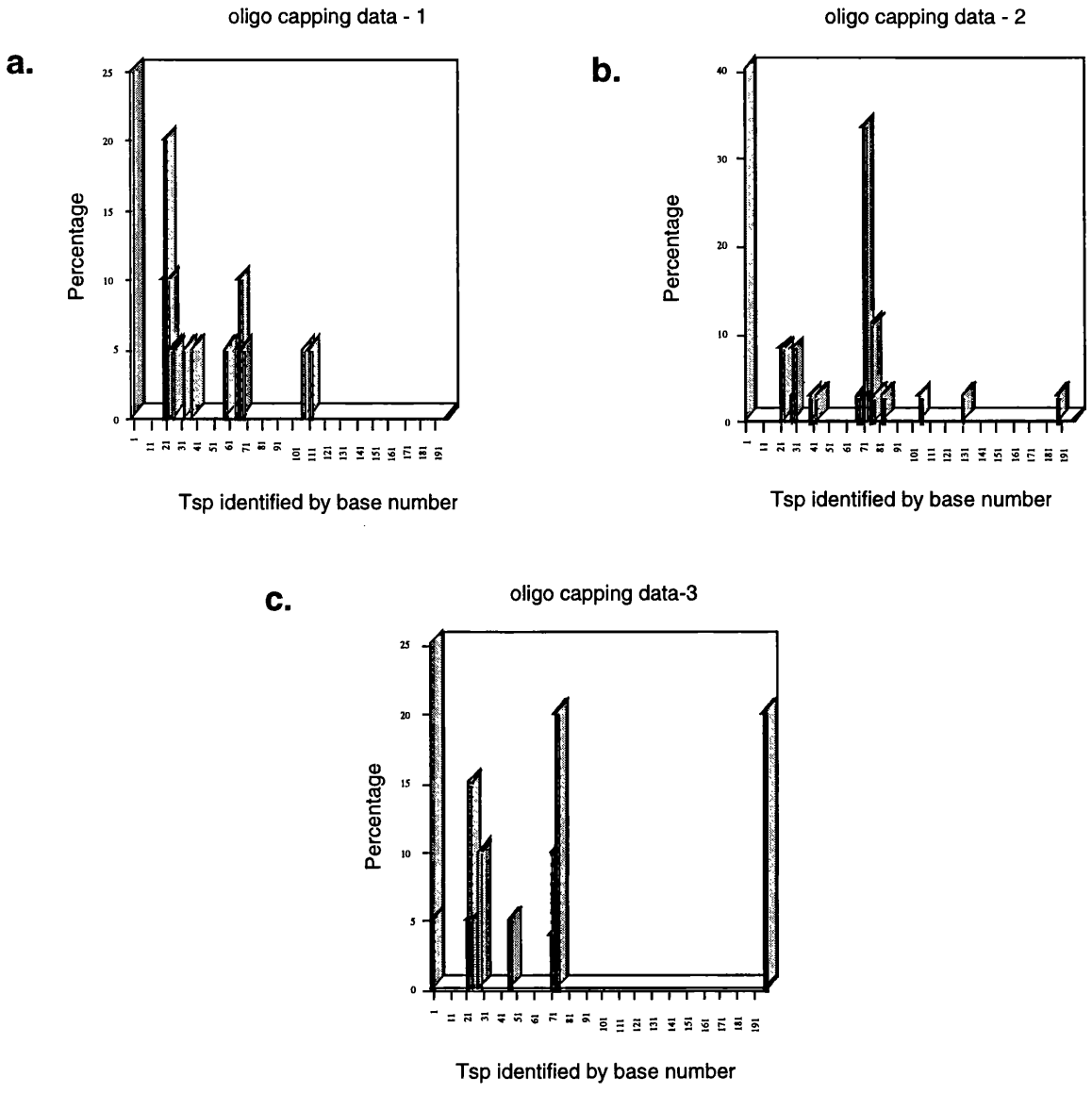


Figure 3.3 Histograms showing transcriptional start point data

a. Histogram showing results of the first oligo - capping experiment. PCR primers used were TAG135, BRδ01 and BR006. 2 major start site clusters were observed.

b. Histogram showing results of the second oligo - capping experiment. PCR primers used were TAG135, BROCI and BROCII. 2 major start site clusters were observed.

c. Histogram showing results of the third oligo - capping experiment. PCR primers used were TAG135, BROCI and BROCII. 2 major start site clusters were observed.

X - axis = 1 indicates the 5' - most start site identified. Y - axis = percentage of tsp identified at a specific base.

Abbreviations used : tsp = transcription start point.

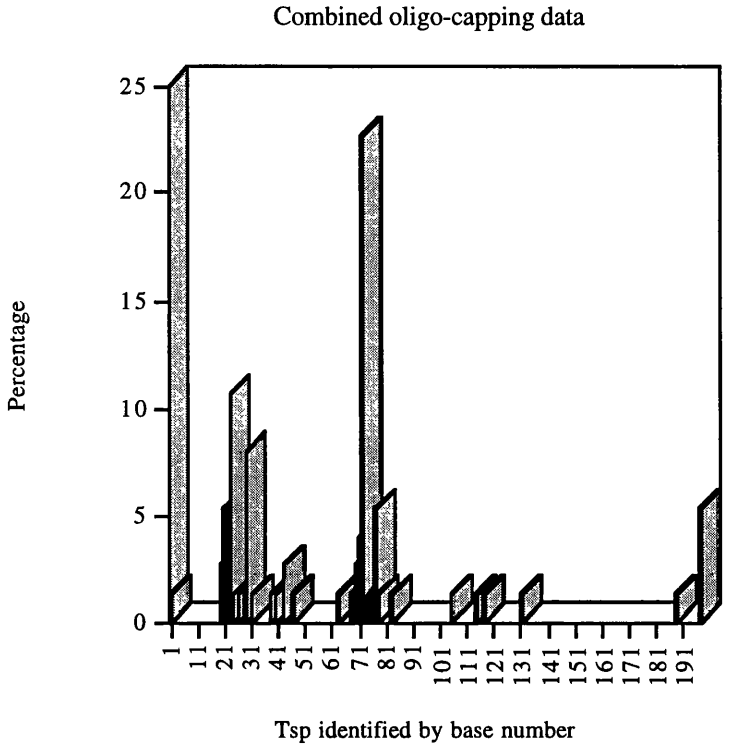


Figure 3.4 Histogram showing combined transcriptional start point data

Histogram showing the combined results of the three oligo - capping experiments. 2 major start site clusters were observed.

X - axis = 1 indicates the 5' - most start site identified. Y - axis = percentage of tsp identified at a specific base.

Abbreviations used : tsp = transcription start point

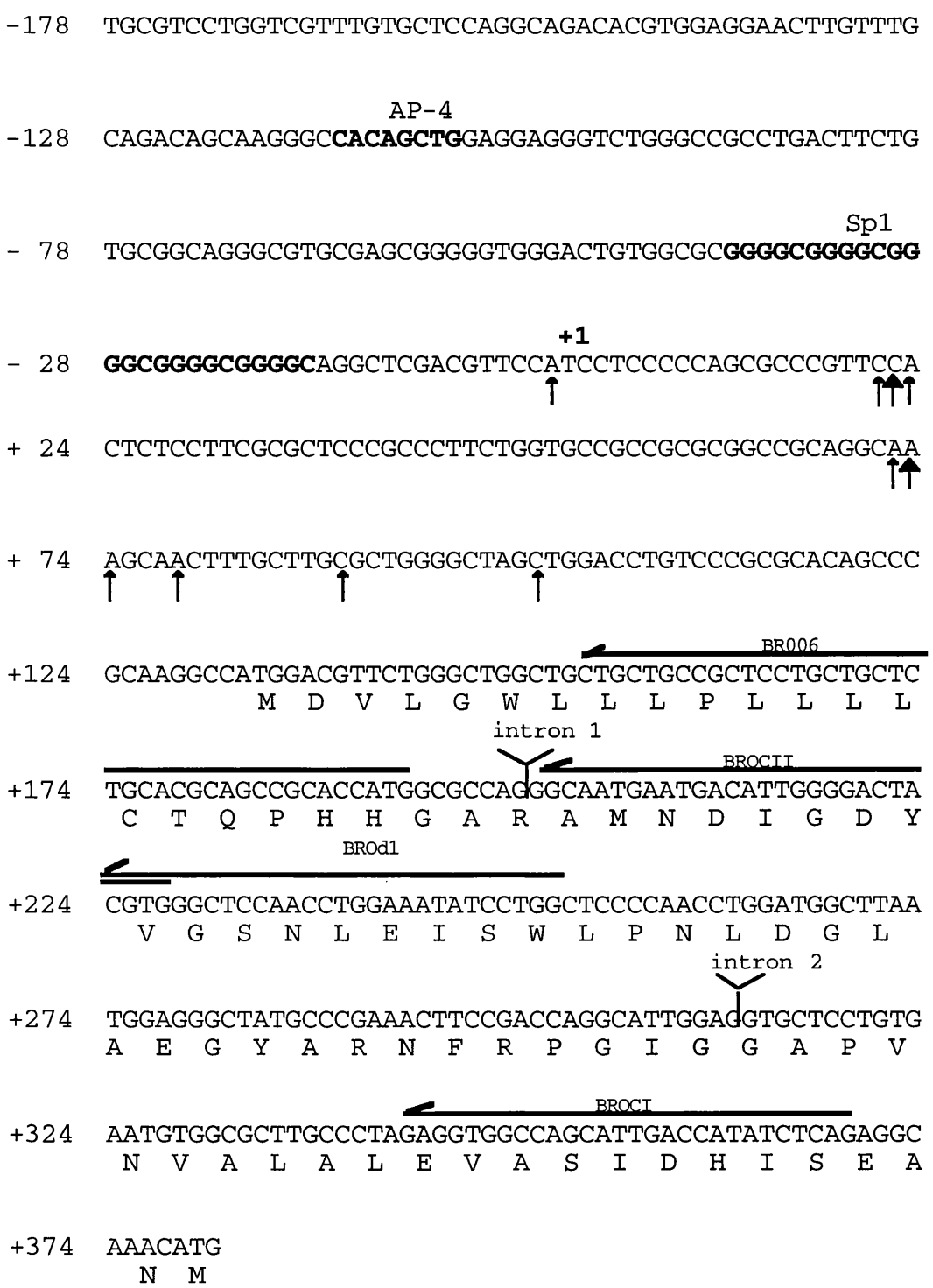
Figure 3.5 Sequence showing the identified transcription start points

Figure 3.5 Sequence showing transcription start points

The sequence shows the position of the transcription start points identified by oligo - capping. There are two major start points (large arrows) and several minor ones (small arrows). The two sets of 3' - nested oligonucleotide primers used were :

BR0δ1 (exon 2) and BR006 (exon 1)

BROCI (exon 3) and BROCII (exon 2) and these are indicated as solid lines above the sequence. Intron/exon boundaries are shown. Consensus sequences for the transcription factors AP-4 and Sp1 are in bold. +1 (base A) relates to the 5' - most transcription start point identified.


-178 TGCGTCCTGGTCGTTTGTGCTCCAGGCAGACACGTGGAGGAACTTGTTTG
AP-4
-128 CAGACAGCAAGGGCCACAGCTGGAGGAGGGTCTGGGCCGCCTGACTTCTG
Sp1
- 78 TGCGGCAGGGCGTGCGAGCGGGGGTGGGACTGTGGCGCGGGGCGGGGCGG
+1
- 28 GGCGGGGCGGGGCAGGCTCGACGTTCCATCCTCCCCCAGCGCCCGTTCCA
+ 24 CTCTCCTTCGCGCTCCCGCCCTTCTGGTGCCGCCGCGCGGCCGCAGGCAA
+ 74 AGCAACTTTGCTTGCGCTGGGGCTAGCTGGACCTGTCCCGCGCACAGCCC
BR006
+124 GCAAGGCCATGGACGTTCTGGGCTGGCTGCTGCTGCCGCTCCTGCTGCTC
M D V L G W L L L P L L L L
intron 1
BROCII
+174 TGCACGCAGCCGCACCATGGCGCCAGGGCAATGAATGACATTGGGGACTA
C T Q P H H G A R A M N D I G D Y
BROd1
+224 CGTGGGCTCCAACCTGGAAATATCCTGGCTCCCCAACCTGGATGGCTTAA
V G S N L E I S W L P N L D G L
intron 2
+274 TGGAGGGCTATGCCCGAAACTTCCGACCAGGCATTGGAGGTGCTCCTGTG
A E G Y A R N F R P G I G G A P V
BROCI
+324 AATGTGGCGCTTGCCCTAGAGGTGGCCAGCATTGACCATATCTCAGAGGC
N V A L A L E V A S I D H I S E A
+374 AAACATG
N M

3.2.3 Sequencing of the 5' - flanking region

Automated double - stranded DNA sequencing (**Section 2.2.3**) was used to sequence the 5' - flanking region. The universal forward and reverse primers and also primers specific to the δ promoter region were used to obtain sequence from both strands (**Table 2.5**). Two plasmids containing 6.3 kb (pBR502) and 10.6 kb (pBR702) (**Chapter 4**) of 5' - upstream DNA were used to sequence the overlapping region between the subcloned DNA fragments. In total,10.6 kb of double - stranded sequence was obtained (**Figure 3.6**).

Figure 3.6 Sequence of the 5' - upstream region of the $GABA_A$ receptor δ subunit gene

Figure 3.6 Sequence of the 5' - upstream sequence of the $GABA_A$ receptor δ subunit gene

The figure shows the complete sequence of approximately 10.6 kb of 5' - upstream region from the $GABA_A$ receptor δ subunit gene. +1 relates to the 5' - most transcription start point identified and is indicated by a vertical arrow below the base (A). The sequences highlighted in red are recognition sites for known transcription factors. These are listed and described in **Tables 3.1 and 3.2**. The blue sequences are repeat regions e.g. SINES and LINES, and the regions highlighted in green are simple repeat sequences. These are listed and described in **Table 3.3**. The translational start point is indicated.

-10602 GGTACCTCTGCACTCCCTTCAGATACTTTGCCTGTGAAATACCACTGCAATACTGTATAGGTGTGTGTGT

-10532 GGCTGGGGATCGAACCCAGGTGTTCTATAAGTTTAGAGCTTTGTGTATGGAAGCCGGATGTCCTAGTAAA

-10462 GCAATTTGGGAAAGATAGCCAGTTGGTTTGTAACCTTTAGCTGAGGGAGAATGTGAACTGGGCCTCCTAC

-10392 TCTGGGTTGACCCTGGTATATGGCTGAGCAGAGAGGGTTGTGAAGTAACCATATCCAATACTAATATTTG

-10322 TGGTATGAAGTATAATCCCCACTATAGTAAAATAAGTGAATAAATTGGTCTTCTCTTTCACGTTGCCTCA

-10252 AATATTTGTTTACAGGGACAAAACTCTGACTAACACATGTAATTTTCTCTGGGGTATACTATACTCCTCT

-10182 GTTAGGCTGGCCAAGCCTGTCAGGTATACATTAGGTTTGCCTCCCTTCCACTTTTTTTGTTTTGTTTTGT

-10112 TTTTTGAGACAGGGTTTCTCTGTGCAGCCCTGGCTGTCCTGGAACTCACTCTGTAGACCAGGCTGGCCTC

-10042 GAACTCAGAAATCCACCTGCCTCTGCCTCCCAAGTGCTGGGATTAAAGGCGTATGCTACCATGCCCGGCT

-9972 CCCTTCCACTTTTATCTCAATATGCCAGTTGGGAATCAAGAACCAAAGGATTTCTCAGTTCAATTCTTCT

-9902 TCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTT

-9832 CTTCTTCTTCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTTCTTTCCTCCTCCTTTTCTTCTT

-9762 CTTCTCCTTCTCCTTCTTCTCCTTCTTCTTCATCTTCTTCCTTCTTCGATCTCTCCAGCCCCTACTCAGT

-9692 TCAATTCTTTTTCCTCTGCTAATCAATCCAAGATGCCTACTAAATTTCTGGCGTACCTAGTTCTATCCCA

-9622 CGACTTTGAACTAGAGGACATGGGCTGACACCATTGGTGCAAGAAATGGTCTGAGAGAGATGACTGCAGG

-9552 GGATTTGGAAATGGGTTACTCACTGTCTGTTTGGCAACGAGGAGCCCTGCCTAAGCAGCATGTGAATTCC

-9482 TTGTCTTAAGGAAGCAGCTCATCTGTGAGACTTTTACCAGCAACAAATTGGAGTGGTAATTCCACTCCAC

-9412 CTGTAATTCAGTTGTATTAATACCCTACAGAGGGATCATGAAAAGTTGGAGGTCAGTAGGATGCTAACAC

-9342 TGCTGTGAAATCAGGTGTTGTCTTGGTAGTTGGTAGGAGCCTGATCTCTCACAGCGAAAGCAAAAGACAA

-9272 GCAAAGCCCGAGTAGGCTATTGAACAGGCTTTACCAAAACTAGGCCACAGTCCTAACCTGTGGACAGTAC

-9202 CTGGGCCTGTGCGGTGGGCACTCACTGACACCCTTGTGTCCTTGAGTGTACGGCTGTCCAGACTCTGAAA

-9132 TGACTTCAGAGCATTCCAAGGTAACTCCTGCCTTACTAGGAAGAGATAGCACCCCAAAATGTTCACGAAG

-9062 ACACAGTAGAGGTTTCTGCCCAGGGGTAACAAGATTCCCTTTGGTGCCTTATTTTAGCTCCTCTCCTGGT

-8992 TGCCATGAGGCCAATAAGCATCGAAATATTCTCTAGACAACTAATAAATAAAATCCACGGGGCTGCTGAC

-8922 TTGTACTGAACTCCCACACCCCAACCGACCAGATCAAACTGGAGCGATTCACACATCACCACAAAGAGCA

-8852 GGGTGTTTATTTGACCTGAGAAACTGGGAAAGCAATGTGATAGCCAAGGAGTTTTACAAGTATCAAGGCA

-8782 AATAAATCAGGAAATGTCACACCAACACAAAATAAACAAACTGTGATGGTTTGTATGTGCCTGGCTCAGG

-8712 GAGTGTGGCCTTGTTGGAGTAAGTGTGTCTTTTTGGGCGTGGGCTTTAAGACCCTACTCATAGCTTCTAG

-8642 CTCACTAGCAGCCCTCAGATGAAGATGTAGAACTTTCATCGATGCCTGCACCATGCTTGCCTGGATGCTG

-8572 CCCTGCTCCCACCTTGATGATGATGGACTGAACCTCTGAACCAGTAAGCCAGCCCCAATTAAATATCCTT

-8502 TATAAGACTTGCCTTGGTCATGGTGTCTGTTCACGGCAGTAAAACCCTAACTAAGACACAAACAGATAGA

-8432 AATAAGACATAAATAAAAACTCCTTAAAACAACCAAAGGAAAGGCCCAGTTTTACAGAGTGCACCAAGAG

-8362 AAACGGAGCCACACTCCAAGGGAGAGACATCCGAAAACACCGAGGAGAACAAGCAAAAGACAGACTGACT

-8292 GACAGGCTCACCAATAACCTAACGACCTATGCCCTGGGAAGCTGACTGTTGAAAATGAACACAATGTTAT

-8222 GGAATGCAAACCACTGGCAGTAAAGTTGTTTTTGTTTAAAAAATAATAGTTAGGCAAACGGTGTAGCTCA

-8152 GTTGACAGAGTGCTTGCCTAGCATGCACAAGACCCTCTGTTAAATCACTAACACTGCATAAAACAGGCAT

-8082 GGTGGCACTCACCATTAATCCCAGCACTTGGGAGACAGACAGGAGGATCAGAGTTCTGCATTATGCTTAA

-8012 TTACATAGTGAGTTTAAGGACATCCTGGGCTTCATGAGACCCTGTCTCAAAAAGCATAAATTTAAAAAGT

-7942 GTGTGTGTGCTGGGCATGGTGGCGCACGCCTTTTATCCCAGCACTTAGGAGGCAAAGGCAGGCAGATTTC

-7872 TGAGTTTTGAGGCCAGCCTGGTCTACAAGTGAGTTCCCAAGAACAGCCAGGGCTACAGAGAAACCCTGTC

-7802 TTGAAAAAAAAAAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGAGTGTGT

-7732 GTGTGTGTGTGTGTGAGTGTGTGTGTGAGTGTGTGTGTGTGCGTGCGTGTGTGTGCGTGCGTGTGTGTGT

-7662 GTGTGTGCGTGCGTGCGTGTGTGCGTGTGTGTGTGTGTGTGTGTGTGCGTGCGCACACTCAGAGGTTA

-7592 TGAGTGTTTTCTCAGAAAACCTAAGTTTGGTTCACACTCAGCACTTACACTGGGCAGCTTACAGCTGCCT

-7522 GTAACTTTAGCAACAGGTGTGGACACACCCTCTGGTCTCTGAAGGCACCTACATACACATGGCATATGCA

-7452 CACACAGACATACACTCATATGTATAAATAAACATTACAAGTTAAAAAAAAAATGAAGGGCTATAGAGATG

-7382 TCTCAGAGGTTAAGAGCACTTGCTGCTCTACCAGAGGACCACAGTTACGTTCCCAACACCCAAGTCAGTG

-7312 GCTCAAAACCCTCTGTAATTCCAGCTCCAGGAGATCTGATGCCTTCTTCTGGGCTCCGAAGGGAGCCTGA

-7242 ACACTCTCTCTCTCTCTCTCACACACACACACACACACACACACACACTTAAAAATAAAATTATTAAG

-7172 GAAAAATAAATTTATCTTTACATTTATAATTTTTATGTTGTGTGTATGAGTGAGTGTTTTGCCTGTACGT

-7102 ACATACGTGTGCTAACATGCATGCCTGGTGCCTTCAGAAGTGAGAAGATGGTATCAGATCGCCTAAAACT

-7032 GGAGTCACAGATGTCTGTAAGCCACCATGTGGGCCCTGGGAACTGAATCCAGGATTTTTGTAAGAGCAAC

-6962 AAATGCTCCTAAGTGCTGAACCATATCTCTAGCCCCTAAAATAAATTTTAAAAGAAAATGAAGATGGATA

-6892 GGCAGTGTAACTTGTTGTGCAGCAATCATATATAGCCTGTGTAGGGCCCTAGAGAGCAACCTTCTGTGCT

-6822 GCAAAACATACAAATAAATACATGAAAAAATAAAGGGAAATTTCTGTTACTTTCCTCCTATCTGGAACAA

-6752 CTTTTAAAAACTGAACAAAACACACAAAGCAGTAACAGTTCTCAGTTATTGGGCATCAGGTCATCTTGGA

-6682 GATGGTAAGTGGGCGAGGGAGCCTATCTTAATTATTTTCATGTTGCCATAACAAAACACCATGATGTAGG

-6612 CAACTTATAGAAGAAAGCTTTTAGTTTGTATCTCACAGTTCCAGAGGGCTGGAGTCCATGACCAAAAATG

-6542 GTGGAGCATGGCAGCAGGCAGTCCAGGGGCTGGAGATGTAGCTGGGAACTAACATCTGACCCACAAGGAT

-6472 GAGTCAGAGAAGAGGAGCTCTGCAGGTAACCTGAGTCGGGAACACCCCTCCTCTAAAAAGGCCACACCTC

-6402 CTCATCCTTCCCAAACAGCTCCACCCAATGGGAACCGAGCATTCAAATATAGGACCCCATGGGAGCAATT

-6332 CTCATTCATACCACCACAGAGCCCTCAGACGATGCCAACTTCTACCAAGGAAAGTGTCCAGGCCATGTTT

-6262 GAGGCAAGAGAGCAGTAATAAAAGAAAATAGATGATGTGGCTTATTTAATAAAGATTAAAGATTCTGGGC

-6192 CAGTACAGTGTTGCCATGAGCCTTTAATCTCAGCACTCGAGAGACAGAGGTAGGAGGATCTCTGAGTTCG

-6122 AGGCCAGCCTGGTCTACAGAGTGAGTTCTAGGACAGTTAGGGCTATGCAGAGAAGCCCTGTCTAGCAAAC

-6052 AAACAAACAAACAAACAAATGAAAACAACAAACTCTGGGCCTGTAAAAGTTTGGCTGAAATGGAAACTAA

-5982 ATAGAGAAGTCCCAGACTGGGCGAGAATATATGTAAAACATCTATCTGAAACAAACATAAGTACCCAGTT

-5912 CAATGAATAGGCAAATAACCTCATTATTTTACTTAGTAAGAAATTAAAAGTAAACACCAAAAAAGTTACT

-5842 CAGCATTTTATTCGTCGAAAAATTTCTCATTATATCTGTAACGAGATAGCACTACAGAGCTTTAGATTGG

-5772 TCCAAATTACAGATTTGCAAGGTTAATTGTCGGTGAGGAGGCGAGGCAACATGAGACCTCACACCTCTCT

-5702 TTAGGTGGGAGCATTGGATGGTGCAGCCACTTTGGAGAGCAGATTGGAAGTCTCTTGTAAATTAAGCAGT

-5632 CGTTCCCCTAAAACCCAACAATCCTACGCCTACATATTTATCCAAAAGAAATGAAAACATATGTCCACTC

-5562 CAGGACTTGTACTTGAACGTTCAAAGCACTTTTACTCATAATAGCCTAACGACTGCCAACAGGTGGGAAG

-5492 TTGAACCAATTAAAGTACATTCAGGGCAATGCAGTTCAGCCATAAAAAATACTGCTGGTGGCCAGCCATGG

-5422 TCCCAGCTCTCAGGAGGCAAAGGTGGGCAGCTTTCTGTGAGTAAGGGTAAGAATCTATCGTCCTCCCCAG

-5352 CTCCCCCCCCCCCCAAATGTGGATGCATGCAGCCACTAAATGTTTGTTGGTTCCCAGCTCTGCAGTCACC

-5282 TGTCACCTGAGATTCAGGGGACTCAAGGGAAATTCAGGCTTATCCACCTTGAAATTCCATGAAAGGAGGG

-5212 GGCAGGATTCAGTTCCTCTATCCAGGGACTGGGGACTGGGGCTGAGGCTGAGGCTGGGGCTGGGGCTGAG

-5142 GCTGAGGGCTGAGGCTGAGGCTGAGGCTGGGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCT

-5072 GAGGCTGAGGCTGGGGCTGAGGCTGAGGCTGGGGCTGGGGCTGGGGCTGGGACTGGGGACTGGGGCTGAG

-5002 GCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGAGGCTGGGGCTGAGGCTGAGGCTGAGGCTGAGGCTG

-4932 AGGCTGAGGCTGGGGCTGAGGCTGAGGCTGGGGCTGGGGCTGGGGCTGGGGCTGAGGCTGAGGCTGAGGC

-4862 TGAGGCTGAGGCTGAGGCTGGGGCTGAGGCTGGGGCTGAGGCTGGGGCTGAGGCTGAGGCTGGGGCTGAG

-4792 GCTGGGGCTGAGGCTGGGGCTGGGGCTGGGGCTGGGGCTGGGGCTGAGGCTGGGGCTGGGGCTGAGGCTG

-4722 GGGCTGGGGCTGAGGCTGAGGCTGGGGCTGGGGCTGATGTCCCTCCTGGCTATGAGTATGTCAAAGCTAC

-4652 CCTCATTTACCTGGTCTGCCATCTGTCAGCTCACGATGAGTATGAAGTACTCTTCATCAGAGAATGGAAA

-4582 CACCAGATGTGCCTGCAGGACTTAAGCCCATGGGTCTTGTGACCAAAGAGATTCCTTTACACCCCAGGAA

-4512 TAGCACTGGGTCCAGATCCTATAAAGGGAAGCTGGCTTTAGGAACCCAAGGCAGGATCATTGGAAGCAAC

-4442 TTTGGAAGGCTACCTATACCAGGTTCATACTGTGTGACTCCATTTATAAAGTAAAAAGAAATGACAGCAA

-4372 CTTGTTATCTAAACTACAAGGCCTGGAACATGTAGCCCAGTTGGGTCTTGAACGCCTCCTGCCTCAGCCT

-4302 TCTGAGCGCTAGGATTGCAGGTATTCGCAACCATCCCTGGCTAGCAAACCCTCACTGACGTGTTAGACAT

-4232 TAGCACACATAATCAAAGAACCAGGGGGCTGGTGAGATGGCTCAGTGGGTAAGAGCACCCGACTGCTCTT

-4162 CCGAAGGTCCAGAGTTCAAATCCCAGCAACCACATGGTGGCTCACAACCATCTGTGACAAGATCTGACGC

-4092 CCTCTTCTGGAGTGTCTGAAGACAGCTACAGTGTACTTACATATAATAAATAAATAAATCTTAAAAAAAA

-4022 AAAAAAAAAGAACCAGGTCCAGGAGGCAGGCCAATGGCAAACCTTTCTCACAAGGTGCTCCAGGGCAGCC

-3952 ACAGAAGTTCTGGTCTGACCCCTTGTATATTAAGTCTAGTATAGGATCTCCTTAGCGCCATTTTTAGGGG

-3882 TTTTAGAACATAGACCACTCATGCTAACATCTAGCATAGAGTATTTTTCACTGGTTGACAGCTCTTTGGT

-3812 TAGAAATTTGTGTCCAACATTTTCTATCATGTGAAACACTTCAATATGTCACTTTCAAAACAGAATTTGA

-3742 GAGTAGAAATTCAAATAATGGTACTTTACTTTGATGGCTGTCTTGCTCCGGGCAGAAATAAATGTTCTGG

-3672 GTGTCCTGGGTAACTTTTCAGTGGGGCATGGGATTGAGATTATTGAAGAAGCGATAATTAAGCCCTTTTC

-3602 TGAGCGGACTGCATGCTTTCTTTGAGCAGCCTGTGGAGTTCAGTCTGCTCTAAGTATTTTGCACTTGATC

-3522 CTTTCTGCCAAGTTTCCCCTGCTTATCCAGATGAACAACTCTATCTGAAAATCACAAGATTAATAATTAC

-3462 GCTCTAGGCTCAATGAAACCCTATTTGGATCACTGTCATTTTTTTTTTTTTTTGTATTTAGTGCATTTCCCC

-3392 AGGAAAATAGCAAAGCAACTTTATAAATGCATCAGGCAAGGAAGGATTTTAAATTTAGCCCATTGTCGTT

-3322 TCCTTTTGCAGGCTGTGTGACTCACCTCCACCTCCTGGGGCCAACCTGATGCAGTAATGGGCTTAGGCCA

-3252 GCTCCCCTGCCCTGGTCTCACGTTTGTACCATCCCTTCCCATGTGCTGTGTGCGCCGATAAAGTGAACGT

-3182 CCAAATCTAAGACAAAGAAACCTGCAGGCCAGTCATGATTAGTGAGCTGCCCAGAAGACCATACCTCTGT

-3112 GGCTTTGTCAGTGTCGCACGGGGCTTTGTTATCCATGCATAAACTGAGCTGATGGCTCCTGGGGTTGCCT

-3042 ATGGAGGCAGAATTTTCAACCCTCCAGCTCTCCTGCTCAGCACCACATCAGGAGAACAGCATGCCTCTCT

-2972 CCCTGGAATGAAGAGGCCTTGATCATCCAACAACAGATTCTACCCAATGGAAATGCCAGAGTTTTGCATG

-2902 TCAAATAAGTAGTAGTGGACGTGATTTAGTGGTCTCATTTCTCTGATGAAATACCCGAAAGGACAGTTTA

-2832 AGGAAGAAAGAGTTTATTTTAGCTCACAGTTTAAAGGTGCGGTCCACCACAGTGGAGGAAGCTTGACAGT

-2762 GAGAACAGTGGGACCAGGAGCGAGGCAGCTGGTCACACTGCATCTGCAGTCAGGAGGAAGCATAGAGTCT

-2692 CTCAAAGGTGCATCTCCTAGGCCATTCCAATCTAATCAAATGACAGTGAAAATCAGCCATGGGTGCCAGC

-2622 CACTAAAGAACGCATAGCTCTGTGGAGATATGAGCAGGCATGAAGTCCTATGTTCATTTTCACTATTGCT

-2552 ATGGTTTAAAGTTTTATATATTTTAACTTTTTTGTGCATGAATATTAGCATGCATGTATACAAGTGTACT

-2482 ATGCATGCTTAGTGCCCGAGAAGATCAGAAGAAAGCTGTTGATCCCTGGAAACTAGTTTGTAAGATGCTA

-2412 TGTGGATGCTGGGGCTCAAACTCATCTCCTCTGCAACTGTAGCAAGTGTTGTGGGGCAGTGGTGGTGCAT

-2342 GCCTTTAATCCCAGCCCATGGGAGGCAAAGGCAGGCAAATCTCTGTGAATTCCAGGCCAGTCTGGTCTGC

-2272 AGAGCACGATCTAGCACAGCCAGTGTGACACACAGAAACTCTGCCTCAAAAAAAAAGAGAGAGAGAAAAG

-2202 AAAGAGAGAGTACCAATTGCTTTGAACCACTAAGACATCTCCCCAGCCCCACCTTACTACTGCTCTATCA

-2132 GGGTAGCAGGTCCCCTAACCCATACCCTAGTCTTGGTTCACCTAAAGGGGGGGGGTGTTCTTTAAGTATT

-2062 TGGAAAAGGATACACACCTCTGAGATGGACAGCCTTTCTGTGAGAAAGGCCTTGAGGTGTTAGACAGTGA

-1992 GTGAGGCTCTCCCTCAGAGCAAGAATAGGAGTGGATGGAGAGATGGAGAGAGTACAAGAACAATCTATAG

-1922 TGTCTATAGTGGAAGAAGCAGGCCAGGCATGTTAGAAACAGCCTAAGAGGTCTGGAGAGATGGCTCAGAG

-1852 GTTGAACACACTAACCGCACTTTCAGAGGTACTTGAGTTCAATTCCCAGCAACCACATGGAGGCTCACAA

-1782 CCATCTGTAATATGACCTTGGTGCCCTCTTCTGGCGTGTAGTACAGAACACTGTACATAACAAACAAACA

-1712 AATAATAAATAAATAAATAATTTTTTTTTAAAAAAAGAAAAAGAAAAGAAAGAGCCAAAGACTATAACAG

-1642 CCGTGCAAGACCAAGGCTTTGTTTCCTGACCTTGGATGAGTCAGCTCCATAGTTATCTCTCCTAATTCCC

-1572 TGAGTCGAACCCAGGTCAGAGGTAAAGACAGACCCATAACTAGTTCAAAATGTATACATACAACCTATAT

-1502 ATGTATATGTGTAGGAAGGAACCAAAGCTCAGAAGCTACACACAAATCTTGCTAGGAACAAAATTTTGAA

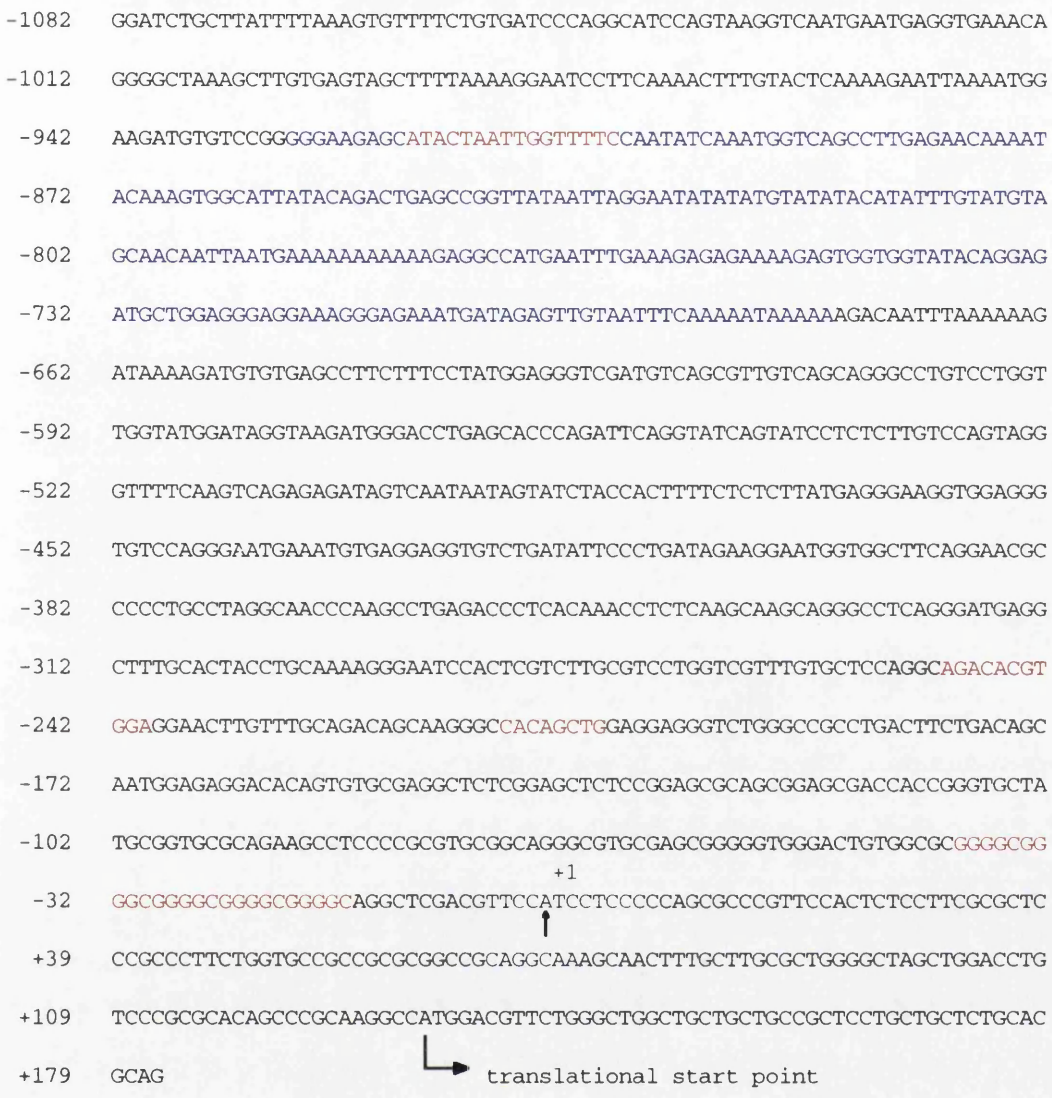
-1432 AGCTTGTAACCCCGGGACTTGAGGCGAAGCCGGATGGGTGGCCAGTAGTTCAGAGTCATCCTTGACTATA

-1362 TAGCAAGTTCAAAACCAGCCTGAACTTCATCAGACCCTGTCTGAAAAATGAAAACAAAACAAAACAAAAC

-1292 AAAAAAAAAACCATATCTAAGCAGTTCCTTGTGCTAGAAGCTCTATAAGTCATCTTTTTCAACACACCTG

-1222 ACTGGCCAGTTTCCAGGCATCCCCAGGGCACCTGAGCATAGGGCATCCTTACTATGTCGATTAGGCTGTA

-1152 TCTAAACCTCTCATCTTCTGCTTCAGTCTCTGAAGTAGCTGGAACTCCACGTGTTCACCACTGGTGGGTG


-1082 GGATCTGCTTATTTTAAAGTGTTTTCTGTGATCCCAGGCATCCAGTAAGGTCAATGAATGAGGTGAAACA
-1012 GGGGCTAAAGCTTGTGAGTAGCTTTTAAAAGGAATCCTTCAAAACTTTGTACTCAAAAGAATTAAAATGG
-942 AAGATGTGTCCGGGGGAAGAGCATACTAATTGGTTTTCCAATATCAAATGGTCAGCCTTGAGAACAAAAT
-872 ACAAAGTGGCATTATACAGACTGAGCCGGTTATAATTAGGAATATATATGTATATACATATTTGTATGTA
-802 GCAACAATTAATGAAAAAAAAAAAAGAGGCCATGAATTTGAAAGAGAGAAAAGAGTGGTGGTATACAGGAG
-732 ATGCTGGAGGGAGGAAAGGGAGAAATGATAGAGTTGTAATTTCAAAAATAAAAAAGACAATTTAAAAAAG
-662 ATAAAAGATGTGTGAGCCTTCTTTCCTATGGAGGGTCGATGTCAGCGTTGTCAGCAGGGCCTGTCCTGGT
-592 TGGTATGGATAGGTAAGATGGGACCTGAGCACCCAGATTCAGGTATCAGTATCCTCTCTTGTCCAGTAGG
-522 GTTTTCAAGTCAGAGAGATAGTCAATAATAGTATCTACCACTTTTCTCTCTTATGAGGGAAGGTGGAGGG
-452 TGTCCAGGGAATGAAATGTGAGGAGGTGTCTGATATTCCCTGATAGAAGGAATGGTGGCTTCAGGAACGC
-382 CCCCTGCCTAGGCAACCCAAGCCTGAGACCCTCACAAACCTCTCAAGCAAGCAGGGCCTCAGGGATGAGG
-312 CTTTGCACTACCTGCAAAAGGGAATCCACTCGTCTTGCGTCCTGGTCGTTTGTGCTCCAGGCAGACACGT
-242 GGAGGAACTTGTTTGCAGACAGCAAGGGCCACAGCTGGAGGAGGGTCTGGGCCGCCTGACTTCTGACAGC
-172 AATGGAGAGGACACAGTGTGCGAGGCTCTCGGAGCTCTCCGGAGCGCAGCGGAGCGACCACCGGGTGCTA
-102 TGCGGTGCGCAGAAGCCTCCCCGCGTGCGGCAGGGCGTGCGAGCGGGGGTGGGACTGTGGCGCGGGGCGG
+1
-32 GGCGGGGCGGGGCGGGGCAGGCTCGACGTTCCATCCTCCCCCAGCGCCCGTTCCACTCTCCTTCGCGCTC
+39 CCGCCCTTCTGGTGCCGCCGCGCGGCCGCAGGCAAAGCAACTTTGCTTGCGCTGGGGCTAGCTGGACCTG
+109 TCCCGCGCACAGCCCGCAAGGCCATGGACGTTCTGGGCTGGCTGCTGCTGCCGCTCCTGCTGCTCTGCAC
+179 GCAG
translational start point

3.2.4 Analysis of the 5' - flanking region sequence

The sequence was analysed using various computer programs e.g. TFSEARCH, TESS and RepeatMasker 2 (**Section 2.2.3.3**) designed to allow comparisons with known transcription binding factors and repeat regions and also to databases of known sequences, e.g. EMBL. **Table 3.1** catalogues the identified recognition sequences that share homology with consensus sequences for transcription factor binding sites and **Table 3.2** gives a brief description of the transcription factors. **Table 3.3** lists the repeat regions identified.

Table 3.1 ***Recognition sequences for transcription factors identified in the 5'-upstream region of the $GABA_A$ receptor δ subunit gene***

Transcription factor (orientation +/-)	Position	Consensus sequence	Actual sequence	Percentage match
Sp1 (+)	-39 to -15	KRGGCGKRRY	GGGGCGGGGC	95.2%
AP-4 (+)	1. -113	YCAGCTGYGG	ACAGCTGGAG	1. 97.3%
(+)	2. -2738		GCAGCTGGTC	2. 95.8%
USF (-)	1. -241	NCACGTGN	CCACGTGT	98%
(+)	2. -1106		CCACGTGT	98%
N-Myc (-)	1. -240	NNNCACGTGNNN	CTCCACGTGTCT	94.7%
(+)	2. -1108		CTCCAGGTGTTC	93.3%
S8 (-)	1. -906	NNANYYAATTANYNN	AAAACCAATTAGTAT	94.6%
(+)	2. -5491		TGAACCAATTAAAGT	97.1%
GATA-1 (+)	1. -1401	SGATAR	GGATGG	94.3%
(-)	2. -2133		TGATAG	93.9%
AP-1 (+)	1. -1606	TKAGTCAG	TGAGTCAG	100%
(+)	2. -6473		TGAGTCAG	100%
HNF-3b (-)	1. -1706	NNNTRTTTRYTY	TATTATTTGTTT	94.2%
(+)	2. -10253		AAATATTTGTTT	94.8%
C/EBP (+)	1. -2066	NNTKTGGWNANNN	TATTTGGAAAAGG	94.6%
(+)	2. -9551		GATTTGGAAATGG	93.1%
Nkx-2 (-)	1. -3535	TYAAGTG	TCAAGTG	100%
(-)	2. -3611		TTAATTA	94.1%
(+)	3. -8016		TTAATTA	94.1%

Transcription factor (orientation +/-)	Position	Consensus sequence	Actual sequence	Percentage match
MZF1 (-)	1. -5344	NGNGGGGA	GGGGGGGA	94.8%
(-)	2. -10300		AGTGGGGA	100%
MyoD (+)	-9331	CAACTGAC	CAGGTGTT	93%
GATA-2 (+)	-8815	WGATAR	TGATAG	92.5%
p300 (+)	-8717	NNNRGGAGTNNNNS	TCAGGGAGTGTGGC	95%
CDP CR (+)	-8605	NATYGATSSS	CATCGATGCC	95.9%
Lyf-1 (-)	1. --6388	TTTGGGAGR	TTTGGGAAG	94.8%
(+)	2. -10458		TTTGGGAAA	93.5%

The table lists the recognition sequences for transcription factor binding sites that were identified in the 5' - upstream region of the $GABA_A$ receptor δ subunit gene by sequence analysis using the TFSEARCH database. The location of the sites are given and relate to **Figure 3.6**, where +1 is equivalent to the 5' - most transcriptional start point. The consensus sequences for the various elements and the percentage match to that sequence is indicated. The actual sequences present are listed and the matches to the consensus sequence is indicated in bold

IUB code : R = A, G; M = A, C; W = A, T; Y = T, C; K = T, G; S = G, C; B = T, G, C; V = A, G, C; H = A, T, C; D = A, T, G.

Table 3.2 ***Transcription factors whose recognition sequences have been identified in the $GABA_A$ receptor δ subunit gene 5' - upstream region***

Transcription factor	Protein type	Description	Reference
AP-1	bZIP	Positive or negative regulating factor of various cellular and viral promoters	Lee *et al.*, 1987
AP-4	b-HLH-Zip	Activates both cellular and viral genes	Mermod *et al.*, 1988
CDP CR	Homeodomain protein with 3 CUT repeats	Cut-like homeodomain protein acting as a negative regulator of gene expression	Harada *et al.*, 1995

Transcription factor	Protein type	Description	Reference
C/EBP	bZIP	Transcription factor family; bind as dimers; show tissue- and stage-restricted expression	Grange *et al.*, 1991
GATA-1	ZF	Developmentally regulated; plays a role in erythroid cell-specific transactivation. Activates globin genes.	Merika *et al.*, 1993
GATA-2	ZF	similar to GATA-1, but has a more restricted expression pattern.	Merika *et al.*, 1993
HNF-3b	FHD	Involved in transcriptional activation of liver specific genes	Lai *et al.*, 1990
Lyf-1	-	May be a general transcriptional activator for genes with restricted expression.	Lo *et al.*, 1991
MyoD	b-HLH	Transcriptional activator	Murre *et al.*, 1989a
MZFI	ZF	May have role in regulating transcription during developmental process. Thought to be involved in regulating hematopoiesis	Morris *et al.*, 1994
Nkx-2	b-HLH	Transcriptional activator	Chen *et al.*, 1995
N-Myc	b-HLH-Zip	Belongs to myc family; involved in cell proliferation and differentiation.	Alex *et al.*, 1992
p300	-	selectively binds DNA sequences related to the enhancer elements recognised by NF-κB	Rikitake *et al.*, 1992
S8	HTH	Homeobox gene involved in autoregulation and trans-regulation of other homeobox genes and unknown target genes.	de Jong *et al.*, 1993
Sp1	ZF	Developmentally regulated; participates in the assembly of active transcription complexes.	Briggs *et al.*, 1986
USF	b-HLH-Zip	Upstream stimulatory factors implicated in the regulation of tissue-specific and developmentally regulated genes.	Carthew *et al.*, 1985

The table lists the transcription factors whose recognition sequences have been identified in the δ 5' - upstream region (**Table 3.1**). The protein type and a brief description of the

factor is included. They are referenced. Abbreviations used : ZF = zinc finger; bZIP = basic leucine zipper; b-HLH = basic helix loop helix; b-HLH-Zip = basic helix - loop - helix - leucine zipper; FHD = fork head domain.

Table 3.3 ***Repeat sequences identified in the 5' - upstream region of the $GABA_A$ receptor δ subunit gene***

Repeat	Position (bp)	Complete/Incomplete	Strand
B1-MM (SINE/Alu)	-9974 to-10117	Complete	Minus
(GAA)n (Simple repeat)	-9716 to -9770	-	Minus
(GGA)n (Simple repeat)	-9771 to -9909	-	Minus
ORR1A2 (LTR/MaLR)	-8741 to -8445	Incomplete	Plus
B4 (SINE/B4)	-8159 to -7957	Incomplete	Plus
B1-F (SINE/Alu)	-8089 to -7953	Incomplete	Plus
B1-MM (SINE/Alu)	-7934 to -7791	Complete	Plus
(CA)n (Simple repeat)	-7990 to -7614	-	Minus
B3A (SINE/B2)	1. -7603 to 7404 2. -7396 to -7170	Incomplete Incomplete	Plus Plus
B3 (SINE/B2)	-7144 to -6927	Complete	Minus
ORR1D (LRT/MaLR)	-6659 to -6316	Complete	Minus
B1-MM (SINE/Alu)	-6185 to -6046	Incomplete	Plus
L1M4-orf2 (LINE/L1)	-5990 to -5915	Incomplete	Plus
L1M2 (LINE/L1)	-5959 to -5517	Incomplete	Plus
B1-F(SINE/Alu)	-5438 to -5386	Incomplete	Plus
PB1D10 (SINE/Alu)	-4343 to -4262	Incomplete	Minus
B2 (SINE/B2)	-4208 to -4011	Complete	Plus
MTE (LTR/MaLR)	-2865 to -2696	Incomplete	Minus
B3 (SINE/B2)	-2537 to 2363	Incomplete	Minus
B1-MM (SINE/Alu)	-2359 to -2213	Complete	Plus
B2 (SINE/B2)	-1874 to -1660	Complete	Plus
B1-F (SINE/Alu)	-1431 to -1309	Incomplete	Plus
PB1 (SINE/Alu)	-1138 to -1092	Incomplete	Minus
Lx9 (LINE/L1)	-929 to -679	Incomplete	Plus

The table lists the repeat sequences identified using the RepeatMasker 2 computer program. The position of the repeats is shown and relates to **Figure 3.6** where +1 is the 5' - most transcription start point. The direction of the repeats is shown. The repeats account for 36.6% of 10.6 kb of 5' - upstream sequence. References : LINEs = Smit *et al.*, 1995; SINEs = Krayev *et al.*, 1982; LTR/MaLR = Smit, 1993.

3.2.5 Comparison of murine and rat 5' - promoter regions

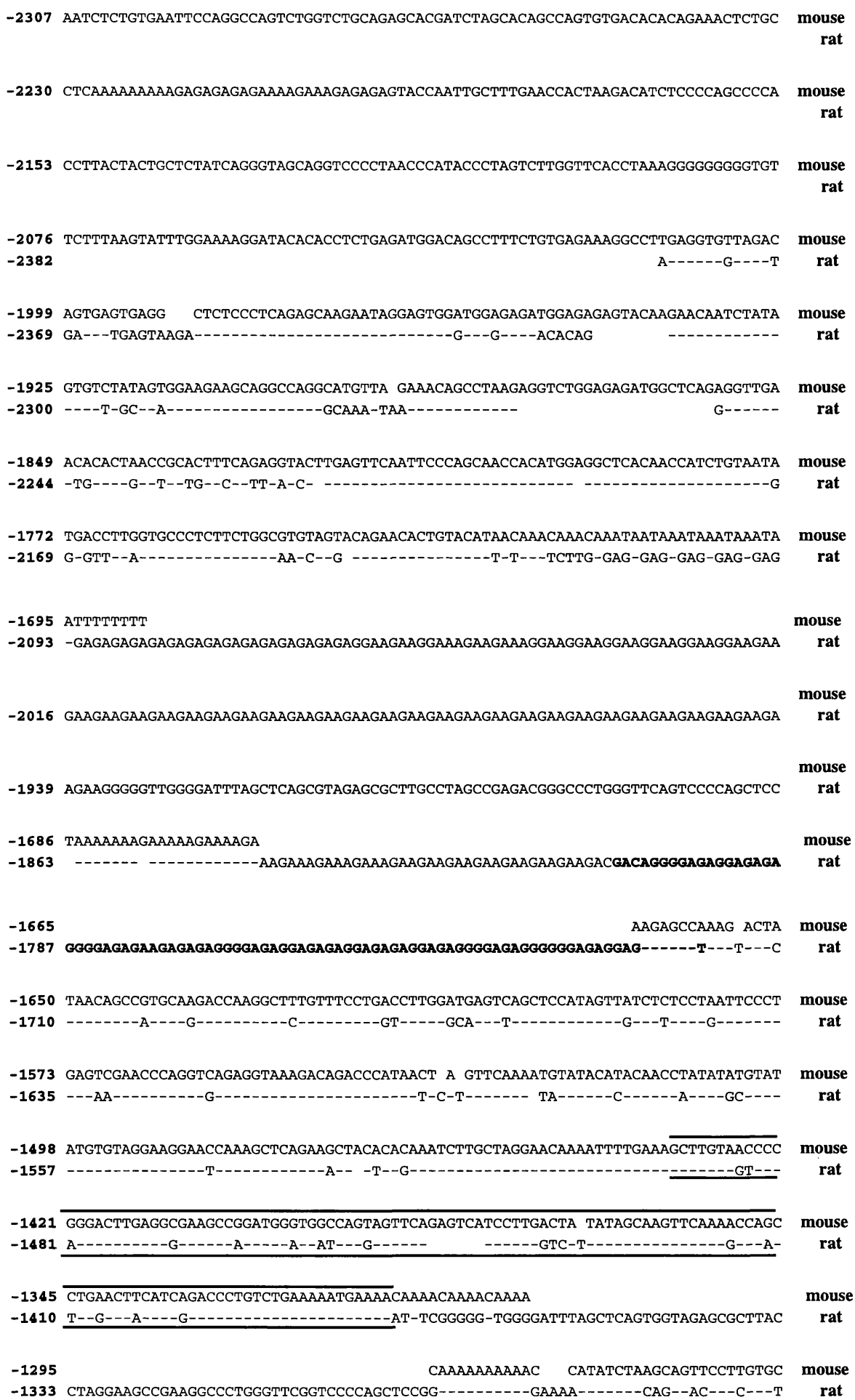
As 3 kb of rat δ subunit gene promoter DNA sequence was available on the data base (Motejlek *et al.*, 1994, EMBL Accession no X69986), comparison of the rat and mouse sequences was possible. This was achieved using the GeneJockey II (Biosoft) software program (**Figure 3.7**). Putative transcription factor recognition sequences and repeat regions common to both sequences are indicated, as are the transcription start points.

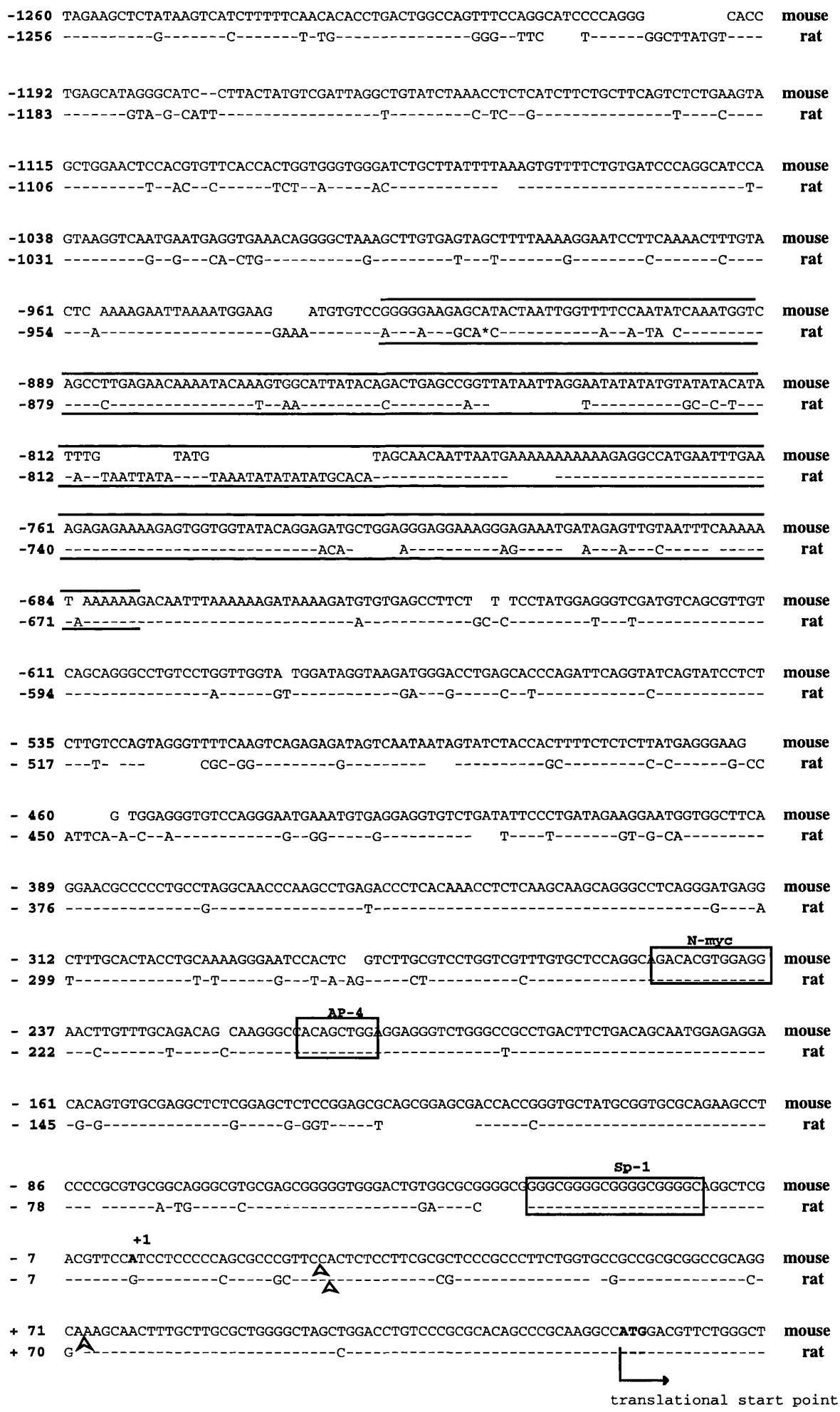
Figure 3.7 Sequence comparison of mouse and rat 5' - upstream region

Figure 3.7 Sequence comparison of mouse and rat 5' - upstream region

3.75 kb of mouse 5' -upstream sequence was compared to 3 kb of rat 5' -upstream sequence using GeneJockey II (Biosoft). Homologous bases are indicated by a dash. Bases that differ are written. Sequence that is absent is indicated with a gap. Putative transcription factor binding sites that are common to both sequences are boxed. Repeat regions that are present in both sequences are underlined for rat, and overlined for mouse. The numbering system relates to **Figure 3.5** where +1 is the 5' - most transcriptional start point.

```
-3752 GAATTTGAGAGTAG AAATTCAAAT AATGGTACTTTACTTTGATGGCTGTCTTGCTCCGGGCAGAAATAAATGTTC  mouse
-3031 -----C-GACT-T-CT-CACT----TC-AACA-TGGC--------AC----------A--------C--------  rat

-3677 TGGGTGTCCTGGGTAACTTTTCAGTGGGGCATGGGATTGAGATTATTGAAGAAGCGATAATTAAGCCCTTTTCTGAG  mouse
-2954 -AAT-----C--------C--T----A--T-----G---------C---G---C-T--------A----C-------  rat

-3600 CGGACTGCATGCTTTCTTTGAGCAGCCTGTGGAGTTCAGTCTGCTCTAAGTATTTTGC              ACTTG  mouse
-2877 T----C-------- ------              G-------TGG-GTT--G-C--ATCCAAGTATTTTGT-----  rat

-3537 ATCCTTTCTGCCAAGTTTCCCCTGCTTATCCAGATGAACAACTCTATCTGAAAATCACAAGATTAATAATTACGCTC  mouse
-2815 ----------------------------------------------------C------------------A---  rat

-3460 TAGGCTCAATGAAACCCTATTTGGATCACTGTCATTTTTTTTTTTTTGTATTTAGTGCATTTCCC CAGGAAAATAG  mouse
-2738 ---------------------------------------C       -------------------A----------  rat

-3384 CAAAGCAACTTTATAAATGCATCAGGCAAGGAAGGATTTTAAATTTAGCCCATTGTCGTTTCCTTTTGCAGGCTGTG  mouse
-2668 ----A-GCT-CATA-G-C---------    -------------------A-----G-------C---------A--  rat

-3307 TGACTCACCTCCACCTCCTGGGGCCAACCTGATGCAGTAATGGGCTTAGGCCAGCTCCCCTGCCCTGGTCTCACGTT  mouse
-2595 --------------T----------------T--------C-------- G--------------A--------A--  rat

-3230 TGTACCATCCCTTCCCATGTGCTGTGTGCGCCGATAAAGTGAACGTCCAAATCTAAG ACAAAGAAACCTGCAGGCC  mouse
-2519 ----------------T------AG----A-----C------C-A-----------G-GC--TGTG-T-C-CCC-T  rat

-3154 AGTCATGATTAGTGAGCTGCCCAGAAGACCATACCTCTGTGGCTTTGTCAGTGTCGCACGGGGCTTTGTTATCCATG  mouse
-2442 CC--                                                                          rat

-3077 CATAAACTGAGCTGATGGCTCCTGGGGTTGCCTATGGAGGCAGAATTTTCAACCCTCCAGCTCTCCTGCTCAGCACC  mouse
                                                                                    rat

-3000 ACATCAGGAGAACAGCATGCCTCTCTCCCTGGAATGAAGAGGCCTTGATCATCCAACAACAGATTCTACCCAATGGA  mouse
-2438                          C-------GCC-TT  ---------------------------------A-G  rat

-2923 AATGCCAGAGTTTTGCATGTCAAATAAGTAGTAGTGGACGTGATTTAGTGGTCTCATTTCTCTGATGAAATACCCGA  mouse
-2388 --AAA-                                                                       rat

-2846 AAGGACAGTTTAAGGAAGAAAGAGTTTATTTTAGCTCACAGTTTAAAGGTGCGGTCCACCACAGTGGAGGAAGCTTG  mouse
                                                                                    rat

-2769 ACAGTGAGAACAGTGGGACCAGGAGCGAGGCAGCTGGTCACACTGCATCTGCAGTCAGGAGGAAGCATAGAGTCTCT  mouse
                                                                                    rat

-2695 CAAAGGTGCATCTCCTAGGCCATTCCAATCTAATCAAATGACAGTGAAAATCAGCCATGGGTGCCAGCCACTAAAGA  mouse
                                                                                    rat

-2615 ACGCATAGCTCTGTGGAGATATGAGCAGGCATGAAGTCCTATGTTCATTTTCACTATTGCTATGGTTTAAAGTTTTA  mouse
                                                                                    rat

-2538 TATATTTTAACTTTTTTGTGCATGAATATTAGCATGCATGTATACAAGTGTACTATGCATGCTTAGTGCCCGAGAAG  mouse
                                                                                    rat

-2461 ATCAGAAGAAAGCTGTTGATCCCTGGAAACTAGTTTGTAAGATGCTATGTGGATGCTGGGGCTCAAACTCATCTCCT  mouse
                                                                                    rat

-2384 CTGCAACTGTAGCAAGTGTTGTGGGGCAGTGGTGGTGCATGCCTTTAATCCCAGCCCATGGGAGGCAAAGGCAGGCA  mouse
                                                                                    rat
```


-2307 AATCTCTGTGAATTCCAGGCCAGTCTGGTCTGCAGAGCACGATCTAGCACAGCCAGTGTGACACACAGAAACTCTGC mouse
rat
-2230 CTCAAAAAAAAAAGAGAGAGAGAAAAGAAAGAGAGAGTACCAATTGCTTTGAACCACTAAGACATCTCCCCAGCCCCA mouse
rat
-2153 CCTTACTACTGCTCTATCAGGGTAGCAGGTCCCCTAACCCATACCCTAGTCTTGGTTCACCTAAAGGGGGGGGGTGT mouse
rat
-2076 TCTTTAAGTATTTGGAAAAGGATACACACCTCTGAGATGGACAGCCTTTCTGTGAGAAAGGCCTTGAGGTGTTAGAC mouse
-2382 A------G----T rat
-1999 AGTGAGTGAGG CTCTCCCTCAGAGCAAGAATAGGAGTGGATGGAGAGATGGAGAGAGTACAAGAACAATCTATA mouse
-2369 GA---TGAGTAAGA----------G---G----ACACAG ---------- rat
-1925 GTGTCTATAGTGGAAGAAGCAGGCCAGGCATGTTA GAAACAGCCTAAGAGGTCTGGAGAGATGGCTCAGAGGTTGA mouse
-2300 ----T-GC--A----------GCAAA-TAA---------- G------ rat
-1849 ACACACTAACCGCACTTTCAGAGGTACTTGAGTTCAATTCCCAGCAACCACATGGAGGCTCACAACCATCTGTAATA mouse
-2244 -TG----G--T--TG--C--TT-A-C- ---------- ----------G rat
-1772 TGACCTTGGTGCCCTCTTCTGGCGTGTAGTACAGAACACTGTACATAACAAACAAACAAATAATAAATAAATAAATA mouse
-2169 G-GTT--A----------AA-C--G ----------T-T---TCTTG-GAG-GAG-GAG-GAG-GAG rat
-1695 ATTTTTTTT mouse
-2093 -GAGAGAGAGAGAGAGAGAGAGAGAGAGGAAGAAGGAAAGAAGAAAGGAAGGAAGGAAGGAAGGAAGGAAGAA rat
mouse
-2016 GAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGA rat
mouse
-1939 AGAAGGGGGTTGGGGATTTAGCTCAGCGTAGAGCGCTTGCCTAGCCGAGACGGGCCCTGGGTTCAGTCCCCAGCTCC rat
-1686 TAAAAAAAGAAAAAGAAAAGA mouse
-1863 ------- ----------AAGAAAGAAAGAAAGAAGAAGAAGAAGAAGAAGAC**GACAGGGGAGAGGAGAGA** rat
-1665 AAGAGCCAAAG ACTA mouse
-1787 **GGGGAGAGAAGAGAGAGGGGAGAGGAGAGAGGAGAGAGGAGAGGGGAGAGGGGGGAGAGGAG------T**---T---C rat
-1650 TAACAGCCGTGCAAGACCAAGGCTTTGTTTCCTGACCTTGGATGAGTCAGCTCCATAGTTATCTCTCCTAATTCCCT mouse
-1710 --------A----G----------C---------GT-----GCA---T----------G---T----G------- rat
-1573 GAGTCGAACCCAGGTCAGAGGTAAAGACAGACCCATAACT A GTTCAAAATGTATACATACAACCTATATATGTAT mouse
-1635 ---AA----------G----------T-C-T------- TA------C------A----GC---- rat
-1498 ATGTGTAGGAAGGAACCAAAGCTCAGAAGCTACACACAAATCTTGCTAGGAACAAAATTTTGAAAGCTTGTAACCCC mouse
-1557 ----------T----------A-- -T--G----------GT--- rat
-1421 GGGACTTGAGGCGAAGCCGGATGGGTGGCCAGTAGTTCAGAGTCATCCTTGACTA TATAGCAAGTTCAAAACCAGC mouse
-1481 A----------G------A-----A--AT---G------ ------GTC-T----------G---A- rat
-1345 CTGAACTTCATCAGACCCTGTCTGAAAAATGAAAACAAAACAAAACAAAA mouse
-1410 T--G---A----G----------AT-TCGGGGG-TGGGGATTTAGCTCAGTGGTAGAGCGCTTAC rat
-1295 CAAAAAAAAAAC CATATCTAAGCAGTTCCTTGTGC mouse
-1333 CTAGGAAGCCGAAGGCCCTGGGTTCGGTCCCCAGCTCCGG----------GAAAA-------CAG--AC---C---T rat


-1260 TAGAAGCTCTATAAGTCATCTTTTTCAACACACCTGACTGGCCAGTTTCCAGGCATCCCCAGGG CACC mouse
-1256 ----------G-------C-------T-TG---------------GGG--TTC T------GGCTTATGT---- rat
-1192 TGAGCATAGGGCATC--CTTACTATGTCGATTAGGCTGTATCTAAACCTCTCATCTTCTGCTTCAGTCTCTGAAGTA mouse
-1183 -------GTA-G-CATT-----------------T---------C-TC--G---------------T----C---- rat
-1115 GCTGGAACTCCACGTGTTCACCACTGGTGGGTGGGATCTGCTTATTTTAAAGTGTTTTCTGTGATCCCAGGCATCCA mouse
-1106 ---------T--AC--C------TCT--A-----AC------------ ------------------------T- rat
-1038 GTAAGGTCAATGAATGAGGTGAAACAGGGGCTAAAGCTTGTGAGTAGCTTTTAAAAGGAATCCTTCAAAACTTTGTA mouse
-1031 ---------G--G---CA-CTG----------G---------T---T-------G--------C-------C---- rat
-961 CTC AAAAGAATTAAAATGGAAG ATGTGTCCGGGGGAAGAGCATACTAATTGGTTTTCCAATATCAAATGGTC mouse
-954 ---A-----------------GAAA--------A---A---GCA*C-----------A--A-TA C--------- rat
-889 AGCCTTGAGAACAAAATACAAAGTGGCATTATACAGACTGAGCCGGTTATAATTAGGAATATATATGTATATACATA mouse
-879 ----C----------------T--AA---------C--------A-- T----------GC-C-T--- rat
-812 TTTG TATG TAGCAACAATTAATGAAAAAAAAAAAGAGGCCATGAATTTGAA mouse
-812 -A--TAATTATA----TAAATATATATATGCACA--------------- ------------------------ rat
-761 AGAGAGAAAAGAGTGGTGGTATACAGGAGATGCTGGAGGGAGGAAAGGGAGAAATGATAGAGTTGTAATTTCAAAAA mouse
-740 ----------ACA- A----------AG----- A---A---C----- ----- rat
-684 T AAAAAAGACAATTTAAAAAAGATAAAAGATGTGTGAGCCTTCT T TCCTATGGAGGGTCGATGTCAGCGTTGT mouse
-671 -A----------A------------GC-C---------T---T---------- rat
-611 CAGCAGGGCCTGTCCTGGTTGGTA TGGATAGGTAAGATGGGACCTGAGCACCCAGATTCAGGTATCAGTATCCTCT mouse
-594 ----------A------GT----------GA---G-----C--T----------C---------- rat
- 535 CTTGTCCAGTAGGGTTTTCAAGTCAGAGAGATAGTCAATAATAGTATCTACCACTTTTCTCTCTTATGAGGGAAG mouse
- 517 ---T- --- CGC-GG---------G--------- ----------GC---------C-C------G-CC rat
- 460 G TGGAGGGTGTCCAGGGAATGAAATGTGAGGAGGTGTCTGATATTCCCTGATAGAAGGAATGGTGGCTTCA mouse
- 450 ATTCA-A-C--A----------G--GG-----G---------- T----T-------GT-G-CA--------- rat
- 389 GGAACGCCCCCTGCCTAGGCAACCCAAGCCTGAGACCCTCACAAACCTCTCAAGCAAGCAGGGCCTCAGGGATGAGG mouse
- 376 ----------G----------T----------G----A rat
N-myc
- 312 CTTTGCACTACCTGCAAAAGGGAATCCACTC GTCTTGCGTCCTGGTCGTTTGTGCTCCAGGCAGACACGTGGAGG mouse
- 299 T----------T-T------G---T-A-AG-----CT----------C---------- rat
AP-4
- 237 AACTTGTTTGCAGACAG CAAGGGCCACAGCTGGAGGAGGGTCTGGGCCGCCTGACTTCTGACAGCAATGGAGAGGA mouse
- 222 ---C-------T-----C---------T---------- rat
- 161 CACAGTGTGCGAGGCTCTCGGAGCTCTCCGGAGCGCAGCGGAGCGACCACCGGGTGCTATGCGGTGCGCAGAAGCCT mouse
- 145 -G-G----------G-----G-GGT-----T ------C---------- rat
Sp-1
- 86 CCCCGCGTGCGGCAGGGCGTGCGAGCGGGGGTGGGACTGTGGCGCGGGGCGGGGCGGGGCGGGGCGGGGCAGGCTCG mouse
- 78 --- ------A-TG-----C----------GA----C ---------- ------ rat
+1
- 7 ACGTTCCATCCTCCCCCAGCGCCCGTTCCACTCTCCTTCGCGCTCCCGCCCTTCTGGTGCCGCCGCGCGGCCGCAGG mouse
- 7 -------G---------C-----GC----------CG---------- -G----------C- rat
+ 71 CAAAGCAACTTTGCTTGCGCTGGGGCTAGCTGGACCTGTCCCGCGCACAGCCCGCAAGGCCATGGACGTTCTGGGCT mouse
+ 70 G----------C---------- rat
translational start point

3.3 DISCUSSION

Identification of the transcription start points (tsp) was achieved using the oligo - capping technique (Maruyama *et al.*, 1994). This was performed 3 times. The first experiment used 3' - oligonucleotide primers from the 2nd and 1st exons. Two major and several minor start sites were identified. In the second oligo - capping experiment, 3' - oligonucleotide primers from the 3rd and 2nd exons were used. It was possible that the primers used in the first experiment could miss some tsp e.g. if there was an alternative 1st exon. Using primers from the 3rd and 2nd exons would eliminate that possibility. Again two major and several minor start sites were identified. Due to the large number of sites identified, a new column - purified 5' - primer (Cruachem) was used in the 3rd experiment to eliminate "wobble" at the 3' - end of the oligonucleotide. Twenty clones were sequenced and the same tsp were identified. However, 4 clones gave putative tsp at the 5' - end of exon 2. Further investigation of these sites and of intron 2 showed that these were errors.

When the data from the three experiments were combined (**Figure 3.4**), two major sites within two main clusters were identified. Only one of the sites coincided with those detected in the rat. The other major cluster at position + 73 (**Figure 3.7**) has not been identified in the rat.

The subcloning of small fragments of the promoter region into pBluescript allowed the sequencing of both strands of DNA to be carried out more easily. 10.6 kilobases of double - stranded DNA sequence were obtained for the murine 5' - flanking region of the $GABA_A$ receptor δ subunit. Computer analysis of this sequence revealed the presence of several recognition sites for known transcription factors (**Table 3.1**). Many of these factors have been shown to be involved in the regulation of tissue - specific and developmentally - regulated genes. Gel shift assays, DNase I footprinting analysis and mutational analysis could be performed to test for their functionality.

A large percentage (36.6%) of the 5' - upstream region is composed of repeat regions (**Table 3.3**). While most of this will be junk DNA, recent evidence has suggested that these repeat regions may actually have some function. They may interact with

surrounding sequences and nearby genes, serve as recombination hotspots, or may be involved in the stabilisation of chromatin structure. Several papers have described B1 or B2 elements affecting the transcription of genes. Arranz *et al.* (1994) demonstrated that two B2 sequences upstream from the MOK-2 promoter (a murine TFIIIA-related zinc - finger protein) exert a negative cis - acting effect. Also, Bladon and McBurney (1991) demonstrated that a B2 element present in the 5' -untranslated region of a reporter gene reduced expression, while in the 3' - region, it increased expression.

As 3 kb of rat δ subunit gene promoter DNA sequence was available on the data base (Motejlek *et al.*, 1994, EMBL Accession no X69986), comparison of the rat and mouse sequences was possible (**Figure 3.7**). There appears to be a high degree of homology within the 5' - flanking regions. Several of the putative regulatory elements are common to both i.e. the Sp1, AP-4 and N - Myc. A proposed binding site identified in the rat as binding BSF1 (Motejlek *et al.*, 1994), is not present in the mouse promoter. Some interspersed repeat elements are present in both species - B2 (SINE B2), B1-F (SINE Alu) and Lx9 (LINE L1). Conservation implies functionality and so it is possible that important regulatory regions are present in the large areas of homology that exists between the mouse and rat. Mutational analysis and subsequent gel shift assays involving these regions of conservation could be performed to determine their importance, if any, in gene regulation.

Chapter 4

Generation of $GABA_A$ Receptor δ Subunit Gene Promoter Constructs

4.1 INTRODUCTION

All methods devised to study regulation of gene transcription rely on "reporter" constructs. These contain the putative DNA regulatory regions under investigation linked to a promoterless reporter gene. Examples of commonly used reporter genes are: β - galactosidase gene (*lac*Z), luciferase (luc), chloramphenicol acetyl transferase (CAT) and green fluorescent protein (GFP). These genes are expressed at very low or undetectable levels in the cells of interest and so the activation of the reporter gene can clearly be distinguished from the promoter activity of the endogenous gene. This approach has led to the identification of distinct promoter, enhancer and silencer elements in many eukaryotic genes and has characterised sequences which mediate cell - type specific expression.

Utility vectors were designed and constructed, in collaboration with Dr T. A. Glencorse, to allow for the insertion of a range of promoter fragments upstream of reporter genes to form transcriptional fusions. These promoter constructs could then be used to identify putative regulatory elements in the promoter fragments. Initially, all constructs would be made with the *lac*Z reporter gene. If there was a problem with endogenous β - galactosidase activity, then some of the constructs could be prepared with the modified luciferase vector. After construction of these vectors, a range of $GABA_A$ receptor δ subunit gene promoter fragments were introduced upstream of the *lac*Z gene. These were then used to investigate regulatory elements in neuronal cell lines (**Chapter 5**) and in the production of recombinant viruses (**Chapter 6**).

4.2 RESULTS

4.2.1 Design and generation of reporter - gene vectors for promoter - deletion constructs

A vector was selected (pGL3 basic; Promega) and modified to allow for the construction of transcriptional fusions with either the luciferase reporter gene (pTG22), or the β - galactosidase gene (pTG08). A control vector (pGL3 control; Promega) was also modified to produce control plasmids containing the SV40 promoter fused to either the luciferase (pTG34) or *lac*Z (pTG18) genes. Each reporter vector was subject to the same modifications. In this way any differences in expression would be due to the promoter fragments, and not to the presence of extraneous fragments of DNA.

A luciferase vector - pGL3 basic (Promega, **Figure 4.1a**) was used to form luciferase fusions, and also as a backbone for β - galactosidase fusions whereby the luciferase gene was replaced by the *lacZ* gene from the pNASS β vector (Clontech, **Figure 4.1b**). Standard control vectors for the luciferase and *lacZ* constructs were designed using the pGL3 control vector as a backbone (**Figure 4.1c**).

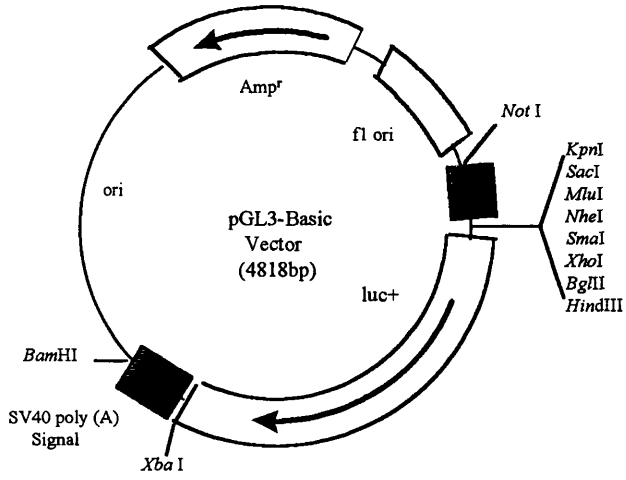


Figure 4.1a pGL3 basic plasmid

The pGL3 basic plasmid (Promega) used in the construction of a utility luciferase vector, and as a backbone for the utility β - galactosidase vector.

Abbreviations used are : Amp^r = ampicillin resistance gene, Poly (A) = polyadenylation site, *luc*$^+$ = luciferase gene, ori = origin of replication.

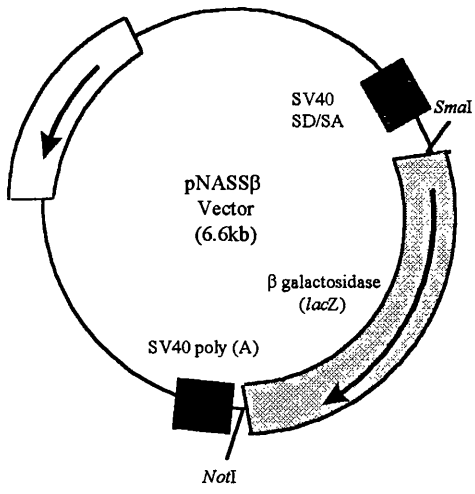


Figure 4.1b pNASS β plasmid

The pNASS β plasmid (Clontech) used as a source of the *lacZ* gene for construction of a utility *lacZ* vector. Abbreviations used are : Amp^r = ampicillin resistance gene, Poly (A) = polyadenylation site, ori = origin of replication, *lacZ* = β-galactosidase gene.

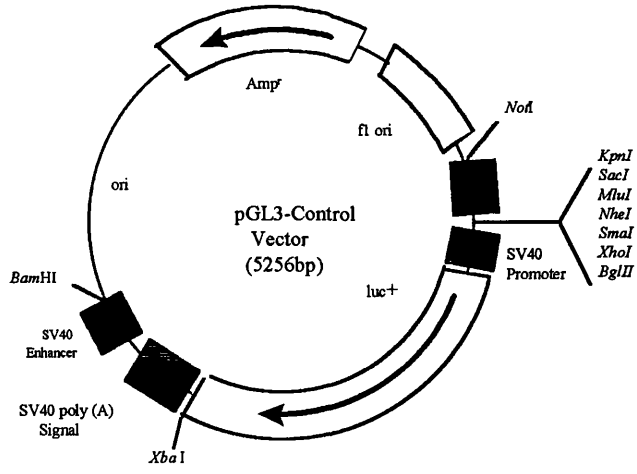


Figure 4.1c pGL3 control plasmid

The pGL3 control plasmid used in the construction of a luciferase control vector, and as a backbone for the *lacZ* control vector. Abbreviations used are : Amp^r = ampicillin resistance gene, Poly (A) = polyadenylation site, luc^+ = luciferase gene, ori = origin of replication.

4.2.1.1 Luciferase vector construction

Figure 4.2 is a schematic diagram of the construction of the luciferase vector - pTG22, using the pGL3 basic plasmid (Promega, **Figure 4.1a**). Initially a *Bgl* II - *Pac* I - *Bgl* II linker (TAG117/8, **Table 2.3**) was introduced into the *Bam* HI site at the 3' - end of the luciferase gene. A *Not* I site was removed by restriction with *Not* I, filling in with Klenow fragment and then re - ligating. The multiple cloning site (mcs) was removed by restriction with *Hin*d III and *Sac* I, filling in with Klenow and then re - ligating. A mcs (TAG138 and TAG139, **Table 2.3**) was inserted into the *Kpn* I site and orientated. The *Xba* I site in the mcs was replaced by an *Spe* I site by insertion of an *Xho* I - *Spe* I - *Eco* RI linker (BRXSE1 and BRXSE2, **Table 2.3**). All these alterations were confirmed by DNA sequencing.

4.2.1.2 Luciferase control vector construction

Figure 4.3 is a schematic diagram of the construction of the luciferase control vector - pTG34, using the pGL3 control plasmid (Promega, **Figure 4.1c**). Initially a *Bgl* II - *Pac* I - *Bgl* II linker (TAG117/8, **Table 2.3**) was introduced into the *Bam* HI site at the 3' - end of the luciferase gene. A *Not* I site was removed by restriction with *Not* I, filling in with Klenow fragment and then re - ligating. The mcs was removed by restriction with *Sac* I - *Bgl* II, filling in with Klenow, and re - ligation. A mcs (TAG138 and TAG139, **Table 2.3**) was inserted into the *Kpn* I site and orientated. The *Xba* I site in the mcs was replaced by an *Spe* I site by insertion of an *Xho* I - *Spe* I - *Eco* RI linker (BRXSE1 and BRXSE2, **Table 2.3**). The alterations were confirmed by DNA sequencing.

4.2.1.3 lacZ vector construction

Figures 4.4a and **4.4b** are schematic diagrams showing the construction of the *lacZ* vector - pTG08. Initially pBluescript KSII+ was altered by insertion of a *Sac* I - *Nhe* I-*Not* I - *Sma* I - *Kpn* I linker (TAG119 and TAG 120, **Table 2.3**). The *lacZ* gene was removed from pNASS β (**Figure 4.1b**) as a *Sma* I - *Not* I fragment and subcloned into the altered KSII+. The *Not* I site was then removed by restriction, end - filling with Klenow and re-ligation (**Figure 4.4a**). The altered luciferase vector (with the *Bgl* II linker and minus the *Not* I site (**Section 4.2.1.1**) was used as the backbone for the *lacZ* vector. The luciferase gene was removed by restriction with *Kpn* I and *Xba* I. The *lacZ* gene was excised as a

Kpn I - *Nhe* I fragment from pBluescript and subcloned into the *Kpn* I - *Xba* I restricted luciferase backbone. A mcs (TAG138 and TAG139, **Table 2.3**) was inserted into the *Kpn* I site and orientated. The *Xba* 1 site in the mcs was replaced by an *Spe* I site by insertion of an *Xho* I - *Spe* I - *Eco* RI linker (BRXSE1 and BRXSE2, **Table 2.3**), (**Figure 4.4b**). All these alterations were confirmed by DNA sequencing.

4.2.1.4 lacZ control vector construction

Figure 4.5 is a schematic diagram showing the construction of the control *lacZ* vector - pTG18. The altered pGL3 control plasmid (with the *Bgl* II linker and minus the *Not* I site (**Section 4.2.1.2**), was used as a backbone for this vector. The luciferase gene was removed as a *Hind* III - *Xba* I fragment. A *Kpn* I - *Hind* III - *Sma* I linker (TAG126 and TAG127, **Table 2.3**) was inserted into the *Kpn* I and *Sma* I sites of the KSII+ - *lacZ* plasmid (**Section 4.2.1.3**). The *lacZ* gene was excised from this as a *Hind* III - *Nhe* I fragment and subcloned into the *Hind* III - *Xba* I restricted luciferase control vector backbone. The *Hind* 111 site was removed by restriction, end - filling with Klenow and re-ligation. A mcs (TAG138 and TAG139, **Table 2.3**) was inserted into the Kpn I site and orientated. The *Xba* I site in the mcs was replaced by an *Spe* I site by insertion of an *Xho* I - *Spe* I - *Eco* RI linker (BRXSE1 and BRXSE2, **Table 2.3**). All these alterations were confirmed by DNA sequencing.

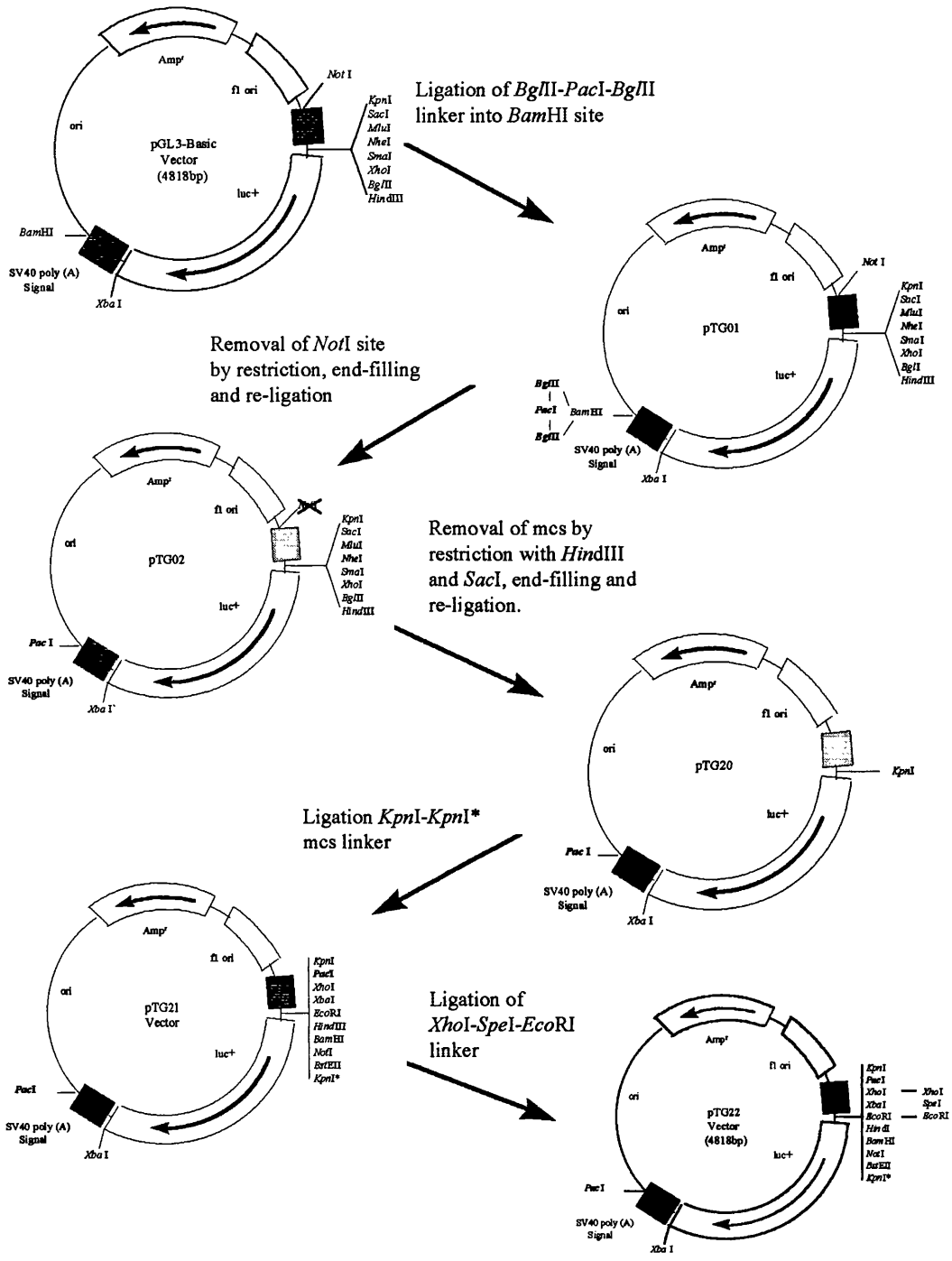


Figure 4.2 Utility luciferase vector

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, luc^+ = luciferase gene, ori = origin of replication.

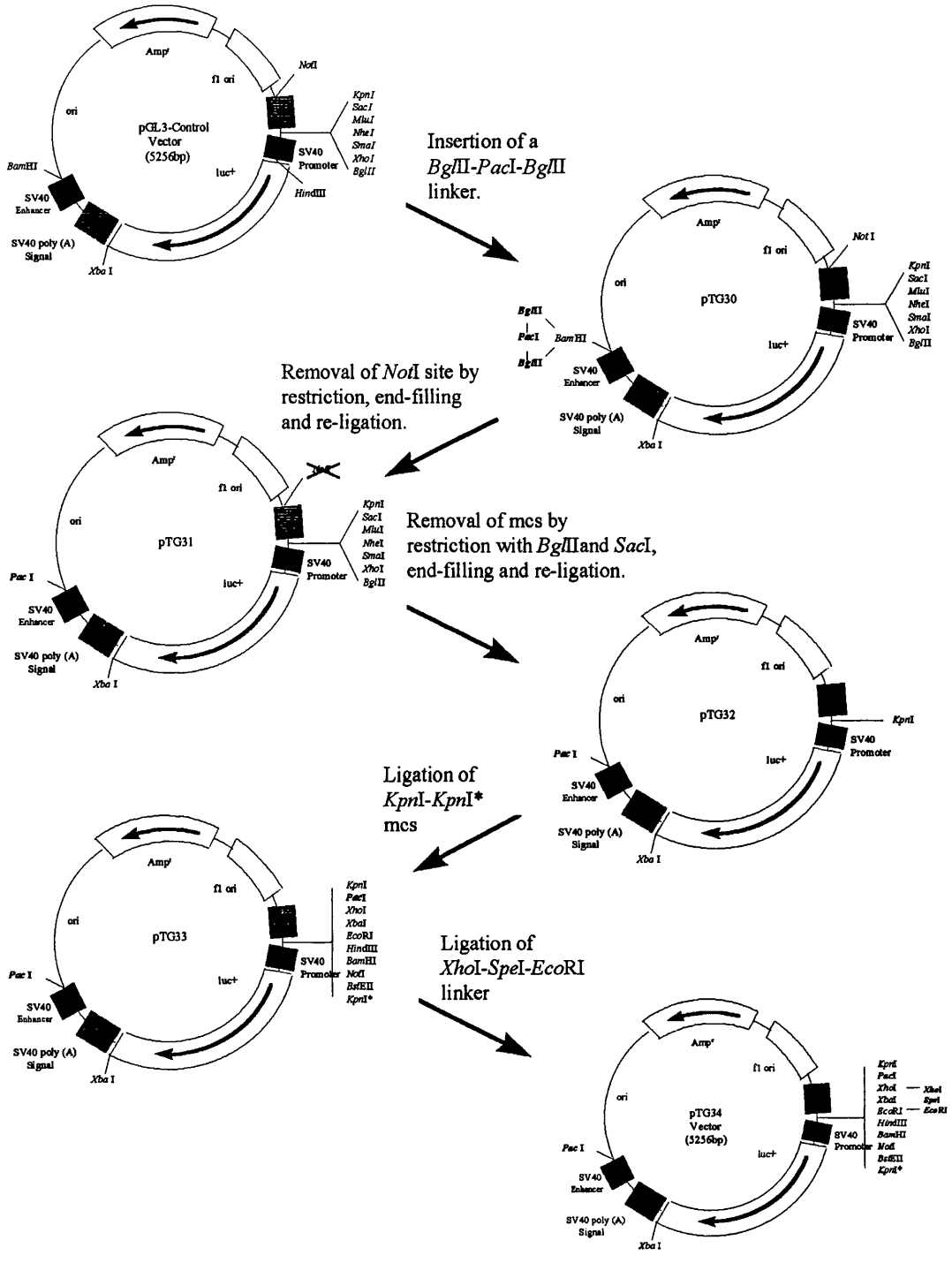


Figure 4.3 Luciferase control vector

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, *luc*$^+$ = luciferase gene, ori = origin of replication.

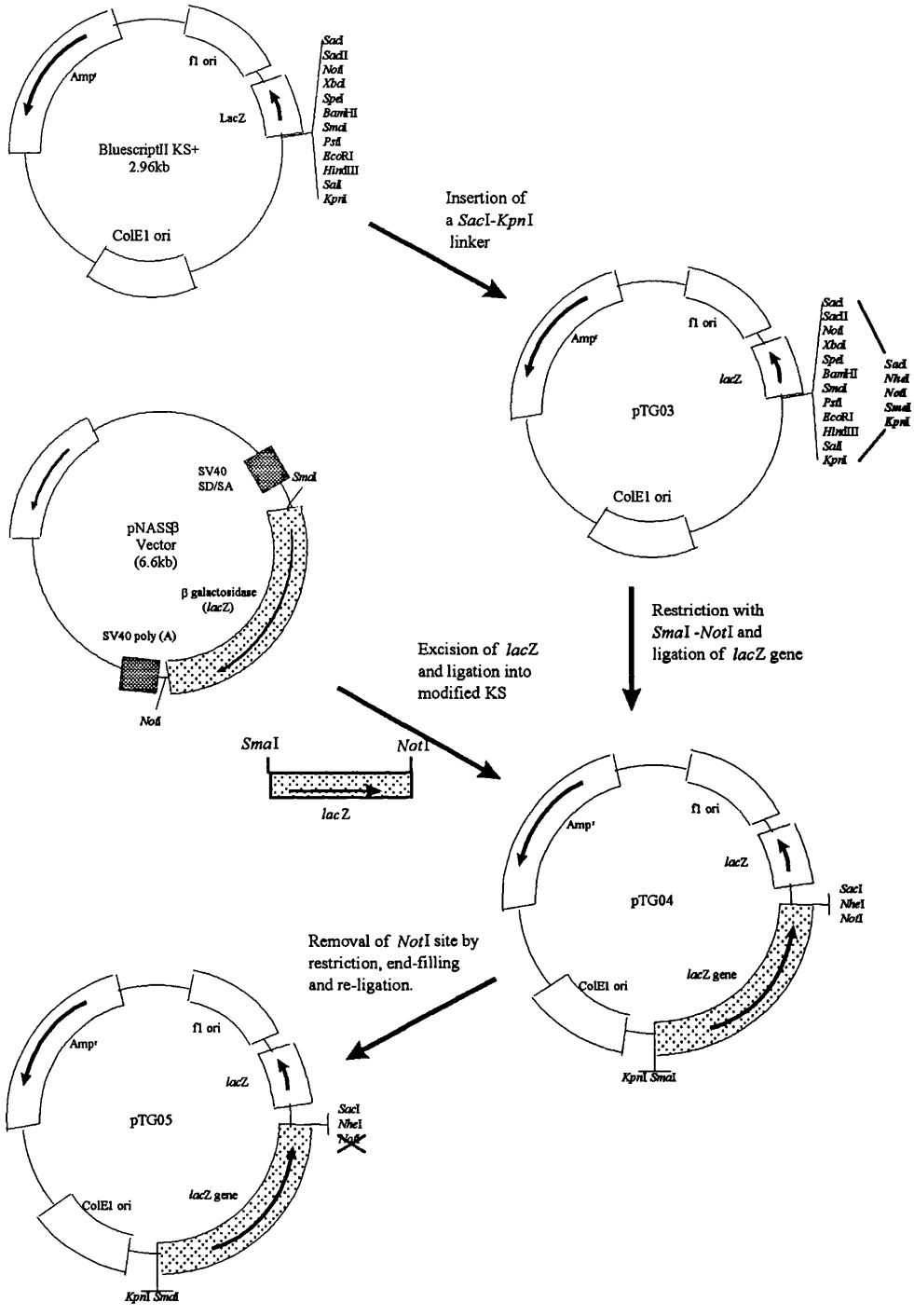


Figure 4.4a Utility lacZ vector

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, luc^+ = luciferase gene, ori = origin of replication, *lacZ* = β-galactosidase gene.

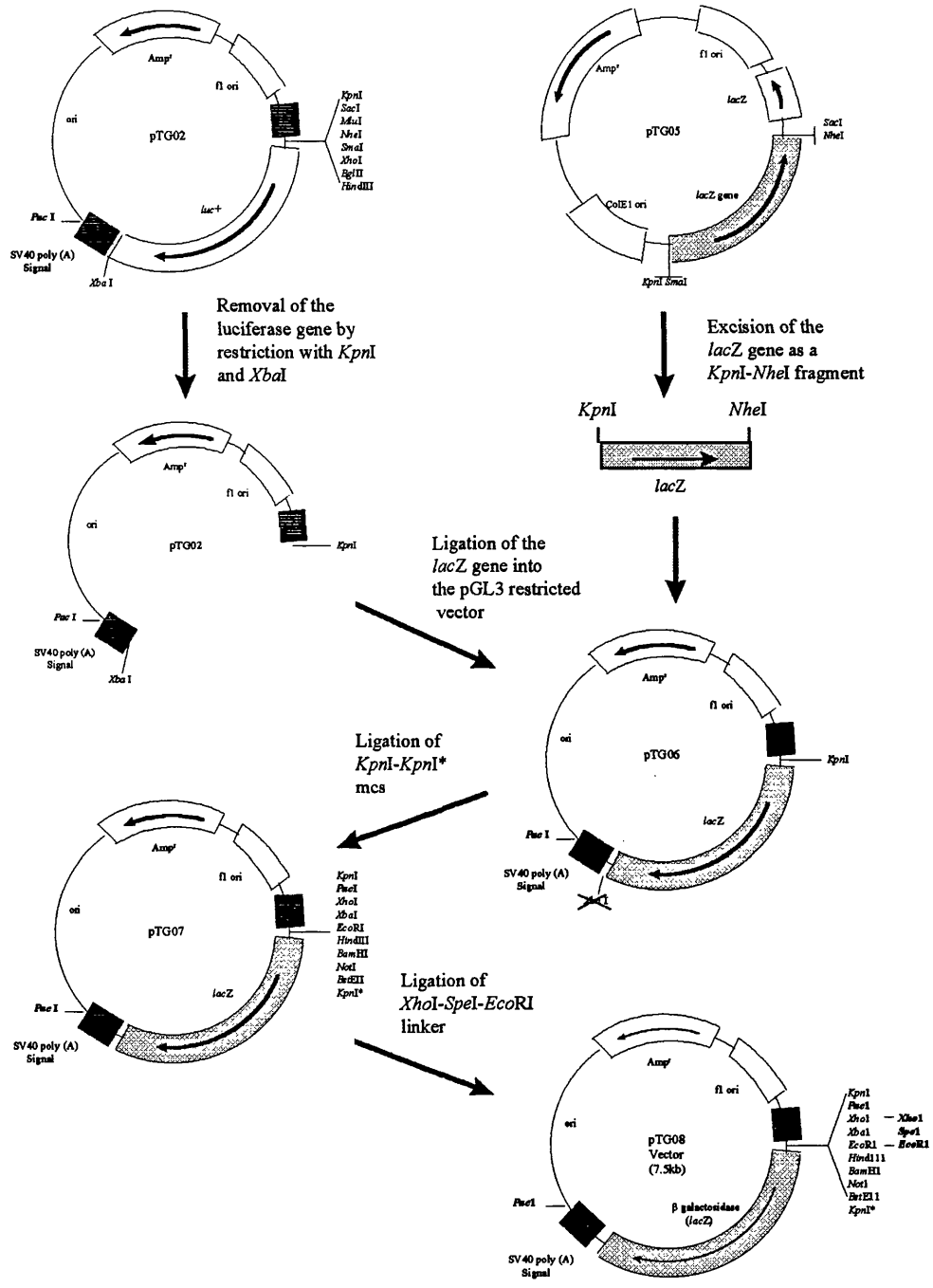


Figure 4.4b Utility lacZ vector

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, luc^+ = luciferase gene, ori = origin of replication, *lacZ* = β galactosidase gene.

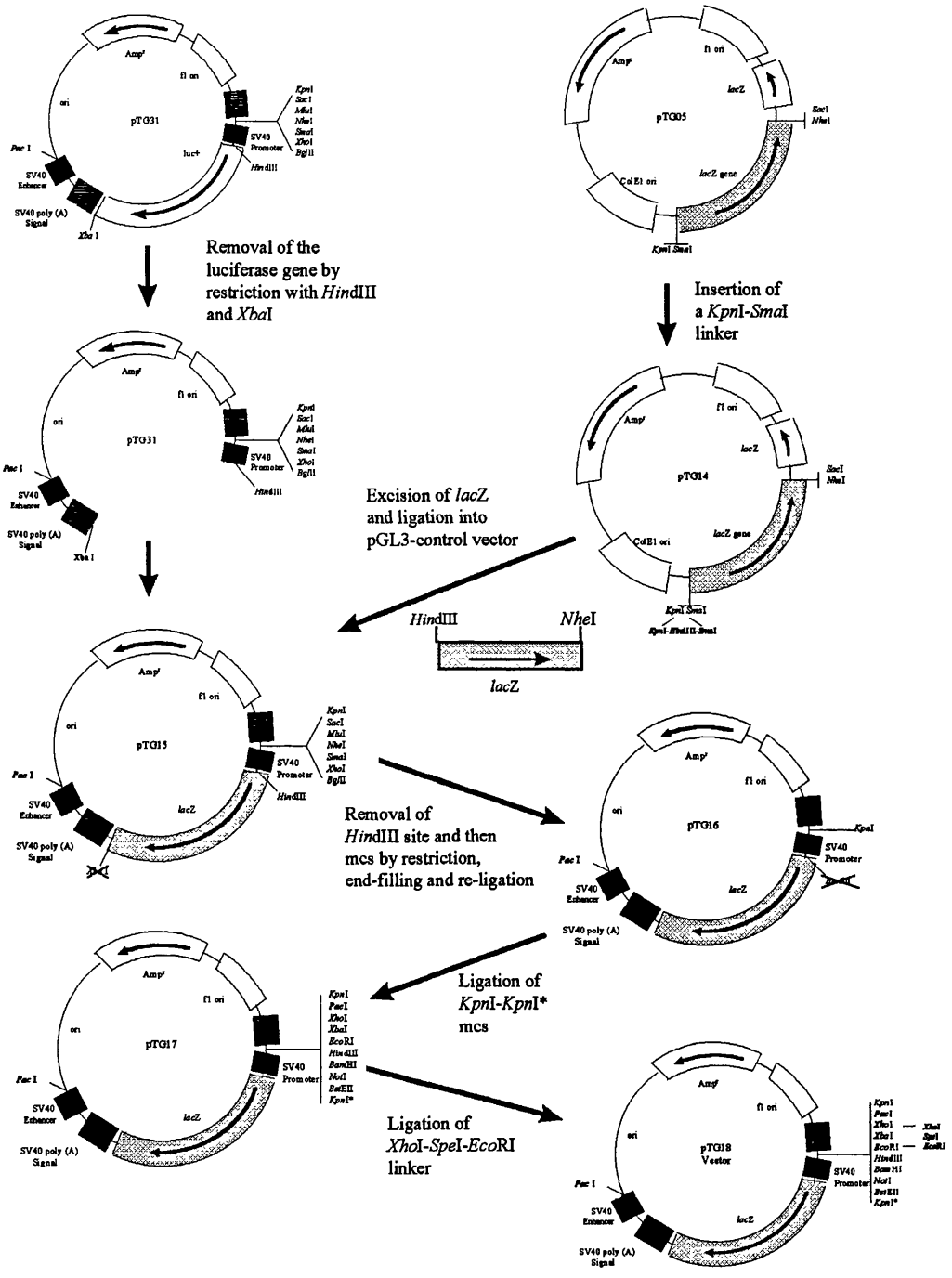


Figure 4.5 lacZ control vector

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, luc^+ = luciferase gene, ori = origin of replication, *lacZ* = β galactosidase gene.

4.2.2 Construction of promoter - deletion vectors

A range of promoter fragments from 1.2 kb up to 10.5 kb were used to form transcriptional fusions (**Figure 4.6**). For the 10.5 kb construct, the *Pac* I site had to be replaced at the 5' - end and so a *Kpn* I - *Pac* I - *Kpn* I linker (BRKPK, **Table 2.3**) was inserted into the *Kpn* I site.

A second set of promoter constructs were designed (**Figure 4.7**) to re - introduce a second cluster of transcription start sites located 3' - to the *Not* I site (see Discussion). Initially a 1.15 kb *Hin*d III - *Nco* I fragment from the δ promoter was subcloned into pLitmus 29 (Promega). An *Nhe* I - *Bst*E II - *Kpn* I linker (BRNK1 and BRNK2, **Table 2.3**) containing δ sequence was inserted between the *Nhe* I site in the δ and the *Kpn* I site of pLitmus 29. The δ promoter was then excised as a *Hin*d III - *Bst*E II fragment and subcloned into the *lac*Z (pTG08) vector. The other promoter fragments (**Figure 4.6**), were then subcloned into this construct using the *Not* I site at the 3' - end. For the 10.5 kb construct, the *Kpn* I - *Pac* I - *Kpn* I linker (BRKPK, **Table 2.3**) was inserted into the *Kpn* I site in order to replace the *Pac* I site.

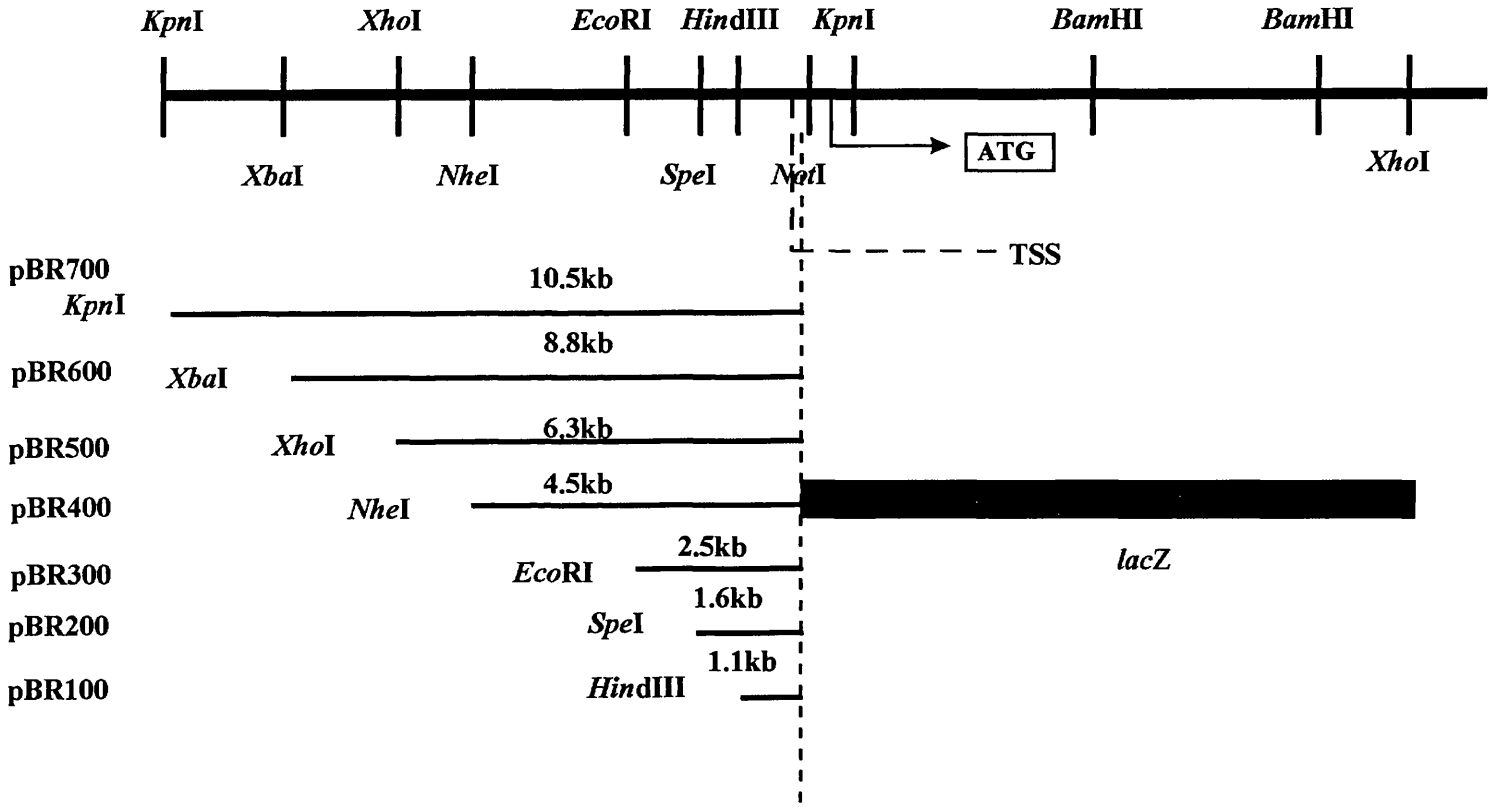


Figure 4.6 Range of promoter fragments inserted into reporter vectors

ATG indicates the translational start site. TSS indicates the position of the transcription start sites. The promoter fragments are given in kilobases. The box represents the β-galactosidase gene (*lacZ*). The plasmid names are given at the side of the diagram, i.e. pBR700 etc. This diagram is not drawn to scale.

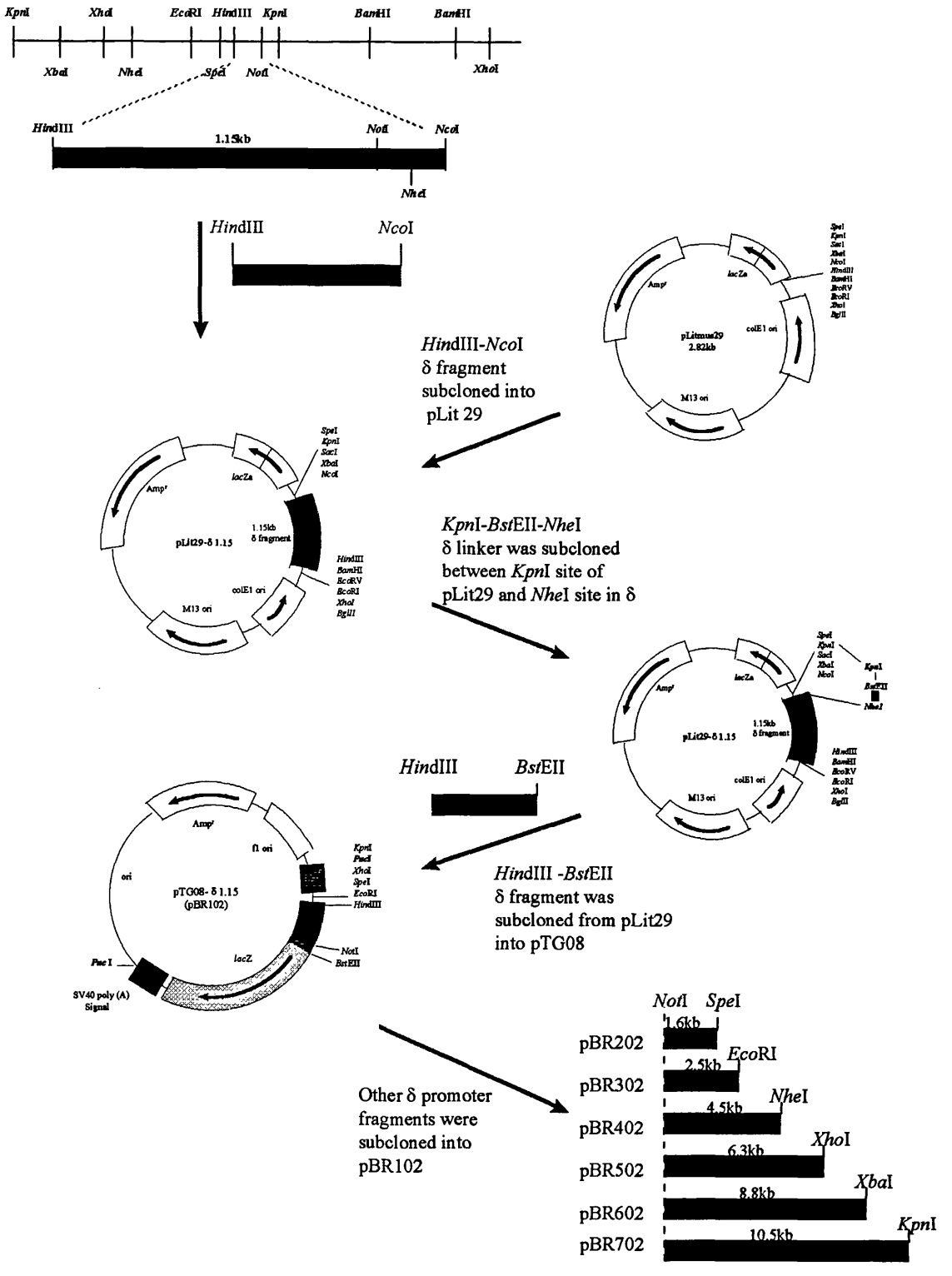


Figure 4.7 Alteration of promoter constructs

Abbreviations used are : Amp^r = ampicillin resistance gene, mcs = multiple cloning site, Poly (A) = polyadenylation site, ori = origin of replication, *lacZ* = β-galactosidase gene.

4.3 DISCUSSION

As a detailed restriction map of the promoter region of the $GABA_A$ receptor δ subunit gene was available (**Figure 3.1**), it was possible to design a range of promoter - deletion constructs that covered the available 10.5 kb of 5' - flanking DNA. Initially utility reporter vectors were constructed. These allowed for the insertion of a range of promoter fragments upstream of the reporter genes. Control plasmids were also constructed whereby the SV40 promoter was fused to the luciferase or β - galactosidase genes. The plasmid pGL3 basic (Promega) which was used as a backbone for both the luciferase and the *lacZ* reporter vectors, was modified extensively.

In the construction of the luciferase vector pTG22, the first modification of pGL3 basic was the insertion of a *Bgl* II linker. This served a dual purpose in that it resulted in the loss of the *Bam* HI site which was to be included in the multiple cloning site (mcs), and also it inserted a *Pac* I site at the 3' - end of the reporter gene. The *Not* I site was deleted as it was also to be included in the mcs. A large mcs was inserted which would allow for all the chosen promoter fragments to be subcloned. The mcs contained a *Pac* I site close to it's 5' - end. This would allow for the excision of the promoter - reporter gene cassette as a *Pac* I fragment, if required. As there was an *Xba* I site at the 3' - end of the luciferase gene, the *Xba* I site in the mcs was replaced with an *Spe* I site which has ends compatible to *Xba* I and so would still allow for the insertion of *Xba* I promoter fragments

The *lacZ* vector pTG08 was constructed from two plasmids, pGL3 basic and pNASSβ (Clontech). The β - galactosidase gene was first subcloned from pNASSβ into a modified pBluescript vector. This altered the restriction sites at the 5' - and 3' - ends to allow for it's subsequent insertion into the modified pGL3 basic vector. The other modifications were the same as those described previously for the luciferase vector.

The control vectors pTG34 and pTG18 were subject to the same alterations as described for the utility vectors.

Using the published information about the location of the transcription start sites (Sommer *et al.*, 1990), the promoter fragments were subcloned into the *lacZ* reporter vector with

Not I at their 3' - ends (**Figure 4.6**). However the results of the oligo - capping experiment described in **Chapter 3** indicates a second cluster of transcription start sites just 3' - to the *Not* I site. Thus it was necessary to replace this region with a linker and re - subclone the promoter fragments (**Figure 4.7**). The 5' - and 3' - ends of the promoter fragments were sequenced to confirm that the junctions were correct. The promoter constructs were cut with *Pac* I to ensure that the promoter - reporter cassettes could be released from the vector backbone if required (**Figure 4.8**).

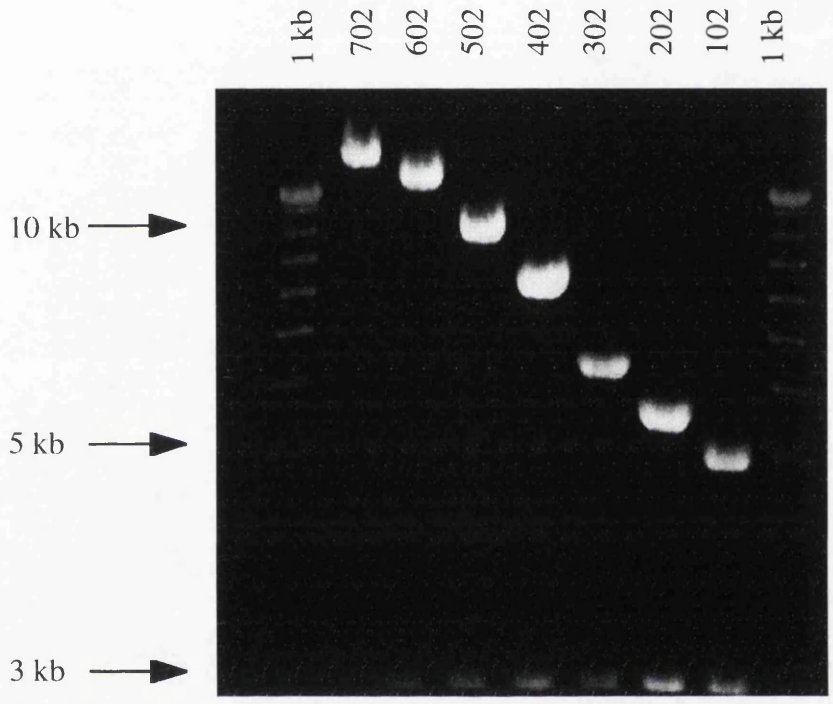


Figure 4.8 Pac I digestion of promoter - reporter constructs

0.5% TBE agarose gel showing the release of the promoter - reporter gene casstte from the plasmid backbone upon digestion with Pac I enzyme. The 3 kb plasmid backbone is indicated.

Chapter 5

Analysis of Promoter Constructs in Model Cell Lines

5.1 INTRODUCTION

While it would be ideal to study the expression of neuronal promoters *in vivo*, it has proved extremely difficult to transfer DNA into neuronal cells and the methods available are expensive, time consuming and to a large extent unsuccessful. An alternative expression system available is the use of neuronal cell lines. The advantages of cell lines are speed, reproducibility and ease of application. A large number of promoter - constructs can be analysed in a relatively short time, to produce information about possible enhancer and silencer regions present in specific promoter fragments. Thus this method provides a preliminary screen for putative *cis* - acting regulatory regions.

Primary neuronal cultures can also be utilised, however they have proved difficult to transfect with DNA using standard methods. Modifications of current techniques do appear to be resulting in a higher percentage of transfected cells (Xia *et al.*, 1996)

In this chapter, expression from the promoter - reporter plasmids (**Chapter 4**) was assayed using a neuronal cell line and primary cerebellar granule cell cultures. The cell line selected for expression studies was NB4 1A3 (**Table 2.6**), which had been previously shown to express the δ subunit (Tyndale *et al.*, 1994). Cerebellar granule cell cultures were established in collaboration with Dr T. Glencorse. Transfection of these cultures was attempted using the promoter constructs.

5.2 RESULTS

5.2.1 Expression of promoter - reporter constructs in cell culture

The construction of a range of promoter - deletion plasmids was described in **Chapter 4**. The transfection procedure is described in **Section 2.2.9.1**. The transfection procedure was repeated 3 times. Two separate DNA preparations (A and B) of each plasmid were used. For the first two experiments, the A set of plasmids was used and each plasmid was transfected in triplicate. For the third transfection, set B was used and each plasmid was transfected 5 times.

Figure 5.1 shows the results of the 2 experiments involving the expression of one set (A) of purified promoter - reporter constructs in the NB4 1A3 cell line.

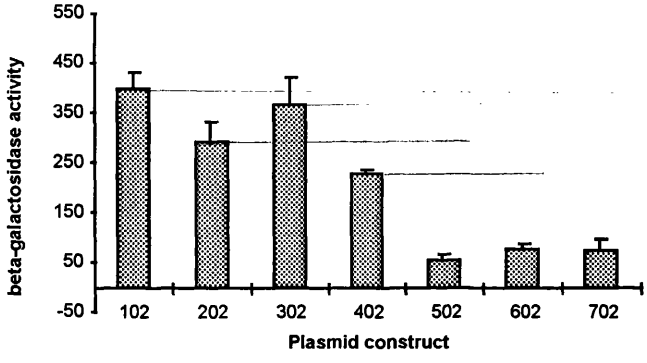


Figure 5.1a ***Experiment 1 - β-galactosidase activity of NB4 1A3 cells transfected with reporter construct set A***

The β-galactosidase activities have been corrected against an internal luciferase control using co - transfection with the pGL3-Control vector (**Figure 4.3**). The x-axis shows δ - promoter constructs with increasing length from left to right. Values shown are from the means from one experiment where each plasmid was transfected in triplicate. Error bars represent standard errors of the mean.

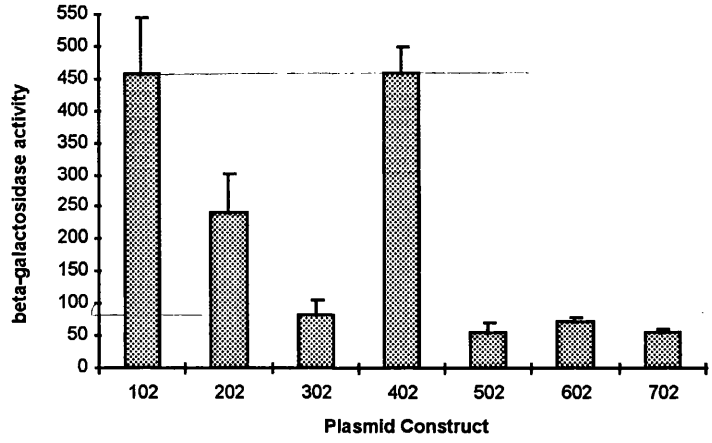


Figure 5.1b Experiment 2 - β-galactosidase activity of NB4 1A3 cells transfected with reporter construct set A

The β-galactosidase activities have been corrected against an internal luciferase control using co - transfection with the pGL3-Control vector (**Figure 4.3**). The x-axis shows δ - promoter constructs with increasing length from left to right. Values shown are from the means from one experiment where each plasmid was transfected in triplicate. Error bars represent standard errors of the mean.

Figure 5.2 shows the results of expression of the second set (B) of purified promoter - reporter constructs in the NB4 1A3 cell line.

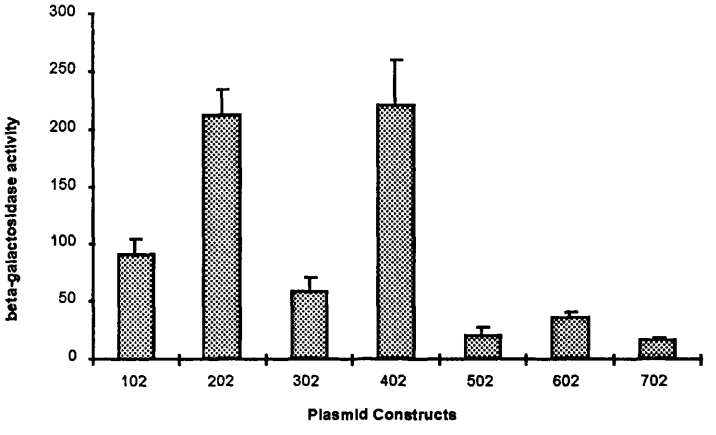


Figure 5.2 ***β-galactosidase activity of NB4 1A3 cells transfected with reporter constructs set B***

The β-galactosidase activities have been corrected against an internal luciferase using co - transfection with the pGL3-Control vector (**Figure 4.3**). The x-axis shows δ - promoter constructs with increasing length from left to right. Values shown are from the means from one experiment where each plasmid was transfected 5 times. Error bars represent standard errors of the mean.

The 3 sets of data were combined and the results shown in **Figure 5.3**.

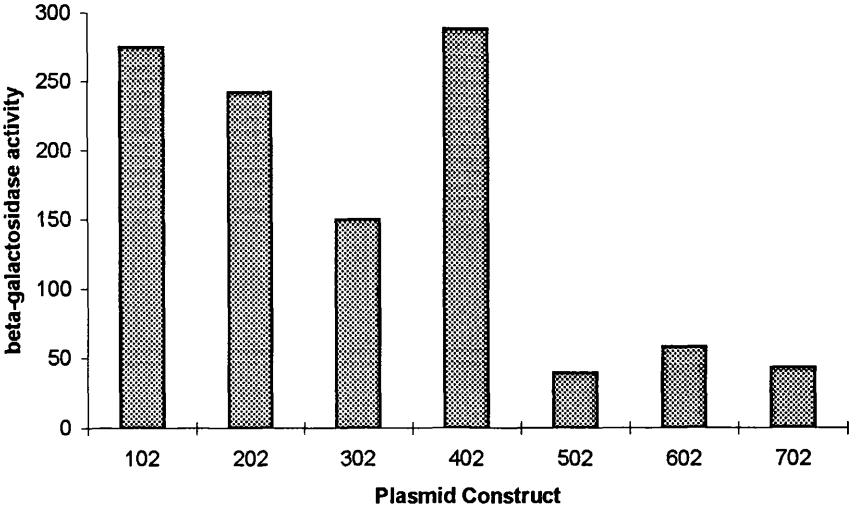


Figure 5.3 ***Graph showing the β-galactosidase activity of the three transfection experiments combined***

The β-galactosidase activities have been corrected against an internal luciferase control - pGL3-Control vector. The x-axis shows δ - promoter constructs with increasing length from left to right. Values shown are from the means from three experiment where each plasmid was transfected a total of 11 times.

The data indicate that there is a reduction of activity between the plasmid constructs pBR402 and pBR502. Student's *t* - distribution calculated between each pair of plasmids show this difference to be significant.

5.2.2 Expression of promoter - reporter constructs in primary cell cultures

This work was performed in collaboration with Dr T. Glencorse and Dr G. Westrop who performed most of the transfections. The culturing of the granule cells and their subsequent transfection is described in **Section 2.2.9.2**. Initially, 2 plasmid constructs were selected for transfection, BR202 and BR502. These were transfected using DOTAP or calcium phosphate precipitation. However both methods proved to be unsuccessful.

There was a very high death rate with DOTAP. No blue cells were detectable upon fixing and staining the cells with X - gal. β - galactosidase activity of the harvested cell cultures was detected but did not differ significantly from the controls. Heating of the extracts to reduce endogenous β - galactosidase activity reduced all the readings to zero.

5.3 DISCUSSION

Expression of a range of promoter - reporter constructs (**Chapter 4**) was analysed *in vitro*. The neuronal cell line NB4 1A3 was transfected on 3 separate occasions with the plasmids and the β - galactosidase activity was assayed. Each transfection gave different levels of expression which made it difficult to combine the data. Overall, the pattern was similar. Student's t - tests performed on the pairs of plasmids demonstrated that the only significant difference observed was between the plasmids pBR402 and pBR502. The promoter fragments in these two plasmids differ in that pBR502 contains an extra 1.8 kb of 5' - upstream sequence (-6157 to - 4363; **Figure 3.6**). It is possible that this effect is due to the presence, in this region, of a binding site for a regulatory element. However, as there was great variability between the transfections, these differences could also be caused by the quality of the plasmid DNA used in the transfections. Thus the transfections should be repeated with newly prepared high quality DNA.

Analysis of this sequence does not reveal any known neuronal silencer elements. Two recognition sites for known transcription factors have been identified in this region (**Table 3.1**) - MZF1 and S8. MZF1 (myeloid zinc finger gene 1) is a member of the C_2H_2 zinc finger family of genes which are DNA binding proteins many of which have been demonstrated to have roles in regulating transcription during developmental processes. MZF1 is thought to play a role in hematopoiesis regulation (Morris *et al.*, 1994). S8 (de Jong *et al.*, 1993) is a murine member of the homeodomain protein family and is highly homologous to another murine homeodomain gene - MHox. They are expressed in a developmentally specific and tissue restricted manner. Thus while it is unlikely that these sequences provide recognition sites for MZF1 and S8, they could bind related proteins. Mutational analysis and subsequent gel shift assays using oligonucleotides designed to these sequences could provide answers about their functionality.

possibility is that there is a recognition site in this region for a novel transcription factor. Oligonucleotide PCR primers could be designed to give overlapping products across this region which could then be used in DNA/protein binding assays.

The transfection of the granule cells has so far been unsuccessful. However, this work will continue with adaptations of the transfection procedures being attempted.

These results provide preliminary evidence about possible regulatory regions in the $GABA_A$ receptor δ subunit gene upstream region. Expression of the plasmid constructs should be performed in another cell line e.g. GT1-7 as the differences observed may be attributable to factors present only in the NB4 1A3 cell line. Future work also will involve attempting to identify the putative recognition site for the proposed silencing element, possibly using gel shift assays, DNase I footprinting assays or mutational analysis.

Chapter 6

Herpes Simplex Virus as a Delivery System

6.1 INTRODUCTION

The second main aim of this project was to develop an HSV - 1 viral vector system to deliver DNA to neurons, the eventual goal being the development of a safe HSV - 1 gene therapy vector for neuron - specific delivery. Most current gene therapy technology has involved the use of retroviral vectors. Retroviruses integrate their genomes into the chromosome of the host cell providing a method to stably introduce genes into cells removed from a patient which can then be returned by cell transplantation procedures following gene transfer. Retroviral vectors carrying therapeutic genes have proved useful for transferring genes to rapidly dividing neoplastic cells, fibroblasts and bone marrow stem cells (Anderson, 1992). However, as they require dividing cells to enter the nucleus and integrate the therapeutic gene, they cannot infect postmitotic cells such as neurons.

Both adenovirus (Alki, *et al.*, 1993; Davidson *et al.*, 1993; Le Gal La Salle *et al.*, 1993) and HSV - based vectors (Anderson *et al.*, 1992; Fink *et al.*, 1992; Chiocca *et al.*, 1990; Glorioso *et al.*, 1992, 1994) possess a wide host cell range and can efficiently infect nondividing cells. HSV may be particularly useful for gene therapy to the CNS as it is naturally neurotropic and has evolved a mechanism for remaining latent in neurons. It can exist without expression of viral proteins or apparent cell damage. Genetic alteration of the HSV virus would be required to ensure that it could not express lytic functions and that it could efficiently express transgenes during the latent phase.

The other requirement for a neuronal gene therapy vector is a suitable neuronal - specific promoter which would restrict expression of the therapeutic gene to the required cell type. The $GABA_A$ receptor δ subunit gene promoter serves as a protoype neuronal promoter as it has a well characterised restricted cell - type specific expression pattern in the CNS. We shall also examine the utility of $GABA_A$ receptor δ promoter fragments for long - term cell specific expression in the context of a safe HSV - 1 vector.

6.1.1 Herpes Simplex Virus type 1

HSV - 1 has a large cloning capacity (McGeoch *et al.*, 1988; Longnecker *et al.*, 1988), remains episomal (which may be advantageous as integration could cause disruption of target cell genes) and can infect many types of neurons. It has a wide cellular host range,

but during natural infection is neurotropic. The virus can be transported by retrograde and anterograde transport within neurons and can cross synapses to neighbouring neurons (Kuypers and Ugolini, 1990). In neurons, it may begin a cycle of lytic replication or enter a latent state in which the viral genome persists without the expression of any viral proteins (Rock and Fraser, 1983; Mellerick and Fraser, 1987; Stevens, 1989). While latent genomes can be reactivated, HSV - 1 vectors have been constructed where the ability to reactivate has been severely impaired (Robertson *et al.*, 1992; Steiner *et al.*, 1989). Also, vectors have been produced that are incapable of initial replication in the epithelium or in neuronal cells, but can establish latency.

6.1.2 Molecular biology of HSV - 1

The HSV - 1 genome is a linear double - stranded DNA molecule composed of two unique segments [unique long (U_L) and unique short (U_S)] each flanked by a pair of inverted repeats (I_R) (**Figure 6.1**).

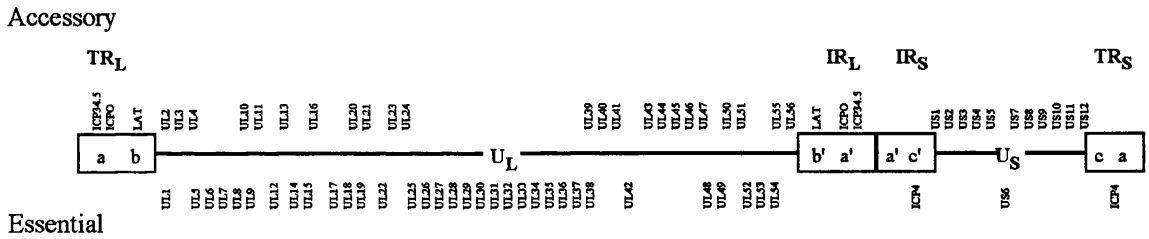


Figure 6.1 Schematic representation of the HSV - 1 genome

Diagram of the HSV - 1 genome showing the unique long (U_L) and unique short (U_S) regions bounded by inverted repeat elements (I_R). The non - essential or accessory genes, which may be deleted without preventing replication *in vitro* are indicated on the upper line. The essential genes which are required for viral replication *in vitro*, are indicated on the lower line (Figure adapted from Fink *et al.*, 1996).

More than 80 genes are encoded in the 152 kb genome and they are expressed in a well - ordered temporal cascade of immediate early (IE or α), followed by early (E or β) and then late (L or γ) gene products (Honess and Roizman, 1974). The viral genes have been categorised as either essential or non - essential according to whether or not they are required for the production of infectious viral particles *in vitro*. Non - essential genes may however contribute to the ability of the virus to effectively replicate and spread *in vivo*. Deletion of non - essential genes could increase the potential cloning capacity of the virus, but may affect viral tropism.

6.1.3 HSV - 1 latency

HSV - 1 can enter a latent state in neurons. While no viral proteins can be detected during this phase, a region of the HSV - 1 genome does remain transcriptionally active, producing a family of latency - associated transcripts (LATs) (Croen *et al.*, 1987; Deatly *et al.*, 1988; Rock *et al.*, 1987; Spivack and Fraser; 1987; Stevens *et al.*, 1987). Three major LAT transcripts can be detected during latency - 2 kb, 1.5kb and 1.45kb (Spivack and Fraser, 1987; Spivack *et al.*, 1991; Wagner *et al.*, 1988). The major species is the 2 kb non - polyadenylated RNA that remains intranuclear and is transcribed from the repeat regions flanking the U_L segment of the genome (**Figure 6.2**). It appears to be a highly stable intron spliced from a large (8.3 kb) poly $(A)^+$ mRNA species (Devi-Rao *et al.*, 1991; Dobson *et al.*, 1989; Farrell *et al.*, 1991). However this putative 8.3 kb LAT transcript has yet to be identified in latently infected neurones.

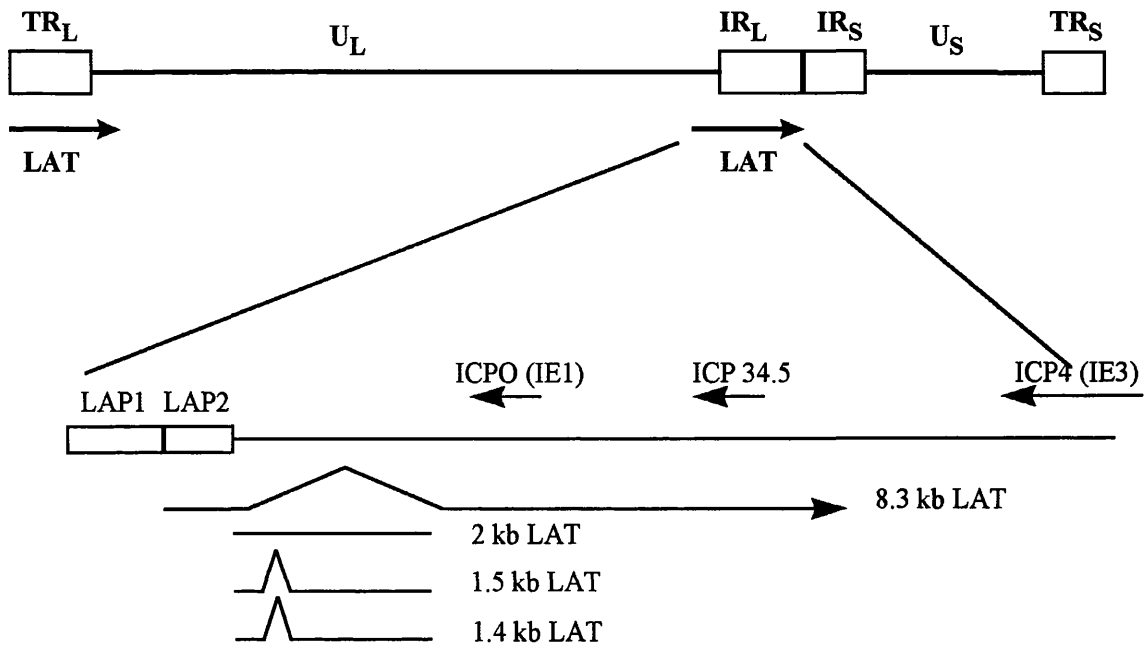


Figure 6.2 Schematic representation of the LAT locus

The diagram shows the structure of the LAT locus. The two LAT promoters (LAP) are indicated as are the products of LAT transcription and splicing. Other genes in the region are also shown.

There are two promoter elements (LAP1 and LAP2) upstream of the 5' - end of the 2 kb LAT (Dobson *et al.*, 1989; Goins *et al.*, 1994). Expression studies using LAP1 and 2 to drive expression of the *lacZ* reporter gene in trigeminal ganglia, have shown that both promoter elements are required for full expression (Goins *et al.*, 1994; Lokensgard *et al.*, 1994; Margolis *et al.*, 1993).

6.1.4 Selection of a viral vector

Two types of HSV - 1 viral vectors can be utilised, either replication - deficient viruses in which the viral genome can be manipulated (Post and Roizman, 1981), or plasmid - based amplicons which require helper virus for DNA replication (Spaete and Frenkel, 1982; Geller and Breakenfield, 1988; Geller *et al.*, 1990). Replication - deficient viruses have essential genes deleted or inactivated and thus require growth in a complementing cell line. One problem is that reversion to wild - type could occur through the cell line complementing the deleted function. These viruses can accommodate up to 15 kb of

foreign DNA and can be purified to relatively high titres (Breakenfield, 1993). However, the large size of the HSV - 1 genome makes production of these vectors difficult, and recombinant viruses can be cytotoxic (Johnson *et al.*, 1992; During *et al.*, 1994; Pakzaban and Chiocca, 1994).

HSV - 1 recombinants have been successfully introduced, *in vitro*, into a range of cultured neurons derived from e.g. spinal cord, cerebellum, basal ganglia, hippocampus and neocortex of rats (Geller and Breakenfield, 1988; Gellar and Freese, 1990; Geschwind *et al.*, 1994). *In vivo*, HSV - 1 recombinants containing reporter genes driven by either HSV - specific or foreign promoters have infected a variety of neuronal cell populations of the rat brain both at the primary injection sites and also at secondary sites (Anderson *et al.*, 1992; Kaplitt *et al.*, 1994; Ridoux *et al.*, 1994; Song *et al.*, 1997).

Amplicons (Spaete and Frenkel, 1982; Geller and Breakenfield, 1988; Geller *et al.*, 1990) are generated from plasmids that contain an expression cassette and minimal viral DNA sequences, including an HSV - 1 origin of replication and packaging sequence. The amplicons do however require helper virus to allow them to propagate. Thus one disadvantage of the amplicon system is that the vectors have to be purified away from the potentially cytotoxic replication competent helper virus.

Our choice of a replication - deficient HSV - 1 vector was determined by our eventual goal which was to develop a viral vector system suitable for use in gene therapy.

6.1.5 Choice of HSV - 1 mutant

Wildtype HSV - 1 causes a lytic infection in the CNS and this must be abolished without affecting the establishment of latency. Two attenuated viral mutants were selected for the generation of recombinant viruses : 1716 (MacLean *et al.*, 1991b) and 1764 (Coffin *et al.*, 1996).

1716 lacks the ICP34.5 gene (Ackermann *et al.*, 1986) which is located in the long repeat regions of the HSV - 1 genome (**Figure 6.1**). ICP34.5 is expressed late in infection and is implicated in neurovirulence (Ackermann *et al.*, 1986; Choi and Roizman, 1986, 1990;

Chou *et al.*, 1990; Dolan *et al.*, 1992; MacLean *et al.*, 1991b). It is not required for replication *in vitro* or for the establishment of latency *in vivo* in at least some neuronal cell types. Deletion or mutation of this gene results in variants that are non - neurovirulent in mice (Bolovan, *et al.*, 1994; Chou *et al.*, 1990; MacLean *et al.*, 1991b; Robertson *et al.*, 1992; Valyi-Nagy *et al.*, 1994). The ICP34.5 mutants are replication - defective in sensory ganglia and the CNS of mice (Bolovan, *et al.*, 1994; Chou *et al.*, 1990; MacLean *et al.*, 1991b; McKie *et al.*, 1994; Whitely *et al.*, 1993), but replication is normal in many (but not all) tissue culture cell types. Thus 1716 is capable of limited replication in the footpads of mice, but is replication - defective in the mouse peripheral nervous system (Robertson *et al.*, 1992). It has been shown to establish latency in dorsal root ganglia (DRG), but is reactivation impaired (Robertson *et al.*, 1992).

A second HSV - 1 viral variant utilised was 1764. It also lacks the ICP34.5 gene and has an additional insertional mutation in the transactivational domain of the Vmw65 (U_L48) gene (Batterson and Roizman, 1983; Campbell *et al.*, 1984). Vmw65 is a virion protein which is required to transactivate HSV gene transcription after infection (Ace *et al.*, 1989; Gerster and Roeder, 1988; McKnight *et al.*, 1987; Preston, *et al.*, 1988). These Vmw65 mutants result in viruses that are unable to transactivate IE gene expression. Replication of these mutant viruses during infection is dependent on the multiplicity of infection (MOI) being high. Steiner *et al.*, (1990) investigated the role of Vmw65 *in vivo* during primary infection, establishment of latency and explant reactivation from latently infected mouse trigeminal ganglia. Replication was not detected, however latency was established and reactivation was noted. Mutants lacking the Vmw65 gene can still be grown in culture as the transactivating activity can be complemented by the inclusion of hexamethylene bisacetamide (HMBA) in the medium (MacFarlane *et al.*, 1992). Viral mutant 1764 was originally generated as it was hoped that its inherent inability to transactivate IE genes would force the virus into the latent pathway of infection, and would reduce the levels of potentially cytopathic IE gene products. Coffin *et al.* (1996) showed that 1764 produced similar effects to 1716 in the PNS and CNS.

6.1.6 Choice of insertion site for $GABA_A$ δ promoter - reporter gene expression constructs

Two loci within the HSV - 1 genome have been targeted as sites of insertion of promoter - reporter gene constructs : U_L43 and LAT (**Figure 6.1**). Our collaborators on the virus work : Prof Clements and Dr MacLean (Dept. of Virology, University of Glasgow) had viral vectors containing promoter - *lacZ* constructs at the U_L43 locus which were known to give successful *lacZ* expression in mouse dorsal root ganglia (DRG). U_L43 is a non - essential gene situated within the unique long region of HSV - 1. Its role was investigated by MacLean *et al.* (1991a). They disrupted the gene by inserting the *lacZ* gene into the U_L43 locus. They also isolated a U_L43 deletion mutant. They found that the absence of the U_L43 polypeptide appeared to have no noticeable effect on the virus phenotype *in vitro* or *in vivo*. Thus the function of the U_L43 gene product remains unknown. U_L43 gene expression has been reported (Carter *et al.*, 1996) and it appears to be transcribed for a short time during lytic infection.

The LAT locus has been discussed in **Section 6.1.2** and may prove useful for long - term expression studies since it represents the only transcriptionally active region during latency. Shuttle vectors were available for insertion of promoter - reporter gene constructs into both the U_L43 (p35) and the LAT (pBL) loci of the HSV - 1 genome by standard recombination methods. As the promoter constructs were designed to be excised as *Pac* I fragments (**Chapter 4**), the shuttle vectors were altered (**Figure 6.3a and 6.3b**) by insertion of *Pac* I linkers within the HSV regions of homology (Dr A. McGregor).

Only 3 GABA δ promoter - reporter gene constructs were initially selected for insertion into HSV - 1 : 1.6kb - *lacZ* (pBR202), 4.5kb - *lacZ* (pBR402) and 10.5kb - *lacZ* (pBR702) (**Chapter 4, Figure 4.7**). It was hoped that these promoter fragments would provide information about the ability of the $GABA_A$ receptor δ subunit gene promoter fragments to direct long - term neuron - specific expression.

Figure 6.3 HSV - 1 Shuttle Vectors

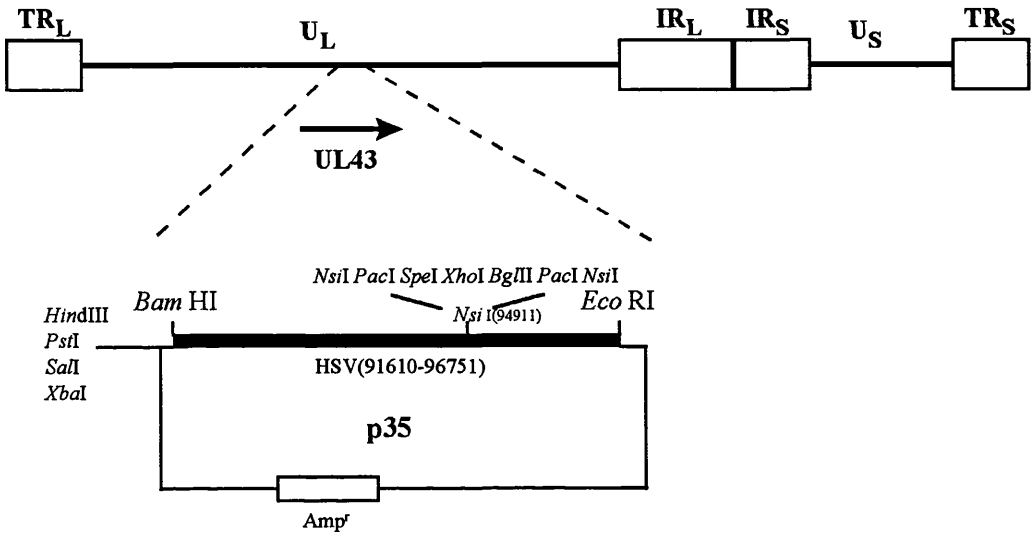
Figure 6.3 HSV - 1 Shuttle Vectors

a. p35 (*Pac* I) vector : pGem - 2 backbone containing a 5.1 kb *Bam* HI -*Eco* RI DNA fragment (91610-96751) from the HSV - 1 UL43 gene. Oligonucleotide linker insertion at the *Nsi* I site generates the *Pac* I site required for insertion of promoter - reporter constructs.

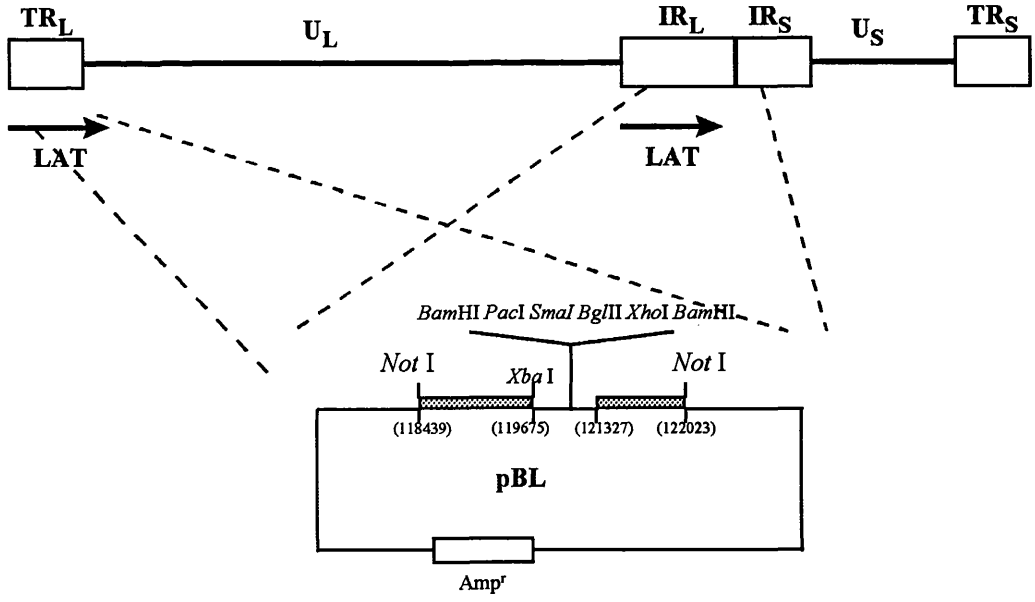
b. pBL (*Pac* I) vector : pGem - 2 backbone containing 2 *Not* I DNA fragments - 1.2 kb (118439-119765) and 0.7 kb (122023-121327) from the HSV - 1 LAT region. Recombination with these fragments results in the loss of a 1.6 kb HSV fragment (121327-119675). Oligonucleotide linker insertion at the *Bam* HI site generates the *Pac* I site required for insertion of promoter - reporter constructs.

Abbreviations used : HSV = herpes simplex virus, Amp^r = ampicillan resistance gene, TR_L = terminal repeat (long) region, TR_S = terminal repeat (short) region, IR_L = internal repeat (long) region, IR_S = internal repeat (short) region, U_L = unique long region and U_S = unique short region.

a. **p35 (*Pac* I)**



b. **pBL (*Pac* I)**



6.2 RESULTS

6.2.1 Generation of promoter construct - shuttle vectors

6.2.1.1 p35 and pBL promoter construct - shuttle vectors

The construction of the promoter - reporter genes is described in **Chapter 4**. The 1.6kb and 4.5kb promoter - reporter gene cassettes were excised from the vectors pBR202 and pBR402 (**Chapter 4**) respectively as *Pac* I fragments and inserted into p35 (*Pac* I) and pBL (*Pac* I). It proved impossible to isolate a shuttle vector containing the 10.5 kb promoter - reporter gene cassette. Attempts to purify the cassette from the plasmid backbone prior to ligation and transformation were repeatedly unsuccessful, with only empty vectors or the original plasmid pBR702 being isolated.

The 1.8 kb NSE promoter fragment was isolated from vector pBluescript. It was subcloned upstream of the *lacZ* gene and the gene cassette was subsequently subcloned into the shuttle vectors.

Table 6.1 lists the promoter - reporter gene shuttle vectors prepared in p35 (*Pac* I) and pBL (*Pac* I).

Table 6.1 p35/pBL promoter - reporter gene shuttle constructs

Shuttle Construct	Promoter Fragment	Shuttle Vector
p35-1.6 *lacZ*	GABA δ 1.6kb	p35 (*Pac* I)
p35-4.5 *lacZ*	GABA δ 4.5kb	p35 (*Pac* I)
p35-NSE *lacZ*	NSE 1.8kb	p35 (*Pac* I)
p35-CMV *lacZ* *	CMV	p35 (*Pac* I)
pBL-1.6 *lacZ*	GABA δ 1.6kb	pBL (*Pac* I)
pBL-NSE *lacZ*	NSE 1.8kb	pBL (*Pac* I)

Constructs marked with a star were prepared by Dr A. McGregor. Abbreviations used : CMV = cytomegalovirus promoter, NSE = neuron specific enolase promoter, *lacZ* = β-galactosidase gene.

6.2.1.2 pZ43 and pZLAT promoter construct - shuttle vectors

A second set of shuttle vectors were designed, using pZErO™-2.1 (Invitrogen) as a vector backbone, to overcome the problems encountered with isolation of the larger promoter - reporter cassettes (**Section 6.2.1.1**). This was performed in collaboration with Dr T.A. Glencorse. The 5.1kb *Bam* HI - *Eco*RI HSV fragment was excised from p35 (*Pac* I) and inserted into the pZErO™-2.1 plasmid to form pZ43. The 2kb *Not* I HSV fragment was excised from pBL (*Pac* I) and inserted into pZErO™-2.1 to form pZLAT. These plasmids use Kanamycin selection as opposed to Ampicillin. This will prevent contamination of the ligation and transformation reactions by the original Ampicillan - containing plasmid backbone.

The 1.6kb and 10.5kb promoter - reporter gene cassettes were excised from the vectors pBR202 and pBR702 (**Chapter 4**) respectively as *Pac* I fragments and inserted into pZ43. The 1.6kb, 4.5kb and 10.5kb promoter - reporter gene cassettes were excised from the vectors pBR202, pBR402 and pBR702 (**Chapter 4**) respectively as *Pac* I fragments and inserted into pZLAT.

Table 6.2 lists the promoter - reporter gene shuttle vectors prepared in pZ43 and pZLAT

Table 6.2 pZ43/pZLAT promoter - reporter gene shuttle constructs

Shuttle Construct	Promoter Fragment	Shuttle Vector
pZ43-1.6 *lacZ*	GABA δ 1.6kb	pZ43
pZ43-10.5 *lacZ*	GABA δ 10.5kb	pZ43
pZLAT-1.6 *lacZ*	GABA δ 1.6kb	pZLAT
pZLAT-4.5 *lacZ*	GABA δ 4.5kb	pZLAT
pZLAT-10.5 *lacZ*	GABA δ 10.5kb	pZLAT

The table lists the shuttle constructs prepared, which promoter fragments were subcloned and which shuttle vector was used.

Abbreviations used : *lacZ* = β-galactosidase gene.

6.2.2 Generation of recombinant HSV - 1 viruses

6.2.2.1 1764 viral variants

Using standard recombination techniques (**Section 2.2.7.2**), the 1.6kb GABA - *lacZ* cassette was introduced into the HSV - 1 genome at the LAT locus. **Figure 6.4** illustrates the banding pattern expected from Southern blot analysis of a recombinant LAT virus.

96 viral plaques were isolated and the DNA prepared from these (**Section 2.2.7.4**). The first 72 viruses were loaded onto a dot blot apparatus. The membrane was probed with a random primed *lacZ* fragment. There were 4 strong putative positives : 27, 47, 52 and 62 (**Figure 6.5a**). These positives were analysed by dot blotting again, along with samples 73 - 96. The membrane was probed with a 1.2 kb *Hin*d III - *Not* I GABA δ fragment. Samples 27, 47 and 91 were positive (**Figure 6.5b**).

The positive viruses were further investigated by Southern blot analysis (**Section 2.2.1.9**). The positive viral DNA samples were digested with *Bam* HI and Southern blotted. The membranes were probed with a random primed HSV *Bam* HI b fragment.

Sample 47 appeared to be a recombinant, of about 20% purity (**Figure 6.6**). After 3 rounds of purification by isolating single plaques, this virus was still a mixture of wild - type and recombinant. 4 further rounds of purification by isolating single plaques was required before the recombinant virus was pure (**Figure 6.7**). The banding pattern was consistent with the GABA - *lacZ* cassette having been inserted at both LAT loci. A high titre virus stock of this recombinant virus was prepared (**Section 2.2.7.6**). The titre of the 1764 LAT (1.6) GABA - *lacZ* virus (1782) was calculated (**Section 2.2.7.7**) to quantitate the amount of infectious virus present in the stock. The viral titre is 1.77×10^9 pfu/ml.

Figure 6.4 Schematic representation of the recombinant LAT - GABA(1.6) - lacZ locus

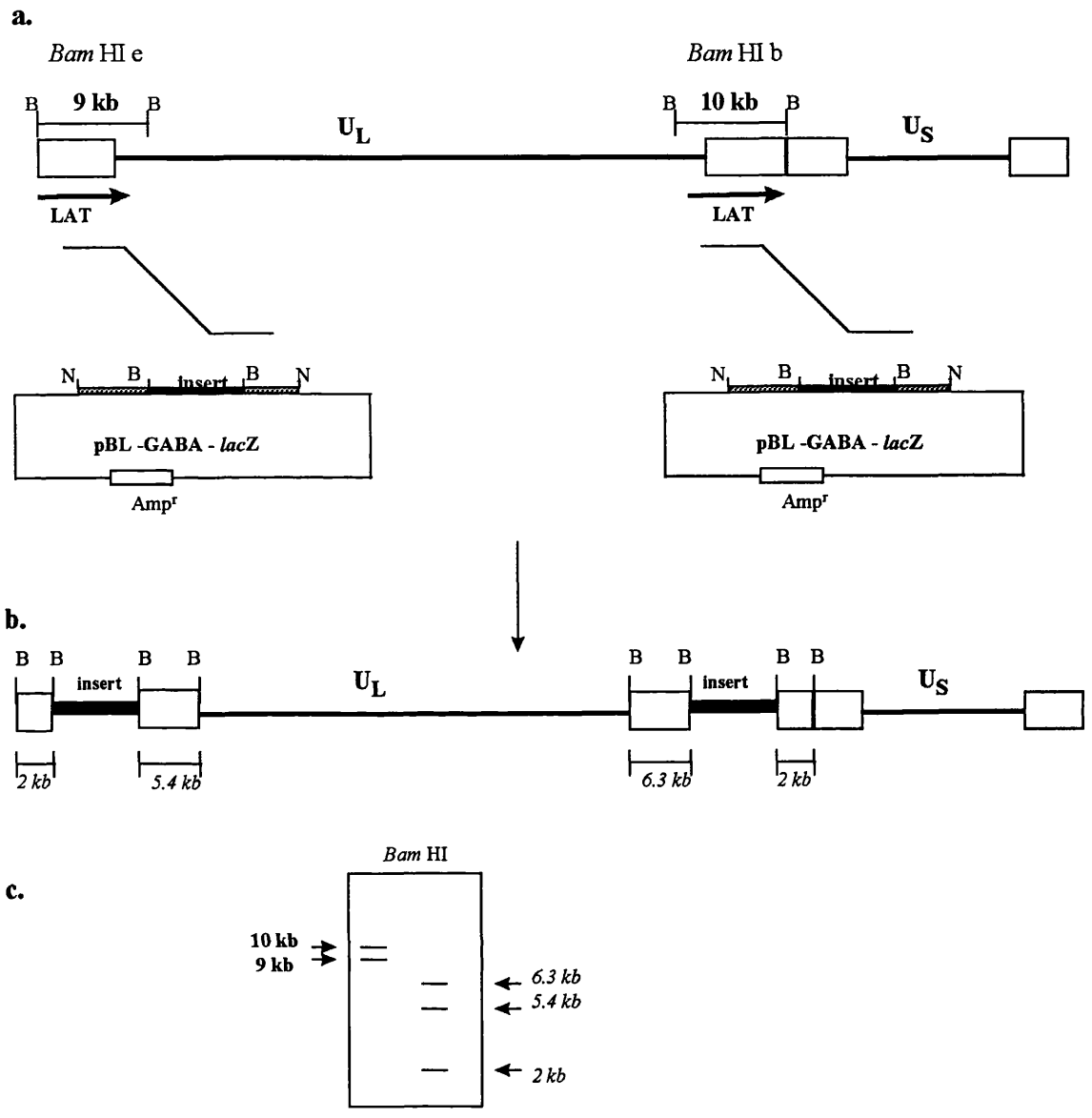
Figure 6.4 ***Schematic representation of the recombinant LAT - GABA(1.6) - lacZ locus***

a. The figure shows the expected recombination event. The location of the *Bam* HI sites in the LAT region, and the expected fragment sizes upon digestion with *Bam* HI are indicated. *Bam* HI b is the HSV - 1 probe used in the Southern blot analysis. This probe will also hybridise to *Bam* HI e due to the presence of homologous sequences.

b. The figure shows the structure of the recombinant LAT virus. The location of the *Bam* HI sites in the LAT region, and the expected fragment sizes upon digestion with *Bam* HI are indicated.

c. Expected banding pattern upon Southern blot analysis of recombinant viral DNA digested with *Bam* HI and probed with *Bam* HI b. The promoter/reporter cassette would be excised.

Abbreviations used : U_L = unique long, U_S = unique short, B = *Bam* HI, N = *Not* I, Amp^r = ampicillin resistance gene.


a.
Bam HI e
Bam HI b
B
9 kb
B
B
10 kb
B
U_L
U_S
LAT
LAT
N
B
insert
B
N
pBL -GABA - lacZ
Amp^r
b.
B B
insert
B B
U_L
B B
insert
B B
U_S
2 kb
5.4 kb
6.3 kb
2 kb
c.
Bam HI
10 kb
9 kb
6.3 kb
5.4 kb
2 kb

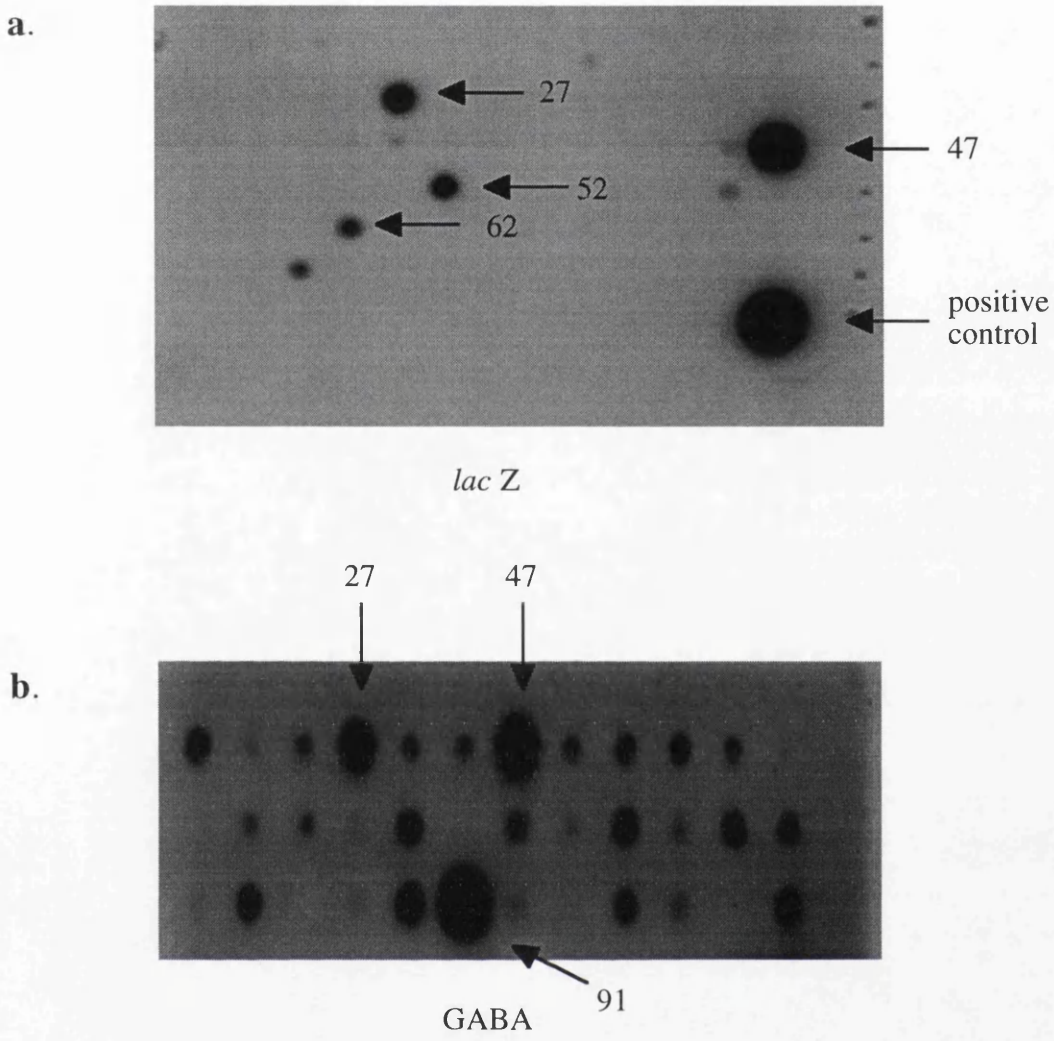


Figure 6.5 ***Dot blot analysis of putative recombinant LAT-GABA(1.6) - lac Z viruses***

Dot blots showing putative positive recombinant viruses for the LAT-GABA(1.6)-*lac* Z construct.

a. The blot was probed with a random - primed *lac* Z DNA fragment. 4 viruses (27, 47, 52, 62) gave strong signals and these are indicated with arrows.

b. The blot was probed with a 1.2 kb *Hin*d III - *Not* I GABA fragment. 3 viruses (27, 47, 91) gave strong signals and these are indicated with arrows.

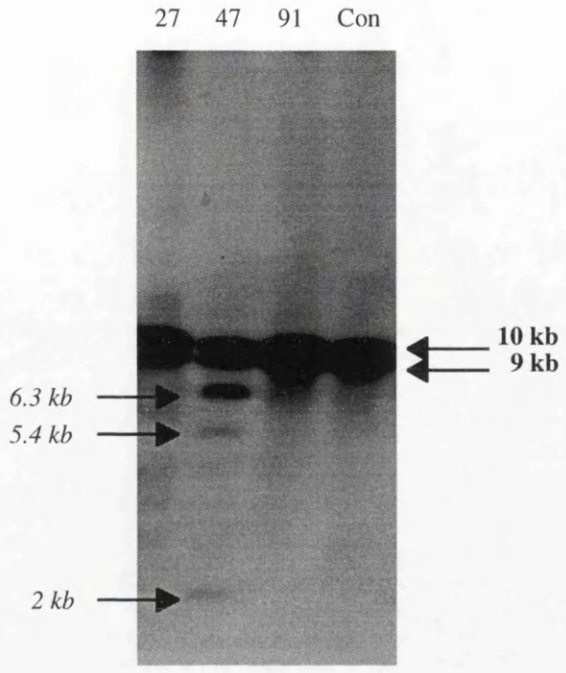


Figure 6.6 ***Southern blot analysis of putative recombinant LAT-GABA(1.6)-lac Z viruses***

The 3 positive viruses from the dot blot analysis were analysed by Southern blotting. They were digested with *Bam* HI and the blot was probed with an HSV-1 *Bam* HI b DNA fragment. The wild - type bands are indicated in bold, the mutant bands are indicated in italics. Only virus 47 showed recombinant bands. The 5.4 kb and 2 kb bands are less intense as they are not fully homologous with the *Bam* HI b probe (**Figure 6.4**)

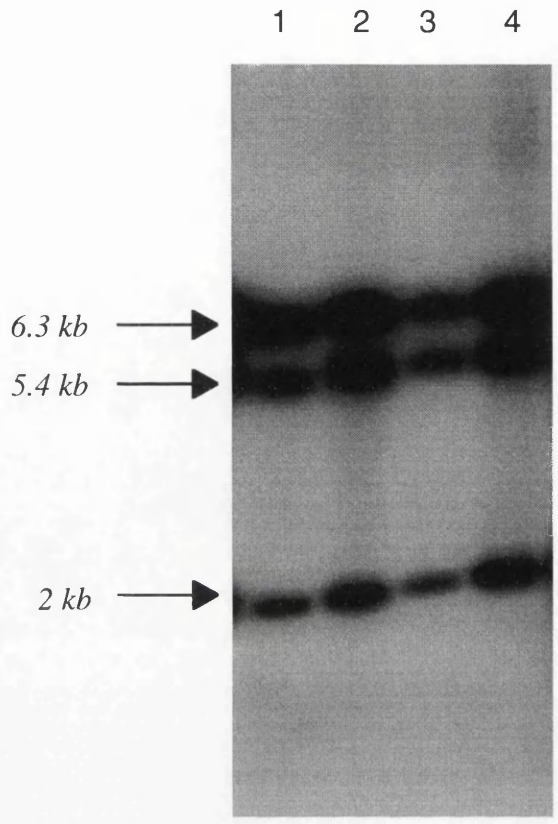


Figure 6.7 Southern blot analysis of LAT-GABA(1.6)-lac Z recombinant viruses
The blot shows 4 viral DNA samples isolated after several rounds of purification. The viral DNA samples were digested with *Bam* HI and the blot was probed with a random primed HSV-1 *Bam* HI b DNA fragment. The mutant bands are indicated in italics. All samples isolated from this round of purification were recombinant.

6.2.2.2 1716 viral variants

Using standard recombination techniques (**Section 2.2.7.2**), attempts were made to introduce promoter - *lacZ* cassettes - 1.6kb and 10.5kb into the HSV genome at the U_L43 locus and promoter - *lacZ* cassettes - 1.6kb, 4.5kb and 10.5kb into the HSV genome at the LAT locus. **Figure 6.8** illustrates the banding pattern expected from Southern blot analysis of a recombinant U_L43 virus

6.2.2.2.1 pZ43 - 1.6 - lacZ

For the pZ43 - 1.6 construct, 96 plaques were isolated and screened by dot blot analysis using the 1.2 kb *Hind* III - *Not* I GABA probe. 7 out of the 96 plaques were positive - 6, 18, 27, 28, 29, 42 and 92 (**Figure 6.9**). These samples were further investigated by Southern blot analysis (**Section 2.2.1.9**). The positive viral DNA samples were cut with *Bam* HI, Southern blotted and probed with a random primed HSV *Bam* HI - *Eco* RI fragment.

All of the samples contained recombinant bands, however 2 samples - 6 and 18 appeared to be pure (**Figure 6.10**). Sample 6 was taken through 2 further rounds of purification by isolating single plaques before a high titre virus stock (**Section 2.2.7.6**) was prepared. The titre of the 1716 UL43 (1.6) GABA - *lacZ* virus (1787R) was calculated (**Section 2.2.7.7**) to quantitate the amount of infectious virus present in the stock. The viral titre is 2×10^{10} pfu/ml.

Figure 6.8 Schematic representation of the recombinant UL43 - GABA(1.6) - lacZ locus

Figure 6.8 Schematic representation of the recombinant UL43 - GABA(1.6) - lacZ locus

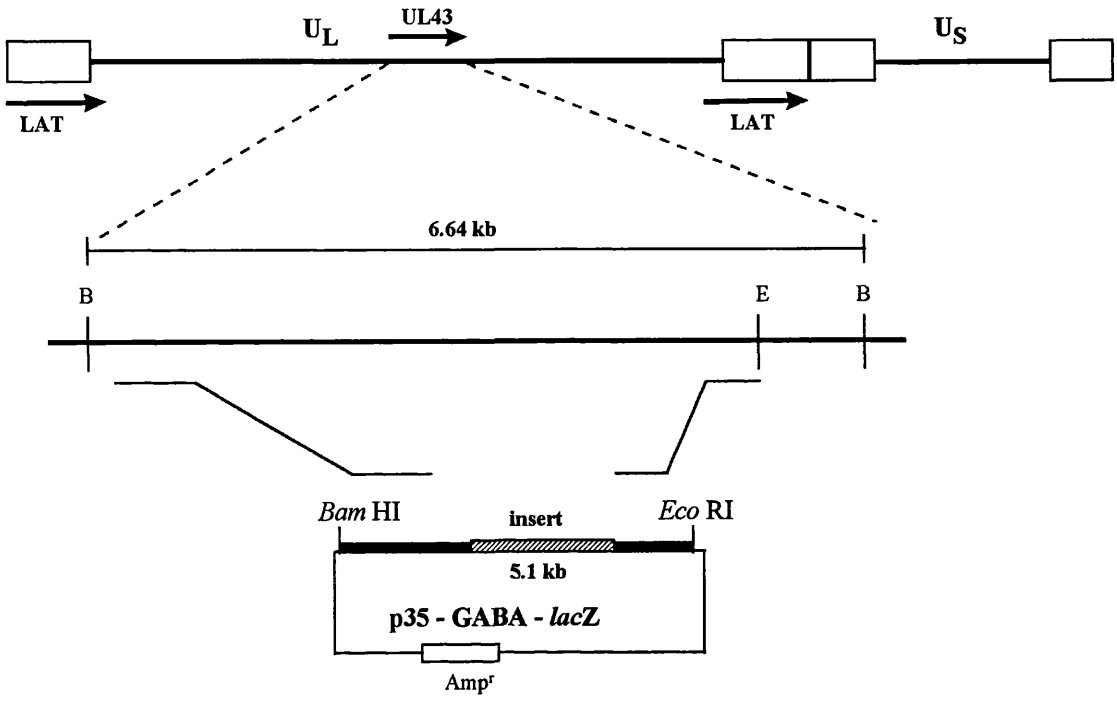
a. The figure shows the expected recombination event. The location of the *Bam* HI sites in the U_L43 region, and the expected fragment size upon digestion with *Bam* HI is indicated. *Bam* HI - *Eco*RI is the HSV - 1 probe used in the Southern blot analysis.

b. The figure shows the structure of the recombinant U_L43 virus. The location of the *Bam* HI sites in the U_L43 region, and the expected fragment size upon digestion with *Bam* HI are indicated.

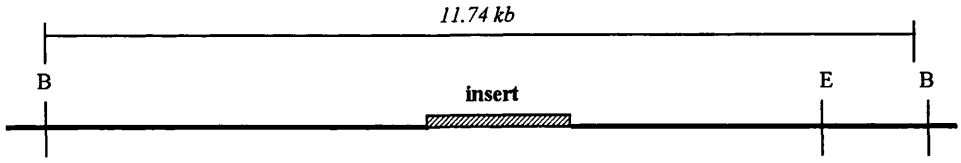
c. Expected banding pattern upon Southern blot analysis of recombinant viral DNA digested with *Bam* HI and probed with the *Bam* HI - *Eco* RI HSV DNA fragment.

Abbreviations used : U_L = unique long, U_S = unique short, B = *Bam* HI, E = *Eco* RI, Amp^r = ampicillin resistance gene.

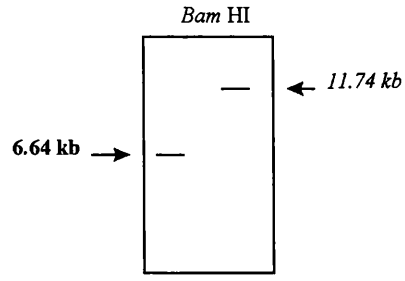
a.


U_L
UL43
U_S
LAT
LAT
6.64 kb
B
E
B
Bam HI
insert
Eco RI
5.1 kb
p35 - GABA - *lacZ*
Amp^r

b.


11.74 kb
B
insert
E
B

c.


Bam HI
11.74 kb
6.64 kb

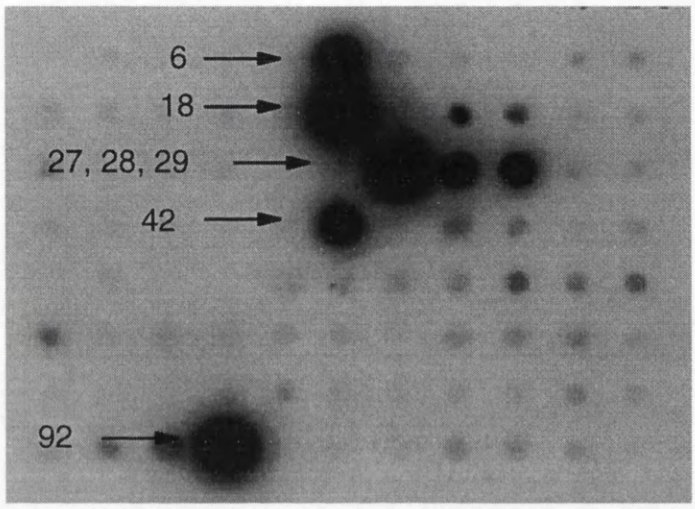


Figure 6.9 ***Dot blot analysis of putative recombinant UL43 -GABA(1.6)-lac Z viruses***

Dot blots showing putative positive recombinant viruses for the UL43-GABA(1.6)-*lac* Z construct.

The blot was probed with a 1.2 kb *Hind* III - *Not* I GABA fragment. 6 viruses gave strong signals : 6, 18, 27, 28, 29, 42, 92 and these are indicated with arrows.

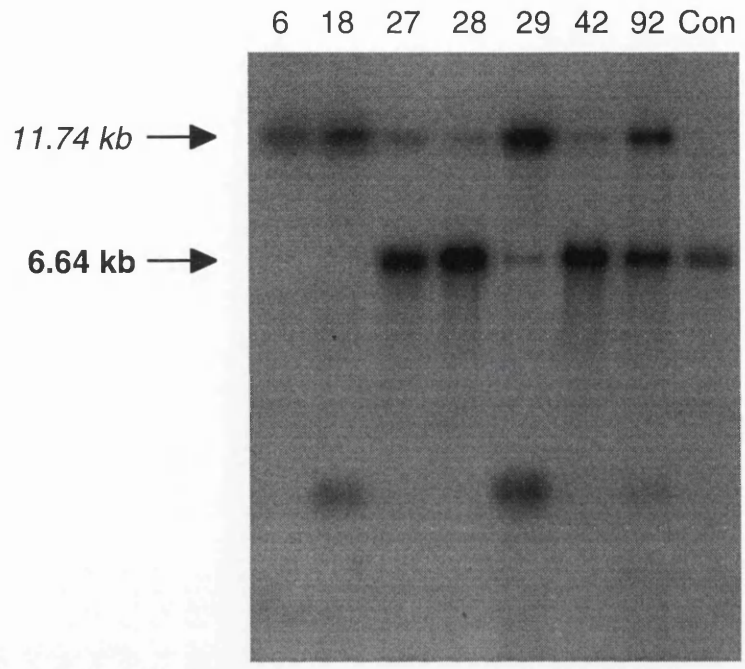


Figure 6.10 Southern blot analysis of putative recombinant UL43-GABA(1.6)-lac Z viruses

The 7 positive viruses from the dot blot analysis were analysed by Southern blotting. They were digested with *Bam* HI and the blot was probed with an HSV-1 UL43 *Bam* HI-*Eco* RI DNA fragment. The wild-type band is indicated in bold, the mutant band is indicated in italics. All viruses showed the mutant band, samples 6 and 18 looked pure.

6.2.2.2.2 pZ43 - 10.5 - lacZ

For the pZ43 - 10.5kb construct, 576 plaques were isolated and screened by dot blot analysis using the 1.2 kb *Hind* III - *Not* I GABA probe. One virus gave a positive result from the dot blot (**Figure 6.11**). This was further analysed by Southern blotting. The DNA was cut with *Bam* HI, blotted overnight and then probed with a random primed HSV *Bam* HI - *Eco* RI fragment. The expected banding pattern would have seen the wild - type 6.64 kb band increase to 20.64 kb. However, it did not give the correct banding pattern (**Figure 6.12**) and was therefore discarded.

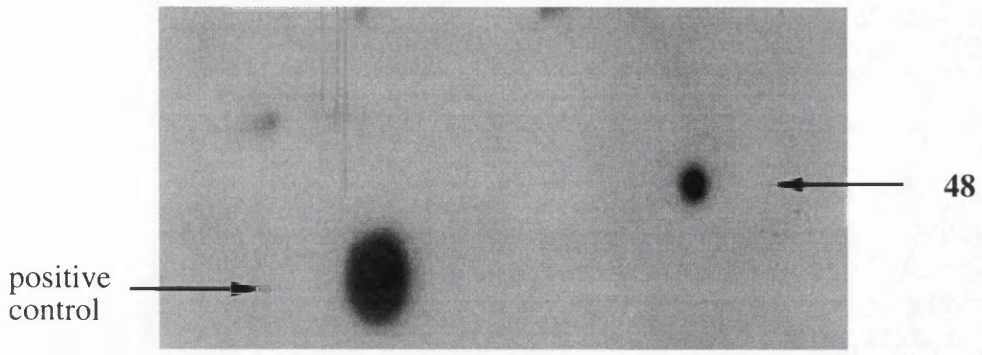


Figure 6.11 Dot blot analysis of putative recombinant UL43-GABA(10.5)-lac Z viruses

Dot blot showing putative positive recombinant viruses for the UL43-GABA(10.5)-*lac* Z construct. The blot was probed with a 1.2 kb *Hin*d III - *Not* I GABA fragment. 1 virus (48) gave a strong signal.

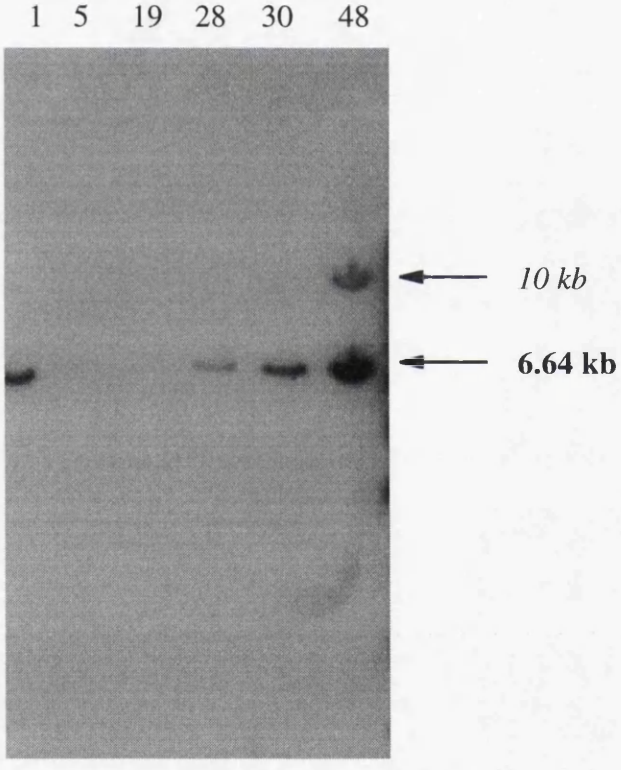


Figure 6.12 Southern blot analysis of putative recombinant UL43-GABA(10.5) - lac Z virus

The putative positive virus (48), and several faint positive isolates, from the dot blot analysis were further analysed by Southern blotting. They were digested with *Bam* HI and the blot probed with an HSV-1 UL43 *Bam* HI - *Eco* RI fragment. The wild - type band is indicated in bold, the mutant band is indicated in italics. Virus 48 does not give the expected 20 kb DNA fragment. The smaller band results in all probability, from an aberrant recombination event.

6.2.2.2.3 Other pZ43 and pZLAT recombinant viruses

pZLAT constructs - 1.6 kb - *lacZ* (100 viral DNAs) and 4.5 kb - *lacZ* (200 viral DNAs) were screened by dot blot analysis for recombinant viruses. No positive viruses were detected.

Table 6.3 lists the recombinant viruses isolated.

Table 6.3 Recombinant 1764 and 1716 viruses

Recombinant Viruses	Viral variant	Promoter Fragment	Insertion Locus
1780	1764	CMV	U_L43
1781	1764	NSE	U_L43
1782	**1764**	**1.6 GABA**	**LAT**
1783	1764	4.5 GABA	U_L43
1786	1764	gD	U_L43
1780R	1716	CMV	U_L43
1781R	1716	NSE	U_L43
1782R	1716	1.6 GABA	LAT
1783R	1716	4.5 GABA	U_L43
1786R	1716	gD	U_L43
1787	**1716**	**1.6 GABA**	U_L43

Viruses in normal type were prepared by Dr A. McGregor. Viruses marked in bold were isolated by myself. Abbreviations used : CMV = cytomegalovirus promoter, NSE = neuron - specific enolase promoter, gD = glycoprotein D (HSV specific promoter), R = viruses were prepared by recombination between the 1764 mutants listed and the 1716 HSV - 1 virus. This corrected the Vmw65 mutation and gave the recombinant 1716 viral mutants listed.

6.3 DISCUSSION

There were several problems encountered during this work. Initially the promoter - reporter cassettes had to be subcloned into the shuttle vectors. While this was straightforward for the cassettes containing the smaller promoter fragments, it proved to be impossible for the larger promoter constructs. Eventually a second set of shuttle vectors based on the pZErO^{TM}-2.1 vector, were designed and constructed (in collaboration with Dr T. Glencorse). The advantage of this vector was that Kanamycin could be used for the selection of positive clones, and this successfully overcame the problems encountered with the original vector at the ligation stage with contamination from the promoter - reporter cassette backbone vector, which was ampicillin resistant. A positive clone containing the 10.5kb promoter - *lacZ* cassette was eventually isolated for the U_L43 and LAT shuttle vectors, with this system.

Another major problem encountered was with the purification of the recombinant viruses. Initially the 1764 viral variant was used. It proved difficult to purify recombinant viruses away from the wild - type virus. It took many rounds of purification to obtain the 1782 (1.6 - *lacZ* into LAT locus) virus. With one virus, the 1.6 - *lacZ* into U_L43, even after repeating the transfection twice and going through numerous rounds of purification, I was unable to isolate a pure recombinant virus. Dr A. McGregor investigated different approaches for obtaining higher proportions of recombinant viruses. These were generally more successful (**Table 6.3**), however, he was also unable to completely purify this recombinant virus.

Another viral variant utilised was 1716. This virus, unlike 1764, does not require chemical help in order to plaque. However, it may produce more cytotoxic viruses. Recombinant viruses were isolated using this variant (**Table 6.3**).

I was unable to isolate a recombinant virus for the 10.5 kb promoter cassette. While one virus was positive when the DNA was analysed by dot blotting and was probed with a GABA fragment, this gave an aberrant restriction pattern upon Southern blotting (**Figures 6.11** and **6.12**). This suggests that deletions or rearrangements were occurring. Dr McGregor was also unable to isolate the 10.5 - *lacZ* recombinant virus. Thus it may be

necessary to delete some of the HSV - 1 genome to achieve insertion of the larger promoter cassette. However at 14 kb, the cloning capacity of HSV - 1 should not have been reached. It is possible that the actual sequence of the $GABA_A$ receptor δ subunit gene promoter has caused these problems. Analysis of the 5' - upstream region (**Chapter 3**) of the $GABA_A$ receptor δ subunit gene revealed an imperfect repeat region (**Figure 3.6**; -5275 to - 4689) that is highly homologous to a region of the HSV - 1 genome. Thus it is possible that recombination events were occurring between the δ and the HSV -1 DNA resulting in the observed deletions or rearrangements. Insertion of another large promoter cassette (e.g. $GABA_A$ receptor α6) would perhaps answer some of these questions.

In summary, there are two major problems with making recombinant viruses using the standard recombination method.. The first is the necessity to transfer promoter - reporter cassettes into shuttle vectors. The second is the low frequency of recombination encountered. The systems being developed by Dr A. McGregor will, to a certain extent, overcome these problems. One advantage of these new methods is that the shuttle vector stage can be omitted. Another is that there should be a lower background of non - recombinant viruses. These systems may be further improved by the use of directional cloning.

The purified recombinant viruses have been assessed for their ability to target specific neurons and for the expression of the various promoter fragments in rats and mice. This is discussed in **Chapter 7**.

Chapter 7

Infection of cerebellar granule cell cultures and rat brains with HSV - 1 recombinant viruses

7.1 INTRODUCTION

The main aim of the viral work was to develop an HSV - 1 viral vector that would enter neurons, establish a latent infection and allow long - term expression of a reporter gene from neuron - specific promoters. A selection of the recombinant viruses (Chapter 6) were steroetactically injected into the cerebellum of adult male rats. The cerebellum was chosen as the site of injection of the viruses as it contains cells which express the $GABA_A$ receptor δ subunit gene. The δ gene has a restricted expression pattern in the cerebellum in that it is expressed only in the granule cells. The Purkinje cells will act as a negative control for neuron - specific expression from the δ promoter as they do not express the endogenous gene. Thus it was hoped that cell - type specific expression would be observed with the δ promoter, i.e. we would expect to see β - galactosidase expression restricted to the granule cells.

This chapter describes the infection of cerebellar granule cell cultures with recombinant HSV - 1 viruses and the stereotactic injection of the viruses into the cerebellum of rats. The isolation of recombinant viruses was described in **Chapter 6**.

7.2 RESULTS

The methods for the introduction of the recombinant viruses into granule cell cultures and rat brains are described in **Section 2.2.10**.

7.2.1 Infection of cerebellar granule cells by recombinant viruses

This work was carried out in collaboration with Dr T.A. Glencorse and Dr G. Westrop. A selection of the purified recombinant 1716 viruses were introduced into cerebellar granule cells : 1786R (gD), 1780R (CMV) and 1782R (1.6 - δ) (**Section 6.2.2.2**). A 17+ (wild - type) gD - *lacZ* control virus was also used in the infection. There appeared to be no cytopathic effect of the viruses on the granule cells. They were fixed and stained 24 hours and 48 hours after infection. **Figures 7.1 - 7-3** show the results of the infection. Only the wild - type gD - *lacZ* and 1716 gD - *lacZ* viruses showed specific β - galactosidase expression.

Figure 7.1 Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses

Figure 7.1 ***Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses***

Cerebellar granule cell cultures were infected with wild - type HSV - 1 virus containing the *lacZ* gene driven by the HSV gD promoter

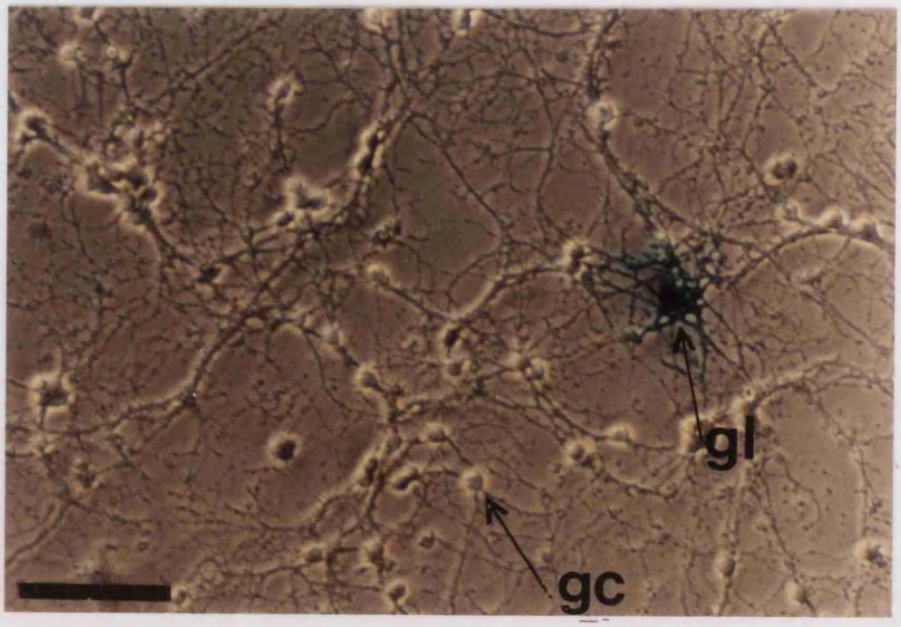
a. Cells were stained 24 hours post - infection. *lacZ* expression can be seen in a glial cell (magnification = 10x).

b. Cells were stained 48 hours post - infection. *lacZ* expression can be seen mainly in glial cells. Some granule cells are positively stained, however these are connected to stained glial cells (magnification = 10x).

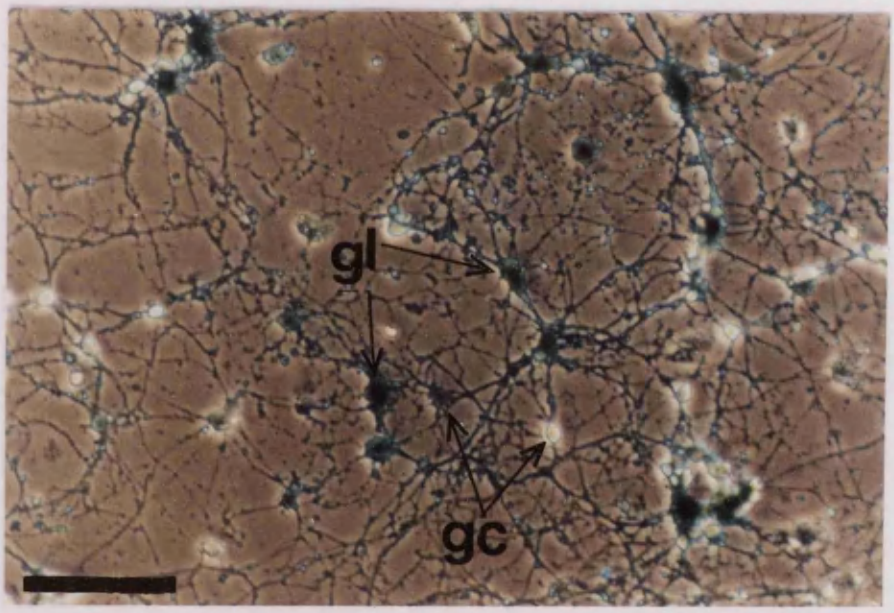
c. A higher magnification of the cells shown in **b**. above. Cells were stained 48 hours post - infection. *lacZ* expression can be seen mainly in glial cells. Some granule cells are positively stained, however these are connected to stained glial cells. (magnification = 20x)

Abbreviations used : gc = cerebellar granule cells; gl = glial cells

a.



b.



c.

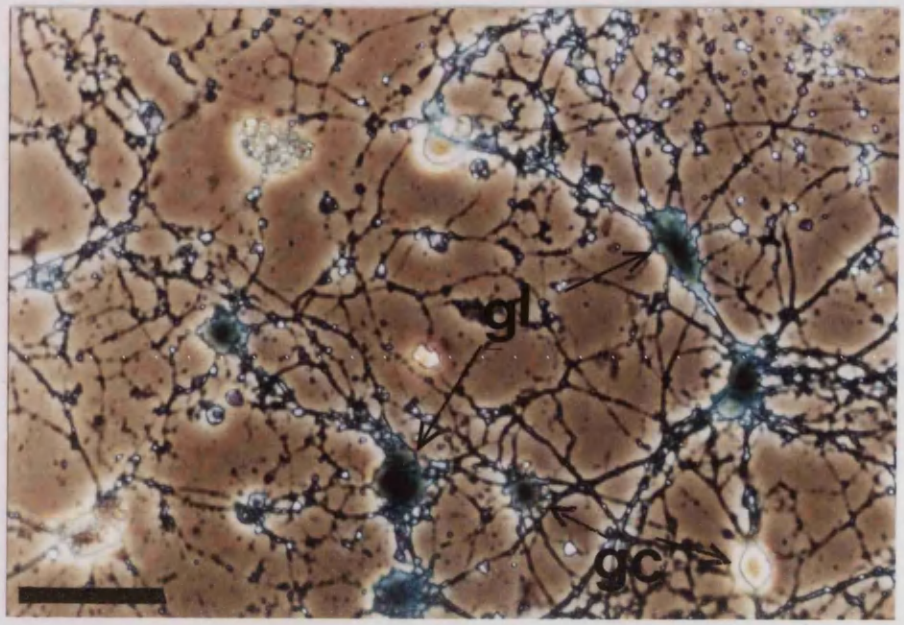


Figure 7.2 Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses

Figure 7.2 ***Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses***

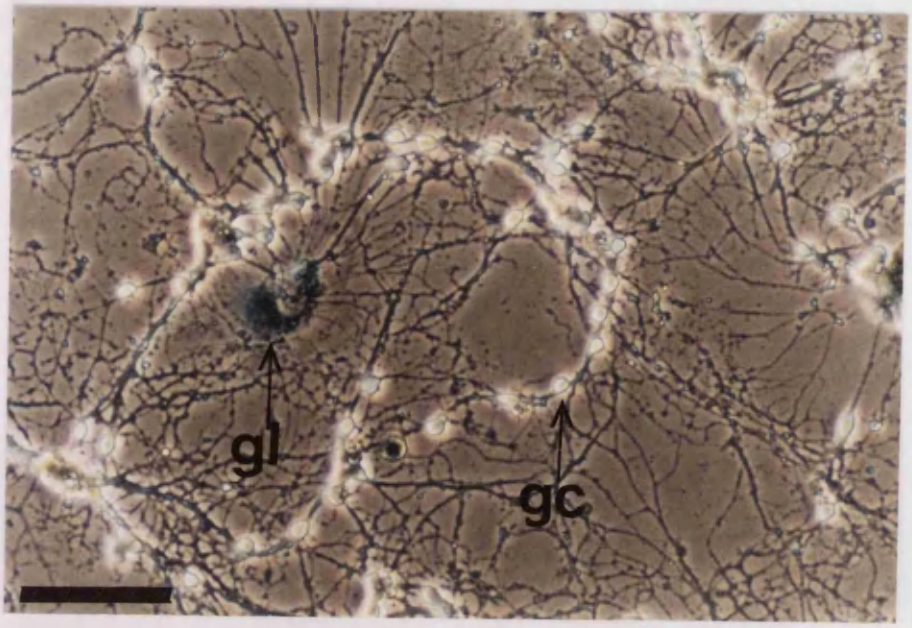
a. Cerebellar granule cell cultures infected with the HSV - 1 1716 gD - *lac*Z recombinant virus. *lac*Z staining can be seen in a glial cell (magnification = 10x).

b. Cerebellar granule cell cultures infected with the HSV - 1 1716 CMV - *lac*Z recombinant virus. No *lac*Z staining can be seen (magnification = 10x).

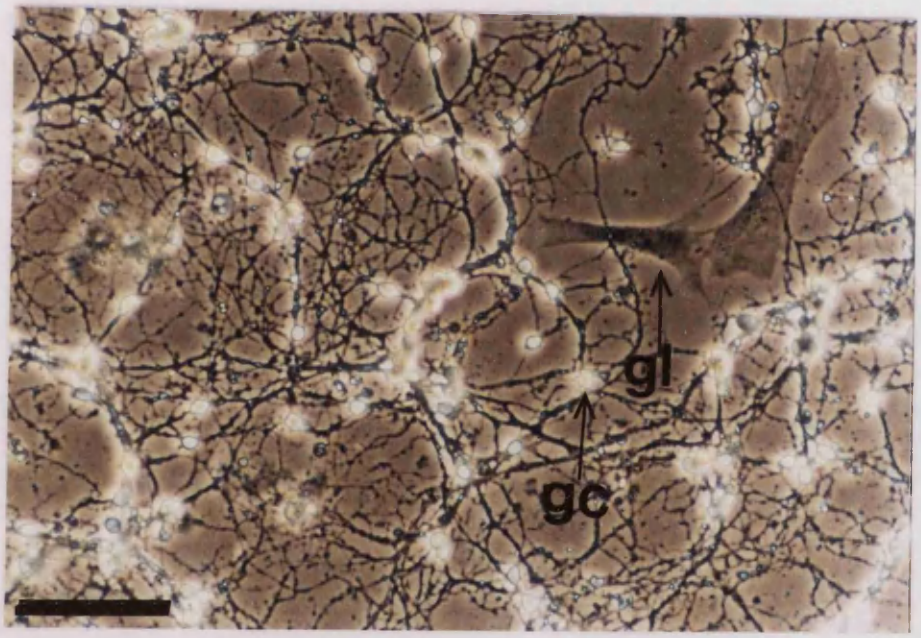
c. Uninfected cerebellar granule cell cultures used as a control for infection. *lac*Z staining can be seen. This is endogenous β - galactosidase activity (magnification = 10x).

Abbreviations used : gc = cerebellar granule cells; gl = glial cells

a.



b.



c.

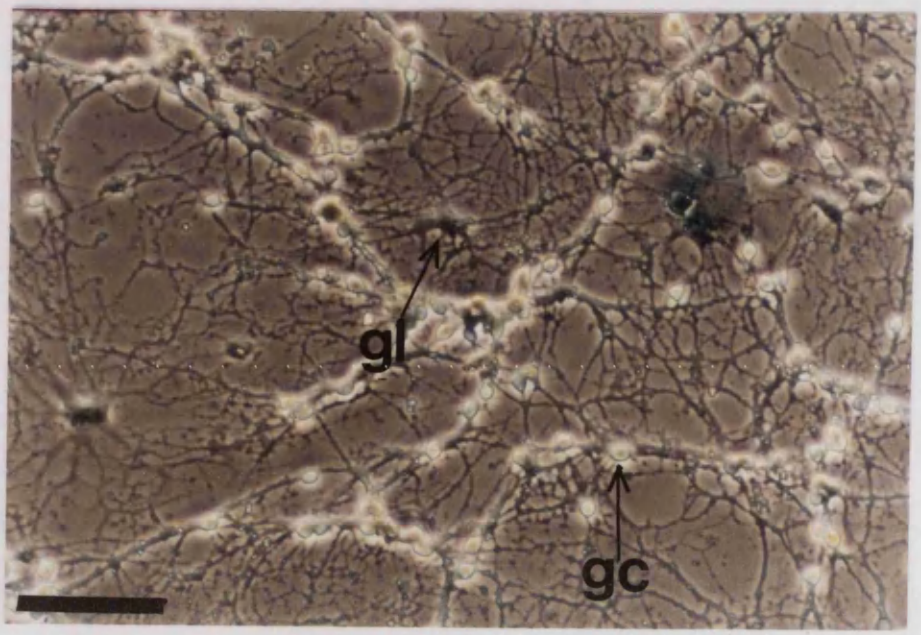


Figure 7.3 Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses

Figure 7.3 ***Photomicrographs showing cerebellar granule cell cultures infected with recombinant viruses***

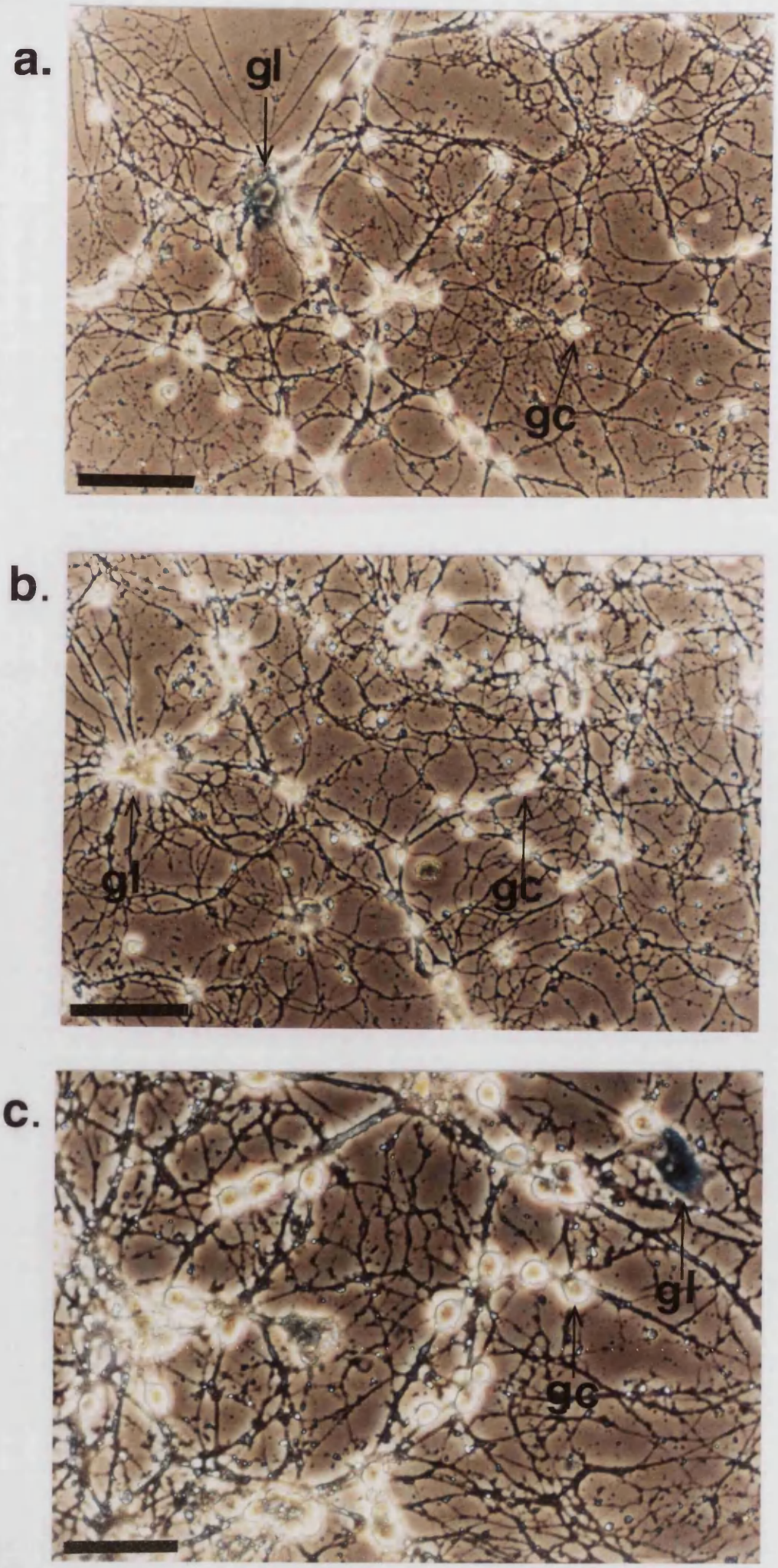
Cerebellar granule cell cultures were infected with the HSV - 1 1716 UL43 - GABA(1.6) - *lac*Z virus

a. No *lac*Z staining was observed in the glial or granule cells (magnification = 10x).

b. No *lac*Z staining was observed in the glial or granule cells (magnification = 10x).

c. One positively stained cell was observed, however this was probably due to endogenous β - galactosidase activity as seen for the control cells (magnification = 20x).

Abbreviations used : gc = cerebellar granule cells; gl = glial cells


a.
gl
gc
b.
gl
gc
c.
gl
gc

7.2.2 Infection of the cerebellum of rat brains by recombinant viruses

This work was carried out in collaboration with Dr A. MacLean and Dr A. McGregor (Dept of Virology, University of Glasgow). Dr J. Campbell and Mr N. Bennett (Dept of Anatomy) carried out the stereotactic injections and subsequent care and sacrifice of the animals.

Recombinant viruses 1780R (CMV - *lacZ*), 1781R (NSE - *lacZ*), 1787 (U_L43 GABA) and 1882R (LAT GABA) (**Chapter 6, Table 6.3**) were stereotactically injected into the cerebellum (co - ordinates : AP 10.5mm, L 2mm, V 2mm from Bregma) of adult rats. The rats were monitored daily for abnormal behaviour or signs of distress. The rats were sacrificed after 5 days. Horizontal sections of the brain were stained for β - galactosidase activity. The results are shown in **Figures 7.4 - 7.8**. The staining pattern was similar for all the recombinant viruses with slight differences in intensity. As can be seen from the control sections, this was due to endogenous β - galactosidase activity. No β - galactosidase staining was observed in the cerebellar granule cells.

Figure 7.4 Photomicrographs showing sections of adult rat brains infected with the UL43-CMV-lacZ virus

Figure 7.4 ***Photomicrographs showing sections of adult rat brains infected with the UL43 - CMV - lacZ virus***

Horizontal sections were taken through adult rat brains infected (in the cerebellum) with the 1716 CMV - *lacZ* virus. Three regions are shown :

a. **Cerebellum** Section shows *lacZ* staining in cells in the Purkinje cell layer. No staining was observed in the granule cells. (magnification = 40x)

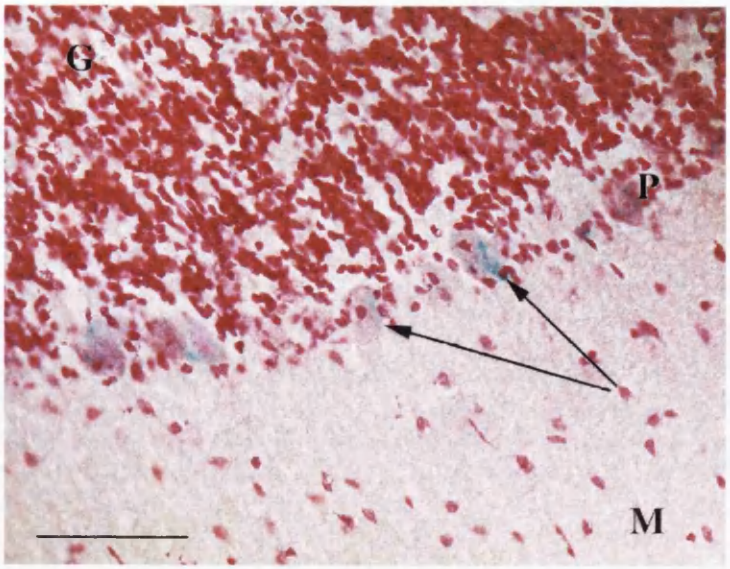
b. **Ventricles** Section shows *lacZ* staining in the ventricles. (magnification = 20x)

c. **Hippocampus** No *lacZ* staining was observed in the hippocampal cells. (magnification = 10x)

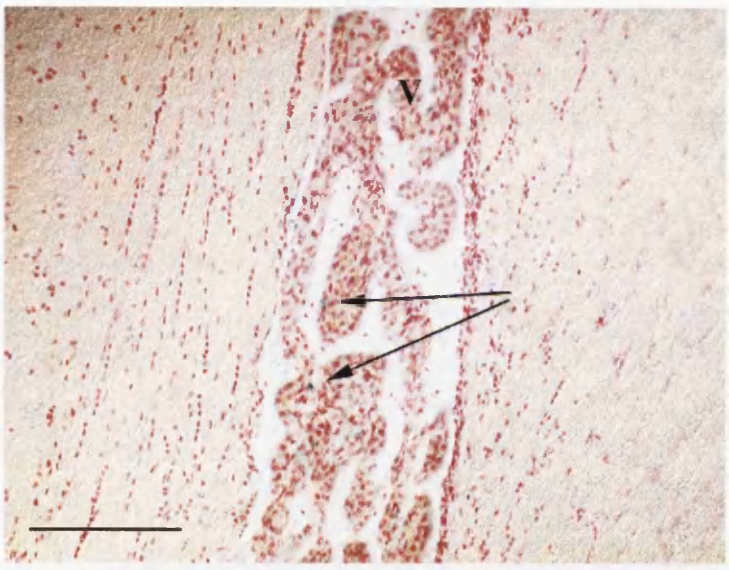
Arrows indicate cells showing β - galactosidase activity.

Abbreviations used : G = cerebellar granule cells; P = Purkinje cell layer; M = cerebellar molecular layer; V = ventricles; DG = dentate gyrus; PoDG = posterior area of the dentate gyrus.

a.



b.



c.

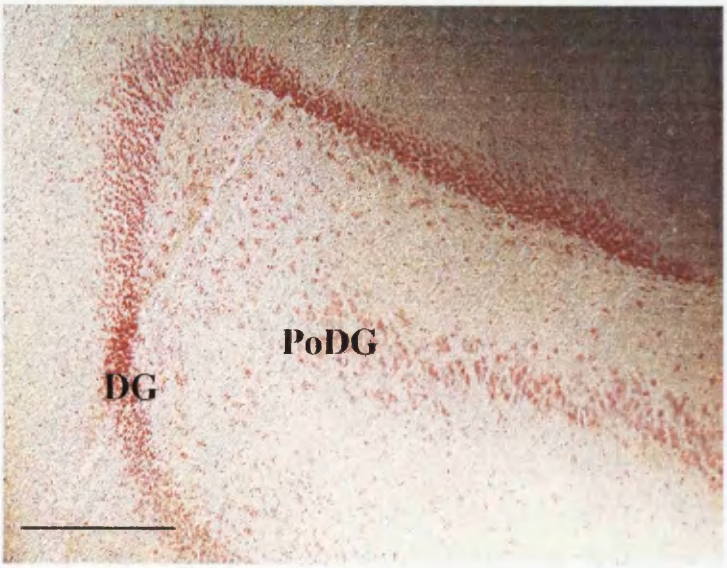


Figure 7.5 Photomicrographs showing sections of adult rat brains infected with the UL43-NSE-lacZ virus

Figure 7.5 ***Photomicrographs showing sections of adult rat brains infected with the UL43 - NSE - lacZ virus***

Horizontal sections were taken through adult rat brains infected (in the cerebellum) with the 1716 NSE - *lacZ* virus. Three regions are shown :

a. **Cerebellum** Section shows *lacZ* staining in cells in the Purkinje cell layer. No staining was observed in the granule cells. (magnification = 40x)

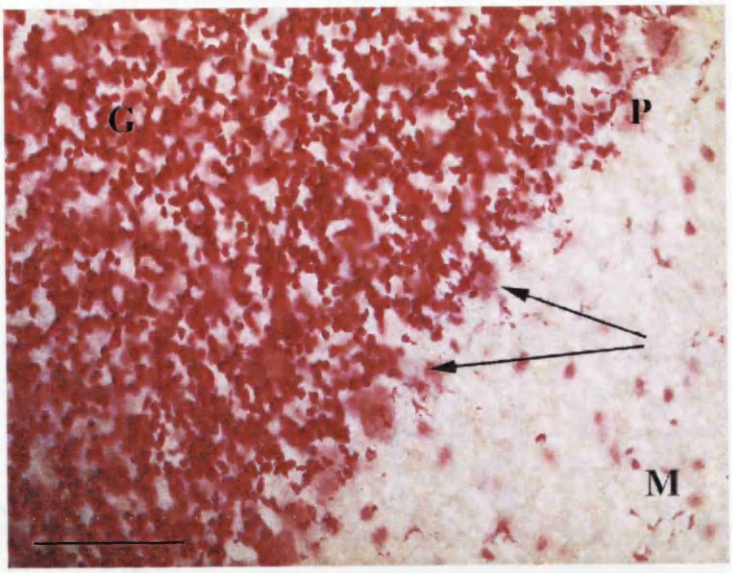
b. **Ventricles** Section shows *lacZ* staining in the ventricles. (magnification = 20x)

c. **Hippocampus** No *lacZ* staining was observed in the hippocampal cells. (magnification = 10x)

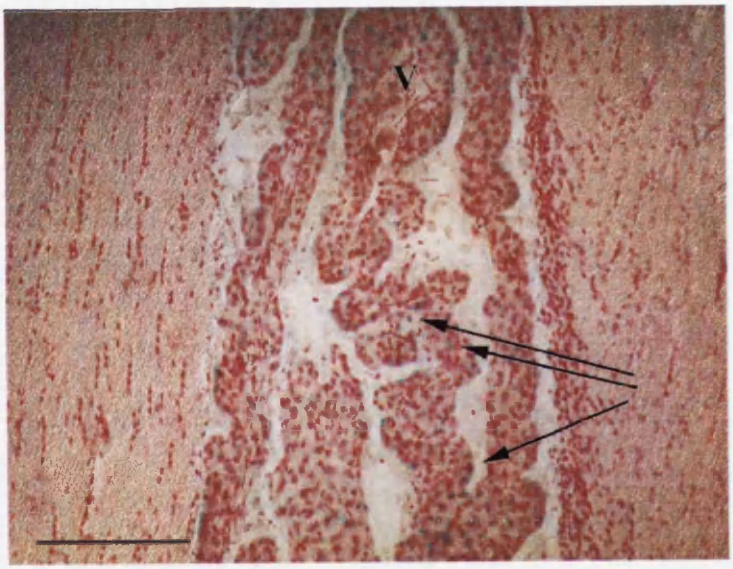
Arrows indicate cells showing β - galactosidase activity.

Abbreviations used : G = cerebellar granule cells; P = Purkinje cell layer; M = cerebellar molecular layer; V = ventricles; DG = dentate gyrus; PoDG = posterior area of the dentate gyrus.

a.



b.



c.

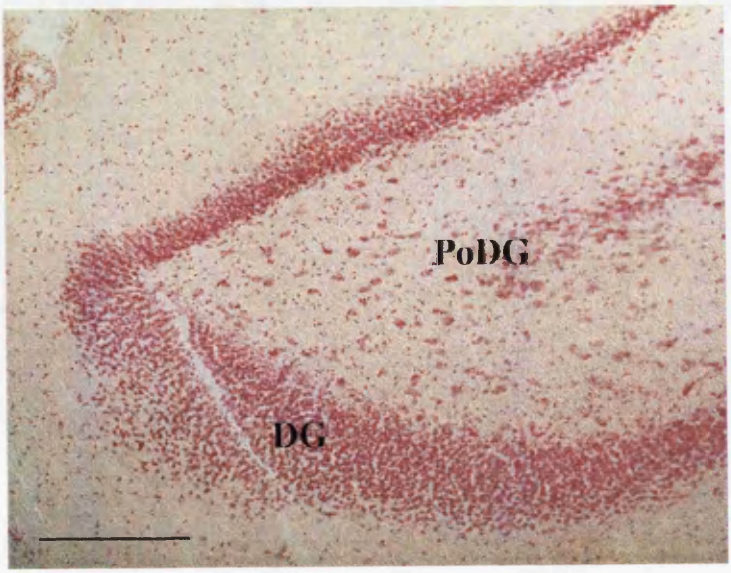


Figure 7.6 Photomicrographs showing sections of adult rat brains infected with the UL43-GABA(1.6)-lacZ virus

Figure 7.6 Photomicrographs showing sections of adult rat brains infected with the UL43 - GABA(1.6) - lacZ virus

Horizontal sections were taken through adult rat brains infected (in the cerebellum) with the 1716 UL43 - GABA(1.6) - *lacZ* virus. Three regions are shown :

a. **Cerebellum** Section shows *lacZ* staining in cells in the Purkinje cell layer. No staining was observed in the granule cells. (magnification = 40x)

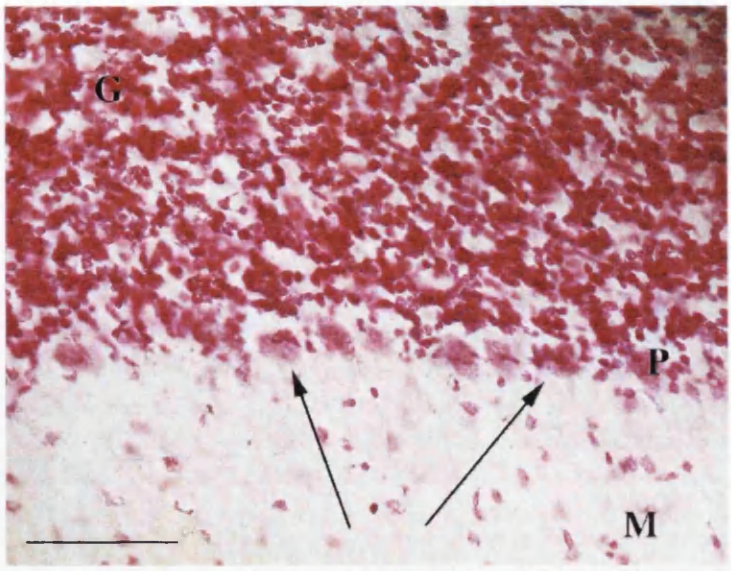
b. **Ventricles** Section shows *lacZ* staining in the ventricles. (magnification = 20x)

c. **Hippocampus** No *lacZ* staining was observed in the hippocampal cells. (magnification = 10x)

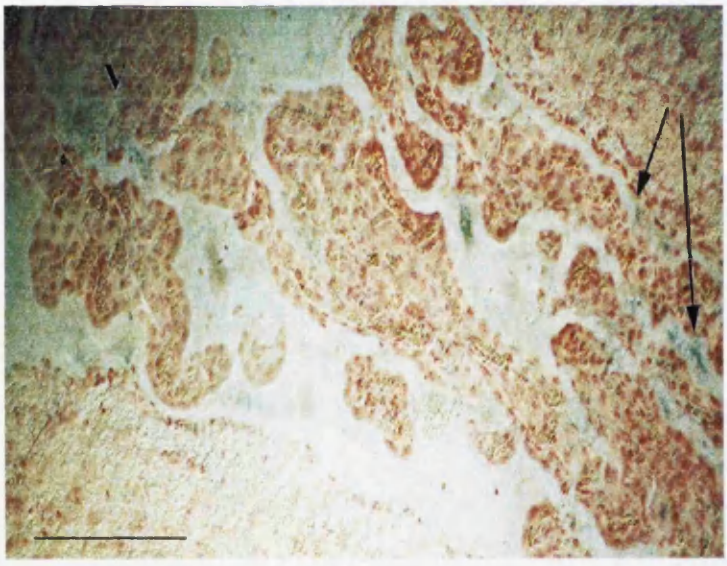
Arrows indicate cells showing β - galactosidase activity.

Abbreviations used : G = cerebellar granule cells; P = Purkinje cell layer; M = cerebellar molecular layer; V = ventricles; DG = dentate gyrus; PoDG = posterior area of the dentate gyrus.

a.



b.



c.

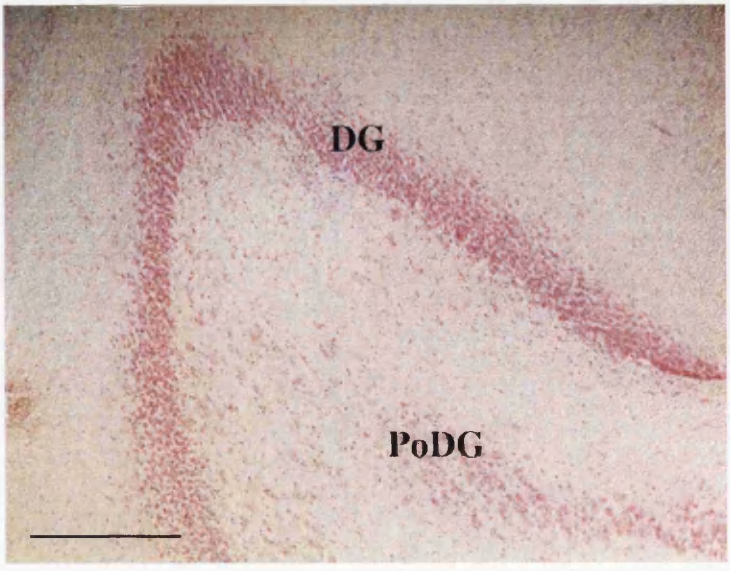


Figure 7.7 Photomicrographs showing sections of adult rat brains infected with the LAT - GABA(1.6) - lacZ virus

Figure 7.7 ***Photomicrographs showing sections of adult rat brains infected with the LAT - GABA(1.6) - lacZ virus***

Horizontal sections were taken through adult rat brains infected (in the cerebellum) with the 1716 LAT - GABA(1.6) - *lacZ* virus. Three regions are shown :

a. **Cerebellum** Section shows *lacZ* staining in cells in the Purkinje cell layer. No staining was observed in the granule cells. (magnification = 40x)

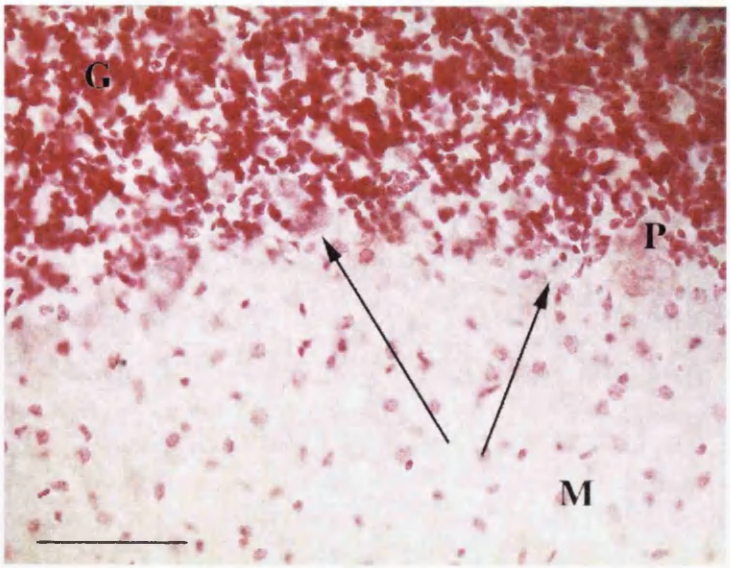
b. **Ventricles** Section shows *lacZ* staining in the ventricles. (magnification = 20x)

c. **Hippocampus** No *lacZ* staining was observed in the hippocampal cells. (magnification = 10x)

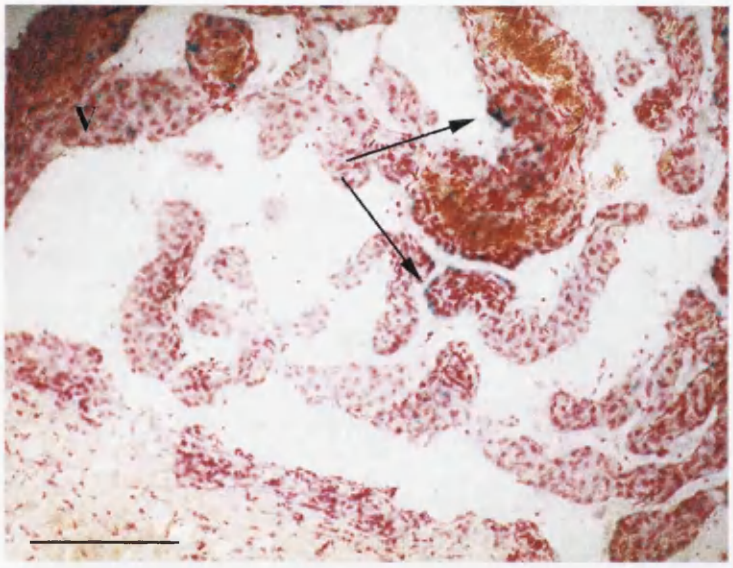
Arrows indicate cells showing β - galactosidase activity.

Abbreviations used : G = cerebellar granule cells; P = Purkinje cell layer; M = cerebellar molecular layer; V = ventricles; DG = dentate gyrus; PoDG = posterior area of the dentate gyrus.

a.



b.



c.

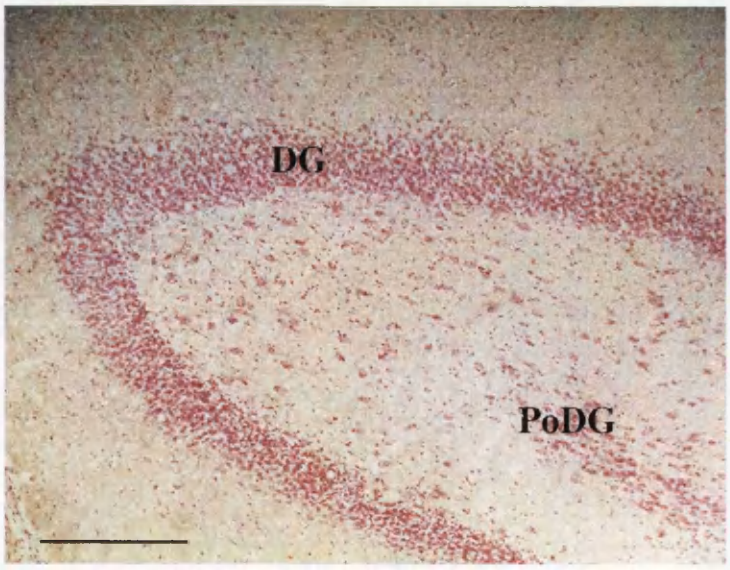


Figure 7.8 Photomicrographs showing sections of uninfected adult rat brains used as experimental controls

Figure 7.8 ***Photomicrographs showing sections of uninfected adult rat brains used as experimental controls***

Horizontal sections were taken through adult rat brains uninfected by virus. Three regions are shown :

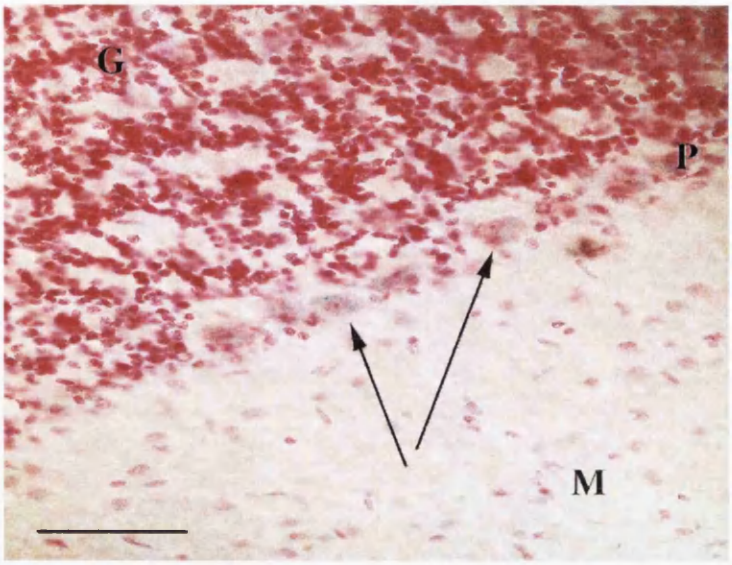
a. Cerebellum Section shows *lacZ* staining in cells in the Purkinje cell layer. No staining was observed in the granule cells.

b. Ventricles Section shows *lacZ* staining in the ventricles.

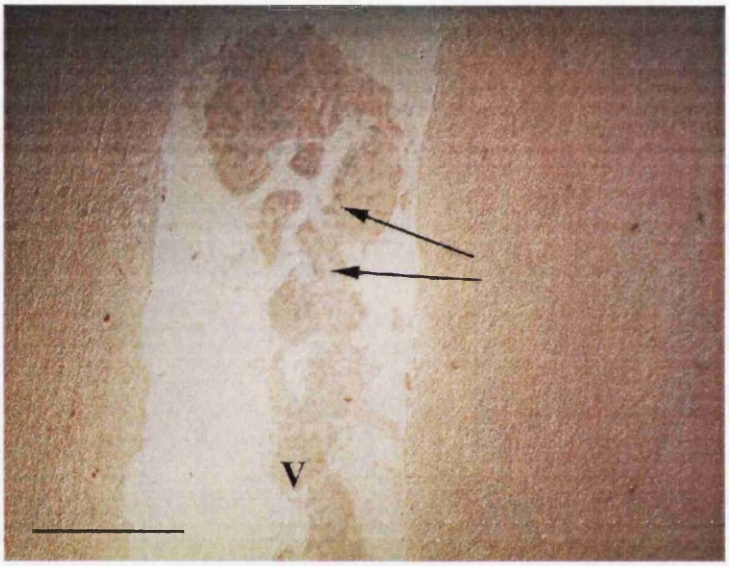
Arrows indicate cells showing β - galactosidase activity.

Abbreviations used : G = cerebellar granule cells; P = Purkinje cell layer; M = cerebellar molecular layer; V = ventricles; DG = dentate gyrus; PoDG = posterior area of the dentate gyrus.

a.



b.



7.3 DISCUSSION

Recombinant viruses containing the *lacZ* gene driven by different promoters were used to infect cerebellar granule cell cultures and rat brains. It was hoped that they would yield information about the ability of neuronal promoters in HSV - 1 vectors to drive neuron - specific, long - term expression.

In the granule cell cultures, a wild - type virus (17+) which contained the *lacZ* gene driven by the gD HSV promoter was used as a positive control for the infection. β - galactosidase activity was detected with this wild - type virus, but mainly in the remaining glial cells. There was some blue staining in granule cells, but only in ones that were connected to the infected glial cells (**Figure 7.1**). The 1716 gD - *lacZ* virus gave blue staining of a few glial cells, but expression from the CMV and δ promoters was not detected **(Figures 7.2 and 7.3)**

Brain sections from the stereotactically injected rats were fixed and stained for β - galactosidase activity. All the sections looked similar with only the 1716 CMV - *lacZ* virus giving a slightly more intense blue colour. The blue staining appeared to track through the brain to the ventricles, perhaps in blood vessels. Upon comparison with a control (uninfected) brain, the staining appeared to be due to endogenous β - galactosidase activity. Although the site of injection appeared visible on the surface of the brains, there did not appear to be needle tracts through the brains. There was no obvious necrosis of the tissue as might be expected from a viral infection.

There are several possibilities to explain these results. The lack of neuronal infection in the granule cells suggests that these viruses are possibly gliatropic and are not able to enter the granule cells except through connections to the glial cells. The lack of infection in the brains could be due to the several factors. If the co - ordinates were inaccurate then the viruses may not actually been inserted into the brain, but merely touched the surface. Another possibility is the titre of the viruses used to infect the brain being too low. It has been reported that HSV -1 only infects a very small percentage (~ 2%) of neurons in the rat brain. Therefore if the viral titre was ten or fifty - fold too low, then little or no

infection would occur. The lack of tissue damage around the site of innoculation would indeed suggest that little viral infection has occurred.

A novel HSV - 1 gene has recently been identified (Ward *et al.*, 1996) which maps antisense to the U_L43 gene - $U_L43.5$. Other HSV - 1 gene pairs that are antisense to each other have been identified e.g. ORF P and ICP 34.5 (Lagunoff and Roizman, 1994); LAT 2kb and 1.5kb and ICP0 (Perry *et al.*, 1986). While it has been postulated that this antisense arrangement contributes to the regulation of gene expression, the mechanism of action is unknown. Thus it is possible that the presence of this antisense gene may interfere with the expression of the reporter gene in the U_L43 locus.. However, as the recombinant LAT virus gave similar results, this is not the only cause for lack of expression. In retrospect, the U_L43 site may not have been the best choice, however at the time of selection no opposing transcript was known.

The granule cell infections are being repeated and the cells analysed at a range of time points after infection. If any of the promoters are shown to drive expression from the *lacZ* gene, then further infections will be performed to allow for analysis of short - or long - term expression from the promoters. The stereotactic injections will be repeated. The co - ordinates will be altered and also a range of viral titres will be used. However, if these particular recombinant viruses infect only a specific type of neurons, and only a small percentage of those, then they are not particularly useful for use with neuronal promoters.

Chapter 8

Gene Targeting of the $GABA_A$ Receptor δ Subunit Gene

8.1 INTRODUCTION

An alternative way to study neuronal gene regulation in the long term is to make genetic alterations *in vivo*. This is now feasible due to recent developments in gene targeting technology which are described below. As an initial experiment preparatory to undertaking the longer term goal, we decided to use gene targeting technology to create a mutation of the $GABA_A$ receptor δ subunit gene that eliminated all protein production yielding information on δ gene function, and eliminate all transcriptional activation to provide support for localisation of the transcriptional start points and identification of other basal promoter elements of the gene.

Targeted gene inactivation has been used to produce mouse models for a range of human genetic deficiency diseases. When DNA is introduced into mammalian cells, it primarily integrates at random. In gene targeting, mutations can be directed to specific mammalian genes by homologous recombination in mouse embryonic stem (ES) cells between DNA sequences residing in the chromosome and newly introduced DNA sequences contained in targeting vectors. Microinjection of the mutant ES cells into mouse blastocysts can then be used to generate germ - line chimaeras (Bradley *et al.*, 1984). Finally, interbreeding of heterozygous siblings would lead to animals homozygous for the desired mutation.

Two types of vectors can be used for targeting mutations to individual loci : replacement vectors or insertion vectors (**Figure 8.1**) (Thomas and Capecchi, 1987). With replacement vectors, a positive - negative selection procedure is used to select against non - homologous recombination (Mansour *et al.*, 1988). Thus the targeting vector may contain a neomycin resistance gene cassette (neo^r) as a positive selection marker for transfection and a herpes simplex virus thymidine kinase gene (*tk*) as a negative selection against cells that have randomly integrated the vector into their genomes. The overall effect is to enrich for cells containing the targeted mutation. With insertion vectors, homologous recombination leads to the duplication of genomic sequences.

The rate of homologous recombination can be affected by certain factors. Higher rates are observed when larger regions of vector homology are used (Thomas and Capecchi,

1987). Also, te Riele *et al*, (1992) observed that using DNA isogenic to the strain of mice from which the ES cells were derived promotes high recombination rates.

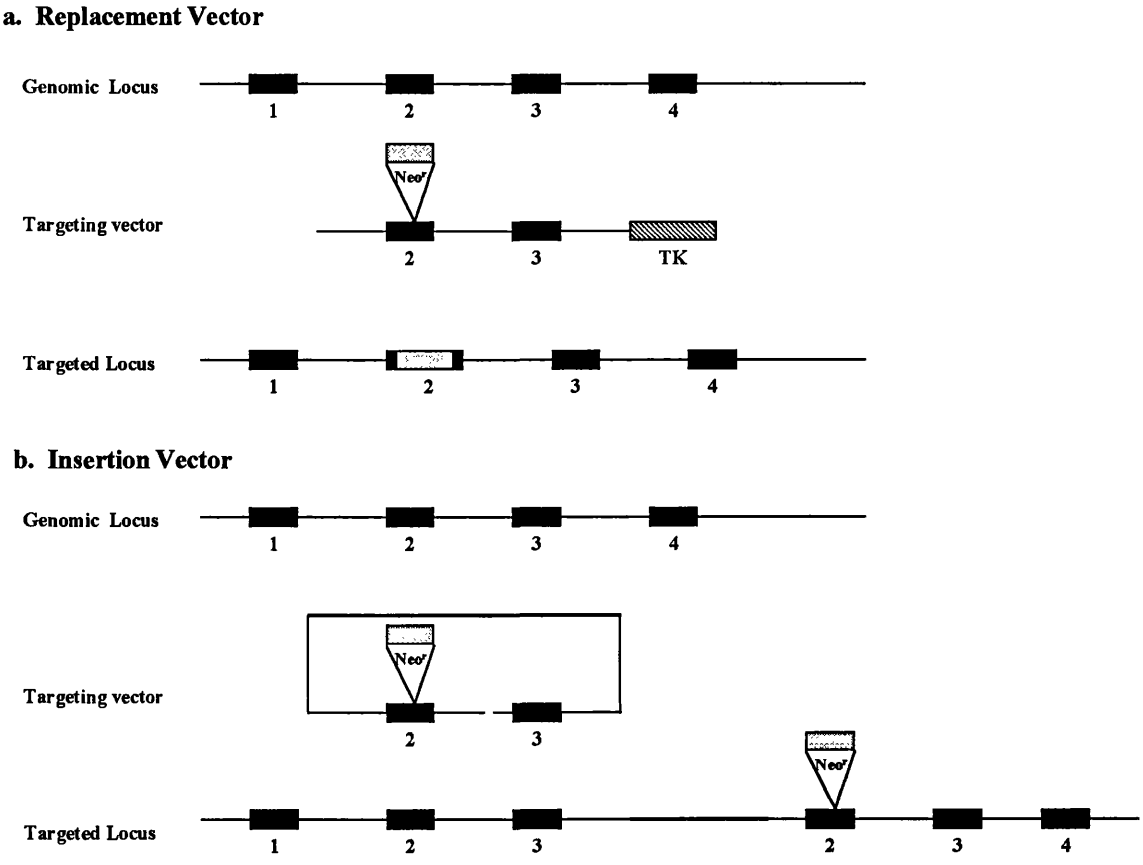


Figure 8.1 Targeting Vectors

a. Replacement Vector and **b.** Insertion Vector. Shaded boxes represent exons, thin lines introns and thick lines plasmid DNA. Abbreviations used : Neo^r = neomycin resistance gene: TK = herpes simplex virus thymidine kinase gene. (Reproduced from Soriano, 1995).

While the methods described above will generate mice with null alleles and allow for functional studies of genes, our longer term goal is to study gene regulation. This requires the ability to generate more subtle site - specific alterations of the genome, the goal being to create a mutation that in every other way mimics the normal expression pattern of the gene. Several methods have been devised to achieve this aim e.g. microinjection of DNA

into cells; co - transfection where the targeting construct and the selectable marker are on different plasmids; using a two - step strategy in which the selectable marker gene is removed from the targeted locus by a second homologous recombination event. Several two - step strategies have been devised including the hit - and - run method (Hasty *et al.*, 1991) and the tag - and exchange method (Askew *et al.*, 1993) (**Figure 8.2**).

More recently site - specific recombinases such as *cre* from bacteriophage P1 and FLP from yeast have been utilised (Kilby *et al.*, 1993) (**Figure 8.3**). These have the advantage of allowing for tissue - specific gene targeting by driving *cre* expression from tissue - specific promoters. If a mutation results in a lethal phenotype as a result of loss of expression in cells, then by restricting the mutation to a specific cell type, this lethality can be overcome. Both recombinases recognise short stretches of DNA in a sequence specific manner and catalyse the deletion or integration of DNA flanked by these recognition sites. The placement and orientation of these recognition sites determines the nature of the recombination event. For example, the *cre* recombinase can catalyze recombination between two *lox*P sites. If the *lox*P sites are in the same orientation, the intervening sequences will be deleted. If the sites are in opposite orientations, then the intervening sequences will be inverted.

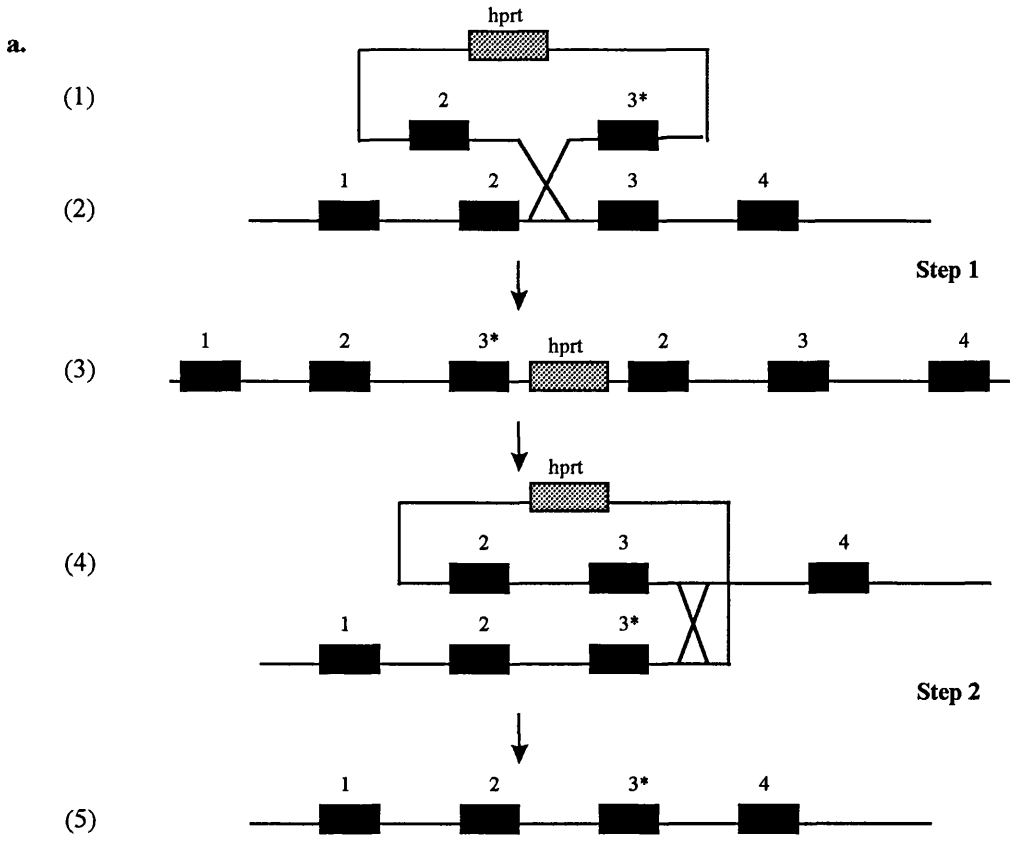
This chapter describes the targeted disruption of the $GABA_A$ receptor δ subunit gene. Two different vector backbones were used to generate replacement targeting constructs. Initially pSSC9 was selected as a vector (**Table 2.2**). It contains a neomycin resistance gene (neo^r) driven by the HSV thymidine kinase (*tk*) promoter and also 2 copies of *tk*. It has several unique sites on either side of the neo^r gene for cloning of genomic DNA fragments. A second vector utilised was pNT (**Table 2.2**). This vector contains a single copy of *tk* and the neo^r is driven by the phosphoglycerate kinase promoter (PGK). This vector also contains unique sites flanking the neo^r for insertion of genomic fragments.

Figure 8.2 Two - step strategies for gene targeting

Figure 8.2 Two - step strategies for gene targeting

a. Hit and run. (1) Targeting construct with two arms of homology to the locus and a selectable marker gene (*hprt*) that can be used for both positive and negative selection. The construct also has a mutation (*) in exon 3. (2) The endogenous locus. (3) The locus following targeted integration of the construct. Positive selection is used to identify recombinants carrying the marker gene. (4) Intrachromosomal recombination event results in the removal of the marker gene and duplicate sequence. Negative selection is used to identify recombinants that have lost the marker gene. (5) The locus following the second recombination event. The locus will either contain the original exon 3 or the mutated exon 3*.

b. Tag and exchange. (1) Targeting construct with two arms of homology and a selectable marker gene for positive and negative selection. (2) The endogenous locus. (3) The locus following targeted integration of the construct. . Positive selection is used to identify recombinants carrying the marker gene. (4) A second targeting construct which lacks a marker gene and carries the desired mutation (*) in exon 2. (5) The locus following the second recombination event. Negative selection is used to identify recombinants that have lost the marker gene and contain the desired mutation.


a.
(1)
hprt
2
3*
(2)
1
2
3
4
Step 1
(3)
1
2
3*
hprt
2
3
4
(4)
hprt
2
3
4
1
2
3*
Step 2
(5)
1
2
3*
4

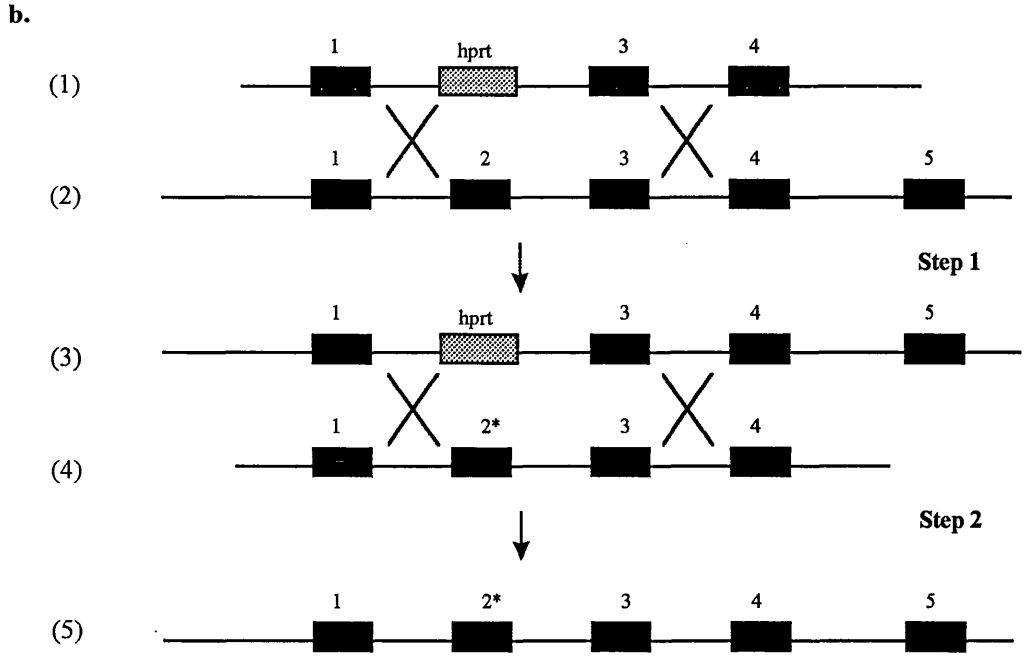

b.
(1)
1
hprt
3
4
(2)
1
2
3
4
5
Step 1
(3)
1
hprt
3
4
5
(4)
1
2*
3
4
Step 2
(5)
1
2*
3
4
5

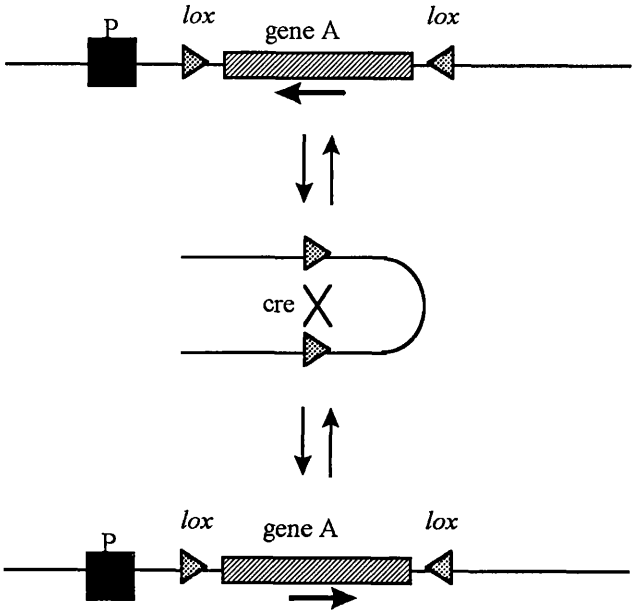
Figure 8.3 Site - specific recombinase reactions

Figure 8.3 Site - specific recombinase reactions

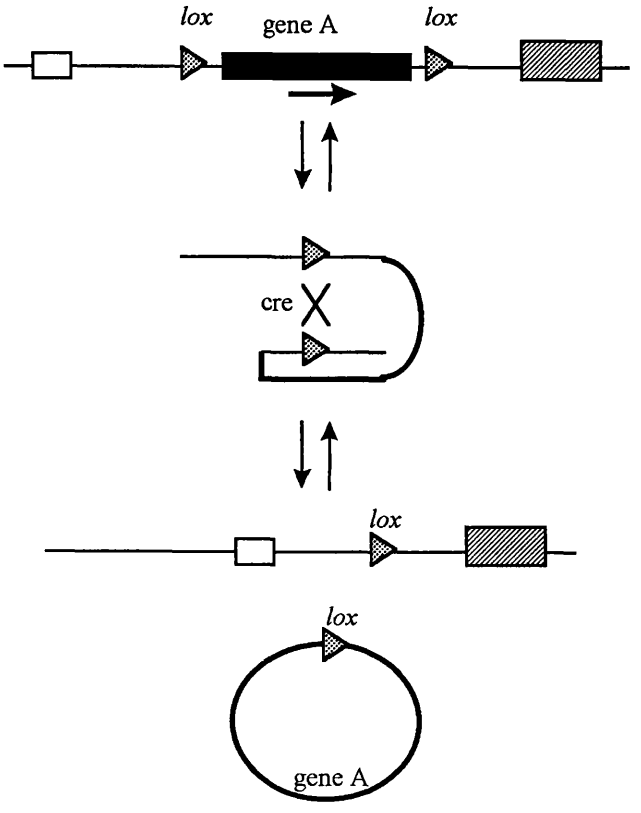
a. Inversion. Site - specific recombination between two inverted target sites (*lox*) on a linear molecule inverts the intervening DNA. Before recombination the coding region of gene A was inverted with respect to the promoter (P). Cotransfecton with a plasmid that expresses *cre* results in recombination and activation of gene A.

b. Deletion. Recombination between two sites in direct orientation leads to excision of the intervening DNA.

a.


P
lox
gene A
lox
cre
lox
gene A
lox
P

b.


lox
gene A
lox
cre
lox
lox
gene A

8.2 RESULTS

8.2.1 Construction of gene targeting constructs

8.2.1.1 pSSC - δ construct

Figure 8.4 illustrates the construction of the pSSC9 replacement targeting vector and the expected targeted disruption of the $GABA_A$ receptor δ subunit gene. A 4kb *Xba* I - *Nhe* I promoter fragment was subcloned from lambda clone λ21 into the *Xba* I site of pSSC9. A 5 kb *Bam* HI fragment (from the first intron) was then excised from lambda clone λ27 and inserted into the *Bam* HI site. DNA was prepared using a Qiagen kit, and 50 μg was digested with *Sfi* I in order to linearise the DNA prior to transfection.

8.2.1.2 pNT - δ construct

Figure 8.5 illustrates the construction of the pNT replacement targeting vector and the expected targeted disruption of the δ gene. A 2 kb *Xho* I fragment was subcloned from pSSC9 - δ (**Section 8.2.1.1**) into the *Xho* I site of pNT. A 5 kb *Bam* HI fragment (from the first intron) was then excised from lambda clone λ27 and inserted into the *Bam* HI site. DNA was prepared using the Qiagen kit, and 50 μg was digested with *Kpn* I in order to linearise the DNA prior to transfection.

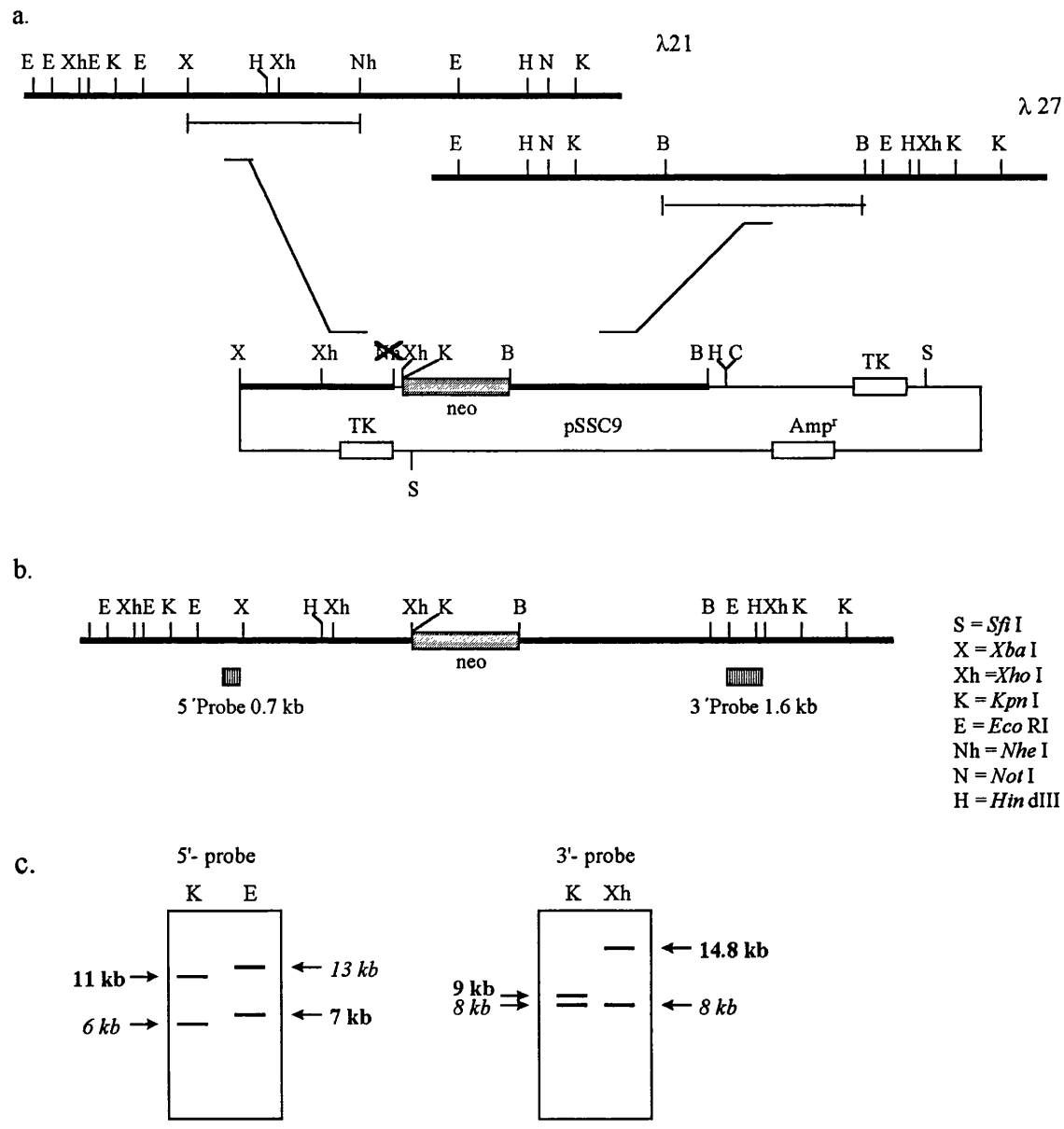


Figure 8.4 ***Targeted disruption of the $GABA_A$ receptor δ subunit gene using vector pSSC9***

a. 2 genomic fragments were excised from overlapping lambda clones and used in the construction of a replacement targeting vector.

b. The targeted locus of the $GABA_A$ receptor δ subunit gene in which exon 1, 4.5 kb of promoter and 2 kb from the first intron were replaced by a *tk* - neo cassette. A 0.7 kb *Pst* I - *Xba* I 5' - fragment and a 1.6 kb *Eco* RI - *Hin*d III 3' - fragment were used as probes.

c. Banding patterns expected from Southern blot analysis of a successful targeting event. Wild - type bands in bold, mutant bands in italics.

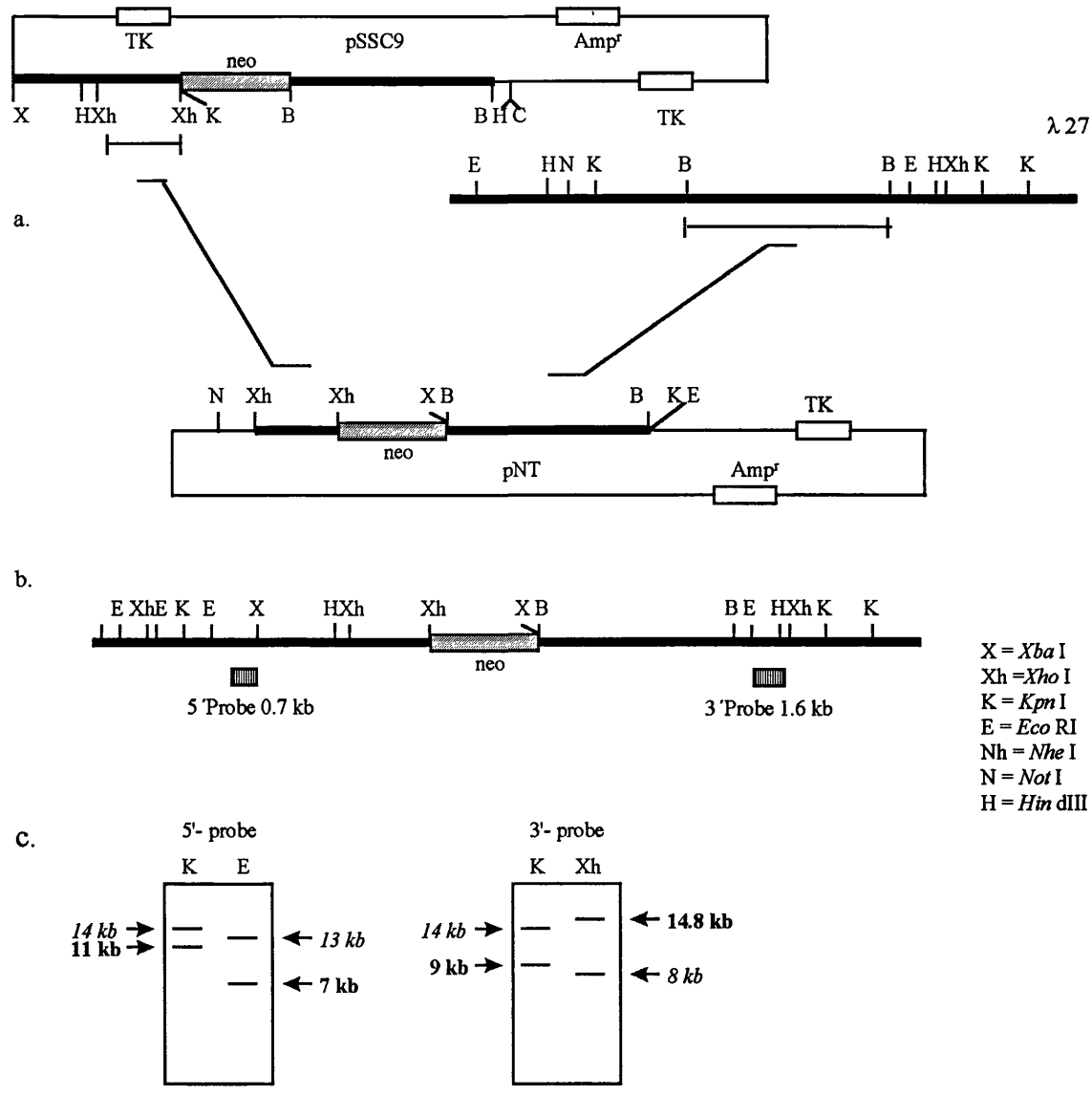


Figure 8.5 ***Targeted disruption of the $GABA_A$ receptor δ subunit gene using vector pNT***

a. A 2 kb *Xho* I fragment was excised from pSSC9 - δ and a 5 kb *Bam* HI excised from a lambda clone and used in the construction of a replacement targeting vector.

b. The targeted locus of the $GABA_A$ receptor δ subunit gene in which exon 1, 4.5 kb of promoter and 2 kb from the first intron were replaced by a PGK - neo cassette. A 0.7 kb *Pst* I - *Xba* I 5' - fragment and a 1.6 kb *Eco* RI - *Hin*d III 3' - fragment were used as probes.

c. Banding patterns expected from Southern blot analysis of a successful targeting event. Wild - type bands in bold, mutant bands in italics.

8.2.2 Establishment of recombinant cell lines

The same transfection procedure was used for each construct (**Section 2.2.8**)

8.2.2.1 pSSC9 -δ transfection

330 G418 resistant colonies were isolated. The enrichment factor due to the counter selection was calculated by comparing the number of colonies obtained with G418 plus gancyclovir and with G418 alone. The enrichment factor was estimated to be about fourfold. The DNA from the clones was isolated (**Section 2.2.8.4.3**), cut with *Kpn* I and *Xho* I, and analysed by Southern blotting (**Section 2.2.8.4.4**). The blots were probed with a 1.6 kb *Eco* RI - *Hin*d III DNA fragment from the 3' - end of the gene (**Figure 8.6**). Initially, 5 clones gave bands of the size expected for a successful targeting event, however the band intensities did not look correct.

These 5 DNAs were further analysed by Southern blotting using a 5' - DNA probe. They were cut with *Eco* RI and then probed with a 0.7kb *Pst* I - *Xba* I DNA fragment from the 5' - promoter region. These did not give the expected banding pattern for a successful targeting event (**Figure 8.7**).

8.2.2.2 pNT - δ transfection

368 G418 resistant colonies were isolated. There did not appear to be any enrichment of the transfection. The DNA from 300 of these clones was prepared (**Section 2.2.8.4.3**), and 240 samples were cut with *Eco* RI and analysed by Southern blotting (**Section 2.2.8.4.4**). The blots were probed with a 0.7kb *Pst* I - *Xba* I DNA fragment from the 5' - promoter region. Two clones (89 and 102) gave the expected restriction pattern. (**Figure 8.8**).

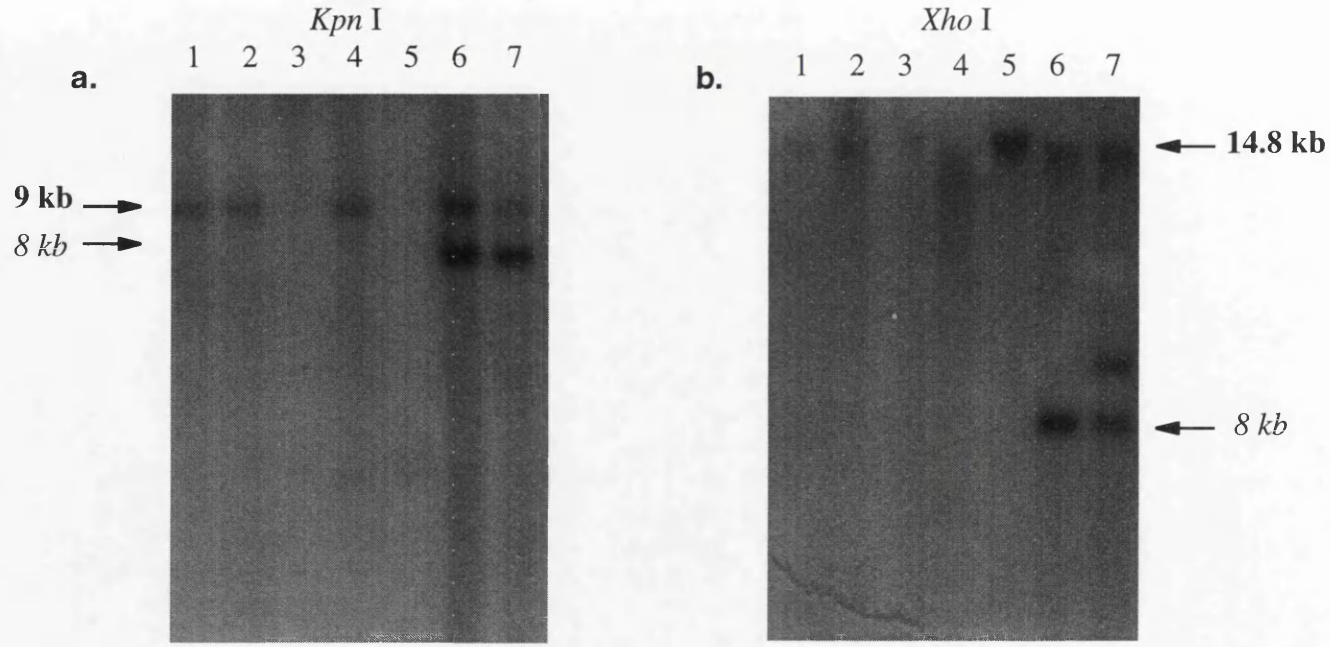


Figure 8.6 Southern blot analysis of putative targeted cell lines

Southern blots showing putative positive targeted cell lines. Wild - type bands are indicated in bold, mutant bands are indicated in italics. Both blots were probed with a 1.6 kb *Eco* RI - *Hind* III 3' - DNA fragment

a. *Kpn* I digest : samples 6 and 7 give the expected banding pattern for a successful targeting event.

b. *Xho* I digest : sample 6 gives the expected banding pattern for a successful targeting event. In sample 7, the extra band could be due to a partial digestion.

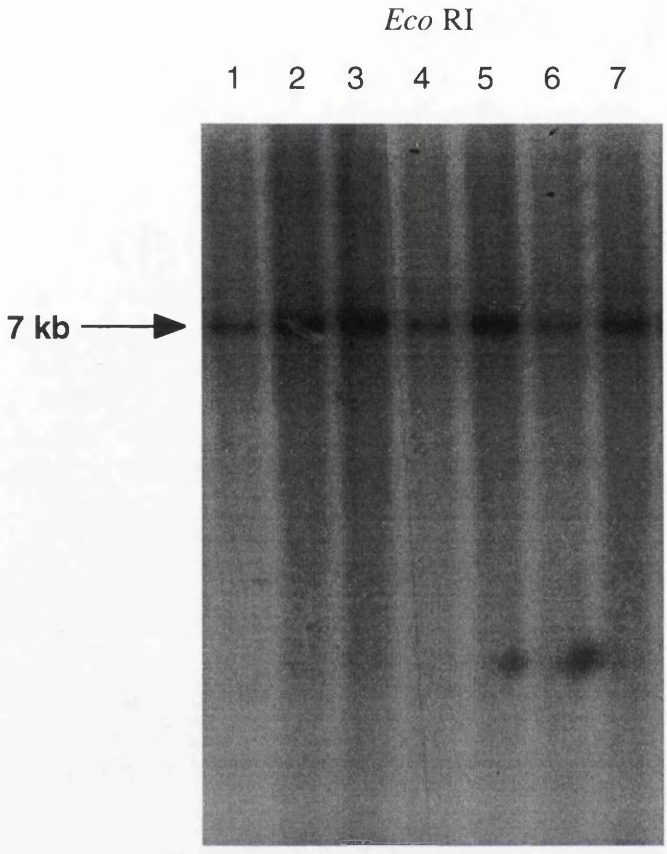


Figure 8.7 Southern blot analysis of putative targeted cell lines

Southern blot of 2 (samples 6 and 7) of the putative positive clones selected after first round screening using a 3' - probe (**Figure 8.6**). These samples were digested with *Eco* RI and the blot was probed with a 0.7 kb *Pst* I - *Xba* I 5' - DNA fragment. Only wild - type bands were visible.

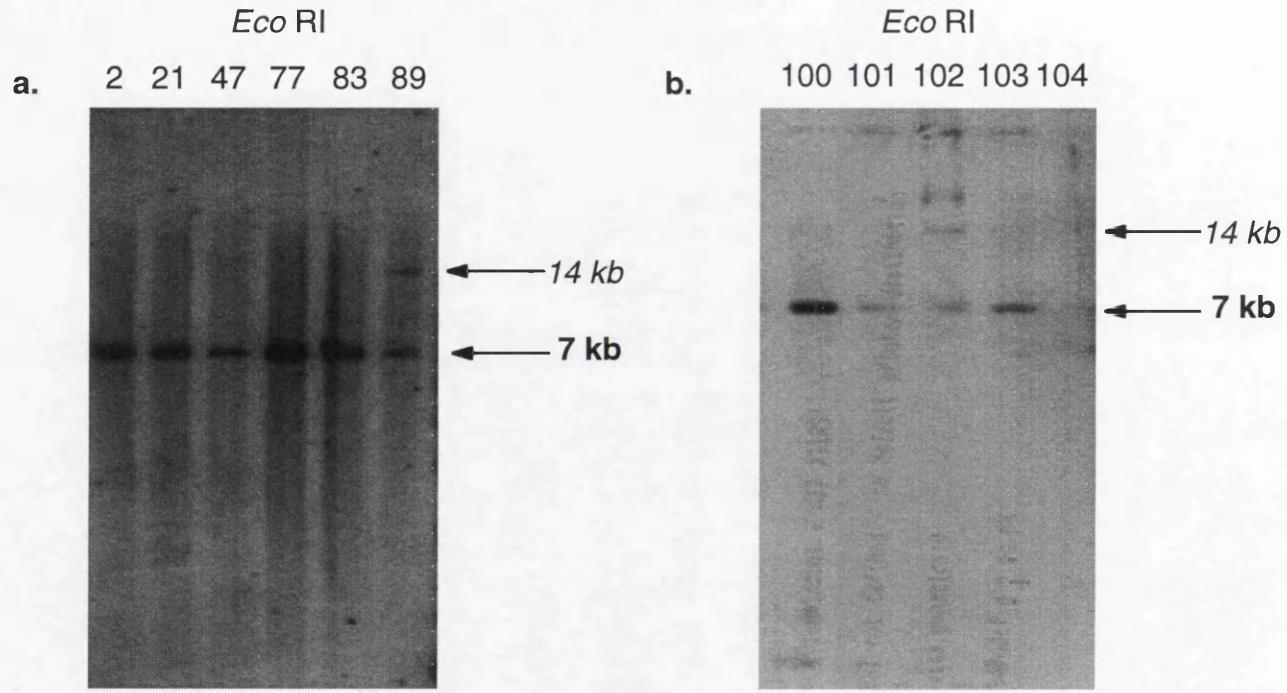


Figure 8.8 Southern blot analysis of putative targeted cell lines

Southern blots showing putative positive targeted cell lines. Wild - type bands are indicated in bold, mutant bands are indicated in italics. These samples were cut with *Eco* RI and the blots probed with a 0.7 kb *Pst* I - *Xba* I 5' - DNA fragment. Samples 89 (blot **a**) and sample 102 (blot **b**) give bands of the correct size and intensity for successful targeting events.

8.3 DISCUSSION

Initially, the pSSC9 plasmid was selected as a targeting vector due to its apparent convenience. The availability of restriction sites in the plasmid allowed for relatively straightforward cloning of suitable genomic DNA fragments. The two fragments selected for insertion (**Figure 8.4**) would result in the removal of 7 kb of DNA including the first exon, 4.5 kb of promoter sequence and 2 kb from the first intron.

The transfection of this construct resulted in 330 G418 resistant colonies. However, upon Southern blot analysis these proved to be non - recombinant cell lines. One reason for this could be due to the selection procedure. The conditions used in the selection process had actually been optimised for the PGK - neomycin cassette. It was assumed that these would also be suitable for the *tk* promoter. However, the *tk* promoter is a weak promoter and it is possible that, under the selection procedure, cell lines containing a single neomycin insertion were sensitive to the G418. This would result in mainly aberrant recombinant events. Another possibility is that this is a low frequency recombination event and too few colonies were analysed. As the reason for the lack of recombinant cell lines was not obvious, we decided to construct a new targeting vector as opposed to repeating the transfection per se.

A second targeting vector was then selected. In this vector, the neomycin gene is driven by the strong PGK promoter. The same 3' - DNA fragment was used as the long arm for recombination, but only 2 kb of the 5' - fragment was used as the short arm due to the limited availability of sites. Recombination with this construct (**Figure 8.5**) would yield the same targeting event as described for the pSSC9 vector i.e. the removal of 7 kb of DNA including the first exon, 4.5 kb of promoter sequence and 2 kb from the first intron.

Transfection with this construct resulted in 368 neomycin resistant colonies being isolated. The negative selection procedure using gancyclovir to select against the presence of the HSVTK gene did not appear to work. Thus, there was no enrichment for targeted clones. DNA was prepared from 300 of these colonies of which 240 were analysed by Southern blotting. Only 2 clones - 89 and 102 (**Figure 8.8**) gave the expected banding pattern with the 5' - probe. The wild type and recombinant bands were of equal

intensity as expected for a successful targeting event. The DNA from these putative positive cell lines will be further analysed using the 3' - probe indicated in **Figure 8.5**, to confirm that the correct homologous recombination event has occurred.

The targeted ES cells containing the mutated δ gene will then be used to produce mice homozygous for the mutation. The cell lines will be injected into host blastocycts which will be introduced into pseudopregnant female mice to generate chimaeric founder mice. Subsequent mating of these chimaeras with wild - type mice will establish if the mutation has gone germ - line. Germ - line progeny will be crossed to obtain mice homozygous for the mutated $GABA_A$ receptor δ subunit gene. These null mice (if they survive) can then be assessed for phenotypic effects caused by the deletion of the gene, and may yield information about the functionality of the δ gene.

Recently, Jones *et al.* (1997) demonstrated that a knock - out of the $GABA_A$ receptor α6 subunit gene appeared to affect the production of the δ protein in cerebellar granule cells. Analysis of the expression of all the $GABA_A$ receptor subunit genes in δ knock - out mice would perhaps verify any association between the δ and α6 genes, or indeed between the δ and any other subunit.

Future work would involve achieving the long term goals which are to use gene targeting technology for studying gene regulation. This would require the use of a two - step strategy as described earlier. Several putative transcription factor recognition sites have been identified in the 5' - upstream sequence of the δ gene (**Chapter 3**). If experimental studies indicated that any of these sites were involved in regulation, then their functionality *in vivo* could be assessed using this technology.

Chapter 9

Genetic Mapping of the $GABA_A$ Receptor δ Subunit Gene in Mouse and Rat

9.1 INTRODUCTION

Most of the $GABA_A$ receptor subunit genes (GABAR) have been assigned to specific chromosomes in man and the mouse (Glencorse and Davies, 1997). To date however, no $GABA_A$ receptor subunit genes have been mapped in the rat. **Table 9.1** contains a list of the localisation of GABAR subtypes to chromosomes in humans and mice.

Table 9.1 Localisation of GABAR genes in Humans and Mice

$GABA_AR$ subunit subtype	Human Chromosome	Mouse Chromosome	References
α1	5q33	11A2-B1 (19)	Buckle *et al.*, 1989 Keir *et al.*, 1991
α2	4p13-12	5C-E2 (35)	Buckle *et al.*, 1989 Danciger *et al.*, 1993
α3	Xq28	XA6-B (32)	Bell *et al.*, 1989
α4	4p14-q12	7 (26.9)	McLean *et al.*, 1995 Danciger *et al.*, 1993
α5	15q11-13	7C-D3 (29)	Knoll *et al.*, 1993
α6	5q31.1-35	11 (23)	Hicks *et al.*, 1994
β1	4p13-12	5C-E2 (35)	Dean *et al.*, 1991 Danciger *et al.*, 1993
β2	5q34-35	?	Russek and Farb, 1994
β3	15q11.2-12	7C-D3 (30)	Wagstaff *et al*, 1991a Wagstaff *et al*, 1991b
γ1	4p14-q21.1	?	Wilcox *et al.*, 1992
γ2	5q31.1-33.2	11A2-B1 (19)	Wilcox *et al.*, 1992 Buckwalter *et al.*, 1992
γ3	15q11.2-12	7	Phillips *et al.*, 1993
δ	1p	?	Sommer *et al.*, 1990
ρ1	6q14-21	4A1-A5 (13)	Cutting *et al.*, 1992
ρ2	6q14-21	4A1-A5 (13)	Cutting *et al.*, 1992

The table lists the chromosomal localisation of the $GABA_A$ receptor subunit genes on human and mouse chromosomes. Human chromosomes are divided into 2 regions : p and q. All 19 autosomal mouse chromosomes, including the X chromosome, are telocentric. The figures given in brackets are the map positions in centimorgans (cM).

As can be seen from the table, with the exception of the ρ subunit genes (GABRR1 and GABRR2), the α3 subunit gene (GABRA3) and the δ subunit gene (GABRD), the other known $GABA_A$ receptor subunit genes appear to be clustered on chromosomes in both man and mouse. Distinct α–β-γ gene clusters are located on human chromosomes 4 (GABRA2, GABRB1, GABRA4 and GABRG1), 5 (GABRA1, GABRA6, GABRB2 and GABRG2) and 15 (GABRA5, GABRB3 and GABRG3), and mouse chromosomes 5, 11 and 7 respectively. These observations correlate well with known regions of conserved synteny between man and mouse (Nadeau, 1989).

Although the origin of these gene clusters is unknown, it has been suggested that they have arisen from the duplication and translocation of an ancestral cluster, and in fact, gene clustering has also been shown for other ligand - gated ion channel superfamilies (Conley *et al.*, 1996). Thus it is possible that the original $GABA_A$ receptor subunit gene cluster consisted of one α–, one β- and one γ- subunit as is found on human Chromosome 15. Duplication of an ancestral cluster would have preceded the translocation to different chromosomes (Russek *et al.*, 1994). Whether the duplication of the α gene occurred prior to the translocation or as a result of tandem duplication of α genes of two α–β-γ clusters located on two different chromosomes is unknown, although the former would appear more likely.

The fact that $GABA_A$ receptor subunit genes have remained in clusters indicates that there could be some sort of co-ordinate expression occurring. An example of this is seen for the developmental expression of the genes clustered on human Chromosome 4. Here the α4, β1 and γ1 subunit mRNAs are the only ones expressed in the undifferentiated neuroepithelium at embryonic day 13 (Ma *et al.*, 1993). During postnatal development, a region - specific depletion occurs such that these subunit mRNAs become barely

detectable. It is possible that these patterns of co - expression occur due to there being common regulatory elements between genes within a cluster. However, it has been shown that there is independent regulation of transcription of clustered genes. The α4 subunit mRNAs are very strongly expressed in the thalamus, while the α2, β1 and γ1 mRNAs are barely detectable (Wisden *et al.*, 1992). While it is plausible to accept the argument for duplication and translocation, it still does not fully explain the presence of the δ subunit gene on human Chromosome 1 or the α3 gene on the X Chromosome. It is possible that this occurred due to fragmentation of a cluster and subsequent translocation of genes not under co - ordinate control.

This chapter describes the localisation of the $GABA_A$ receptor δ subunit gene to chromosome 4 in mouse and chromosome 5 in rat. The mouse mapping was achieved by PCR screening of consomic mouse lines, and the rat by PCR screening of a rat/mouse somatic cell hybrid panel. These methods utilise simple sequence length polymorphisms (SSLP), or "microsatellites" (short tandem repeats e.g. $(CA)_n$ or $(CAG)_n$ randomly distributed along the genome) as genetic markers. They are highly abundant (10^6 copies for CA repeats), are usually found in non - coding regions, are stable throughout generations and can be typed with simple PCR based assays.

9.1.1 Mouse consomic lines and EUCIB resource

The consomic mouse lines were provided by Guénet (personal communication). These were generated by crossing 2 mouse strains : C57BL/6 and *Mus spretus* over several generations until populations of C57BL/6 mice were produced that contained fragments of specific *Mus spretus* chromosomes. Thus mouse line sets were generated whereby each C57BL/6 chromosome contained fragments of *Mus spretus* chromosomes, a large proportion of the *Mus spretus* genome being represented in 19 consomic lines.

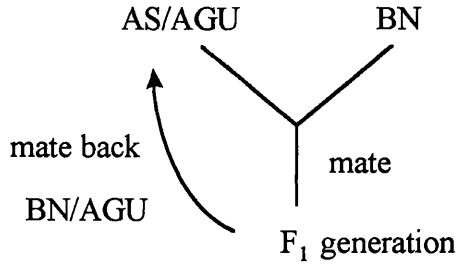
A subset panel of DNAs from the European Collaborative Interspecific Backcross (EUCIB) were used for precise localisation of the mouse gene on a particular chromosome. EUCIB DNAs have been genotyped using panels of genetic markers which provide whole genome coverage. This then allows for the stepwise refinement of the map

position of a particular gene by selecting subset panels which demonstrate recombination events within a region of interest.

9.1.2 Rat somatic cell hybrid panel and interstrain backcross

A somatic cell hybrid panel (Szpirer *et al.*, 1984) was generated by fusing rat and mouse cell lines from parental strains : mouse - BWTG3 and rat - HRSD until a panel of mouse cell lines containing fragments of specific rat chromosomes was obtained (**Table 9.2**). This panel could then be used to assign genes to rat chromosomes by PCR screening for the presence of a specific marker on a particular chromosome.

The interstrain backcross (AS/AGU x BN) F1 x AS/AGU (Shiels *et al.*, 1996) was generated as follows :



This was used for precise genetic mapping of the gene.

9.2 RESULTS

9.2.1 Mouse Genetic Mapping

9.2.1.1 Chromosome localisation

PCR analysis of consomic mouse lines (Guénet, personal communication.) was used to assign the mouse $GABA_A$ receptor δ subunit gene to a chromosome. A B2 repeat element has been identified in the 5' - promoter sequence of the δ gene (**Chapter 3**). It has been established that these elements can be polymorphic between different strains of animals (Krayev *et al.*, 1982). PCR primers (BR018F4 and BR018R4, **Table 2.6**) were therefore designed around this region. Amplification of genomic DNA templates of the 2 parental mouse strains (C57BL/6 and *Mus spretus*) used in generating the consomic lines and

EUCIB DNAs demonstrated that this element was present for C57BL/6 but not for *Mus spretus*. Sequencing of the 2 PCR products confirmed this.

Nineteen members of a consomic mouse line set from parental strains C57BL/6 and *Mus spretus* were used in the initial screen. For the PCR reaction, 50 ng of each primer was used with 120 μM deoxyribonucleotides, 100 ng/ml genomic DNA template, 1 mM Mg^{2+}, 1 x thermophilic DNA polymerase buffer and 0.2 units of Taq polymerase in a final volume of 10 μl. The samples were denatured for 10 min at 94°C and, after the addition of Taq, 25 amplification cycles were performed (denaturation 94°C for 15 sec; annealing 65°C for 30 sec; extension 72°C for 30 sec). A final elongation step was performed at 72°C for 2 min. PCR products were visualised on 2% TBE agarose gels (**Figure 9.1**).

7 members of the line set (1, 2, 13, 16, 17, 18 and 19) demonstrated heterozygosity for these alleles. The mouse $GABA_A$ receptor δ subunit gene was assigned to chromosome 4.

9.2.1.2 Precise genetic mapping

Precise genetic mapping of *Gabrd* to known markers on chromosome 4 was achieved using a subset panel of DNAs from EUCIB. A EUCIB subset panel of 10 members was selected. The recombination events between anchor markers for these animals had been well characterised and subdivided chromosome 4 into 6 regions. PCR based genotype analysis was performed on these DNAs as described in **Section 9.2.1.1**. 4 members of the panel (LB057, LB117, LB169 and LB298) demonstrated heterozygosity of these alleles (**Figure 9.2**). The location of *Gabrd* would appear to be telomere distal to marker *D4DsiI* (64cM). Dr Martin (Pasteur Institute, Paris) performed PCR analysis on further subset panels of DNAs in the *D4DsiI - DNds16* (82 cM) range and mapped *Gabrd* to a 2 cM region between markers *D4Mit209* and *D4Nds16*.

A diagram of the linkage map of mouse chromosome 4 with respect to *Gabrd* is shown in **Figure 9.3**.

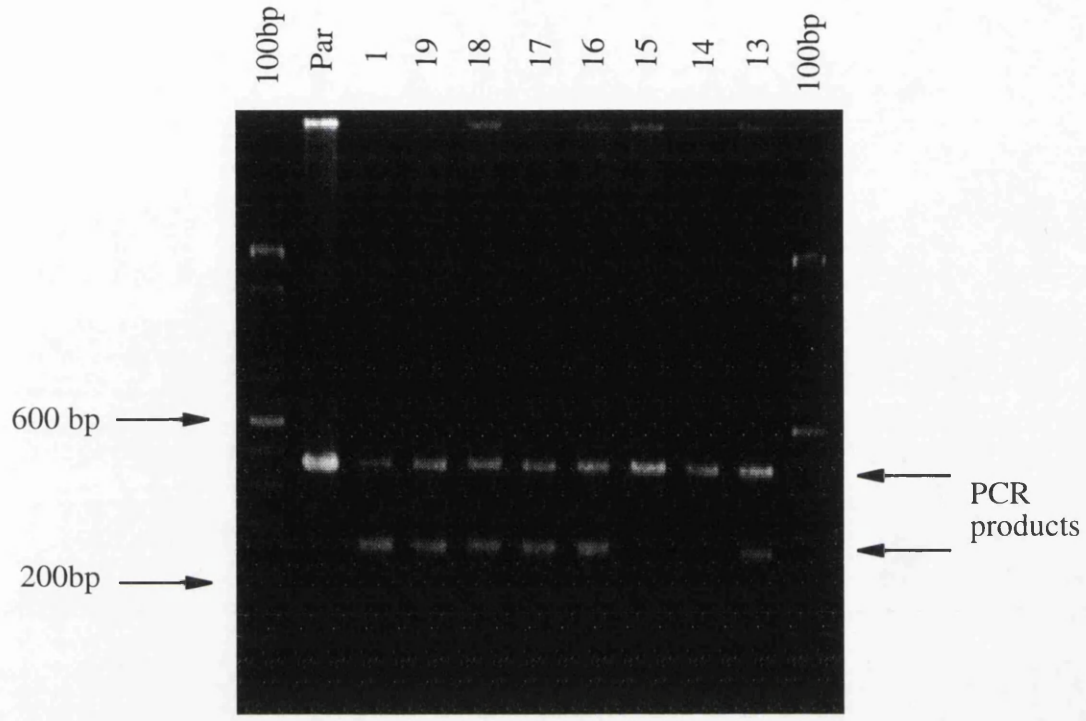


Figure 9.1 ***Chromosomal localisation of the mouse $GABA_A$ receptor δ subunit gene***

2% TBE agarose gel showing PCR products resulting from PCR analysis of some members of consomic mouse lines derived from parental strains C57BL/6 and Mus spretus. Par = *Mus spretus* genomic DNA. 1, 13-19 = represent 8 out of 19 members of a consomic mouse line set. The PCR primers used were BR018F4 and BR018R4 (**Table 2.6**). The PCR products are indicated and are approximately 450 bp (upper band-C57BL/6) and 260 bp (lower band-*Mus spretus*) in size. 1, 13, 16-19 demonstrated heterozygosity for these alleles.

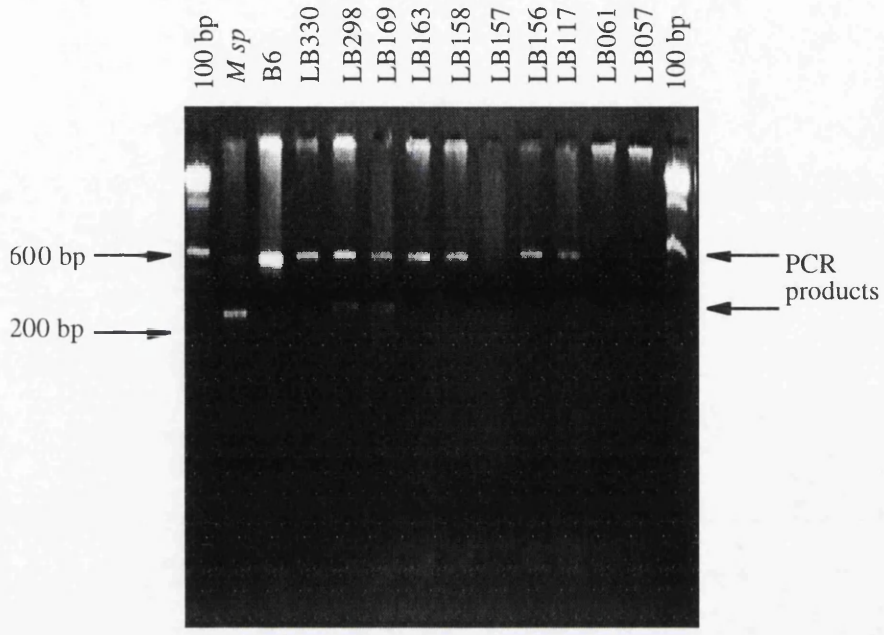


Figure 9.2 PCR screening of EUCIB subset panel of DNAs

2% TBE agarose gel showing PCR products resulting from PCR analysis of a EUCIB subset panel of 10 animals. *M sp* = *Mus spretus* genomic DNA, B6 = C57BL/6 genomic DNA. The PCR primers used were BR018F4 and BR018R4 (**Table 2.6**). The PCR products are indicated and are approximately 450 bp (upper band- C57BL/6) and 260 bp (lower band- *Mus spretus*) in size. LB057, LB117, LB169, LB298 demonstrated heterozygosity for these alleles.

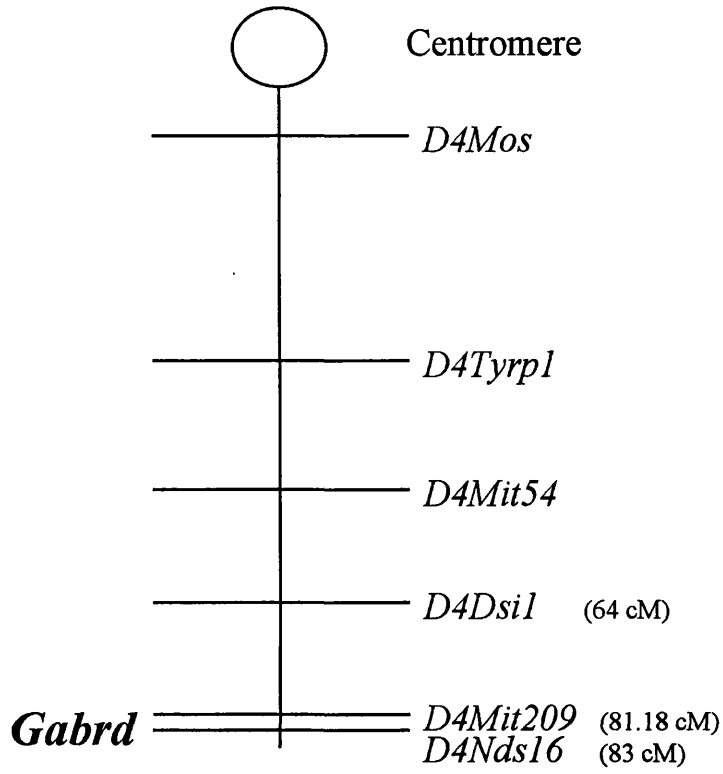


Figure 9.3 Genetic map of mouse chromosome 4

This map is based on the EUCIB MBx database (http://www.hgmp.mrc.ac.uk/MBx/MBxHomepage.html) and shows the location of the $GABA_A$ receptor δ subunit gene. It has been mapped to a 2 cM region between markers *D4Mit209* and *D4Nds16*.

9.2.2 Rat Genetic Mapping

9.2.2.1. Chromosome localisation

PCR analysis of a somatic cell hybrid panel (Szpirer *et al.*, 1984) was used to assign the rat $GABA_A$ receptor δ subunit gene to a chromosome. Ten members of the somatic cell hybrid panel were used in the PCR screen. PCR primers (BRBSF3 and BRBSF4 - **Table 2.5**) were designed to the rat $GABA_A$ receptor δ subunit gene promoter region to amplify a region containing incomplete di- and tri-nucleotide repeats (Motejlek *et al.*, 1994). This region had previously been shown to show length variation between rat and mouse as the repeat region is absent in the mouse sequence (**Chapter 3**).

For the PCR reaction, 50 ng of primers were used with 120 μM deoxyribonucleotides, 100 ng/ml genomic DNA template, 1 mM Mg^{2+}, 1 x thermophilic DNA polymerase buffer and 0.2 units of Taq polymerase in a final volume of 10 μl. The samples were denatured for 10 min at 94°C and, after the addition of Taq, 30 amplification cycles were performed (denaturation 94°C for 15 sec; annealing 58°C for 30 sec; extension 72°C for 30 sec). A final elongation step was performed at 72°C for 2 min. PCR products were visualised on 2% TBE agarose gels (Flowgen) (**Figure 9.4**). Three members of the panel (161, 630 and 600) gave both rat and mouse PCR products and so the rat $GABA_A$ receptor δ subunit gene was assigned to chromosome 5 (**Table 9.2**).

9.2.2.2 Precise genetic mapping

Precise genetic mapping was performed on the interstrain backcross (AS/AGU x BN) F1 x AS/AGU (Shiels *et al*, 1996) by screening progeny from the backcross for strain differences by PCR. Initially, PCR analysis (using the Gabrd primers) of the rat strains involved in the backcross (AS/AGU, BN, F344) showed strain differences between AS/AGU and BN (**Figure 9.5**). These primers could thus be used to precisely map the $GABA_A$ receptor δ subunit gene in the rat.

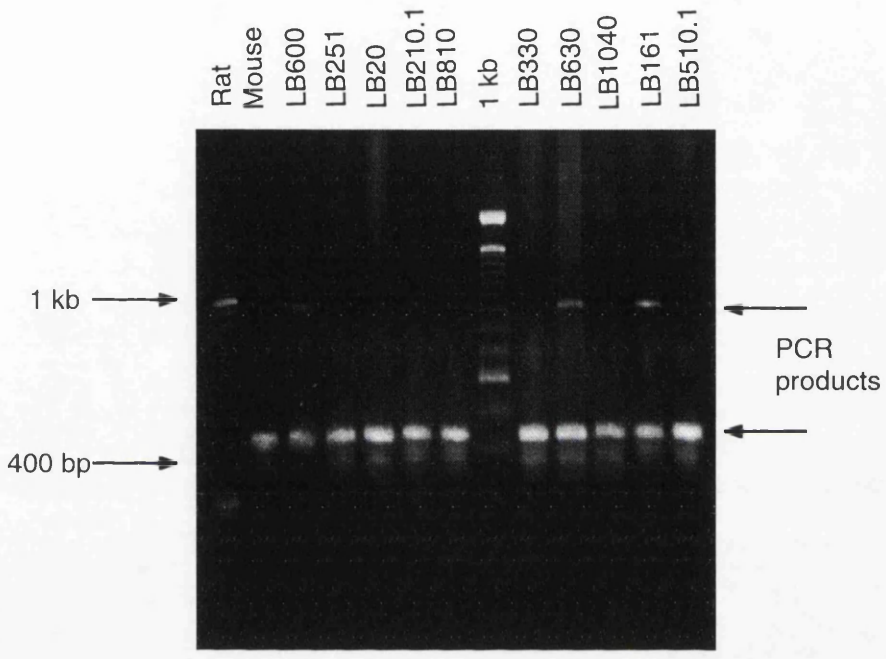


Figure 9.4 Chromosomal localisation of rat $GABA_A$ receptor δ subunit gene

2% TBE agarose gel showing PCR products resulting from PCR analysis of a rat/mouse somatic cell hybrid panel. PCR primers used were BRBSF3 and BRBSF4 (**Table 2.5**). The PCR products are indicated and are approximately 950 bp (upper band-rat) and 450 bp (lower band-mouse) in size. 3 members of the panel (LB161, LB630, LB600) demonstrated the presence of the rat allele.

	X	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
LB150.1	+			+	+			+		+	(+)	+	+	+			(+)	(+)		+	(+)	
LB161	+		+	+	+	+	+	+		+	+		(+)	+	+	+	+	+	+	+	(+)	+
LB1040	+			+	+	(-)	+	+			+	+	+			+	+		+		+	
LB630	+	(-)		+	+	(+)	+	+		+		+	+	+	(+)	+	+		+	+	(-)	+
LB330	+		+	+	+		+				+		+					+				
LB810	+		+	+	+		+	+	+		+	+	+	+	+	+	+	+		+	(+)	
LB210.1	+														+				+			
LB20	+		(+)	(+)			(-)	+					+	+			+	(+)	+	+		
LB251	+	+	+		+		(+)	+			+		+	+				+		+		
LB600	+	+	+	+	+	+	(+)	+			+	+	+	+	+	+	+		+		+	+

Table 9.2 Detection of the rat $GABA_A$ receptor δ subunit gene in cell hybrids

This table shows the presence (+ symbols) of fragments of specific rat chromosomes in members of the cell hybrid lines. Symbols in brackets have to be confirmed. The 3 panel members that gave PCR products are indicated (LB161, LB630, LB600). It has been deduced from the table that the $GABA_A$ receptor δ subunit gene is located on rat Chromosome 5.

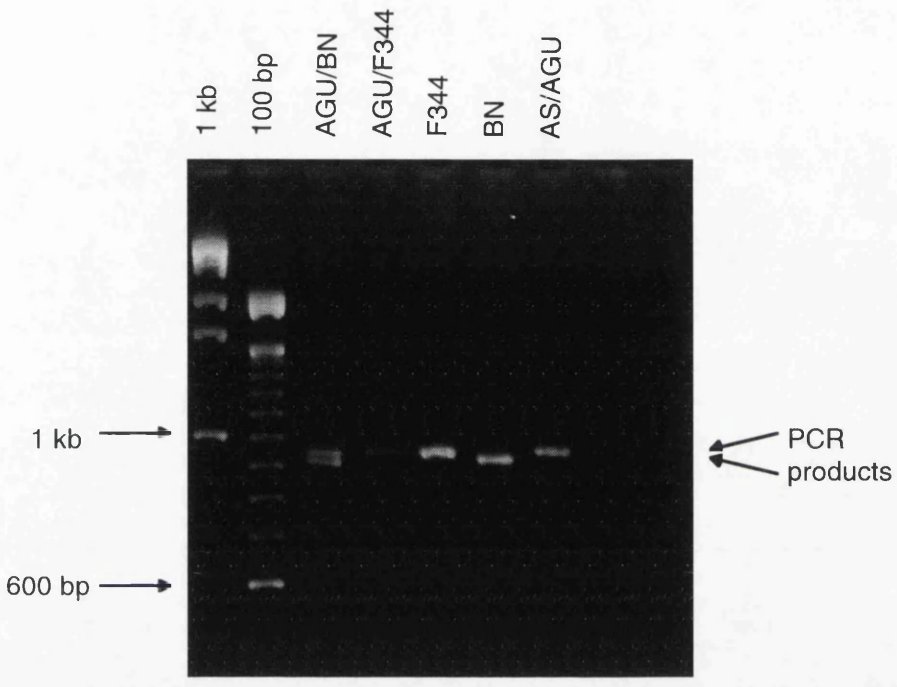


Figure 9.5 PCR analysis showing rat strain differences with Gabrd primers

2% Nusieve 3 :1 agarose gel showing product length variation between rat strains : AS/AGU and BN. The GABA δ primers used were BRBSF3 and BRBSF4 (**Table 2.5**). The PCR products are indicated. The sizes of the bands are approximately 950 bp (upper band) and 920 bp (lower band).

59 progeny were screened using BRBSF3 and BRBSF4 (Gabrd) primers and 2 sets of markers, D5Mit7 and D5Mit9 (**Table 2.5**), which were known to give different product lengths for the parental rat strains. The PCR reactions were essentially as described in **Section 9.2.2.1**. For the D5Mit7 marker, the annealing temperature was 53°C and the PCR products were visualised on 4% Nusieve 3.1 agarose gels (**Figure 9.6**). The annealing temperature for D5Mit9 was 55°C and the PCR products were visualised on 4.5% Nusieve 3:1 gels (**Figure 9.7**). The Gabrd primers were used as described in **Section 9.2.2.1** (**Figure 9.8**). The results of the analysis are shown in **Table 9.3**. A score of less than 50% indicates that the markers may be linked, 50% or greater means that they are unlikely to be linked.

Table 9.3 Interstrain backcross mapping data

Locus 1	Locus 2	Number of recombinants	Genetic Distances (cM)
D5Mit7	Gabrd	18/59	30.5cM +/- 9.9
D5Mit9	Gabrd	30/59	50.8cM +/- 10,7
D5Mit7	D5Mit9	24/59	40.7cM +/- 10.6

The table shows the results of the genetic mapping of Gabrd with respect to two other markers : D5Mit7 and D5Mit9. 59 progeny were screened with each marker primer pair.

These results indicated that Gabrd may be linked to marker D5Mit7, but it is unlikely to be linked to D5Mit9 and so a third marker pair D5Mgh9 (**Table 2.5**) was selected to screen the above progeny. The PCR annealing temperature for this was 63°C and 6% Nusieve 3:1 gels were used for analysis (**Figure 9.9**). The results of this are shown in **Table 9.4**.

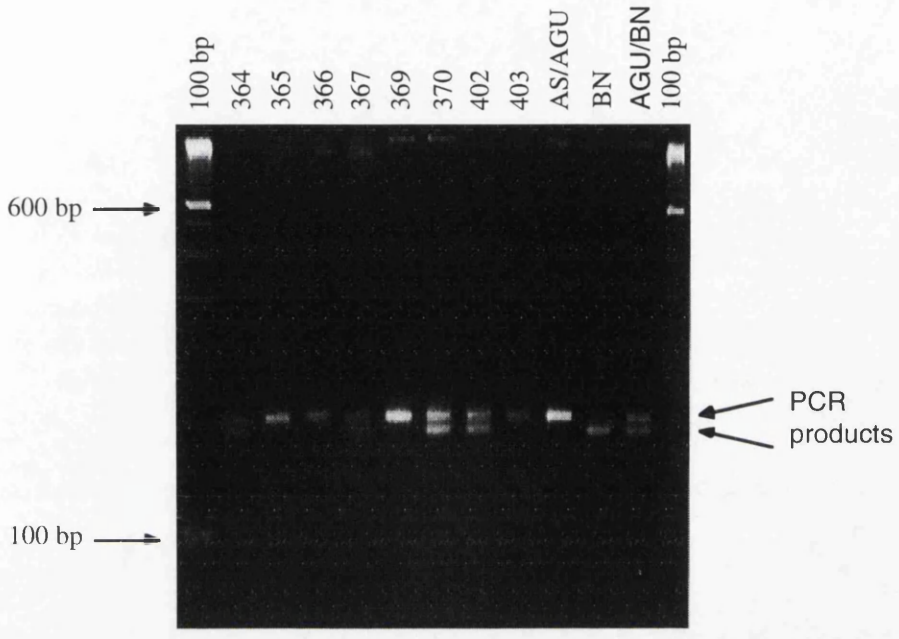


Figure 9.6 PCR analysis of rat interstrain backcross using D5Mit7 primers

4% Nusieve 3.1 agarose gel showing PCR products resulting from PCR analysis of members of a rat interstrain backcross (AS/AGU x BN)F1 x AS/AGU (Shiels *et al.*, 1996). Primer set used was D5Mit7 (**Table 2.5**). The PCR products are indicated and are approximately 180 bp (upper band-AS/AGU) and 160 bp (lower band-BN) in size. AS/AGU, BN and AGU/BN are parental DNAs. 364, 367, 370 and 402 demonstrated heterozygosity for these alleles.

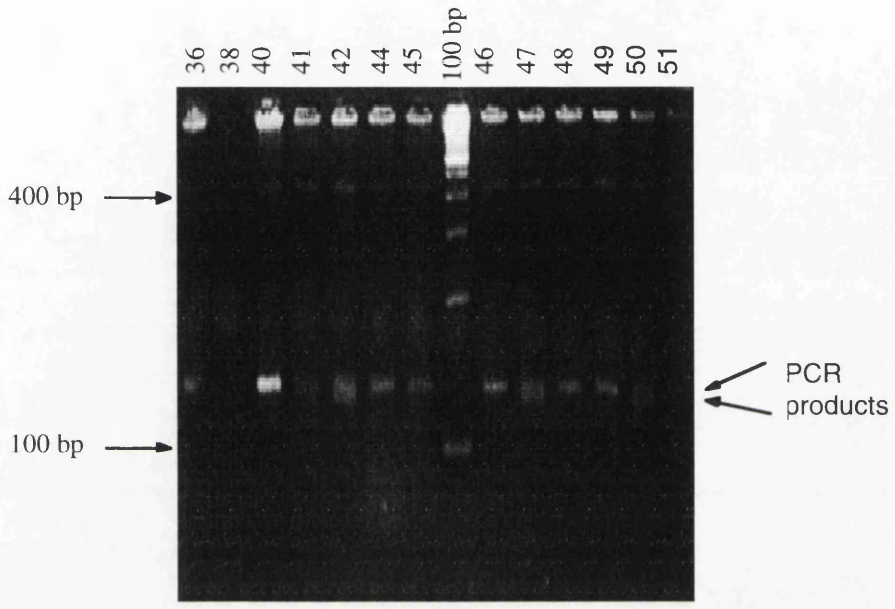


Figure 9.7 PCR analysis of rat interstrain backcross using D5Mit9 primers

4.5% Nusieve 3.1 agarose gel showing PCR products resulting from PCR analysis of members of a rat interstrain backcross (AS/AGU x BN)F1 x AS/AGU (Shiels *et al.*, 1996). Primer set used was D5Mit9 (**Table 2.5**). The PCR products are indicated and are approximately 140 bp (upper band-AS/AGU) and 130 bp (lower band-BN) in size. 42, 47 and 50 demonstrated heterozygosity for these alleles.

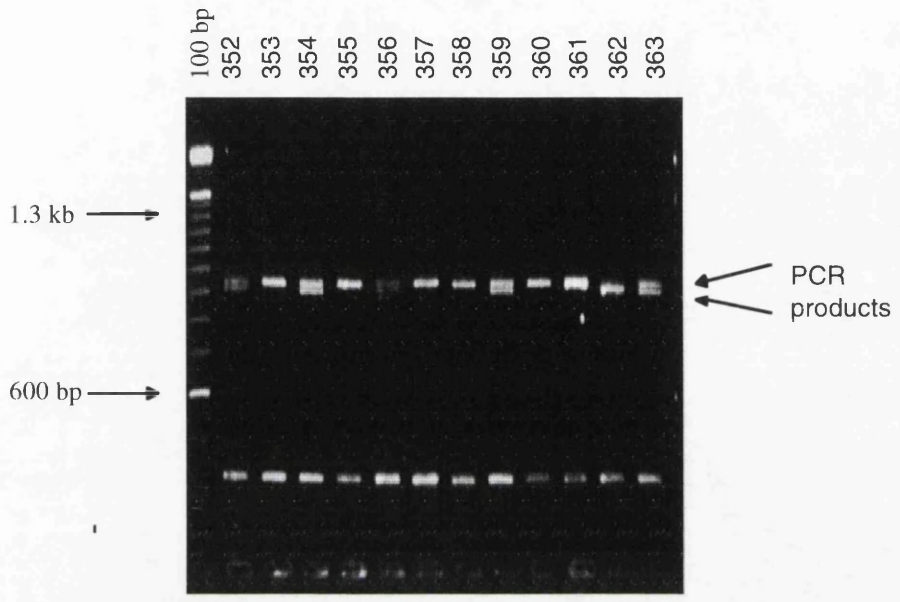


Figure 9.8 PCR analysis of rat interstrain backcross using Gabrd primers

2% Nusieve 3.1 agarose gel showing PCR products resulting from PCR analysis of members of a rat interstrain backcross (AS/AGU x BN)F1 x AS/AGU (Shiels *et al.*, 1996). Primers used were BRBSF3 and BRBSF4 (**Table 2.5**). The PCR products are indicated and are approximately 950 bp (upper band- AS/AGU) and 920 bp (lower band- BN). 352, 354, 356, 359 and 363 demonstrated heterozygosity for these alleles.

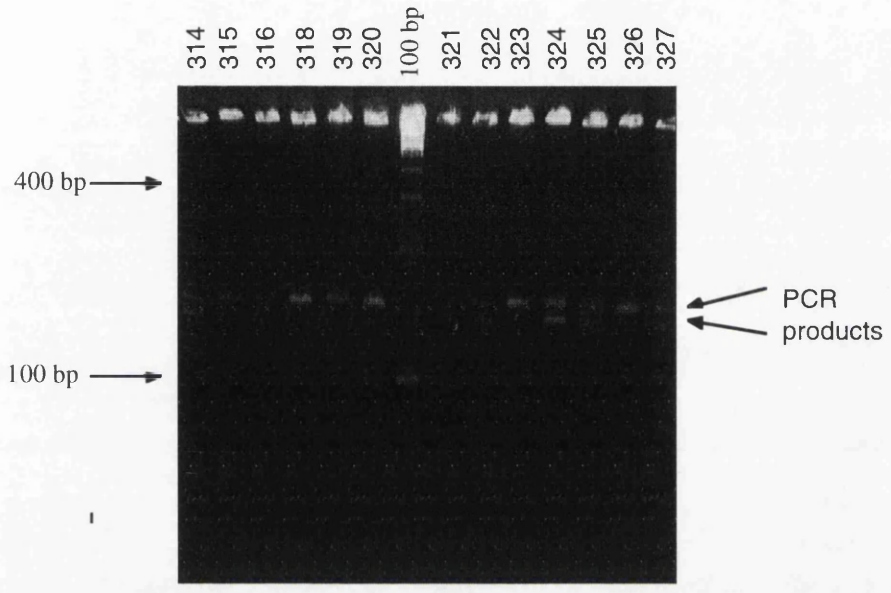


Figure 9.9 PCR analysis of rat interstrain backcross using D5Mgh9 primers

6% Nusieve 3.1 agarose gel showing PCR products resulting from PCR analysis of members of a rat interstrain backcross (AS/AGU x BN)F1 x AS/AGU (Shiels *et al.*, 1996). Primers set used was D5Mgh9 (**Table 2.5**). The PCR products are indicated and are approximately 170 bp (upper band-AS/AGU) and 160 bp (lower band-BN) in size. 314, 316, 322, 324, 325 and 327 demonstrated heterozygosity for these alleles.

Table 9.4 Interstrain backcross mapping data

Locus 1	Locus 2	Number of recombinants	Genetic Distances (cM)
D5Mit7	Gabrd	20/61	32.8cM +/- 9.9
D5Mgh9	Gabrd	0/61	0.00
D5Mit7	D5Mgh9	20/61	32.8cM +/- 9.9

The table shows the results of the genetic mapping of Gabrd with respect to two other markers : D5Mit7 and D5Mgh9. 61 progeny were screened with each marker primer pair. No recombinants were observed for the Gabrd and Mgh9 markers.

These results indicate that Gabrd and D5Mgh9 could be closely linked, however only a small pool of animals was tested. A further 37 progeny were screened with the Gabrd and D5Mgh9 markers and still no recombinants were observed. A diagram of the linkage map of chromosome 5 with respect to Gabrd is shown in **Figure 9.10**.

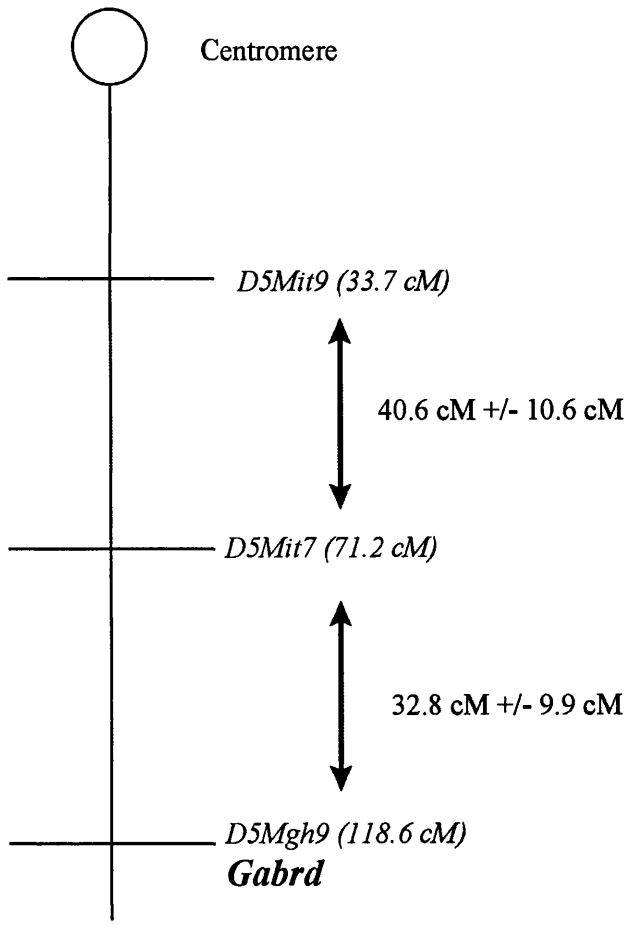


Figure 9.10 Genetic map of rat chromosome 5

This map is based on Jacob *et al.* (1995) and shows the location of the $GABA_A$ receptor δ subunit gene. The distances shown with the markers are from this map. The distances given on the right are based on recombination estimates of the Gabrd locus typed using an interstrain backcross (AS/AGU x BN) F1 x AS/AGU.

9.3 DISCUSSION

The mouse $GABA_A$ receptor δ subunit gene has been localised to chromosome 4 by PCR analysis of consomic mouse lines (Guénet, personal communication). Mouse chromosome 4 is estimated to be about 82cM in length (Mouse Genome Database, http:/www.informantics.jar.org/). A subset of DNAs from the European Collaborative

Interspecific Backcross (EUCIB) was used for precise genetic mapping of *gabrd* relative to known markers on chromosome 4.

A schematic diagram of mouse chromosome 4 is shown in **Figure 9.3.** The gene/marker order on mouse chromosome 4 is : centromere - *D4Dsi1 -D4Mit209 - Gabrd - D4Nds 16*

The rat $GABA_A$ receptor δ subunit gene has been localised to Chromosome 5 by PCR analysis of a somatic cell hybrid panel (Szpirer *et al.*, 1984). Rat Chromosome 5 is telocentric and has been estimated to be 136 cM in length (Jacob *et al.*, 1995). The percentage coverage by the established linkage groups is 87.4%. An interspecies backcross (Shiels *et al.*, 1996) was used for mapping of the rat gene in relation to other known markers for Chromosome 5. All the markers used for the mapping were taken from Jacobs *et al.* (1995). These results are approximations and were from only a small pool of animals tested.

The 2 markers selected for the mapping (D5Mit7 and D5Mit9) were linked, and had been placed on the chromosome at 71.2 cM and 33.7 cM respectively. Gabrd was shown to be linked to D5Mit7 but not to D5Mit9. Thus we were able to place Gabrd downstream of D5Mit7 at position 101.7 cM +/- 9.9 cM. Another marker, D5Mgh9 was selected in order to obtain a three point cross. This marker was known to be linked to D5Mit7 and had been placed at position 118.6 cM on the chromosome. Our results placed Gabrd and D5Mgh9 at the same position on the chromosome within the limits of this analysis.

A further 37 progeny were screened for Gabrd and D5Mgh9 with no recombinants being scored. A schematic diagram of chromosome 5 is shown in **Figure 9.10**. The gene/marker order on chromosome 5 is centromere - D5Mit9 -D5Mit7 - D5Mgh9/Gabrd - telomere.

These results agree with expected regions of synteny between human, mouse and rat i.e. regions of human Chromosome 1p, mouse chromosome 4 and rat chromosome 5 have been shown previously to be syntenic. In fact, upon comparison of the available mapping data for human and mouse, the position of human GABRD may be more precisely mapped to Chromosome 1p35-36. The mapping of the rat $GABA_A$ receptor δ subunit gene is

significant as it is the only $GABA_A$ receptor subunit gene, as yet, to be genetically mapped in the rat.

Chapter 10

General Discussion

10.1 INTRODUCTION

In the past 10 years, gene therapy has progressed from molecular and cellular biology to a broad field of experiments in mammals and even to clinical trials for conditions ranging from cancer to cystic fibrosis. Despite recent advances, technical problems remain, such as the need to target the foreign gene to the appropriate tissues or cells, long - term expression and the necessity to overcome the immune response related to some of the vectors. Viral vectors are the preferred method of gene transfer into target cells of the CNS. The HSV vectors are of particular interest as they can transduce neurons (Horellou *et al.*, 1994). Improvement of current vectors is vital in order to overcome current problems concerning the spread of the virus *in vivo*, and also to remove viral - induced cytotoxic functions (Johnson *et al.*, 1992).

Current strategies to limit expression to a specific class of neurons depends largely on the use of cell - type specific promoters. In this work we chose to use the $GABA_A$ receptor δ subunit gene promoter to drive expression of the β - galactosidase gene, as it has a well characterised restricted expression pattern in the mammalian brain. While much is now known regarding the gene structure and function of $GABA_A$ receptor subunits, little is known about the regulation of subunit gene expression.

10.2 GENE REGULATION

Gene regulation can be achieved at several different levels e.g. mRNA stability, RNA editing, DNA methylation, chromatin structure, gene copy number, transcriptional initiation, elongation and termination and translational efficiency. However, transcriptional control has been implicated as the major method by which eukaryotic gene regulation is achieved (He and Rosenfeld, 1991; Struhl, 1991). Expression of all $GABA_A$ receptor subunit genes occurs in a regional -, developmental - and cell - type specific pattern (Laurie *et al.*, 1992; Wisden *et al.*, 1992). Furthermore, $GABA_A$ receptor gene expression varies in response to chronic exposure to ligands including GABA (Montpied *et al.*, 1991), certain benzodiazepines (Primus and Gallager, 1992) and NMDA (Memo *et al.*, 1991). The localised expression of different subunit combinations is likely to involve a complex system of gene regulation. However, little is known of these processes and even

less is known of the regulatory elements involved in the control of expression of the $GABA_A$ receptor subunit genes, or any neuronal genes.

Partial characterisation of several $GABA_A$ receptor subunit gene promoters has been performed : human and chick α1 (Kang *et al.*, 1994; Bateson *et al.*, 1995), human α5 (Kim *et al.*, 1997), human and rat β3 (Kirkness and Fraser, 1993), mouse and rat α6 (Jones *et al.*, 1996) and mouse and rat δ (Sommer *et al.*, 1990; Motejlek *et al.*, 1994) in an attempt to understand how this regulation is achieved.

The 5' - flanking regions of the rat, chick and human $GABA_A$ receptor α1 subunit genes were sequenced (Ultch *et al.*, 1990; Bateson and Paetsch, 1993; Kang *et al.*, 1994) and show extensive sequence homology. Kang *et al.* (1994) looked for minimal promoter elements in the human α1 gene 5' - region. Initially they assayed promoter - deletion plasmids fused to the luciferase reporter gene in primary cell cultures and identified a 60 bp region located within 250 bp of the translational start point as being essential for promoter activity. Bateson *et al.*, (1995) identified a single transcription initiation site in the chick α1 by primer extension and RNase protection, and this corresponds to the 60 bp promoter region identified for the human α1 gene. A putative TATA box (-24 to -30, where +1 relates to the transcription initiation point) and reverse CAAT box (-69 to -65) were also identified in both the human and chick sequences as were recognition sites for the Sp1 transcription factor (-70 to -75) and a classic cAMP response element (CRE, -2 to -8). However, deletion of the CAAT and Sp1 sequences in the human α1 did not significantly alter the promoter activity (Kang *et al.*, 1994) suggesting that they are non - functional. Gel mobility shift assays were performed using the identified 60 bp promoter fragment and nuclear extracts from HeLa cells. One specific band was identified which could be competed for by oligonucleotides containing the sequences for GRE (glucocorticoid response element), TFIID and CREB (cyclic AMP responsive element binding protein). However, little can be deduced from these assays as only nonneuronal extracts were utilised.

More recently, Gerner *et al.* (1997) investigated the effects of CREB and GR binding to the $GABA_A$ receptor α1 subunit gene basal promoter. They inserted the 60 bp region into a plasmid that contained the luciferase gene under the control of a MMTV promoter in which a hormone response element (HRE) had been deleted. Analysis of luciferase activity in neuronal and nonneuronal cell lines revealed that a neuronal factor which can be activated by forskolin interacts with this region and suppresses expression of the adjacent gene. As the 60bp region contains a CRE element, it is possible that this repression is caused by a CRE binding protein interacting with this site. Gel mobility shift assays were performed with nuclear extracts from neuronal cells. While specific binding was observed, competition with a CRE oligonucleotide did not affect this binding. Also, an antibody against CREB did not bind to the DNA - protein complex. Mutation of the CRE sequence resulted in a weaker binding complex and deletion of the sequence upstream of the CRE element gave the same binding pattern as observed with the non - mutated sequence. Deletion of sequence 3' to the CRE element resulted in DNA - protein binding that could be competed by CRE oligonucleotides. Unfortunately, antibody binding to this complex was not reported. This could possibly have confirmed their conclusions that a CRE binding protein was associated with another factor as a heterodimer and that in the complex, this cofactor was covering the antigen site for the CREB antibody. Leach *et al.* (1997) identified an 18 bp silencer element in the human α1 promoter that represses promoter activity in nonneuronal cells. They have also isolated a REST - like transcription factor that binds to this element.

Kirkness and Fraser (1993) investigated the 5' - regions of the human and rat $GABA_A$ receptor β3 subunit genes. They found a high degree of sequence homology between the two genes in the 5' non - coding region. This conservation suggests functionality, for example the sequence may be involved in the maintenance of DNA or RNA secondary structures. They also revealed the existence of an alternative first exon, exon 1a. This displayed little sequence homology to exon 1, but was a similar length and also had characteristics of a signal peptide (von Heijne, 1990). The relative abundances of the transcripts of exons 1 and 1a were investigated in various brain tissues. While the transcripts of exon 1a were the minor product in all regions, transcript levels were found to vary between brain samples. Exon 1a transcripts were found to be enriched in foetal

brain, but depleted in adult hippocampus. It could therefore be concluded that the expression of the two mRNAs is selectively controlled, but the mechanism of control is unknown.

The transcription start sites for exon 1 were identified by RNase protection. Transcription is initiated from multiple sites within a pyrimidine - rich region located about 100 to 180 bp upstream of the translational start point. Analysis of the immediate 5' - region revealed the absence of TATA and CAAT boxes and showed that the region is rich in GC sequences. While the TATA box is the principal core element of many gene promoters, in a variety of genes this motif is known to be replaced by an initiator element (Roeder *et al.*, 1991). Initiator elements have been found in pyrimidine - rich sequences, although comparison of sequences of initiator regions of several different genes displays little homology (O'Shea - Greenfield and Smale, 1992).

The minimal sequence requirements for functional promoter activity were investigated. Initially, promoter - deletion constructs fused to the chloramphenicol acetyltransferase (CAT) gene were assayed in several cell lines (HeLa, GT1-7, PC12, 293). This revealed strong promoter activity within a 143 bp region (-191 bp to -49 bp, where +1 relates to the ATG of exon 1). Binding of nuclear factors to this region in DNase I footprinting experiments resulted in protection of a 23 bp segment (B3F1) which included the transcription start sites and also an inverted recognition sequence for the Sp1 transcription factor. Further analysis by gel mobility shift assays was performed using a variety of oligonucleotides that contained binding sites for common transcription factors and also for B3F1. This data showed that both the Sp1 and B3F1 sequences caused band shifts which were unrelated to each other. This would suggest that if Sp1 is involved in transcription, it binds to the promoter at a different location. To date, information about the basal promoter region of exon 1a has not been published.

The 5' - upstream region of the human $GABA_A$ receptor $\alpha 5$ subunit gene was characterised (Kim *et al.*, 1997), and three alternative first exons (1A, 1B and 1C) were identified. These share little sequence homology with each other, however exons 1B and 1C show a high degree of homology to the equivalent regions in the rat $\alpha 5$ gene

(Malherbe *et al.*, 1990). Exon 1A does not reveal any significant sequence homology to the rat gene, implying that either the corresponding rat exon 1A has not been identified or that exon 1A is species - specific. A single major transcription start point was identified by primer extension for each of the three mRNA isoforms. No TATA or CAAT boxes were observed but recognition sites for the Sp1 transcription factor were present, the initiation regions were pyrimidine - rich and the basal promoter region was rich in CpG dinucleotides. Expression studies showed that all three mRNA isoforms were expressed in the brain, and that their expression varied significantly in different brain regions.

The 5' - regions of the rat and mouse $GABA_A$ receptor α6 subunit genes were sequenced and their transcription start points identified (Jones *et al.*, 1996) by RACE and RNase protection. A cluster of transcription start points were noted for both the rat and mouse within roughly the same region. The α6 upstream region bears little sequence homology to the other $GABA_A$ receptor genes, but comparison of 500 bp of rat and mouse upstream sequences revealed a high degree of homology, as has been observed for the upstream regions of the other characterised $GABA_A$ receptor subunit genes. TATA and CAAT boxes are absent as are recognition sites for Sp1 transcription factors. This is unusual as all the other genes have at least one Sp1 site close to the area of transcription initiation, although only the δ subunit gene Sp1 sites have been shown to be essential for expression (Dr D. Livingstone, pers. comm.). Recognition sites for several other transcription factors were identified including homeobox - containing proteins, a CK8mer motif and an E - box.

The 500 bp promoter fragment was assayed for activity using transgenic mice (Jones *et al.*, 1996). The rat fragment was linked to a *lacZ* gene and randomly integrated into the mouse genome. Fourteen independent founder mice were generated of which only four expressed β - galactosidase. Only one of these founder mice gave almost the expected expression pattern with predominant expression in the cerebellar granule cells. β - galactosidase expression was also observed in the inferior colliculi, with the rest of the brain being negative. This transgene also differed from the native α6 gene in that expression varied between the cerebellar lobules. The other 3 founder mice gave neuronal but ectopic expression.

Little can be deduced from these transgenic experiments. The observed expression patterns could be due to a number of factors. The regions of insertion of the transgenes were not identified and are likely to have influenced expression patterns. Also, the use of rat sequence in a mouse background is not ideal. While there is a high degree of homology between species, real differences have been observed between the rat and mouse δ promoters (this work) and could be affecting expression in this instance.

The knowledge of the 5' - upstream region of the δ subunit gene prior to my work was discussed in detail in the General Introduction (**Section 1.3**). While most of the characterised $GABA_A$ receptor subunit gene promoters share several common features, they display little sequence homology. There is however a high degree of sequence conservation of the different subunits between species. It is possible that this high sequence conservation in noncoding DNA suggests an important regulatory function for this region, which may have been conserved during the course of evolution. The putative core promoter regions of the various genes have similarities and differences. The δ, β3 and α6 have multiple transcription start points, while the α1 and α5 genes have a single start point. The δ, β3 and α5 genes all lack TATA and CAAT boxes, are rich in CpG dinucleotides, and contain potential transcription factor binding sites for Sp1. Pyrimidine - rich initiator elements are also present. The α6 gene also lacks the TATA and CAAT boxes, but is not CpG - rich and does not contain putative Sp1 binding sites. The α1 promoter exhibits TATA and CCAAT box sequences in the 5' - proximal region.

What conclusions, if any, can be drawn from the above information about the regulation of the various $GABA_A$ receptor subunit genes, and the δ gene in particular? The δ subunit is the only isoform of its class and as such may form functionally unique $GABA_A$ receptor subtypes (Shivers *et al.*, 1989). It has been found to co - exist with the α1, α3, β2/3 and γ2 subunits (Mertons *et al.*, 1993) and Quirk *et al.* (1994) found an exclusive co - localisation of the δ with the α6 polypeptide in the cerebellum. Thus if it forms cell - type specific receptor subtypes with these subunits, it may be postulated that they are co - regulated and as such share regulatory elements. An alternative theory is that the δ gene is regulated in a manner distinct from the other $GABA_A$ receptor subunit genes. Many of the

$GABA_A$ receptor subunit genes appear to be clustered on chromosomes e.g. in humans, α5, β3 and γ3 are found on Chromosome15; α2, β1, α4, γ1 on Chromosome 4 and α1, α6, β2 and γ2 on Chromosome 5, while the δ gene is situated on the short arm of Chromosome 1. It has been proposed that the clustering of the genes facilitates co - ordination of gene expression. Thus the transcriptional regulation of the δ gene would differ from the other subunit genes. Arguments for and against co - ordinate regulation can be drawn from the information available. The β3 and α5 genes are clustered on Chromosome 15 and share several similarities including the presence of alternative exon 1s. Conversely, the α1 and α6 subunits which reside on Chromosome 5 have promoters that are distinct from each other.

While some characterisation of the first 800 bp of the murine $GABA_A$ receptor δ subunit gene promoter had been performed (Sommer *et al.*, 1990), only basal promoter elements were identified. In the rat, similar elements were detected (Motejlek *et al.*, 1994). Dr D. Livingstone (Prof. RW Davies laboratory) performed *in vitro* expression studies using luciferase fusions with short (< 2 kb) δ promoter fragments. Using a series of promoter - deletion constructs in 2 cell lines, GT1 - 7 and NB4 1A3, he found that the Sp1 sites were critical for expression.

In this work a total of 10.5 kb of $GABA_A$ receptor δ subunit gene 5'- upstream sequence was isolated and analysed and the transcription start points were identified. Thirty six percent of the upstream region was composed of repeat sequences, and several putative transcription factor binding sites were identified. 3.75 kb of this sequence (spanning the transcription start points (tsp) and putative core promoter) showed significant homology upon comparison with 3 kb of previously published rat sequence (Motejlek *et al.*, 1994). This conservation between species has also been observed for the other subunits, only over much smaller regions. No significant regions of sequence homology were observed between the $GABA_A$ receptor subunit gene promoters characterised to date.

A novel *cis* sequence element and associated DNA binding protein, BSF1, were identified in the 5' - region of the rat δ gene (Motejlek *et al.*, 1994). From analysis of the sequence,

a 22 bp purine repeat element was identified as being present in seven tandem copies located at the 3' - end of a large purine repeat region. A consensus sequence for this element was generated. Examination of other $GABA_A$ receptor subunit gene promoters revealed related sequences in the mouse γ2, rat α1, human β3 and rat α6 genes. Protein binding to this element was investigated by bandshift assays using nuclear extracts from brain and liver tissue. Using an oligonucleotide probe that matched the consensus sequence exactly, two bands were detected. A single base change to the probe resulted in only one of the bands being detected. This alteration still left the probe with a 100% match to the consensus sequence. A second alteration which changed the sequence from the consensus resulted in loss of all bands. Not suprisingly, no binding was detected for the other GABA probes used, α1 and γ2. The various binding experiments are not conclusive due to the presence of many extraneous bands, many of which are attributed to single - stranded binding proteins. Also, the sequences of the oligonucleotide probes used were altered by the addition of extra bases at each end to allow for future ligation experiments. It is highly probable that these alterations to the consensus sequences would affect binding of proteins.

Sequencing of the mouse upstream region revealed that this sequence element is absent in the mouse. If the BSF 1 protein is a specific binding protein for this sequence, there are several reasons to explain its absence. The binding sequence could be present in another area of the gene e.g. further 5', in an intron or at the 3' - end of the gene. This could be investigated by probing mouse genomic DNA with oligonucleotides designed to hybridise to the BSF 1 recognition sequence. Another possibility is that the BSF 1 factor could bind to another sequence present in the mouse. Binding assays with mouse DNA and the BSF 1 protein would determine if it is binding to an alternative sequence. Finally, if this factor is specific to the rat, it is possible that its role in regulation is controlled by a related factor in the mouse

Having the sequence of a large promoter region will hopefully allow the identification of novel control elements. Analysis of the mouse sequence using databases (EMBL), highlighted regions of homology with other genes. Most of these homologous regions were repeat sequences (LINES, SINES) found throughout the mammalian genome. It has

been proposed that these sequences can play a role in gene regulation. By comparing genes that contain these particular repeats, it may be possible to draw conclusions about their functionality or cell - type specificity.

In vivo promoter analysis has been performed by a number of groups using DNA transgenics. Luscher *et al.* (1993) analysed δ promoter activity using transgenic mice. They illustrated neuron - specific expression from a 6.3 kb promoter fragment driving *lacZ* expression in most regions of the CNS, but were unable to show the correct developmental profile of the native gene. Thus this region lacked important regulatory elements. An α6 knock - out mouse has been produced in which the mutation appears to affect the δ subunit protein (Jones *et al.*, 1997). Homologous recombination in embryonic stem cells was used to create a mouse line in which the α6 subunit gene was disrupted at exon 8. Translation of α6 subunit mRNA from the mutant allele should terminate after the TM2 region resulting in a 300 amino acid protein. Thus this is not a true gene knockout and it is likely that the truncated protein is binding to the δ subunit protein in some way. Mihalek *et al.* (1997) recently reported the generation of a δ knock - out mouse, but have not yet examined the effects, if any, on the other $GABA_A$ receptor subunit genes.

In this work, *in vitro* analysis of a range of promoter - reporter constructs in a neuronal cell line identified a 1.8 kb region (between 4.5 kb and 6.3 kb upstream) that appeared to downregulate expression of the *lacZ* gene. Examination of the sequence of this region revealed several putative transcription factor binding sites. Several repeat regions are also present in this fragment. Future work will involve identifying the putative binding site(s). This can be achieved in several ways. Oligonucleotides may be designed to the identified transcription factor binding sites and these can then be tested for functionality in DNA binding assays (gel retardation and DNA footprinting). PCR oligonucleotides primers could also be designed across putative control regions and the resulting PCR products also tested in gel shift assays.

10.3 VIRAL VECTORS

The major difficulty in studying neuron - specific gene expression *in vivo* is transferring the DNA into the cells. Two techniques being developed for effective gene transfer *in vivo* are transgenics and viral vectors. While the viral vectors may be useful for studying gene expression it should be noted that there may be the possibility of interference from the viral genes and their promoters. On a wider scale, they are particularly relevant for gene therapy. To date, 18 gene therapy trials have been agreed in the UK, with a further 5 still under review (Oxford BioMedica). A range of different viral vector systems which include retroviruses (RV), adenoviruses (AV), adeno - associated viruses (AAV), herpes simplex viruses (HSV), parvoviruses (PV) and more recently lentiviruses are currently being developed as viral vectors.

We chose to develop an HSV - 1 viral vector as a gene transfer vehicle given its ability to establish long - term latent infections in neuronal cells. Gene transfer to neurons using HSV - 1 vectors has significant potential advantages for gene therapy for neurological diseases. It has a potentially large cloning capacity, it remains episomal and it is largely neurotropic. Investigations with HSV -1 based vectors have not yet reached the stage of human phase I trials. Further manipulation of the HSV genome is required, to ensure a truly apathogenic vector is engineered.

Several groups employing HSV - 1 mediated gene delivery have reported significant cellular cytotoxicity with these viral vectors (Anderson *et al.*, 1992; During *et al.*, 1994; Pakzaban and Chiocca, 1994) most likely attributable to the simultaneous expression of a number of viral products. Thus to establish an effective HSV vector system, it is necessary to remove virally induced cytotoxic functions including those required for lytic replication and to promote the establishment of latency without the possibility of reactivation. In an effort to reduce cytotoxicity, HSV - 1 vectors have been constructed in which the pathogenicity of wild - type HSV - 1 has been reduced. These have either been based on disabled viruses in which essential genes have been deleted and which require growth in a complementing cell line, or on defective viruses (amplicons) (Geller *et al.*, 1990; Johnson *et al.*, 1992; Ho *et al.*, 1993; Geschwind *et al.*, 1994). Although the use of amplicons should eliminate the toxicity associated with viral gene products, the presence of helper

virus in amplicon stocks complicates this goal as it must also be disabled in some way. Presently disabled and helper virus systems do give a low level of reversion to a pathogenic phenotype by recombination of the disabled viral DNA with overlapping integrated helper sequences. Future studies on the source of HSV - 1 mediated cytotoxicity and improved vector development are required before this viral vector system can be used for gene therapy.

Development of vectors that contain appropriate gene - specific promoter - enhancer elements to achieve long - term gene expression is also necessary. Early studies demonstrated that HSV - 1 vectors carrying the E coli *lacZ* gene can infect many neuronal populations both *in vitro* and *in vivo* (Geller and Breakefield, 1988). Later reports appeared demonstrating physiological effects resulting from HSV - 1 - mediated gene delivery. An example of this was demonstrated by During *et al.* (1994). They reported that an HSV - 1 - derived vector expressing tyrosine hydroxylase (TH) could rescue, both behaviourally and biochemically, the effects of a 6 - hydroxydopamine lesion in rats when delivered to the partially denervated striatum. More recently, replication - deficient HSV - 1 vectors have been used for overexpression of the apoptosis - inhibitory protein Bcl-2 in primary cultures of hippocampal neurons and in the hippocampus *in vivo.* This resulted in the protection of neurons from oxygen radical accumulation *in vitro* and adriamycin toxicity and focal ischemia *in vivo* (Lawrence *et al.*, 1996).

While the HSV - 1 vector systems had been shown to transduce neurons in the brain (Geller and Breakefield, 1988; Dobson *et al.*, 1990), they had not been capable of targeting expression of foreign genes to a specific type of neuron. Several groups have used cell - type specific promoters in an attempt to target recombinant gene expression to specific neuron populations.

Anderson *et al.* (1992) generated a recombinant HSV-1 virus containing the *Ecoli lacZ* gene under the control of a rat NSE promoter (1.8 kb) inserted within the HSV - 1 thymidine kinase (*tk*) gene. To determine whether the NSE promoter would retain its selective expression in neurons, the NSE - recombinant virus was used to infect a number

of different cell types in culture including neuronal, nonneuronal and primary cultures. Another recombinant virus that contained the *lacZ* gene under the control of an HSV - 1 promoter was used as a control for neuron - specific expression. With the NSE - virus, only neuroblastoma cells and cells resembling neurons in the primary cultures showed β - galactosidase staining 3 days after infection, whereas the control virus showed staining in all cell types infected. When the neuroblastoma cell line was infected with each virus, both gave β - galactosidase stained cells after 3 days. However the NSE - virus gave 10 fold fewer blue cells than the control virus at a comparable multiplicity of infection (moi = 1). To acertain whether this was due to the differing strengths of the promoters or to the lack of viral infection, the NSE - virus was used at a moi = 10. All cells even those showing no blue staining showed marked cytopathic effects after 3 days indicating that lack of detectable *lacZ* expression did not reflect failure of the virus to infect the cells. Infection of a variety of neuron - enriched primary cultures with these viruses gave β - galactosidase staining. Rat dorsal root ganglion (DRG) cultures infected with the NSE - virus showed β - galactosidase staining at 3 and 14 days post - infection, as opposed to the control virus which showed staining at 3 days but not at 14 days.

Expression was also studied *in vivo* by stereotactic injection of the viruses into the frontal lobe of the brains of adult male rats. With the NSE - virus, β - galactosidase staining was observed along the needle tract in neuronal - like cells at 30 days post - inoculation. This β - galactosidase expression pattern was confirmed by immunocytochemical staining with antibodies to *lacZ*. When the control virus was injected using comparable titres, blue staining was observed at 3 days, but not 14 days post - infection. While many of the β - galactosidase stained cells appeared to be neuronal based on size and morphology, it was not shown conclusively that the virus was actually entering neurons. No co - localisation studies were performed making it difficult to evaluate true cell - type specificity of expression. However, this NSE - recombinant virus was the first described to use a mammalian promoter to obtain extended expression of a foreign gene in the adult mammalian CNS.

Another group (Kaplitt *et al.*, 1994) utilised an HSV - 1 amplicon vector to study expression from a foreign gene in the mammalian CNS. They selected the rat

preproenkephalin (PPE) promoter (2.7 kb) to drive expression of the *lacZ* reporter gene. PPE was chosen as it has a well characterised cell - type specific expression pattern in the brain, including the caudate nucleus, amygdala, piriform cortex, olfactory tubercle and ventromedial hypothalamus (Khachaturian *et al.*, 1983; Harlan *et al.*, 1987). The defective PPE virus was stereotactically injected into regions of the rat brain that normally express endogenous PPE mRNA. Two regions that contain few cells that express endogenous PPE mRNA were also injected as negative controls. They noted that β - galactosidase staining was observed in several cells in regions where the endogenous gene is expressed. The efficiency of expression in these regions was 1 - 5% which did not differ significantly from the efficiencies observed with studies using the CMV promoter (Kaplitt *et al.*, 1991). Very few cells were stained in regions where endogenous PPE transcripts are not normally found. β - galactosidase - positive cells were also observed two months after injection. This indicates that HSV - 1 amplicon - based vectors are stable for at least two months and that long - term expression can be achieved through the use of specific promoters.

More recently, a defective HSV - 1 vector was constructed that contained the rat tyrosine hydroxylase (TH) promoter (6.8 kb of 5' upstream sequence) linked to the *lacZ* gene (Song Song *et al.*, 1997). The TH promoter was selected to see if it could target expression of a foreign gene to catecholaminergic neurons in a specific brain region, such as the substantia nigra pars compacta (SNc). The expression of β - galactosidase from the TH promoter was analysed *in vitro* and *in vivo*. Cell lines both positive and negative for TH were used as were primary cultures. They showed that expression in TH positive cells was 20 fold higher than in TH negative cells. Stereotactic injection of the virus into the rat midbrain demonstrated mainly catecholaminergic cell - type specific expression up to six weeks after infection, although some aberrant expression was noted. One reason for this lack of specific expression could be that the 6.8 kb TH promoter fragment lacks some of the regulatory elements required for cell - type specific expression. Another cause could be interference from the HSV - 1 vector or helper virus. It is possible that specific HSV - 1 proteins that are either present in the HSV - 1 particle or transiently expressed from the helper virus may interact with the TH promoter or regulatory elements in the cells. A

helper - virus free packaging system has recently been developed and initial results show an improved cell - type specific expression from the same TH promoter fragment.

The three examples given above demonstrate that either replication - deficient or HSV - 1 defective viral vectors can be used for long - term expression of a foreign gene by a non HSV - 1 promoter. However they have been unable to show faithful cell - type specific expression from these promoters. This could be due to the promoters themselves lacking specific regulatory elements or to interference from the viral genes.

In this work, we utilised two attenuated HSV - 1 viral mutants for the generation of recombinant viruses. The first variant (1716) lacked the ICP34.5 gene which expressed late in infection and is implicated in neurovirulence, but is not necessary for replication *in vitro* or for the establishment of latency *in vivo*. The ICP34.5 mutants have been shown to be replication defective in sensory ganglia and the CNS of mice (Bolovan, *et al.*, 1994; Chou *et al.*, 1990; MacLean *et al.*, 1991b; McKie *et al.*, 1994; Whitely *et al.*, 1993), but replication is normal in most tissue culture cell types. Another variant (1764) also lacked the ICP34.5 gene and contained a second mutation of the Vmw65 (U_L48) gene. This gene causes transactivation of immediate early (IE) genes and inactivating it should force the virus into the latent pathway of infection.. It was hoped that this would reduce the levels of cytopathic IE gene products as compared with 1716. Replication of these mutant viruses during infection is dependent on the multiplicity of infection being high. These variants had previously been shown to allow short - term expression of the β - galactosidase gene after footpad or intracranial inoculation of mice (Coffin *et al.*, 1996).

In this work, many problems were encountered with both viral variants used. It proved extremely difficult to purify recombinant viruses away from the wild - type variants, and the frequency of recombination was very low (< 1 %to < 0.01%). Generation of a recombinant virus containing a large promoter fragment linked to the *lacZ* gene proved impossible. Out of 600 viruses screened, only one gave a positive signal with a GABA probe. Southern blot analysis revealed that this virus contained a deletion of the promoter fragment. This may have been due to the actual sequence of the $GABA_A$ receptor δ subunit gene promoter. A large incomplete repeat region that is highly homologous to an

HSV - 1 sequence is present in the promoter about 5 kb upstream from the transcriptional start sites. It is possible that some aberrant recombination was taking place between the HSV -1 and δ promoter sequences. Infection of cerebellar granule cell cultures and stereotactic injection of these viruses into rat brains showed that these viruses were unable to target the cells of interest and little transgene expression was observed. While the $GABA_A$ receptor δ subunit gene promoter is only a moderately strong promoter, even the use of the strong HSV - 1 gD promoter or the CMV promoter failed to give specific *lac*Z expression. Thus while it is obvious that the particular HSV - 1 variants used in this work are unsuitable for further development in this area, other vectors are being developed that do appear to have targeted neurons and have allowed expression of reporter genes from foreign promoters

The $GABA_A$ receptor δ subunit gene promoter - *lac*Z fusion vectors constructed in this work have been designed in such a way that with very little manipulation they can be inserted into other vectors. Thus, they can now be transferred to new HSV - 1 vectors with better growth characteristics thus furthering the original goal of this work.

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