Targeted Disruption of the Neurotensin Receptor Gene

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

By

Edward Jude Gallagher

Division of Molecular Genetics
Institute of Biomedical and Life Sciences
The University of Glasgow

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

The research in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.

Edward J. Gallagher

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Dedicated
to the memory of
Michael Robert McNulty

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Abbreviations

Measurements

bp	base pair
Ci	curie
cm	centimetre
cpm	counts per minute
°C	degrees celsius
dpm	disintegrations per minute
fg	femtogram
fmol	femtomoles
g	gram
kb	kilobase pair
kDa	kiloDalton
l	litre
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
ng	nanogram
nm	nanometre
nM	nanomolar
pg	picogram
rpm	revolutions per minute
s	second
µCi	microcurie
µg	microgram
µl	microlitre

Alphabetical List

aa	amino acid
amp	ampicillin

Amp	ampere
ATP	adenosine 5'-triphosphate
BRL	buffalo rat liver
BSA	bovine serum albumin
cAMP	cyclic adenosine 5'-monophosphate
CCK	cholecystokinin
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CNS	central nervous system
CRE	cAMP response element
DA	dopamine or dopaminergic
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DIG	digoxigenin-11-dUTP
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine 5'-triphosphate
dUTP	deoxyuracil 5'-triphoshate
EcR	ecdysone receptor
EcREs	ecdysone response elements
EDTA	ethylenediaminetetraacetic acid (disodium salt)
e.g.	for example
E/GRE	hybrid edysone response element
ES	embryonic stem
EtBr	ethidium bromide
Exo III	exonuclease III
FSB	final sample buffer
FRT	Flp recombination target sequence
G418	geneticin^{tm}, Gibco
Ganc	gancyclovir
GCG	genetics computer group, (Wisconsin U.S.A.)
Gly	glycine
GRE	glucocorticoid response element
hprt	hypoxanthine phosphribosyltransferase
HSV	herpes simplex virus

IPTG	isopropyl-β-D-thiogalacto-pyranoside
λ	lambda
LMP	low melting point
*lox*P	the target sequence for Cre recombinase
mRNA	messenger ribonucleic acid
N. Acc.	nucleus accumbens
Neo	neomycin
NRM	nucleus raphe magnus
NT	neurotensin
NM	neuromedin N
NTR	neurotensin receptor
OD	optical density
PA	SV40 polyadenylation site
PAG	periaqueductal gray
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
p.f.u.	plaque forming unit
RNase	ribonuclease
RVM	rostral ventral mendulla
RXR	retinoid X receptor
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SV40	simian virus 40
TEMED	N,N,N',N'-tetramethyethylenediamine
*tet*O	tetracycline operator sequence
*tet*R	tetracycline repressor
Thr	threonine
TH	tyrosine hydroxylase
TK	thymidine kinase
tTA	tetracycline *trans* activator
TVP	cell disaggregating solution
Tyr	tyrosine
U	units
UV	ultraviolet light
V	volts
vol	volume
VpEcR	synthetic ecdysone receptor
VTA	ventral tegmental area

X-gal	5-bromo-4-chloro-3-indolyl β-D-galactoside
X^R	Xresistant
X^S	Xsensitive

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Abstract

The primary aim of the work presented in this thesis was to investigate the neurotensin transmission system of the mouse CNS by employing a reverse genetic approach. The neurotensin peptide and receptor distribution within the CNS has been extensively mapped, implicating a role of neurotensin in many brain functions but little is known of the exact functional relationship of neurotensin transmission with respect to other neuronal pathways. However, increasing biochemical, electrophysiological and neuropharmacological data have highlighted a neuromodulatory role of neurotensin over the dopaminergic mesolimbic and nigrostriatal pathways. These pathways are involved in the regulation of movement and motivational behaviour, which are believed to underlie the mechanism of reward reinforcement for such drugs as cocaine, as well as being important to the pathophysiology of schizophrenia.

Two gene targeting approaches were undertaken to create a null mutation of the neurotensin high affinity receptor. The first strategy was based on the 'Hit and Run' procedure of Hasty *et al.*, 1992a. An integrative vector was designed based on a 2.8 kb *Bam*HI DNA fragment of the neurotensin receptor gene subcloned from a λ EMBL3 Balb/c mouse genomic library. Sequence analysis of the *Bam*HI fragment revealed a large open reading frame encoding sequence encompassing the first four transmembrane domains of the receptor. Subsequent vector construction involved the introduction of a mutagenic linker which introduced a stop codon within the sequence encoding the first transmembrane domain of the receptor. PCR analysis of ES cell clones transfected with the integrative vector, (pKLF), indicated the targeted integration of pKLF at the neurotensin receptor gene locus. However, selection of these ES clones for the desired reversion event did not result in the identification of any ES cell clones containing the linker mutation within the NTR gene. Re-evaluation of the 'Hit and Run' technique, in light of the increased understanding of the factors governing gene targeting in ES cells, led to a change in approach.

The second strategy utilised a replacement type vector in combination with the 'positive negative selection' regime, (Thomas and Capecchi, 1987), to target the neurotensin receptor gene. The replacement vector used was constructed from a 5' 9.2 kb *Eco*RI fragment of the neurotensin receptor gene subcloned from the λ Dash 129J mouse genomic library. The targeting construct introduced a deletion and a disrupting neomycin cassette into the receptor gene within the sequence encoding the

fourth transmembrane domain. This targeting construct was successfully used to target a number of ES cell lines; E14, R1, CGR8 and CGR8.8. Of the 4 targeted R1 ES sublines, 2 produced weakly chimaeric mice, (10-15% ES cell derived). No germline transmission of the null NTR allele was obtained from repeated matings of these chimaeric mice. A targeted CGR8 ES cell subline was used to generate 3 highly chimaeric male mice, (80-90% ES cell derived). However these mice were apparently sterile, with no progeny being produced from repeated matings. Two other targeted sublines were produced from the CGR8.8 ES cell line, and are to be tested for germline transmission. In addition, an *in situ* hybridisation analysis of the neurotensin receptor expression within the developing mouse embryo, revealed specific localisation of the probe to an unidentified structure of the developing forebrain.

Chapter 1

Introduction

1.1 Overview of objectives

Neurotensin was accidentally isolated over 20 years ago during the course of work intended to isolate substance P from bovine hypothalamus tissue, (Carraway and Leeman, 1973). Since then a wealth of data has accumulated on the possible biological function of neurotensin, (NT), as a hormone of the intestinal tract and as a neurotransmitter or neuromodulator of the central nervous system, (CNS). Increasingly over the years, the focus of endeavour has moved from the possible trophic functions of NT to its functions within the CNS. This is principally because of its occurrence within particular brain structures involved in the mediation of pain, the regulation of body temperature, the regulation of sleep, the processing of movement and motivational behaviour. The majority of this research has detailed the distribution and roles of NT and its binding sites with particular neuronal pathways, particularly the role of NT within the nigrostriatal and mesolimbic dopaminergic pathways, for a number of reasons. The control of movement is mediated through the nigrostriatal pathway as demonstrated by the progressive lack of ability to execute movement in Parkinson's sufferers; as a consequence of the progressive loss of dopaminergic neurons from this pathway. The mechanism underling the rewarding and reinforcing effects of drugs such as cocaine and amphetamines is also thought to be mediated through these dopaminergic pathways. Furthermore, the development of dopamine D2 receptor antagonists led to their clinical use as antipsychotics and the implication that a defective DA transmission underlying the pathophysiology of schizophrenia. Interestingly, the action of neurotensin when administered centrally mimics the profile of antipsychotic drugs, where the beneficial effects are thought to be mediated through the mesolimbic system. For these principal reasons, the background data on neurotensin and its receptor in the first part of this introductory chapter will concentrate on briefly reviewing the data concerning dopamine-neurotensin interactions and the accumulating evidence implicating neurotensin as an endogenous neuroleptic that may be involved in the pathology of schizophrenia.

Considerable insight into the CNS functions of NT has been gained using traditional neuroscience approaches, of neuroanatomy, neuropharmacology and neurophysiology. However, these approaches do not offer the same analytical power for the delineation of a neuropeptide's function compared to the modern reverse genetic technology of the mouse, (Davies *et*

al., 1994). The classical neuroscience tools of intervention have been chemical agonists and antagonists, which have been used to obtain a functional understanding of particular neuronal transmission systems. These pharmacological methods can be of great use when investigating a particular molecules function, and when that function can be assayed. However, there are a number of limitations. In the case of neuropeptides, there is a lack of chemical antagonists due to the time and cost in synthesising such inhibitors. The non-peptide antagonists also tend not to be exclusively specific for their intended target. Often a range of receptor types will bind these antagonists with varying affinities, which complicates the analysis of a specific peptide/receptor function(s). Moreover, this type of analysis, typically performed in the adult CNS, does not allow for the lack of function to be assessed with respect to the significance that lack of function may have as a result of ontogeny. Furthermore, the reverse genetic approach now has the flexibility to create a conditional gene specific defect which can be analysed within the adult CNS. This can be a genetic defect that can be investigated in the adult in light of its developmental background or conditionally induced to allow adult function to be analysed separately. It is also now possible, in principle, to disrupt NT transmission in any tissue specific, (neuronal specific), and developmental manner desired. Furthermore, precise mutations can be introduced into the receptor or the neuropeptide encoding precursor gene to address specific functional questions of the protein molecule. Induction and repression of the expression of a precisely mutated gene in a temporal and spatial manner is now possible. However, with respect to this introduction only those gene targeting approaches that were available for the disruption of the receptor within the time frame of this project will be discussed in the second part of this introduction.

1.2 Dopamine and neurotensin interactions

1.2.1 Introduction

There is substantial information from anatomical, neurochemical, electrophysiological, behavioural, and pharmacological studies that demonstrates NT interaction with dopamine systems of the CNS, (reviewed in Kasckow and Nemeroff, 1991). A brief overview of such studies follows, and

Figure 1.1 presents a simple diagram of the major projections of dopaminergic neurons of the mesolimbic and nigrostriatal pathways.

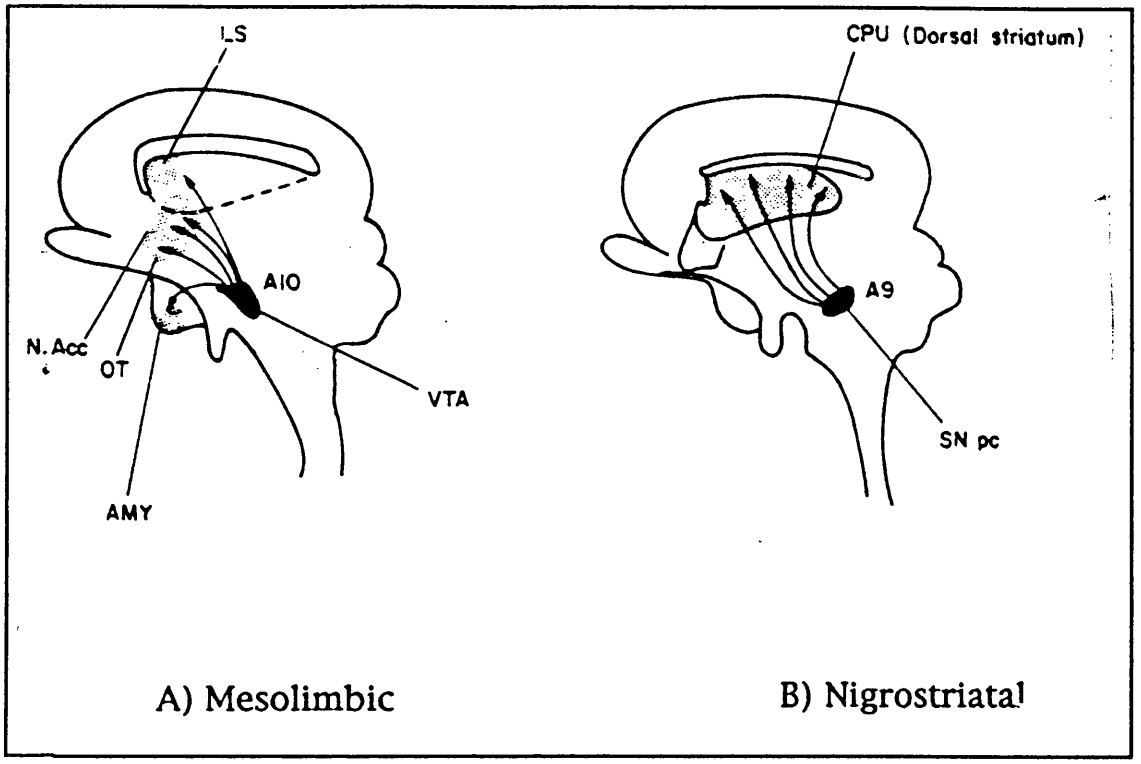


Figure 1.1 The dopaminergic neurons of the nigrostriatal and mesolimbic pathways. The shaded areas indicated the major regions of innervation of the dopaminergic neurons; it should be noted that there is considerable overlap and some collateralization of these pathways. The caudate and putamen (CPU) form one structure in rodents, but are separate in primates. A distinction is usually drawn between the dorsal striatum (the major portion of the caudate-putamen) and the ventral striatum (incorporating the nucleus accumbens, olfactory tubercle and the ventro-medial parts of the caudate-putamen). Abbreviations: A9, cell group of dopamine neurons in the substantia nigra, pars compacta (SNpc); A10, cell group of dopamine neurons in the ventral tegmental area (VTA). The A8 cell group and the A10 neurons of the mesocortical pathway are not shown. N. Acc., nucleus accumbens; OT, olfactory tubercle; AMY, central amygdaloid nucleus; LS, lateral striatum This Figure was adapted from Robbins, 1992.

1.2.2 Anatomical studies

Many studies have documented the association of NT and its receptor with dopaminergic neurons. Principally, this association is with the mesolimbic and nigrostriatal dopaminergic systems. Double labelling studies using 125I-neurotensin and TH-immunocytochemistry, (specific for catecholamine neurons), have localised binding sites, (putative NTRs), to the dopamine neurones of the rat substantia nigra and ventral tegmental area, (Szigethy and Beaudet, 1989). These studies have been confirmed more recently by *in situ* hybridisation of rat brain slices using oligonucleotides derived from the rat cDNA, (Nicot *et al.*, 1994). Neurotensin-immunoreactivity has also been demonstrated in the rat striatum, the nucleus accumbens and the olfactory tubercle. The close association of NTRs with the dopaminergic neurons was emphasised by *in situ* studies on brains from patients who had suffered from Parkinson's disease, (Yamada *et al.*, 1995). The authors observed a reduction in NTR mRNA levels that paralleled the loss of the melanised (most likely dopaminergic) neurons from the substantia nigra and the nucleus paranigralis, (the VTA of the rat brain). Confirmation that the neurotensin is localised to the dopaminergic neurons was obtained indirectly by chemical means, (see below).

1.2.3 Neurochemical studies

A number of chemical lesion studies have been performed on dopaminergic neurons of the CNS which localise NT and its receptor to these neurons. 6-hydroxydopamine can selectively destroy the dopaminergic neurons of the nigrostriatal and mesolimbic systems. These lesions not only remove the dopaminergic terminals, but a large percentage of the NT receptors are lost from the striatum, the nucleus accumbens and the olfactory tubercle, thus indicating the presence of presynaptic NTRs on dopaminergic neurons, (Fuxe *et al.*, 1986). Furthermore, NT has also been shown to be co-localised in the dopamine neurons of the mesolimbic system, (Bean *et al.*, 1989). 6-OHDA lesion studies also demonstrated the depletion of these co-localised neurotransmitters.

A number of biochemical studies have documented a marked increase in striatal NT concentrations in response to DA D_2 receptor

antagonists such as haloperidol, (Govoni, *et al.*, 1980, Bean *et al.*, 1989). Evidence also exists for the increase in NT-immunoreactive neurons in the striatal complex following DA D_2 antagonist administration, (Zahm, 1992). Furthermore, haloperidol has been shown to increase the levels of NTR mRNA in the rat substantia nigra. This increase in NTR density could lead to increased excitability of DA cells as a compensatory mechanism to the effects of DA receptor blockade since the primary action of NT on dopaminergic neurons is to increase dopamine turnover, as has been demonstrated by many studies. For example, the microinjection of NT into the VTA increases the extracellular level of dopamine and its metabolites in the nucleus accumbens, a major terminal field of these dopaminergic neurons, (Laitinen *et al.*, 1990).

1.2.4 Electrophysiological studies

From the biochemical data, NT administered centrally has a stimulant effect on dopaminergic systems in that it increases DA turnover. Therefore, it was surprising to observe that *in vivo* NT generally had no effect on the basal firing rate or the firing pattern of the DA neurons of the midbrain, (Shi *et al.*, 1990). However, in the VTA injection of NT was shown to attenuate the action of D_2/D_3 DA agonists, suggesting that NT has an indirect neuromodulatory role on DA neurons. Analysis of the effects of microionotophoretically applied NT and DA revealed that for almost all cells treated, NT attenuated DA induced inhibition presumably mediated by an antagonistic action of the NTRs on the DA D_2 receptors. This neuromodulatory effect could also be demonstrated when DA was replaced with D_2/D_3 agonists, (Shi, 1991). However, some DA cells did register an increased firing rate on the application of NT. This observation is typically made of midbrain DA neurons analysed *in vitro*, (Seutin *et al.*, 1989, Pinnock, 1985). These type of experiments have indicated that the excitatory effect produced by NT occur at higher concentrations of NT, (>10 nM), than those needed for the neuromodulatory action of NT. The increased firing rate exhibited a linear dose response up to a concentration of 300 nM, (Seutin *et al.*, 1989); above this a cessation of neuronal firing was observed. In conclusion the stimulation of DA neurons occurs by two pathways, an unknown indirect neuromodulatory mechanism, (discussed in section 1.2.6), and directly by increasing the firing rates of DA neurons.

1.2.5 Behavioural studies

The central administration of NT can produce a number of distinct physiological and behavioural effects: NT produces hypothermia, muscle relaxation, antinociception, reduced food consumption , catalepsy and alterations in locomotor activity, (see Bissette *et al.*, 1991, for a review). The majority of these effects are similar to the action of neuroleptic drugs and are mediated through the dopaminergic mesolimbic and nigrostriatal pathways, (discussed in section 1. 4). The decrease in body temperature is mediated by the effects of NT on the DA neurons of the hypothalamus, (Kalivas *et al.*, 1982). The analgesic quality of NT is elicited by its injection into the periaqueductal gray (PAG) and is discussed in section 1.5.3.

1.2.6 Intramembrane interaction between neurotensin and dopamine receptors

The mechanism(s) by which NT exerts its predominantly stimulatory neuromodulatory effects upon dopaminergic neurons is not known. However, an increasing amount of data suggests that this regulation occurs by a NTR-mediated antagonistic action on D_2 receptors. This neuromodulatory mechanism is thought to involve the stimulation of the G-protein linked neurotensin receptor secondary messengers, (probably cGMP and inositoltriphosphate), which act directly or indirectly to alter the state of DA receptors. It has been proposed that activation of the NTR is antagonistic to D_2 mediated transduction, therefore increasing the D_1 receptor-mediated DA transmission by removing the D_2 receptor-mediated inhibition. Figure 1.2 presents the possible pre- and post-synaptic organisation of D_1, D_2 and NT receptors within the terminal field of the nigrostriatal pathway.

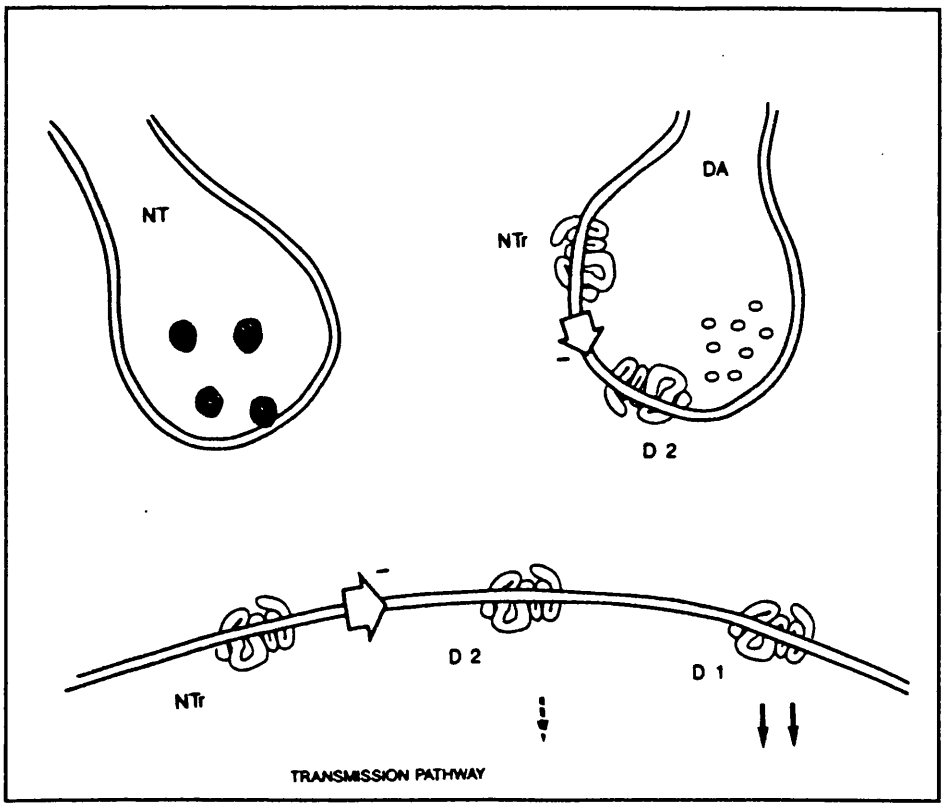


Figure 1.2 Possible pre- and post-synaptic interaction between neurotensin receptors and dopamine D_1 and D_2 receptors within the neurons of the striatum. Activation of neurotensin receptors causes a reduced affinity in D_2 agonist binding sites and enhanced effects of D_1 agonists. The broken arrow illustrates the reduced D_2-mediated DA signal and the two uninterrupted arrows the increased transmission of the D_1 mediated signal.

The diagram presented, (Figure 1.2), predicts that the selective regulation of DA receptors mediated by the NTR exerts an antagonistic effect on signal transduction via the D_2 receptor subtype, favouring signal transduction via the D_1 receptor subtype and thus postsynaptic excitation. Similarly presynaptic NTR-mediated antagonism of D_2 autoreceptors is thought to lead to increased DA release and enhanced dopaminergic neuronal activity. *In vitro* microdialysis studies have provided support for the intramembrane interaction of presynaptic NTRs and D_2 receptors of the striatum by measuring DA release from the nigrostriatal DA neurons, (Tanganelli *et al.*, 1989). The inhibition of DA release by D_2 agonists was antagonised by the action of NT, presumably mediated by NTRs to counteract the transduction of the D_2 autoreceptors. Evidence for the antagonistic action of NT/NTR action on postsynaptic D_2

receptors of the nigrostriatal pathway was also provided by microdialysis studies, (Fuxe, *et al.*, 1992). The inhibitory action of D_2 agonists was attenuated by NT application, as measured by the increase in extracellular levels of GABA released from the GABA neurons of the striatum. The increase in GABA release is also thought to be a result of selective action of D_1 transmission in response to antagonism of D_2 transduction.

Further, microdialysis studies on the effects of NT on DA transmission in the rat nucleus accumbens have documented an interaction with D_2 autoreceptors different from that observed in the striatum. The perfusion of NT into the N. Acc. produced a long lasting GABA out flow from the GABAergic neurons. This is thought to be by a mechanism similar to that proposed for the action of NT on postsynaptic D_2 receptors of the GABAergic neurons of the striatum. However, the presynaptic action of NT, (at subthreshold levels of 10 nM), was to elicit an inhibition of DA release from the DA neuronal terminals of the N. Acc. This effect of NT inhibition is thought to be mediated by a local increase of GABA outflow. This local GABA release could either directly inhibit DA release or indirectly reduce the excitatory input of the DA neurons of the N. Acc. In summary, the postsynaptic inhibition of D_2 receptors mediated by NTR on application of NT is observed in the striatum and the N. Acc. However, the presynaptic antagonism of D_2 receptor transduction is only observed in the striatum and not in the N. Acc when subthreshold levels of 10 nM NT are administered.

The previous studies have demonstrated the complexity of interaction between the NT and DA transmission. However, other factors can influence the action of the NT transmission system such as the type and density of receptors on the particular synaptic membranes. In particular, the interaction of the NT-NTR transmission with the full complement of DA receptor subtypes remains to be determined. This is particularly important in view of the observation that NTR action resembles that of atypical neuroleptics such as clozapine, (section 1.4), which are thought to act preferentially on the dopamine D_4 and not the D_2 receptor subtype. Furthermore, the input of other neuropeptide systems must be considered. This has been demonstrated for the neuropeptide cholecystokinin, (CCK). An inhibitory neuromodulatory action of CCK on D_2 receptor-mediated transduction has also been observed within the presynaptic dopaminergic terminals of the striatum. CCK perfused into the striatum by a microdialysis probe was able, in a concentration related manner, to counteract the inhibitory action of the DA agonist apomorphine, (Tanganelli *et al.*, 1993). This antagonism is mediated by the presynaptic CCKB receptor

on the D2 autoreceptors, and produces an increased DA release. Furthermore, CCK and NT were observed to synergistically antagonise the DA agonist apomorphine induced inhibition of DA release NT at subthreshold concentrations of 0.1-1 nM in the presence of 1 nM CCK-8, counteracts the inhibitory action of apomorphine on DA release by 70%. However, these low concentrations of NT have no effect on apomorphine inhibition when applied in the absence of CCK-8.

In summary, the previous discussion has highlighted the complexity of interaction the NT transmission system has over DA transmission. In particular its neuromodulatory role mediated by an antagonistic action on D_2 receptor signal transduction. However, this does not take in to account the influence of DA transmission on neurotensinergic systems. The effect of DA administration into the SNc is to upregulate NT and NTR density in the striatum, (Deutch and Zahm *et al.*, 1992). The current data suggests that this effect is mediated via DA-D1 receptor transmission. In support of this, the application of D_2 receptor antagonists also upregulates NT concentrations and NTR density, (see section 1.4.). Therefore, the DA-D_1 receptor action leads to an activation of the NTR mechanism, which in turn may operate as an inhibitory feedback loop on D_2 receptor-mediated DA transmission.

1.3 The NT precursor gene, the NTR genes, possible receptor subtypes and transduction pathways

1.3.1 The neurotensin and neuromedin N precursor gene

The neurotensin cDNA was first cloned from a canine cDNA library using a degenerate oligonucleotide designed from the 13 aa NT sequence of purified bovine neurotensin, (Dobner *et al.*, 1987). Sequence analysis of the isolated cDNAs revealed an open reading frame encoding a 170 aa protein. This precursor protein not only encoded the sequence for the 13 aa neurotensin but also the 6 aa neurotensin-like peptide known as neuromedin N. There is also another neuromedin N-like sequence encoded within the gene. In fact, there is the possibility that a number of undefined biologically active peptides may be present within this precursor protein. The amino terminal portion of the

protein contains aa residues typical of signal peptides that precede most secreted proteins.

Subsequently the bovine cDNA was isolated, revealing a 95% sequence identity to the cloned rat cDNA, (Dobner *et al.*, 1992). Furthermore, genomic sequences were obtained for the rat and human neurotensin gene, (Kislauskis *et al.*, 1988). The identity at the aa level was 73% in all four species. The NT/NM gene spans 10.2 kb of rat DNA and the coding sequence is divided into 4 exons as revealed by the rat genomic sequence. Exon 1 encodes the putative signal peptide sequence, exon 4 encodes the NT and NM coding sequence with exons 2 and 3 encoding the remainder of the precursor protein. The fact that exon 4 contains the coding domain for NT and NM precludes the possibility that these peptides are differentially regulated at the level of alternative mRNA splicing. Although, 2 major RNA species are produced by this gene, approximately 1.0 kb and 1.5 kb in length, they are the result of utilising 2 alternative consensus poly (A) addition signals as revealed by northern blot experiments. The different mRNA species are present at equal concentrations throughout the NT expressing brain areas. However, the 1.0 kb species was predominant in the gastrointestinal tract. Not all species displayed this pattern of expression. The bovine hypothalamus tissue exclusively expressed the 1.5 kb mRNA whereas, the canine intestine only expressed the 1.0 kb mRNA form, (Dobner *et al.*, 1987).

Further characterisation of the neurotensin/ neuromedin N gene was undertaken at the promoter level. Mutation and expression studies of the putative NT/NM gene promoter has defined a number *cis* regulatory elements that can influence the gene expression in PC12 cells, (Kislauskis *et al.*, 1990). The rat NT/NM promoter would appear to consist of a number of *cis*-regulatory elements defined within sequences from -216 to +56, that are responsible for the complete synergistic regulation of the gene. These elements include a consensus cAMP response element (CRE) site, two AP-1 like sites, a glucocorticoid response element (GRE) related sequence and a sequence identical to the human c-*jun* site (JARE). Nerve growth factor acts cooperatively with glucocorticoids and adenylate cyclase to increase NT/NM gene expression in PC12 cells, (Dobner *et al.*, 1992). This induction of gene expression requires the simultaneous use of inducers in combination. Individual exposure of PC12 cells to inducers has no or little effect on NT/NM gene expression. Lithium is an inducer molecule that can potentiate the expression of NT/N in combination with other inducers, (Dobner *et al.*, 1988).

This is particularly interesting since lithium is used in the treatment of manic depression, yet the mechanism of its physiological action is not understood. Sequence alterations of the CRE or the GRE elements results in the selective reduction of gene expression. However, mutational analysis of the AP-1 site severely reduces gene expression in response to all combinations of inducer molecules, (Dobner *et al.*, 1992). Therefore, the action of the various AP-1 transactivator complexes would appear to play a crucial role in regulating the synergistic actions of the various stimuli that influence the expression of the NT/NM gene expression. More recently Harrison *et al.*, 1995, have shown by transient transfection of c-Jun and c-Fos expressing plasmids in PC12 cells that the various activator complexes potently activate the NT/NM promoter in a cooperative manner by an average of 50 to 100-fold.

The previous studies have all analysed the expression of the NT/NM gene expression in PC12 cells. However, there is accumulating evidence that regulation of the NT/NM gene in the CNS may be governed by similar factors. The administration of pertussis toxin to striatal neurons, which increases the levels of adenylate cyclase, results in an increased expression of NT/NM in striatal neurons, (Augood *et al.*, 1992). This suggests that cAMP may also up regulate the NT/NM gene. Expression of the NT/NM mRNA has been observed to be at high levels in the striatum in the new born rat, (Kiyama *et al.,* 1992). This expression declines after postnatal day 10 which coincides with a drop in c-*jun* expression, (Pennypacker *et al.*, 1993). A more direct link between the expression of c-*fos* and NT mRNA on the administration of the neuroleptic haloperidol, has been established. Both genes show a direct increase in mRNA levels in the striatum as a result of haloperidol administration. However, this response can be attenuated by antisense oligonucleotides directed against the c-*fos* mRNA. The subsequent decline in c-*fos* gene expression is paralleled by a reduction in NT mRNA, (Robertson *et al.*, 1995).

1.3.2 Introduction to the super family of G-protein linked receptors

Communication between almost all cells in an organism is mediated by extracellular signals such as neurotransmitters, neuromodulators, hormones etc. These signals are transduced to the interior of the cell via diffferent classes of receptor. The vast majority of receptors belong to the super family of G-protein coupled receptors. On binding their native ligand these receptors

undergo a conformational change resulting in the activation of distinct G-proteins that are attached to the surface of the plasma membrane. Ligand binding catalyses the exchange of guanosine 5'-triphosphate (GTP) to from guanosine 5'-diphosphate (GDP) on the G-protein causing it to dissociate from the G-protein receptor complex. The released G-protein subunits, the a subunit bearing GTP and the βγ dimer, can then act on effector systems that produce an intracellular signal within the cell such as cAMP, cyclic guanosine 5'-monophosphatec (GMP) calcium and inositol phosphates. In this way G-protein coupled receptors not only transmit the signal, but also amplify and integrate other extracellular signals to coordinate the cellular response.

1.3.3 Molecular structure of G-protein linked receptors

A large number (> 200) G-protein coupled receptors have been cloned and share a large degree of structural homology. They are characterised by 7 transmembrane domains, predicted to form a helices, connected by altering extracellular and intracellular loops. Figure 1.3 depicts the 2-dimensional topology of the NTR high affinity receptor, Le *et al.*, 1997. There are currently no high resolution X-ray crystallography images to accurately predict the structure of G-protein coupled receptors. However, a number of G-protein coupled receptor models have been proposed based on the structure of the non-G-protein coupled receptor bacterorhadopsin, (Henderson *et al.*, 1990), and to the rhadopsin receptor model, proposed by Findlay and Pappin, 1986. Theses models predict that all G-protein linked receptors form a transmembrane helices that are arranged in a ring like fashion to form a tightly packed helical bundle.

The G-protein coupled superfamily of receptors have been classified into 3 groups. The rhadopsin like group which includes NTR and the majority of G-protein coupled receptors ; secondly the glucagon like group which includes the receptor for glucagon, secretin, calcitonin, growth hormone-releasing hormone and thirdly the group of metabotropic glutamate and calcium sensor receptors. There is no significant homology between these sub-families and relatively little is known about the 2nd and 3rd groups. The primary amino acid sequence identity within the rhadopsin G-protein coupled receptor family is observed over the transmembrane domains. The receptors can be classified by the sequence homology between the transmembrane domains. For species homologues of a receptor this can be in the range of 80-90%: to 60-80% for subtypes of the same receptor to 35-45% for unrelated

receptors, Strader *et al.*, 1994. A similar trend has been predicted for species homologues of the NT receptor see Chapter 3 Figure 3.11.

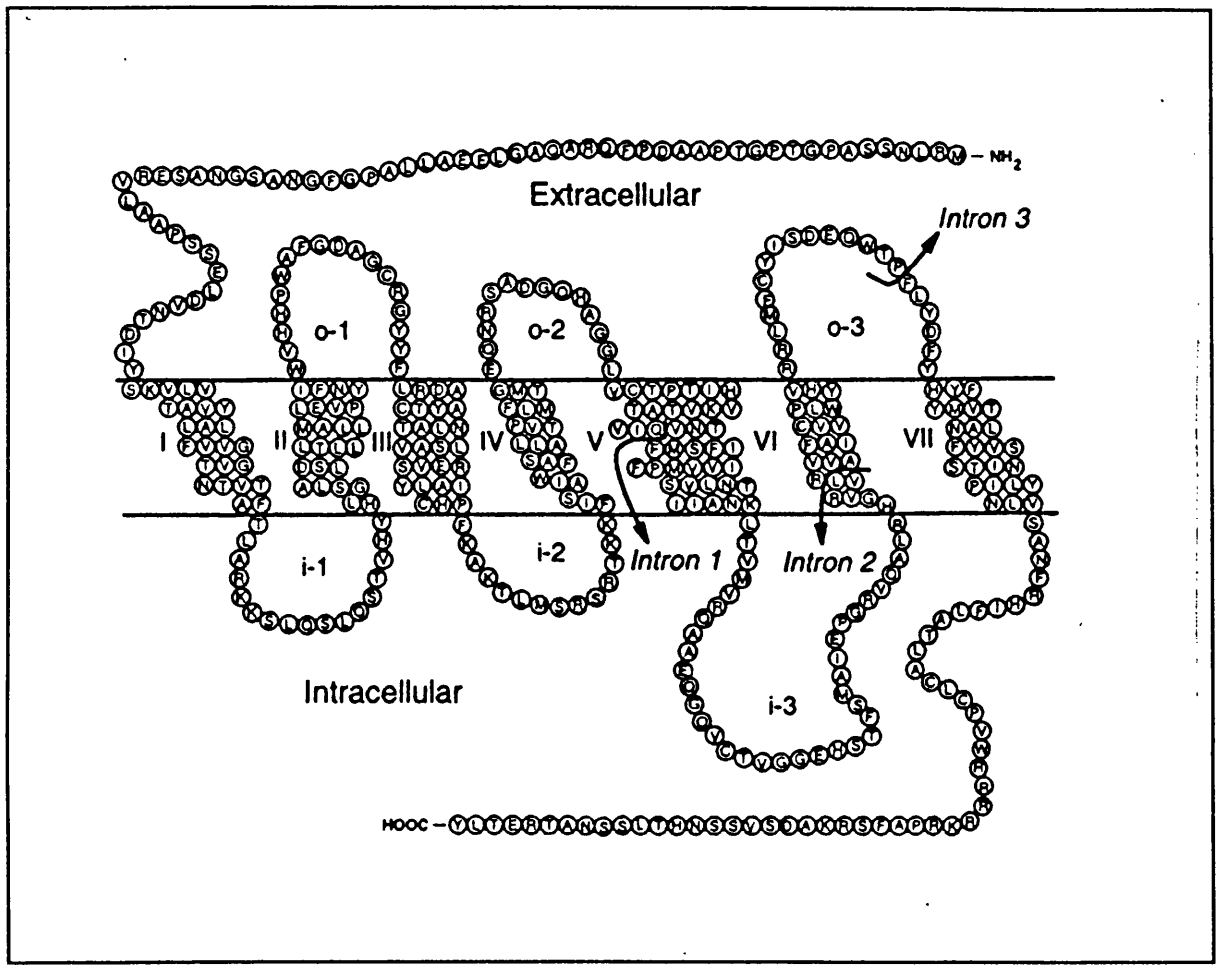


Figure 1.3 The primary structure of the NTR with the proposed topology of the seven transmembrane domains. The amino acid sequence was derived from the the human NTR gene. The sites at which the genomic coding sequences are interupted by introns are indicated by arrows at the relevant amino acid sites. i-1, i-2 and i-3 are intracellular loops 1, 2 and 3 respectively; o-1, o-2 and o-3 are extracellular loops, 2 and 3 respectively, the figure was adapted from Le *et al.*, 1997.

1.3.4 Structure function relationships of G-protein linked receptors

A number of mutational type studies have been performed to delineate functional domains of G-protein linked receptors. Some investigators have taken a chimaeric receptor approach, whereby various domains of one receptor have been interchanged with another receptor, or even whole domains deleted to dissect functional domains. Alternatively, individual amino acid residues can be mutated to assess their individual impact on a receptors function, reviewed by Strader *et al.*, 1994.

For receptors that bind small molecule neurotransmitters like dopamine, the binding site is within the transmembrane domains. Several studies have indicated that portions of the extracellular and intracellular loops have little or no effect on ligand binding, reviewed by Ostrowski *et al.*, 1992. In general mutational analysis has pinpointed a small number of transmembrane residues that are important for ligand binding, and tend to be conserved between homologues. In contrast, receptors that bind neuropeptide ligands have a number of extracellular residues that are also critical for ligand binding as well as transmembrane residues. The high affinity NT receptor primary binding site is proposed to consist of 8 residues within the 3rd extracellular loop, Pang *et al.*, 1996. Although, replacement of residue Asp139 or Arg143 (1st extracellular loop) and Arg327-Arg328 (transmembrane domain VI) abolishes binding. Apparently residues 139 and 149 are directly involved with neurotensin interaction with its receptor. Where as the mutation of Arg327-Arg328 results in a gross conformational change of the protein preventing its activation. Other residues have been reported that have a lesser impact on the receptors ability to bind NT and elicit a response. Mutation off Asp113 of transmembrane I, and Arg149 or Asp150 of transmembrane III lower the binding affinity by 2-3 fold but do not abolish function, Botto *et al.*, 1997. In general, receptors of this type have conserved amino acid residues which are critical for receptor binding and a second set of residues which are involved but to a lesser extent. Furthermore, mutational analysis of the neurokinin receptor has revealed that some residues affect the binding of one agonist type but not another. Implying that several different combinations of residues interacting with the respective ligand can bind and elicit a receptor response.

On binding the respective ligand, a conformational change of the receptor is produced resulting in a conformational change in the G-protein and its activation. The mechanism(s) by which the receptor induces a

signal to the G-protein is still unknown. It may be that the conformational change involves an opening of the intracellular receptor surface, to allow G-proteins to interact with amino acid residues that could not previously be accessed. This view is supported by studies on the a factor pheromone receptor of yeast. The intracellular domains of the receptor become more sensitive to the action of trypsin cleavage when the receptor is activated, Bukusogulu and Jenness, 1996.

Many studies have also implicated transmembrane loops V and VI to be critical in the mechanism of conformational change. Receptor proteins have been produced that bind the appropriate ligand but do not activate the receptor. Such defective receptors can be produced by disulphide cross-linking of transmembrane domains VI and III. These results support the view that relative movement between the two domains is essential to produce a conformational change and thus activate the receptor. Conversely, rhodopsin constitutively active mutant receptors have been isolated that implicate transmembranes VII and III, (Robinson *et al.*, 1992). It has been suggested that a salt bridge between these transmembrane domains holds the receptor in an inactive state. Further studies have begun to unravel domains that are apparently critical for the coupling and selectivity of the receptor to its G-protein. In general residues within the 1st and 3rd intracellular loops and to a lesser extent the 4th intracellular loop, Dohlman *et al.*, 1991. Further evidence for the G-protein/receptor interface derives from a series of experiments using short synthetic peptides that mimic the receptor. The synthetic peptides derived from he 1st and 3rd intracellular loops can mimic or inhibit receptor interaction with G-proteins, Okamoto and Nishimoto, 1992. This is further evidence that a conformational change of the receptor reveals amino acid residues that were not previously exposed to the G-protein. Further studies have defined conserved residues that are critical to the receptor G-protein interaction, such as the charged residues of the intracellular loop 3 at the COOH-terminal domain and the Asp-Arg pairing commonly found at the junction of transmembrane III and intracellular loop 2. In a similar manner a limited number of primarily hydrophobic residues at the NH_2 terminus of intracellular loop 3 are crucial for G-protein recognition, Hill-Eubanks *et al.*, 1996. In addition residues of intracellular loop 2 have been implicated, Blin *et al.*, 1995.

In summary the majority of receptor function studies suggest that much of the cytoplasmic facing surface of the receptor is involved in G-protein interaction. Inparticular, residues at the cytoplasmic loops close

to the plasma membrane and the residues at the ends of all the transmembrane domains are implicated in G-protein /receptor interaction and specificity. The extracellular domains specify a receptors ligand specificity and transduce this signal to the G-protein by a conformational change that permits the necessary intracellular residues to interact. Although G-protein linked receptors display a common architecture it could have been thought that a receptors primary sequence would be sufficient to predict the critical residues for its action, however, further conformational studies are required to define functionality.

1.3.5 The neurotensin receptor genes

A functional clone of the rat neurotensin receptor cDNA was first isolated using an electrophysiological assay in Xenopus oocytes, (Tanaka *et al.*, 1990). Sib-selection from a pool of cDNAs, eliciting an electrophysiological response to the application of NT, resulted in the isolation of a single 3.6 kb cDNA encoding the NTR. Sequence comparisons revealed that the cDNA encoded an open reading frame for a 424 amino acid protein that belonged to the G-protein coupled superfamily of receptors with seven transmembrane domains. Subsequently a human NTR was cloned from a cDNA expression library derived from the HT29 cell line, (Vita *et al.*, 1993). The human cDNA encodes a 418 aa receptor with 84% sequence identity to the cloned rat cDNA and 92% homology at the amino acid level. These receptors exhibit similar binding profiles and affinities for NT and its analogues. These data suggest that these receptors are species variants of the same receptor. Furthermore, binding studies and biological assays using NT and its analogues in a variety of tissue types display similar profiles for these cloned receptors. Until recently there was no molecular data for the existence of NTR subtypes, a number of pharmacological and biochemical reports indicated the existence of other receptor subtypes; these data will be presented in the next section. Interestingly, Mazella *et al.*, have recently cloned the first low affinity neurotensin receptor from mouse brain, (Mazella *et al.*, 1996). The 417 amino acid peptide is 39% homologous to the cloned rat receptor of neurotensin. It displays a distinct pharmacological profile similar to that presented, in the following section, for the proposed levocabastine sensitive low affinity NTR.

1.3.6 Evidence for NT receptor subtypes

The theoretical molecular mass of the rat and human receptors is approximately 47 kDa. However, a report on the photoaffinity labelling of mouse NTRs on neuronal cells indicates the presence 3 different binding sites for NT, (Charby *et al.*, 1993). The authors reported 50 kDa, 60 kDa and 100 kDa receptor species that were able to bind and internalise NT. However, treatment with phenylarsine oxide, an internalisation blocker, results in the 100 kDa protein no longer being detectable on cell membranes. Furthermore, the 100 kDa species can not be detected in the absence of NT. This apparent 100 kDa receptor has been purified from mouse, (Mazella *et al.*, 1989), and human brains, (Zsürger *et al.*, 1994). Binding studies using NT analogues reported the affinity profile of the 100 kDa protein to be indistinguishable from the cloned receptor. Therefore this 100 kDa protein may be the result of dimerisation of the authentic NTR on binding to NT.

Levocabastine, the histamine antagonist, has been used to classify the NTR into two binding sites; site 1 and site 2, (high affinity and low affinity respectively). Sites 2 are sensitive to levacobastine and will not bind NT in its presence, whereas sites 1 are unaltered by the presence of the antagonist. Sites 1 also display a greater affinity for NT, (Kd = 0.13 nM), as opposed to sites 2, (Kd = 2.4 nM). Sites 1 display a lower binding capacity for NT, (66 fmol/mg), compared to sites 2, (160 fmol/mg), as reported by Mazella *et al.*, 1983. It has also been observed that GTP increases the binding capacity of the high affinity sites but not the low affinity sites. The non-peptide antagonist of NT, SR48692, has a higher affinity for site 1 than site 2 receptors, (Gully *et al.*, 1993). Furthermore, a number of studies have reported a greater proportion of the high affinity receptors in new-born rat and mouse brains than adult, (Schotte and Laduron, 1987). The high affinity receptor was reported at a 10-fold higher density in the adult human brain than the new-born, (Zsürger *et al.*, 1992).

Evidence for NT receptor heterogeneity has been suggested by a number of electrophysiological studies. Behbehani *et al.*, 1984, have observed two types of excitatory response elicited by NT action on the periaqueductal gray, (PAG), brain structure. The slow and fast acting responses are believed to be mediated by two receptor subtypes. Similarly the effects of NT on the evoked and spontaneous release of DA from cat striatal slices was suggestive of the existence of two types of NT receptor, (Markstein and Emson, 1988).

Perhaps the most convincing evidence for the existence of two receptor subtypes is provided by studies using the SR48692 NT antagonist. Dubuc *et al.*, 1994, demonstrated that this antagonist could not attenuate the antinociceptive actions of NT administered into the PAG brain region. Furthermore, the same investigators showed that the hypothermia induced by centrally administered NT could not be suppressed by the use of the SR48692 NT antagonist. This was in contrast to the antagonism produced by SR48692 of the hypokinetic effect normally observed on central administration of NT, (Gully *et al.*, 1993). Therefore it may be that the neurotensin induced analgesia and hypothermia are mediated through a NT receptor subtype that is distinct from the receptor mediating the DA response. It has been reported that SR48692 did not antagonise the increase of DA metabolism in the VTA after NT injection, (Steinberg *et al.*, 1994). This was surprising in light of SR48692 ability to antagonise the potassium evoked DA release from striatal slices produced by NT, (Gully *et al.*, 1993). These type of studies have led investigators to propose the existence of at least two types of NT receptor.

1.3.7 Signal transduction mechanisms of the NT receptor

A number of signal transduction mechanisms have been implicated in mediating the intracellular effects of NT binding to its receptor. Various cell lines, both neuronal and non neuronal that express NTR, have been used to demonstrate alterations in the cellular content of inositol phosphates, cyclic AMP, cyclic GMP and calcium produced by the action of NT, (reviewed by Vincent, 1995). Studies involving rat brain slices have demonstrated an increase of inositol phospholipid metabolism and calcium mobilisation on NT binding to many different cell types, (e.g. Sato *et al.*, 1991). Deletion studies have located the functional active domain of the receptor for inositol phospholipid metabolism to the third intracellular loop, (Yamada *et al.*, 1994). The effect on intracellular cGMP levels depends upon the cell type under investigation. Rat brain slices and the N1E-155 cell line show an increase in cGMP, (Pugsley *et al.*, 1995), whereas fibroblast cells transfected with the rat NTR cDNA show no change. The most confusing results concern the actions of NT relative to cellular cAMP levels. The N1E-155 neuroblastoma cell line exhibits an inhibition of cAMP formation on binding NT, (Bouzou *et al.*, 1989). However, in the case of the CHO cell line, NT stimulates an increase in cAMP formation, (Yamada *et al.*, 1993). The vast majority of these studies have been performed *in vitro*. Therefore, the association of the NTR with any

of these secondary messenger systems still has to be accurately established as functional mechanisms of transmembrane signalling in neurons.

1.3.8 Retrograde axonal transport of NT

Another mechanism of cellular communication is by the internalisation of NT on binding to its receptor and the subsequent axonal transport to the cell body, where after a time it becomes predominantly associated with the nucleus, (Chabry *et al.*, 1993). Furthermore, this retrograde transport of NT has been demonstrated within the nigrostriatal dopaminergic neurons of the rat brain, (Catsel *et al.*, 1994). Iodinated NT was injected into the striatum, where NT receptors are located presynaptically on dopaminergic neurons. Two hours after injection the iodinated NT started to accumulate in the neuronal cell bodies of the substantia nigra pars compacta. After four hours the labelled NT was predominantly associated with the nucleus. Similarly, the binding, internalisation and perinuclear association of NT was demonstrated in the cholinergic neurons of the rat basal forebrain, (Faure *et al.*, 1995). One proposed explanation for the internalisation of NT was that it is part of the mechanism for down regulation of the NT receptor. Indeed, rapid disappearance of the receptor was observed from rat embryonic neurons in culture on binding NT, (Vanisberg *et al.*, 1991). However, this does not account for the axonal retrograde transport of the neurotensin/receptor complex to the nucleus. Castel *et al.*, 1994, have observed a 39% increase in tyrosine hydroxylase mRNA expression in dopaminergic cells that exhibit retrograde axonal transport of NT. The same investigators reported that the increase of mRNA expression coincided with the arrival of labelled NT at the nucleus 4 hours after injection. It has also been observed that chronic treatment of rats with the NT antagonist SR48692 decreases the tyrosine hydroxylase mRNA expression by 40%, (Laduron, 1995). Therefore, this mechanism may be a means by which NT can exert long lasting effects over neurons by influencing gene expression. Indeed, the presynaptic action of NT on the nigrostriatal neurons may be a mechanism by which NT acts as a feedback signal on these neuron cell bodies to increase the production and replemish the stores of DA. NT action on the presynaptic dopaminergic terminals is to stimulate the release of DA, but at the same time NT, via retrograde axonal, may increase the biosynthesis of TH which in turn increases the production of DA. In support of this hypothesis the NT receptor is believed to encode a sequence with

similarity to a nuclear localisation signal, (Laduron, 1995). Whether NT or part of the NT/NTR complex interacts specifically at the genomic level or via another messenger molecule is not known.

Castel *et al.*, 1994, also reported that the quantity of retrogradely transported NT was reduced in aged rats. The rate at which NT was transported from the striatum to the SNc remained unchanged only the quantity diminished. This result may be due to fewer numbers of NTR in aged rats. Govoni *et al.*, 1980 have documented a 20% reduction in NTRs of older rats. Nevertheless, the down regulation of this signalling system may have important consequences in ageing or in the pathology of degenerative diseases such as Parkinson's or in the pathology of illnesses such as schizophrenia, (discussed in next section). Lastly, mutational analysis of the rat cDNA receptor and expression in COS7 cells revealed that the carboxy terminus is critical to the ability of the receptor to internalise. Point mutation of aa residues Thr-422 and Tyr-424 almost completely abolished receptor internalisation.

1.4 Neurotensin as an endogenous neuroleptic

There is a considerable amount of data accumulating that implicates NT in the pathophysiology of schizophrenia. Analysis of the NT levels in the cerebrospinal fluid of a group of 21 drug free schizophrenic patients identified a subset of 9 patients who had lower levels of NT than control patients. However, when these patients were treated with antipsychotic drugs, the 9 patients who had below normal levels of NT experienced a 5 to 8-fold increase of NT concentration in their cerebrospinal fluid. These were the first clinical data to suggest that NT may function as an endogenous neuroleptic in the CNS, (Winderlov *et al.*, 1982). The neuroleptic drugs used in the treatment of schizophrenia are DA antagonists that preferentially bind to dopamine D_2 receptors, (Nemeroff *et al.*, 1992). The beneficial effects of these drugs are thought to be mediated by the blockade of the D_2 receptors in the dopaminergic mesolimbic system. However, these drugs take weeks before they work, which implies a complex readjustment within the dopaminergic neurons at the molecular level. Furthermore, neuroleptics also antagonise D_2 receptors of the nigro-striatal pathway causing a number of detrimental side effects such as catalepsy, akathisia, dystonia and Parkinsonian like symptoms. Similarly, blockade of D_2 receptors associated with the hypothalamus results in

neuroendocrine effects such as prolactin secretion. Furthermore, due to the non specific nature of some neuroleptics, blockade of various different receptors can result in neurovegetative disturbances, (Liegeois *et al.*, 1995).

The physiological effects of centrally administered NT demonstrates a pharmacological profile that is similar to that produced by neuroleptic drugs. NT produces catalepsy, poteniates barbiturate and ethanol induced sedation, induces hypothermia, inhibits the amphetamine induced hyperactivity, decreases locomotor activity and increases DA turnover in the nucleus accumbens, (Bissette *et al.*, 1991). Furthermore, injection of NT into the nucleus accumbens antagonises amphetamine induced hyperactivity. However injection of NT into the striatum did not block this hyperactivity, (Jolicoeur *et al.*, 1993). This is further evidence that NT neuroleptic like qualities are mediated via the mesolimbic system. In addition the ability of neurotensin to cause the efflux of dopamine from the nucleus accumbens *in vivo* was similar to the effect caused by the neuroleptic haloperidol, (Blaha *et al.*, 1992). This effect may in part be mediated by the ability of NT, (at concentrations >10 nM but <300 nM), to increase the firing rate of dopaminergic neurons from the VTA, (Seutin *et al.*, 1989).

Even more compelling evidence for NT acting as an endogenous neuroleptic comes from the observation that all clinically effective antipsychotic drugs have been shown to selectively alter the neurotensin system, (Myers *et al.*, 1992). However, not all antipsychotic drugs have the same effect on the NT system. Neuroleptic drugs can be classified into two groups, namely typical and atypical. The atypical drugs display less side effects than the typical drugs. These two groups of drugs can be differentiated by their consequent action on NT expression, (Gygi *et al.*, 1994). The atypical neuroleptics only increase NT concentrations in the N. Acc., (a terminal field of the DA neurons of the mesolimbic system). The typical neuroleptics increase NT levels in the N. Acc. and the caudate nucleus of the striatum, (a terminal field of the nigrostriatal pathway). It has been postulated that the extrapyramidal side effects associated with typical neuroleptics are mediated by their action on the nigrostriatal pathway, hence the increase in NT within the caudate nucleus. The beneficial effects of atypical neuroleptics are mediated via the mesolimbic system, hence the increase of NT expression preferentially in the N. Acc. and not in the caudate nucleus.

More recently it was demonstrated that the increase in NT expression was mediated by the c-*fos* gene, (Robertson *et al.*, 1995). The expression of c-*fos* was increased within the striatum, on administration of the

neuroleptic haloperidol. Furthermore, the authors demonstrated that 80% of the cells displaying Fos-like immunoreactivity also expressed the NT mRNA. The connection between c-*fos* gene expression and the upregulation of NT was demonstrated using antisense oligonucleotides for the c-*fos* gene. These oligonucleotides bind to c-fos mRNA preventing expression which in turn attenuates NT mRNA expression. This result was somewhat expected in light of the expression studies performed on the NT/NM promoter in PC12 cells, (described in section 1.3.1).

In summary, there is growing evidence implicating NT in the pathophysiology of schizophrenia. Central administration of NT displays a profile similar to neuroleptic drugs. A subset of drug free schizophrenic patients were observed to have below normal levels of NT prior to neuroleptic treatment. All clinically effective neuroleptics increase NT levels in the dopaminergic mesolimbic system, (particularly the atypical neuroleptics). It is also interesting to observe that a rare allele of the tyrosine hydroxylase, (TH), gene is associated with the occurrence of schizophrenia, (Mallet, 1996). Two separate populations, of unrelated chronic schizophrenics, displayed the variant allele at a frequency of 5% and 9% compared to 0% within the control samples. The functional significance of this allele is unknown but, the implication is that it alters or disrupts TH gene expression in a manner that is important to the development of schizophrenia. As mentioned in the previous section, NT was demonstrated to undergo retrograde axonal transport in the nigrostriatal pathway of rats. The transported NT becomes targeted to the nucleus whereupon an increase in TH mRNA was observed. If indeed TH is a downstream target for NT, then the existence of a variant allele of the TH gene being associated with schizophrenia, is interesting in light of the evidence implicating NT transmission in the pathology of schizophrenia.

1.5 Other important functions of NT transmission

1.5.1 Neurotensin as a regulatory hormone

Neurotensin is a regulatory hormone of the gut that mediates several physiological responses and is secreted from endocrine N cells on luminal ingestion of nutrients, (Read *et al.*, 1984). Bombesin and other neurotransmitters are also capable of stimulating the secretion of neurotensin into the circulatory system from endocrine N cells, (Rokaeus, 1984). The

neuromedin N peptide which has similar properties to NT and is encoded within the same gene is also co-localised with neurotensin in the cells of the rat ileum. However, neuromedin N is not co-released on administration of the usual stimulants, (Cuber *et al.*, 1990). The exact physiological role(s) of neurotensin in the intestinal tract is uncertain but many observations of its action suggest it may coordinate the digestive processes. For example, neurotensin is released from the N-cells of the ileum upon the ingestion of fat, but glucose and amino acids have negligible effects upon the secretion of NT, (Rosell and Rokaeus, 1979). Moreover, neurotensin has been shown to stimulate (3H) oleic acid translocation across the rat small intestine, (Armstrong *et al.*, 1986). Furthermore, circulating neurotensin plays a role in the stimulation of pancreatic exocrine secretion, (Sakamoto *et al.*, 1984). In addition, neurotensin can inhibit gastric acid secretion, (Anderson *et al.*, 1980). A number of studies have shown that the infusion of neurotensin into the gastrointestinal tract increases colonic motility and defaecation, (Thor and Rosell, 1986). Trophic effects of neurotensin have been observed on the mucosa of the colon in young and aged rats, (Evers *et al.*, 1992). In young rats the increased weight/growth of the colon was associated with an increase in mucosal cellularity. However, in aged rats this growth of colon tissue was not accompanied by an increase in DNA content, suggesting the growth was the result of hypertrophy.

More recently the expression of neurotensin has been associated with 1 in 4 human colon cancers, (Evers *et al.*, 1996). Furthermore, the developmentally regulated transient expression of neurotensin has been recorded in the human and rat foetal colon, (Evers *et al.*, 1993). Interestingly, this is when the developing colon has a morphological structure similar to the small intestine. The authors suggest that the neoplastic transformation of the colon cells could be the result of the reversion of neurotensin expression to a foetal like profile, in certain types of colon cancer. Another study by Sehgal *et al.*, 1994, provides evidence for neurotensin functioning as an autocrine trophic factor in human prostate cancer. In this investigation they postulated that the expression of endocrine peptides, such as neurotensin, may be responsible for the proliferation of androgen independent prostrate cancer. Normal growth of these cancers is stimulated by androgen and does not require neurotensin expression. Therefore, hormone depletion is the normal clinical treatment for these types of cancer. However, if this treatment fails progression of the cancer is thought to be through an alternative growth regulatory pathway distinct from that mediated by androgen. Analysis of an androgen dependent

prostrate cancer cell line, registered an up regulation of neurotensin expression on withdrawal of androgen. Administration of neurotensin stimulated the growth of androgen deprived cells in culture, but did not have an affect on cells supplemented with androgen. Therefore, neurotensin may provide an alternative autocrine trophic factor for the proliferation of androgen independent prostate cancer.

1.5.2 Neurotensin and the cholinergic neurons of the basal forebrain

NT immunoreactive neurons have been shown to innervate the cholinergic neurons of the nucleus basalis of Meynert (NBM), (Zahm *et al.*, 1987). Furthermore, NTRs are located on the perikarya and dendritic terminals of the NBM cholinergic neurons, (Szigethy *et al.*, 1990). Moreover, intracerebroventricular administration of NT has been shown to produce an awakening effect and an increase in limbic theta rhythm activity, (Castel *et al.*, 1989). *In vivo* stimulation of the nucleus basalis leads to cortical arousal; therefore NT may play a role in the mechanism of cortical arousal. This brain region is also thought to be important to the learning and memory processes. These neurons provide the major cholinergic innervation of neocortex and the amygdala and are prone to degeneration in Alzheimer's disease and other dementias, (Whitehouse *et al.*, 1991). However, this degeneration is secondary to the loss of neurons from the hippocampus which provide the cholinergic neurons with nerve growth factor. Cholinergic neurons from the NBM were grown in culture and the effects of NT analysed by the patch clamp technique, (Farkas *et al.*, 1994). It was shown that NT acting via a pertussis toxin insensitive G protein produces a long lasting excitation of nucleus basalis cholinergic neurons by reducing inwardly rectifying K^+ conductance. NT is also thought to briefly excite these neurons by inducing an inward current, mediated in part by Na+ ions. Furthermore, Alonso *et al.*, 1994, demonstrated that NT promotes oscillatory bursting behaviour within the cholinergic neurons of the basal forebrain magnocellular complex. In conclusion these results provide strong evidence that NT has an excitatory neuromodulatory action on cholinergic neurons.

1.5.3 The antinociceptive effects of neurotensin

The central administration of NT and its inferred involvement in pain inhibition was first demonstrated by Clineschmidt *et al.*, 1979. Subsequently a number of behavioural and electrophysiological studies have substantiated these initial observations. Injection of NT into the periaqueductal gray (PAG) brain structure produced a dose dependent analgesia which was not blocked by the opioid antagonist naloxone. These effects of NT on PAG are mediated through the nucleus raphe magnus (NRM). This was demonstrated by the lesioning of NRM which abolished the analgesic effects of NT, (Behbehani and Pert, 1984). The same study showed that the electrophysiological effect of NT treatments on PAG neurons was excitatory, which in turn activated the pain inhibitory pathway of the NRM that innervates the spinal cord. Furthermore, studies have documented the innervation of the rostral ventral medulla (RVM), an area of the brain that contains the NRM, by neurotensinergic neurons originating from the PAG, (Beitz, 1982), and the presence of NTRs in the PAG, (Behbehani, 1992). However, the source of NT-mediated response within the PAG has not been totally identified. One possible source is from the neurotensinergic cells of the lateral hypothalamus (LH) that innervate the PAG, (Kahn *et al.*, 1980). Studies have shown that electrical stimulation of the LH neurons and chemical stimulation of PAG with exogenous NT elicited similar responses.

1.6.1 Introduction to gene targeting and embryonic stem cell technology

The ability of classical genetical research to yield information on the function of complex organs such as the mammalian CNS, becomes increasingly more difficult the more complex the organism of study. In the case of the mouse, limitations are imposed by the attainable mutation rate, and the ability to isolate mutations relevant to the particular phenotype of study. Until recently, investigators were dependent on random mutagenesis to generate 'interesting' mutations by chance. It is now possible via gene targeting and ES technology to create a mouse of any desired genotype. In principle, it is possible to take any cloned murine gene and modify it in any required manner. A mutation is first engineered into a cloned fragment of the gene of interest. The mutated gene construct is used to transfer the specific alteration to the genome of mouse by first modifying the gene of interest within ES cells by a process known as gene targeting.

ES cells are pluripotent stem cells derived from the epiblast of mouse blastocysts, Brook and Gardner 1997. Under defined culture conditions they retain their ability to colonise the somatic tissues of a host embryo to form a chimaeric mouse, following their re-introduction into host blastocysts. Frequently the introduced ES cells colonise the germ cell lineage of the chimaeric mouse. Thus allowing the modified genotype to be transmitted to subsequent generations. This totipotent potential, combined with the stable growth *in vitro* of ES cells permits the introduction of specific modifications into the murine germline. A number of cell culture selection regimes allows the relative rare event of homologous recombination between the incoming modified gene construct and the target locus to be enriched allowing easier detection.

Breeding of chimaeras produced from the modified ES cells and subsequent breeding of their progeny can result in the production of homozygote mice for the desired genetic modification. Therefore, the effect of a specific modification on the phenotype of an animal can be analysed. This back to front method of genetic analysis, from genotype to phenotype, has become known as the 'reverse genetic' approach to studying gene function. Thus reverse genetics gives the neurobiologist the tools to dissect the functional role of any particular brain molecule in a precise manner never possible by traditional pharmacological methods. Figure 1.4 overviews the general principles of gene targeting and ES cell technology.

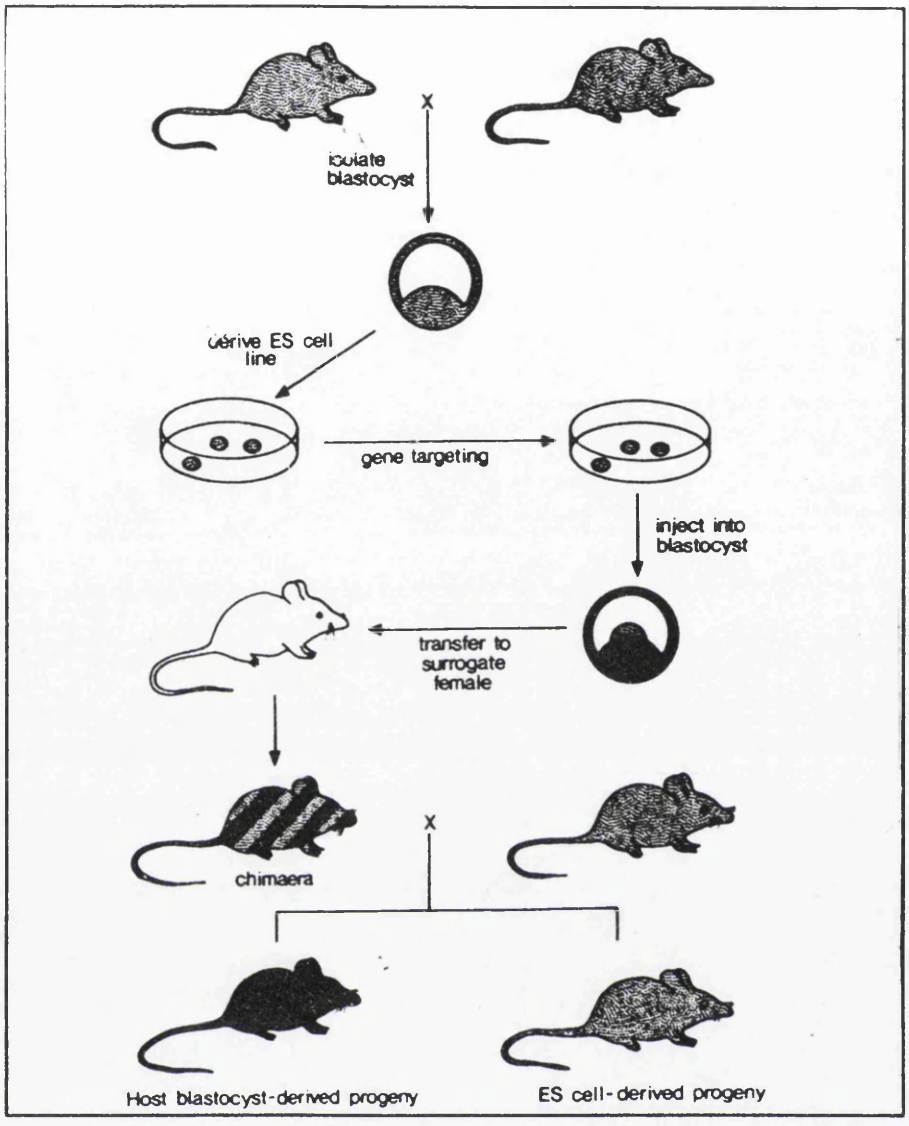


Figure 1.4 Strategy for germline modification using ES cells. Blastocysts derived from an inbred mouse strain with a light coat colour, (usually 129/sv), are used to derive ES cells. ES cells are then modified by techniques known as gene targeting to specifically alter the genome. The altered ES cells are introduced into host blastocyst of a dark coat-colour genotype. The host blastocysts are introduced into a surrogate mother where they develop to term. The resulting chimaeric offspring are derived from the 2 genotypes i.e. the ES cell and the host blastocyst, light and dark coat-colour respectively. Chimaeras are tested for germline transmission by mating to the genotype from which the ES cell line was derived, (light coat-colour). Progeny resulting from ES cell colonisation of the germline are of a light coat-colour, and half will statistically carry the specific genetic modification introduced into the ES cells. Recombinant DNA techniques are used to identify such individuals and conventional breeding used to derive mutant stocks. Adapted from Hooper 1992.

The remainder of this introduction focuses on vector design and the parameters that are known to govern the frequency of gene targeting. Only those vector designs that were available for this study will be considered within this introduction. Since the strategy presented in Chapter 4 for targeting the receptor was designed in respect of the available technology at the beginning of 1992. The second strategy of Chapter 5 was undertaken in light of the advances made in gene targeting up until the end of 1994. However, some consideration will be given to more recent advances within Chapter 9. There were essentially two basic types of vector that could be used for gene targeting; replacement vectors and insertion vectors, (Thomas and Capecchi, 1987). However variations on these vector types exist, some of which will be considered in due course. Firstly, the general features of replacement vectors and the positive negative selection system will be presented.

1.6.2 Replacement vectors and the positive negative selection strategy

The most widely used approach to creating a null mutation via gene targeting has been to create a replacement vector for the desired gene and employ the positive negative selection strategy; see Figure 1.5. A replacement vector consists of a portion of gene homology interrupted within its exonic sequence with a selectable marker, such as a neomycin cassette. This cassette typically includes promoter and polyadenylation sequences to create a functional neomycin phosphotransferase gene. The cassette, as well as being mutagenic, provides positive selection for the genomic integration of the vector into ES cell chromosomal DNA using G418 selection. In addition, one arm of the gene homology, sometimes both (Chauhan and Gottesman, 1992), is flanked by a thymidine kinase cassette derived from the herpes simplex virus. The gene replacement vector is believed to homologously integrate via 2 reciprocal crossover events between the 2 arms of homology and the endogenous gene, although integration may infact proceed through a single strand exchange within one region of homology, followed by branch migration and resolution within the second arm of homology, (Ellis and Bernstein, 1989). The result is that homologous integration of the vector does not extend to the integration of the thymidine kinase cassette. However, the mechanism of non-homologous, (random), integration is independent of homology, thus gene sequence and marker gene sequence can not be distinguished. Thus, random integration of the vector, generally results in the genomic integration of the thymidine kinase cassette. This allows ES cells that have randomly integrated the vector to be

selected against using a toxic nucleotide analogue such as gancyclovir or FIAU, (1-(2-deoxy-2-fluro-b-D-arabinofuranosyl)-5-iodouracil, which are substrates for the vector but not the cellular thymidine kinase. This system of simultaneously selecting for integration and against non-homologous integration is known as the positive negative selection strategy (PNS). The enrichment for targeted clones using this technique was originally reported to be up to 2000-fold, (Mansour *et al.*, 1988). However more typically enrichments of 2 to 20-fold are normally observed. It is also possible to use the diphtheria toxin A fragment gene for negative selection instead of the herpes thymidine kinase gene. Since the product of the diphtheria toxin A fragment gene is toxic to ES cells, then there is the added advantage of not having to select the cells with toxic nucleosides such as gancyclovir. An enrichment factor of about 10-fold was obtained when targeting the c-*fyn* locus in the original study, (Yagi *et al.*, 1990).

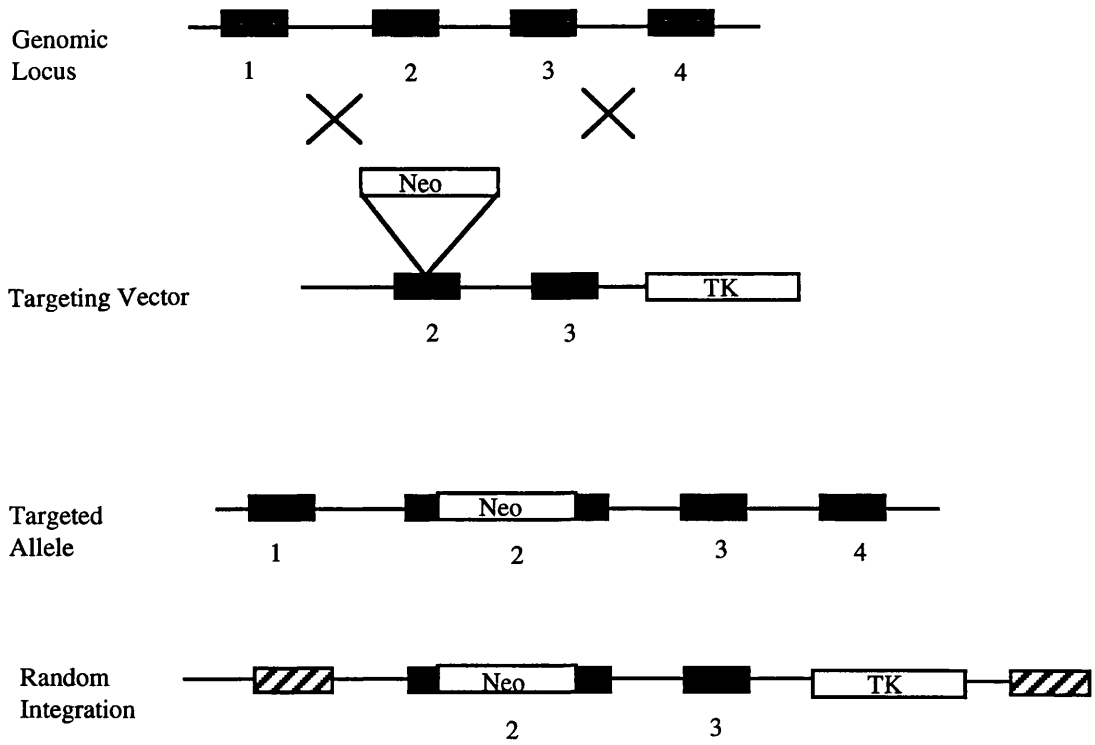


Figure 1.5 Gene targeting with a replacement vector used in the positive and negative selection strategy. The 4 exons of the gene are represented by black boxes. The thin black lines represent introns; NEO, the neomycin cassette; TK, the thymidine kinase cassette and the striped boxes represent exons of another gene. The targeting vector consists of exons 2 and 3 flanked by a thymidine kinase cassette with exon 2 interrupted by a neomycin cassette.

Homologous recombination results in the integration of the neomycin cassette within exon 2 of the endogenous gene, but the thymidine kinase cassette does not integrate. However, non-homologous recombination generally results in both selectable markers being integrated.

Enrichment for targeted clones can also be achieved by using engineered neomycin cassettes, i.e. the use of enhancerless type cassettes, (Jasin and Berg, 1988), or neomycin cassettes devoid of a polyadenylation signal, (Donehower *et al.*, 1992), or promoterless neomycin cassettes, (Jeannotte *et al.*, 1991). Such vector constructs require the chromosomal integration site to provide the necessary sequences for the creation of a functional neomycin gene and for the target gene to be transcriptionally active in ES cells. The Donehowe study reported a 50-fold increase in targeting frequency for the p53 tumour suppresser gene targeted with a neomycin gene lacking a polyadenylation signal. For a promoterless neomycin construct, approximately only 1% of random integrants will provide promoter sequences for neomycin activation. Therefore, this approach potentially provides a 100-fold enrichment for homologous integrations. The Jeannotte *et al.*, 1991 study, further demonstrated that high levels of the target gene transcription are not necessary to provide adequate levels of G418 resistance. In this case the Hox 1.3 gene RNA expression could only be detected in ES cells after PCR amplification of poly $(A)^+$ selected RNA, and not by northern blotting. However this approach can be prone to failure if the fusion protein of the target gene produces non-functional neomycin phosphotransferase. This problem can be alleviated by not relying on the translation start signal of the target gene but, incorporating a viral internal ribosomal entry site within the 5' end of the neomycin cassette, (Mountford and Smith, 1995). However it must be noted that low transcription levels at a gene locus in ES cells may not result in effective levels of drug resistance for a selectable marker. Te Riele *et al.*, 1992, observed that the use of a mutated version of the neomycin gene reduced the recovery of homologous recombinants more than the recovery of random integrants. They concluded that the expression of their neomycin gene at the retinoblastoma susceptibility gene locus is around or even below the threshold required for drug resistance.

1.6.3 Insertion type vectors

This vector is constructed from a region of gene homology with a neomycin cassette. However, unlike the replacement vector, the neomycin cassette is placed outwith the region of homology in the plasmid backbone and the vector is linearised within the region of homology prior to ES cell transfection. The result is that homologous recombination results in the complete insertion of the vector into the target locus creating a duplication of the region of homology, as presented in Figure 1.6. This disruption of a gene normally produces a null allele. However, if read through occurs at the neomycin polyadenylation site then a transcript may result that could be spliced to produce a product, functionally similar to that of the wild type gene, as demonstrated by Moens *et al.*, 1992.

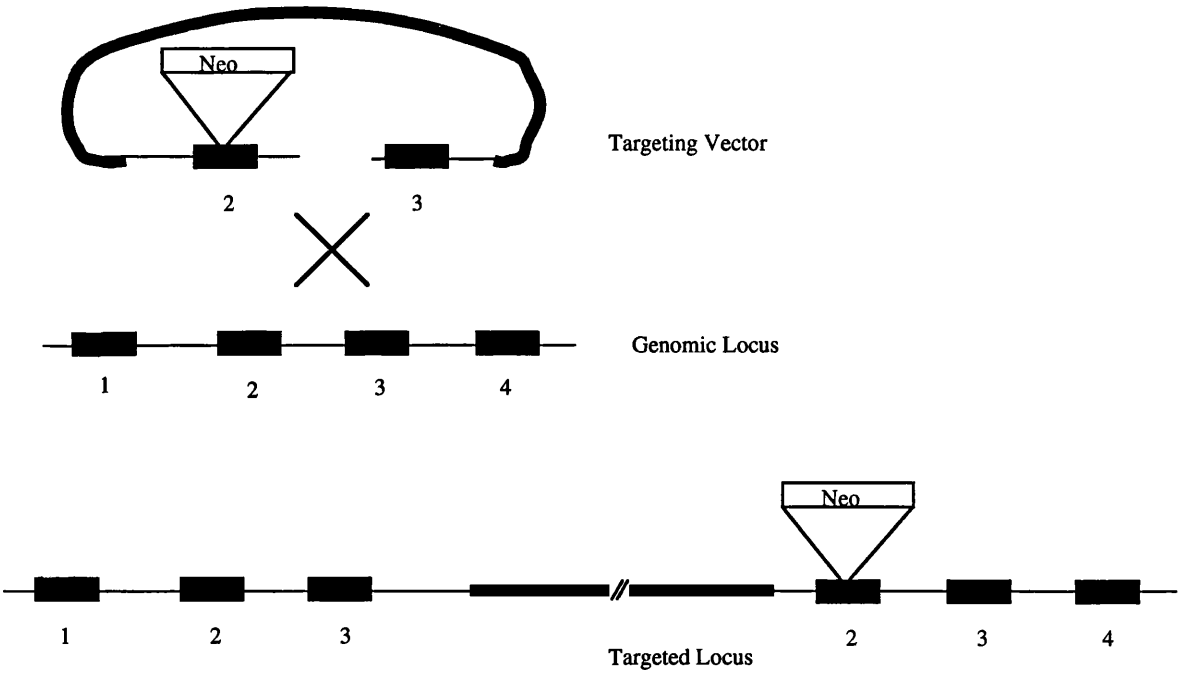


Figure 1.6 Gene targeting with an insertion type vector. The 4 exons of the gene are represented by the black boxes; introns by thin lines; and the plasmid back bone by thick black lines. The vector consists of exons 2 and 3 with a neomycin cassette, (Neo), inserted within exon 2, and is linearised within the region of homology. Homologous recombination results in the insertion of the entire vector creating a duplication of exons 2 and 3.

1.6.4 Creation of subtle mutations using insertional vectors.

The majority of gene targeting studies have been concerned with the disruption of the gene using the neomycin selectable marker stably integrated into the gene locus of interest. However, it may not be desirable to introduce enhancer/promoter elements into a gene locus as these elements may deregulate the transcription of neighbouring genes, thus producing a difficult phenotype to analyse. The introduction of specific subtle mutations can be engineered using insertional type vectors like those used in the 'Hit and Run' or 'In-Out' procedures, (Hasty *et al.*, 1991a; Valancius and Smithies, 1991). This method uses a two step recombinational approach to introduce subtle mutations within the target gene locus, see Figure 1.7. An insertion type vector is constructed with a region of gene homology bearing the desired mutation, with the neomycin and thymidine kinase selectable markers positioned within the plasmid back bone of the vector. In the first step, selection is applied for those cells that have integrated the vector by selecting for neomycin gene expression. If homologous integration occurs then a duplication of the gene homology separated by the plasmid backbone and the selectable markers will normally be created with the desired mutation present. The second step of the procedure applies negative selection against the thymidine kinase selectable marker. This can result in the excision of the vector and one copy of the duplicated sequences such that the mutation is retained within the gene locus, due to intrachromosomal recombination between the duplication. The frequency at which the mutation is retained within the genome during this reversion event depends upon the location of mutation relative to the vector linearisation site. Hasty *et al.*, 1991a, observed that 2 of the 15 colonies from the study of the *Hprt* locus contained the *Bam*HI mutation after selection. Further discussion of this strategy is presented in Chapter 4.

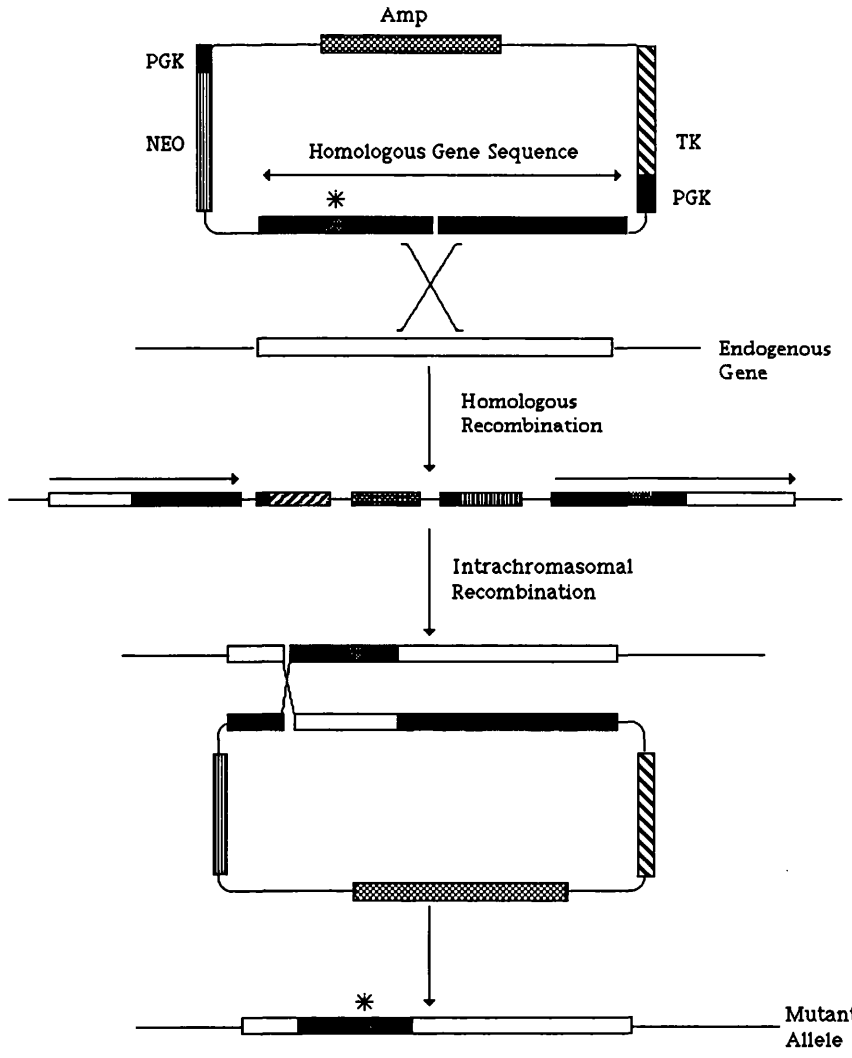


Figure 1.7 The 'Hit and Run' procedure. The diagram shows an insertional vector containing a neomycin cassette (NEO), a phosphoglyceratekinase promoter (PGK), a thymidine kinase cassette (TK) and the plasmid b-lactamase gene conferring ampicillin resistance (Amp). The mutated gene site is indicated by an *, and the open and filled boxes represent the gene homology. The method is a two step recombination process; homologous recombination of the vector at the gene locus results in a duplication that can be subsequently resolved by intrachromosomal recombination. In the example shown the reversion event has resulted in the mutated sequence being retained within the endogenous gene. Refer to the text for a more detailed explanation, (section 1.6.4).

1.6.5 Creation of subtle mutations using replacement type vectors

Replacement type vectors have been used to create subtle mutations using a gene targeting approach known as 'Tag-and-Exchange', (Askew *et al.*, 1993). This technique employs a strategy of sequential gene targeting. The first step is to tag the gene of interest with selectable markers, and secondly to replace the tagged sequence with sequence carrying the desired mutation(s). In the original study a neomycin phosphotransferase gene and the herpes simplex virus thymidine kinase gene were used as positive and negative selectable markers. These genes were fused into one cassette which was then flanked on either side with gene homology to construct a 'tagging vector'. G418 was used to select for homologous integrants which were detected at a targeting frequency of 1 in 40. The second step requires the use of an 'exchange vector'. This vector consists of gene homology with the engineered mutation(s). This vector is used to target the tagged locus by homologous recombination and negative selection against the thymidine kinase marker gene. Therefore, targeting the tagged locus results in the removal of the selection cassette and the introduction of the site specific mutation(s). This was achieved in the Askew *et al.*, 1993 study, at a targeting frequency of 1 in 54 for the second replacement event. This system provides a method of introducing subtle mutations without introducing unwanted enhancer/promoter elements into the target locus, as outlined in Figure 1.8. There is also the added advantage that once a tagged cell line has been generated, then various exchange vectors can be designed to introduce a variety of different mutations.

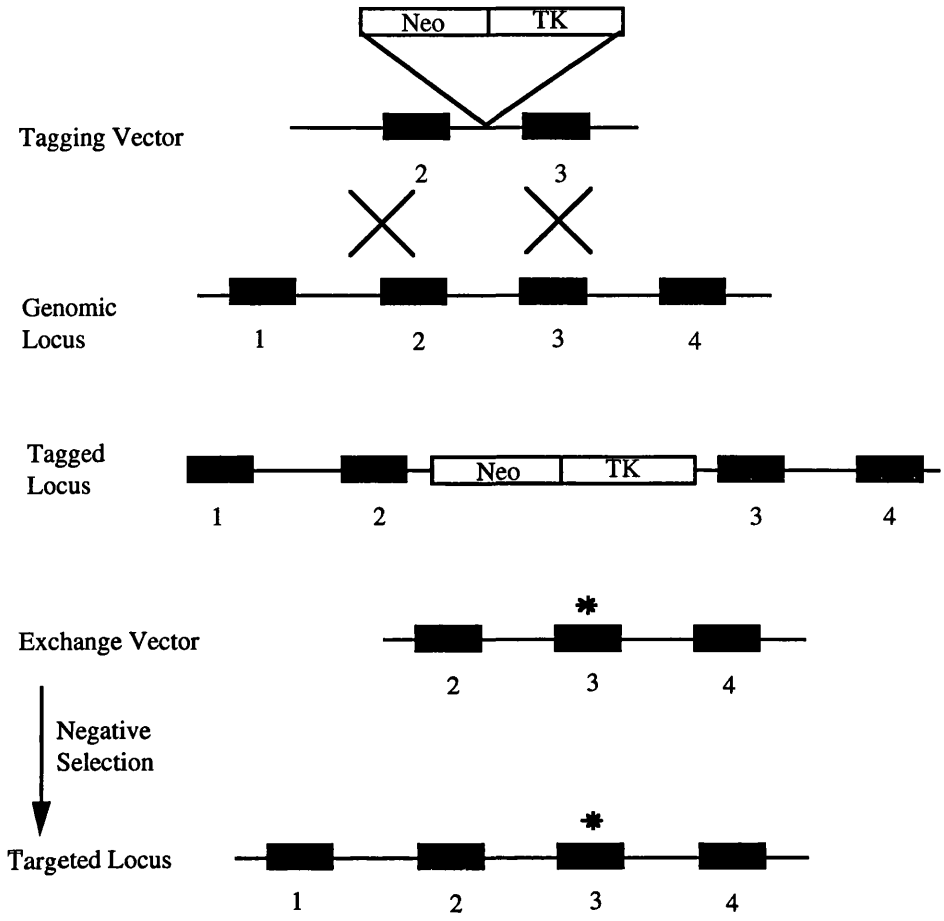


Figure 1.8 The 'Tag-and-Exchange' approach to gene targeting. The 4 exons of the gene are represented by black boxes, the intronic sequences by the thin lines. The tagging vector consists of exons 2 and 3 with the fused selection cassette, (Neo and TK), inserted between the two exons. The asterisk denotes the mutation in the exchange vector and the targeted locus.

1.7 Factors that influence gene targeting frequencies in ES cells

Early gene targeting experiments showed great variation in the number of homologous integrants versus the total number of integrations, ranging from 1 in 20 to 1 in 40,000, (Hasty *et al.*, 1991b). Several factors are known to be critical to gene targeting frequencies such as the design of the vector, overall length homology and the use of isogenic as opposed to non-isogenic DNA. Each of these factors will be considered in the following sections.

1.7.1 The effect of homology length on gene targeting frequencies

The influence the length of homology has on homologous recombination was known to be important for sometime, from studies by Rubnitz and Capecchi, 1984. They observed the rates of extrachromosomal intramolecular recombination in mammalian cells, and showed a linear increase in recombination frequency with increased length of homology from 0.25 kb to 0.5 kb. However, such studies do not address the question of vector/chromosome relationships, which may not be governed by the same criterion. The first insights were gained from the Thomas and Capecchi, 1987 study, who noticed that a 2-fold increase in homology length resulted in a 20-fold increase in homologous recombination at the *hprt* gene locus. A more detailed study by Hasty *et al.*, 1991b, demonstrated even greater increases of gene targeting frequencies at the *hprt* locus with increasing targeting homology length. 9 replacement and 3 insertion vectors with gene homology ranging from 1.3 kb to 6.8 kb were used to target the gene. They observed an increase in gene targeting frequency, compared to random vector genome integration, of greater than 190-fold for replacement vectors and 250-fold greater for insertion vectors over this size range. Furthermore, their results suggest that there is no added advantage in using vectors containing gene homologies greater than 6 kb. No increased targeting frequency accompanied vectors utilising greater than 6 kb homology, in their study. However, the relevance of targeting frequencies using such a small fragment of gene homology as 1.3 kb must be doubted especially when this homology is split in two for the construction of a replacement vector. The most informative study was performed by Deng and Capecchi, 1992. The targeting efficiency at the *hprt* locus was investigated using a total of 22 replacement and insertion vectors. They concluded that there was a strong dependency of the targeting frequency on the length of homology between the targeting vector and the target locus. The relationship between length of homology to targeting frequency between the range of 2 kb to 10 kb was exponential, in fact a 100-fold increase in targeting frequency was observed. Unlike the Hasty *et al.*, 1991b study, these authors observed increasing gene targeting frequencies with constructs using 14 kb of gene homology as opposed to 6 kb in length.

1.7.2 Isogenic and non-isogenic DNA influence on gene targeting efficiency

Observations such as the decrease in the sensitivity of the *E.coli* homologous recombination frequency due to base pair mismatches between the substrates of homologous recombination, (Shen and Huang, 1986), prompted the investigation of this parameter in ES cells. Furthermore, in mammalian cells it has been shown that base pair mismatches strongly affect the efficiency of intrachromosomal recombination (Bollag *et al.*, 1989).

The first of these studies by Te Riele ,1992, analysed gene targeting at the retinoblastoma susceptibility gene. They used 2 identical replacement vectors containing 17 kb of gene homology. However, one vector consisted of DNA isogenic to the ES cell line, derived from the 129J mouse strain. The other vector was constructed of DNA derived from the BALB/c mouse strain. They achieved a 20-fold increase in gene targeting efficiency using the isogenic DNA derived vector, in fact they reported a targeting frequency of 78%. The total extent of sequence divergence between the two 17 kb homologous fragments was not determined. However, a total of 1687 nt from each fragment was sequenced for comparison. 9 base pair substitutions, three small deletions, (1, 4 and 6 nt), and a polymorphic CA repeat, (a 14 nt deletion), were detected in the BALB/c derived sequence relative to the 129 sequence.

A similar study for the efficiency of isogenic versus non-isogenic DNA was undertaken using the creatine kinase M gene locus as a target, (van Deursen and Wieringa, 1992). This study involved the comparison of two replacement vectors consisting of 8.1 kb of gene homology derived from the 129 and BALB/c mouse strains. The isogenic construct targeted at a frequency of 12%, whereas, the non-isogenic construct produced no homologous recombinant clones for the 357 colonies analysed. The extent of sequence divergence at this locus was estimated at 2%, based upon sequencing data generated from 4 regions of the gene.

It is now well accepted within the gene targeting field that the allelic differences observed between the vector and the endogenous gene can reduce targeting frequencies. However, this information was not published when the decision to construct the insertional vector, (pKLF), used in Chapter 4 was made.

1.7.3 Vector design

There has been some confusion concerning the relative merits of using replacement vectors or insertion vectors for gene targeting with respect to their targeting frequencies. Thomas and Capecchi, 1987, reported no significant difference between insertion and replacement constructs on targeting frequencies containing gene homology of the same size. However, the Hasty *et al.*, 1991b study, claims to observe a difference, in that they suggest insertion vectors to be more efficient at targeting the *hprt* locus than the replacement vectors. A 250-fold increase of gene targeting frequency compared to random genome integrations was observed with the insertion vectors, whereas replacement vectors produced a 190 fold increase in gene targeting frequency over the range assayed. The analysis was based on 3 insertion vectors; two with homology of 1.3 kb and one with 6.8 kb, 9 replacement vectors; ranging from 1.3 kb to 6.8 kb of gene homology. Therefore, major conclusions were based largely on vectors with small amounts of gene homology. In particular, doubts exist regarding data for gene targeting frequencies of replacement vectors with less than 1 kb of gene homology in an arm of the vector, for reasons discussed later.

A second study by Hasty *et al.*, 1991c, suggests higher targeting frequencies for insertion vectors over replacement vectors. Again they target the *hprt* locus using 6.8 kb of gene homology for the vector constructions. Overall they observed a 5 to 9 fold higher targeting frequency using insertion vectors. The proposed reasons for these differences were the advantage of presenting a substrate for homologous recombination with two ends of homology by creating a double strand break within the region of homology. Secondly the gene homology of the vector is not interrupted by a selectable marker gene and apparently requires only one cross-over to recombine at the target locus. Replacement vectors may require two cross-over events to homologously integrate.

At that point in time, the summer of 1991, insertion vectors appeared to undergo homologous recombination at greater frequencies than replacement vectors, based on the above data. The 'Hit and Run' procedure which allowed for the introduction of subtle site-specific mutations was developed using insertion vectors. These apparent advantages influenced my decision to generate an insertional targeting vector for the neurotensin receptor gene, with a view to using the 'Hit and Run' method of gene targeting. However, the publication of two particular papers the following year, shed

considerable doubt on whether insertion vectors were as a general rule more efficient at gene targeting than replacement vectors.

The first of these papers, by Thomas *et al.*, 1992, analysed the frequency of gene targeting for replacement vectors. More specifically the study directed attention towards the minimum length of gene homology within the short arm of the vector required for effective gene targeting. In conclusion, the results suggested a minimum size of 1 kb gene homology should be contained within the short arm of a replacement vector. Using less than 1 kb of homology severely decreases the ability of a replacement vector to homologously integrate. These results suggest that 1 kb of homology is the minimum length the mammalian recombinational system will recognise as a substrate for homologous recombination between extrachromosomal DNA and chromosomal DNA. Moreover, unpredictable patterns of integration and rearrangement are observed at the target locus if the vector has less than 1 kb of homology at the end. Therefore, if these results can be extrapolated as a general rule, the results of Hasty *et al.*, 1991b and 1991c have to be re-evaluated. A number of the replacement vectors in these studies had short arms of homology which were less than 1 kb in the length. Furthermore it became apparent as discussed in Chapter 1, (section 1.7.2), that mismatches between vector and target gene due to non-isogenic DNA decrease the efficiency of gene targeting. Together these observations may explain the higher targeting frequencies observed with insertional vectors as opposed to replacement vectors in the Hasty *et al.*, studies.

The most comprehensive analysis of vector design and influence on gene targeting frequencies was performed by Deng and Capecchi, 1992. 13 replacement and 9 insertion vectors consisting of isogenic and non-isogenic DNA were designed against two sites of the *Hprt* gene. The major observations for all vectors (except one replacement construct) were: i) no significant difference in targeting frequencies was observed between insertion and replacement vectors; ii) the overall length of homology, between 2 and 10 kb, exhibited an exponential relationship, exhibiting a 100-fold increase in gene targeting frequencies over this range; iii) constructs of isogenic DNA target at 4 to 5-fold more efficiently than non-isogenic DNA constructs of the same length; iv) no increase in gene targeting frequencies are oberved using > 14 kb of homology. The one exception to this rule was a replacement vector constructed of non-isogenic DNA, which targeted at an approximately 10-fold lower frequency than expected. The explanation proposed was that an unfavourable distribution of heterology between the endogenous gene and the

vector severely handicapped its ability to target the locus. It is also known that a significant degree of allelic variation exists at the *hprt* locus. The results of this paper combined with the enrichment advantages gained by using the positive negative selection strategy, greatly influenced the decision to create a replacement vector as a second strategy to targeting the neurotensin receptor gene.

1.8 Screening procedures for targeted ES clones

Many elements of good vector design can be used to enhance the frequency of homologous recombinant ES cell clones e.g. the use of isogenic DNA, large portions of gene homology up to about 14 kb, positive negative selection using replacement vectors, and the use of more than 1 kb of homology the short arm of a replacement vector. Nevertheless, a screening regime is required to detect the homologous recombinants relative to the background of random integrants. The most common procedure is to screen the clones by Southern blot analysis. Typically, a restriction site within the disrupting marker provides a new restriction pattern for a targeted locus. The probe in such an analysis is generally derived from a flanking fragment of the gene exclusive of the homology used in the targeting vector, see Chapter 6 (section 6.3) for an example. However, some investigators have been known to base the screening procedure on the difference in restriction fragment size caused by the insertion of the selectable marker at the target locus, e.g. Endelman, *et al.*, 1996.

Another common approach is to use a PCR screen to detect homologous recombinants. This strategy was first outlined by Kim and Smithies 1988, and involved screening pools of colonies with a pair of PCR primers specific for the targeted event. Positive pools subsequently undergo a PCR/sib-selection protocol until individual targeted colonies can be identified. A variation of the original protocol is outlined in Chapter 4.

1.9 Future directions for increasing gene targeting frequencies in ES cells

1.9.1 Introduction

The frequency of non-homologous integration of targeting vectors is generally accepted to be 3 to 5 orders of magnitude more frequent than integration by homologous recombination. Improvements in gene targeting frequency maybe achieved by incorporating sequences, that stimulate homologous recombination, into the targeting vector. Furthermore, the identification of the enzymes that promote homologous recombination should provide methods for increasing gene targeting frequencies in ES cells.

1.9.2 Double strand breaks and homologous recombination

Current models of homologous recombination in eukaryotes, are primarily deduced from experiments in *Saccharomyces cerevisae*, and involve double strand breaks (DSBs) at the site of recombination, (Petes *et al.*, 1989). A natural example of homologous recombination being initiated by DSBs is observed at the *MAT* locus of *Saccharomyces cerevisae*. This reaction is initiated by DSBs initiated by the mating type Endonuclease HO at the *MAT* locus, (Strathern *et al.*, 1982). Therefore, by extrapolation the introduction of a DSB at a target gene should increase the frequency of homologous recombinants generated during a gene targeting experiment. This has been demonstrated by Smith *et al.*, 1995. Their study involved, placing a I-*Sce*I restriction site at the target locus. This locus was then re-targeted in the presence of transient expression of the I-*SceI* restriction enzyme. They found that the introduction of DSBs at the target locus by I-*Sce*I stimulated the gene targeting frequency about 50-fold. Those two step strategies for gene targeting that involve a second replacement or exchange event would benefit from the incorporation of a I-*Sce*I site within the first targeting vector. The second targeting event would then be more efficient due to DSBs created within the target sequence. The feasibility of this approach is supported by the observations of Brenneman *et al.*, 1996. They studied the frequency of homologous recombination on a duplicated portion of the *hprt* gene with enzymes that cut within and outwith the duplicated region of the gene. Homologous recombination was increased 10-fold for enzymes that cut within the region of homology but not outwith. Therefore in conclusion, the

introduction of DSBs at the target locus is a method by which the frequency of homologous recombination may be increased.

1.9.3 *Cis*-acting elements that influence the frequency of homologous recombination

In a number of distantly related organisms, certain *cis*-acting elements have been identified that influence the frequency of recombination. For example, the M26 sequence of *Schizosaccharomyces pombe* increases homologous recombination by introducing DSBs, (Schar *et al.*,1994). Analysis of the recombinicity of the IgH locus indicates that an element(s) within the VH-Cm intron influences the rate of recombination by 10-fold. However, the investigators were unable to define further the genetic element(s) that was specifically responsible for this influence on recombination. Nevertheless, progress is being made towards identifying *cis* like elements that may be used in the construction of more efficient gene targeting strategies. Inclusion of such elements within a targeting construct may not increase initial targeting frequencies. However, once integrated into the chromosome their benefits may be realised for two step targeting systems.

1.9.4 Possible approaches to altering gene targeting frequencies

The continuing elucidation of the eukaryotic mechanism of homologous recombination will create opportunities for the improvement of gene targeting frequencies. In particular, the enzymes which mediate this process, may be used to increase the frequency of gene targeting. One such enzyme is the product of the *Rad51* gene, (Plug *et al.*, 1996). This gene is believed to be the mouse homologue of the *E. coli RecA* gene. This enzyme has been demonstrated to play a central role in recombination both as a structural protein and as reaction catalyst, (reviewed by West, 1992). The mouse RecA homologue could possibly be used to increase gene targeting frequencies. The gene could be either transiently expressed in ES cells or its protein electroporated with the targeting vector into ES cells, which would hopefully increase the frequency of homologous integration of the targeting vector .

In addition, it may also be advantageous to introduce the targeting vector as ssDNA. The double strand break repair model, (Orrweaver and Szostak, 1983), predicts that ssDNA is essential to the homologous recombination process, as the reaction is initiated by the invasion of single

strand 3' DNA end into the region of homology of the reciprocal duplex. The ability of single stranded DNA to participate in gene targeting in mammalian cells has been investigated by Fujioka *et al.*, 1993. They targeted the *APRT* gene with ssDNA vectors and dsDNA vectors in CHO cells. Their results suggested that both types of vector target with equal efficiency. Unfortunately, the selection regime employed did not allow for comparison of random integrants to homologous integrants. Therefore, it is possible that ssDNA vectors target the locus with a greater efficiency than dsDNA vectors, since ssDNA may be more prone to cleavage and degradation by nucleases. Nonetheless, these results demonstrate the feasibility of using ssDNA vectors for gene targeting. In combination with *cis* and *trans* components of the mammalian recombination machinery they may provide methods for more efficient gene targeting procedures in ES cells. Moreover, the elucidation of the critical factors for homologous recombination may alleviate the need to use laborious selection procedures for ES cells altogether. Further development of cell transfection methods combined with more efficient gene targeting procedures, could possibly create systems whereby gene targeting would be achievable at the mouse embryo stage, therefore circumventing the need to use ES cells at all.

Chapter 2

Materials and Methods

2.1 Introduction

The chapter is divided into four main sections for convenience: general information, (2.2); Growth, Maintenance, and Storage of *E.coli* and bacteriophage λ, (2.3); DNA molecular biological techniques, (2.4); and general ES cell culture methods, (2.5).

2.2 *General Information*

2.2.1 Chemicals and biochemicals

It is not practical to list every supplier of every chemical or reagent used in this thesis. However, an attempt has been made to list all those most commonly used. Furthermore, when a particular reagent is deemed to be crucial to the success of an experiment the supplier will be indicated within the text.

Antibiotics were obtained from Sigma Chemical Co. (Poole, UK).
Bactotryptone, yeast extract and Bactotryptone (agar) were obtained from Difco (Oxford, England).
General chemicals of analytical grade, biochemicals, and organic solvents were obtained from one of the following suppliers: BDH Chemicals Ltd., (Poole UK); Formachem Ltd., (Strathaven, Scotland); FSA Laboratory Supplies, (Loughborough, UK); Koch-Light Ltd., (Haverhill, UK); Sigma Chemical Co., (Poole UK).
Oligonucleotides were synthesised on an Applied Biosystems Model 280A DNA synthesiser at the Institute of Genetics, University of Glasgow, using reagents from Cruachem, (Science Park, Glasgow).
All restriction enzymes, T4 DNA ligase, and T4 Polynucleotide kinase were obtained from BRL and Promega Corporation.
The sequencing kit used was the Sequenase™ sequencing kit (USB Biochemicals, La Jolla, Ca., USA).
Cuvettes for electroporation were supplied by Bio-Rad
Nucleon I DNA extraction kit: Scotlab, Kirkshaws Road, Coatbridge, Strathclyde, Scotland, ML5 8AD.
QIAEX Gel Extraction Kit: Qiagen Inc., 9259 Eton Avenue, Chatsworth, CA 91311, USA.
Plastic pipettes 1 ml, 5 ml, 10 ml, 25 ml, for ES cell culture, (Bibby Sterilin Ltd, Staffordshire, England.

Plastic flasks for ES cell culture: Corning Inc. Corning, NY 14831, USA.
The various constituents for ES cell medium were obtained from BRL (Gibco Ltd. Paisely, Scotland), with the exception of foetal calf serum for CGR8 and CGR8.8, (selected batches from Globerpharm Surrey).

2.2.2 Bacterial strains

Strain	Genoype	Reference/Source
***Escherichia coli* strains**		
LE392	Lsdr514(r k^-,mk^+) supE44 supF58 LacY galKZ galT22 metB1 trp55 mcrA.	Borck *et al.*, 1976.
DH5α	F^-, supE44 lacU169 (f80lacZ M15) hsdR17 (r k^-,mk^+) recA1 endA1 gyrA96 thi-1 relA1 D(lacZYA-*arg*F), λ^-	Hanahan *et al.*, 1983.
DH10B	F^- mcrA D(mcr-hsd RMS-mcrBC) f80d lacZDM15 Dlac X74 deo RecA1 end A1 ara D139 D (ara, leu) 7697 gal U gal K λ^- rpsh hupG	Bio-Rad

2.2.3 Plasmid and bacteriophage vectors

Plasmid	Description	Reference/Source
pBluescript™ II SK+/-	Cloning vectors	Mead *et al.*, 1985 Stratagene USA
pGem 3, 5Zf(+), +11Zf(-), 9Zf(-)	Cloning vectors	Promega Corporation
prNTR2	The rat NTR cDNA cloned into pBluescript II KS(+).	Tanaka, *et al.*, 1990
p2.8B	A 2.8 kb *Bam*HI fragment of the NTR gene subcloned from the λ ced1 clone into pBluescript II SK-.	Chapter 3
p4.5B	A 4.5 kb *Bam*HI fragment of the NTR gene subcloned from the λ ced5 clone into pBluescript II SK-.	Chapter 3
pNT	Insertion vector used for the 'Hit and Run' procedure. It contains a thymidine kinase and a neomycin cassettes.	Hasty *et al.*, 1991a
pKLF	A mutated 2.8 kb *Bam*HI fragment of the NTR gene inserted into pNT.	Chapter 4
pNeo	A neomycin cassette (*Xho*I/*Xba*I fragment from pNT) cloned into the *Hinc*II site of pBS™ II SK-.	Chapter 5

pTK	A thymidine kinase cassette, (*Hin*dIII /*Eco*RI fragment from pNT), cloned into pGem 9Zf(-) plasmid.	Chapter 5
p9.2EiG7	A 9.2 kb *Eco*RI fragment of NTR gene subcloned from the l T12 clone into the pGem 7Zf(-) plasmid.	Chapter 5
p4.8EiG7	A 4.8 kb *Eco*RI fragment of NTR gene subcloned from the l T12 clone into the pGem 7Zf(-) plasmid.	Chapter 5
p9.2EiL	A deletion of the 1.5 kb *Nco*I fragment from p9.2EiG7 plasmid and insertion of a *Sal*I linker.	Chapter 5
p9.2EiNeo	A neomycin cassette from pNeo subcloned into the *Sal*I linker site of the p9.2EiL plasmid.	Chapter 5
p9.2EiNeoTK	The *Eco*RI fragment of p9.2EiNeo subcloned into the *Eco*RI site of pTK to generate a replacement vector.	Chapter 5
pX650	A 650 bp *Xho*I 5' fragment of the NTR gene, subcloned into the pBS SK II$^-$.	Chapter 5
p9.2EiNeoC	The *Eco*RI fragment of p9.2EiNeo subcloned into the pTK plasmid to generate a control replacement type vector.	Chapter 5

Bacteriophage	Description	Reference/Source
λ DASH mouse genomic library	Genomic DNA derived from E14 ES cells	Dr. J. Rossant
λ EMBL3 Mouse genomic library	Genomic DNA derived from adult liver of the Balb/c mouse line. Average insert size of 15 kb.	Clontech laboratories Inc.

2.3 Growth, maintenance and storage of *E.coli* and bacteriophage λ

2.3.1 Media for propagation of *E.coli*

The most common suppliers for the following media were BDH Chemicals Ltd.; Sigma Chemical Co. Ltd. and Difco Laboratories, (Detroit, Michigan, USA).

L-broth
1% (w/v) tryptone, 0·5% (w/v) yeast extract, 0·5% (w/v) NaCl, 0·1% (w/v) glucose, 0·002% (w/v) thymine, made up in distilled water and adjusted to pH 7·0 with NaOH.

L-agar
As L-broth, but without glucose and with the addition of 1·5% (w/v) bacto-agar.

2X YT medium
1·6% (w/v) bacto-tryptone, 1% (w/v) bacto-yeast extract, 0·5% (w/v) NaCl, made up in distilled water and adjusted to pH 7·0 with NaOH.

SOC medium
2% bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl, 10 mM $MgSO_4$, 20 mM glucose

Top agar
As L-agar, but with only 1% (w/v) bacto agar.

Top agarose
As L-Agar, but with 0·65% agarose.

NZCYM medium
To 950 ml of dH_2O add 10 g of NZ amine, 5 g of NaCl, 1 g casamino acids, 2 g $MgSO_4 \cdot 7H_2O$, adjust to pH 7.0 with 5 M NaOH and finally adjust volume to 1 litre with dH_2O.

2.3.2 Growth of *E.coli*

Liquid cultures of *E. coli* strains from which plasmids were to be isolated were grown in L broth with the appropriate antibiotic selection, (typically ampicillin at 100 mg/ml). For the preparation of electro-competent cells, liquid cultures of *E. coli* DH5a or DH10B were grown in L-broth with no antibiotic selection. The volume of broth inoculated depended on the quantity of plasmid required. Routinely, 3 ml and 100 ml cultures were used for small and large scale plasmid preparations, (sections 2.3.8 and 2.3.9 respectively). All cultures were incubated at 37°C in an orbital shaker at 250 rpm.

2.3.3 Storage of *E.coli*

E.coli strains were stored in glycerol at -70°C. A 1 ml aliquot of an overnight culture was mixed with an equal volume of 100% glycerol, and snap frozen in liquid N_2. The strains were revived by scraping the surface of the frozen suspension with a sterile inoculating loop and streaking onto a L-agar plate to isolate single colonies.

2.3.4 Antibiotic selection and indicators

When necessary ampicillin, the only antibiotic used for bacterial work in this study, was used at a final concentration of 100 μg/ml, (the stock was stored at -20°C at a concentration of 20 mg/ml in sterile water). Where possible the selection of recombinant clones was aided by the use of X-gal and IPTG to indicate the disruption of the *lac*Z gene present in many of the plasmids used in this study. Typically when the *lac*Z gene is disrupted the resulting recombinant clones appear as white colonies as opposed to the blue non recombinant colonies. X-gal was dissolved in dimethylformamide and IPTG was dissolved in sterile water. Both solutions were stored -20°C at a stock concentration of 20 mg/ml and used at a final concentration of 20 μg/ml.

2.3.5 Sterilisation

All growth media and glassware were sterilised by autoclaving at 121°C for 15 minutes. Buffer solutions were autoclaved at 108°C for 8 minutes. Plastic ware was autoclaved at 121°C for 9 minutes. Heat sensitive reagents were sterilised by filtration using membrane filters of 0.45 μm pore size, (Millipore).

2.3.6 Preparation of electro-competent *E.coli*

The preparation of these cells was essentially as described by Dower D. J.. (Bio-Rad Laboratories), with the exception of centrifuge times being reduced to 7 minutes. Furthermore, all plasticware was washed thoroughly by steeping in sterile distilled water overnight to remove any traces of detergent and pre-chilled on ice prior to use. The *E.coli* strains DH5α and latterly DH10B were used for this procedure.

2.3.7 Transformation of *E.coli* by electroporation

This was performed according to the manufacturers instructions using a Bio-Rad Gene Pulser® with the following alterations. The electroporation cuvette size used was 0.1 cm, (Bio-Rad), and the apparatus was set at 25 μF capacitance, 1800 V and the pulse controller unit set at 200 Ω. If SOC

medium was not available for the re-suspension of cells then 2X YT medium was used supplemented with glucose to a final concentration of 20 mM.

2.3.8 Small scale plasmid preparation

Small plasmid preparations or 'mini-preps' were performed according to the 'Wizard mini-prep' procedure, (Promega). The procedure is similar to the alkaline lysis method, (Sambrook *et al.,* 1989). The primary difference is that no phenol-chloroform or ethanol precipitation steps are required. Instead the 'Wizard mini-prep' procedure employs a silica-based binder to selectively remove the plasmid DNA from solution as an alternative. The solutions used were that of the alkaline lysis method i.e. solutions BDI, BDII, and BDIII, (section 2.4.1). The yields for a high copy number plasmid were typically 3-5 μg per ml of culture.

2.3.9 Large scale plasmid preparation

Large plasmid preparations were performed according to the 'Wizard midi-prep' procedure, (Promega). This is similar to the procedure outlined above; however, volumes of 100-200 ml of bacterial culture can be processed as opposed to 3 ml.

2.3.10 'One Step' plasmid preparation

1 ml of culture was pelleted by centrifugation at 12000 rpm for 2 min and resuspended in 200 μl of FSB solution, (1 ml 10% SDS and 2 ml of 10X loading buffer in 8 ml of distilled water). The cell pellet was resuspended by 1 min of vigorous vortexing and allowed to stand on the bench for a further 15 min. Samples were then centrifuged at 12000 rpm for 20 min. After which 10-20 μl of the supernatant was carefully removed for further analysis by agarose gel electrophoresis,, (section 2.4.6).

2.3.11 Storage of bacteriophage lambda

High titre bacteriophage lambda library stocks and single clone isolates were maintained in phage buffer at 4°C. Lambda plugs from primary secondary or tertiary rounds of library screening were also stored at 4°C in 1 ml phage buffer with 50 µl of chloroform. Long term storage of lambda at -70°C was achieved by supplementing the phage buffer with 7% DMSO prior to storage. Lambda library plates, sealed in plastic bags, were also stored at -70°C after the plaque lawn had been overlaid with Whatman 3 MM paper impregnated with 60% glycerol in phage buffer.

2.3.12 Preparation of lambda plating cells

An overnight culture of the *E.coli* strain LE392 was grown from a single colony. A 1:100 dilution of the culture was made with LB-broth and grown for approximately 3 hours to an OD_{600}nm of 0.5. The culture was then centrifuged at 4000 rpm. for 10 min at room temperature. The cells were then resuspended in 10 mM $MgSO_4$ to an $OD_{600} = 2$. These cells can be stored at 4°C for up to 1 week with little loss of viability.

2.3.13 Plating bacteriophage lambda to determine titre

The method used was that detailed in Ausbel *et al.*, 1990, with minor modifications. It was necessary to make serial dilutions of phage lysates to determine the titre of the stock phage. Typically 10-fold dilutions were made, of which 10 µl were added to 200 µl of LE392 plating cells, (section 2.3.12), and incubated at 37°C for 20 min. The cell phage mix was then added to 3 ml of top agar pre-melted to 48°C and spread onto LB bottom agar plates, (82 mm in diameter), pre-warmed to 37°C. After 6 to 8 hours of incubation at 37°C visible plaques formed, thus allowing the viable number of phage in the starting stock to be calculated.

2.3.14 Liquid lysates

Liquid lysates were performed by infection of bacteria at high multiplicity of infection. Typically, 5 µl of a high titre stock, (section 2.3.15), was incubated with 200 µl of plating cells, (section 2.3.12), at 37°C for 20 min. The phage cell mix was then added to 50 ml of pre warmed NZCYM medium in a 250 ml flask, and incubated with vigorous shaking at 37°C for up to 6 hours. 1 ml of chloroform was added to the resulting lysed culture and incubated for a further 10 min.

2.3.15 Plate lysates and high titre stocks

Prior to making plate lysates it was necessary to determine the titre of the phage stock, (section 2.3.13). Approximately 1 X 10^5 p.f.u are mixed with 500 µl of plating cells, (section 2.3.12), and incubated at 37°C for 20 min. The phage cell mix was added to 5 ml of top agar pre melted to 48°C, and spread onto bottom LB-agar plates, (10 cm x 10 cm), pre warmed to 37°C. Plaques will be visible after 6-8 hours but were typically left to grow for 10 hours until they reached confluence. Then 5 ml of phage buffer was added and the phage allowed to elute overnight. The remaining phage buffer, (1-2 ml), was collected and stored as a high titre stock, (section 2.3.11).

2.3.16 Plating bacteriophage lambda libraries and transferring to filter membranes

The methods used were essentially those detailed in Ausbel *et al.*, 1990, with minor modifications. Bacteriophage were plated at high density on to LB-agar plates and replica copies of the plates were transferred on to nylon membranes. The filter membranes were processed to fix the lambda DNA and subsequently screened by nucleic acid hybridisation to identify homologous clones.

Prior to plating the library it was necessary to determine the titre of the library by serial dilution as described in section 2.3.13. The library was plated on to large 245 mm X 245 mm plates (Nunc), containing 400 ml of bottom LB agar, dried in a 37°C incubator and pre incubated to 37°C. The phage were then mixed with 2 ml of plating cells, (section 2.3.12), allowed to

adhere at 37°C for 20 min, mixed with 30 ml of top agar/agarose 0.65% (w/v) and plated to give 100,000 p.f.u./plate. A total of 10 plates were used to allow 1 X 10^6 p.f.u. to be screened. The plates were then incubated at 37°C for 8-12 hours in an inverted position until plaques were visible but not confluent. The plates are then incubated at 4°C for at least 1 hour prior to making filter lifts.

Bacteriophage were then transferred onto Hybond-N nylon filters, (Amersham), by placing the filter directly on to the surface of the cold plate and allowing the phage to absorb for 1 min. During this time the orientation of the filters was recorded by piercing the filters/LB-agar at several asymmetrical points with a 20-G needle that had been dipped in Indian ink. The second filter was applied to the phage for 2 minutes and pierced with a needle at exactly the same positions. The resulting filters were then allowed to dry at room temperature for 10 min and placed phage side up onto 3 MM paper saturated with 0.2 M denaturing solution for 7 min. The filters were then transferred sequentially to 3 MM paper soaked with neutralising solution, (twice), and 2X SSC for 3 min each. The filters are then baked in a oven at 80°C for 2 hours and stored in a dry environment at room temperature.

2.3.17 Small scale preparation of lambda DNA

Liquid lysates were prepared as described in section 2.3.14. 1.6 µl of liquid lysate was centrifuged at 10000 rpm for 5 min. The supernatant was removed to a fresh tube and treated with 3 µl of DNase/RNase, (20 mg/ml each), at 37°C for 30 min. Precipitation of the phage particles was achieved by adding 400 µl of PEG solution (30% PEG, 3 M NaCl), mixed by inversion and placed on ice for 1 hour. The phage particles were pelleted by centrifuging at 12000 rpm for 20 min. The supernatant was removed and the remaining pellet was resuspended in 340 µl of reagent B, (400 mM Tris-HCl pH to 8.0 using 2M NaOH, 60 mM EDTA, 150 mM NaCl, 1% SDS). 2 ml of 10 mg/ml RNase A was added and incubated at 37°C for 15-20 min. A 100 µl of 3M sodium perchlorate was added to each tube and incubated at 37°C for 20 min with occasional inversion. Samples were then incubated for a further 20 min. at 65°C with occasional inversion. 580 µl of chloroform chilled on dry ice was added, and the samples rotary mixed at room temperature for 20 min. Centrifugation at 12000 rpm for 20 min was followed by the removal of the

supernatant to a fresh microfuge tube and the above chloroform step was repeated. 2 volumes of 100% ethanol, about 750 µl, were added to the supernatant to precipitate the DNA. Samples were subsequently centrifuged at 12000 rpm for 10 min. Supernatants were removed and 500 µl of 70% ethanol was used to wash each pellet followed by a 12000 rpm. spin for 5 min. Again supernatants were removed and the DNA pellets allowed to dry at room temperature. The DNA's were resuspended in 60 µl of H_2O. 5 µl of each sample was used to check the integrity and estimate the concentration of DNA by agarose gel electrophoresis, (section 2.4.6).

2.3.18 Large scale preparation of lambda DNA

The large scale preparation of lambda DNA was achieved by adapting the method detailed above, (section 2.3.17). 16 ml of liquid lysate was centrifuged at 10,000 rpm for 5 min. The supernatant was removed to a fresh tube and treated with 50 ml of DNase/RNase, (20 mg/ml each), at 37°C for 30 min. Precipitation of the phage particles was achieved by adding 400 µl of PEG solution, (30% PEG, 3 M NaCl), mixed by inversion and placed on ice for 1 hour. The phage particles were pelleted by centrifuging at 12000 rpm for 20 min. The supernatant was removed and the remaining pellet was resuspended in 680 ml of reagent B, (400 mM Tris-HCl pH to 8.0 using 2 M NaOH, 60 mM EDTA, 150 mM NaCl, 1% SDS). 5 µl of 10 mg/ml RNase A was added and incubated at 37°C for 15-20 min. A 200 µl of 3 M sodium perchlorate was added to each tube and incubated at 37°C for 20 min with occasional inversion. Samples were then incubated for a further 20 min at 65°C with occasional inversion. 650 ml of chloroform chilled on dry ice was added, and samples rotary mixed at room temperature for 20 min. Centrifugation at 12000 rpm for 20 min was followed by the removal of the supernatant to a fresh microfuge tube and the above chloroform step was repeated until no debris was observed at the interface. 2 volumes of 100% ethanol, about 1.5 ml, were added to the supernatant to precipitate the DNA. Samples were subsequently centrifuged at 12000 rpm for 10 min. Supernatants were removed and 500 ml of 70% ethanol was used to wash each pellet followed by a 12000 rpm spin for 5 min. Again supernatants were removed and the DNA pellets allowed to dry at room temperature. The DNAs were re-suspended in 100 µl of H_2O. 2 ml of each sample was used to check the integrity and estimate the concentration of DNA by agarose gel electrophoresis, (section 2.4.6).

2.3.19 Picking and processing of phage plugs

Positively identified plaques were picked for further processing using cut sterile 1 ml micropipette tips. Firstly, 1.5 cm of the tip was removed using a heated scalpel blade. The wide bored tip was accurately forced into the library plate at the point indicated by the positive signals on the replica filters, (the filters were aligned by their asymmetric ink marks to the phage plates with the aid of a light box). The phage agar plug was recovered and placed in a 1.6 ml microfuge tube with 1 ml of phage buffer and 60 µl of chloroform. The phage were allowed to elute from the plugs at room temperature for at least 4 hours.

2.3.4 DNA molecular biological techniques

2.4.1 Commonly used Buffers

TE buffer (10x)
100 mM Tris-HCl pH 8·0, 10 mM EDTA.

TAE buffer (50x)
242g Tris-HCl, 57.1 ml glacial acetic acid, 37.2g of $Na_2EDTA{\cdot}2H_2O$, in 1 litre distilled water, pH adjusted to 8·5 with glacial acetic acid.

TBE buffer (10x)
10·9% (w/v) Tris, 5·5% (w/v) boric acid, 0·93% (w/v) $Na_2EDTA.2H_2O$ pH adjusted to 8.0, in distilled water.

Agarose gel loading buffer (10x) pH 7·4
0·5% (w/v) bromophenol blue, 0·05% (w/v) xylene cyanol, 50% (w/v) Ficoll, 1% (w/v) SDS, 100 mM EDTA.

l *Hin*d III DNA markers
l DNA cleaved with *Hin*dIII generating 8 fragments, 23.1 kb to 125 bp, (Boehringer Manheim).

1 kb DNA Marker
Mixture of 1018 bp fragment, its multimeres and pBR322 fragments. A total of 23 fragments, from 12216 bp to 756 bp. (Boehringer Manheim).

Denaturing solution
1·5 M NaCl, 0·5 M NaOH.

Neutralising solution
1·5 M NaCl, 0·5 M Tris-HCl pH 7.4.

Birnboim Doly I (BDI) solution
50 mM glucose, 25 mM Tris-HCl pH 8·0, 10 mM EDTA.

Birnboim Doly II (BDII) solution
0·2 M NaOH, 1% (w/v) SDS which was stored in a plastic container.

Birnboim Doly III (BDIII) solution
5 M KOAc pH 4·8; prepared by mixing equal volumes of 3 M CH_3COOK and 2 M CH_3COOH.

SSC (20X)
3 M NaCl, 0·3 M tri-sodium citrate, pH 7·0.

SSPE (20X)
175.3 g NaCl, 27.6 g NaH_2PO_4, 7.4 g EDTA, in 800 ml H_20, pH adjusted to 7.4 with 10 M NaOH, final volume adjusted to 1 litre.

Denhardt's solution (100X)
2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin, (Pentax Fraction V), filtered and stored at -20°C.

Phage Buffer
20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$.

2.4.2 Quantification of DNA

DNA concentrations were calculated by spectrophoretically measuring the OD at 260 nm. At this wavelength an OD = 1 corresponds to a concentration of 50 µg/ml for double stranded DNA and approximately 33 µg/ml for oligonucleotides. However, when necessary a more accurate estimation of oligonucleotide concentration can be calculated using extinction coefficients as described by Sambrook *et al.*. 1989. When samples had limiting quantities of DNA< 200 ng, then a small portion of the sample was run on a 1% (w/v) agarose gel containing EtBr, (0.5 mg/ml), with known DNA concentration standards. The gel was then photographed, (section 2.4.8), and by comparing the intensity of fluorescence of the sample to the known standards, an estimation of DNA concentration was made.

2.4.3 Preparation of genomic DNA from ES cells

Genomic DNA was prepared by a method similar to the 'Nucleon I' DNA extraction kit method of Scotlab. The following method is optimised for the extraction of DNA from the confluent wells of a 24 well tissue culture plate. Each well of the 24 well plate was washed twice with 1X PBS. Cells were then trypsinised with 100 µl TVP (section 2.5.2) for 3 minutes. 1 ml of ES cell growth medium (section 2.5.2) was added to each well. The cell suspension was placed in a 1.6 ml microfuge tube and centrifuged at 2000 rpm. for 5 min. The cell pellets were resuspended by gentle vortexing in 340 µl of reagent B (400 mM Tris-HCl pH to 8.0 using 2 M NaOH. 60 mM EDTA. 150 mM NaCl. 1% SDS). 2 µl of 10 mg/ml RNase A was added and incubated at 37°C for 15-20 min. A 100 µl of 5 M sodium perchlorate was added to each tube and incubated at 37°C for 20 min with occasional inversion. Samples were then incubated for a further 20 min at 65°C with occasional inversion. 580 µl of chloroform chilled on dry ice, was added and samples rotary mixed at room temperature for 20 min. Centrifugation at 12000 rpm. for 20 min was followed by the careful removal of the supernatant to a fresh microfuge tube and the above chloroform step was repeated. This time 2 volumes of 100% ethanol, (750 µl), were added to precipitate the DNA which was obvious upon inversion of the tubes. Samples were subsequently centrifuged at 12000 rpm. for 10 min. Supernatants were carefully removed and 500 µl of 70% ethanol was used to wash each pellet followed by a 12000 rpm. for 5 min spin. Again,

supernatants were removed and the DNA pellets allowed to dry at room temperature. The DNAs were resuspended in 60 µl of H_2O; to help resuspension, samples were placed at 65°C for 10 min or alternatively placed at 4°C overnight. Subsequently, 20 µl of each sample was used for restriction digestion as this volume typically contained approximately 10 µg of DNA.

2.4.4 Preparation of genomic DNA from tissue

It was necessary to isolate genomic DNA from rat and mouse livers. The method used was essentially that detailed in Sambrook *et al.*, 1989, 'Protocol I'. This procedure is based on a method first reported by Blin and Stafford, 1976.

2.4.5 Restriction enzyme digestion

Restriction enzymes were used according to the manufacturers instructions with the appropriate buffer unless otherwise specified in the text. For restriction digests, a 2-3 fold excess of enzyme was used i.e. 2-3 units/µg of DNA with the enzyme concentration not exceeding 1/20 of the total reaction volume. Digestions were incubated at 37°C for 1 hour in the case of plasmid or lambda DNA, however for genomic DNA incubation times were increased to 3 hours.

2.4.6 Agarose gel electrophoresis

DNA was electrophoresed on agarose gels of a concentration range of 0.5% to 2%, depending on the size of the fragments to be resolved using a 1X TBE buffer, (Sambrook *et al.*, 1989). The applied voltages depended on fragment sizes being analysed and duration of running time, however they did not exceed 10 V/cm. It was necessary to use low melting point agarose gels for DNA band isolation. Therefore 1X TAE buffer was used and the gels were electrophoresed at 4°C in the cold room. All agarose gels contained 0.5 µg/ml EtBr for the visualisation of DNA, (section 2.4.8).

2.4.7 Polyacrylamide gel electrophoresis for sequencing

Sequencing reactions were prepared as detailed in section 2.4.16. The products were separated on denaturing polyacrylamide gels; 6% (w/v) acrylamide (N, N'-methyleneisacrylamide, 19:1), 7 M urea, in 1X TBE. Acrylamide polymerisation was achieved by the addition of 1 ml of 10% (w/v) ammonium persulphate and 25 µl of TEMED (N,N,N'N', -tetramethylenediamine) to 100 ml of acrylamide/urea mix. The gels were poured and run on the IBI 'Base Runner' sequencing apparatus according to the manufacturers specifications. Gels were pre-run at 60 watts for 30 min. prior to the addition of the samples and subsequently run at 60 watts for the required length of time. Gels were finally transferred to 3 MM Whatman paper and dried at 80°C under vacuum for 1 hour prior to autoradiography, (section 2.4.25).

2.4.8 Gel visualisation and photography

All agarose gels contained 0.5 µg/ml of EtBr for visualisation of double stranded DNA. However, if further staining of gels was required then they were placed in 1X TBE containing 0.5 µg/ml of EtBr for 15 min after which they were washed in 1X TBE for 10 min. The DNA bands could then be visualised by fluorescence under a 254 nm or 302 nm transilluminator. Gels were photographed using a Polaroid camera loaded with type 667 land film fitted with a Kodak Wratten filter No. 23A. Alternatively a picture image could be made using a Mitsubishi video copy processor attached to a UVP video camera, fitted with a Kodak Wratten filter No. 23A.

2.4.9 Purification of DNA from LMP gels

Purification of DNA from LMP gels was achieved using the 'QIAEX Gel Extraction Kit' from Qiagen, based on principles first reported by Vogelstein *et al.*, 1979. Typically, DNA fragments were separated on 1% LMP gels and processed as described in sections 2.4.6 and 2.4.8. The DNA bands were then excised from the gels using sterile scalpel blades and placed in 1.6 ml microfuge tubes. The gel slices were then processed according to the manufacturer's instructions. Briefly the gel slices were placed in solubilisation

buffer at 50°C with 10 µl of DNA binder. This buffer contains a high concentration of sodium perchlorate which not only facilitates solubilisation but binding of DNA molecules to the silica particles due to the high salt concentration. The bound DNA is then washed in a high salt, ethanol containing buffer. The pellet is then allowed to dry and the DNA eluted from the silica gel particles using H_20.

2.4.10 Ligation of double-stranded DNA

The ligation of double-stranded DNA molecules was performed as described in the Promega Protocols and Application Guide, (Promega). Typically about 200 ng of vector and a vector to insert molar ratios of 1:1 and 1:3 were used for ligations. Most ligations in this study also used vectors which had been dephosphorylated as selection of recombinant clones using X-gal/IPTG selection, (section 2.3.4), was often not an option. Unlike the Promega protocols, Shrimp Alkaline Phosphatase, (SAP, (USB Biochemicals)), was used as opposed to Calf Intestinal Alkaline Phosphatase (CIAP), as detailed by the supplier. SAP is reliably inactivated at 65°C for 15 min unlike CIAP. The only other major modification was that the transformation of *E. coli* was achieved by electroporation, (section 2.3.7). The ligations were performed overnight at 16 °C, unless otherwise stated.

2.4.11 Exonuclease III deletion library

This procedure was performed essentially as detailed in Promega Protocols and Application Guide, (Promega), with minor modifications. The method is based on the procedure developed by Henikoff *et al.,* 1984. The Exonuclease III enzyme is used to digest double stranded DNA from a 5' protruding or blunt end, while leaving a 4 base or larger 3' overhang intact. Alternatively, 5' protruding overhangs can be protected from digestion by incorporating α-phosphorothioate deoxynucleotide derivatives onto the overhang. The doubly cut linear molecule can now be subjected to progressive Exo III digestion from the blunt or 5' overhang closest to insert while the 3' or 5' protected end will remain resistant to Exo III digestion. Samples of the digesting DNA of study are removed at convenient time points since digestion proceeds at a rate of 450 bp/min when incubated at 37°C. These samples are

then subjected to S1 nuclease digestion to remove the single-stranded DNA generated by Exo III digestion. Klenow DNA polymerase was added to generate blunt ends which are then ligated to circularise the plasmids. The resulting ligations are then transformed into *E.coli.* producing a variety of colonies containing different sized subclones.

2.4.12 Generation of template DNA for exonuclease III digestion

10 mg of supercoiled plasmid was prepared as described in section 2.3.7. A *Sal*I restriction digest was performed as described in section 2.4.5. Completion of digestion was checked by agarose gel electrophoresis, (section 2.4.6). The resulting 5' protruding end had to be protected using α–phosphorothioate, (thio), base derivatives. Therefore, thio base derivatives of dATP and dGTP along with normal dCTP and dTTP were added at a final concentration of 10 mM and incubated at 30°C for 30 min in the presence of 10 U/ml Klenow DNA polymerase, (Boehringer), to fill in the 5' overhang. The completed digest and fill in reactions were subjected to phenol-chloroform extraction and isopropanol precipitation, (sections 2.4.30 and 2.4.29 respectively). The DNA was then re-suspended in *Eco*RI restriction enzyme buffer and digested for 1 hour. However, an aliquot (200 ng) of single cut DNA was retained for a test Exo III digestion. The completed digest was subjected to phenol-chloroform extraction followed by ethanol precipitation, (sections 2.4.30 and 2.4.29 respectively). The DNA was resuspended in 60 µl of 1X Exo III buffer, (10X: 660 mM Tris-HCl pH 8.0, 1 mM EDTA).

2.4.13 Exonuclease III test digestion

The single enzyme cut thio protected template DNA and the double enzyme cut template DNA were subjected to Exo III test digests. 200 ng of each DNA in 10 µλ 1X Exo III buffer, (10X 660 mM Tris-HCl pH 8.0, 1 mM EDTA), with 50 units of Exo III were incubated for 15 min at 37°C. The reaction products were then checked by agarose gel electrophoresis, (section 2.4.6). The protected thio filled template should remain resistant to digestion but the double cut template appears as a smear as a result of digestion.

2.4.14 Exonuclease III digestion and SI nuclease digestion

The DNA template in 60 µl of 1X Exo III buffer was pre warmed to 37°C. The digestion was started by the addition of 750 units Exo III and allowed to incubate at 37°C. At 40 s intervals 2.5 µl samples of the reaction were removed and placed into 1 of the 17 microfuge tubes on ice containing 7.5 ml of S1 mix, (45 mM KOAC pH 4.6, 350 mM NaCl, 1.3 mM $ZnSO_4$ and 7% glycerol), and 2 Units of S1 nuclease. All the samples were then incubated at room temperature for 20 min. The reactions were stopped by adding 1 µl S1 stop buffer, (0.3 M Tris base 0.05 M EDTA), and incubated at 70°C for 10 min.

2.4.15 Ligation and transformation of Exo III deletions

All time point samples received 1 µl of Klenow mix, (30 µl Klenow buffer (20 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$) and 3-5 Units Klenow DNA polymerase), and were incubated at 37°C for 10 min in the presence of 1 µl of the dNTP mix, (0.125 mM each of dATP, dTTP, dCTP, and dGTP). The ligations were then performed using 4 µl of each Klenow reaction combined with 20 µl of ligation mix (1X Promega ligase buffer, 5% PEG, 1 mM DTT 5U/ml T4 ligase). Samples were then incubated at room temperature for 3 hours and 2 µl of each reaction was used for transformation into DH5α, (section 2.3.7).

2.4.16 Double-stranded DNA sequencing

DNA sequencing reactions were performed using the Sequenase version 2.0 kit exactly as described by the manufacturers, (USB Biochemicals). 5 µg of double stranded plasmid was used as the template and was prepared as described in section 2.3.8.

2.4.17 End-labelling of oligonucleotides using T4 kinase

The radioactive labelling of oligonucleotides was catalysed by bacteriophage T4 polynucleotide kinase using $\gamma^{32}P$ ATP as a label. The reaction was performed in a total reaction volume of 10 µl containing 10 pmoles of oligonucleotide, 30 µCi of $\gamma^{32}P$ ATP at 3000 Ci/mmole, 10 units T4 kinase in 1X T4 kinase buffer, (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine). The reaction was stopped by adding 1 µl of 0.5 M EDTA. Purification of labelled oligonucleotide from unincorporated label was achieved by gel filtration, (section 2.4.19).

2.4.18 Random priming DNA templates

The random priming method for DNA labelling was performed using a Random Primed DNA Labelling Kit, (Boehringer). The labelled DNA is synthesised with the klenow enzyme after the hybridisation of all possible combinations of random hexanucleotides to the denatured DNA template. Typically 25 ng of template was combined with 50 µCi of $\alpha^{32}P$ dCTP (3000 Ci/mmole) to produce probes with a specific activity of $1\text{-}1.5x10^9$ dpm/µg. The labelled templates were purified from unincorporated $\alpha^{32}P$ dCTP by gel filtration section, (2.4.19).

2.4.19 Purification of radioactive probes

Sephadex G-50 and G-25 columns, (Pharmacia), were routinely used to purify radioactively labelled random primed and end-labelled oligonucleotide probes respectively. This method uses gel filtration chromatography to separate labelled molecules from unincorporated ^{32}P label. The columns were equilibrated with 1 ml of 1X TE prior to use. The probe reactions with 50 µl of bromophenol blue, (0.05% w/v), were loaded directly onto the column and 100 µl aliquots of 1X TE were added to run the sample in. A further 1.5 ml of 1X TE was added, allowing the column elutant to be collected in 3 drop samples. The labelled probe was present in the blue dye fraction with the unincorporated ^{32}P being present in later fractions.

2.4.20 Preparation of non-radioactive probes

Non-radioactive probes were also produced using the random priming method detailed in section, (2.4.18), except that dideoxygenin-11-dUTP was used instead of α^{32}P dCTP. The probed filters were processed according to the manufacturer's protocols, (Boehringer).

2.4.21 Southern blotting by capillary transfer

Southern blotting was performed as described by Sambrook *et al.* 1989, this method was first reported by Southern *et al.*, 1975. The gels were prepared for transfer by sequentially soaking for 1 hour in denaturing solution, 30 min in neutralising solution, 10 min in 20X SSC and then transferred to Hybond-N nylon membranes, (Amersham), by capillary action. Nylon membranes were pre-wetted in distilled water for 10 min and equilibrated in 20X SSC prior to DNA transfer. DNA was fixed to the membranes by UV cross-linking using a Stratalinker, (Stratagene), on the auto cross-link mode as instructed by the manufacturer or by baking at 80°C for 2 hours.

2.4.22 DNA transfer by electro-blotting

Gels for transfer to nylon membranes were prepared as described in section 2.4.21, except that the gels and membranes were equilibrated in 1X TBE as opposed to 20X SSC prior to transfer. Electroblotting was performed with the 'Electrophor' apparatus, (Hoefer Instruments), in 1X TBE at 50 V, 1.5 milliAmps with constant agitation of the buffer using a magnetic stir bar for a minimum of 4 hours or overnight. The DNA was then fixed to the membrane as described in section 2.4.21.

2.4.23 Pre-hybridisation and hybridisation of membrane fixed DNA

Nylon membranes were routinely pre-hybridised for 1 hour at 42°C with hybridisation buffer, (50% formamide, 6X SSPE, 5X Denhardt's solution, 1% SDS and 100 µg/ml sheared salmon sperm DNA), at a volume of 0.2 ml/cm^2 of membrane. Pre-hybridisation was performed in a rotary oven, (Techne), or in

plastic bags placed in a shaking water bath. Hybridisation was performed with at 42°C for 16 hours with denatured radioactive DNA probes at 1-3 X 10^6 dpm/ml of hybridisation buffer.

2.4.24 Washing of membranes

Membranes were washed once with 2X SSPE, 0.1% SDS at room temperature, once with 2X SSPE, 0.1% SDS at 60°C and twice with 0.25X SSPE, 0.1% SDS at 60°C. Membranes were then sealed in plastic bags prior to autoradiography, (section 2.4.25).

2.4.25 Autoradiography

Auotoradiograph was performed at -70°C with intensifying screens when necessary. Fuji NIF RX X-ray film was exposed to the radioactive membranes for as long as required. Development of X-ray film was performed using the X-Ograph (Compact X2). Processed sequencing gels were treated as above except that intensifying screens were not used and autoradiography was performed at room temperature.

2.4.26 Re-use of DNA bound membrane filters

DNA bound membrane filters were 'stripped' of probe by immersion in a boiling solution of 0.1% SDS, which was allowed to cool to room temperature, where-upon the procedure was repeated. Removal of radioactive probes was verified by autoradiography, (section 2.4.25).

2.4.27 Oligonucleotide synthesis and deprotection

Oligonucleotides were synthesised using the automated PCR-MATE 391 DNA synthesiser in accordance with the manufacturer's instructions, (Applied Biosystems). To separate the oligonucleotides from the glass bead synthesis matrix, the beads were treated with 1 ml of 30% NH_4OH and left at room temperature for 1 hour. The glass beads are then removed by centrifugation at

12000 rpm for 10 min. A further 1 ml of 30% NH_4OH was added to the solution and incubated at 50°C for 16 hours. The oligonucleotide was subsequently ethanol precipitated by the addition of 0.11 vol of 3 M NaAc pH 5.2 and 3 vol of absolute ethanol at -20°C for 2 hours. The oligonucleotide was centrifuged at 12000 rpm for 20 min, and the pelleted oligonucleotide was washed in 70% ethanol. The oligonucleotide was repelleted by centrifugation at 12000 rpm, the 70% ethanol was removed and the pellet was dried at room temperature. The oligonucleotide was now resuspended in 200 µl of 1X TE or sterile dH_2O, of which 10 µl was sacrificed to accurately measure the optical density at 260nm, (section 2.4.2).

2.4.28 PCR reactions

The PCR reactions were performed as detailed in "PCR protocols: a guide to methods and applications, (Innis *et al.*, 1990). Reactions were performed on a Perkin Elmer 9600 machine. A typical reaction mix contained the following reagents in a total volume of 100 µl in thin walled microfuge tubes; typically 100 ng of template DNA, 100 pmoles of each primer, 1X reaction buffer (20 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl), 0.2 µM of each dNTP and 2 units of Taq DNA polymerase, (Promega). Reaction mixtures were heated to 95°C for 5 min prior to the addition of Taq. The PCR reaction cycle conditions were specific to the target sequence to be amplified and are therefore detailed within the text. One tenth of the reaction products were analysed by agarose gel electrophoresis, (section 2.4.6).

2.4.29 Ethanol or isopropanol precipitation of DNA

The precipitation of DNA was generally achieved by the addition of 0.1 vol of 3 M NaAc pH 5.2 and 2 vol of absolute ethanol to the DNA sample and stored at -20°C for 30 min. Instead of using ethanol, 0.7 vol of isopropanol was added and incubated at room temperature for 15 min. The precipitated DNA was centrifuged at 12000 rpm for 20 min. The supernatant was discarded and 70% ethanol was added; after brief vortexing the sample was centrifuged at 12000 rpm for 5 min. The supernatant was again discarded, the pelleted DNA was dried at room temperature and resuspended in a suitable volume of 1X TE or sterile dH_2O.

2.4.30 Phenol-chloroform extraction of DNA

Phenol-chloroform extractions were performed to deproteinise aqueous DNA solutions. For this purpose commercially supplied distilled phenol was used, (Aldrich). The phenol was melted at 55°C and buffered by sequentially adding fresh 0.1 M Tris-HCl pH 8.0 until the pH of the phenol reached 8.0. The phenol was saturated by mixing with an equal volume of 1X TE. The saturated phenol was added to an equal volume of chloroform and 0.1% 8-hydroxyquinoline antioxidant, (w/v). The DNA solution was extracted with an equal volume of phenol-chloroform (v/v) and using gentle vortexing for 1 min. The sample was centrifuged at 12000 rpm for 5 min to separate the phases and the upper aqueous phase was carefully removed leaving the interface undisturbed. The extraction was generally repeated until no interface was observed. The DNA was then recovered by ethanol precipitation of the supernatant as described in section 2.4.29.

2.4.31 *In situ* hybridisation

Sections were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS), ph 7.4, for 10 mins, rinsed twice in PBS, and placed in 2X SSC for 10 mins. at room temperature, dehydrated in ethanol, defatted in chloroform, rinsed in 95% ethanol and air dried. Radioactive probes were generated by end labelling of 45mer oligonucleotides: 15ng of oligonuleotide was labelled with 20 µCi ^{35}S dATP using terminal transferase according to the manufacturers instrucions, (Pharmacia). Labeled probe was purified from unincorporated nucleotide by centrifugation of the reaction through a 1 ml G-50 sephadex column,(4 min 12000 rpm). All hybridizations were standardized such that each contained 2.0 $x10^3$ dpm/ml of probe in hybridization buffer (50% (v/v) formamide, 4 x SSC, 25 mM sodium phosphate (pH 7.0), 1 mM sodium pyrophosphate, 20 mM dithiothreitol, 5 x Denhardt's solution, 10% (w/v) dextran sulphate, 200 µg/ml acid-alkali hydrolyzed salmon sperm DNA, 100 mg/ml polyadenylic acid, 120 mg/ml heparin). Sections were hybridized in 200 µl buffer, under parafilm coverslips, overnight at 42°C. Control sections were hybridized in the presence of a 50-fold molar excess of the appropriate unlabelled oligonucleotide. After hybridization, coverslips were removed in 1 x SSC at room temperature, and the sections were washed in 0.3x SSC for 60

min at 55°C. They were rinsed at room temperature in 1x SSC, followed by a rinse in 0.1x SSC, dehydrated sequentially in 70% (v/v) and 95% (v/v) ethanol and air-dried. All wash solutions contained 10 mM dithiothreitol. Sections were exposed to Kodak X-Omat film at room temperature for 15 days before being dipped in Ilford K-5 emulsion. After exposure for 50 days at 4°C, sections were processed in Kodak D19 developer for 2 min at 17°C, briefly rinsed in distilled water, fixed in 30% (w/v) sodium thiosulphate for 4 min, and finally washed in distilled water for 10 min. Sections were dried overnight at room temperature before being counterstained with 0.05% (w/v) thionin, differentiated in an ascending ethanol series (70%, 95% and 100% (v/v)), cleared in Histo-clear (National Diagnostics), and mounted in DPX (BDH).

2.6.32 Photography for *in situ* hybridisation

Autoradiographic images generated on X-ray film were printed with an enlarger directly onto Kodak Kodabrome II RC photographic paper (grade F5 M), giving reverse images. All sections were photographed onto Kodak Tech-Pan film using a Leitz microscope and 35mm camera with lightfield and darkfield condensers at 200 ASA and 1600 ASA settings respectively. Negatives were processed using Kodak HC-110 developer at 20°C for 4.5 minutes. Both lightfield and darkfield negatives were printed onto Kodak Kodabrome II RC photographic paper (grade F5 M).

2.5. ES cell tissue culture, media, maintenance and methods

2.5.1 ES cell lines

ES Cell Lines	Source
EI4	Handyside *et al.*, 1989.
RI	Nagy *et al.*, 1993.
CGR8	Mountford *et al.*, 1994.
CGR8.8	Skarnes W. C., CGR in Edinburgh, (personal gift).

2.5.2 ES cell tissue culture media and materials

E14 ES cell medium

E14 ES cells were grown on the Glasgow modification of Eagle's medium with the supplements detailed below after the medium had been conditioned on Buffalo Rat Liver, (BRL), cells as detailed by Handyside *et al.*, 1989.

CMB medium;	
Sterile Water	375 ml
10X BHK21 medium, (Glasgow MEM;Gibco)	45 ml
7.5% Sodium bicarbonate, (Gibco)	16.5 ml
100X MEM non-essential aa, (Gibco)	5 ml
100 mM Na pyruvate, (Gibco)	5 ml
200 mM L-glutamax, (Gibco)	5 ml
0.1 mM b-mercaptoethanol, (Gibco)	5 ml
100X Penicillin-streptomycin, (Gibco)	5 ml
Foetal calf serum, (selected batches; Gibco)	25 ml

Newborn calf serum, (selected batches; Gibco)	25 ml

The CMB medium was conditioned by incubating in the presence of confluent BRL cells. 175 cm^2 tissue culture flasks were seeded with BRL cells and grown to confluence using CMB medium. Upon reaching confluence, fresh CMB medium was added for conditioning at 60 ml/ 175 cm^2 flask and incubated at 37°C/5% CO_2 for 1 week. Then the medium was passed through a membrane filter of pore size 0.8 mm or smaller. The filtered medium was stored at -20°C until required. This medium was designated '100% BRL-conditioned medium'. For growth of E14 ES cells, 60% BRL-conditioned medium was used. Therefore 6 volumes of 100% BRL-conditioned medium was mixed with 4 volumes of CMB medium.

R1 ES cell medium

R1 ES cells were grown on the Glasgow modification of Eagle's medium with the supplements detailed below:

Sterile Water	267 ml
10X BHK21 medium, (Glasgow MEM Gibco)	40 ml
7.5% Sodium bicarbonate, (Gibco)	13.2 ml
100X MEM non-essential aa, (Gibco)	4 ml
100 mM Na pyruvate, (Gibco)	4 ml
200 mM L-glutamax, (Gibco)	4 ml
0.1 mM b-mercaptoethanol, (Gibco)	4 ml
100X Penicillin-streptomycin, (Gibco)	4 ml
Foetal calf serum, (selected batches Gibco)	60 ml
Murine leukaemia inhibitory factor, (Gibco) (1 X 10^7 units/ml)	40 ml

CGR8 and CGR8.8 ES Cell Medium

CGR8 and CGR8.8 ES cells were grown on the Glasgow modification of Eagle's medium with the supplements detailed below:

Sterile Water	336 ml
10X BHK21 medium, (Glasgow MEM Gibco)	40 ml
7.5% Sodium bicarbonate, (Gibco)	13.2 ml
100X MEM non-essential aa, (Gibco)	4 ml
100 mM Na pyruvate, (Gibco)	4 ml
200 mM L-glutamax, (Gibco)	4 ml
0.1 mM b-mercaptoethanol, (Gibco)	4 ml
100X Penicillin-streptomycin, (Gibco)	4 ml
Foetal calf serum, (selected batches from Globerpharm, Surrey)	40 ml
Murine leukaemia inhibitory factor, (Gibco), (1 X 10^7 units/ml)	40 ml

TVP (cell disaggregating solution)

0.025% (w/v) trypsin, (Gibco);1 mM Na_2 EDTA, (Sigma); 1% (v/v) chick serum, (Flow Labs); in (Ca^{++}, Mg^{++}-free) 1X PBS, (Gibco). The solution is sterilised by membrane filtration using filters of 0.4 mm pore size, (Millipore), and stored as -20°C aliquots.

1% Gelatin Stock Solution

Gelatin, (Sigma, swine skin), was dissolved in distilled water at 10 g/l and autoclaved. The resulting solution was allowed to cool at room temperature and re-autoclaved. The stock was stored in 20 ml aliquots at 4°C.

Freezing Medium

ES cell growth medium supplemented with 10% (v/v) DMSO, (Sigma).

2.5.3 Maintenance of ES cells

The following procedures apply to the maintenance of E14, R1, CGR8 and CGR8.8 ES cell lines. All cells were grown on a feeder cell independent system. Medium was generally changed every day and cells were not allowed

to grow to confluence. All ES cell manipulations were performed in a laminar flow hood. All ES cells were grown in humidified 37°C/5% CO_2 incubators and all solutions were warmed to 37°C prior to use.

2.5.4 Growth and passage of ES cells

ES cells are grown on plastic tissue culture flasks, (Corning Inc.), precoated with 0.1% gelatin, (1% stock solution was warmed to 37°C to liquefy and further diluted to 0.1% with sterile water). Typically, 2 ml of 0.1% gelatin was added to a 25 cm^2 flasks and allowed to sit for 5 min at room temperature. The gelatin solution was then aspirated off prior to the addition of medium on to cells. When growing cells from frozen, an aliquot of frozen cells (0.5 X 10^7) was quickly thawed at 37°C and 10 ml of pre-warmed medium was added and pelleted by centrifugation at 1200 rpm for 5 min. The cell pellet was resuspended in 5 ml of medium and added to a 25 cm^2 flask prepared as described above. The cells were then incubated at 37°C for no longer than 16 hours prior to the change of medium.

ES cells were passed when the cells had neared confluence, typically about every 2-3 days. For a 25 cm^2 flask the medium was aspirated off and 5-10 ml of 1X PBS was added and gently allowed to wash over the cells. The 1X PBS was aspirated off and the above wash step repeated. The cells are then covered with 1 ml of TVP and allowed to incubate at room temperature until a cell suspension forms, (approximately 3 min). 9 ml of medium was added to stop trypsinisation. One tenth of this cell suspension was removed and placed in a freshly gelatinised 25 cm^2 flask with 4 ml fresh medium. Therefore, a 1:10 split ratio was typically used for the passage of ES cells. However, when expanding ES cells for an electroporatation experiment the same procedure was followed but with the cells being passed at twice the density, (a 1:5 split ratio), into larger flasks.

2.5.5 Freezing of ES cells for long term storage

Cells are washed twice in 1X PBS and trypsinised as described above. 9 ml of medium was added to stop the reaction and the cell suspension centrifuged at 1200 rpm for 5 min. The medium was aspirated off and resuspended in 1 ml of freezing medium and 0.5 ml aliquots were frozen down in cryotubes. When freezing ES cells it is desirable to freeze at 1°C/min. This was achieved by

placing the cell vials in a polystyrene box prior to overnight storage at -70°C The next day the cell vials were placed in the vapour phase of a liquid nitrogen storage vessel.

2.5.6 Short term storage and recovery of ES cells

This method was only used for the storage of ES cells grown on 24 well plates and is a modification of a procedure first presented by McPheat *et al.*, 1991. Individual ES cell clones were grown in 24 well plates to near confluence. The medium was aspirated off and 250 µl of freezing medium was added to each well of the plates. The plates were then placed in polystyrene boxes and stored at -70°C for up to 3 months. The recovery of individual clones from these plates was achieved by transferring the plate to a 37°C incubator. 2 ml of medium was added to a thawed well. This 2 ml of medium was subsequently transferred to 1 well of a six well plate with 8 ml of fresh medium. The original well received 1 ml of fresh medium. Both parts of the sample were maintained in culture until the cells recovered, changing the medium daily.

2.5.7 Preparation of vector DNA for electroporation

Plasmid DNA was isolated using the large scale plasmid preparation method, (section 2.3.9). 25 µg of the vector DNA was linearised by restriction digestion as described in section 2.4.5. The digest was checked for completion by running 200 ng of the digested vector on a 0.7% agarose gel, (section 2.4.6). The digested DNA was ethanol precipitated, (section 2.4.29), and washed with 1 ml of 70% ethanol and centrifuged at 12000 rpm for 5 min. The 70% ethanol was removed by aspiration in a laminar flow hood where the pelleted DNA was allowed to dry at room temperature. The DNA was then resuspended in 100 µl of 1X PBS for several hours at room temperature or overnight at 4°C. Lastly, the vector DNA was heated to 65°C for 5 minutes prior to its use in transfection.

2.5.8 Transfection of E14 and R1 ES cells

It is important that cells are growing well prior to electroporation, therefore actively growing cells that required passage every 2-3 days were used. Typically 5 X 10^7 cells were used, (this approximates to a near confluent 175 cm^2 flask), which were fed with fresh medium 3 hours prior to harvest. The cells were washed twice with 1X PBS, trypsinised with 2.5 ml of TVP, the reaction was stooped by adding 25 ml of growth medium and centrifuged at 1200 rpm for 5 min. The supernatant was aspirated off and the pellet of cells resuspended in 10 ml 1X PBS. The cells were pelleted as described and resuspended in a further 10 ml of 1X PBS and centrifuged. The pelleted cells were resuspended in 0.7 µl of 1X PBS, added to the tube containing the linearised plasmid, (section 2.5.7.), and transferred to a 0.4 cm electroporation cuvette, (Bio-Rad). Electroporation was performed with a Bio-Rad Gene Pulser unit set at 500 µF and 250 volts. Cells were allowed to recover for 20 min in the cuvette at room temperature prior to plating. The electroporation mix was plated onto 6 freshly gelatinised plates, (10 cm in diameter), with 10 ml of fresh medium. Selection was not applied to the cells until 24 hours after electroporation. All plates received fresh medium daily, containing 200 µg/ml G418 and 1 X 10^{-6} M GANC except for the sixth plate, which received only 200 µg/ml G418. This regime continued for 10-14 days, after which colonies of 1 mm and larger were ready to be processed as described in section 2.5.10. Colonies from the plate which were only selected with G418 were not picked. This plate provided a means by which to assess the efficiency of GANC enrichment of targeted clones.

2.5.9 Transfection of CGR8 and CGR8.8 ES cells

CGR8 and CGR8.8 ES cells were electroporated and $G418^R$ $GANC^R$ cells selected as described above with the following exceptions. A cell concentration of 1 X 10^8 cells were electroporated with 150 µg of linearised vector DNA at 3 µF and 800 volts. The electroporation mix was plated onto 20 X 10 cm diameter plates and selected as above with one of the plates not receiving GANC selection. However, these cells grow quicker and only require 9-12 days of selection.

2.5.10 Processing G418/gancyclovir resistant ES colonies

G418-GANC resistant ES colonies were picked and grown on 24 well plates. The plates containing the resistant colonies were washed twice with 1X PBS and a final 10 ml of 1X PBS added. Colonies were picked with a P200 Pipetteman set at 100 µl with sterile tips; colonies were dislodged, the cells pipetted in a volume of 100 ml 1X PBS and transferred to a 24 well plate. Once 24 colonies had been picked, 100 µl of TVP solution was added to each well and incubated at 37°C with frequent tapping until the colonies were dispersed. 2 ml of growth medium was added to each well to stop the trypsinisation. The colonies were expanded until near confluent. The cells were then trypsinised with 4/5 of the cells being harvested for Southern blot analysis and 1/5 of the cells used to re-seed a freshly gelatinised 24 well plate. The plates were then grown to near confluence and stored as described in section 2.5.6, until required.

2.5.11 Production of chimaeric mice by blastocyst injection

This work was performed by Dr. W. C. Skarnes of the Centre for Genome Research, University of Edinburgh, Scotland. The material and methods used, for the blastocyst injection approach to producing chimaeric mice, are well documented in a number of publications such as Papaioannou and Johnson, 1993. Briefly, blastocysts were collected from 3.5 day post coitus C57BL/6J females on the morning of injection. 10-12 ES cells were microinjected into each blastocyst using a hanging drop chamber mounted on a micromanipulator. Injected blastocysts were incubated at 37°C for a 1-2 hour recovery period. These embryos were then transferred into the lumen of the uterus of pseudopregnant foster mothers.

2.5.12 Preparation of metaphase spreads from ES cells

A culture of ES cells was set up in a 25 cm^2 flask such that it was about 50% confluent on the day the cells were harvested. 24 hours prior to to harvest the cells were fed with fresh medium , (this causes partial synchronisation). 2 hours prior to harvest colcemid was added to a final concentration of 0.1 mg /ml. Cells were harvested as described in section 2.5.4. Cells were then gently resuspended in 10 ml of hypotonic solution, (0.075 M KCl), incubated at room

temperture for 4min. Cells were spun at 500 rpm for 5 min, all but one drop of supernatent was removed, in which the pellet was resuspended by gently flicking the tube, and placed on ice. Cells are then fixed by drop wise addition of ice cold fixative, (methanol/glacial acetic acid 3:1 v/v, freshly prepared), to a final volume of 10 ml and left on ice for 30 min. Cells were pelleted by spining at 500 rpm for 5 min. The pellet was resuspended in 10 ml ice cold fixative and left on ice for 5 min. After which, it was re-pelleted as before and resuspended in 5 ml fixative. 2-3 drops of the cell suspension were then droped from a height of about 30 cm on to prepared microscope slides placed at a 45° angle. The slides were prepared in advance by washing thourghly in 70% ethanol and rinsing in cold tap water immediately prior to use. Chromosomes were stained by allowing the slides to air dry for at least 1 hour prior to dipping in a 3% Giemsa solution for 12- 15 min followed by 2 washes in distilled H_2O for 30 s.

Chapter 3

Cloning and Characterisation of Lambda Clones Containing Genomic DNA Encompassing the Neurotensin Receptor Gene

3.1 Introduction

A primary aim of this project was to clone suitable genomic mouse fragments encoding the mouse neurotensin receptor gene (NTR), for the construction of a gene targeting construct. Theoretically, a number of possible strategies could be used to clone the NTR gene. However, a functional cDNA clone of the rat NTR became available, (Tanaka, *et al.*, 1990). Thus the most logical approach was to clone the NTR gene by homologous nucleic acid hybridisation using the rat cDNA to probe a λ EMBL3 Balb/c mouse genomic DNA library.

The resulting positive λ clones, from the mouse genomic EMBL3 library, were subjected to basic characterisation. The clones were restriction digested to release their genomic fragments and subsequent hybridisation with probes derived from the rat cDNA allowed the identification of suitable fragments for vector construction. Such fragments were subcloned and restriction enzyme mapped. The isolation of genomic DNA fragments containing exonic NTR sequences was confirmed by DNA sequencing. The sequencing of the gene fragments provided the necessary data for the construction of an insertional targeting vector, based on the 'Hit and Run' procedure presented in Chapter 4, and subsequently for the construction of a replacement type vector, (Chapter 5).

3.2 Hybridisation conditions of library screens

Suitable hybridisation conditions for screening the λ EMBL3 Balb/c library were determined by Southern blot analysis of digested mouse and rat genomic DNA with a rat NTR cDNA derived probe. Typically in such an analysis the starting point would be hybridisation performed at relaxed stringency with low stringency washings. The specificity of the resulting hybridising bands can be increased by altering the stringency of the procedure. Either increase the stringency of hybridisation by raising the temperature or by adding formamide to the buffer. Alternatively the temperature of the washes could have been increased or the salt concentration lowered. However, for this particular experiment the Southern blot was probed at high stringency with formamide based buffer and subsequently washed at high temperature with washings at low salt concentrations. The strategy was one of starting at high stringency, followed by stepwise reduction in stringency, until Southern analysis resulted in a hybridisation profile indicative of a single copy gene.

Rat and mouse DNAs were restriction digested with *Eco*RI and *Bam*HI enzymes. The resulting digests were ethanol precipitated, resuspended in 10 µl of dH_2O and electrophoresed on a 0.7% agarose gel. The gel was stained, photographed and the DNAs transferred to a nylon membrane by Southern blotting. The membrane was pre-hybridised and hybridised in a 50% formamide buffer at 42°C for 16 hours as described in section 2.4.23. The probe used was derived from the 3.6 kb rat cDNA (Tanaka *et al.*, 1990). Only the 1.4 kb 5' portion of the cDNA was used as indicated in Figure 3.1. This terminal *Sma*I portion of the gene was chosen as it contains the majority of the coding region of the NTR gene, from position 171 nt to position 1257 nt. The 3' non-coding sequence was discarded so as to reduce cross-hybridisation of the probe to spurious sequences.

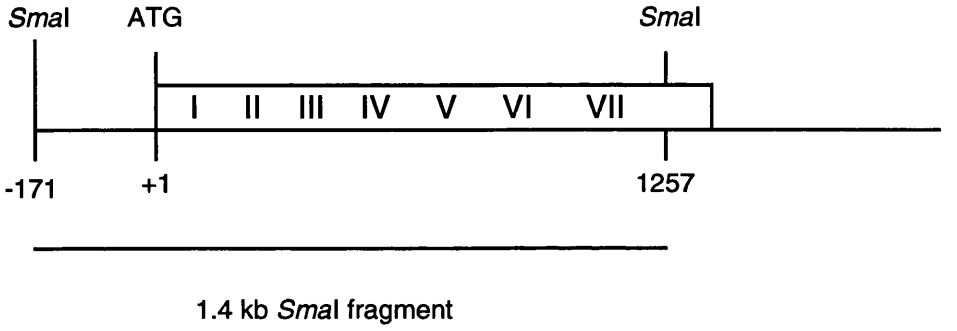


Figure 3.1 Diagram of the 1.4 kb *Sma*I fragment of the rat cDNA. This fragment was used as a probe for Southern analysis and library screening. The nucleotide positions of the *Sma*I sites are indicated relative to the ATG start codon, position +1. The sequence encoding the seven presumptive transmembrane domains are indicated as I-VII.

The 1.4 kb *Sma*I NTR cDNA fragment was purified by first digesting 20 µg of the prNTR2 cDNA with the *Sma*I enzyme in a total volume of 60 µl. The digested DNA was then electrophoresed on a 1.5% LMP agarose gel at 50 volts for 3 hours. The 1.4 kb band was then cut from the gel and purified as described in section 2.4.9. 25 ng to 30 ng of this fragment was then labelled with 50 µCi α ^{32}P dCTP by the random priming method. The resulting probe was then added to the hybridisation mix at 3 X 10^6 dpm/ml hybridisation buffer. The probed membrane was washed as described at a final

wash of 0.2 X SSPE. 0.1% SDS at 60°C for 20 min. The resulting hybridising bands were observed after 3 days autoradiography see Figure 3.2.

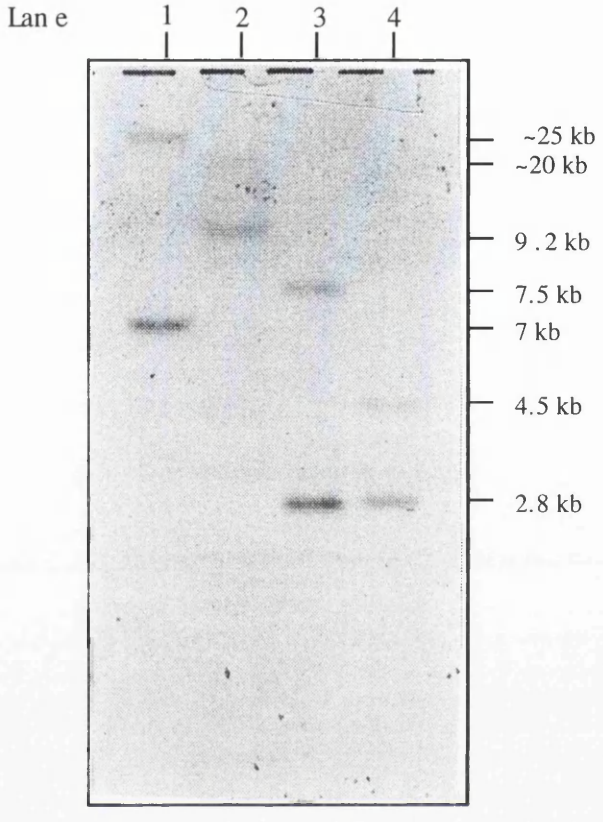


Figure 3.2 Southern blot analysis of mouse and rat gDNAs with the rat NTR cDNA. 10 µg of each sample of DNA was digested with 30 units of either *Bam*HI or *Eco*RI for 3 hours at 37°C. The digested DNAs were resolved on a 0.7% agarose gel. The gel was transferred to a nylon membrane and probed with a ^{32}P labelled 1.4 kb *Sma*I derived fragment of the rat cDNA. The resulting blot was exposed to X-ray film for 3 days. The approximate sizes of the visible hybridising bands are indicated on the right hand side of the Figure.

Lane 1; Rat gDNA digested with *Eco*RI
Lane 2; Mouse gDNA digested with *Eco*RI
Lane 3; Rat gDNA digested with *Bam*HI
Lane 4; Mouse gDNA digested with *Bam*HI

3.3 Results of Library Screen

The number of individual lambda clones from the EMBL3 Balb/c genomic library that had to be screened to give a 99% probability of cloning a single copy gene, was calculated using the formula shown below :

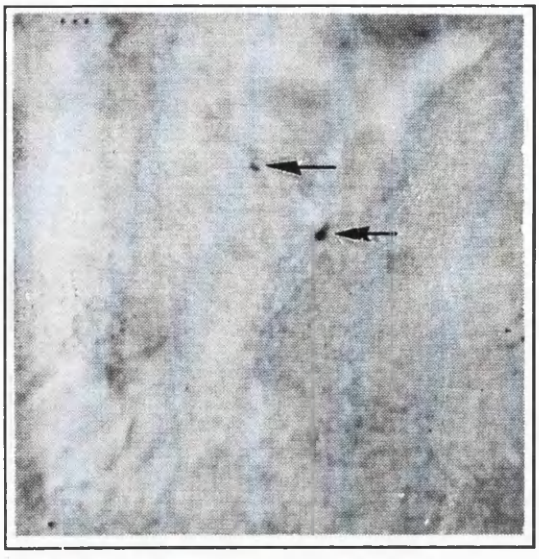
$$N = \frac{\ln(1-P)}{\ln[1-(I/G)]}$$

where: N = the number of clones that must be screened
P = the probability of finding a particular sequence
I = the average insert size of genomic DNA/clone which in this case was 1.5×10^4 bp
G = the haploid size of the genome which in this case is 2.7×10^9 bp for the mouse genome

Thus the number of recombinant clones that had to be screened to give a 99% probability of cloning any single sequence was calculated to be approximately 830,000. Therefore the following library screen was undertaken with a total number of about 1,000,000 clones being screened.

The library was plated onto 10 large plates, (245 mm X 245 mm), as described in section 2.3.16. Replica filters were made and probed with a ^{32}P labelled 1.4 kb *Sma*I fragment of the rat cDNA (specific activity of 1×10^9 dpm/μg) at a concentration of 1.5×10^6 dpm/ml of hybridisation buffer. The filters were washed as described in Material and Methods, (section 2.4.24), with a final wash concentration of 0.2 X SSPE, 0.1 % SDS at 60°C for 20 min. Autoradiography was performed overnight and a total of 10 putative positives were identified; an example of one is shown in Figure 3.3A and its replica filter in Figure 3.3B.

A



B

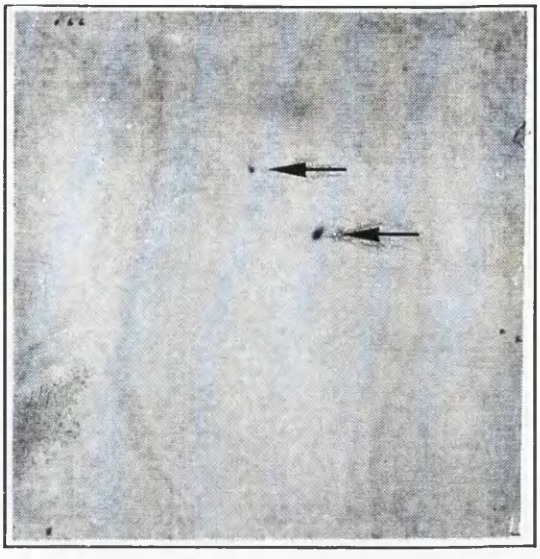
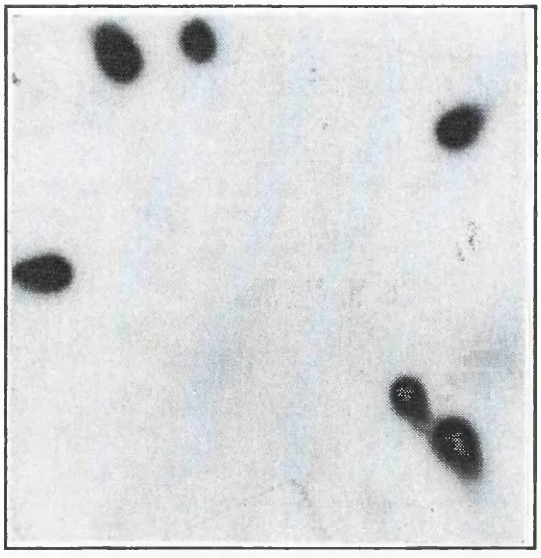


Figure 3.3 Two putative positive plaques on replica filters from the initial library screening. The filters (24 cm X 24 cm) were probed at high stringency in a 50 % formamide based hybridisation buffer with a ^{32}P labelled portion of the rat cDNA, (see text for details). The blot was washed and exposed to X-ray film overnight. The two potentially positive hybridising plaques are indicated by the arrows on filters A and B.

3.4 Secondary and tertiary screening of putative positives clones

The 10 putative positive clones from the library screen were picked as described in section 2.3.19. It was then necessary to purify the λ clones as each plug can contain up to about 85 different p.f.u. The titre of each plug was determined and approximately 300 p.f.u. were plated onto 10 cm X 10 cm plates. Replica filters were made as previously described, with the exception that one set of the replica filters were probed with a dioxigenin (DIG) labelled probe, (section 2.4.20). An example of the resulting autoradiographs are shown in Figure 3.4. Positive plaques were again picked and subjected to a tertiary round of screening. After successful screening, well isolated positive plaques were picked and high titre stocks were made by the plate lysis method described in section 2.3.15. A total of 8 positive λ clones were isolated.

A



B

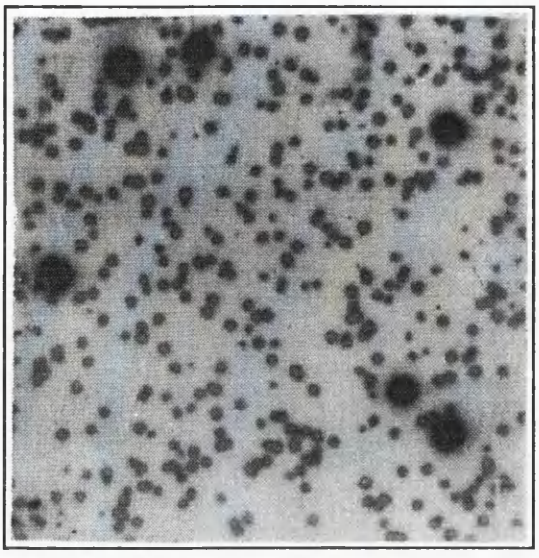


Figure 3.4 Replica filters indicating six positive plaques from a second round library screening of a phage plug taken from the original library. Filter A was prepared and probed as previously described with the exception that only 3 hours autoradiography was needed. Replica filter B was probed with a DIG labelled probe, (Materials and Methods; section 2.4.20), and exposed to X-ray film for only 3 minutes.

3.5 Confirmation of positive lambda clones

The main aim of the following Southern blot analysis was to confirm that the 8 positive λ clones contained DNA inserts that hybridised at high stringency to the rat cDNA. Furthermore, the principle interest was not full characterisation of the l inserts, but the identification of suitable genomic DNA fragments for the construction of a targeting vector. Preferably, a DNA fragment containing 5' coding sequence was sought, since it was intended to mutate the NTR coding sequence as suitably near to the ATG start codon as possible.

DNA was prepared from each of the 8 positive λ clones (ced 1, 2, 4, 5, 7, 8, 9, and ced 10). Each was digested separately with *Eco*RI, *Bam*HI and *Sal*I restriction enzymes. The completed digests were resolved on an 0.7 % agarose gel. The gel was blotted and probed with a 1.4 kb *Sma*I fragment of the rat NTR cDNA, (Figure 3.1). The resulting autoradiograph, (Figure 3.5), confirms the presence of different hybridising insert sizes within the 8 clones. Most interesting was the presence of the 2.8 kb *Bam*HI bands

(lanes 1 and 8), the 4.5 kb *Bam*HI band (lane 4), and the 9.2 kb *Eco*RI band (lane 10), which are identical in size to those observed in the genomic Southern blot, (Figure 3.2).

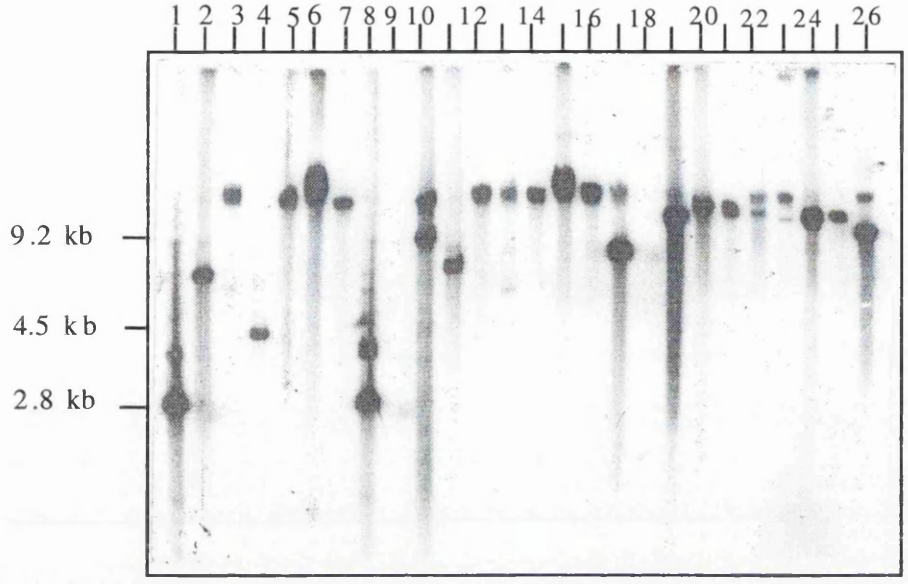


Figure 3.5 Southern blot analysis of the 8 purified λ clones.
About 250 ng of each clone was restricted with *Bam*HI, *Eco*RI and *Sal*I enzymes, resolved on an 0.7% agarose gel, blotted and probed with the ^{32}P labelled 1.4 kb *Sma*I fragment of the rat NTR cDNA at high stringency. The blot was washed at high stringency (final wash 0.2 X SSPE, 0.1% SDS at 60°C). The blot was then exposed to X-ray film for 6 hours and the resulting exposure is shown above. The sizes of some bands are indicated to the left of the figure.

Lane 1; ced1, lane 2; ced2, lane 3; ced4, lane 4; ced5, lane 5; ced7, lane 6; ced8, lane 7; ced9 and lane 8; ced10, are all digested with *Bam*HI.Lane 9; l *Hin*dIII marker. Lane 10; ced 1, lane 11; ced2, lane 12; ced4, lane 13; ced5, lane 14; ced7, lane 15; ced8, lane 16; ced9 and lane 17; ced10; are all digested with *Eco*RI Lane 18; 1 kb markerLane 19; ced 1, lane 20; ced2, lane 21; ced4, lane 22; ced5, lane 23; ced7, lane 24; ced8, lane 25; ced9 and lane 26; ced10; are all digested with *Sal*I.

Unfortunately partial digestion in lanes 1 to 8 has made the results a bit confusing. Nonetheless, the blot was stripped and re-probed with a more defined 458 bp *Pst*I 5' portion of the rat cDNA, (-171 nt to 287 nt). This was done to identify any DNA fragments which may contain sequences

corresponding to the 5' coding region of the NTR gene. The resulting autoradiograph is shown in Figure 3.6

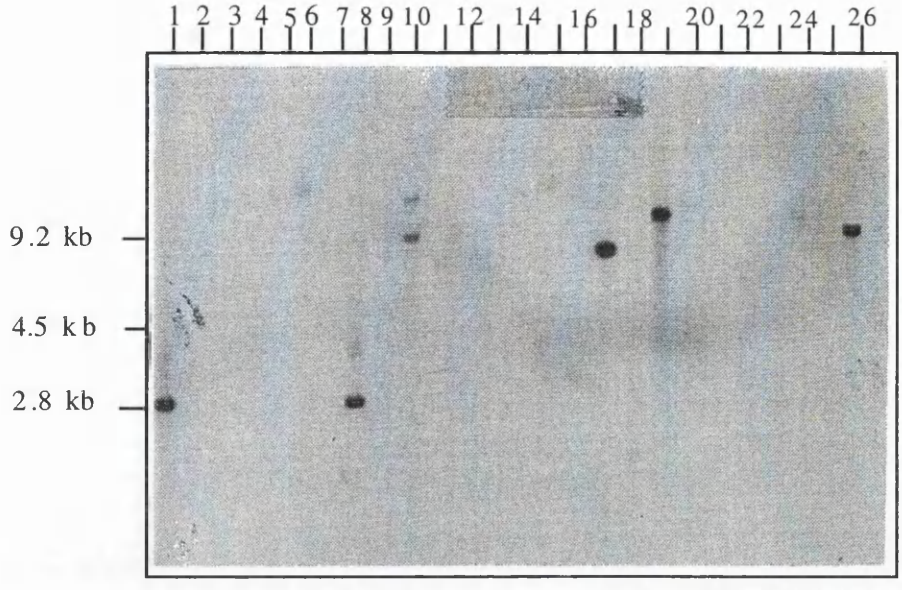


Figure 3.6 Southern analysis of 8 purified λ clones with a defined 5' rat cDNA derived probe. This is the same blot as Figure 3.5 that has been stripped and re-probed with the 458 bp *Pst*I 5' portion of the rat cDNA (-171 nt to 287 nt). Therefore, refer to Figure 3.5 for lane details etc.

3.6 Subcloning of the 2.8 and 4.5 kb *Bam*HI fragment

The initial restriction digest and 5' probing data revealed the presence of a number of different λ clones. Of particular interest was the clones containing hybridising bands of sizes similar to that first observed in the Southern analysis, see Figure 3.2. Therefore the *Bam*HI 2.8 kb band from clone ced1 and the 4.5 kb band from clone ced5 were subcloned into pBluescript II KS^+, (plasmids p2.8B and p4.5B respectively). This was done because if these bands potentially corresponded to coding fragments of the NTR gene, then they would provide large enough segments of DNA for the construction of an insertional type vector similar to that used in the 'Hit and Run' approach to gene targeting, (Hasty *et al.*, 1991a)

Clones ced1 and ced5 were restriction digested with *Bam*HI enzyme and subsequently electrophoresed on a 1% agarose LMP gel. The 2.8 kb and 4.5 kb *Bam*HI bands were then purified from the agarose, (Materials and Methods, section 2.4.9). The purified DNAs were ligated into

the pBluescript II SK$^-$ vector linearised at the *Bam*HI site. The resulting ligations were transformed into electro-competent DH5α cells. The resulting transformed white colonies were picked and grown overnight. The plasmids were purified and subjected to *Bam*HI digestion to verify the presence of the 4.5 kb and 2.8 kb bands.

3.7 Restriction mapping of p2.8B and p4.5B plasmids

The plasmids p2.8B and p4.5B were subjected to basic restriction mapping, as such information would be essential to the design of a targeting construct. The strategy employed was to use 6 base cutter enzymes such as *Eco*RI that were unique to the parental plasmid, (pBluescript II SK$^-$), in the hope of finding restriction sites that occured only once or twice within the insert DNA. The data generated from this initial analysis is summarised in Figures 3.7 and 3.8.

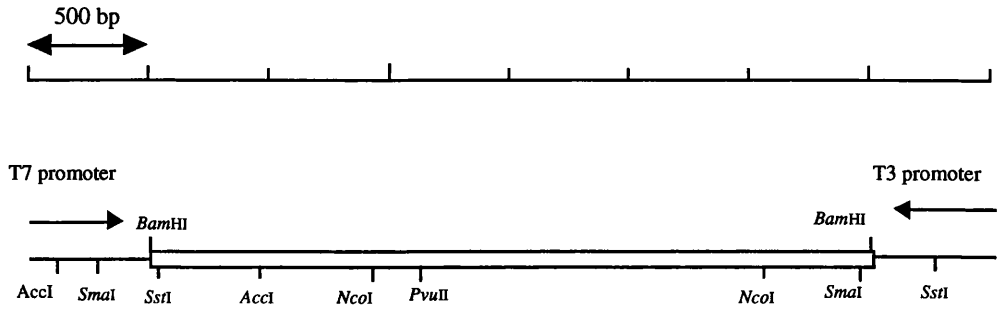


Figure 3.7 Preliminary restriction map of the p2.8B plasmid. The 2.8 kb *Bam*HI fragment was cloned into the *Bam*HI site of the pBluescript SK II$^-$ vector. The known restriction sites within the fragment are shown with selected sites from the vector polylinker, (polylinker sites are not to scale).

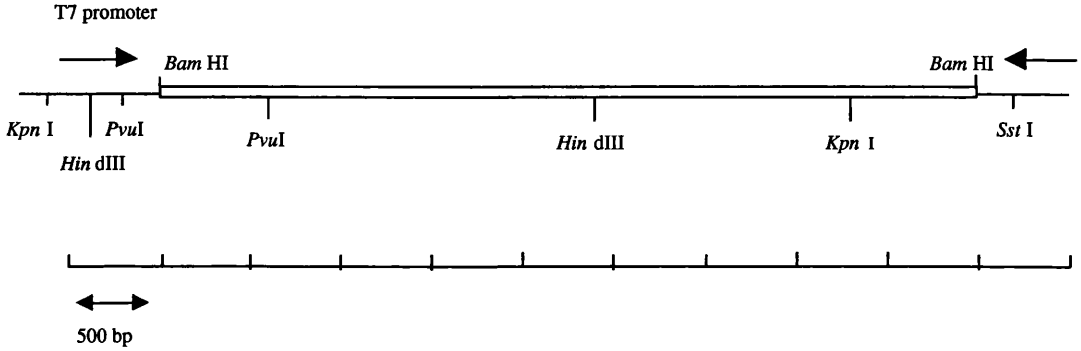


Figure 3.8 Preliminary restriction map of the p4.5B plasmid. The 4.5 kb *Bam*HI fragment was cloned into the *Bam*HI site of the pBluescript SK II$^-$ vector. The

known restriction sites within the fragment are shown with selected sites from the vector polylinker, (polylinker sites are not to scale).

3.8 Generation of a deletion library from plasmid p2.8B

The plasmid p2.8B containing the genomic *Bam*HI 2.8 kb fragment was chosen for further characterisation for the following reasons. Initial double-stranded sequencing from the 5' and 3' ends of the insert revealed sequence homology to the 5' untranslated region of the rat NTR cDNA, (data not shown). Therefore, it was presumed that p2.8B plasmid potentially contained the start of the open reading frame of the mouse NTR gene. Furthermore, the 2.8 kb sized BamHI fragment provided sufficient homology for the design of an insertional vector similar to that used in the approach described by Hasty *et al.* 1991a The p2.8B plasmid was further characterised by sequencing the deletion library created by Exonuclease III digestion of the plasmid.

The procedure used for the generation of the deletion library was based on that of Henikoff *et al.*, 1984. The buffers used were optimised by Tomlinson, 1994, the recipes of which were essentially that detailed in Promega Protocols and Applications guide, (Promega). The complete method used is detailed fully in materials and methods, however a brief outline follows. It was necessary to generate a linear template of the p2.8B plasmid. Typically the DNA template is linearised with two different restriction enzymes; one of which provides 3' protruding ends from which Exonuclease III digestion cannot proceed and the other provides 5' protruding or blunt ended molecules for digestion. A suitable restriction enzyme site leaving a 3' overhang was not available in the p2.8B plasmid . Therefore, the plasmid was linearised with the *Sal*I enzyme which leaves 5' protruding ends. The protruding ends were then filled in using the klenow enzyme to incorporate the appropriate a-phosphorothioate deoxynucleotide derivatives into the overhang. A sample of this protected template DNA was then treated with Exo III enzyme to test the efficiency of the fill-in reaction. The linearised template was then digested with the *Eco*RI enzyme to produce 5' protruding ends. Exo III digestion was then allowed to proceed with the reaction sampled every 40 s. These samples were then subjected to SI nuclease digestion to remove the single stranded tails. The plasmid molecules were then ligated and transformed into DH5α by electroporation. Plasmids were eventually isolated by the 'one step' plasmid prep procedure and resolved on a 1% agarose gel. A number of plasmids were selected on the basis of size and digested with

*Bam*HI enzyme to release the insert DNAs, the results are presented in Figure 3.9.

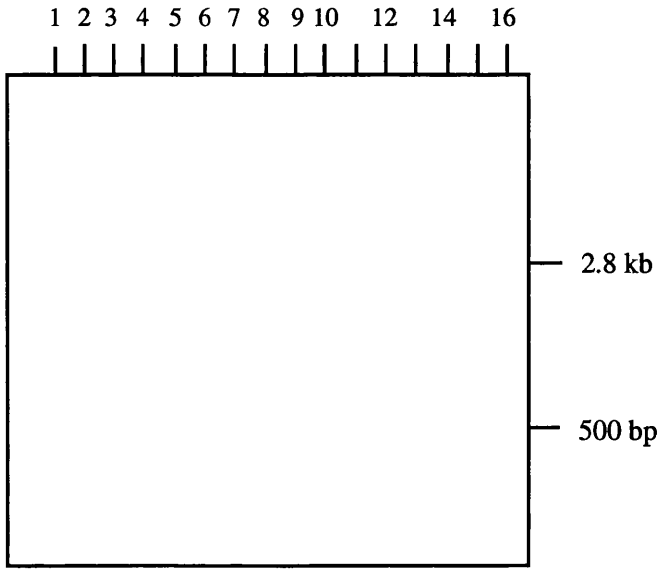


Figure 3.9 The deletion series plasmids generated from the p2.8B plasmid. The deletion plasmids have been digested with *Bam*HI enzyme to free the insert DNA; lanes 3 to 17. The insert DNA ranges in size from 2.8 kb to 500 bp. Lane 1 is λ *Hin*dIII marker.

3.9 Sequence analysis of the p2.8B plasmid deletion library

Creation of a deletion library from the p2.8B plasmid allowed the unidirectional sequencing of individual clones from the plasmids T7 promoter sites to positions progressively within the 2.8 kb *Bam*HI fragment. Miniprep DNA was prepared from each of the differing sized plasmids isolated from the deletion library. The double stranded template DNA was subjected to dideoxy chain termination sequencing method as described in Material and Methods; section 2.4.16. The resulting sequence from the nested set of deleted clones was analysed using the Assembly Align software package from MacVector. This allowed the overlapping sequences to be assembled into a 3 contigs, (Figure 3.10). A nucleotide BLAST search was performed with the 3 contigs; only contig 1 revealed any significant sequence similarity to entries present in the GenBank and EMBL databases. Contig 1 contained sequence that aligned to the rat NTR cDNA, results of the aligned sequences as well as the sequences of contigs 2 and 3 are presented in Figure 3.11., contig 1 was

aligned to the rat cDNA sequence using the GCG Gap Align software programme from GCG.

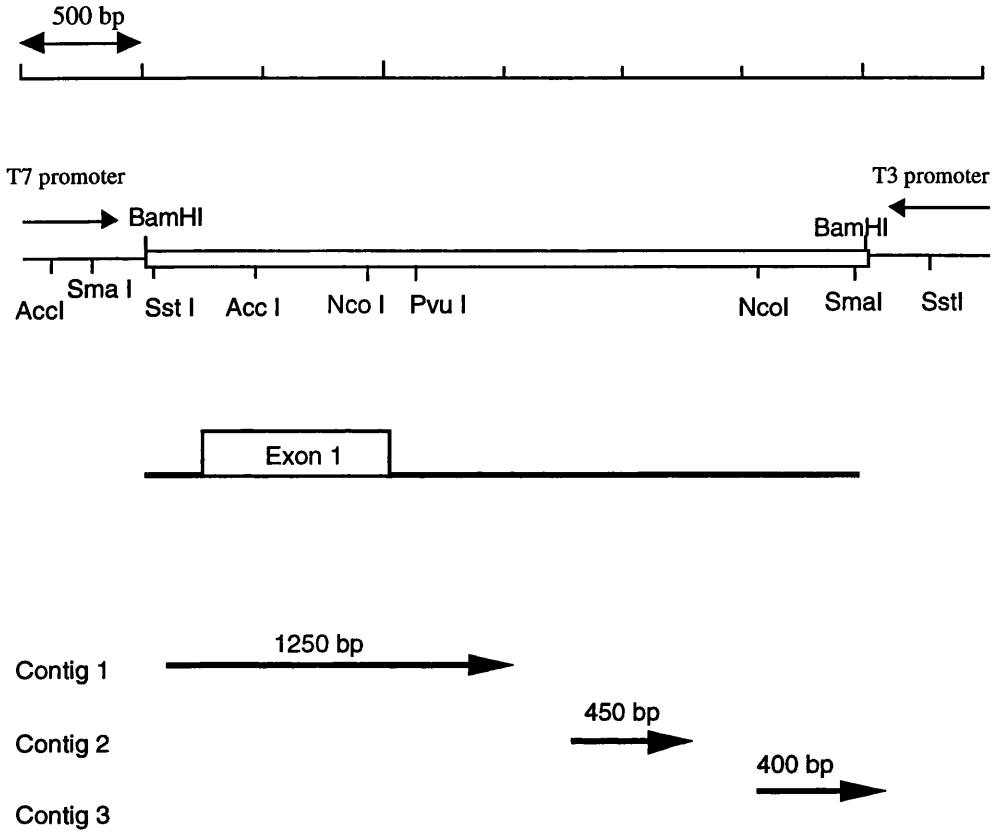
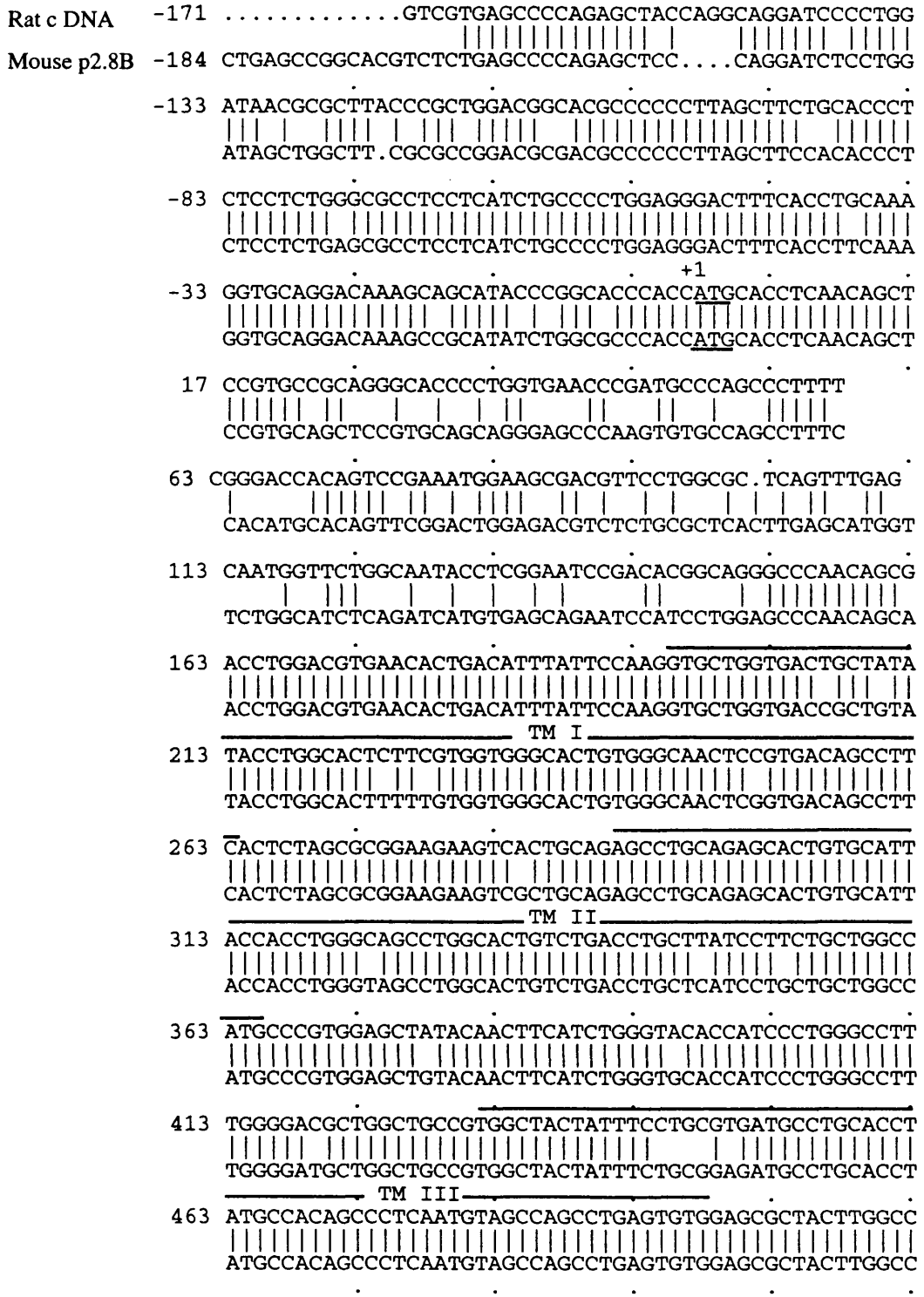


Figure 3.10 The relative position of the 3 contigs derived from the p2.8B plasmid. The sequencing of the subclones derived from the exonuclease deletion library produced 3 non-overlapping clones. There is a gap of approximately 300 nucleotides between contig 1 and contig 2, and a gap of about 200 nucleotides between contigs 2 and 3.


Rat c DNA
Mouse p2.8B
TM I
TM II
TM III

```
612 ATCTGCCATCCCTTCAAGGCCAAGACCCTCATGTGCCGCAGCCGCACCAA
    ||||||||||| |||||||||||||||||||| |||||||||||||
    ATCTGCCATCCCGGCAAGGCCAAGACCCTCATGTCCCGCAGCCGCACCAA
       ┌────────.─────────.────────. TM IV──.────────.
662 GAAATTCATCAGTGCCATATGGCTAGCTTCGGCGCTGCTGGCTATACCCA
    ||||||||||||||||||| |||||| ||||||||||||||| ||||||
    GAAATTCATCAGTGCCATATCGCTAGCCTCGGCGCTGCTGGCTGTACCCA
    ──────────.─────────┐       .         .         .
712 TGCTTTTCACCATGGGCCTG...CAGAACCGCAGTGGTGACGACACGCAC
    |||||||||||||||||||    |||||||||||| || | |  ||||
    TGCTTTTCACCATGGGCCTGACAGAGAACCGCAGTGCCGATGGCCAGCAC
              .         .         .         .         .
759 CCTGGCGGCCTGGTGTG...CACACCCATTGTGGACACAGCCACTGTCAA
    ||||| | || |  |||   ||||||||  |||||||||||||| ||||
762 CCTGGTGACCGGTGGTGTGCCACACCCACGGTGGACACAGCCACCGTCA.
              .         .         .         .         .
806 GGTCGTCATCCAG
     |||||||||||
812 .GTCGTCATCCAGgtaagaac atctaaaagg ggactctctg

     agctgtngca cattagaagg gcacaccang ggcagctggg
     tttcccgtct gggctgtttc tttctattgg arccacacag
     aaacttttag cagtgtcacc tcagaaatyc cggtngattc
     naggcaccac ctggggana acacagccgt gttgccttgt
     tggaaagaac tgtncctcaa aancacttta ttcttcnccn
     ntgtcccaaa gtnggcccnn tnacnnannt nnnnannnan
     nnantcnnnn ancnnrgncn cttagagttg gaanggnggg
     gggcatnacc atcactctct cacttttcat ccatttgcat
     gccctcctn.................................

     .......gap of about 300 nucleotides........

     ..................................ggaattgtcc
     ccntttgagt tacactngcc ctgnctgtga agggangtgg
     atcngnactt ggtcnngagg gcncanagag gggtttgncn
     gcncccatnc taagtcggtc caggncntng ntgttgttan
     ttcagnttct ncataacnnt ctgaactgtc tggggnctga
     ctaggtgccc aggttacnat tgggattatn nacnccattc
     cnctgnggtc tganctttagn tgcaccggan canatgggat
     cccaccncgg taagttcccn ncatactaac ncgacanggn
     ctactncncn tcaggagctg accttgatgt gngaaggggg
     catcggcggg gtcccnanag catattccac ntcncaacct
     catgcatggt tnaanggtgc cgntactcna aaangctnac
     gcatgcanga ttaaccagtt ntgaa................

     .......gap of about 200 nucleotides........

     ..................................gaggtcgact
     cctgccatgt tagagggtcc tccagggaag ctggctgtgc
     catctgtctg tttctttctt caccaagcc  atctatag
     actcccagat cctcacagtg agattctgtg gtcacatggt
     atgtccaggc aacacccaga caccagaaat gctgggtcag
     ttgtaaccca cattccgatg ccaggcaact caacagtgca
     tctctgaggt gggccatggg gataagtgtc cctgtgtgag
     gtcccccctg agctgccact cctcaggggg actgagtcag
     tgcccaatgt ttctaacaag gtcctctgca tttaggcaa
     gtgcacattc gttgggtgtt ccacgtgaca agcagta
```

Figure 3.11 Sequence alignment of the rat NTR cDNA with 5' sequence of the mouse 2.8 kb *Bam*HI fragment. **The sequence on the top line represents the rat cDNA, with the lower line sequence being derived from mouse genomic DNA.** The sequences are numbered relative to the predicted underlined ATG start codons, i.e. +1. The putative transmembrane domains are indicated, I-IV, on the basis of the hydropathic profile predicted from the rat cDNA sequence. The 3' sequence of contig 1 and all of contigs 2 and 3 are presented in lowercase and do not reveal significant similarity to the cloned neurotensin receptors or any other sequences present in the GenBank or EMBL databases.

Comparison of the rat cDNA sequence to the mouse genomic sequence, revealed a similarity of 84% over the first 890 nucleotides of rat sequence. However, the majority of sequence divergence occurred in the region of the gene encoding the putative N-terminal portion of the receptor. Generally this is a highly variable region of G-protein linked receptors, (Burbach and Meijer, 1992). When the sequence encoding the putative transmembrane domains I to IV, the first two intracellular domains and the first extracellular domain are considered separately from the N-terminal sequence, then the sequence similarity between the 2 species rises to 97%.

The mouse sequence was translated using GCG software to provide the predicted amino acid sequence. The peptide sequence was used to search the EMBL and GenBank databases with the GCG BLAST programme. The closest matches are presented as a table of amino acid identities at the foot of Figure 3.12. which also provides an alignment of the 4 receptor sequences. The derived amino acid sequence shares a high level of identity with the human and rat high affinity neurotensin receptors 84% and 79% respectively. The next highest significant identity was to the low affinity mouse receptor (41%). The database search also revealed lower identities (<25%) to other members of the G-protein linked family of receptors.

```
      1                                                  50
rat NTR-1     MHLNSSVPQG TPGEPDAQPF SGPQSEMEAT FLALSLSNGS GNTSESDTAG
human NTR-1   MRLNSSAP.G TPGTPAADPF QRAQAGLEEA LLAPGFGNAS GNASERVLAA
mouse NTR-X   MHLNSSVQLR AAGSPSVPAF PHAQFGLETS LRSLSMVLAS QIMSAESILE
mouse NTR-2   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~METSSLWP PRPSPSAGLS
Consensus     M-LNSS---- --G-P----F ---Q---E-- ---------S ---S------

              51                                              100
rat NTR-1     PNSDLDVNTD IYSKVLVTAI YLALFVVGTV GNSVTAFTLA RKKSLQSLQS
human NTR-1   PSSELDVNTD IYSKVLVTAV YLALFVVGTV GNTVTAFTLA RKKSLQSLQS
mouse NTR-X   PTSNLDVNTD IYSKVLVTAV YLALFVVGTV GNSVTAFTLA RKKSLQSLQS
mouse NTR-2   LEARLGVDTR LWAKVLFTAL YSLIFALGTA GNALSVHVVL KART..GRPG
Consensus     P-S-LDVNTD IYSKVLVTA- YLALFVVGTV GN-VTAFTLA RKKSLQSLQS
                                   TM1

              101          +                          *    *     ++ 150
rat NTR-1     TVHYHLGSLA LSDLLILLLA MPVELYNFIW VHHPWAFGDA GCRGYYFLRD
human NTR-1   TVHYHLGSLA LSDLLTLLLA MPVELYNFIW VHHPWAFGDA GCRGYYFLRD
mouse NTR-X   TVHYHLGSLA LSDLLILLLA MPVELYNFIW VHHPWAFGDA GCRGYYFCGD
mouse NTR-2   RLRYHVLSLA LSALLLLLIS VPMELYNFVW SHYPWVFGDL GCRGYYFVRE
Consensus     TVHYHLGSLA LSDLL-LLLA MPVELYNFIW VHHPWAFGDA GCRGYYF-RD
                        TM2

              151                                             200
rat NTR-1     ACTYATALNV ASLSVERYLA ICHPFKAKTL MCRSRTKKFI SAIWLASALL
human NTR-1   ACTYATALNV ASLSVERYLA ICHPFKAKTL MSRSRTKKFI SAIWLASALL
mouse NTR-X   ACTYATALNV ASLSVERYLA ICHPGKAKTL MSRSRTKKFI SAISLASALL
mouse NTR-2   LCAYATVLSV ASLSAERCLA VCQPLRARRL LTPRRTCRLL SLVWVASLGL
Consensus     ACTYATALNV ASLSVERYLA ICHP-KAKTL M-RSRTKKFI SAIWLASALL
                     TM3                                TM4

              201                                       242
rat NTR-1     AIPMLFTMGL ..QNRSGDDT HPGGLVC.TP IVDTATVKVV IQ
human NTR-1   TVPMLFTMGE ..QNRSADGQ HAGGLVC.TP TIHTATVKVV IQ
mouse NTR-X   AVPMLFTMGL T.ENRSADGQ HPGDRWCATP TVDTATVSVV IQ
mouse NTR-2   ALPMAVIMGQ KHEMERADGE PEPASRVCTV LVSRASSRST FQ
Consensus     A-PMLFTMG- ---NRSADG- H-G---C-TP -V-TATV-VV IQ
                                                  TM5
```

Receptor	Description	Human NTR-1	Mouse NTR-X	Mouse NTR-2
Rat NTR-1	High affinity neurotensin receptor	73.2%	78.2%	41.0%
Human NTR-1	High affinity neurotensin receptor	-	79.0%	40.0%
Mouse NTR-X	Putative high affinity neurotensin receptor	79.0%	-	41.0%
Mouse NTR-2	Low affinity neurotensin receptor	40.0%	41.0%	-

Figure 3.12 Sequence alignment of the putative mouse NTR to other members of the NTR family. The amino acid sequences of the human, rat and mouse neurotensin receptors were aligned with the putative mouse receptor sequence using the GCG multiple alignment programme PILEUP. A table of identitites was also produced using the GCG programme DISTANCES. The predicted transmembrane domains of the receptors are underlined, based on the rat

receptor cDNA sequence (Tanaka *et al.* 1990). Those amino acid residues that have been identified as critical for the ligand binding are highlighted with *, and those that lower binding activity by 2-3 fold with +, Botto *et al.*, 1997.
NTR-1, high affinity neurotensin receptor; NTR-2, low affinity neurotensin receptor; NTR-X, putative high affinity mouse neurotensin receptor (sequence derived from clone p2.8B).

Primary sequence identity of G-protein linked receptors typically display sequence identity across the transmembrane domains of 85-95% for species homologues, to 60-80% for related receptor subtypes, down to 35-45% for related family members and down to 20-25% for unrelated G-protein linked receptors, (Strader *et al.*, 1994). Similarly, when the sequence identity of the putative mouse NT receptor was compared to the rat and human high affinity receptors, for the amino acid sequence across the first 4 transmembrane domains, then a sequence identity of 96% and 94% respectively was recorded. These data suggests that the sequence encoded within clone p2.8B is an orthologue of the high affinity neurotensin receptor or at the very least a subtype of the receptor.

In general, most of the variability between G-protein linked receptors is in the N-terminal extracellular domain, the third intracellular loop and the C-terminal domain. It is also apparent from Figure 3.11, for the portion of the neurotensin receptor depicted, that the most variable part of the protein is the N-terminal domain as highlighted by the lack of consensus between all 4 sequences. However, this region of G-protein linked receptors has been shown to be critical, as in the case of neurokinin receptors for the binding of the respective ligand, (Fong *et al.*, 1992). A number of residues namely N23, Q24 and F25 have been shown to be necessary for high affinity binding. In contrast, the lutropin receptor has a long N-terminal domain (338 amino acids), and when expressed in the absence of this domain the mutant receptor still possess high affinity and specificity for its ligand luteinizing hormone, (Tsai-Morris *et al.*, 1990). In general peptide binding G-protein linked receptors do not have stretches of conserved amino acids that are critical for ligand binding but rather specific amino acids within domains. Binding affinity studies of neurotensin to its receptor have also identified a number of residues which are essential for high affinity binding. Eight residues located within transmembrane domain III have been demonstrated by mutation studies to be important for neurotensin binding, (Pang *et al.*, 1996). A more recent study, Botto *et al.* 1997, has extended this work to identify two residues Asp139 and Arg143 that when mutated abolish the ability of

neurotensin to bind to the receptor. These residues are conserved within the predicted coding sequence derived from the p2.8B clone, Figure 3.12. Therefore, the lack of overall sequence conservation between the species may reflect the involvement of only a small number of amino acid residues in the receptors specificity for neurotensin.

Examination of the peptide sequence derived from the mouse clone p2.8B predicts that the coding sequence terminates within transmembrane IV at residue Q242, (---VIQ.). This termination of coding sequence is in agreement with the defined intron /exon structure of the human high affinity neurotensin receptor, see Figure 1.3. Furthermore, the mouse genomic sequence observes the AG-GT consensus sequence often found at exon-intron boundaries. This is further evidence to suggest that the genomic clone p2.8B encodes part of the high affinity neurotensin receptor.

3.10 Future confirmation of the putative mouse neurotensin receptor

The sequence from clone p2.8B suspected to encode the high affinity neurotensin receptor could be used as a probe to screen a mouse cDNA library in order to isolate the corresponding clones. Sequencing and subsequent expression studies of isolated cDNAs would verify the validity of the p2.8 clone encoding the high affinity receptor. Full length cDNAs could be expressed in a cell line that does not normally express the receptor thus allowing ligand binding assays to be performed with neurotensin and its analogues. Alternatively, cDNAs could be expressed in Xenopus oocytes, subsequent electrophysiological measurements could be performed to confirm the receptor's functionality in response to neurotensin. Supporting evidence to authentic the p2.8B clone could also come from chromosomal localisation of this genomic clone. The rat cDNA has previously been used to assign the NTR gene to the H region of mouse chromosome 2, Laurent *et al.*, 1994. It would be expected that clone p2.8B would also map to this region.

3.11 Summary

Conditions for the successful screening of a mouse genomic DNA library, with a rat NTR cDNA derived probe, were defined by Southern blot analysis. A high stringency probing was performed with high stringency washings on digested mouse and rat genomic DNAs. This produced hybridising bands on a Southern blot with a profile typical of a single copy gene. For mouse genomic DNA; *Eco*RI bands of 9.2 kb and about 20 kb, and *Bam*HI bands of 2.8 kb and 4.5 kb were observed. Therefore, these conditions were used to screen a bacteriophage λ library, (EMBL3; Balb/c). After tertiary screening, 8 positive λ clones were isolated. Initial analysis suggested that two of these clones contained the 2.8 kb *Bam*HI band, (ced1 and ced10), and the 4.5 kb band, (ced5), both band sizes were previously observed with the initial Southern blot analysis, (Figure 3.2). The 2.8 kb and the 4.5 kb *Bam*HI bands were subcloned and subjected to sequence analysis. The 2.8 kb band contained a potential large open reading frame encoding sequence with 84% homology to the first 890 nucleotides of the rat high affinity NTR cDNA. The sequence homology increased to 97% when only the sequence encoding the transmembrane domains I-IV was considered. Furthermore, the predicted peptide sequence of the isolated mouse clone p2.8 has a high identity to the human and rat high affinity neurotensin receptors, 84% and 79% respectively.

The principle aim of these experiments was to identify suitable mouse genomic DNA fragment(s) of the NTR gene for the design of a targeting construct. Therefore, the isolation of the 2.8 kb *Bam*HI fragment containing homology to the 5' coding sequence of the rat cDNA satisfied this criterion. It contained suitable coding sequence that could be specifically mutated and presented a sufficiently large piece of homology for the design of an insertional type targeting construct similar to that of Hasty *et al.*, 1991a. The design of such a construct and the principles behind the gene targeting approach employed, are detailed in Chapter 4.

Chapter 4

A 'Hit and Run' Approach to Targeting the Neurotensin Receptor gene

4.1 Introduction

The primary aim of this section of work was to produce a targeting construct that could be used to mutate an allele of the NTR gene in embryonic stem (ES) cells. The various different approaches considered for targeting the NTR gene are detailed in Chapter 1. The most attractive strategy at the time, (the summer of 1992), was to generate an integrative construct for the NTR gene and employ the two-step recombination procedure successfully used by Hasty *et al.*, 1991a.

The two-step recombination strategy, also known as the 'Hit and Run' method was chosen in preference to the other approaches for several reasons. It was reported by Hasty *et al.* 1991b, that higher gene targeting frequencies were obtained using integrative targeting vectors. In their study they observed 5 to 12 fold higher targeting frequencies using integrative vectors as opposed to replacement vectors of the same length. This is because the vector is linearised within the homologous gene sequence providing molecules for recombination with homology at both ends and thus superior to molecules with homology only at one end. Therefore, smaller gene fragments are required using this method. Furthermore, the two-step system allows the creation of site specific mutations within the target gene. Although, the original modification to the NTR gene was to create a null mutation, the development of the 'Hit and Run' procedure was seen to be invaluable to the creation of more specific subtle mutations via a second generation of targeting constructs.

The 'Hit and Run' procedure itself, introduces its site specific mutation into the target gene by a two step process. The targeting construct contains the homologous gene sequence with its engineered mutation. This sequence is flanked on either side with a neomycin and a thymidine kinase cassettes. This allows the positive selection of integration via neomycin, and reversion via selection for the loss of the thymidine kinase cassette. The construct is linearised within the region of gene homology, such that the first step of the targeting requires the insertion of the targeting vector at the gene locus by reciprocal recombination. This integration results in the duplication of the gene sequence used in the targeting construct separated by the neomycin cassette, the thymidine kinase cassette and the accompanying plasmid sequences. The second step of the procedure involves selection for resolution of the duplication by a single reciprocal intrachromosomal event, referred to as reversion. This reversion results in the removal of the neomycin

and thymidine kinase cassettes, leaving behind one copy of the gene. Since recombination can occur at any position between any two homologous sites of the duplication, some of the revertant clones will retain the desired mutation and some will be wild type. This procedure is illustrated in Figure 4.1, with the desired reversion event shown, (reproduced from Chapter 1 for the readers convenience).

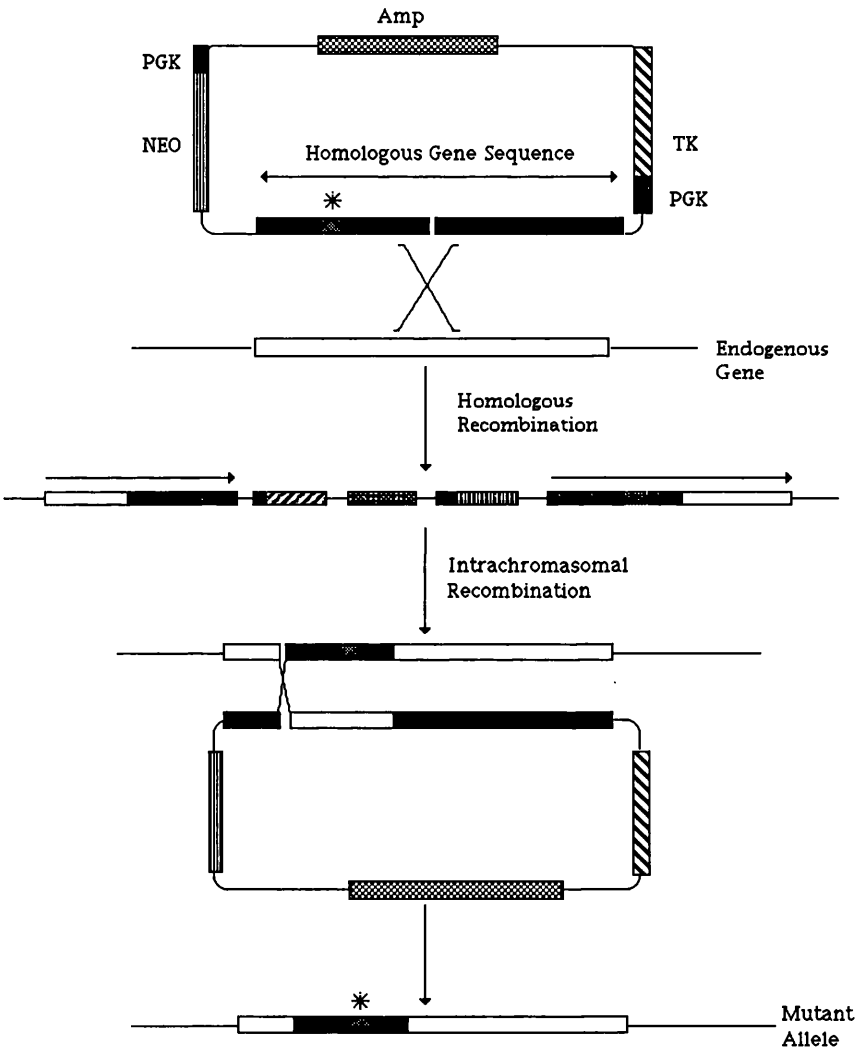


Figure 4.1 The 'Hit and Run' procedure. The diagram shows an insertional vector containing a neomycin cassette (NEO), a thymidine kinase cassette (TK), the plasmid β-lactamase gene conferring ampicillin resistance (Amp), phosphoglycerate kinase promoter (PGK), and the mutated gene site is indicated by an *. The method is a two step recombination process; homologous recombination of the vector at the gene locus results in a

duplication that can be subsequently resolved by intrachromosomal recombination. The reversion event has resulted in the mutated sequence being retained within the endogenous gene. Refer to section 4.1 for a more detailed explanation.

4.2.1 Design of an insertional targeting vector for the NTR gene

The insertional vector for the disruption of the NTR gene was based on the p2.8B plasmid containing the coding sequence for the first 242 aa residues of the receptor. The strategy was to introduce a linker into the coding sequence at the *Acc*I restriction site (GTATAC) at nucleotide position 210-215. The linker would introduce a stop codon within the region of the gene encoding the first transmembrane domain (TM) of the receptor at aa position 71. However, the parental plasmid also contained an *Acc*I site within its polylinker. Therefore, the plasmid *AccI* restriction site had to be removed before the introduction of the mutagenic linker into the unique *Acc*I site of the gene sequence. Finally, the mutated 2.8 kb *Bam*HI fragment was subcloned into the *Bam*HI site of the pNT plasmid. This plasmid contains a neomycin and thymidine kinase cassettes on either side of the *Bam*HI cloning site. The completed construct can then be linearised within the region of homology at the unique *Nhe*I site (GCTGTA), at nucleotide position 603-608, (within transmembrane domain IV). The completed construct and construction steps are summarised in Figure 4.2.

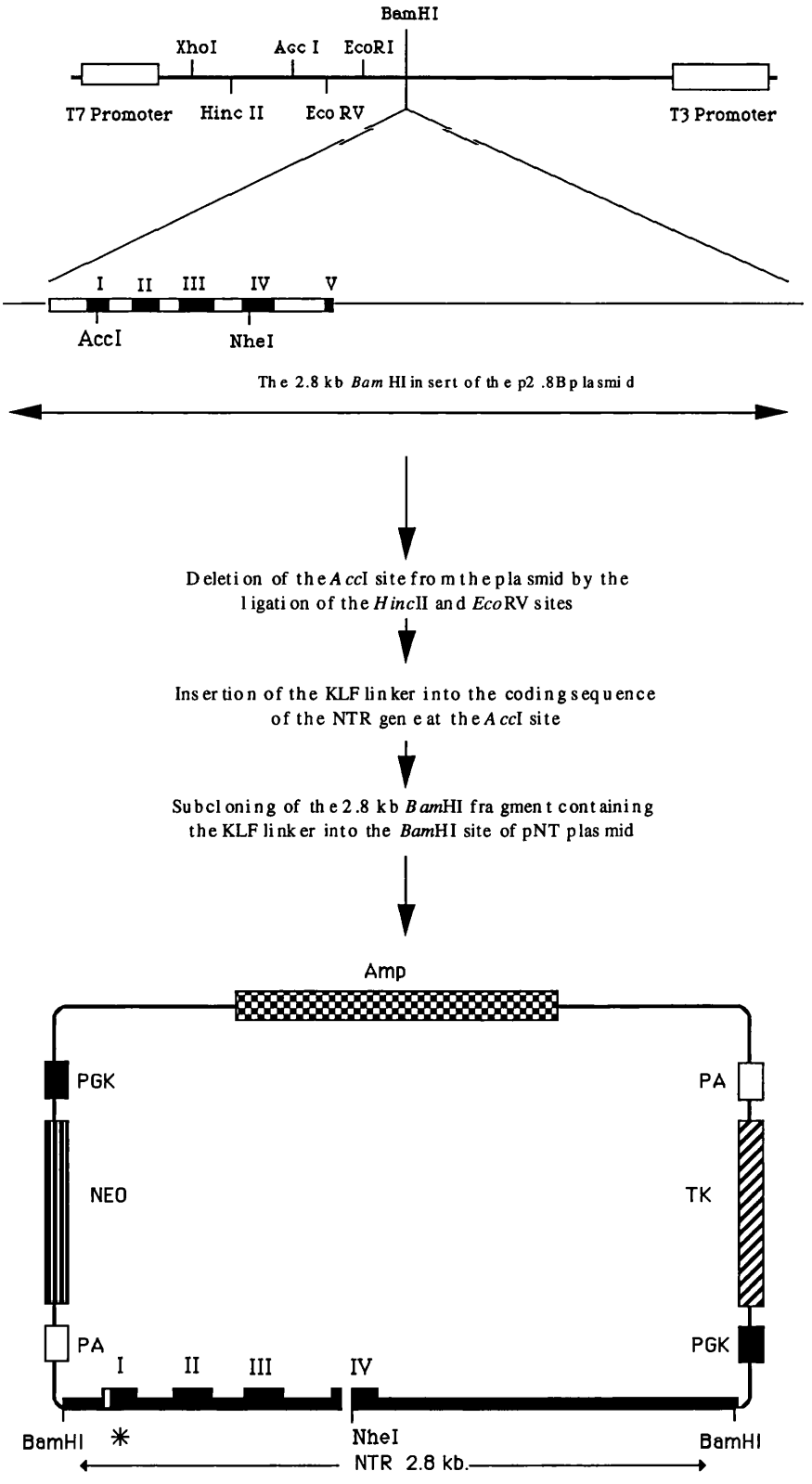


Figure 4.2 The strategy for creating an insertional type vector for targeting of the mouse NTR gene. The completed vector, (pKLF), is shown linearised at the unique *Nhe*I site. The asterisk denotes the linker insertion site. The location of

sequences encoding the transmembrane domains I-IV; PGK phosphoglycerate kinase promoter; PA SV40 polyadenylation site; AMP, b-lactamase gene; NEO, neomycin phosphotransferase gene; TK, HSV thymidine kinase gene.

4.2.2 Verification of the genomic 2.8 kb *Bam*HI fragment

Prior to the construction of the targeting vector it was advisable to check that the mouse 2.8 kb *Bam*HI fragment was homologous to the the rat sequence. This question was addressed by Southern blot analysis of mouse genomic DNA using a probe derived from the non coding sequence of the subcloned 2.8 kb *Bam*HI fragment. The 1.5 kb *Pvu*II fragment that was used as a probe is diagramatically portrayed in Figure 4.3.

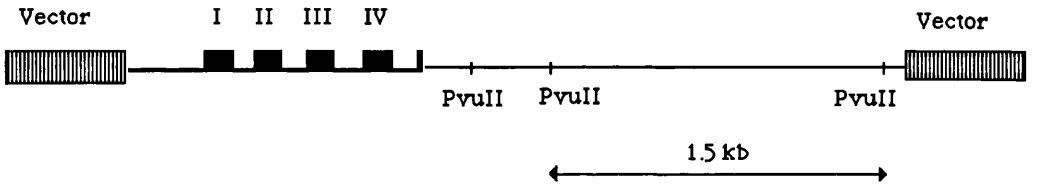


Figure 4.3 The 1.5 kb *Pvu* II probe fragment. The above diagram represents the p2.8B plasmid. The presumptive transmembrane domains are represented (I to IV) and the relative positions of the *Pvu*II restriction sites are shown.

The high stringency Southern blot analysis suggests that the sub-cloned 2.8 kb *Bam*HI mouse fragment is homologous to the genomic fragment of the rat NTR gene. The autoradiograph of the Southern blot of the various digested genomic mouse and rat DNAs with plasmid controls is shown in Figure 4.4. Lane 1 depicts a mouse 9.2 kb *Eco*RI hybridising band of the same size as that previously observed using a probe derived from the rat NTR cDNA, (Figure 3.2). Lane 2 contains a 2.8 kb *Bam*HI rat band which gives a weak hybridisation signal relative to the expected mouse *Bam*HI band of the same size. Presumably this is because of greater sequence divergence between intronic sequences of different species. Lane 4 shows a hybridising 1.5 kb *Pvu*II mouse band as expected. Lane 5 contains a hybridising *Kpn*I band of about 9 kb. The control lanes, (6, 7 and 8), of the *Bam*HI digested p2.8B plasmid produced the expected hybridising bands of 2.8 kb in size.

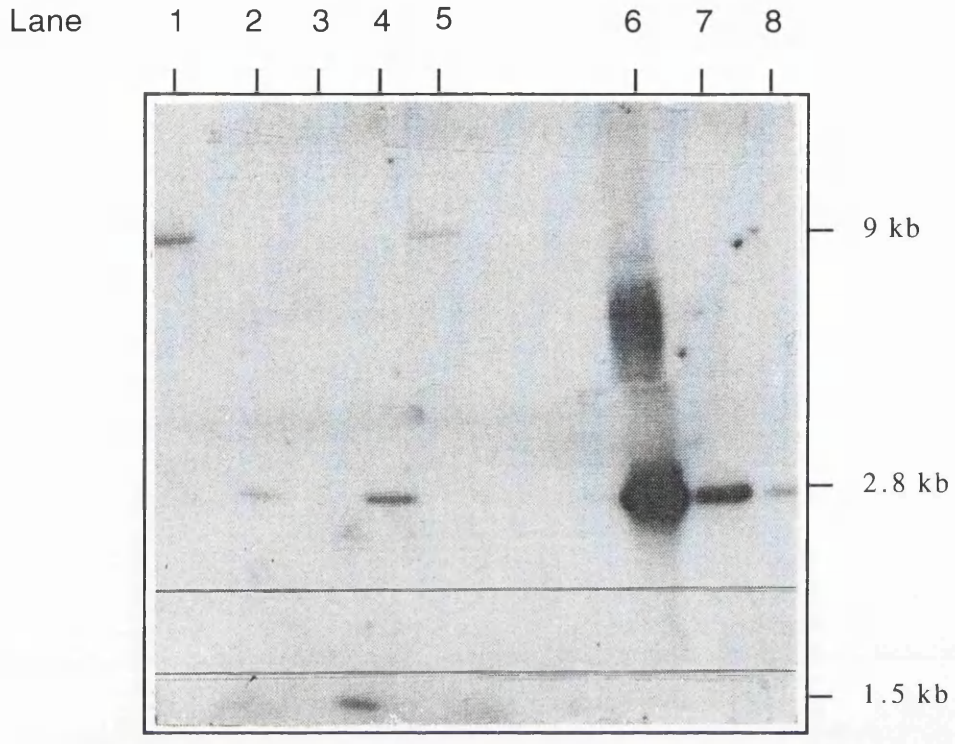


Figure 4.4 Genomic Southern blot analysis of mouse and rat DNAs, probed with a 1.5 kb *Pvu*II fragment derived from the p2.8B plasmid. 10 µg of each genomic DNA was digested with the appropriateenzyme, resolved on an 0.7% agarose gel, blotted on to a nylon membrane and probed with the aforementioned ^{32}P labelled probe. The resulting 3 day autoradiograph is shown above with the hybridising band sizes indicated to the right of the Figure.

Lane 1; 10 µg mouse gDNA/*Eco*RI
Lane 2; 10 µg rat gDNA/*Bam*HI
Lane 3; 10 µg mouse gDNA/*Pvu*II
Lane 4; 10 µg mouse gDNA/*Bam*HI
Lane 5; 10 µg mouse gDNA/*Kpn*I
Lane 6; 5 pg of plasmid p2.8B/*Bam*HI
Lane 7; 0.5 pg of plasmid p2.8B/*Bam*HI
Lane 8; 50 fg of plasmid p2.8B/*Bam*HI

4.2.3 Deletion of the *Acc*I site from the p2.8B plasmid

Prior to the addition of a mutating linker, it was necessary to remove the parental plasmid derived *Acc*I restriction site from the p2.8B plasmid. Removal of the *Acc*I restriction site was achieved by double digestion of the p2.8B plasmid with enzymes flanking the *Acc*I site. The p2.8B plasmid was double digested with *Eco*RV and *Hinc*II enzymes. This resulted in the removal of the *Acc*I site. The resulting linearised plasmid was band purified from a 1% LMP agarose gel to remove it from the intervening 20 bp fragments. The purified plasmid was then blunt-end ligated overnight and transformed into DH5α cells. The resulting colonies were picked, grown overnight, plasmids purified and subjected to *Acc*I restriction digestion. Plasmid with the *Acc*I site removed were only linearised on digestion as opposed to producing a 420 bp band. Before proceeding to the next cloning step, selected plasmids were sequenced to confirm the predicted sequence junction at the site of deletion. The sequence of one such plasmid, (p2.8BA$^-$), is presented in Figure 4.5.

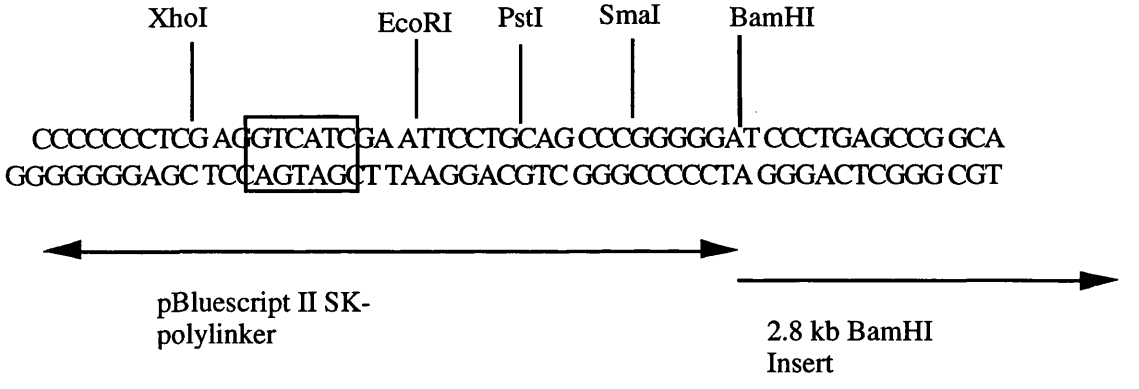


Figure 4.5 Sequence from the p2.8BA$^-$ plasmid polylinker. The sequence shown is that of the vector polylinker where the ligation junction between the *Hinc*II and *Eco*RV restriction sites are indicated as boxed, (5' gtcatc 3'). The start of the 2.8 kb *Bam*HI insert sequence is also shown.

4.2.4 Insertion of the KLF linker into the p2.8BA$^-$ plasmid

Insertion of the linker termed KLF into the p2.8BA$^-$ plasmid was undertaken in order to mutate the coding sequence of the NTR gene. The linker was designed such that it would introduce a stop codon into the NTR gene at nucleotide position 210-215, i.e. at aa position 71 of the receptor, thus

disrupting the gene within the first transmembrane domain. The linker also introduces a unique *Kpn*I restriction site which can be used to facilitate the analysis of potentially targeted clones. The KLF linker was made by designing a single 18 mer oligonucleotide which was partially complementary to itself, such that on annealing it formed a duplex with sticky ends compatible with a cut *Acc*I restriction site.

Firstly, the p2.8BA$^-$ plasmid was linearised at its unique *Acc*I site and treated with shrimp alkaline phosphatase, (SAP), to dephosphorylate its ends . The KLF linker was prepared by kinasing the oligonucleotide to phosphorylate the ends of the molecules. Prior to ligation, the oligonucleotide was heated to 95°C for 5 minutes and then allowed to cool to room temperature slowly. The linker was then added to the linearised p2.8BA$^-$ at 10 and 50 molar excess and ligated overnight. The resulting ligations were transformed, colonies picked, grown and the resulting purified plasmids analysed for successful insertion of the linker by *Kpn*I digestion. The new *Kpn*I site produces a 460 bp band on digestion if the plasmid contains the KLF linker, (Figure 4.6). One in every three plasmids analysed contained the linker. The selected plasmids were subsequently checked for a single correct insertion of the KLF linker by double stranded sequencing using a specifically designed oligonucleotide that hybridised upstream of the insertion site. The plasmid sequence across the site of insertion and details of the KLF linker are given in Figure 4.7

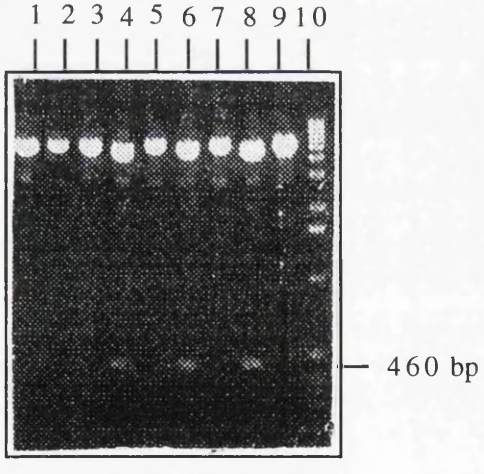


Figure 4.6 Restriction analysis of p2.8B plasmids containing the KLF linker. Plasmids were digested with *Kpn*I to identify those that contained the KLF linker. Lanes 4, 6 and 8 contain plasmids with the KLF linker; lanes 1, 2, 3, 5, 7 and 9 are p2.8 plasmids cut with *Kpn*I; lane 10 is 1 kb marker.

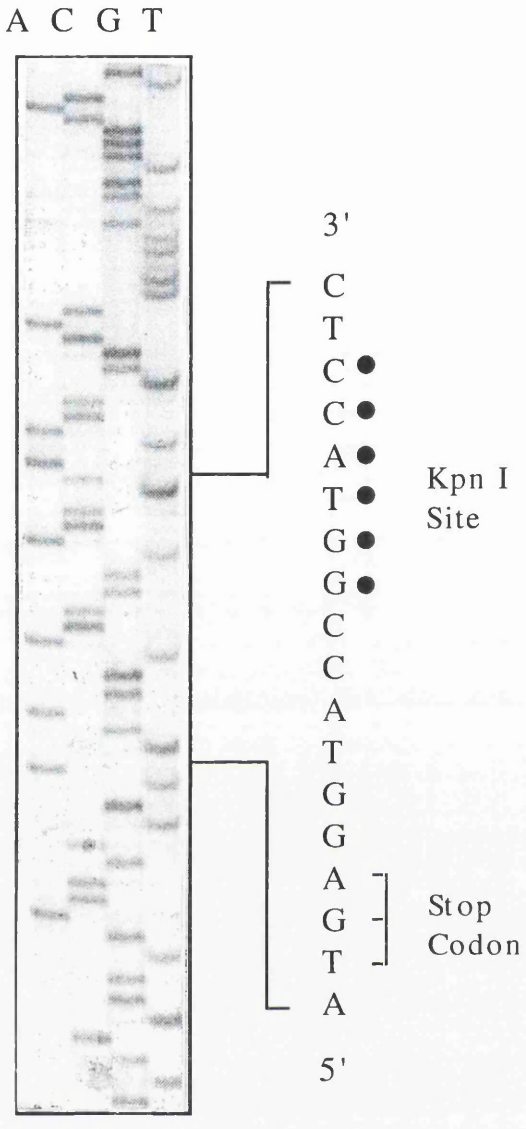


Figure 4.7 Sequence of the KLF linker across the *Acc*I site of insertion. The sequence is from the single insertion of a KLF linker into the p2.8BA$^-$ plasmid. The in-frame stop codon and *Kpn*I restriction site that the linker introduces are indicated.

4.2.5 Subcloning of the 2.8 kb *Bam*HI fragment of the p2.8BA$^-$ plasmid into the pNT plasmid

The final step in the creation of an insertional vector, (pKLF), was to subclone the 2.8 kb *Bam*HI fragment from of the p2.8BA$^-$ plasmid into the pNT plasmid. The p2.8BA$^-$ plasmid was digested with the *Bam*HI enzyme. The resulting digest was resolved on a 1.2% LMP agarose gel and the 2.8 kb *Bam*HI band was purified. This DNA fragment was then ligated into the dephosphorylated *Bam*HI cut pNT plasmid at a vector to insert ratio of 1:3 and 1:1. The ligations were then transformed into DH5a cells; resulting colonies were picked, grown overnight, and plasmids purified by the 'one step' mini-prep method. This allowed plasmids to be resolved on a 1% agarose gel and successful subclones to be isolated on the basis of size. However, presumptive positives were subjected to *Bam*HI digestion to confirm the presence of the 2.8 kb insert. Furthermore, the orientation of the insert was determined by *Sst*I digestion, since the insert contains a single *Sst*I site 35 bp from one end. The pNT plasmid contains 3 *Sst*I sites, producing two distinctive banding patterns on digestion (approximate sizes of either 4.4 kb, 2.8 kb, 1.9 kb and 1.2 kb or 4.7 kb, 4.4 kb, 1.2 kb and 60 bp). Figure 4.8 presents an example of such an analysis. In total, 4 such plasmids were isolated, known as pKLF(A, B, C, and D).

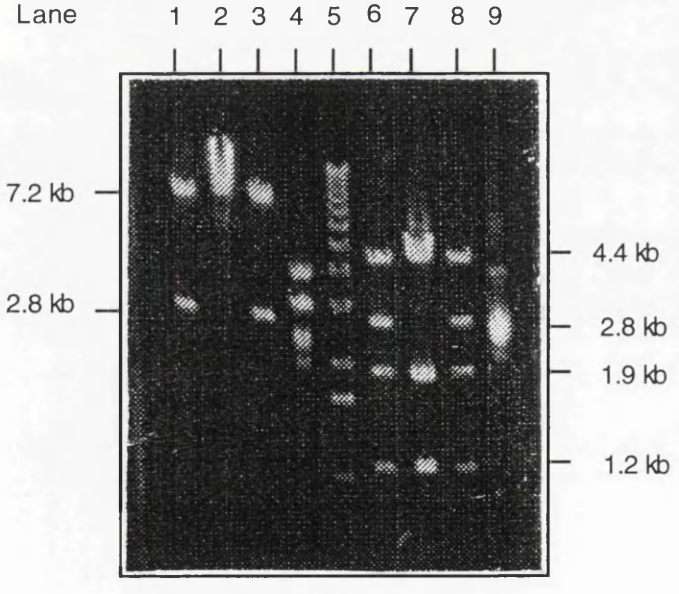


Figure 4.8 Restriction analysis of pNT plasmids containing the 2.8 kb *Bam*HI fragment. *Bam*HI digestions released the 2.8 kb inserts from the pNT

plasmids. *Sst*I digestions revealed the orientation of the inserts in the pNT plasmids.

Lane 1; clone 1 /*Bam*HI
Lane 2; clone 2 /*Bam*HI
Lane 3; clone 3 /*Bam*HI
Lane 4; clone 4 /*Bam*HI
Lane 5; 1 kb Marker
Lane 6; clone 1 /*Sst*I
Lane 7; clone 2 /*Sst*I
Lane 8; clone 3 /*Sst*I
Lane 9; clone 4 /*Sst*I

The *Bam*HI digestions in Figure 4.8 reveal two clones containing the 2.8 kb insert i.e. clone 1 and clone 3. Both clones have the insert ligated into the pNT plasmid in the same orientation as revealed by the *Sst*I digests. This orientation is the same as that presented for the completed plasmid in Figure 4.2. Clone 2 is the wild type plasmid with no insert. Clone 4 is an unknown contaminant.

4.3 Targeting the NTR gene using the pKLF vector

4.3.1 Transfection and selection of E14 ES cells

The pKLF plasmid was linearised within the region of homology at the *Nhe*I site, as detailed in Figure 4.2. The E14 ES cells were expanded to a density of 5 X 10^7, and prepared as described in Material and Methods. After electroporation the cells were plated onto 6 round plates, (10 cm in diameter). Selection for neomycin resistance was achieved by adding 200 μg/ml of G418 to the medium in which they were maintained for the next 14 days. Colonies were then big enough to be picked, (at least 1 mm in diameter). One half of each colony was picked into a 24 well plate, expanded and frozen down, (section 2.5.6), the other half of the colony was harvested for PCR analysis. A total of 650 colonies were processed in this manner.

4.3.2 PCR analysis of G418 resistant colonies

The G418^R colonies should not require to be analysed at this stage , but expanded and selected with gancyclovir to produce revertant Ganc^S colonies. Then the GANC^S colonies would be analysed by Southern blot or PCR analysis, for the correct reversion event. For the NTR gene targeted with the pKLF insertional vector, both possibilities existed for the analysis of ES cell clones because, the KLF linker molecule introduces a new *Kpn*I site for Southern analysis, and also provides unique sequence for the design of a PCR primer pair for a diagnostic PCR. However, the desired reversion event will not be detected unless the insertional vector has integrated at the NTR gene locus. Therefore, the G418^R colonies were screened for the correct insertion of the targeting pKLF vector at the NTR gene locus by PCR.

The diagnostic PCR reaction to identify clones containing the correct insertional event was performed using the primer pair detailed in Figure 4.9. A unique forward primer designed to hybridise to the KLF linker, and a reverse primer to the 2.8 kb *Bam*HI insert at a point down stream of the *Nhe*I cut site. The reasoning for this experimental design was that this PCR reaction could only possibly produce a 900 bp band if the targeting vector had correctly inserted to give a duplication, therefore, creating contiguous sequence between the ES4 and ES6 primer sites. The majority of the G418^R colonies should result from random integrants, thus not allowing the production of the 900 bp PCR band.

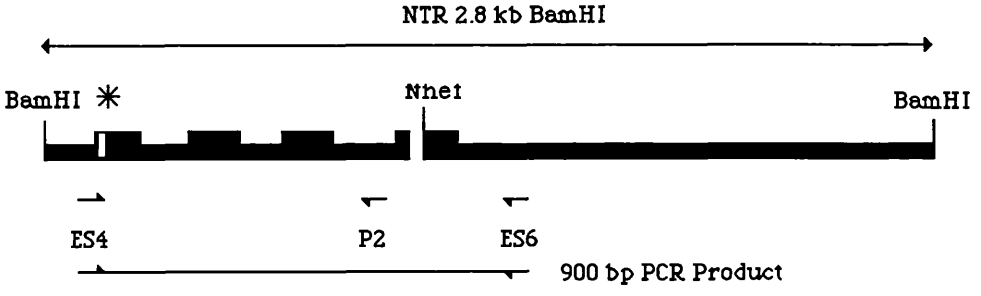


Figure 4.9 Primers for the PCR analysis of G418^R ES colonies. Primer positions are shown relative to the NTR 2.8 kb *Bam*HI fragment of the pKLF vector and its *Nhe*I cut site. The 3' positions of the primers are indicated by arrow heads. PCR analysis of G418^R colonies was performed using the ES4 primer, (5' ctg gtg acc gct gta tga ggt a 3'), and the ES6 primer, (5' ctc tcc ata tgt tgc tca gta t 3'). The ^{32}P end labelled P2 primer, (5' gct agc cat atg gca ctg atg a 3'), was used as a probe in the Southern analysis of the PCR products.

The DNAs of the G418^R colonies were analysed in batches of 12 pooled colonies /PCR reaction. Positive PCR reactions would be followed by PCR/sib-selection to identify individual positive clones, (Kim and Smithies, 1988). The results of one such PCR screen are shown in Figure 4.10. It would appear that some of the PCR reactions have in fact produced the diagnostic 900 bp band as well as some bands of unexpected sizes. Therefore, the gel was Southern blotted and probed with a ^{32}P end labelled primer, (P2), that hybridises internally to the 900 bp band to authenticate the 900 bp band, detailed in Figure 4.10.

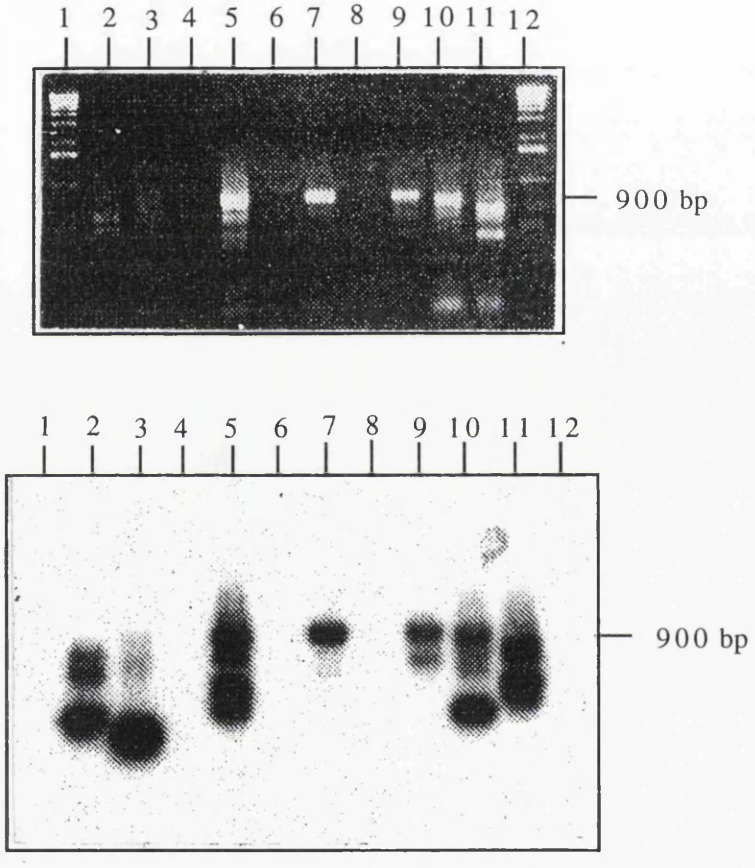


Figure 4.10 The PCR analysis of batches of 12 G418^R colonies, (top panel), and the Southern analysis of the PCR reactions, (lower panel). The PCR analysis of the pooled clones, using primers ES4 and ES6, was performed on 100 ng of DNA denatured at 95°C for 10 minutes. 35 cycles were performed at; 94°C for 30 s, 57°C for 1 minute and extended at 72°C for 1.5 minutes. 10 µl of each reaction were resolved on a 1.2% agarose gel. The gel was transferred to a nylon membrane and probed with the ^{32}P labelled P2 primer, (lower panel). The expected 0.9 kb band is indicated on the right of each picture. Lanes 1 and

12 are 1 kb marker, lanes 2 to 11 are the PCR reaction products for pooled colonies A to J.

The result was somewhat unexpected in that 4, (possibly 5), of the 10 pools produced hybridising bands of the expected 900 bp band, (lanes 5, 7, 9 and 10), and a number of smaller hybridising bands were present in many of the lanes. One of the positive pools was expanded, allowing DNA to be isolated from 10 of the 12 clones for PCR analysis, (figure 4.11). This resulted in 3 potentially positive clones being identified. This result would indicate a targeting frequency of approximately 1 in 10 targeted colonies. Such a high targeting frequency was not anticipated and was presumed to be the result of false positives which are discussed later. Nevertheless, the PCR results indicate the potential presence of positive colonies for the integration of the pKLF vector.

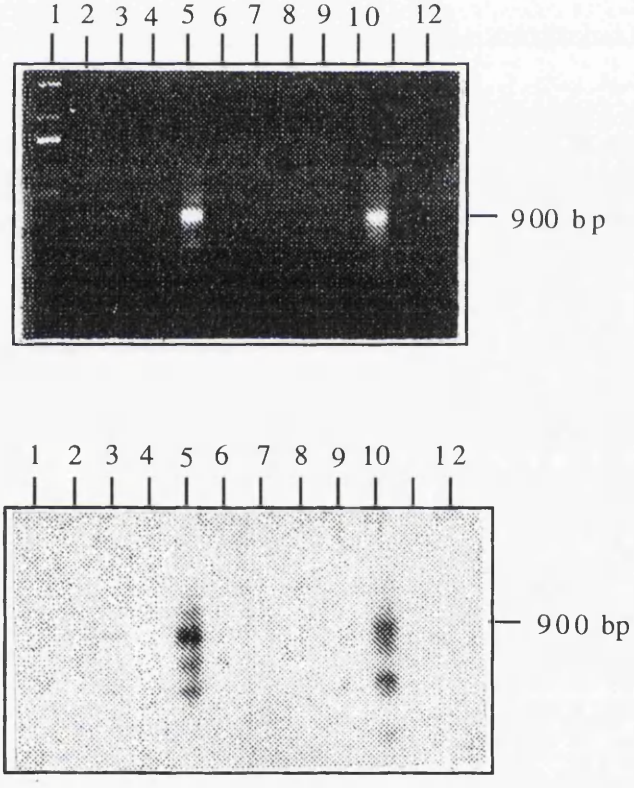


Figure 4.11 The PCR and Southern blot analysis of individual clones from pool D of G418^R colonies, (lane 5 of Figure 4.10). The PCR reaction and Southern blot conditions are the same as those detailed in Figure 4.10. Lane 1 is 1 kb marker; lanes 2 to 12 are the PCR and Southern blot results for clones D1 to D11. The expected diagnostic PCR band of 0.9 kb is apparently present in lanes 3, 5 and 10.

4.3.3 PCR analysis and selection of gancyclovir resistant colonies

The integration of the pKLF insertional vector creates a duplication within the NTR gene which does not necessarily disrupt the gene. Therefore, selection for the reversion event was undertaken to produce revertant ES colonies containing a disrupted NTR allele. The G418^R colonies were expanded to a density of 1.5 X 10^6 cells/colony, and selected in gancyclovir for 10 days. Then the Ganc^R colonies, 150 in total, were picked and analysed in pools of 12 clones as described before. The DNA from each pool was analysed for the presence of a revertant clone carrying the desired mutation i.e. retention of the KLF linker at the *Acc*I site of the NTR gene, by PCR. The PCR strategy is such that the primers selected, (ES1 and ES2), will only permit amplification when the correct gene targeting/reversion event has occured. The ES1 primer is unique to the construct with its 3' end complimentary to the KLF linker. The ES2 primer is gene specific but hybridises upstream of the region of homology used in the targeting construct. Details of the primers used are shown in Figure 4.12.

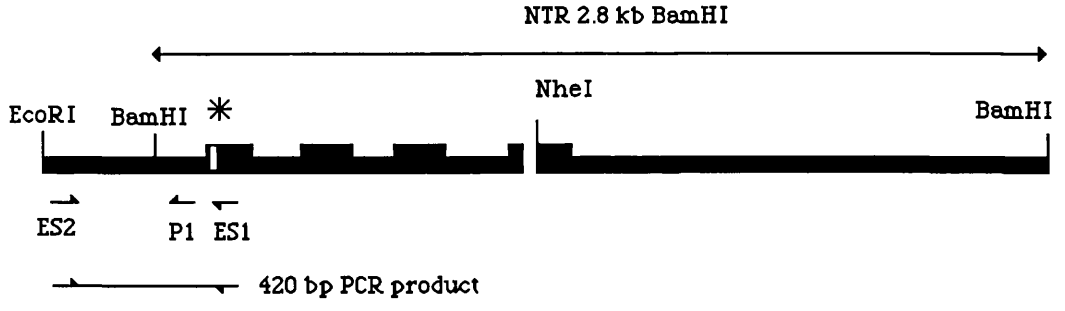


Figure 4.12 The primers for the PCR analysis of Ganc^R colonies. The positions of the primers are shown relative to the 2.8 kb *Bam*HI fragment of the pKLF vector. The ES1 primer, (5' aca aaa agt gcc agg tat gag gta c 3'), is unique for the vector site at the KLF linker. The ES2 primer, (5' a gga gat ccc aga ctc tga agg aga 3'), site is 5' to the region of homology used in the targeting construct. The ^{32}P end labelled P1 primer, (5' gct agc cat atg gca ctg atg a 3'), was used as a probe in the Southern analysis of the PCR products.

The products of the PCR reactions were run on a 1.4% agarose gel, where no visible bands were observed of the correct size, with the exception of the positive control, (Figure 4.13). The gel was Southern blotted and probed with an internally hybridising primer (P1); an example of a typical blot is shown in Figure 4.13. The only visible hybridising band is that of the positive control. This result was typical for all the Ganc^R colonies analysed, demonstrating that no revertant colonies containing the mutagenic KLF linker at the desired location were isolated.

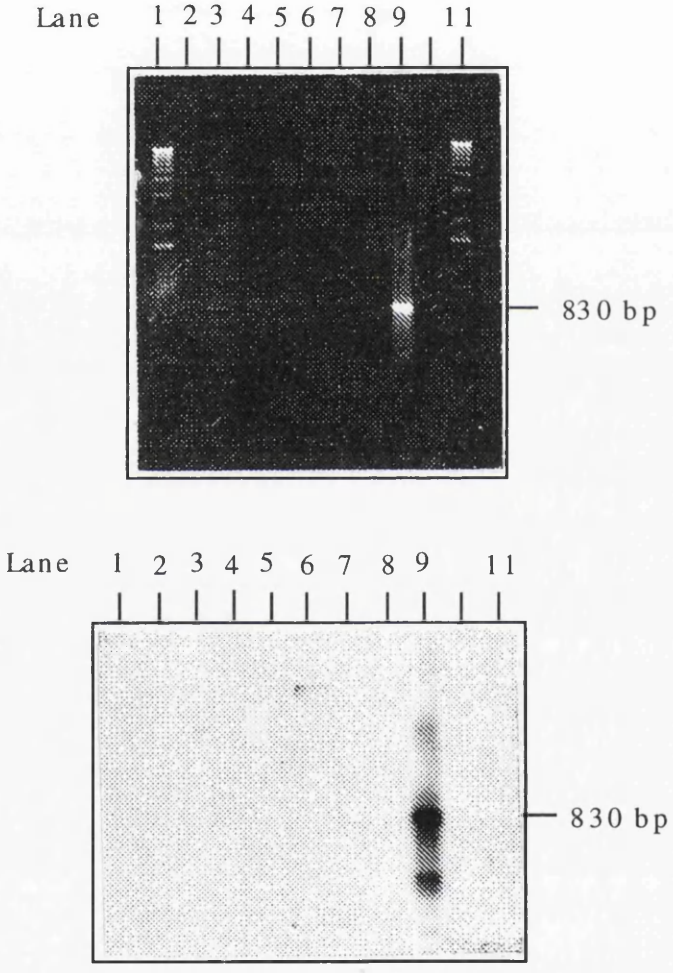


Figure 4.13 The PCR analysis of batches of 12 Ganc^R colonies, (top panel), and Southern blot analysis of the PCR reactions, (lower panel). The PCR analysis of the pooled colonies, using primers ES1 and ES2, was performed on a 100 ng of DNA denatured at 95°C for 10 min. 35 cycles were performed at; 94°C for 30 s, 62°C for 1 minute and extended at 72°C for 1 minute. 10 ml of each

reaction was resolved on a 1.4% agarose gel. The DNAs were transferred to a nylon membrane and probed with a ^{32}P end-labelled primer P1, (details provided in Figure 4.12), the resulting autoradiograph is represented in the lower panel. Lanes 1 and 11 are 1 kb marker, lanes 2 to 8 are 12 clone pools (A to H), lane 9 is a positive control PCR of 100 ng of mouse DNA amplified with primers ES1 and P2 which produces an 830 bp band which contains the P1 primer probe site, lane 10 is a negative control PCR of 100 ng mouse DNA amplified with the ES1 and ES2 primers.

4.3.4 Discussion of the results for the PCR analysis of G418^R and Ganc^R ES colonies

A number of positive PCR results were observed for G418^R colonies, yet no positive PCR results were observed in the analysis of the revertant Ganc^R colonies. The projected number of G418^R colonies, containing the pKLF insertional vector integrated as desired at the NTR locus, was higher than expected at possibly 1 targeted integration for every 10 G418^R colonies. It was probably the case that a number of false positives were observed. Several explanations can be given to explain these results, some of which are presented below.

When employing any PCR screening regime for the analysis of ES colonies, false positives are always a potential problem, since the picking of a positive colony from the culture plate results in the dispersion of cells from that colony throughout the plate. Thus subsequent colonies picked may be contaminated with these 'positive cells', resulting in positive PCR results for negative colonies.

Another source of false positives could come from the 'end extension repair' of the pKLF vector prior to genome integration. This phenomenon was first reported by Adair *et al.*, 1989 and further studied by Aratani *et al.*, 1992. They suggest that the incoming targeting vector has its region of homology extended by some sort of repair mechanism mediated by homologous recombination. One proposed model for this end extension repair involves the pairing of one end of the homologous vector sequence with the target locus, and subsequent extension of the vector sequence from the 3' end of the molecule with the 5' end of the vector molecule being repaired by the enzymes responsible for DNA synthesis of the lagging strand. This would result in the formation of a Holliday junction between the vector and the target

locus. Following branch migration of the Holliday junction, resolution of the junction would result in an extended vector molecule that could then integrate randomly into the genome, (model A is presented in Figure 4.14). The other proposed mechanism for end extension repair, model B, is also presented in Figure 4.14. This mechanism requires a DNA replication fork to pass through the target locus, opening the duplex DNA. The action of endonucleases and exonucleases could create single stranded tails within the replicating duplex. These molecules could then pair with the incoming single stranded vector ends. Following, gap repair and the action of nucleases, the production of end extended vector molecule and a repaired target locus could result.

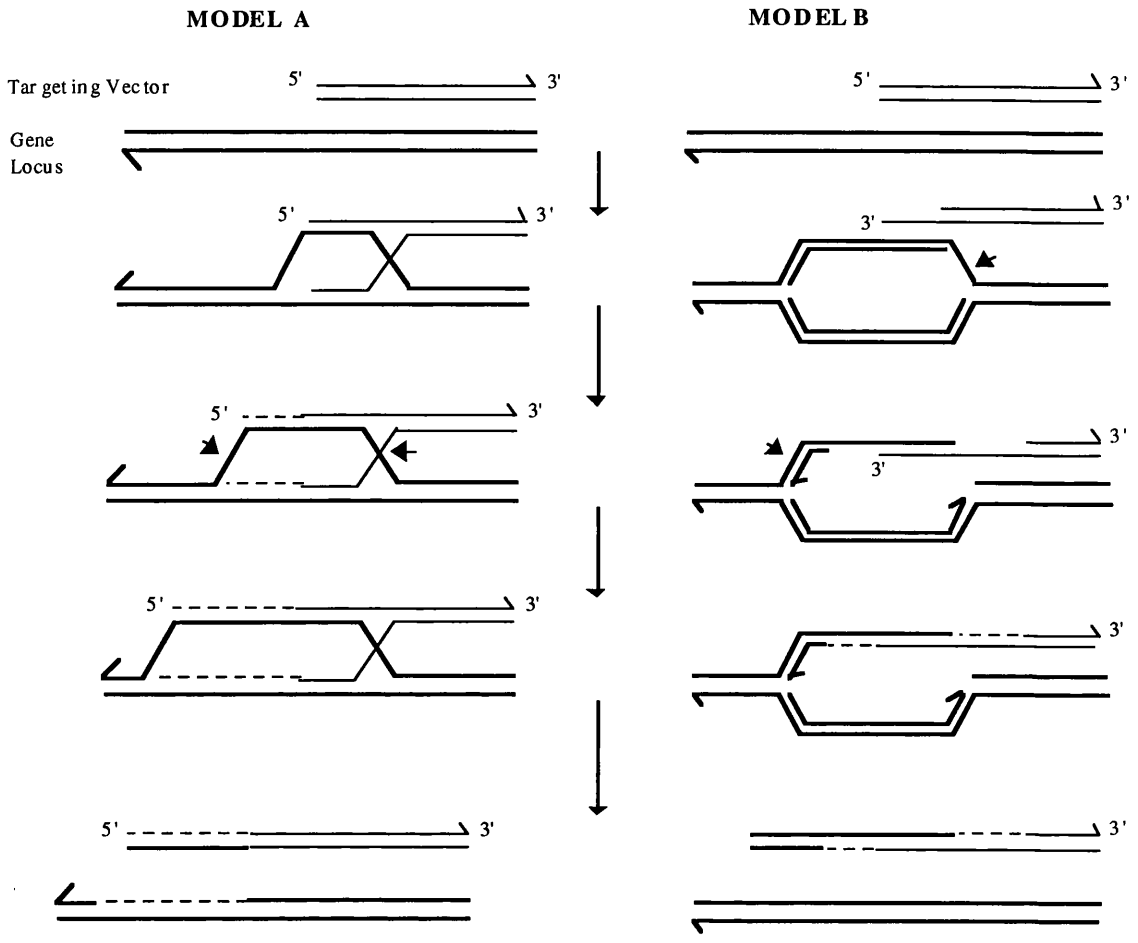


Figure 4.14 Two possible models for end extension repair. Refer to the text for a description of models A and B. The diagrams were adapted from Aratani *et al.*, 1992.

Regardless of the mechanism of end extension repair the Aratani *et al.*, 1992 study, produced 58 potentially targeted colonies with various targeting vectors. However, only half of these were the result of gene integration at the *aprt* gene locus under study. PCR and Southern analysis confirmed that the false positives were the result of the phenonomen known as 'end extension repair' of the incoming vector followed by random genome integration.

Another possibility for the production of false positive PCRs could be by a process known as 'PHLOP', polymerase halt mediated linkage of primers, (Fronham *et al.*, 1990). It has been suggested that the initial rounds of a PCR reaction can give rise to 'extended primers' as a result of truncated linear amplification from a primer target site within the genome. The resulting longer primer molecules could subsequently hybridise to another site within the genome that contained extensive homology, especially to the 3' end of the molecule. If this second site was situated at an amplifiable distance from

one of the original primer sites, then exponential amplification of intervening sequence could occur, generating false positive PCR bands. Thus 'PHLOP', must be considered as a serious source of contamination in PCR analysis of the $G418^R$ colonies. This was principally because of the potential for the generation of those 'extended primer' molecules. Each $G418^R$ clone contains the chromosomally integrated vector, albeit randomly integrated. However, the unique ES4 primer, designed to hybridise to the KLF linker, would have been able to do so for every $G418^R$ clone. The first rounds of a PCR reaction could produce 'extended primer' molecules if the *Taq* polymerase stalled within the region of NTR homology. These molecules would then have sequence homology at their 3' ends to the NTR gene. If the sequence extension of the 3' end is long enough, then molecules of sufficient homology will be produced that could hybridise to the endogenous NTR gene. The ES6 primer which hybridises upstream of the *Nhe*I site is gene specific and not vector specific. Thus the ES6 primer in combination with 'extended primers' could potentially produce target molecules that could be exponentially amplified in subsequent rounds of PCR, by the ES4 and ES6 primers, resulting in the production of the 900 bp band and false positives. Fronham *et al.*, 1990, note that, these artifacts are generally produced after 30 rounds or more of PCR, and especially when the DNA template is isolated from a cell pool of transfectants.

Furthermore, it must be noted that the success of this PCR analysis relies on separating the 2 primer sites, (ES4 and ES6), by linearisation of the *Nhe*I vector site. Linearisation of the molecule followed by random integration of the vector molecule, should result in two juxtaposed primer sites that are not capable of producing the amplifiable 900 bp band. However, if the vector re-ligates prior to integration then the vector sites may no longer be separated when the vector integrates. Thus, a re-ligated vector that integrates without disruption of the intervening sequence between the ES4 and ES6 primer sites, would provide a suitable target sequence for the production of 900 bp molecules and false positive PCR results. A possible solution to this problem would have been to use a primer downstream of ES6, lying outwith the region of the gene that is duplicated. However, this would require the reliable amplification of a PCR product in excess of 2.4 kb.

Therefore, in this experiment a number of sources by which false positive PCR reactions could be produced must be taken into consideration. However, some of the 650 $G418^R$ colonies may be the result of the correct integration of the pKLF vector. Thus the colonies were expanded and selected using gancyclovir for the reversion event to produce $Ganc^R$

colonies containing the mutating KLF linker at the NTR gene locus. The PCR analysis of the 150 $Ganc^R$ colonies did not result in the detection of any desired colonies. This could be explained, in part, by the explanations given for false positive PCR results. However, if colonies carrying the duplication of the NTR gene existed, they may not have been expanded to high enough density prior to gancyclovir selection for the desired reversion event to be produced. When this selection was undertaken only two published examples existed of successful reversion events (Hasty *et al.*, 1991a). The *hprt* gene duplication reverted at a rate of 4.3×10^{-6} per cell generation with two of the fifteen HAT^r colonies retaining the desired mutation. The reversion rate for the *Hox-2.6* gene duplication was at a rate of 3.9×10^{-3} per cell generation. This is a 1000-fold difference between these 2 genes, which the authors suggest could be a function of the recombinational efficiencies of the two gene loci. I proceeded with expanding the cells to a density of 1.5×10^6 cells per colony prior to selecting for the reversion event without any knowledge of at what frequency a targeted NTR gene may revert. Thus the cells may not have been expanded to a great enough density to recover the desired reversion event.

Moreover, the design of the insertional type vector pKLF was unknowingly further compromised by the position of the mutation relative to the double strand break in the vector, as revealed in a recent publication by Hasty *et al.*, 1995. This study investigated the frequency with which the ends of insertion vectors undergo gene conversion and if the reaction has a preference for conversion to vector or chromosomal sequence. A number of insertion vectors for the *aprt* locus carrying a mutation within the gene homology were linearised at various distances from the mutation and the frequency of gene conversion measured. It was observed that the frequency with which the mutation was lost in favour of chromosomal sequence on homologous integration was inversely proportional to the distance between the vector mutation and the double strand break. Loss of chromosomal sequence in preference to vector sequence was a low frequency event. One particular insertion vector was linearised at 0.427 kb from the mutation and underwent gene conversion to chromosomal sequence at 40% of all homologous integrations. Extrapolation to the pKLF insertion vector in this study, predicts that at least 40% of correctly targeted clones would have lost the mutagenic linker sequence by gene conversion. In conclusion, this particular vector construction may have produced targeted clones but, due to the design of the vector and/or duplication reversion rates, it was unlikely to give the desired revertant clones at a practical frequency.

4.4 Summary

An insertional type vector for targeting the neurotensin receptor gene (NTR) by the 'Hit and Run' approach was made using the homologous 2.8 kb *Bam*HI genomic fragment. This *Bam*HI fragment was engineered such that a mutagenic KLF linker was introduced at the *Acc*I site and the homologous gene sequence was flanked on either side by a neomycin and a thymidine kinase cassette. Insertion of this linker created an in-frame stop codon and introduced *Kpn*I restriction sequence into the region of the gene encoding the first transmembrane domain of the receptor. The linker served two purposes, firstly to create a null mutation of the gene and secondly to provide a unique sequence for the identification of targeted clones by a PCR or Southern blot strategy. The gene targeting approach, a two step recombinational procedure known as 'Hit and Run', was attempted. The first step of producing a targeted duplication of the NTR gene may have been achieved. However, the PCR analysis appeared to be complicated by false positive PCR results, since an unusually high targeting frequency of possibly 1 in 10 clones was suggested by the data. The second step of selecting for revertant colonies did not yield any of the desired revertant colonies. This may be a result of vector design and/or not expanding the colonies to a high enough density prior to gancyclovir selection for reversion. The results and conditions of the targeting experiment are summarised in Figure 4.15

ES cell line	Electroporation settings	No. of cells and amount of vector	Transformation frequency	Targeting frequency	No. of revertant targeted clones
E14	500 μF, 250 V	5 X 10^7 cells 25 μg DNA	1.3 X 10^{-5}	1/10*	0 from 150 resistant colonies

Figure 4.15 Summary of the results from the 'Hit and Run' targeting experiment of Chapter 4. *Targeting frequency as determined by PCR analysis, see section 4.3.4 for a discussion on false positives.

Chapter 5

Construction of a Replacement Vector for Targeting the Neurotensin Receptor Gene.

5.1 Introduction

The lack of success in generating a targeted mutation of the NTR gene with the pKLF vector, led to the re-evaluation of the 'Hit and Run' procedure as a method for producing a null mutation of the gene. Principal concerns surrounded the general applicability of this procedure, such as the rate of reversion of the 'average' gene locus. Furthermore, the actual design of the targeting construct (pKLF), with its relatively small amount of gene homology and position of the mutation within that homology, may have compromised the vector's ability to generate the desired mutation. Moreover, data concerning the use of isogenic versus non isogenic DNA, overall length of homology, the selective utilities of insertional and replacement vectors, became available with respect to their influence on targeting frequencies of the *Hprt* gene (Deng, *et al.*, 1992a). For these reasons a replacement construct, utilising the positive negative selection (PNS) strategy (Thomas, and Capecchi, 1987), was designed to target the NTR gene.

The first step in construction of the replacement vector was the isolation of isogenic mouse genomic fragments from a l library derived from DNA of the mouse 129J strain. Subcloning of a suitably large fragment of the NTR gene and restriction map analysis followed. Vector design required the introduction of a neomycin cassette to produce a mutation and provide positive selection of vector integration into ES cells. Secondly, a thymidine kinase cassette was added to provide negative selection against random integrants and hence enrich for targeted events. A PCR screening procedure was designed to detect targeted clones. However, to ensure the success of such a screen a control vector was also made. This vector was based on the replacement vector with additional 5' gene sequence, to that of the replacement construct. Thus the control vector contained homologous sequence that would only be encountered if the targeting vector correctly targeted the NTR gene. This control vector allowed the evaluation of sets of PCR primers designed to detect homologous recombinants as a result of gene targeting with the replacement vector.

5.2 Subcloning of the 9.2 kb *Eco*RI fragment of the NTR gene from a 129J mouse genomic library

5.2.1 Screening of a bacteriophage λ Dash 129J mouse genomic library

The strategy for screening the λ Dash 129J mouse genomic library was essentially identical to that for the isolation of λ clones from λ EMBL3 Balb/c mouse genomic library described in Chapter 3. The probe used was the same as the ^{32}P labelled 1.4 kb *Sma*I derived fragment of the rat cDNA previously used, (Figure 2.1). Prehybridisation and hybridisation of the library filters was performed exactly as before with the same 50% formamide based buffer. The only exception was that the library was plated onto 40 (10 cm X 10 cm) plates at a density of 25000 p.f.u./plate. Therefore a total number of 1 000 000 plaques were screened.

5.2.2 Results of the library screen

Initial screening of the library resulted in the identification of 18 putative positive λ clones. After secondary and tertiary rounds of screening and purification, a total of 16 λ clones were isolated. Figure 5.2 represents the autoradiographs from primary and secondary rounds of the library screen.

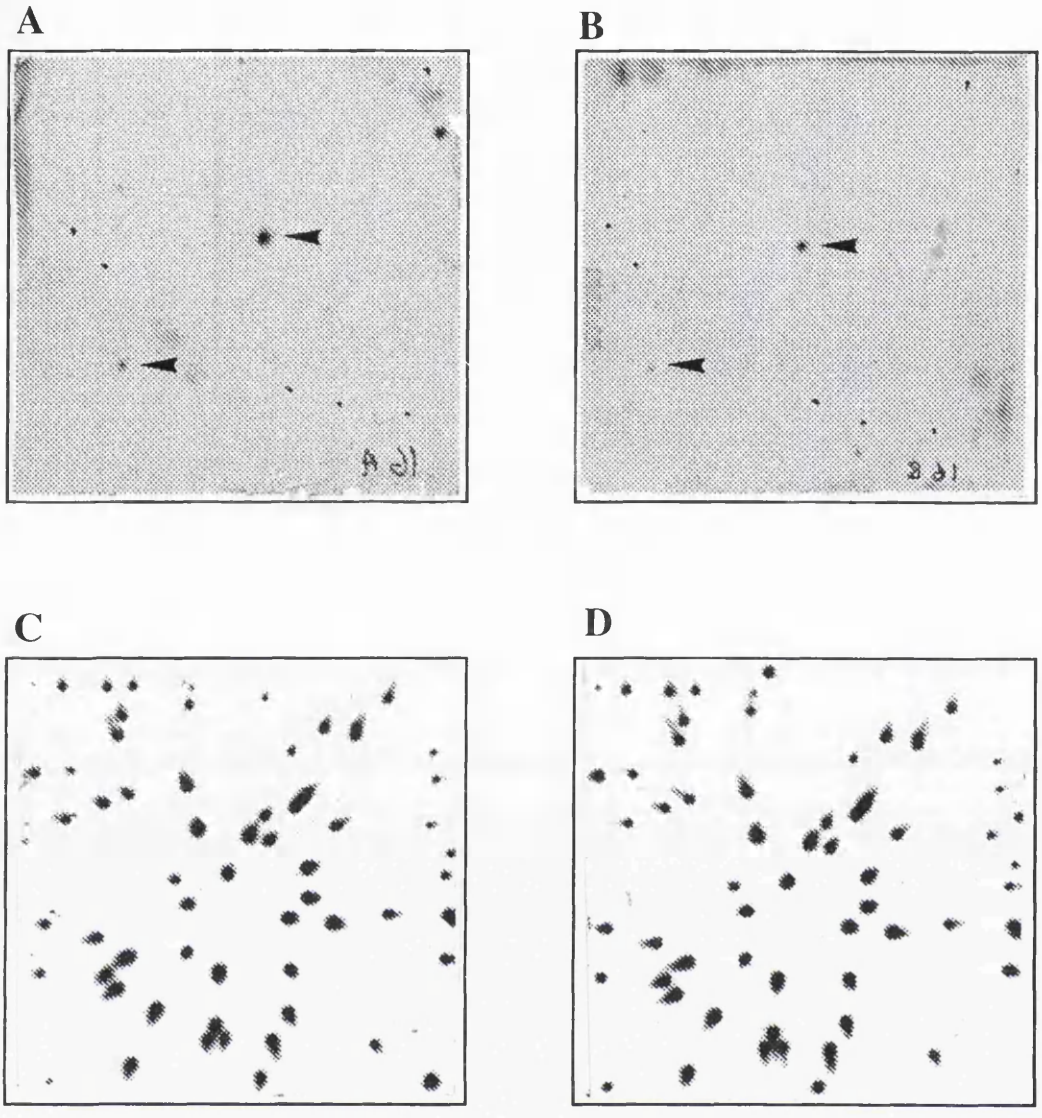


Figure 5.1 Primary and secondary screening of putative positives from the λ Dash 129 J library. Panels A and B are autoradiographs of a set of replica filters from the primary screen; the arrows indicate the positions of two presumptive positive clones. Panels C and D are the autoradiographs from the secondary screening of a positive clone. The probe and hybridisation conditions are those detailed in Chapter 3, (section 3.2).

5.2.3 Identification of a genomic 9.2 kb *Eco*RI fragment

The genomic Southern blot analysis from Chapter 3, Figure 3.2, indicated a hybridising 9.2 kb *Eco*RI band to the 5' derived probe of the rat cDNA. Reference to the subsequent 5' probings of the λ clones Figure 3.5 indicates that the 2.8 kb *Bam*HI fragment of the NTR gene is located within the 9.2 kb

*Eco*RI fragment. The 2.8 kb *Bam*HI fragment referred to was that subcloned (p2.8B) and used for the construction of a 'Hit and Run' type vector. Since the 9.2 kb *Eco*RI fragment contains a 5' portion of the NTR gene, encoding transmembrane domains I to IV, this was considered a suitable fragment of gene homology for the construction of a replacement vector. The immediate goal was to identify such λ clones containing the 9.2 kb *Eco*RI and subclone such a fragment. Two such λ clones were isolated. Figure 5.2 indicates the presence of the 9.2 kb *Eco*RI and 2.8 kb *Bam*HI bands; the 9.2 kb band of clone T12 was subcloned.

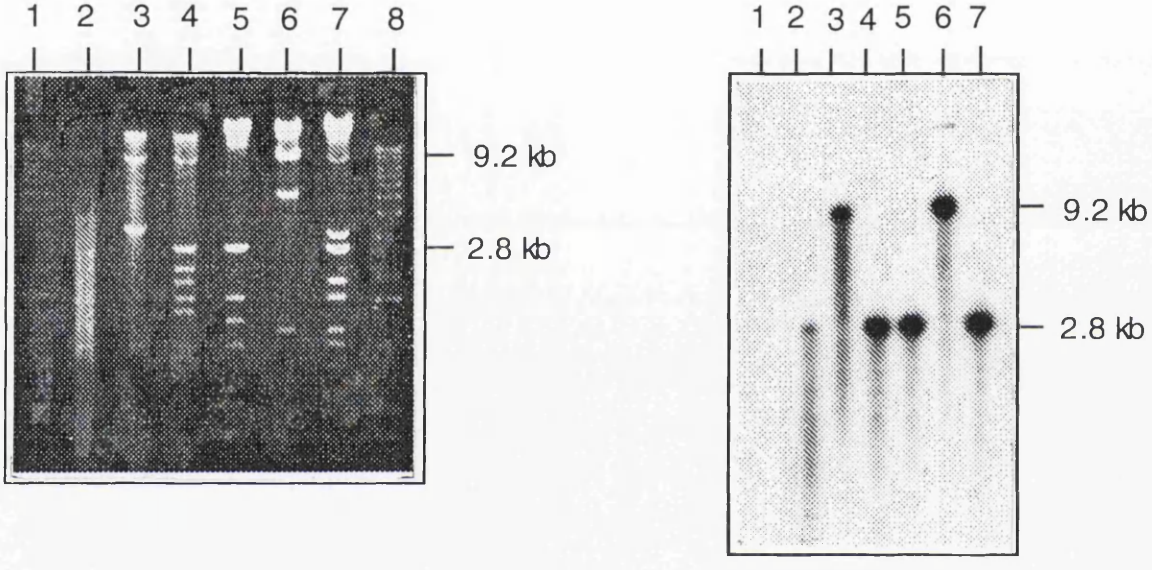


Figure 5.2 Restriction digestion and Southern analysis of λ clones T1 and T12. The digestions were resolved on an 0.7% agarose gel and transferred to a nylon membrane. The probe used was the ^{32}P labelled 1.4 kb *Sma*I fragment derived from the rat cDNA. The hybridisations were performed as described in Chapter 3.

Lanes 1 and 8 are 1 kb marker
lane 2; clone T1 /*Bam*HI (degraded)
lane 3; clone T1 /*Eco*RI
lane 4; clone T1 /*Eco*RI and *Bam*HI
lane 5; clone T12 /*Bam*HI
lane 6; clone T12 /*Eco*RI
lane 7; clone T12 /*Eco*RI and *Bam*HI

Both clones T1 and T12 contain the 9.2 kb *Eco*RI fragment that hybridises to the rat NTR cDNA. This mouse genomic fragment contains the 2.8 kb *Bam*HI fragment as revealed by the double digestions; lanes 4 and 7.

5.2.4 Subcloning and restriction mapping of the 9.2 kb *Eco*RI fragment

The 9.2 kb *Eco*RI fragment from the λ T12 clone was subcloned in plasmid pGem 7Zf. The *Eco*RI cut vector was dephosphorylated using shrimp alkaline phosphatase (SAP), extracted with phenol chloroform twice, precipitated with ethanol and resuspended in dH_2O. The λ DNA was *Eco*RI digested, precipitated with ethanol, resuspended in dH_2O. Ligations were performed overnight and subsequently transformed into DH5α. The white recombinant colonies were analysed by the 'one step' mini prep DNA method, (Chapter 2, section 2.3.10). This resulted in the identification of subclones for the 9.2 kb *Eco*RI fragment as well as the 5' and 3' flanking *Eco*RI fragments, (4.8 kb and 1.2 kb), from the T12 clone.

The 9.2 kb *Eco*RI subclone, (p9.2EiG7), was confirmed by restriction enzyme analysis. Restriction digests were performed with rare cutting enzymes and those enzymes known to infrequently cut the 2.8 kb *Bam*HI fragment. The data generated in this analysis are summarised in Figure 5.3.

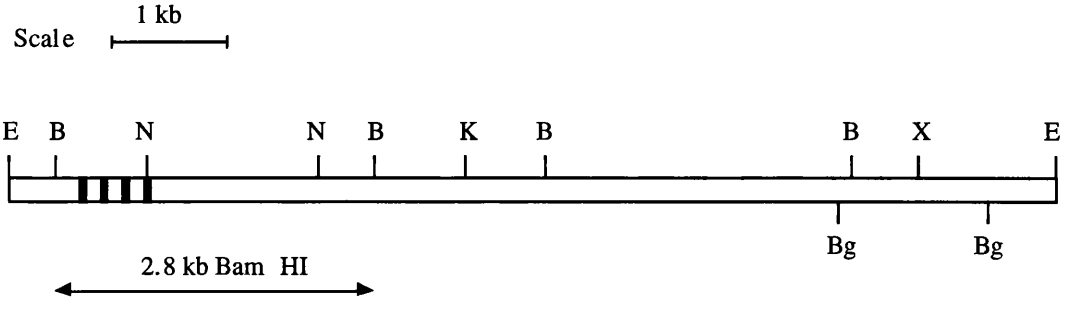


Figure 5.3 A restriction map of the 9.2 kb *Eco*RI fragment. The position of the previously characterised 2.8 kb *Bam*HI fragment is indicated, as are the relative positions of the sequence encoding transmembrane domains by the black boxes. E, *Eco*RI; B, *Bam*HI; N, *Nco*I; K, *Kpn*I; Bg, *Bgl*II; X, *Xba*I.

5.3 Construction of a replacement type targeting vector for the NTR gene.

5.3.1 The strategy for construction of the replacement vector

The replacement vector was generated by deleting part of the coding sequence contained within the 9.2 kb *Eco*RI NTR gene fragment, by inserting a neomycin cassette at the *Nco*I deletion site and placing a thymidine kinase cassette 3' of the large arm of gene homology. Creation of this construct required several cloning steps which are summarised by the flow chart presented in Figure 5.4. Essentially the neomycin and thymidine kinase cassettes were derived from the pNT plasmid, and sequentially introduced into the 9.2 kb *Eco*RI gene fragment. However, insertion of the neomycin cassette was via a *Sal*I site introduced by the insertion of a linker at the *Nco*I deletion site. This extra linker step was necessary due to the presence of an *Xho*I site in the gene fragment. Finally, linearisation of this vector (p2.8EiNeoTk) produced a construct with two arms of homology, 1.3 kb and 6.4 kb, that was used to target the NTR gene in ES cells.

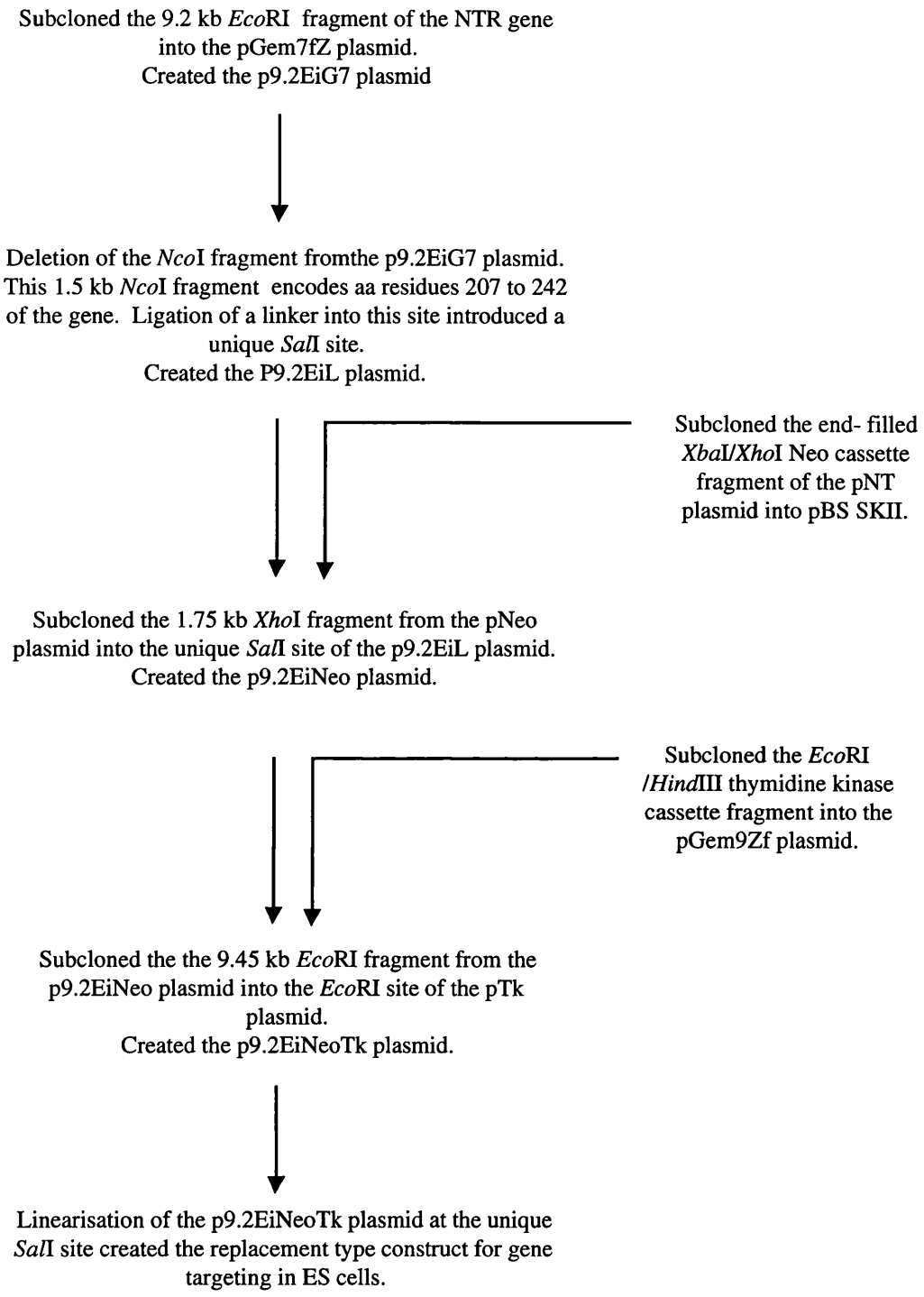


Figure 5.4 Construction of a replacement type vector. The various cloning steps and manipulations involved in creating the replacement type construct used for targeting of the NTR gene in ES cells.

5.3.2 Deletion of the *Nco*I fragment from the 9.2 kb *Eco*RI NTR gene fragment and addition of a *Sal*I linker

Deletion of the *Nco*I fragment creates a mutation within the 9.2 kb *Eco*RI NTR gene fragment. This deletion step was followed by the addition of a linker, which created a unique *Sal*I restriction site, into which a neomycin cassette was subcloned. Deletion of the *Nco*I 1.5 kb fragment removed part of the gene encoding aa residues 207 to 242; the remainder of the fragment was intronic.

The p2.9EiG7 plasmid was digested with *Nco*I and resolved on a 1% agarose gel from which the deleted plasmid was band purified to separate it from the 1.5 kb *Nco*I fragment. The deleted plasmid was then ligated overnight with the *Sal*I linker, (5' cat ggc tcg agc 3'). However, no linker containing plasmids were isolated. Therefore, the deleted plasmid was dephosphorylated using the SAP enzyme. The partially self-complementary oligonucleotide used to make the linker with *Nco*I sticky ends, was phosphorylated with the T4 DNA kinase enzyme prior to its use. The phosphorylated oligonucleotide was heated to 95°C for 10 minutes and allowed to anneal slowly to room temperature prior to the ligation. The deleted plasmid and linker were ligated overnight at vector to linker molar ratios of 1:10 and 1:100.

Successfully transformed ligations were analysed by restriction digestion of the plasmids. *Eco*RI/*Sal*I digestions were performed; the *Eco*RI enzyme frees the 7.7 kb, (9.2 - 1.5), insert from the 3 kb plasmid, and the *Sal*I enzyme cuts the insert in two, if the linker is present, to produce 1.3 kb and a 6.4 kb fragments. The desired modified plasmid, (p9.2EiL), was eventually obtained as is detailed in Figure 5.5. The *Nco*I digestion linearised the plasmid due to the conservation of the *Nco*I sites after linker insertion, (lane 3). The *Sal*I digestion also linearises the vector due to the presence of the *Sal*I linker's restriction site, (lane 4). The *Sal*I/*Eco*RI digestion produces the expected restriction pattern of 3 kb, 1.3 kb and 6.4 kb fragments for the modified plasmid, (p9.2EiL).

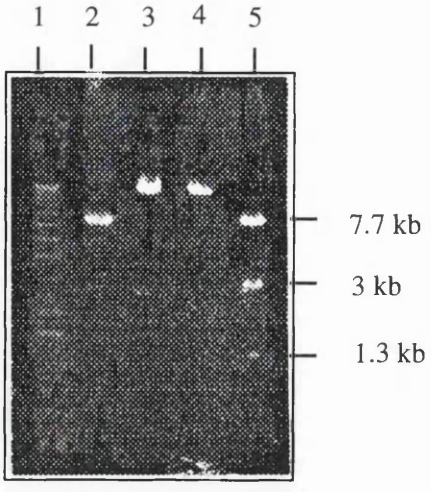


Figure 5.5 Restriction analysis of the p9.2EiL plasmid. The p9.2EiL plasmid was digested with the appropriate enzymes, to detect the presence of the *Sal*I linker, and the reactions were resolved on a 1% agarose gel. Lane 1 is 1 kb marker; lane 2, uncut p9.2EiL plasmid; lane 3, p9.2EiL /*Nco*I; lane 4, p9.2EiL /*Sal*I; lane 5, p9.2EiL /*Sal*I and *Eco*RI.

5.3.3. Insertion of the neomycin cassette into the p9.2EiL plasmid

This stage of the construction involved two separate subcloning steps. Firstly, the neomycin cassette from the pNT plasmid was subcloned into the pBS SK II$^-$ plasmid. This allowed the neomycin cassette to be retrieved from the pNeo plasmid by *Xho*I digestion. The *Xho*I excised neomycin cassette was then subcloned into the compatible *Sal*I site of the p9.2EiL plasmid.

The pNT plasmid was digested with the *Xho*I and *Xba*I enzymes to release the neomycin cassette. The 1.75 kb neomycin fragment was then band purified by resolution on a 1% LMP agarose gel. The purified band was then end-filled using the Klenow enzyme. This fragment was then blunt-end ligated into the dephosphorylated BS SK II$^-$ plasmid, linearised at the *Hinc*II site. White recombinant colonies were initially analysed by agarose gel electrophoresis on the basis of supercoiled plasmid size. However, confirmation of the subcloning was achieved by *Xho*I restriction digestion, which releases the neomycin cassette. This was possible as the 1.7 kb neomycin fragment was flanked by two *Xho*I sites: one site was reconstituted on insertion into of the neomycin cassette into the plasmid and the other was

provided by the flanking polylinker sequence at the *Hinc*II/*Xba*I junction. This subclone was known as pNeo.

The neomycin cassette was recovered from the pNeo plasmid by *Xho*I digestion and band purification on a 1.2% LMP agarose gel. The 1.7 kb *Xho*I fragment was then subcloned into the *Sal*I site of the p9.2EiL plasmid. The recombinant plasmids were identified by *Nco*I restriction digestion. Plasmids containing the neomycin cassette produced two other bands as opposed to the parental plasmid. This is because the neomycin cassette contains an internal *Nco*I site and the *Sal*I linker provides two *Nco*I sites flanking the neomycin cassette. Restriction analysis of some of the clones recovered from this ligation experiment are presented in Figure 5.6.

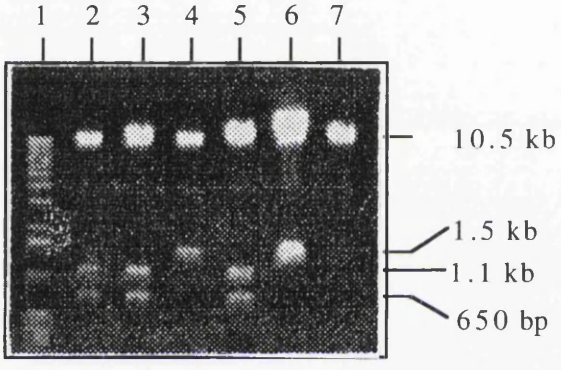


Figure 5.6 Restriction analysis of potential clones containing the neomycin cassette. All candidate clones were digested with the *Nco*I enzyme and the reactions resolved on a 1.2% agarose gel. Lane 1 is 1 kb marker; lanes 2, 3, and 5 are plasmids containing the neomycin fragment; lanes 4 and 6 are the p9.2Ei plasmid; lane 7 is the p9.2EiL plasmid.

The *Nco*I analysis of the recovered plasmids from the transformation consisted of three types as illustrated in Figure 5.6. The original plasmid, (p9.2Ei), containing the 9.2 kb *Eco*RI mouse genomic fragment was detected; lanes 4 and 6. *Nco*I digestion of this plasmid released the genomic 1.5 kb fragment of the NTR gene, which should not be present. The second plasmid type was p9.2EiL, which has its 1.5 kb *Nco*I fragment removed and replaced with the *Sal*I linker; lane 7. Therefore, on digestion with *Nco*I this plasmid was linearised, releasing the *Sal*I linker. Lastly, the desired plasmid construct was isolated containing the neomycin cassette subcloned into the *Sal*I site of the *Sal*I linker. Digestion of this plasmid with *Nco*I results in the release of the neomycin cassette as two fragments, (1.1 kb and 650 bp), due

to an internal *Nco*I site. One of these clones, termed p9.2EiNeo, was used for the final stage of the construction i.e. addition of the thymidine kinase cassette.

Finally, the orientation of the neomycin cassette relative to the NTR gene fragment was determined by *Bgl*II digestion. This was possible as the neomycin cassette contains a *Bgl*II restriction site within the polyadenylation sequence, 150 nt from the end. Thus a distinct banding pattern would be generated depending on the orientation of the cassette relative to the two *Bgl*II sites of the gene fragment. As presented in the diagram of Figure 5.7, a *Bgl*II digestion would yield fragments of 1.3 kb, 6.55 kb and 4.6 kb, (forward orientation). The reverse orientation would result in *Bgl*II fragments of 1.3 kb, 5.1 kb and 6.05 kb.

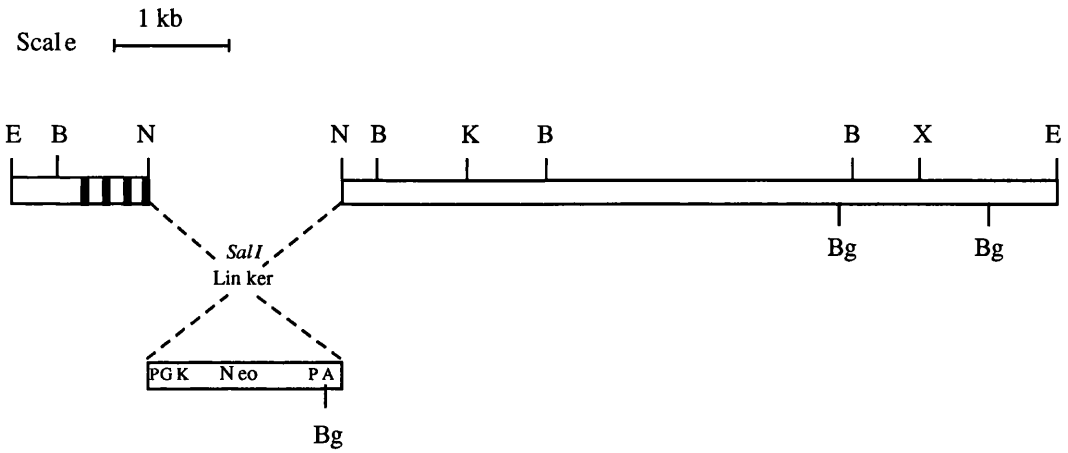


Figure 5.7 A restriction map of the 9.2 kb NTR gene fragment, detailing the position of the *Bgl*II restriction site in the neomycin cassette relative to the *Bgl*II sites of the gene fragment. The neomycin cassette was cloned into the gene fragment via the insertion of a *Sal*I linker. The original 9.2 kb *Eco*RI gene fragment is shown with black stripes indicating the position of the sequences encoding the transmembrane domains I-IV and a neomycin cassette insertion. PGK, phosphoglycerate kinase promoter; PA, SV40 polyadenylation site; Neo, neomycin phosphotransferase gene;E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Bg, *Bgl*II; X, *Xba*I and K, *Kpn*I.

This *Bgl*II restriction analysis was necessary as the orientation of the neomycin cassette was crucial to the design of PCR primers for the analysis of ES colonies. Furthermore, the relative position of the *Bgl*II site allowed the accurate prediction of *Bgl*II fragment sizes for the Southern blot analysis of

targeted ES clones. Figure 5.8 presents such an analysis for the p9.2EiNeo plasmid (lane 2), with the *Bgl*II digestion revealing a banding pattern indicative of the neomycin cassette orientated in the forward direction as displayed in Figure 5.7.

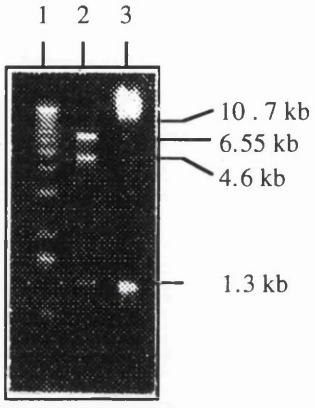


Figure 5.8 *Bgl*II restriction analysis of the p9.2EiNeo plasmid. The restriction digest was resolved on a 1% agarose gel. Lane 1 is 1 kb marker, lane 2 is p9.2EiNeo digested with *Bgl*II, lane 3 is p9.2Ei digested with *Bgl*II.

5.3.4 Addition of the thymidine kinase cassette

The final stage of the replacement vector construction involved two cloning steps. The thymidine kinase cassette was subcloned from the pNT plasmid into the pGem9 vector, generating the pTK plasmid. The gene fragment with the neomycin cassette was subsequently subcloned from the p9.2EiNeo plasmid into the pTK plasmid.

The *Eco*RI/*Hin*dIII 2.7 kb fragment, containing the thymidine kinase cassette from the pNT plasmid, was subcloned into the pGem9 plasmid. The *Eco*RI/*Hin*dIII double digestion of the pNT plasmid was resolved on a 1% agarose gel, where the 2.7 kb fragment was band purified. The purified fragment was sub cloned into the *Eco*RI/*Hin*dIII digested pGem9 plasmid, at vector to insert ratios of 1:1 and 1:3. White colonies from the transformation of the DH5α cells were grown overnight and the recombinant plasmids purified. Conformation of the thymidine kinase cassette subcloning success, was confirmed by *Eco*RI/*Hin*dIII restriction digestion. The orientation of the cassette was dictated by this directional cloning step. One such subclone, termed pTK, was used for the final step of the replacement vector construction.

The pTK plasmid was linearised at its *Eco*RI restriction site. The band purified *Eco*RI fragment from the p9.2EiNeo plasmid and the linearised pTK plasmid were ligated overnight. The desired recombinant plasmid was isolated on the basis of supercoiled plasmid size using the 'one step' plasmid miniprep procedure. Finally, the orientation of the 9.4 kb *Eco*RI fragment of the construct was determined by *Sst*I digestion. If inserted as desired, an *Sst*I restriction fragment profile of 6.85 kb, 4.59 kb, 3.05 kb and two bands of approximately 280 bp. would be produced, as opposed to the reverse orientation of 5.8 kb, 4.59 kb, 3.05 kb, 1.33 kb and a 280 bp band. Therefore, lane 5 of Figure 5.9 shows the digestion profile of a clone with the desired insert orientation. This clone, p9.2EiNeoTK, represents the completed replacement construct used to target the NTR gene in ES cells.

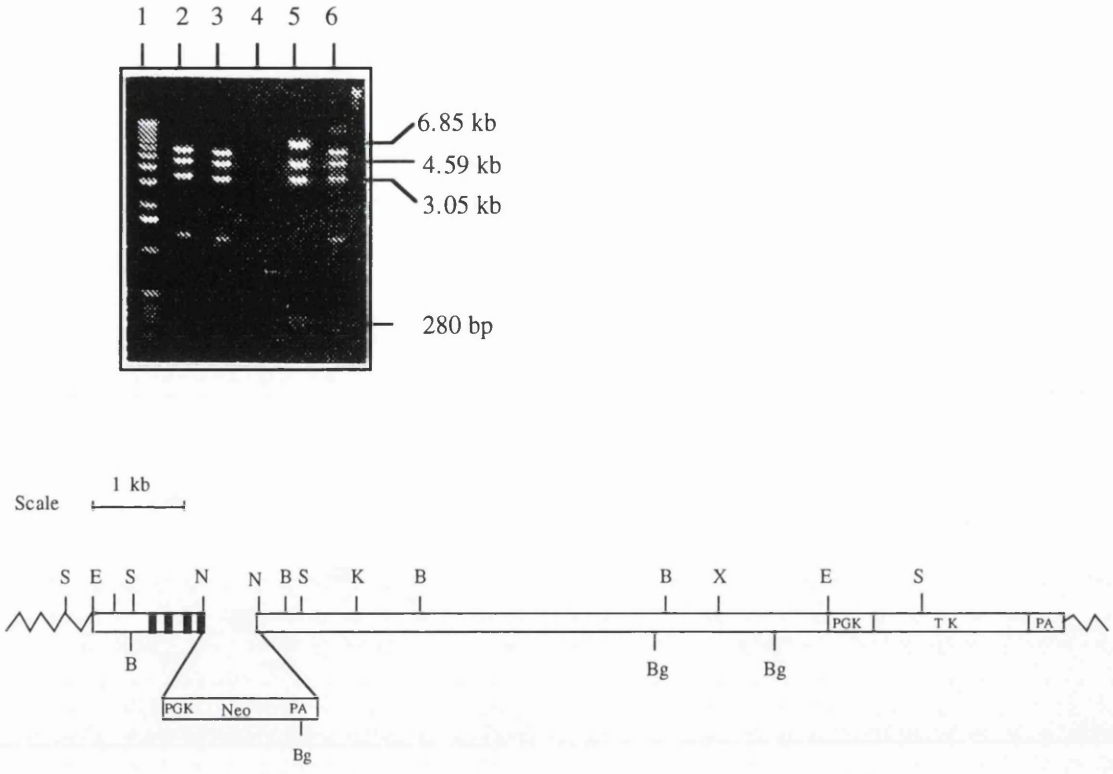


Figure 5.9 Restriction analysis of clones containing the 9.45 kb *Eco*RI fragment of the p9.2EiNeo plasmid subcloned into the *Eco*RI restriction site of the pTK plasmid. *Sst*I digestion of these plasmids allowed the orientation of the 9.4 kb insert relative to the pTK plasmid to be deduced. Plasmids were digested with *Sst*I and the reactions resolved on a 1.2% agarose gel. Lane 1 is 1 kb marker, lane 2 is clone 1, lane 3 is clone 2 lane 4 is blank, lane 5 is clone 3, lane 6 is clone 4. The band sizes on the right of the gel picture refer to clone 3 of lane 5. The restriction map of clone 3, designated p9.2EiNeoTK, is displayed below the gel picture. *Sst*I digestion of clone 3 produces the predicted fragment sizes deduced from the restriction map, i.e. 6.85 kb, 4.59 kb 3.05 kb and 2 bands of about 280 bp. The original 9.2 kb *Eco*RI gene fragment is shown with black stripes indicating the position of the sequence encoding transmembrane domains I-IV, a neomycin cassette insertion and is flanked by the thymidine kinase cassette. PGK, phosphoglycerate kinase promoter; PA, SV40 polyadenylation site; NEO, neomycin phosphotransferase gene; TK, HSV thymidine kinase gene; E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Bg, *Bgl*II; K, *Kpn*I; X, *Xba*I and S, *Sst*I. The wavy line at both ends of the construct represents the pGem9 plasmid sequences (2.9 kb), with an *Sst*I restriction site that is not positioned to scale due to its proximity to the insert DNA.

5.4 A control replacement vector for the development of a diagnostic PCR screen

5.4.1 Construction of a control replacement construct

A control construct was made to test the feasibility of the PCR screen designed to detect targeted ES clones. This construct consists of an additional 175 bp of 5' flanking sequence upstream of the 9.2 kb *Eco*RI fragment of the NTR gene used for the replacement construct.
The subcloned 4.8 kb *Eco*RI fragment from the T12 lambda clone, (this chapter section 5.2.4), contained the 5' flanking DNA of the 9.2 kb *Eco*RI fragment. Partial mapping of this subclone identified a suitable *Xho*I fragment that was used to construct the control vector. A summary of the mapping data and the 650 bp *Xho*I fragment used for the control vector construction are outlined in Figure 5.10.

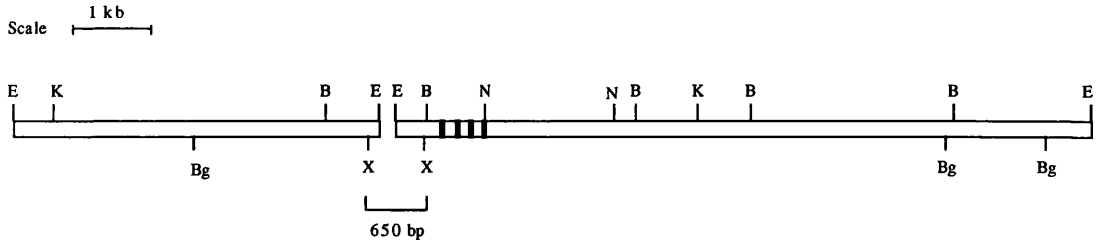


Figure 5.10 Partial restriction maps of the 9.2 kb and 4.8 kb *Eco*RI fragments subcloned from lambda T12. The relative position of the sequence encoding the transmembrane domains are indicated as black stripes within the 9.2 kb *Eco*RI fragment. The 650 bp *Xho*I fragment used for the construction of the control vector has been highlighted. E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Bg, *Bgl*II; X, *Xho*I and K, *Kpn*I.

An outline of the strategy used to construct the control vector is presented in Figure 5.11 The first step requires the subcloning of the 650 bp *Xho*I fragment from the T12 λ clone. The T12 λ clone was digested with the *Xho*I enzyme, resolved on a 1.4% LMP agarose gel and the 650 bp fragment band purified.

This fragment was then ligated into the *Xho*I linearised pBS SK II$^-$ vector, at vector to insert ratios of 1:1 and 1:3. The resulting recombinant plasmids were purified and assayed for the presence of the insert DNA by *Xho*I restriction digestion. Furthermore, the orientation of the insert DNA was determined by *Eco*RI digestion. The correct orientation produces a 475 bp band as opposed to a 175 bp band when digested with *Eco*RI. A successfully ligated plasmid was isolated and named pX650.

The pX650 plasmid was subjected to double-stranded DNA sequencing. This sequence information was used to design primers for the diagnostic PCR screen described in section 5.8 of this chapter. It also confirmed the mapping data generated for the T12 λ clone, as the sequence was in agreement with that produced from the 5' end of the 9.2 kb *Eco*RI fragment. The second stage of the construction involved the subcloning of the 9.45 kb *Eco*RI fragment of the 9.2EiNeo plasmid into the *Eco*RI digested pX650 plasmid. Digestion of the pX650 plasmid with the EcoRI enzyme releases a 475 bp fragment of the NTR gene. However, this portion of the gene already exists at the terminal end of the 9.45 kb *Eco*RI fragment. Therefore, only the remaining unique 175 bp DNA of the pX650 plasmid will be added to the 9.45 kb *Eco*RI fragment when it is ligated into the *Eco*RI digested pX650 plasmid.

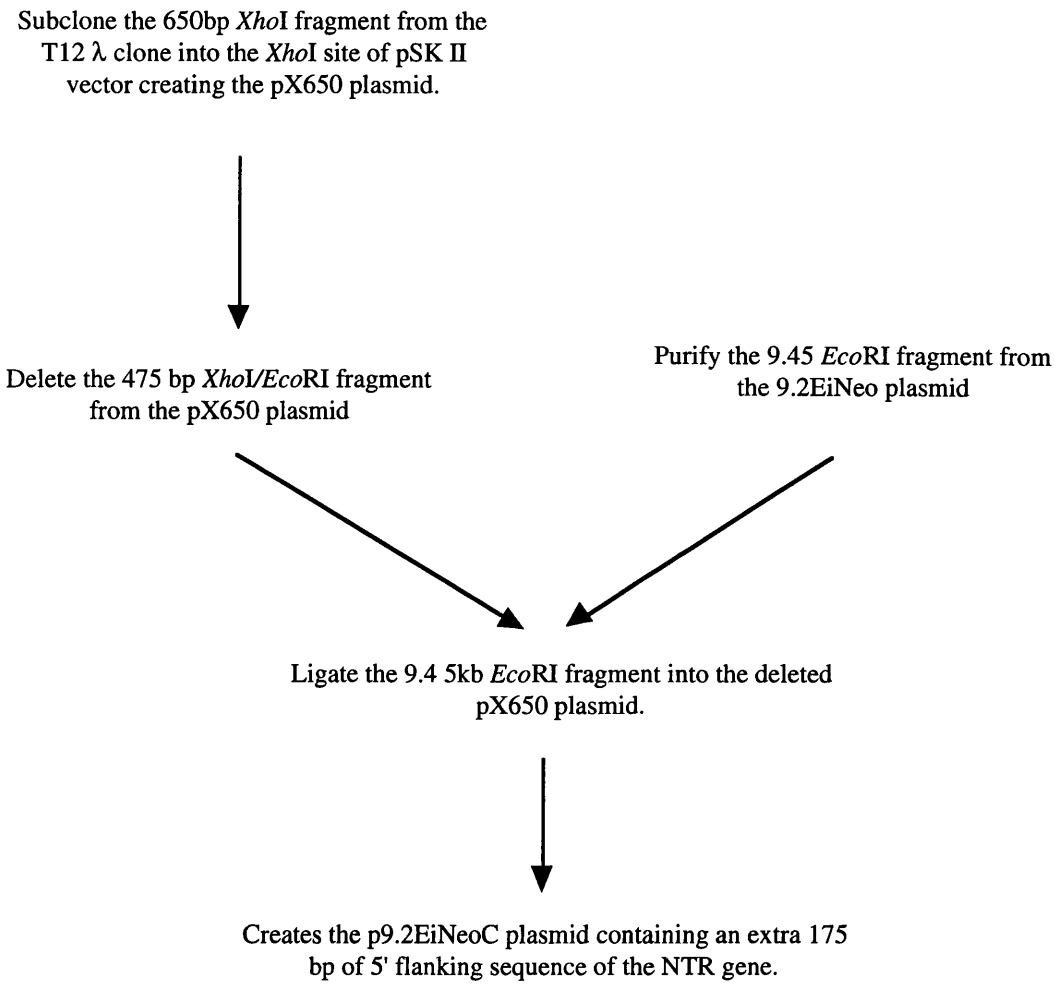


Figure 5.11 A flow diagram summarising the various manipulations involved in the creation of the p9.2EiNeoC control vector.

The pX650 plasmid was digested with the *Eco*RI enzyme and the vector band with its 175 bp of insert was purified from a 1% LMP agarose gel. The 9.45 kb fragment from the p9.2EiNeo plasmid was purified by similar means. The *Xho*I/*Eco*RI digested plasmid DNA and the 9.45 kb DNA fragment were ligated overnight at vector to insert ratios of 1:1 and 1:3. Recombinant plasmids were initially isolated on the basis of the size of their of supercoiled plasmids using the one step plasmid preparation procedure. Candidate clones were analysed by restriction digestion to determine if the insert was subcloned in the correct orientation. Digestion of the intended construct with the *Xho*I enzyme should produce the reconstructed 650 bp *Xho*I fragment. The analysis of one such clone, p9.2EiNeoC is depicted in Figure 5.12.

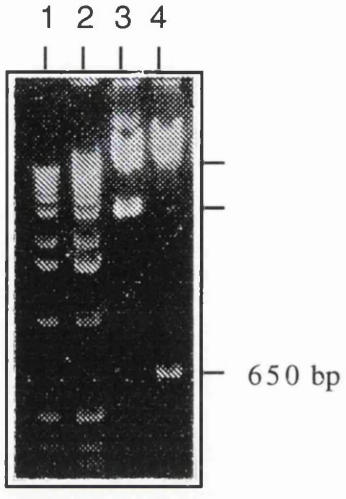


Figure 5.12 Restriction analysis of the p9.2EiNeoC control plasmid. Restriction analysis of this clone confirms the reconstruction of the 650 bp *Xho*I fragment. Lanes 1 and 2 are 1 kb marker; lane 3 is p9.2EiNeoC digested with *Eco*RI, lane 4 is p9.2EiNeoC digested with *Xho*I.

5.4.2 Design and testing of nested primers for the PCR analysis of ES Cell clones

For the successful isolation of targeted ES cell clones using a PCR screen it was advisable to test the fidelity of the PCR reaction. This required the analysis of sets of PCR primers, (22 mers to 25 mers), on the control plasmid. Initially it was hoped that the development of a simple PCR screen involving only one set of primers would provide a reliable screen. However this was not achieved. Instead, a two step PCR procedure was used known as nested PCR. The initial PCR reaction was performed using primers F5 and B4, (Figure 5.13), and 1/40 of this reaction was used as the template for a second PCR reaction using primers F8 and B1. The F8 and F5 primers are derived from the additional *XhoI/Eco*RI 175 bp of NTR gene sequence. The B1 and B4 primers are the 'unique' primers derived from the PGK promoter of the neomycin cassette. The F8/B1 primer pair are internal in location to the first set of primers with respect to the control plasmid template; see Figure 5.13. The rationale for this is that the first PCR reaction would enrich for the target

molecules, if any existed; the second reaction would then amplify the target molecules in preference to any spurious molecules produced by the first PCR reaction.

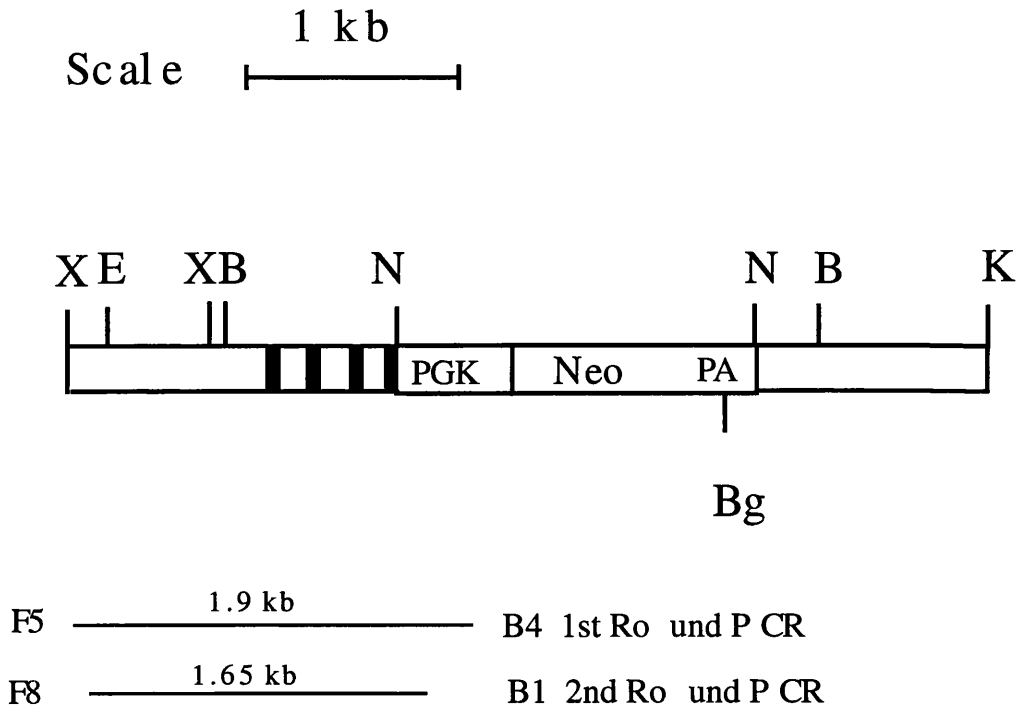


Figure 5.13 The nested primer pair used for the PCR analysis of ES cells. The restriction map depicted is of the 5' end of the control vector. The expected PCR product sizes for the primer pairs are indicated. Primers; F5 (5' taa ggg agc tga aga cgg cta cgg 3'), F8 (5' gat cgt gaa aga aag atc gtg ctg 3'), B1 (5' ctg act agg gga gga gta gaa ggt g 3'), B4 (5' cca ttt gtc acg tcc tgc acg ac 3'). Black stripes indicate the sequence position encoding the transmembrane domains I-IV; PGK, phosphoglycerate kinase promoter; PA, SV40 polyadenylation site; NEO, neomycin phosphotransferase gene; E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Bg, *Bgl*II; K, *Kpn*I and X, *Xho*I .

The sensitivity of this nested PCR screen was tested by using these conditions to amplify the desired band from varying amounts of control plasmid (template target molecules), mixed with genomic DNA. The genomic DNA provides a competitive background against which the primers must seek out and preferentially hybridise/amplify the target molecules for the reaction to be successful.

The sensitivity of the reaction was tested on 300 ng of genomic mouse DNA supplemented with serially decreasing amounts of control plasmid , p9.2EiNeoC. Quantities of plasmid providing 3.3 X 10^5 target molecules, (equivalent to the number of target molecules of a single copy gene in 1 µg of gDNA), ranging to quantities providing in theory single copy number of target molecules were used. The results of the nested PCR screen are presented in Figure 5.14.

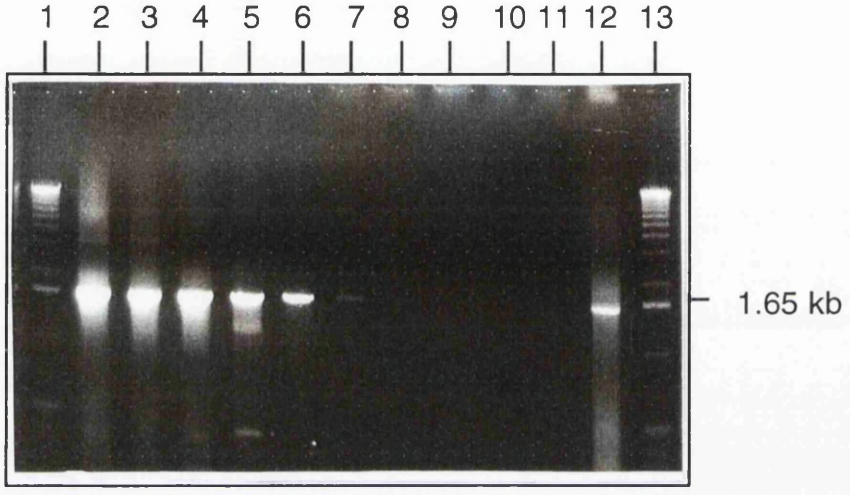


Figure 5.14 Nested PCR on serial dilutions of the control plasmid. Each clone was subjected to 25 cycles of 94°C for 30s, 55°C for 30s and 72°C for 2 min 10s with primers B4 and F5. 1/10 of each reaction was then used as a template for a subsequent reaction of 30 cycles at 94°C for 30's, 52°C for 2 min 10s with the F8 and B1 primer pair. 10 µl of each nested PCR reaction was then resolved on a 1.2% agarose gel. Lane 1 and 13 are 1 kb marker,
lane 2: template DNA was 250 ng mouse gDNA + 40 pg of control plasmid
lane 3: template DNA was 250 ng mouse gDNA + 4 pg of control plasmid
lane 4: template DNA was 250 ng mouse gDNA + 400 fg of control plasmid
lane 5: template DNA was 250 ng mouse gDNA + 40 fg of control plasmid
lane 6: template DNA was 250 ng mouse gDNA + 4 fg of control plasmid
lane 7: template DNA was 250 ng mouse gDNA + 0.4 fg of control plasmid
lane 8: template DNA was 250 ng mouse gDNA + 0.04 fg of control plasmid
lane 9: template DNA was 250 ng mouse gDNA + 0.4 fg of control plasmid
lane 10: template DNA was 250 ng mouse gDNA -ve control
lane 11: template DNA was 250 ng mouse gDNA -ve control
lane 12: template DNA was 4 pg of control plasmid +ve control

These results indicate that this PCR screen was capable of amplifying the desired product band of 1.65 kb from as little as 0.4 fg of control plasmid DNA, p9.2EiNeoC. This translates to a theoretical copy number of about 33 target molecules.

5.4.3 Transfection and selection of E14 ES cells with the control vector.

Transfection of E14 cells was undertaken to evaluate the PCR analysis of ES clones. Isolated G418^R clones were analysed using the nested PCR screen outlined in the previous section.

E14 ES cells were grown to an approximate density of 5 X 10^7/175 cm^2 plate. The cells were prepared for electroporation and electroporated with 40 μg of the control plasmid p9.2EiNeoC linearised at its *Sal*I site. The cells were then allowed to recover for 24 hours prior to G418 selection, which continued for 14 days. Then, 20 of the 1000 G418^R colonies produced were subjected to DNA isolation and PCR analysis. The results of the nested PCR analysis are presented in Figure 5.15. As indicated only 6 of the 10 clones analysed produced the major PCR product of the expected 1.65 kb. The other 4 clones that did not give rise to the expected band are thought to be genuine PCR results for these clones, and not failed PCR reactions. Repeating the nested PCR analysis for these 4 clones and other clones, (data not shown), produced similar results. A probable explanation is that the terminal NTR gene sequences were lost from the control vector during electroporation and genome integration. Loss of the gene sequences from the vector dictates loss of the F8 and F5 primer sites, therefore no randomly integrated vector sequence would produce the desired template for the production of the 1.65 kb PCR fragment. Furthermore, one of these clones was selected to further test the sensitivity of this PCR approach. The DNA from clone 9 was serially diluted and used as template for the nested PCR analysis. No loss of signal was detected when only 1/32 of the same DNA was used as a template, (data not shown). A comparable level of sensitivity was achieved when using the control plasmid as a template for the nested PCR, as little as 0.4 fg of plasmid (about 33 target molecules) was detectable. Thus the results indicate that the nested PCR regime previously outlined was sufficiently sensitive to be used as a screen for targeted ES cell clones.

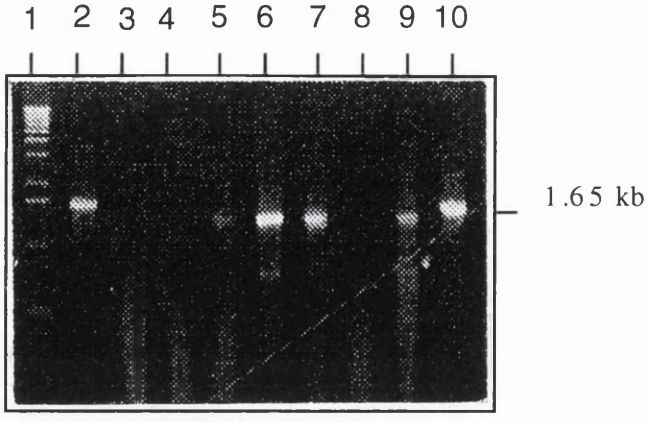


Figure 5.15 Nested PCR analysis of G418^R colonies. 1/10 of the DNA sample from each clone was subjected to 25 cycles of 94°C for 30s, 55°C for 30s and 72°C for 2 min 10s with primers B4 and F5. A tenth of the PCR reaction was then used as a template for a subsequent reaction of 30 cycles at 94°C for 30's, 52°C for 30s and 72°C for 2 min 10s with the F8 and B1 primer pair. 10 µl of each nested PCR reaction was then resolved on a 1.2% agarose gel. Lane 1 is 1 kb marker, lanes 2 to 10 are the PCR reactions of clones 1 to 9 respectively.

5.5 Summary

A replacement vector, (p9.2EiNeoTk,), was constructed to target the NTR gene utilising the positive negative selection strategy. This vector was assembled using a genomic 9.2 kb *Eco*RI NTR gene fragment subcloned from the 129J mouse library. An internal 1.5 kb *Nco*I fragment was deleted from the 9.2 kb *Eco*RI fragment, creating a deletion which removed gene sequences encoding aa residues 207 to 242. The neomycin cassette from the pNT plasmid was subcloned from the the pNeo plasmid into the *Sal*I linker placed between the *Nco*I restriction sites of the p9.2Ei plasmid. The fragment was then flanked by the addition of the thymidine kinase cassette, again subcloned from the pNT plasmid. Linearisation of the vector at the unique *Sal*I site of the polylinker produces a replacement construct with two arms of homology, (1.3 kb and 6.4 kb). Thus a construct satisfying the guidelines suggested by Deng *et. al.*, 1992a, was produced.

In addition, a control construct was generated to evaluate the PCR primers that may be used for the screening of targeted ES clones.

This vector consisted of the same 9.2 kb *Eco*RI gene fragment engineered with the neomycin cassette, plus an additional 175 bp of gene sequence flanking the short arm of homology. This construct was then used to transfect E14 ES cells and generate $G418^R$ colonies for the assessment of the PCR screen. The nested PCR analysis devised, successfully amplified the predicted 1.65 kb DNA fragment from the majority of colonies analysed. Furthermore, this screen was able to amplify the 1.65 kb product from as little as 0.4 fg of plasmid, (approximately 33 target molecules). Thus a reliable PCR screen was generated which would allow the identification of ES clones targeted with the p9.2EiNeoTK replacement vector.

Chapter 6

Targeted Mutagenesis of the Neurotensin Receptor Gene in ES Cells.

6.1 Introduction

Targeted disruption of the NTR gene in ES cells was achieved using the replacement construct p9.2EiNeoTk, and employing the positive negative selection strategy outlined in Figure 6.1, (Thomas and Capecchi, 1987). This approach allows selection to be applied simultaneously for an integrative event and against random integrants. The targeted ES clones were to be initially detected by the nested PCR analysis developed in Chapter 5. Confirmation of targeted clones was achieved by expanding individual clones for Southern blot analysis. The strategy was to generate as many targeted sub lines as possible from different ES cell lines; E14, R1, CGR8 and CGR8.8. The targeted ES cell lines would then be used to generate chimaeric mice via blastocyst injection and reimplantation into pseudo-pregnant mice.

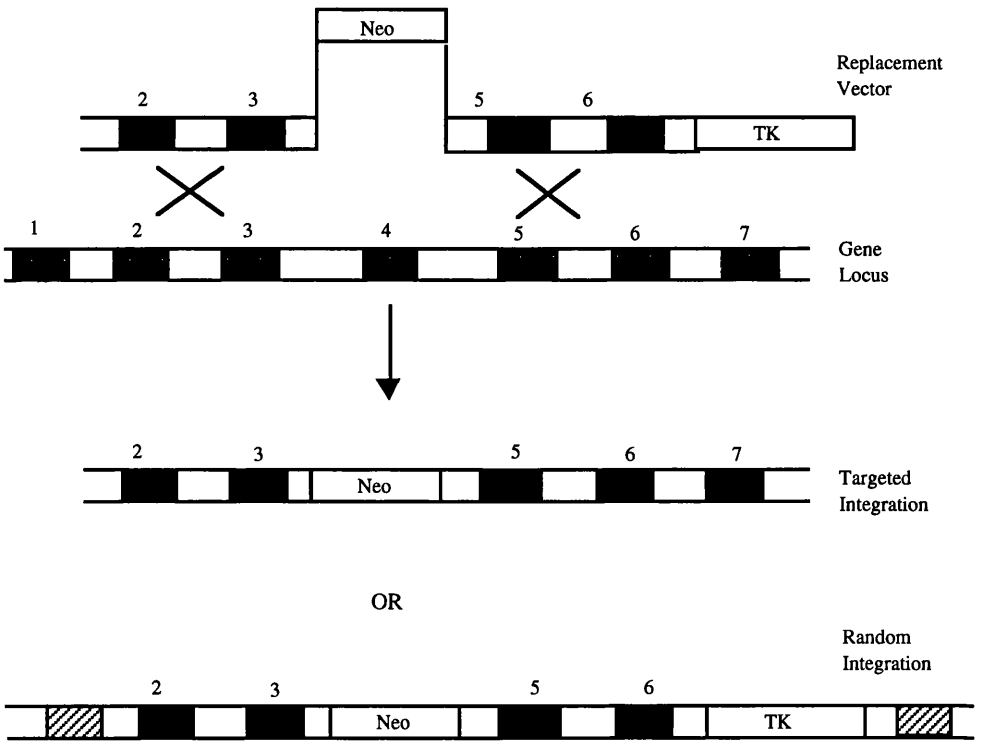


Figure 6.1 The targeted integration and random insertion of a replacement vector as observed in the positive negative selection strategy of gene targeting. The replacement construct consists of exons 2, 3, 5 and 6 with exon 2 being replaced by a neomycin cassette. The region of gene homology is flanked on one side by a thymidine kinase cassette. Targeted integration of the vector by homologous recombination would result in the replacement of exon 4 with a

neomycin cassette at the gene locus, but no integration of the thymidine kinase cassette. However, random integration of the vector into the genome, would generally result in the thymidine kinase cassette also being incorporated into the genome. Thus, integrants can be selected for with G418 due to the neomycin cassette, i.e. positive selection. Enrichment for targeted integrations can be achieved using gancyclovir to select against the presence of the thymidine kinase cassette, i.e. negative selection.

6.2 Transfection and selection of E14 ES cells with replacement construct; PCR results

The first ES cell line used for transfection was the E14 cell line; Handyside *et al.*, 1989. This cell line was derived from the 129/Ola substrain of the 129/J mouse strain, Lyon and Searle, 1989. This is a feeder independent line that was maintained on BRL conditioned medium. The initial strategy for the detection of targeted clones was a nested PCR analysis of batched clones; for this genomic DNAs from $G418^R$ $Ganc^R$ colonies were isolated in batches of 12 and subjected to the sensitive PCR regime developed in Chapter 5 section 5.6. Positive batches of 12 clones could then be expanded and re-analysed by individual PCRs or by Southern blot analysis to identify targeted clones.

E14 ES cells, passage 22, were harvested and prepared for transfection by electroporation. 40 µg of the p9.2EiNeoTK was linearised with the *SalI* enzyme and resuspended in 800 µl of 1 X PBS with 4.9 X 10^7 ES cells. After electroporation the cells were plated onto 5 round plates, 10 cm in diameter. All plates received G418 after a 24 hour recovery period. However, only 4 of the 5 plates received gancyclovir; the fifth plate served as a control for the enrichment of targeted clones using gancyclovir. From the 4 plates to which G418 and gancyclovir were added, a total of 220 $G418^R$ $Ganc^R$ colonies were recovered. The fifth plate produced a total of 224 $G418^R$ colonies. This translates to an apparent enrichment of almost 4-fold for targeted clones over random integrants.

The $G418^R$ $Ganc^R$ colonies were picked into and expanded in 24 well plates. Batches of 12 colonies were pooled for PCR analysis, with one half of each colony used to seed a well of a 24 well plate. The cells of the 24 well plates were grown to near confluence and stored *in situ* at -70°C until required. The DNA was isolated from the batches of clones and subjected to the nested PCR analysis outlined in Chapter 5. The results of this PCR analysis are presented in Figure 6.2.

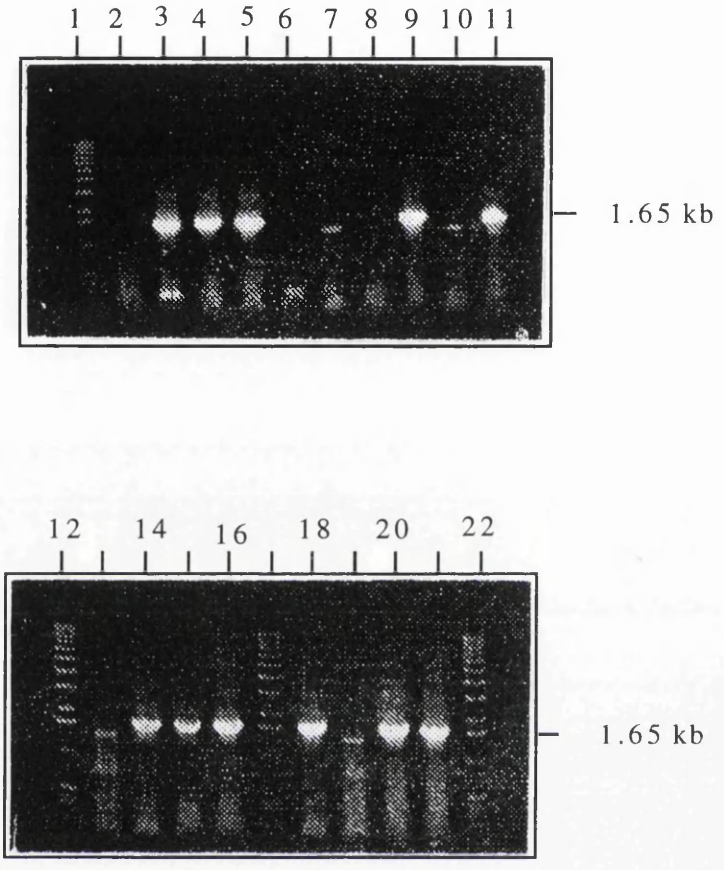


Figure 6.2 Nested PCR analysis of the batched G418^R Ganc^R colonies. 1/10 of the DNA sample from each clone was subjected to 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min 10 s with primers B4 and F5. A second round of PCR was performed on 1/10 of the previous PCR reaction for 30 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 2 min 10 s. Details of the primers used are presented in Figure 5.14. 10 µl of each nested PCR reaction was then resolved on a 1.2% agarose gel. Lanes 1, 12, 17 and 22 are 1 kb marker; lane 2 is the negative control (PCR results for 100 ng template of mouse genomic DNA); lanes 3 to 11 are batches A to I respectively; lanes 13 to 16 are batches J to M; lanes 14 15 and 16 are batches N, O and P; lane 22 is a positive control using DNA from a clone generated during the control transfection, (section 5.7).

The PCR results suggested that 10 of the 16 batches analysed contain at least 1 targeted clone. This translates to a minimum of 10 positive clones in 192, i.e. an approximate targeting frequency of 1 in 19. This was an encouragingly high

targeting frequency, and batches of clones were expanded for individual analysis.

6.3 Southern blot analysis of E14 G418^R Ganc^R colonies

The positive results from the PCR analysis on the batches of clones suggested a high targeting frequency of 1 in 19 targeted to non-targeted clones. Individual targeted clones were then studied by Southern blot analysis. Although it was possible in theory to identify positive pools by individual PCRs, this was not done. Apprehension about false positives PCR results and the ultimate need to verify potentially targeted clones by Southern blot analysis, dictated the following analysis.

Southern blot analysis was performed using the 3 kb *Bgl*II-*Xho*I fragment derived from the p4.8Ei plasmid. This fragment maps 5' to the gene fragment used for construction of the replacement vector. Probing genomic Southern blots with this fragment results in the detection of the 10.45 kb restriction fragment of the NTR gene. However, if the gene is targeted with the p9.2EiNeoTK construct, then a new *Bgl*II restriction site is introduced into the gene via the neomycin cassette. For such targeted clones a 5.95 kb band was observed on Southern analysis. Details of the 3 kb *Bgl*II-*Xho*I probe fragment, its relationship to the replacement vector and sizes of the predicted *Bgl*II restriction fragments are presented in Figure 6.3

Southern blot analysis was performed on the 220 G418^R Ganc^R colonies. The cells were retrieved from -70°C storage, expanded, DNAs isolated for *Bgl*II restriction digestion, the subsequent gel was Southern blotted to nylon membrane and probed using the labelled 3 kb *Bgl*II-*Xho*I fragment. A total of 5 targeted clones were identified, 4 of which are presented in Figure 6.4.

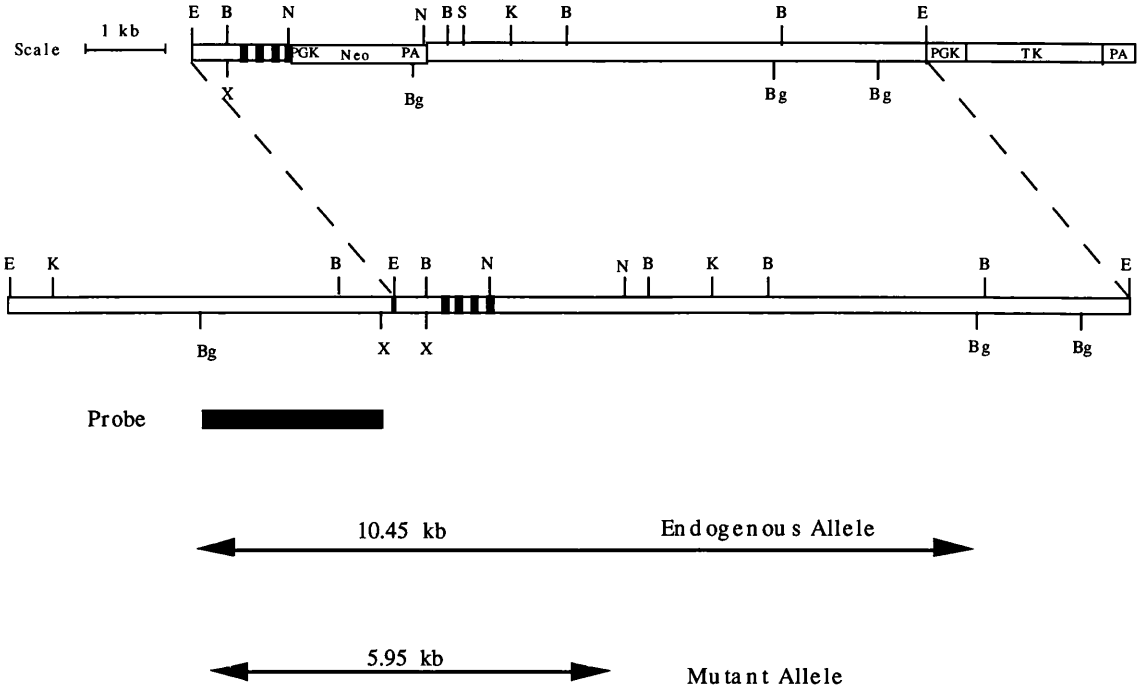


Figure 6.3 Details of the DNA probe fragment used for Southern blot screening of G418^R Ganc^R clones. Restriction maps for the targeting construct, p9.2EiNeoTK, and the NTR gene locus are shown. The sizes of the bands expected from *Bgl*II digested targetedand non targeted alleles are indicated. The *Bgl*II-*Xho*I fragment used as a probe is indicated by the black box. The original 9.2 kb *Eco*RI gene fragment is shown with black stripes indicating the position of the transmembrane domains I-IV, a neomycin cassette insertion and flanked by a thymidine kinase cassette. PGK, phosphoglycerate kinase promoter; PA, SV40 polyadenylation site; Neo, neomycin phosphotransferase gene; TK, HSV thymidine kinase gene; E, *Eco*RI; B, *Bam*HI; N, *Nco*I; Bg, BglII; X, *Xho*I and K, *Kpn*I.

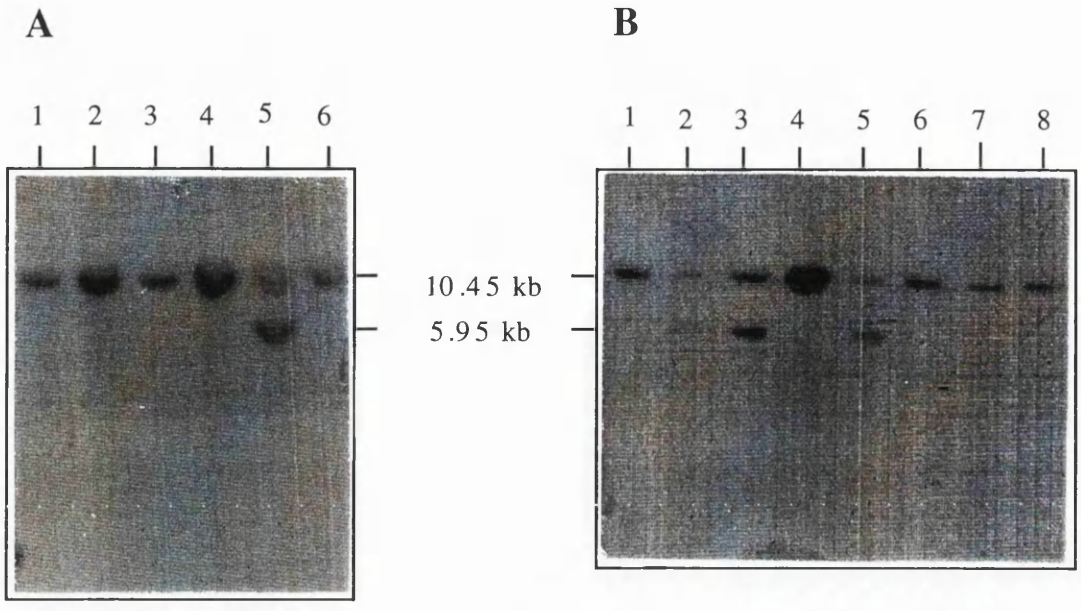


Figure 6.4 Southern blot analysis of G418^R Ganc^R E14 ES cell colonies. 5-15 µg DNA of individual clones was digested with *Bgl*II enzyme, resolved on 0.7% agarose gels and subsequently transferred to nylon membranes. The ^{32}P labelled *Bgl*II-*Xho*I probe was prepared by the random priming method, (Material and Methods; section 2.4.18), and hybridised in a 50% formamide based buffer at 2-3 X 10^6 dpm/ml. The resulting autoradiographs are presented in panels A and B. Panel A: lanes 1,2,3,4 and 6 are non-targeted clones since only the wild type allele of 10.45 kb was present; lane 5 is a targeted clone (E14A), wild type allele of 10.45 kb and mutant allele of 5.95 kb. Panel B: lanes 1, 4, 6, 7 and 8 are non-targeted clones containing only the wild type allele of 10.45 kb; lanes 2, 3 and 5 are targeted clones, (E14B, E14C and E14D respectively).

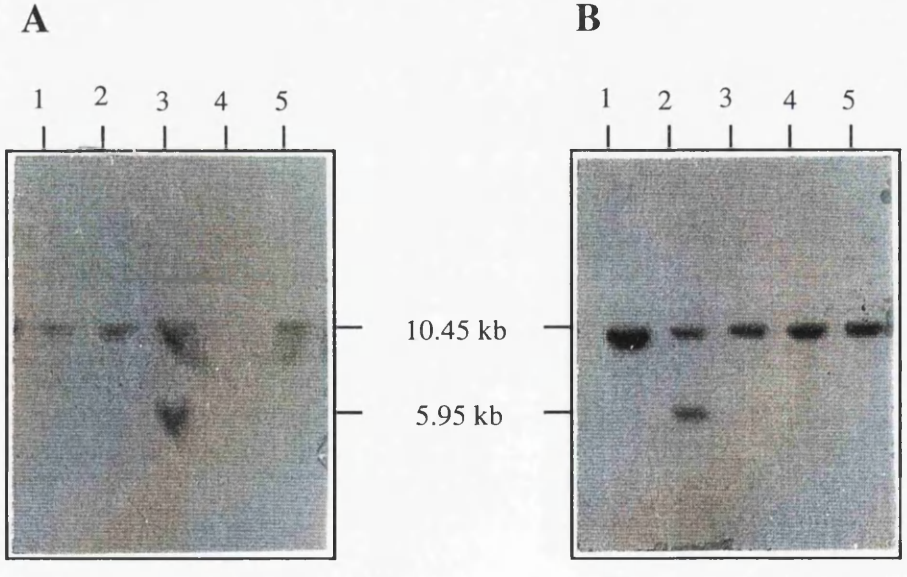
6.4 Transfection and selection of R1 ES cells; Southern blot results

The R1 ES cell line, (Nagy, *et al.*, 1993), was transfected with the replacement construct, (p9.2EiNeoTK). This 129/J derived cell line was chosen for its ability to generate chimaeric mice by aggregation as well as by blastocyst injection, (Wood, *et al.*, 1993). The R1 ES cell line was maintained on a feeder independent system with growth medium supplemented with LIF.

The R1 ES cells were transfected and selected in a similar manner to that described for the E14 cell line. Briefly, 40 µg of *Sal*I

linearised p9.2EiNeoTK plasmid was mixed with approximately 5 X 10^7 cells and electroporated. The cells were plated onto 5 plates with the medium supplemented with 200 µg/ml G418 after a 24 hour recovery period; 4 of the plates also received gancyclovir @ 1 X 10^{-6} M. $G418^R$ $Ganc^R$ colonies were ready to pick after 12-14 days of selection, of which 168 colonies were recovered. The fifth plate which only received G418, produced 161 colonies. This translates to an enrichment factor of about 4 fold for this experiment.

The $G418^R$ $Ganc^R$ colonies were picked and expanded in 24 well plates. When near confluent, 80 % of the cells were harvested for DNA isolation and Southern analysis. The remainder of the cells were grown to near confluence and stored at -70°C until required. Nested PCR analysis was not performed on these clones as the previous experiment predicted a targeting frequency of about 1 in 40 clones, which was suitably high to proceed with Southern analyses of these clones. The approach used was identical to that described in the previous section and resulted in a total of 4 targeted sub lines being isolated. Again, a comparable targeting frequency of about 1 in 40 colonies was observed. The results are presented in Figure 6.5.



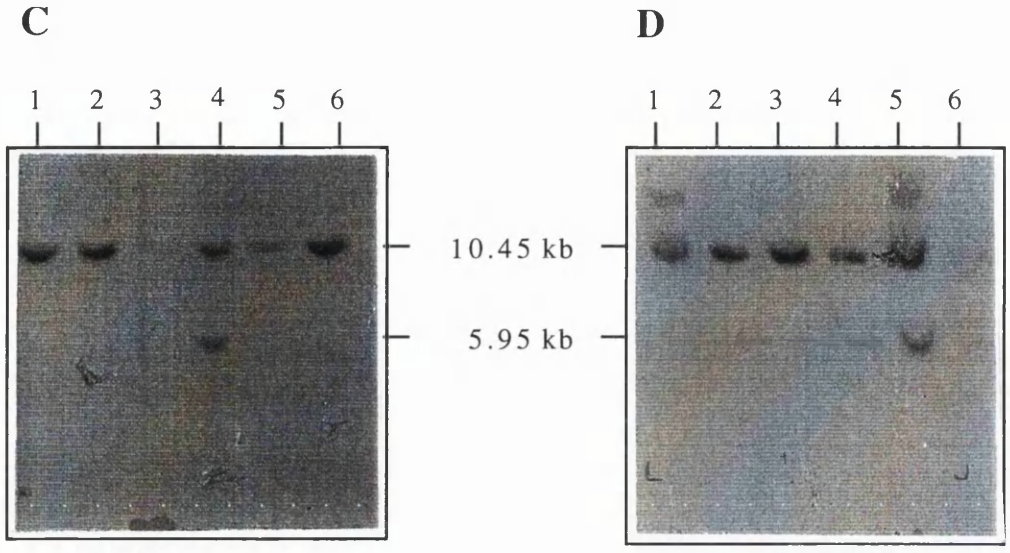


Figure 6.5 Southern blot analysis of G418^R Ganc^R colonies of the R1 ES cell transfection. 5-15 µg DNA of individual clones was digested with *Bgl*II enzyme, resolved on 0.7% agarose gels and subsequently transferred to nylon membranes. The ^{32}P labelled *Bgl*II-*Xho*I probe was prepared by the random priming method, (Material and Methods; section 2.4.18), and hybridised in a 50% formamide based buffer at 2-3 X 10^6 dpm/ml. The resulting autoradiographs are presented in panels A, B, C and D. Presence of the 5.95 kb fragment denotes a targeted allele as observed for; panel A lane 3, panel B lane 2, panel C lane 4 and panel D lane 5. These are targeted clones R1.A to R1.D respectively. All other lanes only display the wild type allele of 10.45 kb.

6.5 Transfection and selection of CGR8 ES cells; Southern blot results

The CGR8 ES cell line was also derived from the 129/Ola mouse strain, (Mountford *et al.*, 1994). This is a highly germline transmissible cell line, approximately 30 % of gene trap generated ES cell lines were germ line transmissible, (personal communication, Dr. W. C. Skarnes, the BBSRC Centre for Genome Research, University of Edinburgh, Scotland). The transfection and selection of the CGR8 cell line was essentially the same as that for E14 and R1 cell lines except a method of electroporation with parameters defined by Dr. W. C. Skarnes was used, as described in Material and Methods, (section 2.5.9).

Approximately 1 X 10^8 ES cells were electroporated with 150 µg of *Sal*I linearised p9.2EiNeoTK plasmid. G418 and gancyclovir

selection were as before except a total of 20 plates were used with one plate only receiving G418, the control for gancyclovir enrichment. A total of 38 $G418^R$ $Ganc^R$ colonies were picked from the other 19 plates with 6 colonies remaining on the 'G418 only' plate. This suggests an enrichment factor of about 3 fold. The electroporation of these cells was abnormal, as a time constant of 0.3 ms was recorded as opposed to 0.1 ms, (Dr. W. C. Skarnes, personal communication). Nevertheless, the 38 $G418^R$ $Ganc^R$ colonies were processed for Southern analysis as previously described, and the results are presented in Figure 6.6.

The results from the Southern analysis revealed only one clone targeted in the expected manner, clone CGR8.A (lane 4). However, lane 2 contains an unexpected band of about 3.5 kb in size which, may be the result of a targeting event accompanied by a rearrangement at this allele causing a deletion. Furthermore, the intensity of this band is not equivalent to that 10.45 kb band of the wild type allele. Therefore, only a portion of the cells from this clone now contain this targeted but rearranged allele. For these reasons the clone was discarded from further analysis.

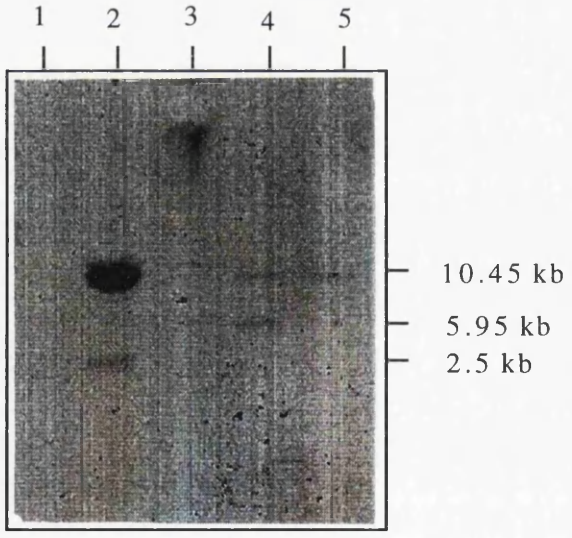


Figure 6.6 Southern blot analysis of $G418^R$ $Ganc^R$ colonies from CGR8 transfection. 5-15 mg of DNA from individual clones wasdigested with *Bgl*II enzyme, resolved on 0.7% agarose gels and subsequently transferred to nylon membranes. The ^{32}P labelled *Bgl*II-*Xho*I probe was prepared by the random priming method , (Material and Methods; section 2.4.18), and hybridised in a 50% formamide based buffer at 2-3 X 10^6 dpm/ml. The resulting

autoradiograph is shown with band sizes. Lanes 1 and 3 are apparently empty; lane 2 contains a targeted but rearranged clone; lane 4 shows a banding pattern indicative of a targeted clone (CGR8.A); lane 5 displays the 10.45 kb band observed for the wild type allele.

6.6 Characterisation of the CGR8.A ES cell line

Occasionally targeted ES cell lines carry a random integration of the targeting construct. For this reason it is good routine practice to check targeted ES lines prior to blastocyst injection. Furthermore, continued passage of ES cells in culture can typically result in abnormal chromosome numbers. Therefore metaphase spreads were prepared from the CGR8.A line to determine chromosome number.

Southern blot analysis of the CGR8.A line was performed using a probe derived from the coding region of the thymidine kinase cassette. An internal 900 bp *Pst*I fragment derived from the HSV thymidine kinase gene (pTK) was used as probe to identify the presence of such sequences in targeted and non targeted ES cell clones; Figure 6.7 displays the results of such an analysis.

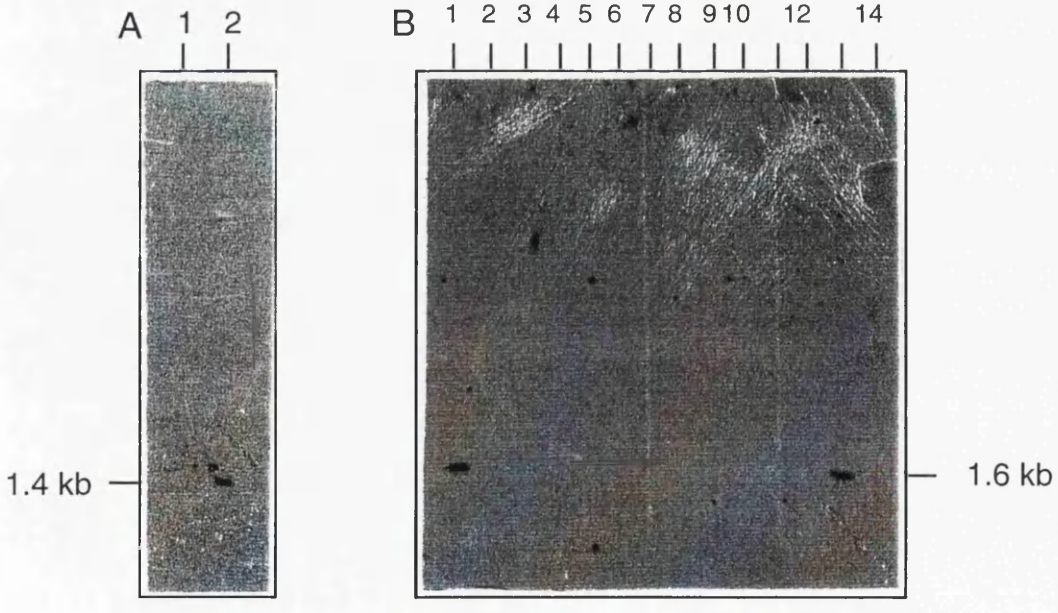


Figure 6.7 Southern blot analysis of the CGR8.A clone with a thymidine kinase derived probe. 5-15 mg of DNA from individual clones was digested with *Bgl*II enzyme, resolved on 0.7% agarose gels and subsequently transferred to nylon membranes. The ^{32}P labelled probe, (a *Pst*I 900 bp fragment derived from

pTK), was prepared by the random priming method , (Material and Methods; section 2.4.18), and hybridised in a 50% formamide based buffer at 2-3 X 10^6 dpm/ml. The resulting autoradiograph is shown with band sizes. Panel A lane 2 contains a 1.4 kb hybridising band from a non-targeted clone. Panel B lane 5 contains DNA from the targeted CGR8.A clone; lanes 1 and 14 are 1 kb marker lanes both displaying a hybridising 1.6 kb plasmid derived band; the remaining lanes are of non-targeted ES clones.

The targeted CGR8.A line does not carry an integrated copy of the thymidine kinase cassette, as suggested by the lack of a hybridising band in lane 5 panel B of Figure 6.7.

The possibility of an aberrant chromosome number for this line was addressed by counting mitotic spreads. A total of 50 spreads were examined with a strong modular distribution of 40 chromosomes being observed. Figure 6.8 represents a typical metaphase spread from the CGR8.A cell line, displaying the normal mouse chromosome complement of 40.

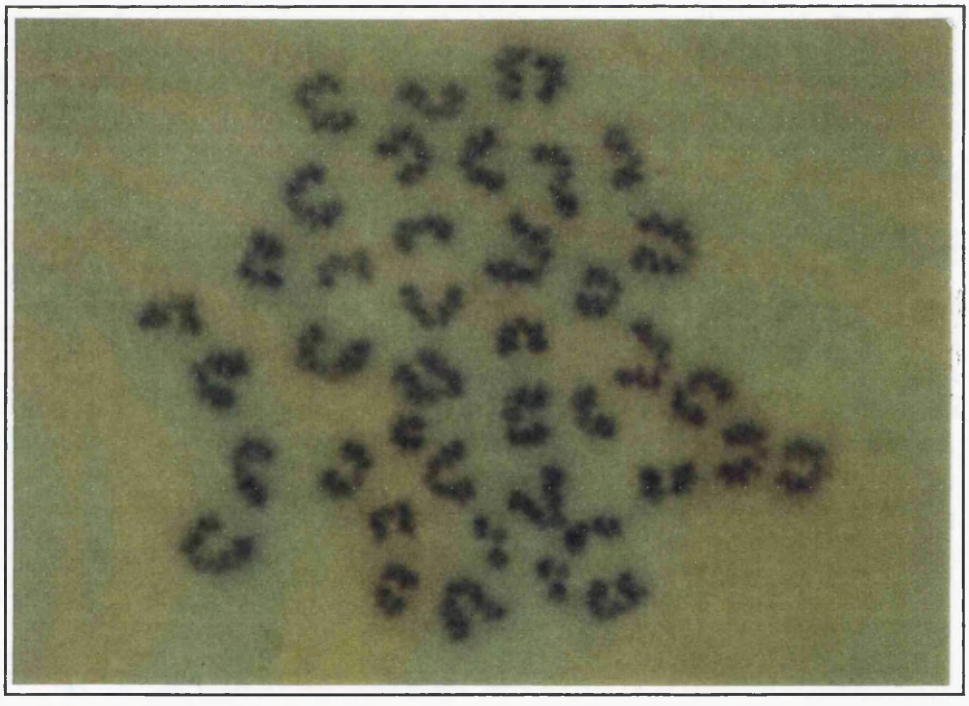


Figure 6.8 Metaphase spread from the CGR8.A ES cell line.

6.7 Transfection and selection of CGR8.8 ES cells; Southern blot results

The CGR8.8 ES cell line was derived from the CGR8 cell line by Dr. W. C. Skarnes of the BBSRC Centre for Genome Research, University of Edinburgh, Scotland. This cell line has a higher germline transmission rate than the parental strain; approaching nearly 70% of gene trap generated cell lines were germline transmissible.

Transfection and selection of the CGR8.8 cell line was performed as described for the CGR8 cell line. On this occasion the observed time constant was as expected, i.e. 0.1 ms. The number of $G418^R$ $Ganc^R$ colonies from the 19 plates totalled approximately 2,700. The plate that received no gancyclovir produced approximately 480 colonies, which translates to about a 3-fold enrichment using gancyclovir selection. The number of $G418^R$ $Ganc^R$ colonies produced was too many to be processed by one person. Therefore, a total of 192 were picked and expanded for Southern analysis. Of the 182 colonies successfully analysed only 2 were apparently targeted, (Figure 6.9). This calculates as a targeting frequency of about 1 clone in 90.

The targeting frequency for the CGR8.8 ES cell line is lower than that previously observed for the E14 and R1 cell lines of 1 in 40 clones. Moreover, the enrichment produced by the gancyclovir selection was less than previously observed with an apparent drop from 4 to 3-fold. It is not possible to attach any statistical significance to these results since a number of other variable factors could have contributed to the differences observed, other than solely being a property of the CGR8.8 line.

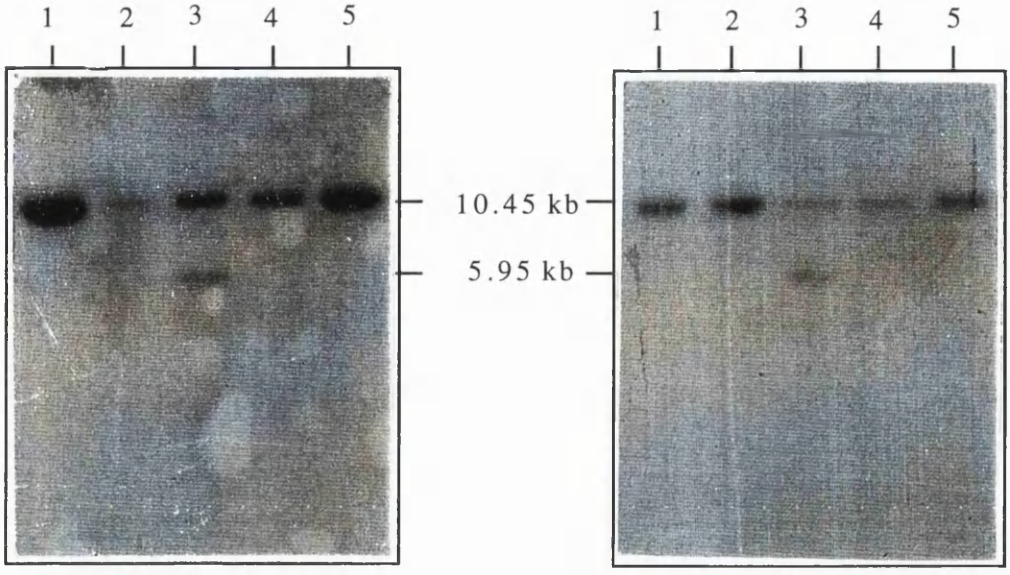


Figure 6.9 Southern analysis of G418^R Ganc^R colonies from the transfection of CGR8.8 ES cells. 5-15 µg of DNA from individual clones was digested with *Bgl*II enzyme, resolved on 0.7% agarose gels and subsequently transferred to nylon membranes. The ^{32}P labelled *Bgl*II-*Xho*I probe was prepared by the random priming method, (Material and Methods; section 2.4.18), and hybridised in a 50% formamide based buffer at 2-3 X 10^6 dpm/ml. The resulting autoradiographs are shown with band sizes indicated in panel A and B. Lane 3 of each autoradiograph represents a targeted clone as indicated by the presence of the 5.95 kb band, (clones CGR8.8A and CGR8.8B). All other lanes contain non-targeted clones.

6.8 Summary

Transfection of the E14 ES cell line with the p9.2EiNeoTK replacement construct produced a targeting frequency of about 1 in 40 targeted to non-targeted clones using the positive negative selection strategy. A gancyclovir enrichment factor of 4-fold was observed. This reasonable targeting frequency resulted in the subsequent omission of the nested PCR screening strategy for targeted clones developed in Chapter 5. The PCR approach was also dropped because false positive results were being produced. The initial PCR screen of the E14 G418^R Ganc^R clones suggested a targeting frequency of about 1 in 19, but this was evidently not the case as revealed by the Southern analysis. Possible explanations for false positive PCR results have previously been presented in Chapter 4; section 4.3.4. Transfection of the R1 ES cell line

resulted in a similar targeting frequency of 1 in 40 with a gancyclovir enrichment factor of 4-fold. The results of transfection of the CGR8 ES cell line produced fewer $G418^R$ $Ganc^R$ colonies than expected due to some unknown error believed to have occurred during the preparation of the cells for the electroporation procedure. The results obtained from targeting the CGR8.8 ES cell line produced a lower targeting frequency of only about 1 in 90, and a lower gancyclovir enrichment factor of about 3-fold. These transfection experiments were performed using different electroporation conditions and in different laboratory environments. Therefore, direct comparison of targeting or enrichment frequencies between the cell lines would be invalid. In summary, the total numbers of targeted sub-lines produced for the NTR gene were: 5 from the E14 ES cell line, 4 from the R1 ES cell line, 1 from the CGR8 ES cell line and 2 from the CGR8.8 ES cell line, see Figure 6.10.

ES cell line	Electroporation settings	No. of cells and amount of vector	Transformation frequency	Enrichment factor	Targeting frequency	No. of targeted clones
E14	500 μF, 250 V	5×10^7 cells 25 μg DNA	2.2×10^{-5}	4	1/40	5
R1	500 μF, 250 V	5×10^7 cells 25 μg DNA	1.6×10^{-5}	4	1/40	4
CGR8	3 μF, 800 V	1×10^8 cells 150 μg DNA	1.2×10^{-6}	4	1/40	1
CGR8.8	3μF, 800 V	1×10^8 cells 150 μg DNA	9.6×10^{-5}	3	1/90	2

Figure 6.10 Summary of the conditions and results from the ES cell transfection experiments of Chapter 6.

Chapter 7

Production of Transgenic Mice

7.1 Introduction

Chimaeric embryos can be produced by either blastocyst injection, (Papaioannou and Johnson, 1993), or by aggregation of morula stage embryos with the targeted ES cell lines, (Wood *et al.*, 1993). The chimaeric mice reported in this chapter were generated by blastocyst injection performed by Dr. W. C. Skarnes of the Centre for Genome Research, University of Edinburgh, Scotland. The E14 targeted ES cell lines were not microinjected into blastocysts as 4 of them were lost due to bacterial contamination of the tissue culture facilities; the fifth line did not recovery from temporary -70°C storage. However, the four targeted RI ES cell lines and the targeted CGR8 ES cell line were successfully microinjected into blastocysts, to produce chimaeric mice.

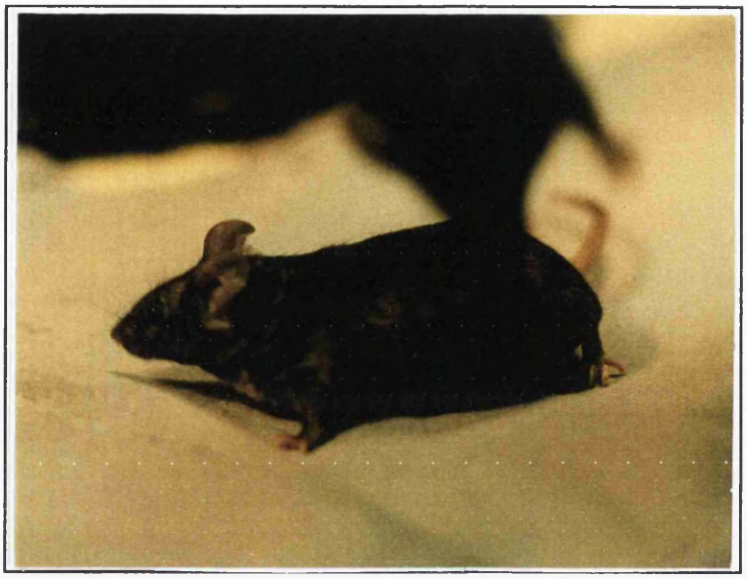
7.2 Results of blastocyst injection for the targeted R1 ES cell sublines

The four targeted ES cell lines RI.A-D were injected into 3.5 day C57BL/6J derived blastocysts. The RI.A and RI.B cell lines were injected into 16 blastocysts, and the embryos were mixed prior to transplanting into two pseudopregnant females. None of the resulting offspring displayed any coat colour chimaerism. Cell lines RI.C and RI.D were injected into 17 blastocysts and transferred to two pseudopregnant females who produced 11 offspring. 9 of the mice were weakly chimaeric, (10-15% agouti coat colour; see Figure 7.1), the other two were not. Breeding of these chimaeric mice did not produce any germline transmission of the null NTR allele, as observed by the lack of offspring with agouti coat colour.

7.3 Results of blastocyst injection for the targeted CGR8 ES cell subline

The targeted CGR8.A cell line was microinjected into 12 blastocysts, resulting in the production of 3 male offspring with 80% to 90% chimaerism; see Figure 7.2. The 3 strongly chimaeric males were mated with C57BL/6 females to test for germline transmission. However, all these males proved to probably be sterile, as no progeny were produced with several different females.

A



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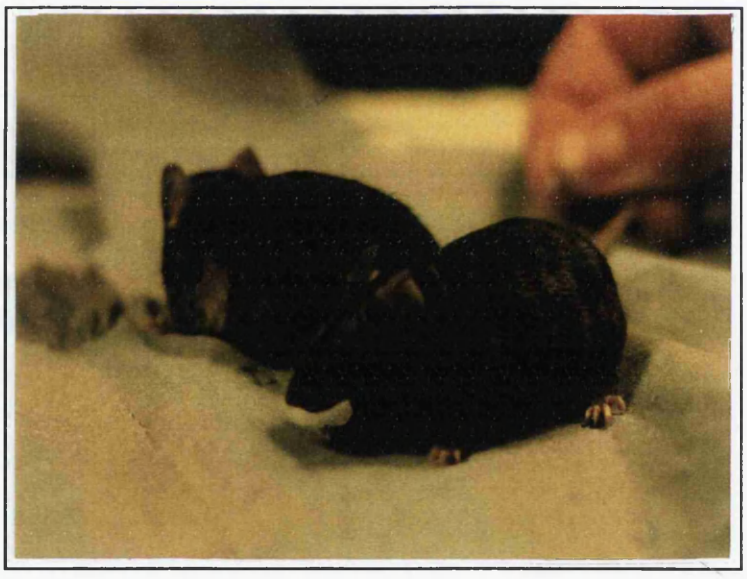


Figure 7.1 R1 derived chimaeric mice. Photographs A and B show a male mouse that was typically representative of the weakly chimaeric mice produced by injection of targeted R1.C and R1.B cell lines, (129/SvJ), into C57BL/6 blastocysts. The agouti coat colour, which covered approximately 10 to 15% of the mouse, was the ES cell derived fraction of the mouse as opposed to the blastocyst derived black coat colour.

A

B

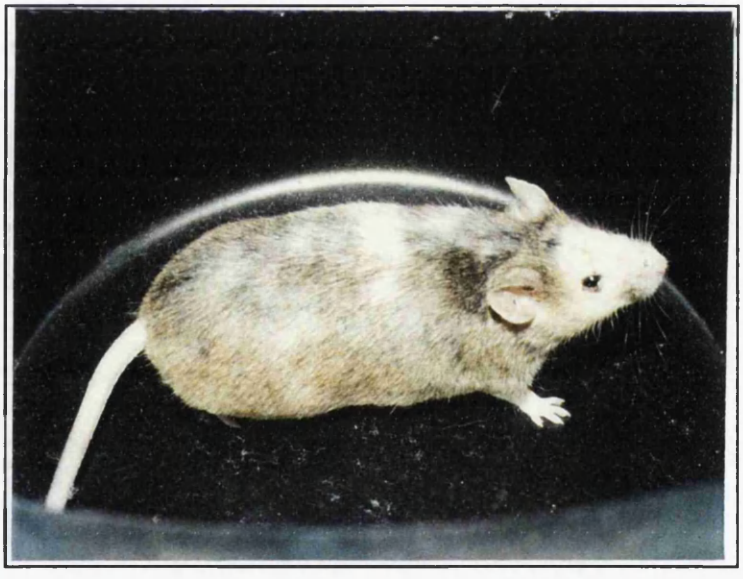


Figure 7.2 CGR8 derived chimaeric mouse. Photographs A and B show one of the male derived offspring from injection of the targeted CGR8.A cell line, (129/Ola), into C57BL/B derived blastocysts. The 80-90% agouti coat colour represents the ES cell derived portion of the mouse, as opposed to the black coat colour of the blastocyst.

7.4 Summary

The production of chimaeric mice was successfully achieved for the targeted R1.A, R1.B ES and CGR8.A ES cell lines. The level of chimaerism achieved using the R1 ES cell lines was estimated at about 10-15%, based on coat colour, for the 9 offspring. Coat colour chimaerism is a good indication of the level to which an ES cell line has contributed to the germline. Therefore, the likelihood of germline transmission of the null NTR allele was not high using these chimaeric mice; not surprisingly germline transmission was not obtained. However, the targeted CGR8.A ES cell line produced 3 strongly chimaeric male mice displaying 80-90% agouti coat colour derived from the ES cell line. Unexpectedly, these mice did not achieve germline transmission of the targeted allele; possible reasons for this lack of transmission are presented in the Discussion, (Chapter 8; section 8.2.3). Finally, the targeted cell lines generated from the CGR8.8 ES cell line await blastocyst injection at the time of thesis submission.

Chapter 8

In Situ Hybridisation of the Neurotensin Receptor in the embryonic mouse

8.1 Introduction

The overall aim of this project was to investigate the function of the NT transmission system within the adult CNS of the mouse using an unconditional gene 'knock out' strategy. However, if NT has an alternative role during development of the CNS, for example as a trophic factor, then mutation of the receptor could result in developmental abnormalities of the CNS that may confuse or limit this approach's ability to unravel the function of the NT system within the adult brain. The following *in situ* hybridisation study was designed to look at the expression of the NTR receptor mRNA within the developing mouse embryo. Previous studies have indicated a post-natal developmental pattern of expression for NTR that differs from the expression observed in the adult rodent brain, Sato *et al.*, 1992, which suggests that neurotensin may have a distinct function within the developing brain.

It must be noted that the work presented within this chapter was produced with the help and supervision of Dr. M. Sutherland from Vanderbilt University, U.S.A.

8.2 *In situ* hybridisation of sagittal mouse embryo sections

A series of mid-sagittal embryo sections taken from days 13.5, 15.5 and 17.5 embryos, were hybridised with an anti-sense ^{35}S labelled oligonucleotide probe, (Chapter 2 sections 2.4.31 and 2.4.32), for the mouse neurotensin receptor. The sequence for the probe was derived from clone p2.8 (Chapter 3 figure 3.11), from nucleotide position 262 to 306,(5'- cag tgc tct gca ggc tct gca gcg act tct tcc gcg cta gag tga 3'). The following Figures 8.1, 8.2, 8.3 and 8.4 present the results of such an analysis. Figures 8.2, 8.3 and 8.4 also include histological sections detailing the expected anatomical development of mid-sagittal sections taken from similar time points, (adapted from Kaufman, 1992).

Figure 8.1 details the results for the hybridisation of the mouse probe derived from the putative mouse NTR sequence of Chapter 2. As expected, specific hybridisation is observed to mid-brain structures corresponding to the location of the substantia nigra and the ventral tegmental areas panel A. A number of negative controls were performed, whereby the same probe, but with a 50 molar excess of unlabeled probe, was hybridised to adjacent sections from the mouse mid-brain; no specific hybridisation was observed. The series of sagittal embryo sections first produced a weak signal

hybridising to a developing structure of the mouse forebrain at day 13.5, Figure 8.2. The hybridisation signal becomes stronger at days 15.5 and 17.5, and the sections display a more definite hybridisation signal possibly corresponding to the developing neocortex, Figures 8.3 and 8.4.

A

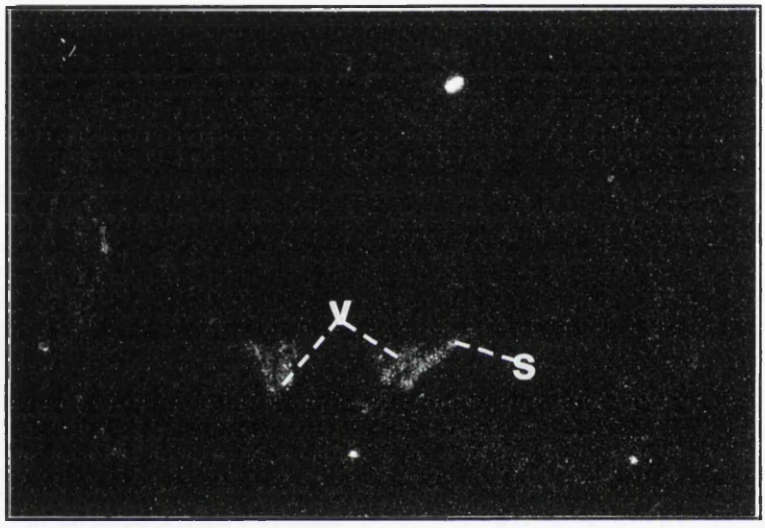
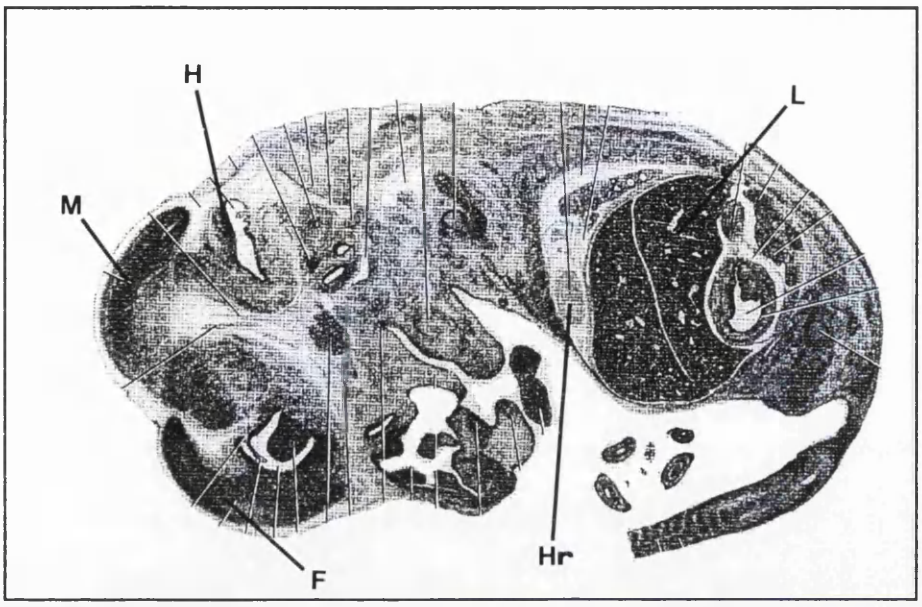


Figure 8. 1 Mid-coronal section from the adult mouse brain. Panel A details the hybridisation of the ^{35}S labelled probe (5'- cag tgc tct gca ggc tct gca gcg act tct tcc gcg cta gag tga 3') to an area of the brain that corresponds to the substania-nigra and the ventral tegmental area of the mouse mid-brain. S, substantia nigra; V, ventral tegmental area.

A



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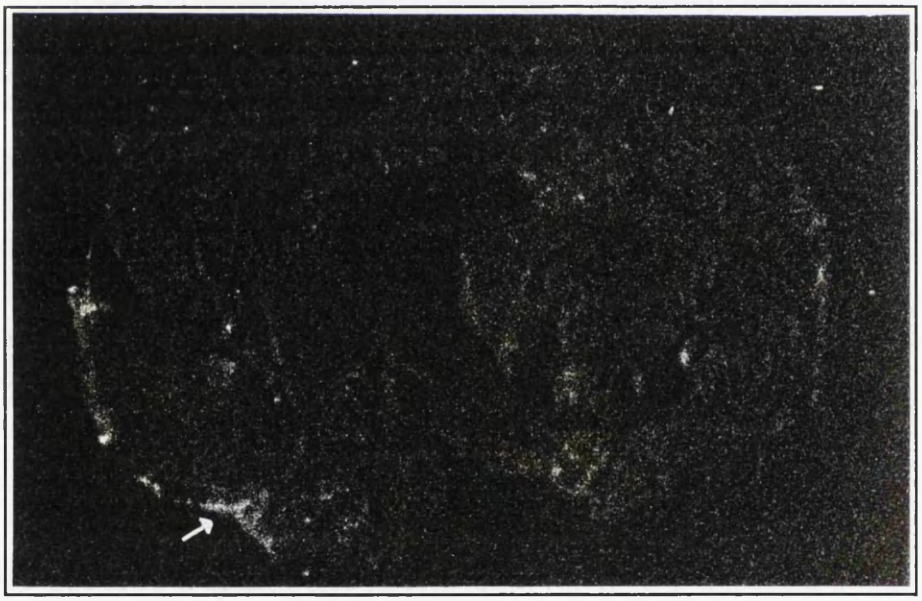
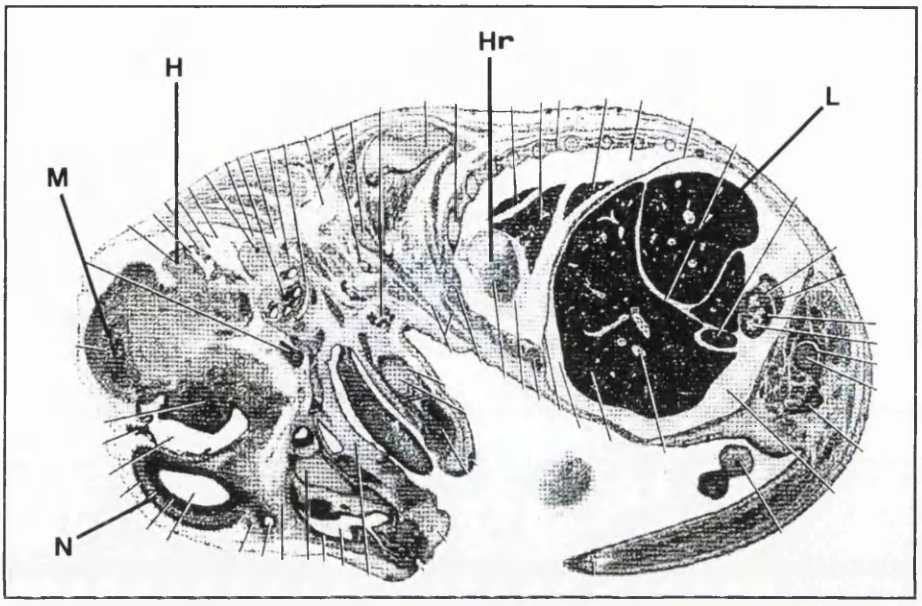


Figure 8.2 Sagittal section from a 13.5 day mouse embryo. Panel A is a histological section of the mouse embryo from approximately the same time point of development and is a mid-section sagittal slice that is roughly equivalent to that of Panel B. The lower panel details the specific hybridisation observed with a NTR specific probe derived from clone p2.8, (5'- cag tgc tct gca

ggc tct gca gcg act tct tcc gcg cta gag tga 3'). Arrow indicates specific hybridisation; F, forebrain; M, midbrain; Hr ,heart; L, liver.

A



B

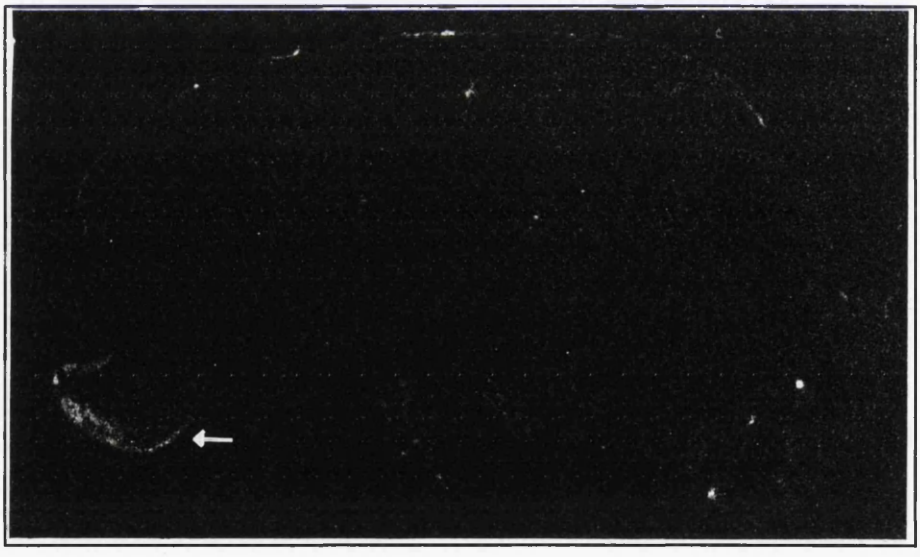
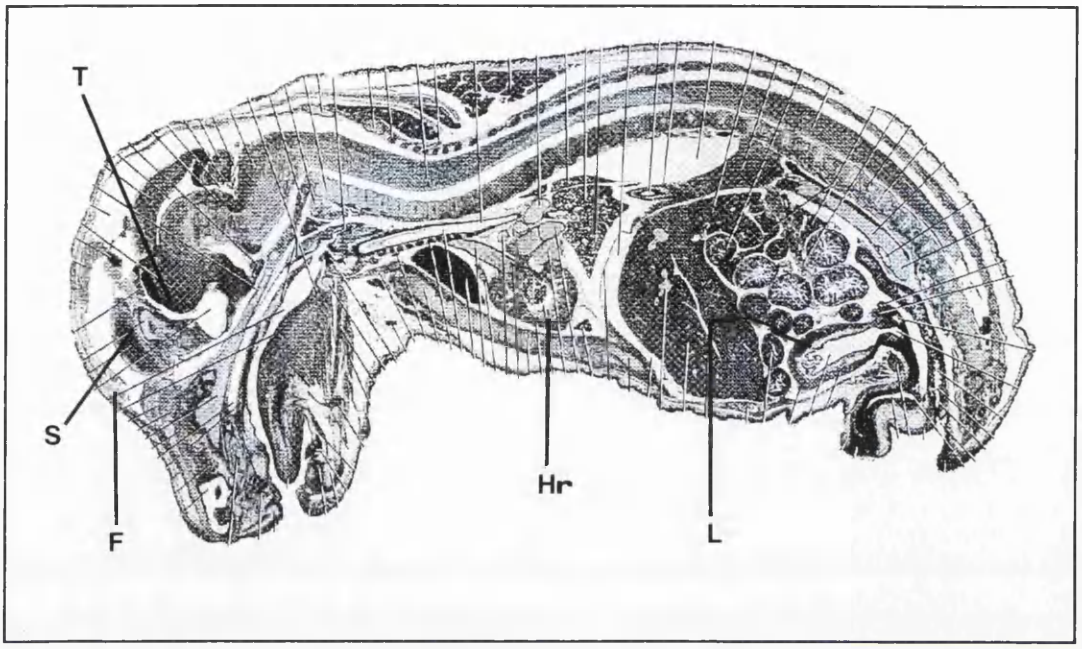


Figure 8.3 Sagittal section from a 15.5 day mouse embryo. Panel A is a histological section of the mouse embryo from approximately the same time point of development and is a mid-section sagittal slice that is roughly equivalent to that of Panel B. The lower panel details the specific hybridisation observed with a NTR specific probe derived from clone p2.8, (5'- cag tgc tct gca ggc tct gca gcg act tct tcc gcg cta gag tga 3'). The arrow indicates specific

hybridisation; N, Neopallial cortex; M, midbrain; H, hindbrain; Hr, heart; L, liver.

A



B

Figure 8.4 Sagittal section from a 17.5 day mouse embryo. Panel A is a histological section of the mouse embryo from approximately the same time point of development and is a mid-section sagittal slice that is roughly

equivalent to that of Panel B. The lower panel details the specific hybridisation observed with a NTR specific probe derived from clone p2.8, (5'- cag tgc tct gca ggc tct gca gcg act tct tcc gcg cta gag tga 3'). The arrow indicates specific hybridisaton; F, forebrain; S, striatum; T, thalamus; Hr, heart; L, liver.

9.3 Summary

The above hybridisation results indicate the probe derived from clone p2.8B hybridised to specific mid-brain structures, Figure 8.1 panel A. Neurotensin receptors are known to be expressed highly within the ventral tegmental and the substantia nigra brain structures located within the mid-brain of the mouse. Therefore, a specific predicted pattern of expression was observed for the probe derived from the genomic sequence of the putative mouse NTR in agreement with published results for the NTR of the rat, (Szigethy and Beaudet, 1989). However, further hybridisation experiments would be desirable to confirm the location of the hybridising brain structures. Adjacent sections could be simultaneously probed with either the NTR specific probe or a dopamine neuron specific probe such as one directed against mRNA of the tyrosine hydroxylase gene. Moreover, the *in situ* hybridisation analysis of embryo sections suggested that the NTR mRNA expression is first detected within the forebrain of the 13.5 day embryo, and within an undefined structure of the 15.5 day and 17.5 day developing mouse brain, (possibly the neocortex). The results of this *in situ* study extends the observations of Sato *et al.*, 1992, who have detailed extensive expression of NTR in the day 7, day 14 and day 21 of the postnatal rat. They observed expression over the neocortex and the limbic cortex at a time when neuronal pathways and connections are being made, which suggests that NT may have a trophic role within the developing CNS distinct from its function in the adult brain.

Chapter 9

Discussion

9.1 Introduction

After a brief review of the experimental achievements presented here, this discussion will briefly consider the immediate future work that needs to be undertaken for the further characterisation of the mouse NTR gene. The progress that has been made in generating 'knock out' mice for the receptor will be reviewed. The possible reasons for the lack of germline transmission, as yet, will be considered. Furthermore, if the replacement strategy described in Chapter 5 for the generation of a null mutation of the NTR in mice is not feasible due to the lack of germline transmission, then alternative strategies that may be employed will be discussed. In the light of recent technological developments a number of more precise receptor mutations could be engineered to ask more specific questions of receptor function in neurotensin transmission in the adult CNS. Therefore, some of the possible avenues of NTR analysis will be presented in the context of present technology.

9.2 Summary of experimental achievements

9.2.1 Characterisation of the NTR gene

High stringency library screening, using the heterologous rat cDNA identified a number of clones (8 λ BALB/c clones and 16 λ 129J clones), containing genomic sequence of the mouse NTR gene. Further restriction and sequence analysis revealed a 2.8 kb *Bam*HI fragment encoding a large exonic fragment for the first 242 aa residues of the receptor. The mouse sequence was 97% identical to the rat cDNA, for the sequence encoding the putative transmembranes I to IV. Furthermore, 5' and 3' probings of the restricted λ clones with defined fragments of the rat cDNA revealed a set of overlapping clones encompassing the coding sequence of the gene. However, further analysis is necessary to reveal the complete genomic structure of the gene.

9.2.2 Developmental expression of the NTR gene

In addition, a developmental *in situ* analysis of the mouse NTR mRNA has been initiated. Embryonic slices from day 13.5 and every day until day 17.5 of gestation have been probed with a 45 mer antisense oligonucleotide designed from the sequence encoding aa residues 87 to 102 of the mouse receptor. Initial data indicates transient expression in, as yet, unidentified brain structures in the developing cortex, which is clearly evident from day 15.5 to day 17.5 of gestation. No published data presently exists on the expression of the NTR in the mouse embryonic CNS. However, Sato *et al.*, 1992, studied the expression of the NTR mRNA in the rat at postnatal day 0, 7, 14 and 21. They classified the NTR mRNA expression pattern into 3 types. The most striking was type I expression where the entire neocortex and most of the limbic cortex exhibited a high level of mRNA expression during the first postnatal week, which decreased greatly thereafter. These results in conjunction with my own observations, Chapter 8, suggest a trophic role of neurotensin in the development of the CNS, separate from its neurotransmitter role in the adult. If such a developmental role exists for neurotensin, then the strategy employed to mutate the receptor may cause developmental defects that obscure the analysis of the neurotensin function within the adult brain. Therefore, a simple 'knockout' approach may not be appropriate and more sophisticated conditional knockout strategies, (such as those detailed in sections 9.3.3 and thereafter) , may be needed to unravel the neurotransmitter role of NT in the adult CNS from the presumed trophic role within the developing CNS.

9.2.3 Generation of a null mutation using the p9.2EiNeoTK replacement vector

The principal aim of this project was to generate null mutant mice for the neurotensin receptor, in order to analyse NT transmission. Significant progress has been made, in that a replacement type construct utilising the positive negative selection strategy has been used to target the NTR gene in several ES cell lines. This was achieved with a reasonable targeting frequency of 1 in 40 targeted to non-targeted clones for the E14, RI and CGR8 ES cell lines. Although, one targeting experiment with the CGR8.8 cell line produced a targeting frequency of 1 in 90. Of these targeted cell lines, 3 sub-lines have been used to successfully create chimaeric mice but unfortunately germline transmission has not yet been achieved. However, two targeted sub-lines, (C10

and K12), of the highly germline transmissible CGR8.8 ES line await blastocyst injection at the time of thesis submission.

9.2.4 Possible reasons for the observed lack of germline transmission and chimaeric male sterility

The targeted ES cell lines generated may not be able to generate 'knock out' mice for a number of reasons. An obvious explanation for the lack of germ line transmission is that the targeted ES cell lines, did not contribute to the germline of the chimaeric mice generated. This is probably the case for the weakly chimaeric mice generated from the targeted RI ES sub-lines. However, this explanation does not satisfactorily explain the apparent sterility of the 3 strongly chimaeric male mice generated from the CGR8.A ES cell line. A more likely explanation is that the targeted ES cell line, (CGR8.A) had a second copy of the replacement vector integrated randomly in the genome. If the thymidine kinase gene was present within the ES cell line, then this would prevent germline transmission as expression of the thymidine kinase gene in male gonads causes sterility. However Southern blot analysis of the ES cell line with a thymidine kinase specific probe proved negative, Chapter 6 section 6.6. Therefore, it is unlikely that the presence of a random copy of the targeting vector is the cause of the apparent sterility. A common cause of the lack of germline transmission can be karyotipic abnormalities. Increased or decreased numbers of chromosomes are often observed for ES cell lines of increased passage. This possibility was addressed by looking at metaphase spreads from the CGR8.8 subline, but the normal complement of 40 chromosomes was present. Nonetheless, this analysis does not rule out smaller genome re-arrangements contributing to the failure of this line to transmit to the next generation.

Another possible explanation is that the heterozygous null mutation of NTR caused the death and readsorption of the developing embryos, therefore the apparent sterility and lack of germline transmission. This phenomenon, commonly known as haploid insufficiency, results when the heterozygous form of the mutant gene causes insufficient production of the gene product for normal development. This is an interesting possibility when speculating on the nature of the mutant receptor produced by the replacement construct. The construct mutates the NTR gene by introducing a neomycin cassette within the gene sequence coding for the fourth transmembrane domain. This mutation may result in the expression of a truncated receptor that

still has affinity for neurotensin. Although the proposed binding site for neurotensin is within the 3rd extracellular loop, residues of the first extracellular loop and transmembrane III have been shown to be important for NT binding to its receptor, see Chapter 1 section 1.3.3. Such a mutant receptor could cause a dominant mutation of the neurotensin transmission system if its affinity was sufficient to bind NT but the truncated receptor was unable to transmit the signal. Thus creating a dominant mutation that had deficit in NT transmission. This may at first seem an unlikely explanation, since the strongly chimaeric male mice were (on limited observation) apparently normal. Unless the lack of neurotensin transmission is specifically crucial to the correct development of the germline. Thus creating the distinct possibility that the lack of germline transmission is probably due to the abnormal development of the germline itself, resulting in sterility of the highly chimaeric mice. This scenario becomes even more intriguing by the knowledge that NT mRNA is known to be expressed in the testes, (Dobner *et al.*, 1993).

9.3 Future perspectives

9.3.1 Targeted ES cell subline testing

The immediate future direction will be to generate more targeted CGR8.8 ES cell lines using the replacement construct (p9.2EiNeoTK), for the production of chimaeric mice and hopefully germline transmission. This is required because it is possible that the apparent observed sterility is due to a property of the particular targeted ES cell subline, (CGR8.A), and independent of the NTR mutation introduced by the replacement vector.

9.3.2 Chimaeric analysis of a null NTR mutation

If germline transmission proved to be a problem using multiple ES cell lines targeted with the NTR replacement vector, then one possible solution would be chimaeric analysis. Typically, ES clones in which both alleles of the gene have been mutated are used to generate highly chimaeric mice. This can be done by targeting the remaining wild type allele in the heterozygous ES cell line, (Mortensen *et al.*, 1991). Alternatively, the heterozygous ES cell line can be selected at elevated drug, (G418), concentrations above the threshold of the

resistance produced by one copy of the gene, (Mortensen *et al.*, 1992). Injection of the homozygous mutant cell lines would be into blastocysts derived from gene trap lines that ubiquitously express the *lacZ* gene. This allows the tissues derived from the ES cell line or the embryo to be distinguished at the single cell level.

The chimaeric analysis offers one approach to the possible problem of not being able to generate viable mice with the null mutation of the neurotensin receptor. However a number of technological advances in the gene targeting field offer a range of other strategies to overcome this difficulty. Furthermore, it is now possible to generate null mutations in a more defined spatial and temporal manner. There have also been improvements for the strategies that create subtle mutations within a gene. These developments and their possible applications to the analysis of neurotensin receptor function will be considered in the following sections.

9.3.3 Conditional Cre-loxP mediated recombination

It may not be possible to generate adult mice with a null mutation of the NTR gene using the replacement strategy outlined in this study for reasons previously presented. Furthermore, a number of studies have highlighted the possible role of neurotensin in early development. Detailed analysis of neurotensin mRNA expression in the human and rat foetal colon, observed varying patterns of expression during gestation but not in the adult, (Evers *et al.*, 1994). Other studies have also recorded developmental expression of the NTR gene in the postnatal rat, (Sato *et al.*, 1992). Both of these studies suggest that neurotensin may have a trophic role during mammalian development. Therefore, to analyse the function of the NTR in the adult mouse CNS by gene targeting, other conditional mutational strategies may have to be employed. One such approach that can create conditional gene 'knock outs' is the Cre-*lox*P system. This technique can be used to precisely knockout the expression of a gene in one particular cell type as demonstrated by Gu *et al.*, (1994). They successfully deleted the promoter and the first exon of the DNA polymerase β gene only in T-cells of mice. This system is based on the Cre-*lox*P recombination system of bacteriophage P1. The Cre recombinase lines up 2 *lox*P sites that are in direct repeat orientation, and performs site specific recombination, excising the intervening DNA and leaving behind a loxP site. Therefore, by controlling the expression of the Cre recombinase such that it is

compartmentalised in a cell type specific and/or a developmentally specific manner a conditional mutation of a target gene can be achieved. This system requires the production of 2 transgenic strains of mice; one carrying the target gene flanked by *lox*P sites, the other with a Cre expressing transgene under specific temporal and spatial promoter control. Such a strategy is outlined in Figure 9.1.

The Cre-*lox*P system is not the only scheme capable of creating a tissue specific mutation. The FLP enzyme from yeast has the potential to manipulate genes in a similar manner, by deleting DNA fragments flanked by frt sequences, (Fiering *et al.*, 1993). This system was recently shown to efficiently mediate site specific recombination in transgenic mice, (Dymecki, 1996). From the point of view of the neurotensin receptor's possible trophic role in development, a complete null mutation may not be possible. Therefore, confining the mutation of the receptor to the adult CNS may alleviate this problem. Using the Cre-*lox*P strategy, conditional mutation of the receptor may be achievable by confining the expression of Cre recombinase to the adult CNS using a neuron specific promoter that is expressed exclusively in differentiated adult neurons. A complete neuronal deletion of the receptor would be obtainable by a breeding program that combined the Cre expressing transgenic line with a transgenic line bearing a NTR gene flanked by *lox*P sites.

Although the approach is apparently straight forward, problems may be encountered in defining a suitably functional portion of the desired promoter for the construction of the *cre* transgene. However, a strategy that targeted the *cre* transgene to the endogenous NTR gene locus, such that the *cre* gene was now under the transcriptional control of the NTR promoter, would alleviate such a problem. However, this approach would knockout the NTR in all tissues and would not be suitable if haploid insufficiency prevents germline transmission. The chromosomal position of the integrated *cre* transgene may result in unpredictable patterns of expression, but individual cre expressing lines can be tested. Moreover, if the transgene is only expressed transiently during development, then the cre recombinase may not have sufficient time to excise all DNA fragments flanked by *lox*P sites. This may explain why only an estimated 40% of T-cells were homozygous for the desired deletion of the DNA polymerase-b gene in the Gu *et al.*, 1994 study. However it must be noted they used the wild type version of the cre recombinase gene. A subsequent publication by Hennet *et al.*, (1995), involved the use of a modified Cre recombinase gene with additional kozak

translational consensus and nuclear localisation signals. They apparently achieved complete Cre-*lox*P mediated recombination in T-cells to create a deletion of a polypeptide N-acetylgalactosaminyl-transferase gene. More recently a report described the efficient expression and action of a *cre* transgene at the one-cell zygote stage of embryonic development, (Lasko *et al.*, 1996). This was achieved using a *cre* transgene under the control of the adenovirus EIIa promoter. Therefore, the choice of promoter, for *cre* transgene expression, is crucial to the success of developing a strategy for the conditional mutation of the NTR gene.

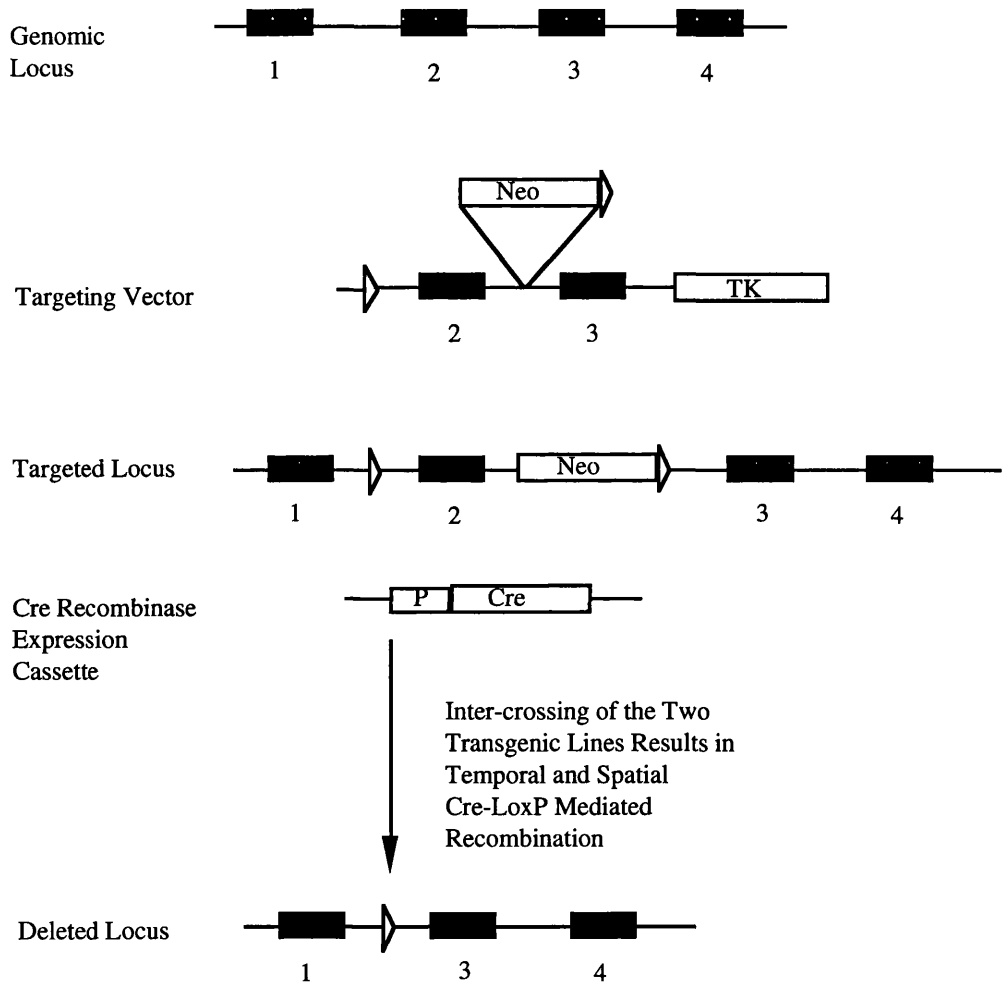


Figure 9.1 Cell specific deletion of exon 2 using the Cre/loxP mediated recombination system. The target gene consists of 4 exons represented by the black boxes, thin lines introns. The replacement construct consists of: exons 2 and 3; a neomycin cassette (Neo); a thymidine kinase cassette (TK); 2 *lox*P sites represented by open triangles, one of which is intronic the other 3' of Neo. The

targeted locus integrates the loxP sites and Neo intronically, such that they do not interfere with gene expression. A second transgenic strain is generated, expressing the Cre recombinase transgene (Cre) under control of a cell specific promoter (P). A breeding program involving the 2 mouse strains and subsequent expression of the Cre recombinase results in the deletion of exon 2 from the target locus only in those cell types expressing the Cre recombinase.

9.3.4 Temporal control of gene expression

A number of groups have been working towards defining systems that would allow the deactivation of genes in specific cell types. Bujard and Gossen, 1992, modified the *cis* and *trans* control elements of the *E.coli* transposon Tn *10* tetracycline resistance operon. The transcription of the operon genes is negatively regulated such that in the absence of the antibiotic, the tetracycline resistance repressor (*tet*R) binds to the operator sequences of the promoter. In the presence of the antibiotic the genes of the operon become transcriptionally active because the tetracycline binds to the *tet*R and presents binding to the operator sequences. However, this system was modified such that tetracycline turns genes off instead of on. This was achieved by combining the tetR gene with HSV c-terminal domain of the VP16 gene, thus creating a hybrid activator protein. This hybrid transactivator (tTA) can bind to and activate mammalian genes that have promoters fused to tetracycline operator (*tet*O) sequences but only in the absence of tetracycline. This system has been shown to work in mice using the reporter genes b-galactosidase and luciferase under the transcriptional control of the minimal promoter from the human cytomegalovirus fused to *tet*O sequences. In the Furth *et al.*, 1994, study several transgenic lines were generated with the reporter constructs described above and also expressing the hybrid transactivator (tTA). Expression of the reporter genes was achieved at several chromosomal loci using this system, demonstrating that the tTA could gain access to the bacterial promoter elements packaged into chromatin. Inhibition of the reporter constructs was achieved by applying tetracycline, which prevents the transactivator from binding to the *tet*O sequences. Basal gene activity was not observed when b-galactosidase activity was assayed. However, measurable levels of luciferase activity were occasionally observed in some tissues. They concluded that basal gene activity is apparently a function of the minimal promoter and the chromosomal location of the transgene. Removal of the tetracycline resulted in the induction of the reporter genes as expected. Therefore, this system offers

the potential to turn genes on and off at will, by the presence or absence of tetracycline.

The temporal control of gene expression using tetracycline, the hybrid transactivator and the *tet*O sequences could possibly be used to perturb the neurotensin transmission system in the mouse. One scenario would involve the neurotensin receptor transgene (cDNA) under control of the hybrid transactivator tTA. These transgenes would have to be introduced into a null NTR background, (refer to Figure 9.2).

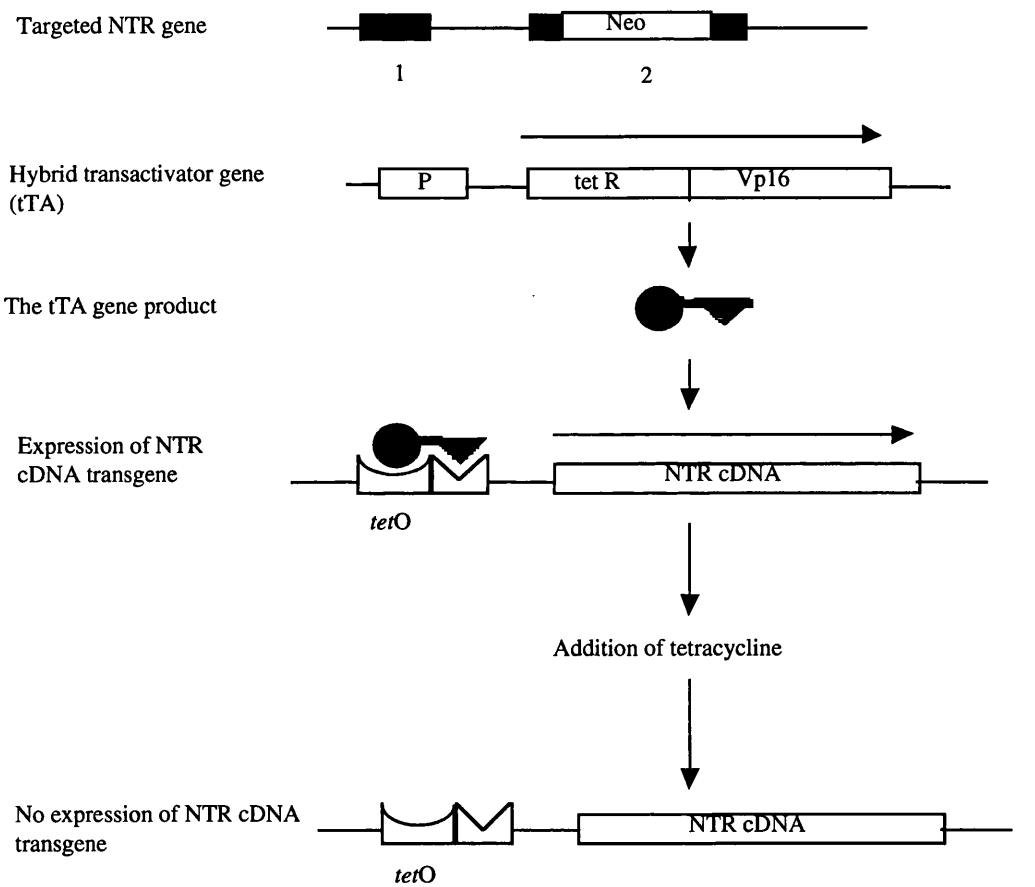


Figure 9.2 Temporal control of the NTR gene expression using the tetracycline system. This strategy requires the expression of the NTR cDNA transgene under transcriptional control of the hybrid transactivator (tTA). These transgenes are introduced into a null NTR background. The endogenous NTR gene has been targeted with a replacement construct that introduces a disrupting neomycin cassette into exon 2 of the gene. The hybrid transactivator gene consists of the tetracycline repressor gene (*tet*R) fused to the HSVc-terminal domain of the VP16 gene (VP16). The gene is controlled by the adenovirus EIIa promoter (P). The product of the hybrid transactivator, (represented by the

black circle joined to the black triangle), binds to the tetracycline operator sequences, (*tet*O), of the cDNA promoter and activates transcription. The above scenario is achieved in the absence of tetracycline, which prevents the hybrid transactivator protein from binding to the *tet*O sequences. The NTR gene is down regulated by the addition of tetracycline. Arrows above genes indicates transcriptional expression.

One difficulty would be in creating a minimal promoter with *tet*O control sequences that would permit the correct neuronal expression of the receptor. If possible administration of tetracycline would down regulate the expression of the receptor. However there would be a lag period before the receptor would be removed from neuronal membranes. Normal neurotensin transmission has shown that the receptor becomes internalised on binding of the ligand and hence is removed from the cell surface, (Charby *et al.*, 1993). However, it is not clear whether this is the case for all neurotensin receptors. Furthermore, basal activity from the minimal promoter may provide sufficient expression of the NTR transgene such that no observable disruption of the neurotensin system is achieved. Some of the problems associated with the minimal promoter and the site of integration of the transgene can be evaluated using the bi-directional promoter developed by Baron *et al.*, 1995. This promoter co-regulates the expression of two transcriptional units from the centrally located *tet*O sequences flanked by two minimal promoters. As before this bi-directional promoter is responsive to the hybrid transactivator tTA, which in turn is negatively regulated by tetracycline. Since two genes can be co-regulated by this promoter, a reporter gene such as b-galactosidase could be included with the NTR gene on the other side. Therefore, a gene such as NTR could be monitored via the expression of a reporter gene and permit the evaluation of particular transgenes in mice. Furthermore, a sensitive reporter gene such as luciferase could be used to assay for basal gene expression from the minimal promoter in the presence of tetracycline. Nonetheless, tetracycline mediated inhibition of the NTR expression is possible in theory using this system.

The strategy outlined above for temporal control of the NTR gene is elaborate, technically demanding and involves a number of uncertainties. Another system offering temporal gene control is based upon the *Drosophila melanogaster* ecdysone hormone and its receptor. Ecdysone mediates its effects through the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene, (USP). Modification of this system has achieved inducible gene

expression within the mouse, (No *et al.*, 1996). In order to create an efficient inducible system for mammalian genes, No and co-workers made several modifications to the original system. They replaced EcR's natural partner with its mammalian homologue, namely the retinoid X receptor (RXR). A 34-fold increase in induction was achieved using this heterodimeric pair. However, a 212-fold increase was achieved by creating a fusion protein of the EcR with the VP16 activation domain fused to the N-terminal truncated version of the EcR, creating VpEcR. In addition, the ecdysone responsive promoter was modified by inserting Sp1 consensus binding sites between the minimal promoter and the ecdysone response elements, (EcREs), to increase transriptional activity. Furthermore, the binding specificity of VpEcR was changed to preclude activation by endogenous frasenoids on synthetic promoters containing EcRE elements. This required the mutation of 3 aa residues of the VpEcR within the P box of the DNA binding domain, such that the altered receptor mimicked the DNA binding specificity of the glucocorticoid receptor. This hybrid receptor named VgEcR is responsive to a hybrid response element termed E/GRE. This element is a hybrid between the glucocorticoid response element (GRE) and that of the type two nuclear receptors such as RXR. When this system was tested in cell lines, maximum induction levels were observed of 20000-fold after 20 hours of exposure to the ecdysone agonist muristerone A. Comparisons were made between the ecdysone and tetracycline systems using transient transfections. The basal activity of the ecdysone responsive promoter was shown to be 20-fold lower than that of the tetracycline repressor promoter and 500-fold lower than the tetracycline transactivator promoter.

It would appear that the ecdysone system offers several advantages over the tetracycline system, such as higher inducible transcription rates and lower basal activity of the promoters. Besides, the lipophilic qualities of ecdysteroid compounds permits rapid penetration to all tissue types including the brain. Also, the short half life of such compounds will allow precise potent inductions and facilitate rapid clearance from tissues. Therefore, this system should be a more controllable than the tetracycline system. Furthermore, these compounds are not known to be toxic or affect mammalian physiology. This system presents a strategy for the efficient temporal induction of a gene in transgenic mice with responsive promoter elements that exhibit low basal activity. One of the more obvious uses for temporal gene induction would be ablation experiments using the diphtheria toxin A gene under control of the ecdysone responsive promoter (E/GRE). The ablation could be spatially regulated by restricting the expression of the synthetic

receptor (Vp EcR) to specific cell types by placing it under the control of a tissue specific promoter.

With respect to analysing the NTR gene, there exists the possibility of using this system in conjunction with the Cre-*lox*P strategy for creating temporally and spatially specific mutations. For example, the receptor could be induced to mutate only in the adult brain thus avoiding any problems associated with expression of a mutant receptor during development. This would require a number of transgenes to be developed and introduced into the mouse germ line as well as targeted modification of the endogenous NTR gene. Firstly the NTR gene would have to be targeted by homologous recombination in ES cells to generate a segment of the gene flanked by *lox*P sites. The induction of the *cre* recombinase transgene could be placed under the control of the modified ecdysone responsive promoter. Therefore, induction of Cre recombinase and hence mutation of the NTR gene would be dependent upon the administration of an ecdysone agonist. The advantage of this strategy to the original approach, outlined in previous section 9.3.2, is that the NTR mutation could be created at many different developmental stages using the same transgenic mouse line. The system could be further modified by restricting the expression of the ecdysone heterodimeric receptor to neuronal cells or a subset of neuronal cells by placing the retinoid X receptor transgene under specific neuronal promoter control. This would create a system for analysing NTR function in the CNS with respect to the developing brain and the adult neurotensin transmission pathways.

The ability to mutate a gene in such a temporal and spatial manner offers numerous possibilities for investigation. However this ability to interfere with neurotensin transmission in the CNS in a temporal and spatial manner is particularly exciting considering the body of evidence implicating the neurotensin transmission in the mesolimbic system with an involvement in the pathology of schizophrenia, (Chapter 1; section 1.4), since the disease is increasingly thought to have some developmental defect in its etiology. Therefore, to perturb the neurotensin system at a particular time point of development may be more informative of the effect such a NTR mutation on mouse development and behaviour.

9.3.5 Creation of subtle mutations within the neurotensin receptor

Three approaches for introducing subtle mutations into any gene have been reported recently. They will be briefly described and the potential mutations that one might want to introduce into the NTR gene will be considered.

The first of these techniques is similar to the tag and exchange strategy, (Askew *et al.*, 1993; refer to Chapter 1: section 1.16), and involves two sequential replacement events. Stacey *et al.*, 1994, refer to their approach as a double replacement strategy which they used to replace the mouse a-lactalbumin gene with its human homologue. However, the general principles of the technique are applicable to introducing specific subtle mutations into any mouse gene. A replacement vector of the target gene is constructed using the Hprt selectable marker inserted within exonic sequence. The gene targeting and selection was performed using a hprt-deficient ES cell line. This cell line exploits the ability to select for and against the *hprt* marker gene. Therefore, unlike the tag and exchange method only one selectable marker is introduced into the target locus after the first replacement event, although the replacement can carry the thymidine kinase cassette as a negative selectable marker for enrichment of homologous integrants. This cell line can then be re-targeted with a second replacement vector bearing the desired gene mutation. This vector does not contain a positive disrupting selectable marker, since positive selection is achieved by selection for the loss of the *hprt* gene at the target locus.

Superficially, this double replacement method appears to be the same as the tag and exchange method but, it offers a number of advantages. Firstly, the thymidine kinase cassette is not integrated into the target locus as a selectable marker for the second replacement step or exchange step. Instead, thymidine kinase is used as a negative selection marker in the traditional manner for a replacement vector designed for the positive negative selection regime. With the tag and exchange approach the thymidine kinase gene becomes integrated into the 'tagged' locus but this is not the case for the double replacement strategy. Therefore, the added advantage of using the ES cell line from the first replacement event to generate a null mutation for the gene of interest exists. This also allows for testing the totipotency of targeted ES cell lines prior to further manipulations of the second replacement step. Thymidine kinase gene expression in male gonads causes sterility, thus precluding the use or testing of the ES cell lines generated in the first step of the tag and exchange method. However, both techniques permit the design of numerous replacement type constructs to introduce various mutations into the target locus in the second step of their procedures.

The next approach to introducing desired specific mutations into a given gene combines components of the 'tag and exchange' and the 'hit and run' strategies. A replacement type construct is generated carrying neomycin and thymidine kinase cassettes internal to a duplicated portion of the target gene. The subtle mutation is carried on one arm of gene homology but does not form part of the duplicated gene sequence, see Figure 9.3. Therefore, the first step involves the targeting of the desired gene with the replacement type vector. This creates a duplication at the target locus which is resolved in the second step by applying negative selection against the integrated thymidine kinase cassette. The resolution is by intrachromosomal recombination between the duplicated portion of the gene.

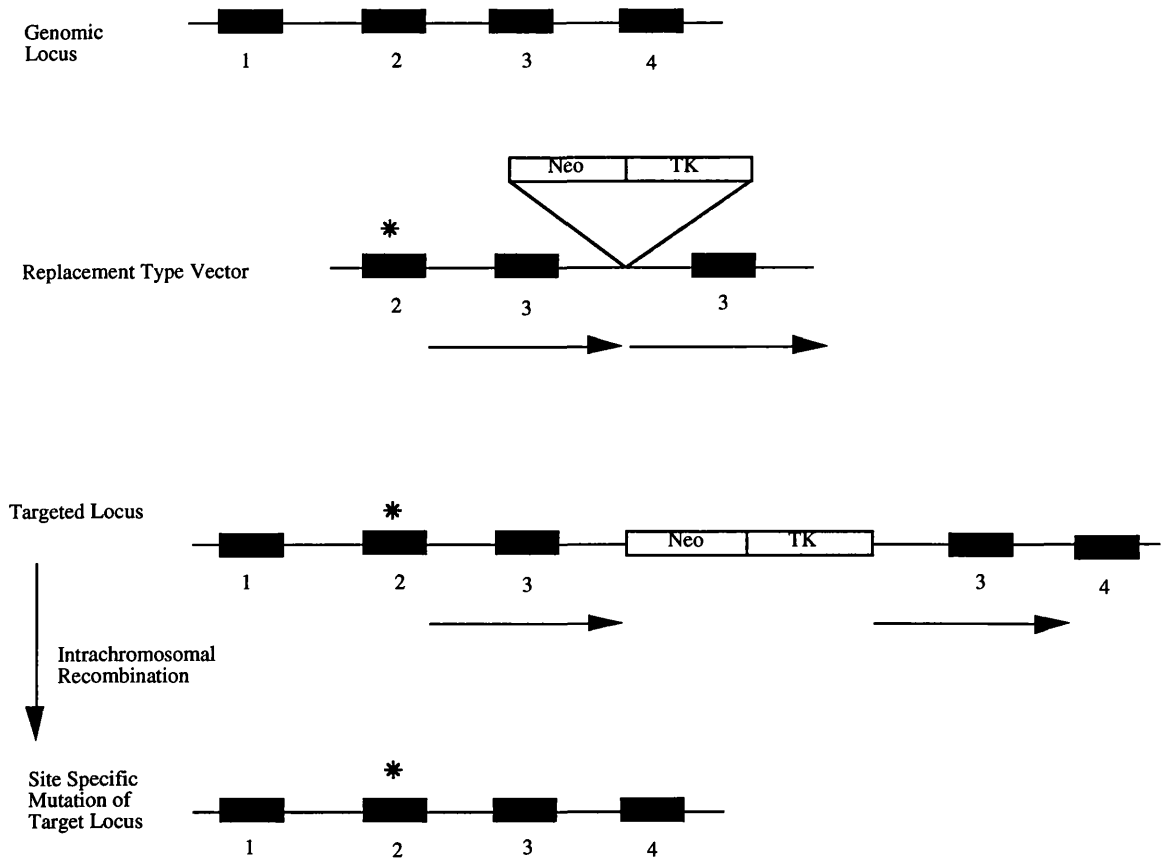


Figure 9.3 A two step replacement/excision technique for introducing subtle mutations into any gene locus. The 4 exons of thegene are represented with black boxes; the thin lines are the intronic sequences. The replacement type vector consists of exons 2 and 3 with exon 3 and its flanking intronic sequences being duplicated; the fused selection cassette, (Neo and TK), inserted between

the duplicated gene homology which is clearly highlighted by the underlining arrows. The asterisk denotes thesite specific mutation with in exon 2. The first step of the procedure involves targeting the locus with the replacement type vector. The targeted locus undergoes negative selection against the presence of the thymidine kinase cassette in order to select for excision of the duplicated sequence by intrachromosomal recombination. Such a reaction should result in the locus retaining the site-specific mutation.

This replacement/excision technique was developed by Horie *et al.*, 1995, using the F9 mouse cell line. The major advantage of this method over the hit and run procedure is that the subtle mutation is more likely to be retained at the chromosomal locus after the excision event, as the mutation is not part of the duplicated gene homology. Horie and co-workers observed excision frequencies as high as 84% for a targeted locus. However, another targeted clone only produced $Ganc^R$ colonies with a 7% frequency of producing the desired excision. This was not due to loss of the desired mutation but a result of inactivation of the thymidine kinase due to methylation of the *Eco*RI site within the promoter of the thymidine kinase cassette (Tasseron-de Jong *et al.*, 1989). Nonetheless, this strategy introduces a new technique for generating subtle mutations at any loci within the mouse genome.

The first technique of a double replacement strategy would appear to be the most attractive of the two techniques primarily because the thymidine kinase cassette is used in the replacement construct for the enrichment of targeted clones in the first step. Furthermore, the targeted cell line can be used to generate a null mutation for the target gene in mice, which would also test the totipotency of the cell line prior to further manipulations. However it must also be noted that the Cre-*lox*P offers great potential for the creation of subtle mutations within the NTR gene. Such a strategy involves a gene targeting event using a replacement vector that places the positively selectable marker within the intronic sequence of the gene flanked by *lox*P sites. The site specific mutation is contained within an arm of homology of the vector. Application of cre recombinase can be *in vitro*, at the ES cell stage, or *in vivo*; mating of the homozygous mutant mouse line carrying the *lox*P-Neo-*lox*P cassette with a transgenic line expressing Cre early in development will result in the deletion of the Neo cassette from the gene locus, leaving behind an intronic *lox*P site and the site specific mutation, (refer to Figure 9.4). Recently the feasibility of such a strategy was demonstrated by Lasko *et al.*, 1996. They created a Cre expressing transgenic line in which the adenovirus EIIa promoter directs expression of the *cre* transgene. This transgene has the ability to delete

DNA, flanked by *lox*P sites, at the zygote stage of mouse development. Therefore, if the Cre recombinase correctly excises the intervening DNA at this stage of development, then all tissue types will acquire the desired mutation. This approach has the added advantage of not requiring extended tissue culture of the targeted ES cells, in order to expose them to Cre recombinase, prior to blastocyst injection and also saves on the labour of screening for the desired mutation.

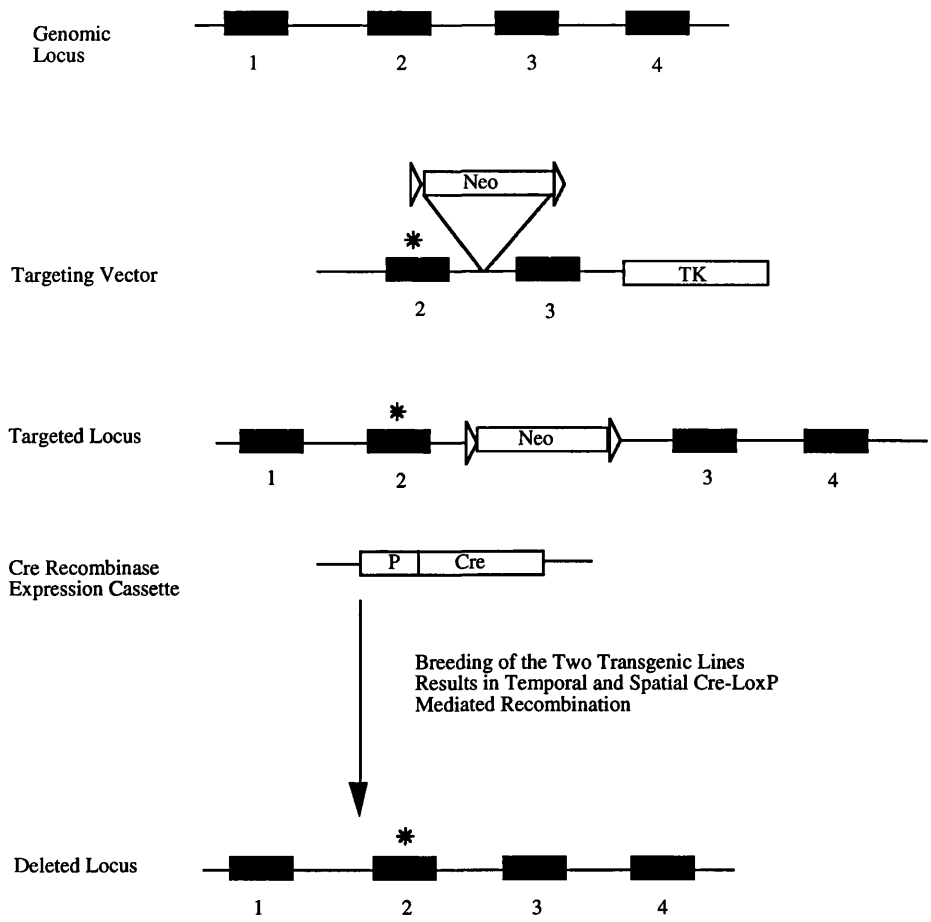


Figure 9.4 Site-specific mutation of a gene using the Cre-*lox*P mediated recombination system. The target gene consists of 4 exons represented by the black boxes, thin lines introns. The replacement construct consists of: exons 2 and 3; a neomycin cassette (Neo); a thymidine kinase cassette (TK); 2 *lox*P sites represented by open triangles, both of which are intronic 3' and 5' of Neo. The targeted locus integrates the loxP sites and Neo intronically, such that they do not interfere with gene expression. A second transgenic strain is generated, expressing the Cre recombinase transgene (Cre) under control of a cell specific promoter (P). A breeding program involving the 2 mouse strains and

subsequent expression of the Cre recombinase results in the deletion of exon 2 from the target locus only in those cell types expressing the Cre recombinase.

Therefore, alternative procedures exist for the generation of subtle mutations within the neurotensin receptor. One particularly interesting function of the NTRs has been the observation that on binding their ligand, the receptor neurotensin complex becomes internalised and subsequently undergoes retrograde axonal transport. Upon reaching the cell bodies an increased expression of the tyrosine hydroxylase enzyme was observed. However, the functional significance of such a process is not known. This has been demonstrated within the rat brain for the NTRs associated with the dopaminergic neurons of the nigrostriatal pathway, (Castel *et al.*. 1994). The NTRs have been shown to be primarily located on the pre-synaptic membranes of these neurons and the cell bodies within the substantia nigra pars compacta. The high density of NTRs associated with the nigrostriatal pathway makes this a desirable system, not only for the functional analysis of this receptor but, the general functional study of the phenomenon of neuropeptide axonal transport in the CNS.

The creation of informative mutations, with respect to the analysis of retrograde axonal transport, has been aided by mutational studies of the receptor in cell culture systems. Charby *et al.*, 1995, performed site directed mutagenesis of the cDNA encoding the NTR. The various constructs were transiently transfected into mammalian COS7 cells and assayed for their ability to internalise iodinated neurotensin. To summarise, they identified two aa residues (Thr-422 and Tyr-424) which when point mutated to Gly virtually abolished the receptor's ability to internalise the labelled neurotensin.

Therefore it would be desirable to generate a transgenic mouse expressing a mutant NTR gene with altered Thr-422 and Tyr-424 aa residues in a similar manner to Chanbry and his colleagues. This could be achieved by employing one of the gene targeting techniques previously presented. However, it may be more informative to create such a mutation in the adult CNS so as not to complicate the analysis. In theory, the available technology exists to create such a mutation in the mouse. One possibility would be to 'switch off' the endogenous NTR gene using the Cre-*lox*P system or by using the tetracycline system, in conjunction with 'switching on' a mutant cDNA NTR transgene which is responsive to ecdysone agonist, or constitutively expressing the mutant cDNA transgene. The following scenario represents a strategy for manipulation of the expression of the NTR gene and

also serves the purpose of elucidating the possibilities that now exist with the present transgenic mouse technologies.

Disruption of the NTR gene could be regulated in a spatial and temporal manner. The endogenous NTR gene could be engineered such that *lox*P sequences flank the first exon. Mutation of the NTR gene in the mouse would require the expression of the Cre recombinase gene. Typically the spatial and temporal control of the mutation is derived from the cell specific promoter driving the Cre recombinase expression. However, the Cre transgene could be placed under the transcriptional control of the ecdysone responsive promoter (E/GRE). Therefore, use of an ecdysone agonist will induce the expression of Cre recombinase and delete the first exon of the endogenous NTR gene. Similarly, a transgene expressing the mutant NTR cDNA, for aa residues Thr-422 and Tyr-424, could be under the inducible expression of the E/GRE promoter. A further two transgenes would have to be introduced into the mouse germline, namely the components of the synthetic heterodimeric ecdysone receptor i.e. the VpEcR gene and the RXR gene. To introduce spatial control to this system one or both of the transgenes for the ecdysone receptor could be placed under control of a dopaminergic expressing promoter such as that from the tyrosine hydroxylase gene or the dopamine transporter gene. To recap, presuming sufficient inducible expression and low basal activity of the Cre recombinase transgene and the mutant NTR cDNA transgene, in conjunction with sufficient tissue specific expression of the ecdysone receptors, it should be possible to create an ecdysone spatially and temporally regulated system for the analysis of a mutant NTR with respect to its ability to internalise within the mouse CNS. An added advantage of the system outlined, is that administration of an ecdysone agonist at any particular time point of development should activate the desired mutant receptor gene and simultaneously 'knock out' the endogenous NTR gene in the CNS, (Figure 9.5).

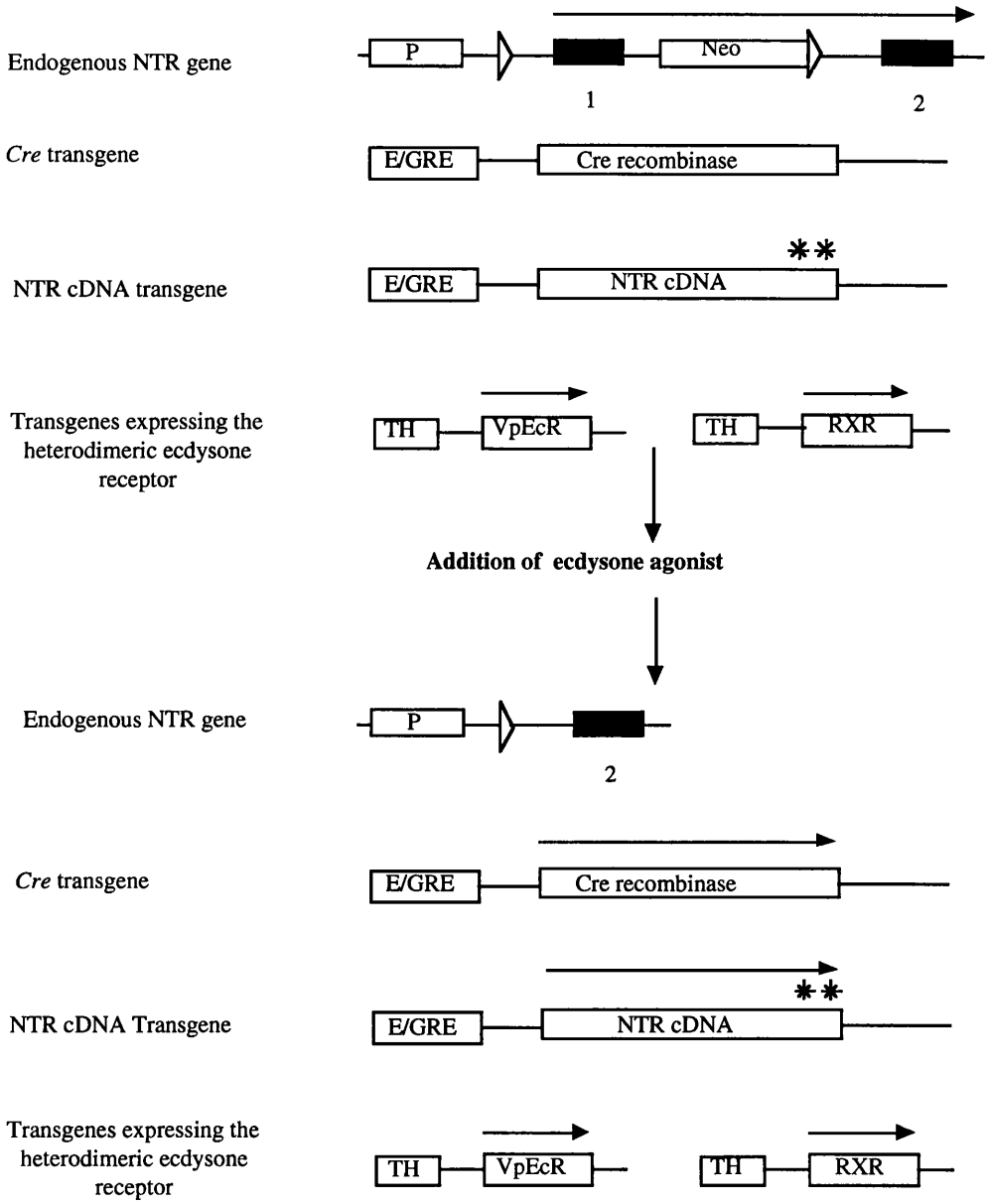


Figure 9.5 Temporal and spatial mutation of the NTR gene and expression of a NTR cDNA with a site specific mutation of the aa residues implicated in retrograde axonal transport of NT in the CNS. This strategy involves the expression of several transgenes within a mouse line which is homozygous for the engineered NTR gene. The transgene is placed under the transcriptional control of the ecdysone responsive promoter, (E/GRE), as is the NTR cDNA bearing the site specific mutation of aa residues 422 and 424, represented by the asterisk. Two other transgenes would be introduced into the mouse germline for the expression of the heterodimeric ecdysone receptor; the retinoid X receptor gene, (RXR) ; the synthetic component of the ecdysone receptor, (VpEcR). These receptor genes are under the transcriptional control of the tyrosine hydroxylase promoter, (TH). Addition of an ecdysone agonist activates the

expression of Cre recombinase, which in turn promotes the site specific recombination of the *lox*P sites and deletes exon 1 of the NTR gene, the expression of the NTR cDNA transgene is also switched on. Transcriptional expression is represented by arrowed lines above the genes.

Another interesting mutation of the NTR, would be a deletion within the 3rd intracellular loop of the receptor, (Yamada *et al.*, 1994). Deletion of aa residues 270-296, apparently abolished the receptors ability to stimulate polyphosphoinositide hydrolysis but, did not reduce its ability to stimulate cAMP formation when assayed in CHO-K1 cell line. This mutation did not affect the binding affinity of neurotensin to the mutant receptor compared to the wild-type receptor. The present mouse technology would allow the expression and analysis of this receptor *in vivo* and the functional significance of this secondary messenger pathway to neurotensin transmission to be evaluated.

9.3.6 Summary of perspectives

The previous sections of this discussion highlighted methods that could be used for the creation of precise specific and temporal mutation of the NTR to manipulate NT transmission within the adult CNS for a number of reasons. For example, the NTR gene knock out may not be capable of germline transmission due to sterility caused by haploid insufficiency. Therefore, homozygous mutant mice may not be produced using any simple knockout approach detailed in this thesis. Secondly, my own observations have recorded the expression of the neurotensin receptor within the embryonic CNS. Early transient postnatal expression of the receptor has also been detailed within the developing rat brain long before a neuronal network is established, Sato, *et al., 1992*. Taken together, these data suggest that the neurotensin system plays an important trophic role in the CNS distinct from its neurotransmitter like role in the adult. However, analogy with the DA system emphasises the point that expression of mRNA does not imply function. DA production and DA receptor expression have been observed in the developing mouse embryo, (day 10 and day 15). Therefore, it was surprising that targeted mutation of the tyrosine hydroxylase gene produced phenotypically DA^-/DA^- mice which developed a normal dopaminergic neuronal network. Although these mice were hypoactive and stopped feeding shortly after birth, this phenotype could

be rescued by administration of the drug L-DOPA, such that nearly normal growth was achieved. Nevertheless, if the null mutation of NTR does not cause sterility, lethality or severe developmental abnormalities it is still desirable to analyse the NT transmission of the adult CNS independently of a complex mutant background caused by abnormal developmental trophic effects or abnormal intestinal function(s).

The effects of a NTR null mutation within the adult CNS may be many fold. NT has been observed to elicit antinociceptive qualities on central administration into the PAG brain structure. Therefore, the lack of NT transmission may perhaps cause an increase in sensitivity to painful stimuli or fail to down regulate pain perception under the appropriate physiological conditions such as fear. NT application to the cholinergic neurons to the basal forebrain (NBM) is excitatory and causes cortical arousal. The NTR^-/NTR^- mouse may have an impairment in this process that may manifest itself as an attention deficit. Furthermore, this brain structure has been implicated in the processing of learning and memory. A number of behavioural tests could be employed to address the question of learning and memory impairment in mutant mice. Other effects such as hypothermia enhanced ethanol and barbiturate sedation have been observed on central injection of NT. Presumably the administration of NT to mice which are neuronally NTR^-/NTR^- will no longer elicit such effects. This is a somewhat contentious conclusion as it implies that there is only one type of NTR. Some pharmacological and electrophysiological data exists suggesting that the hypothermia and antinociceptive effects are mediated by a low affinity NTR subtype. NTR binding study analysis of NTR^-/NTR^- mice will answer the question of multiple NTR subtypes, since labelled NT should not elicit a binding profile within the neurotensinergic systems if only one receptor subtype exists.

There is also doubt concerning the mechanism by which NT interacts with the DA in the nigrostriatal pathway. Most data points to an antagonistic function of the activated NTR on D_2 receptor transduction, such that an increased D_1 receptor-mediated DA transmission is favoured. However, one hypothesis proposes that the mode of action is of NT forming a complex with DA, and in this way preventing DA from binding to D_2 receptors, (Adachi *et al.*, 1990). If so, the NTR^-/NTR^- mice on application of NT should still elicit this NTR independent action and presumably an increase in GABAergic activity in the striatum will be observed.

The effective lack of NT transmission on the nigrostriatal pathway may have implications for the regulation and coordination of movement. If indeed the main neuromodulatory action of NT is to antagonise the action of D_2 receptor transduction, increase presynaptic DA release, mediate D_1 receptor DA transmission then lack of a NT transmission system could conceivably down regulate D_1 receptor mediated transmission, (Xu *et al.*, 1995). In light of this, the null mutation of the D_1 receptor may hold some clues to the lack of NT transmission. The D_1 receptor 'knock out' mice display hyperactive locomotor activity and decreased levels of dynorphin in the striatum. This is not to say that the lack of the NTR would display any obviously similar behavioural phenotype, but perhaps will significantly down regulate levels of dynorphin due to down regulated DA transmission to elicit milder but observable behavioural phenotype. In fact, the modulatory effects from the lack of NT transmission on dopaminergic systems, and other neuronal systems, may cause subtle local changes such as alteration in levels of gene expression, levels of other neuropeptides or alter the reactive state of DA receptors. Therefore, a multidisciplinary analysis of NTR^-/NTR^- mice using molecular, biochemical, electrophysiological and pharmacological will be necessary to accurately reveal the phenotypic effects of a null NTR mutation.

Lastly, and of particular interest, is the potential role NT transmission plays in the antipsychotic action of neuroleptic drugs. The action of the atypical neuroleptics appears to be mediated through the mesolimbic system, causing an increase in NT content primarily within the N.Acc. The lack of NT transmission system within the dopaminergic neurons will probably lead to a down regulation of D_1 receptor mediated transmission. The effects on the postsynaptic neurons may result in reduced transmission of the GABA neurons. However, as stated before fine analysis of the dopaminergic circuitry and those pathways that it innervates will be necessary to realise the phenotypic effects. The treatment of NTR^-/NTR^- mice with neuroleptic drugs will probably result in increased levels of NT expression in the N.Acc. However, without an animal model to assay the behavioural effects of neuroleptics it will not be possible to comment on the possible function NT transmission has on mediating their effects. Latent inhibition of a conditioned emotional response has been proposed as a quantitative measure of selective attention. This involves brain functions related to schizophrenia and targets of therapeutic drugs. Latent inhibition is poteniated on the administration of neuroleptics. However, co-administration of neuroleptics with the NT antagonist SR48692 abolished the observed increase in latent inhibition in a

dose dependent manner. These results suggest that the NT system is required to elicit the latent inhibition response. Therefore, it will be interesting to determine if the same inhibitory response of latent inhibition is observed for NTR^{-}/NTR^{-} mice on neuroleptic treatment. If latent inhibition is not poteniated on the administration of neuroleptics to NTR^{-}/NTR^{-} mice then this will provide further evidence for the involvement of NT transmission in the neuroleptic response.

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