Investigations into Region-Specific and Activity-Dependent Gene Expression in the Mammalian Hippocampus

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Abstract

The hippocampus is a brain region involved in the creation of certain types of memory. The work that contributes to this thesis concerns molecular biological investigations into the functioning of this region and can be divided into two parts.

Firstly, the analysis of genes which are restricted in expression to the hippocampus would not only go some way to explain the functional characteristics of this region, but would also provide genetic elements capable of directing spatially restricted expression of transgenes for the purpose of informatively perturbing the formation of memory. A variety of techniques such as degenerate PCR and subtractive hybridisation were therefore applied in an attempt to identify such transcripts. While two novel genes were cloned together with several exhibiting hippocampus-enrichment, none were exclusively expressed in this tissue.

Secondly, temporary amnesia occurs in patients who have undergone electroconvulsive therapy as a treatment for severe depression. One potential explanation for this is a saturation of the synaptic plasticity that may underlie memory (known as long-term potentiation, or LTP). A subtractive hybridisation technique has been applied to a rat model of this treatment to investigate the possibility that the occlusion of LTP is maintained by long-term changes in hippocampus gene expression; induced gene expression may also be responsible for the anti-depressive effects of this treatment.

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Glossary of Terms and Abbreviations

Chemicals

EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
PEG	Polyethylene glycol
NAD	Nicotinamide-adenine dinucleotide
SDS	Sodium dodecyl sulphate
TRIS	Tris(hydroxymethyl)methylamine
DEPC	Diethyl pyrocarbonate

Measurements

cm	Centimetre
mm	Millimetre
g	Gram
µg	Microgram
l	Litre
ml	Millilitre
µl	Microlitre
s	Second
min	Minute
hr	Hour
A	Ampere (current)
V	Volt (potential)
Ω	Ohm (resistance)
^{o}C	Degree Celsius
M	Molar
mM	Millimolar
µM	Micromolar
cpm	Counts per minute
Ci	Curie (1Ci=37 GBq, 1 Bq=60 dpm)
Bq	Becquerel
dpm	Disintegrations per minute
Hz	Hertz (cycles per second)
g	Acceleration due to gravity
OD	Optical density
A	Absorbance
Tm	Nucleic acid hybrid melting temperature

DNA

A	Adenosine
C	Cytidine

G	Guanosine
T	Thymidine
U	Uridine
I	Inosine
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
cDNA	Complementary DNA
rRNA	Ribosomal RNA
RNase	Ribonuclease
DNase	Deoxyribonuclease
Poly A^+	Polyadenylate
dNTP	Deoxy nucleotide triphosphate
NTP	Nucleotide triphosphate
(d)(A/T/C/G)(T/D)P	(Deoxy)(base)(tri/di)phosphate
cAMP	Cyclic adenosine monophosphate
nt	Nucleotide(s)
bp	Base pair(s)
kb	Kilobase(s)
aa	Amino acid(s)
ORF	Open reading frame

Neurobiology

PCR	Polymerase chain reaction
RT	Reverse transcript(ion/ase)
Taq	Taq polymerase (heat-stable DNA polymerase)
CCLS	Chemical cross-linking subtraction
CNS	Central nervous sytem
LTP	Long-term potentiation
LTD	Long-term depression
STP	Short-term potentiation
PPF	Paired-pulse facilitation
PTP	Post-tetanic potentiation
ECS	Electroconvulsive stimulation
ECT	Electroconvulsive therapy
LTM	Long-term memory
STM	Short-term memory
MTL	Medial temporal lobe
NO	Nitric oxide
CO	Carbon monoxide
AA	Arachidinic acid
PAF	Platelet-activating factor
NMDA	N-methyl-D-aspartate
AMPA	a-Amino-3-hydroxy-5-methylisoxasole-4-propionate
KA	Kainic acid
5-HT	5-hydroxy tryptamine/Serotonin
IP_3	Inositol triphosphate
GPCR	G protein-coupled receptor
mGluR	Metabotropic glutamate receptor
$G\alpha$	G protein α subunit
PTP	Protein tyrosine phosphatase
FN III	Fibronectin type III domain
Ig	Immunoglobulin-like domain

CREB	cAMP response element-binding protein
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
CAMK II	Calcium-calmodulin-dependent protein kinase II
TM	Transmembrane domain
CCLS	Chemical cross-linking subtraction
DZQ	Diaziridinylbenzoquinine
RoB	Rest of brain (brain with hippocampus removed)
ECU	ECS upregulated clone
ECD	ECS downregulated clone
IEG	Immediate early gene

This thesis is dedicated to my grandfather, Dr G.B.R. Feilden.

Chapter I

Introduction

'The lack of definitive answers on brain/mind matters is not a cause for despair...and is not to be seen as a sign of failure of the scientific fields no engaged in the effort. On the contrary, the spirit of the troops is high since the rate at which new findings are accruing is greater than ever...If there is any cause for worry, it comes not from a lack of progress but rather from the torrent of new facts that neuroscience is delivering and the threat that they may engulf the ability to think clearly.'

Antonio R Damasio, in *Descartes' Error: Emotion, Reason and the Human Brain* (Putnam)

1.1: The central nervous system is comprised of distinct regions.

The nervous system has evolved as a means for an organism to sense and interpret environmental stimuli and convert them into behavioural/physiological responses which aid the survival of that organism. Due to its highly specialised biological role and mechanistic complexity, it requires the expression of a large number of genes; a substantial number of which are to be found exclusively in this organ.

There exists a large degree of division of labour within the central nervous system such that anatomically and functionally distinct regions have been well defined (ref. 1). In a caudal to rostral progression through the CNS, the spinal cord is followed by the hindbrain (cerebellum, medulla, and pons), the midbrain, and finally, the forebrain (thalamus and hypothalamus (the diencephalon) together with the basal ganglia, hippocampus, amygdala, and cerebral cortex (the telencephalon)). This progression also charts the increasing functional complexity of these subregions and the increasing relative volumetric change throughout evolution. For example, the medulla and pons, among other functions, regulate blood pressure and respiration, whereas the cerebral cortex is involved in higher order processing of sensory and motor information together with the organisation of complex cognitive behaviour. This latter region, above all others, has undergone the most profound increase in relative and absolute size in the evolution of the human brain.

1.2: Memory

A focus for all disciplines of neuroscience has been the study of memory (refs. 2 and 3). The concept of memory can be summarised as a persistent change within the brain brought about by a stimulus. This definition applies to the psychological study of human individuals displaying cognitive disruption after brain injury, the behavioural study of rats learning the location of the platform in a swim-maze, and the macromolecular changes in a synapse brought about by neuronal stimulation. The study of memory could be seen as the first step towards understanding the more complex cognitive processes that occur in the brain. Indeed, experiments showing the link between emotional state and memory storage efficiency (ref. 4), together with the large body of information concerning the varied effects of psychoactive drugs, have already shown that memory is linked with other psychological phenomena.

The following sections outline the succession of neurobiological research trends that have led to a better understanding of specific kinds of memory and which form the background for the work described in this thesis.

1.3: The hippocampus is a vital brain region for the formation of certain types of memory

The study of memory began in earnest in the 1950's with the detailed psychological evaluation of several patients suffering from amnesic symptoms: the result of brain lesions or injuries. The temporal categorisation of memory into 'short-term memory' (S.T.M.) and 'long-term memory' (L.T.M.) was the first outcome of these studies (refs. 5 and 6). It was observed that amnesic patients exhibited a loss of the latter with the retention of the former. Anterograde amnesia is the term applied to the deficits seen in many of these patients who were unable to form new memories post-lesioning but who had a good recall for events in their lives that occurred prior (although maybe not immediately prior) to the lesion. Generally, memories were unimpaired if they had been formed between 1 and 10 years beforehand.

Post-mortem and PET scan studies of the brains of a number of the patients (ref. 7) showed that the principal regions affected by the lesions were a brain structure known as the hippocampus and the surrounding cortical tissues; collectively this region is described as the medial temporal lobe (M.T.L.). The second major conceptual advance in the study of M.T.L. (and diencephalic) damage was that a particular type of memory was affected, called 'declarative' memory. Declarative memory (also known as conscious, explicit or relational memory) can be distinguished from a

second type of memory, variously called non-declarative, non-conscious, skill, habit, simple classical conditioning, or implicit memory. The former, as the name suggests, relates to the ability to 'know something', as opposed to the latter, which is involved in 'knowing how to'. The two memory types differ in other respects apart from their storage location and informational content. Declarative memory is usually rapidly created but not one hundred percent accurate. Another characteristic of this type of memory is its inherent 'flexibility'; it seems to be able to cope with multi-sensory inputs (modalities) such as phonological and visual information and is able to translate these into different outputs. Under the umbrella of declarative memory come two further categories: episodic and semantic memory. Episodic memory denotes those personal or autobiographical memories that contain within them spatial and temporal pointers that create the contextual background to an event. This memory type seems particularly susceptible to M.T.L. and frontal lobe damage. Semantic memories are second-hand experiences which can be described as general knowledge. M.T.L. damage does not have such a profound effect on this form of declarative memory because multiple repetitions of the information can recover the loss. As mentioned before, short-term memories are not affected by M.T.L. lesions. Tests of declarative memory such as 'delayed non-matching to sample' (ref. 8), when carried out in such a way that the factual presentation and the test of recall are separated by a very short time (0.5 secs. in monkeys; up to 10 secs. in humans) are not affected by such lesions. The explanation for this is that there is a momentary or 'working memory' store outside of the hippocampus which operates for a few seconds (ref. 9). This working memory seems to consist of temporary speech and visual memory systems as well as another system responsible for attention. Recent work on this type of memory has shown that its principal location is the pre-frontal cortex.

Research has been carried out to determine precisely which regions of the M.T.L. are responsible for the creation of memories. This has been approached by performing progressively more selective lesions in animals (usually Macacque monkeys, but rats show similar results) and assessing their performance in a number of tests designed to expose particular facets of declarative memory. Lesions described as H^+A^+ removed the hippocampus, subiculum, amygdala and the associated cortical regions. H^+ lesions removed the hippocampus, subiculum, and some cortical regions, whereas H lesions selectively affect the hippocampus and subiculum. In brief, it was seen that the more extensive the dissection, the more profound the cognitive deficits. However, removal of the amygdala alone did not seem to have an effect on these memory tests and so it was deduced that the hippocampus and directly associated regions were the key sites of this type of memory acquisition.

1.4: The hippocampus is not the final repository for memories

The temporally discriminating nature and localisation of amnesia led to the idea that the hippocampus was not the final resting place of memories but was responsible for their temporary storage until they could be relocated in the neocortex. Thus, the ability of anterograde amnesiacs to recall events in the distant past could be explained because their retrieval and storage are hippocampus-independent processes. Nevertheless, one unexplained aspect of the symptoms of amnesia is the occasional loss of one year or more of declarative memory prior to the hippocampus lesion (retrograde amnesia). It is hard to reconcile the function of the hippocampus as a temporary memory store when it is so clearly required to retrieve memories created up to 12 months previously, even in the least-affected cases. Perhaps this is caused by the collateral damage of the lesions in these patients, but one other explanation is that as the cortex becomes more complex as one progresses up the evolutionary ladder, there is an increase in the time-lag for the transfer of memories from the hippocampus to the cortex (monkey models of retrograde amnesia exhibit a lag of 12-16 weeks and rats even less). This is also a key feature of a recent, and convincing, attempt to explain the role of the hippocampus and, more precisely, the process whereby memories are 'consolidated' in the cortex (ref. 10). In simple terms, the model proposes that the long-term memories are stored in the various cortical regions that were initially responsible for the interpretation of the incoming sensory information. These regions individually innervate the hippocampus which flexibly and rapidly stores the associations of the cortical inputs. Even if it receives excitatory input from just one of the cortical domains in the ensuing period, the hippocampus can re-establish the original cortical pattern of excitation. However, the hippocampal associations are not permanent and fade as a function of time. Thus, it is the emerging intracortical connections between the relevant domains that form the basis of the long-term memory. In contrast to the flexible nature of the associations within the hippocampus these connections are very slow to form (but slow to degrade) and so require the hippocampus to repeatedly recreate the original combinatorial excitations until they are competent to achieve them independently.

1.5: The anatomy of the hippocampus

The anatomical structure of the hippocampus has been studied by many in search for an explanation of its function. Fig. 1.1a shows that the hippocampus is a bilateral limbic brain region intimately associated with the neocortex. The cross-section diagram of the hippocampus clearly shows the arrangement of neurons which forms a 'tri-synaptic' circuit. It is the arrangement of the neuronal pathways within this circuit

Figure 1.1 a Illustration of the position of the hippocampus within the rat brain: anterior parts of the brain are towards the bottom left of the picture. A section of the left neocortex has been removed to reveal the underlying hippocampus (shown in red). The coronal cross-section through the hippocampus illustrates the components of the tri-synaptic circuit. Synapse 1 represents connections between the entirhinal cortex (EC) and the granule cells of the dentate Gyrus (DG). Synapse 2 is the non-Hebbian mossy-fibre synaptic connection in the CA3 region of the hippocampus. Finally, the Schaffer Collateral pathway forms Synapse 3 with the pyramidal neurons of CA1 **b** Summary of the major biochemical events initiated in the post-synaptic neuron in response to glutamate release. Glutamate stimulates the NMDA and metabotropic (mGluR) receptors. Secondary messengers such as phospholipid metabolites (produced by phospholipases A2 and C), cAMP (produced by adenylyl cyclase II), and Calcium ions act on downstream proteins (such as protein kinase C, alpha-calcium/calmodulin-dependent protein kinase II, and cAMP response element-binding protein) to induce the short and long-term events of synaptic potentiation.

Fig. 1.1a

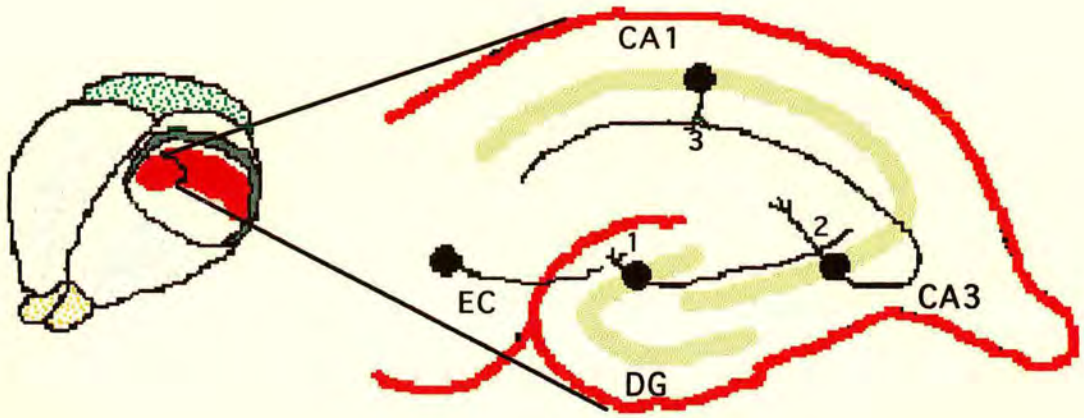
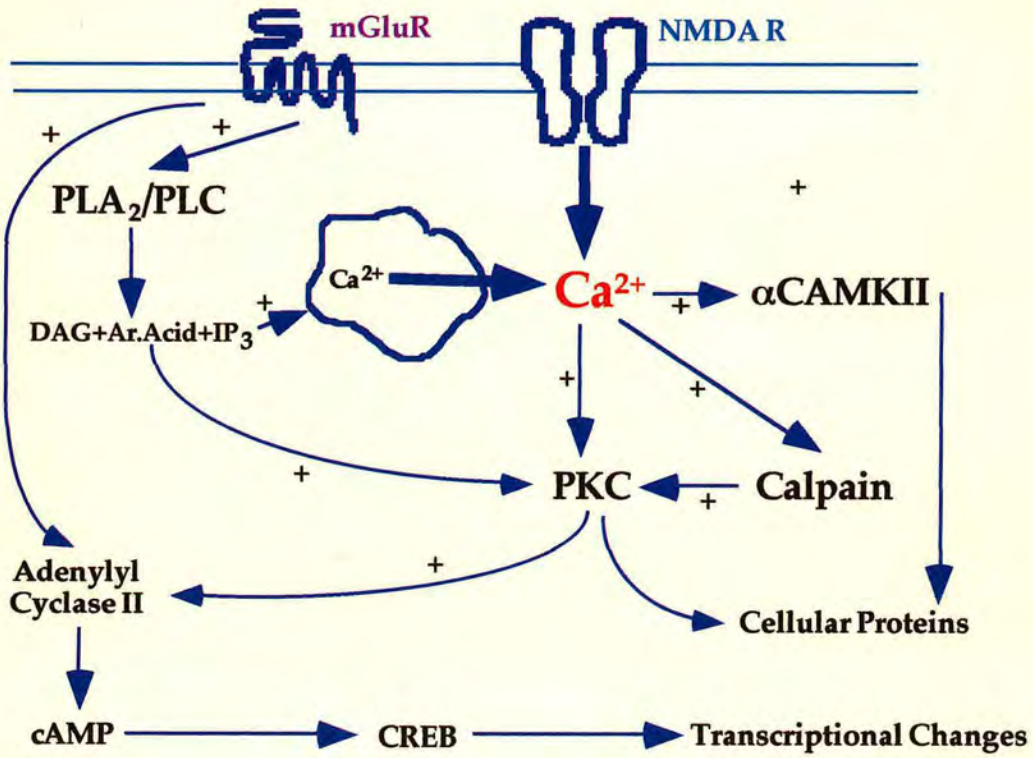


Fig. 1.1b



and their modifiable synaptic transmission efficacies that have not only made this brain region easier than others to investigate by electrophysiological means but have also contributed to hypotheses concerning the creation of memory.

The first projection of the trisynaptic circuit enters the dentate gyrus of the hippocampus from the entorhinal cortex and synapses onto the granule cells localised there. These cells, in turn, form synapses with the pyramidal cells in region CA3 of the hippocampus. Axonal projections from CA3 neurons (collectively termed Schaffer collaterals) enter region CA1 where they form synapses with the dendrites of pyramidal neurons. From here, although not part of the tri-synaptic circuit, projections re-enter the cortex via the subiculum. In recent years this serial circuit diagram has become a less satisfactory model in the light of more detailed anatomical and electrophysiological observations (ref. 11). It is now clear that some regions of the entorhinal cortex send axonal projections into the hippocampus that bypass the dentate gyrus and even the CA3 regions. CA1 and CA3 projections are not targeted as simply as was once thought; an individual axon from these regions has many terminations which form synapses in different, yet well-defined domains along the length of the CA1 region. *In vitro* labeling of individual inhibitory interneurons has highlighted their extensive projections and synapsing within the hippocampus. Also, it was observed that some of these located in the CA1 region can project 'backwards' to the CA3 region (ref. 12). The emerging picture is that there may be functional significance not only to the precise origin of hippocampal innervation from the cortex, but also the precise routing of information throughout the sub-regions of the hippocampus. Particular cognitive tasks may only require the participation of defined regions of the hippocampal 'tri-synaptic' circuit. Furthermore, recent work on hippocampus lesioning has convincingly demonstrated that only the dorsal (septal) region of the hippocampus is required for the accomplishment of spatial memory tasks (ref. 13). Thus, functional divisions exist along the length of the hippocampus as well as within the tri-synaptic circuitry itself.

1.6: Synaptic transmission modifications underlie certain behavioural responses

Donald Hebb, in his book 'The Organization of Behavior' (1949, New York, Wiley) made one of the first proposals as to how the brain's constituent neurons could act to create memories. Subsequent refinements resulted in the hypothetical model known as the 'Hebbian synapse' (refs. 14 and 15). In outline, this model states that the connections between neurons, the synapses, are the site of memory creation. If two connected neurons are activated simultaneously by their respective excitatory inputs then any synaptic connections between the two will be strengthened. This

strengthening allows the neurons to become more permissive in mutual communication such that when one is active then there is an increased likelihood that it will cause activation of the other. The corollary is also deemed to take place: when only one of the pair is active, the shared synapses will be weakened; this is the anti-Hebbian synapse. The Hebbian synapse's critical component is its associative nature; two events have to coincide before there is a change in synapse strength. This hypothetical cellular property has been shown to have an exact behavioural counterpart *in vivo* as will be described.

The neural basis of behaviour has been studied in simple invertebrates such as *Aplysia californica* which have a defined repertoire of reactive behaviours and a simple nervous system consisting of just 20,000 neurons. Three historically well-understood behavioural paradigms are represented in this animal: habituation, sensitization, and classical conditioning (ref. 1).

Habituation is the decreasing responsiveness to a repeated stimulus and is a non-associative form of learning. For example, when the siphon of this animal is touched, there is a reflexive withdrawal of the siphon and the gill apparatus. If this stimulus is repeated a number of times then the response becomes less acute. This decreased reactiveness lasts a few minutes in this case but has a duration of up to 3 weeks when there are 4 rounds of 10 stimulations over a short space of time.

Sensitization is also a non-associative behaviour. If a noxious stimulus, such as an electrical shock to the tail, is administered to the animal then the reflex to siphon stimulation will be much stronger. The animal's behavioural reaction has undergone a 'priming' procedure which makes it respond more vigorously.

Classical conditioning is an associative behaviour as it requires the apposition of two events to elicit a strengthening of a response; a situation very similar to the Hebbian model. In this case, a 'conditioned stimulus' such as the stimulus of the mantle shelf elicits the gill-withdrawal response. If there is also an 'unconditioned stimulus' in the form of a shock to the tail directly after the stimulus (causing a large defensive reaction in the animal) then after a few repetitions of this procedure the large response can be induced by the conditioned stimulus alone. Thus, the animal has learned that the mantle stimulus immediately precedes a noxious event and acts pre-emptively. It can be shown that this is a discriminating response because stimulation of the siphon will not produce the same degree of reaction.

In depth analyses of the neural pathways that contribute to these three behavioural systems together with the biochemical events occurring inside the relevant neurons

have given us insight into their underlying mechanisms, although there has been argument over the extent to which it is possible to infer the processes that take place in higher organisms from those in a mollusc.

Habituation seems to occur through a reduction in neurotransmitter released by the sensory neurons at the synapses with the motor neurons. Thus, a point is reached at which, the level of transmitter released from a stimulated sensory neuron is not sufficient to overcome the threshold of activation of the motor neuron, thus preventing the reflex. One possible mechanism for this reduction is the decrease in calcium influx through calcium channels into the pre-synapse; a critical requirement for vesicle exocytosis and neurotransmitter release. Sensitization requires the same pathway to come under the modulatory influence of synaptic transmission from the sensory neurons of the (shocked) tail. This acts on the pathway by increasing synaptic vesicle/neurotransmitter release onto the motor neuron dendrites. The interaction is therefore known as 'heterosynaptic facilitation'. Serotonin is the principal modulatory neurotransmitter involved and it seems to act in the sensory neuron by causing a chain of short- and long-term events mediated by the increased intracellular production of cyclic adenosine monophosphate (cAMP) by the enzyme adenylyl cyclase. Classical conditioning is brought about by similar process but, in this case, there is an additive effect brought about by the near-simultaneous activation of the two pathways such that the shocked tail facilitator connection elicits a large increase of calcium influx into the mantle sensory neurons. The calcium is chelated by calmodulin protein which then acts as a cofactor for adenylyl cyclase resulting in large cAMP increase. The biochemical cascade which ensues results in the strengthening of synaptic connections onto motor neurons. In comparison with sensitization, the resulting increase in reflex response is much greater and longer-lasting.

1.7: LTP is a model for synaptic transmission changes in the hippocampus

The first concrete evidence of a neuronal mechanism for memory came in 1973. The hippocampus, because of its simple, laminar structure, allows the relatively easy implantation of stimulus and recording electrodes into defined neuronal fields. Bliss and Lømo recorded the change in post-synaptic potentials of neurons from various regions in the rabbit hippocampus and showed that they increased when the presynaptic neurons had undergone trains of high frequency (100Hz) stimulation (ref. 16). This change in the activity of the postsynaptic cell in response to presynaptic stimulation lasted from days to weeks and was dubbed 'long-term potentiation' (LTP: refs. 17-22). The change in potentiation occurring at a synapse is generally measured

as a change in the slope of the excitatory post-synaptic potential (EPSP). Typically, a 100Hz tetanus induces a 2-3-fold increase in this value which remains stable at this new level for a period of time depending on the precise experimental procedures used.

As described earlier, the hippocampus contains three major synaptic domains. All exhibit LTP, but the precise nature of this potentiation is not always the same. The synapses between the perforant pathway and the dentate gyrus and the synapses between CA3 Schaffer collaterals and neurons in the CA1 region were shown to be additive/cooperative, which means that a minimum number of simultaneous neuronal inputs are required to potentiate a postsynaptic cell. They were also shown to be associative and specific, that is, only the connections between those active presynaptic neurons and simultaneously active postsynaptic neurons become stronger. In contrast, the mossy fibre synapses which connect dentate gyrus granule cells with CA3 pyramidal neurons are neither associative nor cooperative; if a sufficiently strong excitatory signal is passed onto a CA3 neuron then the responsible synapse will become potentiated even if the CA3 neuron was not in an active state (ref. 23).

1.8: The molecular mechanisms of memory: glutamate receptors

The discovery of LTP as a possible cellular mechanism for memory has led to the investigation of molecular processes which may cause this synaptic potentiation. Glutamate is the major excitatory neurotransmitter in the CNS and several classes of glutamate receptor were originally defined by virtue of their pharmacological and electrophysiological characteristics (these will be dealt with in detail below). In particular, the 'NMDA' receptor (named after its high affinity agonist, N-methyl-D-aspartate), exhibited unusual physiological parameters which made it an ideal candidate for the molecular mechanism responsible for the cooperative and associative characteristics of LTP (ref. 24). Firstly, unlike the other glutamate receptors, it did not gate ions when stimulated with glutamate under normal neuronal potentials. However, gating did occur with glutamate addition when the postsynaptic cell had been depolarized to a threshold level by the combined action of conventional glutamate ion conductance at numerous cooperatively activated synapses. Therefore, it exhibits an activation profile that matches that required to shift a neuron into a potentiated state. It has since been discovered that the voltage-gating property is mediated by a magnesium ion blocking the channel at normal cellular potentials. Upon sufficient post-synaptic depolarization, this block is removed and ions can pass freely through the channel provided that glutamate is bound to the ligand-binding

portion of the receptor. Interestingly, electrophysiological conditions which may contribute to the alleviation of the magnesium block have been observed in the hippocampus when a rat is actively involved in a hippocampus-dependent task such as the exploration of a novel environment. During such activity, cholinergic fibres from the medial septal nuclei (MSN) induce a regular (4-10 Hz) innervation of the hippocampal inhibitory interneuron network. These neurons in turn provoke theta rhythmicity in pyramidal cells. The presence of this 'theta' rhythm may act to synchronise the activity of the hippocampal neurons and push them into a more suitably depolarized state for the induction of LTP (refs. 25-27). The other key feature of this receptor, in comparison with the other glutamate receptor channels, is that not only does it permit the passage of sodium and potassium ions, but it also allows the gating of calcium ions; a process which is crucial for the stimulation of long-lasting potentiation changes.

The application of molecular cloning techniques to the analysis of glutamate receptor biology has revealed the complexities behind their pharmacological profiles (refs. 28-32). Original classification defined glutamate receptors as being of the 'non-NMDA' class (comprising AMPA and kainate (KA) ligand-gated ion channels), the 'NMDA' class of ligand-/voltage-gated ion channels, and the 'metabotropic' class of G protein-coupled glutamate receptors. Cloning of the genes encoding these functions (initially by expression of cDNA libraries in an assay system such as *Xenopus* oocytes) has revealed that there are at least 7 non-NMDA receptor genes (GluR1-5, KA-1, and KA-2), at least 5 NMDA receptor genes (NMDA R1 and R2A-D), and at least 8 metabotropic glutamate receptor genes (mGluR 1-8). Adding to this complexity are the variety of post-transcriptional changes that modify the structure and functioning of these receptors; for example, the 'flip/flop' splice-forms of the AMPA receptor transcripts and the RNA editing of the AMPA and kainate receptor transcripts. With respect to the correct function of the NMDA receptor, it has been demonstrated by co-transfection experiments that a heteromultimeric (probably pentameric from evidence of molecular weight) structure is required to reproduce the *in vivo* electrophysiological and pharmacological characteristics. Within the channel complex, the requirement for the NMDA R1 subunit seems to be absolute; this is confirmed by expression data which suggests that, of the NMDA R mRNAs, R1 is the only one ubiquitously distributed in the brain (refs. 33 and 34).

The functional parameters of these glutamate channels can be subject to modification by post-synaptic proteins (refs. 35-40). This may be responsible for some of the electrophysiological effects observable after LTP-inducing stimuli. Phosphorylation (mediated by kinases such as *src* and cAMP-dependent protein kinase A) may

increase the activity of these receptors, whereas dephosphorylation (by a variety of phosphatases, including PP1, PP2A, and PP2B/calcineurin) may act in the opposite manner.

The metabotropic glutamate receptors transduce the glutamate-binding signal into cellular responses via the action of G proteins. Therefore, their effects are subject to a slight time-delay in comparison to the channel-forming receptors. In addition, unlike NMDA receptors, there is no associative component to their activation. The section of the hippocampal synaptic circuit between the dentate gyrus and CA3 region is known to exhibit a non-associative LTP called mossy fibre LTP. No NMDA receptors are to be found in this region and so it may be that mGluRs are responsible for this form of potentiation. Alternatively, because the LTP in this region is assumed to be pre-synaptic, the atypical pre-synaptic glutamate receptor, GR33 (see Chapter V) might be involved. A mGluR agonist, ACPD, augments tetanus-induced potentiation in the hippocampus, whereas the antagonist, MCPG, inhibits LTP. This latter effect is notable because it occurs in regions of the hippocampus where LTP can be either NMDA-dependent or NMDA-independent. Metabotropic glutamate receptors seem to represent a necessary component of LTP induction even in regions where LTP was previously considered wholly dependent on the NMDA receptor. This has been rationalised in one report by the suggestion that the mGluR component of NMDA receptor-mediated LTP may act as a competence switch to aid the full induction of LTP (ref. 42).

1.9: The molecular mechanisms of memory: calcium influx

In the post-synaptic neuron, the induction of LTP seems to rely on a transitory increase in intracellular calcium ion concentration. The introduction of the Ca^{2+} chelating agent, EGTA, into the cytoplasm of the post-synaptic neuron prevents the induction of LTP (ref. 43). The NMDA receptor channel itself can act as one source of this Ca^{2+} ion influx but several other systems are also capable of causing this increase (refs. 44-46). Voltage-sensitive calcium channels allow the entry of calcium into the post-synapse after depolarization. It is also evident that the Ca^{2+} ions do not have to originate from the extracellular medium. Ca^{2+} influx can trigger the subsequent release of calcium ions from the intracellular stores: inhibitors of the receptors on the storage vesicles that mediate this Ca^{2+} release also inhibit the induction of LTP. In addition, metabotropic glutamate receptor activation can cause the release of this internal Ca^{2+} by the action of one of its downstream messengers, inositol-1,4,5-trisphosphate (ref. 47). The post-synapse is usually a bulbous spine attached to a dendritic fibre. It has been postulated that the spine operates to limit the

diffusion of Ca^{2+} ions into the dendrite and hence neighbouring post-synapses (ref. 48). Thus, an individual synapse can simultaneously create a high post-synaptic Ca^{2+} ion concentration by being of limited volume as well as maintaining the synapse-specificity required. When sufficient numbers of spines are simultaneously activated, possibly coupled with the opening of the spine neck, the dendritic shaft Ca^{2+} concentration increase might achieve a degree of summation that could cause a somatic change (more specifically, a transcriptional change) in the neuron that would consolidate the potentiation. These kinds of localised and widespread changes in Ca^{2+} concentration have been examined in detail recently with the advent of real-time Ca^{2+} imaging techniques which make use of Ca^{2+} concentration-sensitive fluorescent compounds (ref. 49).

1.10: The molecular mechanisms of memory: synaptic biochemistry

With calcium being accepted as a general trigger for the induction of LTP, much work has centred on its cellular targets (ref. 17). These can be broken down into several groups of enzymes (e.g. protein kinases, phosphatases, phospholipases, adenylyl cyclases, and proteases).

Studies of an invertebrate, the fruit fly (*Drosophila melanogaster)*, have contributed greatly towards our understanding of the biochemistry of the synapse. Initial work involved the creation of specific behavioural tests which could be used to assess the presence or absence of memory capabilities in the fly (ref. 50). Then a large-scale mutagenesis screen was performed from which flies were subjected to the testing procedure. Using this technique two major memory-affecting alleles were discovered; '*dunce*' (ref. 51), and '*rutabaga*' (ref. 52). Subsequently, alleles which include '*amnesiac*', '*radish*', '*latheo*', '*linotte*', '*DCO*', '*turnip*', '*cabbage*', '*zucchini*', '*shaker*', and '*mushroom body damaged*' have been discovered using similar approaches. Interestingly, some of the genes which are affected in these mutants have been cloned and identified. *Dunce* mutants were originally described as having unusually high intracellular concentrations of cyclic AMP. This was due to the fact that *dunce* encodes a cAMP phophodiesterase which, when mutated, was not able to catabolise excess cAMP. Because this secondary messenger has many downstream effectors, the mutant phenotype observed probably arises from the cumulative overactivity of all cAMP-responsive proteins. The gene affected by the *rutabaga* mutation encodes a calcium/calmodulin-sensitive adenylyl cyclase. Hence, the memory phenotype seen in this mutant results from a lack of cAMP production. Other mutant genes isolated include *amnesiac* (a putative neuropeptide involved in a middle-term potentiation step), *DCO* (the catalytic subunit of the cAMP-dependent protein kinase), *shaker*

(voltage-dependent potassium channels), and *linotte* (its protein product has no database homologies but seems to be involved in the acquisition of memory) (refs. 53 and 54). *Latheo* (another acquisition-affecting mutation) and *radish* (which affects anaesthesia-resistant memory) have not yet been cloned.

With the important role of cAMP in memory processes so evident from many of the isolated alleles, further work has analysed the role of cAMP-responsive transcription factors in the creation of memory in *Drosophila*. A dominant negative mutation of the CREB (cAMP-responsive element-binding protein) transcription factor results in the severe impairment of long-term memory whilst short-term retention is unaffected (ref. 55). The complementary experiment of overexpressing a constitutively active form of the transcription factor leads to an enhancement in that particular stage of memory (ref. 56).

The following section (together with fig. 1.1b) briefly outlines the biochemistry of the vertebrate synapse which plays a part in the early stages of long-term potentiation (refs. 17 and 18).

Two protein kinases which have been implicated in the processes underlying LTP are protein kinase C (PKC) and α-Ca^{2+}/calmodulin-dependent kinase type II (CAMK II). PKC has been reported to phosphorylate a large number of neuronal membrane-associated proteins in the presence of Ca^{2+} and phospholipids. These include glutamate receptors, adenylyl cyclase type II, and GAP-43 amongst others. The use of an inhibitory peptide against this kinase blocks the formation of LTP. CAMK II is a highly abundant protein in the hippocampus and its activation seems to be another critical step in the induction of LTP; possibly by the phosphorylation of non-NMDA receptors amongst other proteins. Because both of these kinases are able to become constitutively active (by autophosphorylation, in the case of CAMK II, and calpain protease activity, in the case of PKC) several models have been proposed in which these may act as 'molecular switches' for the induction of memory (ref. 57). While the induction of both kinases is important in the creation of LTP, it would be wrong to attribute all activity-dependent synaptic excitability changes to their actions.

As in other species investigated (such as *Drosophila* and *Aplysia*), the production of cyclic AMP is a necessary step in the production of early and late forms of potentiation. At least five forms of adenylyl cyclase are known to be expressed in the mouse brain, each encoded by a separate gene (ref. 58). Type II seems to be important in the production of cAMP in the context of LTP. cAMP as a secondary messenger has many varied sites of action in the neuron including the stimulation of protein kinase A activity (another important protein kinase) and the triggering of

some transcription pathways via the activation of CREB (cAMP response element-binding protein).

G protein activity (for example, from metabotropic glutamate receptor stimulation) is known to trigger the operation of a number of downstream proteins. Two of these, phospholipases A_2 and C (PLA_2 and PLC) break down membrane lipids into diacylglycerol and arachidonic acid (which activate PKC) and inositol-1,4,5-trisphosphate (IP_3), which results in the release of calcium from intracellular stores.

Hence, the activation of key proteins in activated neurons causes the short-term potentiation events that are detectable in the first few minutes after stimulation and also permits the induction of other events (including some at the nucleus) which will lead to long-lasting potentiation changes.

1.11: Have links between synaptic molecules, LTP, and memory been conclusively demonstrated?

Because memory at the behavioural level might be due to LTP at the synaptic level and LTP seems to rely on the functioning of the NMDA receptor, there have been attempts to unify the interrelationships of these processes by experimentation. In a seminal study of this nature (ref. 59), the effect of the administration of an NMDA R antagonist (AP5) was observed on the ability of rats to learn a swim maze test; a test intended to involve hippocampus-dependent spatial learning (ref. 60). Chronic infusion of the antagonist during training prevented performance improvement in these rats in comparison with untreated control rats. Therefore, by inhibiting the function of a molecule in the hippocampus, effects were seen not only on LTP induction, but also on memory acquisition. Recently, this belief has had to undergo some revision in the light of new evidence that suggests that NMDA R-dependent LTP and hippocampus-dependent spatial learning may not be the only contributors to performance in this behavioural assay. Hence, either additional cognitive systems responsible for the task acquisition must be discovered, or a more specific test devised such that only the cognitive systems under study are required for its completion. Only in this way will causal links be confirmed between gene products, electrophysiological phenomena, and behaviour *in vivo*. (refs. 61-63).

To these experiments must be added the growing list of transgenic perturbations of synaptic molecular function (described in more detail in section 1.13) which have shown, in a number of cases, simultaneous disruption of LTP and spatial learning.

1.12: Changes in transmission efficacy could arise through different effects on synaptic function

Section 1.10 listed the biochemical pathways that are active during LTP induction. However, the precise way in which the synaptic transmission efficacy change is brought about is still somewhat controversial (refs. 64 and 65).

A synapse could become potentiated by a number of mechanisms. In the presynaptic cell there could be an increase in the likelihood of an action potential triggering neurotransmitter release (refs. 66-69), an increase in the amount of neurotransmitter within each synaptic vesicle released, or an increase in the number of vesicles released per stimulus. In the postsynaptic cell, either the number of receptor sites could increase (ref. 70) or their responsiveness.

In an attempt to reveal the locus of expression of LTP many experimental approaches have been taken. It is a feature of this field that appropriate evidence can be found to support the notions that potentiation events are wholly presynaptic, wholly postsynaptic, or a combination of the two. Techniques that have been used to detect changes after potentiation include the monitoring of labeled glutamate release from the presynaptic bouton, quantal analysis of spontaneous miniature synaptic potentials (refs. 71 and 72), sensitivity of receptors to agonists, activity-dependent inhibition rates of NMDA receptors, and the analysis of synaptic transmission failure. With the wide variety of stimulus paradigms, experimental conditions, and interpretations possible it remains to be seen if the locus can be assigned convincingly.

1.13: Associative LTP with a presynaptic component requires a retrograde messenger

In the case of associative LTP, where both pre- and post-synaptic cells are active at the same time, there is a coincidence detection mechanism in the form of the NMDA receptor. If potentiation is to have a pre-synaptic component in this instance, then a post- to pre-synaptic signal must be transmitted to trigger those events. It is with this in mind, that a 'retrograde messenger' has been postulated and recent efforts have concentrated on a number of candidates for this messenger.

Arachidonic ccid is produced by the action of the enzyme phospholipase A_2 and, like the other messenger candidates discussed, has the ability to diffuse freely through cell membranes which is a prerequisite for a trans-synaptic activity. Once in the pre-synaptic neuron, its mode of action maybe the stimulation of PKC or participation in prostaglandin synthesis (ref. 73).

Platelet-activating factor (PAF) is another product of the action of phospholipase A_2. Administering this chemical (even post-synaptically) seems to enhance or even create (*de novo*) synaptic LTP. This effect is inhibited by PAF antagonists such as BN52021 although subsequent high-frequency stimulation can overcome this block which probably means that additional mechanisms are participating (refs. 74 and 75).

Nitric oxide (NO) is a gas which has already been shown to have biological activity in a number of systems (refs. 76-80). These include blood vessel endothelium relaxation in response to bradykinin or acetylcholine and the macrophage response to endotoxins. In the context of the brain, NO was implicated as a retrograde messenger when it was shown that the addition of haemoglobin to the fluid surrounding a synapse could prevent the retrograde message from reaching its goal; NO was therefore a candidate because the haem moiety in this protein chelates NO and similar gases. NO synthase (NOS, also known as NADP diaphorase) is the enzyme responsible for the production of this gas (ref. 81). NO synthase activity is present in the brain and inhibitors of this enzyme, such as L-NMMA, have an inhibitory effect on the induction of LTP. To date, three genes encoding members of the NO synthase family have been cloned; endothelial NOS (eNOS), inducible NOS (iNOS; found in macrophages), and neuronal NOS (nNOS) (ref. 82). The neuronal form has been shown to be activated by calmodulin after an increase in intracellular calcium levels. Unexpectedly, nNOS was shown not to be expressed in the CA1 region of the hippocampus: a region known to exhibit NOinhibitor-sensitive LTP. This was later resolved by the discovery that eNOS is found in high levels in this region. Targeted disruption of all three NOS genes has been reported. Inducible NOS-null mice are immuno-compromised. Neuronal NOS-null mice were expected to display memory deficits (perhaps erroneously, due to the distribution anomaly) but have normal LTP. They do, however, exhibit behavioural disorders such as aggressiveness which may be due to the gene product's absence from other brain regions. In addition, they exhibit increased protection from NMDA-dependent neurotoxicity as well as having a gut disorder which closely resembles the human condition of pyloric stenosis. The deletion of the endothelial form of NOS has a large effect on hypertension regulation in the mouse resulting in a 35% increase in mean blood pressure. It remains to be reported if these mutant mice possess memory impairments.

Observations concerning the functioning of NO in the hippocampus suggest that the Hebbian description of the synapse may be slightly limited in some circumstances. As expected of a retrograde messenger, NO only seems to potentiate a synapse when associative activity is occurring. If NO is administered to a synapse where the NMDA receptor component has not been triggered then inhibitory processes occur such that

the NMDA receptor activation threshold is increased (refs. 83 and 84). This effect is known as 'heterosynaptic depression' and still conforms to the Hebbian model. However, a series of experiments have shown a slight deviation from the traditional Hebbian system. In these experiments two parallel fibres in close proximity were stimulated. Both fibres' synapses were recorded for electrical activity changes, but one of these had been iontophoretically loaded with NOS inhibitor. The observation was that even though this synapse (in isolation) could not become potentiated, when stimulated in conjunction with the other synapse, potentiation was observed in both. To explain this phenomenon a diffusible NO signal (from the uninhibited synapse) must be involved in the potentiation of the inhibited synapse. Thus a synapse which has not met the requirements for LTP can be potentiated if certain 'local' environmental conditions are met (ref. 85).

The routes by which NO can exert its influence are defined by its strong interaction with the iron moieties of several proteins. Chief amongst these is guanylyl cyclase, whose activity is increased in the presence of NO. The subsequent rise in concentration of its product, cyclic guanine monophosphate (cGMP), can be thought of as analogous to the rise in cAMP in the post-synaptic cell with respect to the possibilities for effector activation. Other direct targets probably include aconitase (a citric acid cycle enzyme and mRNA-binding protein; see Chapter V), NADH-ubiquinone oxidoreductase, NAD succinate oxidoreductase, and ribonucleotide reductase; all of which are distinguished by possessing iron cofactors.

Another gas which could behave in a similar way to NO, and which has comparable reactive characteristics, is carbon monoxide, CO (ref. 86). CO is synthesized by haem-oxygenase 2 (HO2) which is present in the brain. ZnPP (zinc protoporphyrin IX) acts as an inhibitor of this enzyme and has been shown to block the synthesis of LTP in some laboratories (ref. 87). Very surprising is this inhibitor's described ability to eliminate previously induced LTP. With the publication of a knock-out of haem-oxygenase 2, (ref. 88) the role of CO in the production of LTP has been thrown into further doubt. No HO2 activity is seen in the homozygote mutant animals and yet they exhibit normal LTP. Furthermore, the mutant mice are as susceptible as wild-type mice to LTP blockage by the administration of ZnPP. This suggests that the inhibitory action of ZnPP is not specific to HO2.

1.14: Later phases in the formation of LTP

The above descriptions of synapse potentiation only go so far as to document the immediate changes that result in an increased level of synapse excitability. These

include the influx of calcium ions and the resultant phosphorylation events that alter the activities of synaptic proteins. However, experiments have shown that there are critical periods of transcription and translation in the minutes and hours subsequent to the induction of LTP.

The role of transcription and translation in activity-dependent synaptic efficacy changes has been investigated in *Aplysia*, *Drosophila* and rat. Much of this work has been associated with the administration of transcriptional and translational inhibitors to explants of neuronal tissue in order to observe their effects on the longevity of stimulated potentiation. In *Aplysia*, the ability to dissect out the principal synaptic components of the gill-withdrawal reflex pathway has enabled investigation into the effects of these inhibitors, which is ruled out in the intact animal in view of their toxicity (ref. 89). The overall conclusion drawn from investigations in this system is that the inhibition of transcription (by drugs such as actinomycin D) or translation (by drugs such as anisomycin or cycloheximide) prevents the conversion of short-term facilitation/sensitization into the long-lasting forms (refs. 90 and 91). Activity-dependent structural changes of synapses (thought to be a contributory mechanism to changes in synaptic efficacy) are also subject to the effects of these drugs. Moreover, the precise time of administration determines the extent of this inhibition (ref. 92). The critical 'time-window' for effective drug administration (and, therefore, the biological processes involved) seems to be within minutes of the training stimulus. It can be concluded that there is both a need for the translation of existing transcripts into proteins and a need for *de novo* transcription in the processes leading to consolidation of potentiation.

Similar work has been performed on rat brain slices with comparable results (refs. 93-95). Tetanus-induced potentiation of hippocampal synapses has been monitored in the presence of the same inhibitors of transcription and translation. Again, they act in a dose-dependent and time-dependent fashion to block the conversion of short-term potentiation into long-term potentiation. Interestingly, the early phase of potentiation is left relatively unaffected by the presence of these drugs; effects only appear approximately ten minutes after the stimulation. This has led to the acceptance of the hypothesis that the immediate electrophysiological sequelae of tetanic stimulation are mediated by 'covalent' processes, such as the phosphorylation of key regulatory molecules, whereas the longer-term effects are dependent upon 'macromolecular synthesis'. The latter must occur for the potentiation to be long-lasting and classified as genuine LTP. If it is disrupted then the potentiation is short-lived and designated 'short-term potentiation' (STP).

Details of the gene expression changes that correlate with full LTP are summarised in the context of experiments designed to examine similar 'activity-dependent' gene expression changes (see Chapter V) and will not be dealt with further here.

1.15: Activity-dependent neuronal morphology changes

One line of investigation into how a synapse can become potentiated is based on the assumption that the nuclear products that are synthesized in response to synaptic activity will have a function in changing the operation of that synapse. It has been proposed, therefore, that these changes might be found in the ultrastructure of the synapse (refs. 96 and 97). Several groups have applied microscopical techniques to the examination of the size, shape and number of dendritic spines before and after the induction of LTP or the exploration of a complex environment. One report has shown an increase in the number of CA1 dendritic spines in rats which have undergone spatial training in such an enriched environment (ref. 98).

Others have focused on the action of proteins that may create or reorganise the cell-cell interactions, intracellular cytoskeletal networks, and the extracellular matrix that, together, form the basis for the connection of one neuron to another. Antisera raised against the NCAM (neural cell adhesion molecule) protein and infused into the brain has an amnesic effect on chicks assayed in a one-trial passive avoidance test. Similar experiments carried out in rats using antibodies against NCAM and L1 (another cell adhesion molecule) also resulted in memory impairments (refs. 99-101). L1 expression also seems to be down-regulated in cell cultures of sensory neurons which have been subjected to low frequency (0.1Hz) electrical stimulation (ref. 102). A large number of serine protease genes are expressed in the hippocampus (at least ten; B. Davies (C.G.R.), personal communication) these may play a role in the restructuring of the extracellular matrix and the cleavage of cell-surface proteins. Two members of this family, neuropsin and tissue-plasminogen activator, are known to be up-regulated in response to LTP-causing stimuli in the hippocampus (see Chapter V). The actions of the intracellular protease, calpain, have been tightly linked with the induction of LTP in the hippocampus through the effect of specific inhibitors (ref. 103). By the cleavage of substrate proteins that make up the cytoskeleton, synaptic and neuronal morphology may be altered such that synaptic transmission efficiency is modified.

1.16: The application of transgenesis to the study of memory

With the advent of techniques permitting the manipulation of the mouse genome, it has become possible to extend the mutagenesis work carried out in flies to vertebrates. In an attempt to probe their roles, several genes which were presumed to be involved in the biochemical pathways that underlie LTP have been disrupted in the mouse genome using homologous recombination (refs. 104-110). Briefly, embryonic stem cells are electroporated with DNA which is, in part, complementary to the locus under study. This DNA is arranged so that if it becomes correctly incorporated into the chromosomal DNA it either deletes the gene's functional exons or results in the production of non-functional transcripts. Selection for these rare recombination events is made possible by the inclusion of antibiotic resistance markers in the exogenous DNA sequence. Mutant stem cells can then be injected into blastocysts which are then implanted in pseudopregnant mice. The chimaeric offspring can be bred to produce the mutant allele in the heterozygous or homozygous state. These 'knock-out' experiments produce mice which are deficient in the gene under study. With respect to the analysis of learning and memory, mice null for a particular gene can be assayed with a battery of electrophysiological, molecular biological, anatomical, and behavioural tests. The phenotype obtained provides evidence for or against that gene's involvement in memory formation.

The following list of knock-out and transgenic experiments is not exhaustive but has been chosen because of the pronounced effects on hippocampal function; other experiments which have a bearing on more specific brain functions are described elsewhere in this thesis.

The role of glutamate receptors in the induction of LTP is central to the models of memory that have been proposed to date and this has been confirmed by studies of mice mutant for the relevant genes. Of the five NMDA receptor subunits identified, NMDA R1 (ζ) is ubiquitously expressed in the brain and is a necessary component of the correctly functioning channel complex. Two groups have described the disruption of this gene in mice and both report that the effect of the homozygous mutation is perinatal lethality due to breathing control difficulties (refs. 111 and 112). One group demonstrated that the whisker-related neuronal barrels fail to develop in the trigeminal brainstem nuclei, demonstrating a developmental role for the NMDA R family. The other group used hippocampal primary cell cultures from neonatal brains to assay glutamate and NMDA responses; as predicted, the latter were completely absent. There was an apparent knock-on effect of the deletion of this gene because

expression of the NMDA R2B subunit was greatly downregulated; this could be evidence for coordinate regulation of subunit expression in order to ensure functional receptors. Deletion of the NMDA R2A subunit (which is normally expressed in the cortex, hippocampus, cerebellum, and olfactory bulbs) resulted in a 50% reduction in elicited LTP and substantial underperformance in the water maze test of spatial learning (ref. 113).

Two groups have also reported the successful disruption of the metabotropic glutamate receptor gene, mGluR1, which is normally expressed in cerebellum, olfactory bulb, and thalamus, together with the dentate gyrus and CA3 regions of the hippocampus (refs. 114 and 115). In both cases the mice were viable but possessed motor coordination deficits which resulted in tremorous movement. Combined, the results indicate that this isoform plays important roles in cerebellum and hippocampus function. Long-term depression (another form of synaptic excitability change) in the cerebellum is decreased in these mutants. In the hippocampus, mGluR1 seems critical for the correct functioning of CA3 neurons; mossy fibre LTP (in which CA3 neurons are post-synaptic) and Schaffer collateral LTP (in which CA3 neurons are pre-synaptic) are both decreased significantly. This is matched by similar deficits in spatial learning (carefully controlled for motor impairment factors).

The brain-specific (γ) isoform of the calcium- and phospholipid-responsive protein kinase C (PKC) has been deleted by homologous recombination (refs. 116 and 117). Whilst LTD and PPF remain unaffected in the hippocampus, a decrease in LTP was observed. However, this can be reinstated by prior treatment of the hippocampal circuit with low frequency stimulation; so-called 'priming'. The cause of this reversible LTP block is unknown but the low frequency stimulation might act to depotentiate 'saturated' neurons permitting subsequent LTP. The authors discount this as a possibility because they see no linear relationship between the extent of priming and the level to which LTP is subsequently inducible. However, the relationship between synaptic saturation and inducible LTP may not be linear (see chapter V, section 5 for a more complete explanation) so this remains a possibility. Spatial learning deficits are also observed in these mice.

In the light of data showing that tyrosine kinase inhibitors prevent the induction of LTP, a systematic survey of four tyrosine kinase mutants (*fyn*, *src*, *yes*, and *abl*) was carried out. Only *fyn* homozygous mutant mice exhibited a memory impairment phenotype (ref. 118). This consisted of a 50% reduction in evoked LTP under strong tetanic stimulation whereas other electrophysiological parameters appeared normal. Spatial learning in a swim maze appeared almost completely absent in mutant mice although they do seem to exhibit some as yet unexplained deficit in the visible

platform test (a test of visual, motivational, or motor capacities), especially in the early stages of training.

Perhaps the most extensively studied knock-out mouse line, in terms of memory phenotype, is one which lacks the gene encoding the α-calcium/calmodulin-dependent kinase II (refs. 119 and 120). This highly abundant protein is localised in the post-synaptic densities of the hippocampus and cortex neurons and is thought to play an important role in the phosphorylation events that immediately follow LTP induction. In most cases, mutant mice show a severe deficiency in hippocampus LTP induction and are also poor performers in swim maze experiments. However, several problems complicate the interpretation of these results (refs. 121-124). Firstly, other experiments have shown that mutant mice are abnormal in behavioural tests designed to examine fear and aggression; and, moreover, the phenotypes observed in some behavioural tests do not follow an expected progression in severity relative to the mutant gene's dosage. Secondly, the mutant mice suffer from 'profound hyperexcitability' resulting in frequent epileptic seizures. Finally, the study of certain electrophysiological constants in these mice revealed unexpected results. PPF is decreased, indicating a presynaptic role for the protein, whereas PTP (post-tetanic potentiation) is actually increased.

Homologous recombination resulting in the loss of the gene encoding the type I adenylyl cyclase (ref. 125) reduces the cAMP production activity by only 40-60% in the hippocampus (indicating that other isoforms of this enzyme exist). Nevertheless, compensation by these other proteins is incomplete because the mice exhibit both LTP induction and spatial learning deficits.

The deletion of one of the four cAMP-dependent protein kinase A regulatory subunits (Rlβ) results in deficits in LTD induction but leaves LTP unaffected, demonstrating the independent nature of these two forms of synaptic excitability modification (ref. 126).

The cAMP-responsive element-binding (CREB) protein is a transcription factor that may affect the transcription of genes responsible for the consolidation of LTP at the synapse. The absence of this protein in the brain (ref. 127) does not affect standard electrophysiological parameters but prevents the formation of long-term memories such that one-trial-a-day swim maze learning is severely disrupted, whereas intensive training over one day does not clearly distinguish between mutant and wild-type mice (this test does not require the long-term storage of information and is probably achieved in a hippocampus-independent fashion). Electrophysiological tests show that at 10 mins after tetanization, mutant hippocampus LTP is detectably attenuated.

NCAM is a cell adhesion molecule which is found, in adult mice, principally in the brain. The consequences of the loss of this gene are that the size of the brain overall, and the olfactory bulbs in particular, is decreased in comparison with wild-type mice. In addition these mutant mice do not perform well in spatial learning tests (ref. 128).

Brain-derived neurotrophic factor (BDNF) is a secreted protein that has been shown to be responsible for the increased survival of explanted neurons in culture. Its expression pattern, which is primarily hippocampal, suggested that it may play a role in the functioning of this region of the brain: indeed, there is some evidence that neurotrophic factors can be secreted from neurons in an activity-dependent manner. Homozygote nulls for this gene do not survive for more than four weeks after birth (ref. 129). Comparative electrophysiology performed on hippocampal slices derived from wild-type, heterozygote, and homozygote mice revealed a novel form of LTP disruption. In mice with either one or both mutant alleles LTP was significantly harder to achieve (in terms of the number of successful experimental attempts) than in wild-type, but was apparently normal when obtained. It could be possible that one role of BDNF is to maintain neurons in a physiological state in which they can become potentiated. Because the homozygous lethal phenotype is affected by gene dosage, it appears that it is independent of the LTP deficit.

Finally, examination of the learning phenotypes of two genes intimately associated with neurodegenerative diseases, the amyloid precursor protein and the prion protein, has shown that both possess some impairments (refs. 130 and 131). APP mutants exhibit deficits of learning in the swim maze assay and PrP mutants appear to have defective LTP. The undefined normal functions of these two proteins makes precise experimental design and assessment of results difficult.

1.17: Some difficulties may exist in the interpretation of knock-out phenotypes in the context of hippocampal LTP and memory

It is clear that the application of knock-out strategies to the analysis of LTP has provided much useful data. However, the interpretations of the mutant phenotypes described above are still open to some criticism (ref. 132). Some question whether the phenotype observed in the adult is a product of the absence of a particular gene in the neurobiological system under study at that time, or in the developmental processes that gave rise to the brain. An anatomical malformation exists in the *fyn* mutant hippocampus; the granule cells of the dentate gyrus and pyramidal cells of the CA3 region have a concertinaed appearance which probably originates from the overabundance of neurons in this region. Therefore, processes which govern the

division or programmed cell-death of these cells must be affected during hippocampal development by the absence of the *fyn* gene product. Can the LTP deficit observed in these mice genuinely be attributed to the lack of the gene product in the functioning adult system or is the morphological disruption to the hippocampus the root cause? 'Rescue' experiments (involving the reintroduction of the deleted gene during adulthood) should differentiate between the two possible origins of the phenotype. Using such an approach, provisional evidence for the ability to separate the morphological phenotype from the behavioural phenotype in the case of the *fyn* mutation has been reported ('Neuroscience' 1995, abstract).

The second major criticism is the widespread nature of the mutation; the gene is disrupted in all cells throughout the body. This means that the phenotype might not be solely due to the absence of the gene product in the hippocampus (the site of the processes under study), but might be as a result of other brain or body defects. The disruption of the CAMK II gene might be such an example. Some behavioural tests designed to assay the function of the hippocampus with respect to its role in spatial learning could be subject to the effects of the mutation in other cognitive systems. For example, the swim maze test requires not only competence in the learning of the location of a platform, but also requires the correct functioning of the visual and motor control systems not to mention a host of other more subtle behavioural properties such as attention and reward comprehension. The CAMK II mice exhibit a wide range of neurological abnormalities which may interfere with or supersede the (hippocampal) cognitive functions required to complete a swim maze test successfully. This kind of criticism is only applicable in cases where the phenotypes are interpreted solely in the context of hippocampal function.

Anther criticism levelled against the published data has been whether the cognitive phenotype of mice would be different if the mutation was in different genetic backgrounds. The contribution of the genetic background can result in the compensation or exacerbation of the mutant phenotype because of the presence of modifier alleles.

The application of a wide selection of control experiments (such as the 'visible platform', 'visual discrimination' and 'transfer' tests) are also a critical part of the correct assessment of the neurological defect present. The direct electrophysiological study of hippocampal slices enables a more specific comprehension of the mutation phenotype because the fibre pathways recorded are separate from the rest of the CNS.

1.18: Hippocampus-specific genes as tools for the precise transgenic manipulation of memory processes

In the light of these interpretational difficulties, it has been a long-term goal of those involved in this type of study to be able to achieve a more concrete analysis of the role of a gene in hippocampus function by the application of more refined techniques. To overcome the drawbacks of a simple knock-out strategy, a system of mutagenesis is required which not only restricts the genetic lesion to the tissue of interest, but which is also capable of being initiated at a set point in the mouse's life. Hence, the temporal and spatial control of a mutation is the key to the unambiguous interpretation of the resulting neurological phenotypes.

In our group, one line of research has focused on the identification of genes which are restricted in their expression to the hippocampus. Such transcripts could prove invaluable in the analysis of this brain region's function. Firstly, the role of such a gene (with its specific localisation) might be linked to the specific function of the hippocampus. Assuming that this brain region performs a distinct cognitive role, it could be hypothesised that it would require a distinct set of molecular components. Secondly, (and more importantly with respect to the problems described above) the genetic elements which dictate the expression pattern of such a gene, such as the promoter and enhancer regions, could be utilised as a means to express transgenes in the same location. Transgenes under the control of such genetic elements could be designed to perturb the normal functioning of neurons in a spatially specific fashion. It can be appreciated that a host of potential experiments based upon such a genetic tool exist and some of these are outlined below.

Modified forms of proteins which are known to play a role in LTP could be expressed in the hippocampus and their effects ascertained. Candidates for this type of study would include the glutamate receptors, kinases, phosphatases, signal cascade constituents, ion channels, transcription factors, neurotrophic factors and their receptors, amongst others. Some mutations might act in a dominant negative fashion, with effects similar to a knock-out, others would be gain-of-function mutations resulting in different phenotypes.

One approach employed in other biological systems is the expression of a toxic gene product in a specified cell-type or organ. Examples of toxins used include ricin and the product of the thymidine kinase gene. Thymidine kinase converts the anti-herpetic drug, ganciclovir, into a cytotoxic product (ref. 133). Therefore, by expressing the thymidine kinase gene in a restricted region of the body and applying the drug at a specified time, it is possible to ablate that tissue in a controlled manner. Lesioning the

hippocampus in this way would be much more precise and complete than the surgical lesions described earlier in this chapter, and would not involve any extrahippocampal damage. In addition, specific subregions within the hippocampus could be targeted individually if appropriate promoters could be isolated. One problem with this approach is that the action of this kinase gene may require the cells expressing it to be actively dividing before the cytotoxic effects manifest themselves. Adult brain neurons, however, are in a terminally differentiated state.

A hippocampus-specific promoter could be used as a means to 'rescue' hippocampus deficits present in knock-out mice by the reintroduction of the deleted gene to the hippocampus. A restoration of LTP or swim maze performance in such an experiment would indicate that the loss of function of this gene in the hippocampus was the cause of the deficit, and not deficits in other brain regions.

1.19: Novel transgenic techniques could employ hippocampus promoters

Several technological advances which have emerged since the inception of this project could be combined with a hippocampus expression-driving promoter. The cre recombinase derived from the P1 bacteriophage acts to excise any DNA sequence bracketed by specific palindromic sequences termed lox P sites. By placing these sites either side of a stretch of DNA and expressing the recombinase in a tissue-specific manner then efficient deletion of the intervening DNA can be achieved solely in that tissue (ref. 134). Tissue-specific knockouts can therefore be performed if a suitable promoter is available: the gene to be disrupted is surrounded by lox P sites ('floxed') by one round of transgenic intervention and the resulting mice crossed with others expressing the cre recombinase in the specific tissue of interest (obtained through a second round of transgenesis). It can be seen that it will be possible in the future simply to take previously created mouse lines which express the recombinase in the desired tissue (or, in a restricted developmental stage) and cross them with the experimental mouse line possessing the lox P sites in a suitable position. Refinements to this protocol have centred on making the cre recombinase inducible. One group have placed the recombinase under the control of an interferon-responsive promoter; at the required time-point this promoter could be exogenously activated permitting the cre-mediated recombination to occur (ref. 135). Another group have created a fusion protein linking the recombinase with the ligand-binding region of the oestrogen receptor. This protein was expressed in cells which possessed no endogenous receptor (ref. 136). Upon treatment with oestrogen, the protein was activated and transported to the nucleus where it could carry out its recombinase activity. If a similar system is to be useful *in vivo* it should be inducible by a non-

physiological signal; perhaps, a xenobiotic and its receptor could be adapted for this purpose.

Another biological system presents itself as a possible tool for the analysis of brain function in a transgenic context. The product of the tTA gene is a modified transcription factor which upregulates expression (by virtue of its VP16 transactivation domain) of genes which possess its binding-site motif (tetO) in their promoters (ref. 137). This upregulation is prevented by the antibiotic, tetracycline, which binds to the factor and prevents DNA-binding. Both tTA and 'X' transgenes, each under the control of promoters possessing the tet-responsive site, could be introduced into a line of mice which have been made null for the endogenous gene 'X'. In this way, expression of the tTA and 'X' genes would be under the control of the tTA gene product. At a defined time-point, tetracycline could be administered to the mice which would result in the cessation of tTA and 'X' expression. In essence, this procedure is an inducible knockout, with respect to gene 'X', and could be used to distinguish between the developmental and functional factors which contribute to the null phenotype.

In summary, the above procedures would allow either the spatial or temporal control of a transgenic manipulation; it still remains to be seen if both of these constraints can be combined in a single intervention.

1.20: The existence of hippocampus-specific gene expression

Possession of hippocampus-specific genes would only be useful for transgenic approaches if they were expressed above a certain level necessary for the transgene product's presence to register an effect on the tissue in question. Even though this will vary according to the function of the transgene, there are still minimum required levels. Genes restricted in expression to the brain are numerous and well-characterised; for example the promoters of 'neural-specific enolase' and 'tyrosine hydroxylase' have been frequently used to direct the expression of transgenes to the CNS or a subregion thereof, respectively.

As mentioned at the beginning of this chapter, the brain requires the utilisation of a disproportionate number of genes encoded in the genome. Current estimates of genome size in humans fall within the range of 50,000 to 100,000 genes (reviewed in ref. 138). It has been difficult to ascertain an exact figure for the number of genes expressed in the brain as the results from the principal technique, that of cDNA/mRNA or cDNA/genomic DNA reassociation kinetics, show wide variability between groups (refs. 139-142). One key point is that, of the genes expressed in the

brain, up to 50% may be employed solely in that tissue. The general trends observed point to the fact that the brain has between two to six times the transcriptional complexity of other tissues such as liver or kidney. This complexity is not due to the combination of sub-regional expression profiles. Dissection and analysis of brain regions such as cerebellum, hypothalamus, hippocampus, and cortex shows that, individually, they have the same order of transcriptional complexity as the brain as a whole (there is some evidence for the hippocampus being marginally less complex than the other regions). Between brain regions, the difference in the identity of genes expressed appears less than 20%. The authors of refs. 141 and 142, using reassociation rate curves, go some way in classifying gene expression in the brain. In essence, there are a small number of genes whose expression is at a very high level, a slightly larger set with lower expression levels, and a very large set of genes whose expression is seen at an extremely low level. Milner and Sutcliffe (ref. 143), through analysis of 191 clones picked randomly from a rat brain cDNA library, tried to assign rules about the type of gene and its expression level and distribution. About 18% showed expression in brain, liver and kidney. 26% showed expression in the brain and liver or brain and kidney. 30% showed expression limited to the brain and 26% were undetectable at Northern sensitivity. The abundance levels for each clone were also quantified and used to estimate that the brain expresses 2,000-3,000 genes at an abundance of 0.01% or more of total. This allowed a prediction of 30,000 genes expressed in the brain to be made. Another feature noted was that as the rarity of expression or the brain specificity increased, so did the average transcript length. These results and others seem to suggest that the higher the expression level of a gene, the more widespread its expression profile. Any attempt to isolate a brain region-specific gene would have to be sensitive enough to detect genes that are expressed at a low level.

1.21: Aims of the experiments described in this thesis

The aim of the work described in the following chapters was the isolation of hippocampus-specific genes.

The techniques that were used for this purpose can be broken down into two groups; degenerate PCR of candidate gene families and subtractive hybridisation. In both cases a progression of modifications was adopted in response to technical problems or to increase sensitivity. Both of the approaches have benefits and disadvantages at the theoretical and experimental levels and these will be discussed where relevant and as a summary in Chapter VI. The approach described in Chapter V is based on the assumption that some hippocampus-specific gene expression may be activity-

dependent, and not present in the basal-activity hippocampus. The subtraction outlined in this chapter also attempted to examine the long-terrm gene expression changes associated with an LTP-like stimulation.

Because of the varied nature of the approaches used in this thesis, it will be necessary to introduce them in more detail where they occur. Chapter II contains an introduction to the degenerate PCR amplification of gene families, Chapter III contains an introduction to differential screening/subtraction and Chapter V describes published attempts to identify activity-dependent neuronal gene expression.

Chapter II

Candidate Gene Family Approach

2.1: Rationale for PCR approach

Many genes, if not the majority, can be placed into distinct groups on the basis of their sequences. The DNA complement of eukaryotes has been subjected, through time, to multiple rounds of chromosomal duplication and reverse transcriptional insertion events (known as retroposition; ref. 1) that have resulted in the scattering of multiple copies of ancestral genes (or domains thereof) throughout the genome. These copies have since undergone mutational changes and concomitant evolutionary selection. Related genes in different regions of the genome diverge at the nucleic acid level as a result of mutation and selection such that the proteins they encode differ in precise functional specificity, if not in overall function. Additionally, the elements controlling the expression of the genes undergo more radical shifts such that different members of a burgeoning gene family might be restricted in their expression to particular tissue types, developmental stages, or physiological conditions. Combined, these changes permit both independent operation and the specific control of a tissue or organ (for example endocrine control of the heart by adrenal gland secretion of adrenaline). The result, in general terms, is that this increase in genetic complexity has permitted an increase in the organisational complexity of higher eukaryotes.

As discussed in the introduction, one aim of the work described in this thesis is to identify regulatory elements that direct hippocampus-specific gene expression. The remainder of this chapter concerns one route to the identification of such elements: the use of degenerate PCR in the search for members of gene families which are selectively expressed in rat hippocampus. It was reasoned that, among the members of the selected gene families, there might exist some specifically expressed in the hippocampus, meeting the particular operational requirements of this brain region.

2.2: PCR technique

The polymerase chain reaction (PCR: refs. 2-5) is the most direct technique for studying multi-gene families. By designing two oligonucleotide primers which are complementary to two regions of sequence bracketing the DNA stretch of interest, the

intervening region can be exponentionally amplified such that direct cloning and analysis is possible.

The precise details of how the PCR technique was applied to the study of candidate gene families is described in the Materials and Methods chapter. Factors affecting the design of the PCR primers are also discussed.

2.3: Application to multi-gene family amplification: Degenerate PCR

PCR has been used in many ways; cloning, mapping, diagnostics, and genotyping among others. The first direction taken in this project was the cloning of members of defined multi-gene families. Between any two members of such a gene family there are regions of conserved amino acid sequence. These regions have been conserved because they define functional domains or structures in the complete protein (for example, a kinase domain in a receptor or a nucleotide-binding pocket in a G-protein). Because this selection for conservation is at the amino acid level and the genetic code is redundant, the PCR primers must be designed to take into account the decreased conservation at the nucleic acid level. Therefore, the primers are synthesized such that the majority of the coding variants capable of producing the relevant amino acid motif are represented in the pool. This mixture of primer variants is known as a 'degenerate primer'. A balance has to be struck to ensure that the degeneracy is sufficient to amplify all possible gene family members but low enough to avoid indiscriminate priming. Often, where any one of the bases A, C, T, or G could be used in a codon, the nucleotide Inosine is specified to prevent excessive degeneracy (inosine can potentially hydrogen bond with all bases although it has a preference for G and A). This excessive degeneracy often occurs at the third position in a codon where most 'wobble' lies. This is especially true for the amino acids leucine, arginine, and serine which can each be encoded by any one of six codons. To reduce degeneracy further, reference can be made to data showing the the relative codon usage specifying a particular amino acid in the organism being studied (ref. 6).

Rat hippocampus cDNA was used as a template in PCR reactions designed to find members of four gene families which were selected for their apparent large sizes and roles in neuronal function. They were:-

1) G protein-coupled receptors
2) Metabotropic glutamate receptors.
3) G protein α subunits
4) Protein tyrosine phosphatases.

2.4: Introduction to G protein-coupled receptors

The first multi-gene family studied by the degenerate PCR technique was the G protein-coupled receptor (GPCR) family. The proteins encoded by these genes have seven hydrophobic regions which function as transmembrane domains (TM domains) to anchor them in the cytoplasmic membrane and to transduce signals across the lipid bilayer (refs. 7 and 8). Extracellular signals are converted into a structural conformation change which, in turn, stimulates members of the heterotrimeric G protein family to become active, setting in motion a chain of secondary messenger events evoking suitable cellular responses.

To date, well over one hundred members of this family have been cloned from numerous species ranging from bacteria to humans (ref. 9). The GPCR signal transduction paradigm is found in many physiological systems, for example, photoreception, gustation, olfaction, and the more standard ligand-/hormone-/neurotransmitter-receptor interactions.

The prototypical seven transmembrane domain receptor is the photoresponsive bacteriorhodopsin (BR). The precise three-dimensional arrangement of the transmembrane domains of this protein in the cell membrane has been elucidated (refs. 10-12). The realisation that bovine rhodopsin (refs. 13 and 14), human rhodopsins, and the β adrenergic receptor had related structures prompted investigation into the GPCR multi-gene family. Nucleic acid homologies are not easily detected between bacteriorhodopsin and other members of the family. Pardo et al. (ref 15) have rectified this confusion by non-sequential sequence comparison between the transmembrane domains of BR and those of other GPCRs. They found that domains II to VII of BR showed homologies with domains 4, 5, 6, 1, 2, and 3 (in that order) of other GPCRs. Thus, the transition from prokaryotic to eukaryotic organisms seems to have coincided with exon-shuffling events resulting in the present-day arrangement of TM domains in higher organisms. Helical-wheel analysis of the alpha-helical TM domains has revealed amphipathic amino-acid residue distribution such that a model of the packing order of the domains in the membrane could be made (ref. 16). Hydrophobic (lipophilic) residues lie on the outside, whereas inside, the residues are primarily hydrophilic, forming a pocket which is involved in ligand binding. This model fits well with the X-ray structure analysis of BR.

Fig. 2.1 shows a diagram of a typical GPCR upon which key residues involved in the functioning of the receptor have been labelled. With respect to the PCR approach to clone GPCRs, there a no large regions of sequence homology between all members of the family (ref 9); this is because different genes have been subjected to different

Figure 2.1 Diagram of a typical member of the G protein-coupled receptor family. Highlighted are the seven transmembrane domains with some of their conserved residues. Arrows represent regions of conserved amino acid sequence which acted as a basis for the design of degenerate PCR primers. Also featured are the N-terminal extracellular glycosylation, the extracellular disulphide bridge linking loops one and two, and the C-terminal palmitoylation.

Figure 2.2 Diagram of a typical member of the metabotropic glutamate receptor family. In comparison to the GPCR proteins there is a longer N-terminal extracellular domain and the interaction with the G-protein complex is probably at a different position.

Figure 2.1

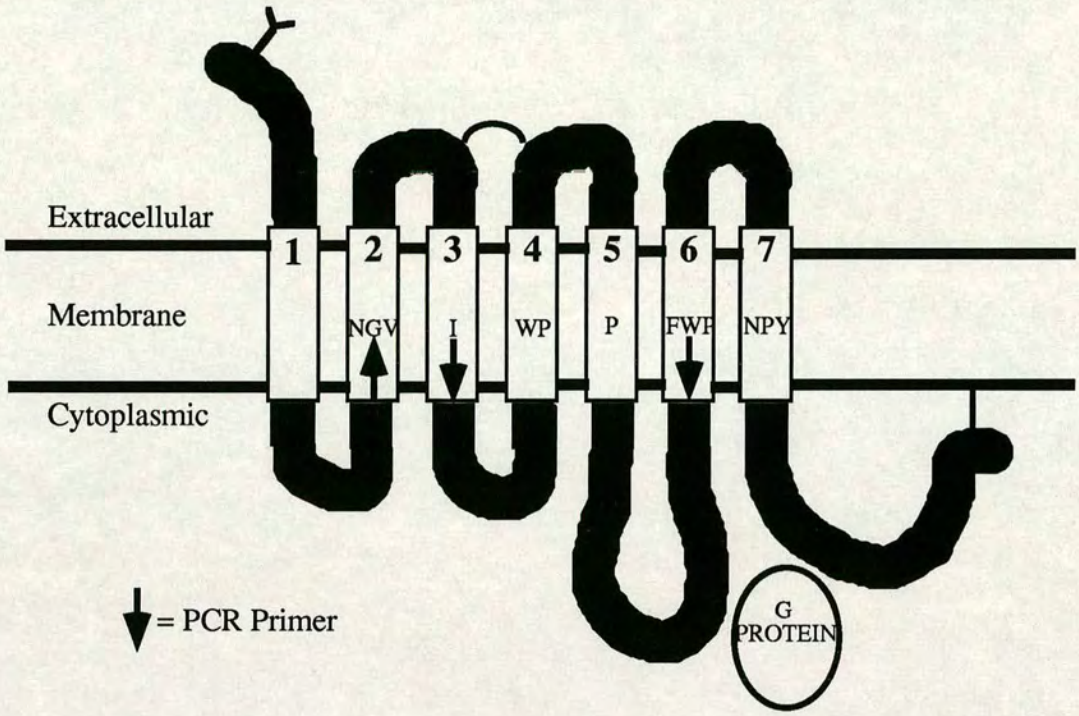
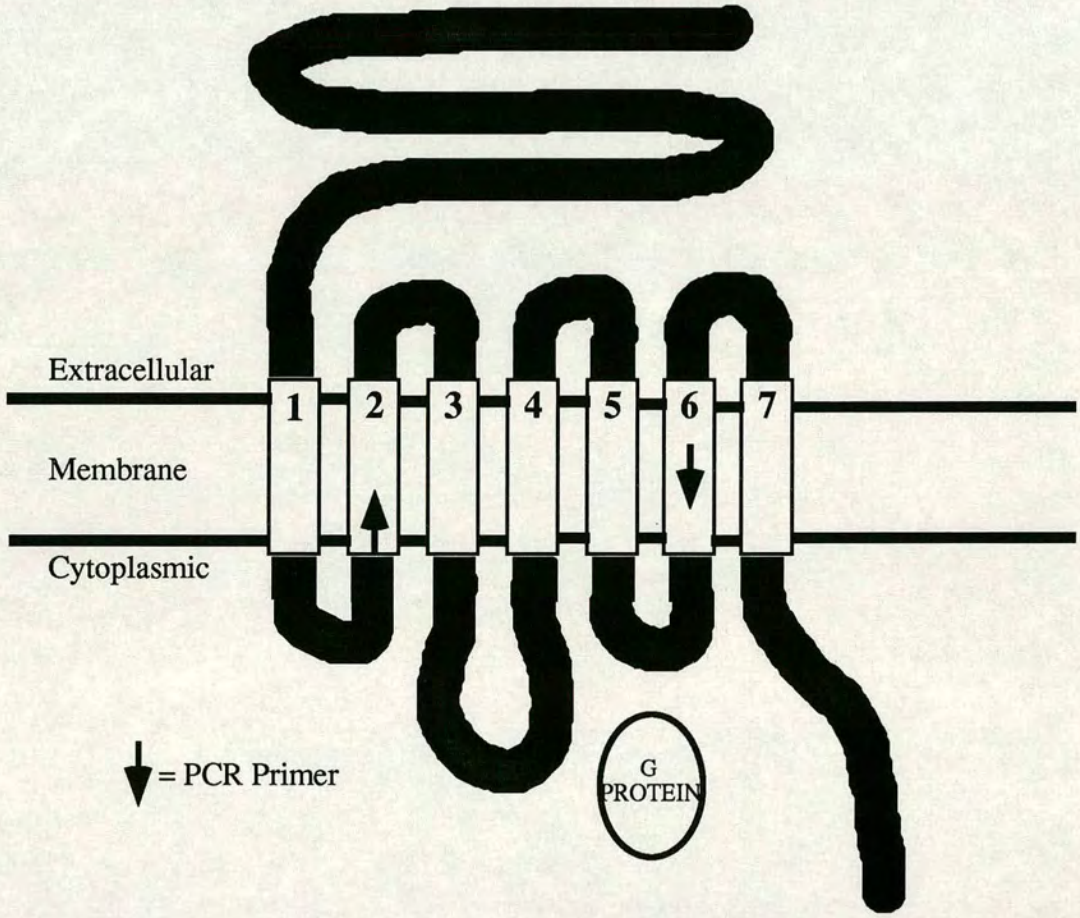


Figure 2.2



lengths of evolutionary drift after successive gene duplication events. Hence, subfamilies of the GPCR family have emerged with higher sequence homology among themselves than with the family as a whole: this will be discussed later in the context of primer design. Despite this, certain key residues are to be found in most members of the family described to date and these are nearly always associated with the transmembrane domains. It is presumed that the role of these TM domains is critical for function, explaining their increased conservation. Proline residues, probably involved in kinking the alpha-helices to create a suitable ligand-binding pocket, are to be found in TM domains 4, 5, 6, and 7. TM 2 contains conserved glycine, asparagine, and valine amino acids. TMs 3 and 4 contain an isoleucine and a tryptophan amino acid, respectively. Phenylalanine and tryptophan residues are conserved in TM 6 and, finally, TM 7 possesses conserved asparagine and tyrosine residues.

Cysteine residues contained within the first and second extracellular loops have been proposed to form a disulphide bridge which may contribute to the structure of the receptor. Another set of cysteines are often found in the intracellular carboxy-terminus of the receptor and have been shown to be modified by palmitoylation. This has the effect of anchoring the tail to the membrane; seemingly a critical requirement in the case of the β2 adrenergic receptor, which becomes hyperphosphorylated and desensitised upon mutagenesis of the relevant cysteine residue (ref. 17).

The large third intracellular loop and the carboxy-terminus appear to contain key regions of phosphorylation on the receptor (ref. 18). Persistent agonist stimulation of some receptors such as rhodopsin and the β2 adrenergic receptor leads to receptor desensitisation. This process requires protein kinase A (PKA) or specific GPCR kinases to phosphorylate serine/threonine residues on the two regions, permitting the binding of arrestin/arrestin-related proteins which prevent receptor-coupling to G proteins (ref. 19). At least seven GPCR kinases have been cloned from a variety of species (ref 20), the most studied of which is the β adrenergic receptor kinase (BARK).

The amino-terminus of a typical GPCR possesses much N-linked glycosylation which, when removed by site-directed mutagenesis, as in the case of the β2 adrenergic receptor (ref. 21), or by glycosylation inhibitors, as in the case of the m1 muscarinic acetylcholine receptor (ref. 22), results in a decrease in the expression levels of the protein but not the agonist affinity: this is probably due to incorrect intracellular trafficking.

As mentioned earlier, the site of agonist binding in GPCRs seems to be the hydrophilic cleft created by the bundling together of the transmembrane domains. Analysis of the retinol moiety in the rhodopsin complex shows that it occupies this region. Point mutation and chimaeric protein experiments have shown that an aspartate residue in the third transmembrane domain is vital for agonist/antagonist binding in the case of the adrenergic (refs. 23 and 24) and m1 muscarinic acetylcholine receptors (ref. 25). Serine residues in the fifth transmembrane domain are known to be involved in hydrogen bonding to the aromatic hydroxyl groups of catecholamine agonists.

Coupling of the activated receptor to the appropriate G protein has been studied by various approaches. Luttrel et al. (ref. 26) showed, in cell culture, that by the addition of a synthetic peptide identical to the α1b adrenergic receptor's third intracellular loop, all coupling from that receptor and the related α1c receptor was blocked whereas other GPCRs remained unaffected. A chimaeric β2 adrenergic receptor was created which possessed a third intracellular loop derived from the α2 adrenergic receptor (ref. 27). This behaved like an α2 receptor by coupling to the G protein $G_{\alpha}i$ instead of the usual $G_{\alpha}s$. Site-directed mutagenesis experiments have shown that many conserved residues present in the intracellular loops have a role in the receptor-G protein coupling process. O'Dowd et al. (ref. 28) showed that the membrane proximal regions of the third intracellular loop and a proline in the second loop were critical for β adrenergic coupling. Others have reported that the aspartate in loop two and the lysine in loop three are also important for this coupling.

Receptors of other types have been shown to be proto-oncogenes. This does not seem to be so evident in the case of GPCRs; perhaps because of the fact that they are not directly involved in phosphorylation events. However, the *mas* proto-oncogene is a member of the GPCR family (ref. 29) and site-directed mutagenesis of three residues on the third intracellular loop of the α1b adrenergic receptor causes it to become deregulated such that injection of cells expressing this mutant form into nude mice results in the formation of tumours (ref. 30). Spontaneous mutations in GPCRs are causative of a number of diseases such as congenital nephrogenetic diabetes (vasopressin receptor), hyperfunctioning thyroid adenomas (thyrotropin receptor), familial male precocious puberty (luteinizing hormone receptor), and night blindness diseases (rhodopsin). In addition, different allelic forms of the melanocyte-stimulating hormone receptor are responsible for different coat colour phenotypes in a variety of mammalian species.

In summary, the diverse roles undertaken by the many members of this gene family (particularly in the central nervous system) led to speculation that there might be

found members restricted in expression to the hippocampus. These might be involved in the memory formation processes of this brain region.

2.5: GPCR primer design

The first set of primers designed to amplify members of this family were a gift from Dr. S. Morley (Centre for Genome Research) and were based upon those published by Libert et al. (ref. 31). By analysing the amino acid sequences of six GPCRs (β1 adrenergic, β2 adrenergic, α2 adrenergic, 5-HT 1a, muscarinic acetylcholine 1, and substance K receptors) they selected two conserved regions; one associated with transmembrane domain 3 and the other with transmembrane domain 6. To avoid excess degeneracy in the primers, not all possible codon variations were included for a particular amino acid; merely those most frequently occurring. Inosine residues were also included to reduce degeneracy. Also, HindIII and SalI sites were included in the primer sequences in order to facilitate cloning into plasmid vectors.

The second pair of primers directed against this gene family were biased in sequence so that the products would contain members of the neuro-peptide/bio-active peptide receptor subset of GPCRs. Those members which formed the basis for the design of the primers included neurotensin, neurokinin A/substance K, substance P, bradykinin, bombesin, and angiotensin II receptors. The 5' primer was directed at a region of conserved sequence located in the second transmembrane domain whereas the 3' primer utilised the previously described region of homology in domain VI; albeit with a slightly different nucleic acid composition. Restriction sites for the enzymes ClaI and XbaI were included in the primers.

The precise primer sequences are listed in table 2a. As with all of the primers created for this approach, the following rationale was used: the conserved amino acid motif was chosen from a comparison of family members and then all possible codon variants encoding each amino acid were listed. In order to decrease the resulting degeneracy, codon usage in previously cloned family members was analysed in order to discount rare codon variants. Although this is a necessary step in the design of a competent primer, there is always the danger of a 'circular argument' ensuing; that is, by basing the primers so very closely on sequences already known, slightly divergent members might escape detection. This is especially true if the previously cloned members were obtained by the use of PCR primers designed in a similar fashion.
Using the equation described in the Materials and Methods chapter, it is possible to estimate the melting and annealing temperatures for the primers. These are listed in the figure legends.

Table 2 a Table listing the degenerate PCR primers used to amplify members of the GPCR family and the conserved amino acid sequences from which they were derived. Conserved amino acid residues are shown above the degenerate nucleic acid sequence of the primer. Also shown (where included) are the 5' restriction sites which were intended to aid cloning of amplified products. For each primer an approximate annealing temperature can be calculated using the equation described in the Materials and Methods chapter. Because these primers are degenerate, there are a range of values. The GPCR primers are longer than the other listed PCR primers which may result in the annealing figures being an overestimation. Primer 1; 69-81°C, primer 2; 81-87°C, primer 3, 55-69°C, and primer 4; 61-81°C.

Table 2a

Primer	Sequence												
	Amino Acid (N-C)	SAL I	L	C	V	I	A	L	D	R	Y		
1	Nucleic Acid	5'_GTCGAC	CTG	TG(C/T)	G(C/T)(C/G)	AT(C/T)	GCI	IT(G/T)	GA(C/T)	(C/A)G(C/G)	TAC_3'		
	Amino Acid (N-C)	_	F	I	L	C	W	L	P	F	F	I	HIND III
2	Nucleic Acid	3'_	AAG	(T/C)(G/A)(G/C)	IAG	ACG	ACC	GAC	GGG	A(A/T)G	A(A/T)G	(T/G)A	TTCGAA-3'
	Amino Acid (N-C)	XBA I	N/s	L	A	V/L	A	D/e	L/f				
3	Nucleic Acid	5'_GC TCTAGA	T(G/C)	A(G/A)(T/C)	CT(G/A)	GCI	(C/G)T(G/C/T)	GC(C/A/T)	GA(T/C/G)	(C/T)T_3'			
	Amino Acid (N-C)	_	F	A/v/f	L/I/v/f	C	W	L	P	Y/L	H/q/n	CLA I	
4	Nucleic Acid	3'_	AA(A/G)	(C/A)(A/G)(G/C)	IA(G/C)	AC(G/A)	ACC	(G/A)A(C/G/T)	GG(G/A)	(G/A)(T/A)(G/C/A)	(T/G)T	AGCTA CC_5'	

2.6: GPCR PCR results

Five slightly different approaches were used to amplify and clone members of the G protein-coupled receptor family from hippocampus cDNA. These were necessary because of the extent of degeneracy of the PCR primers used; this resulted in amplification products which contained only a small percentage of genuine GPCR species, the remainder comprising of misprimed artifacts. Another major obstacle was the inability to achieve large-scale cloning of the PCR products into plasmid vectors. In part, this was due to the small quantities of material obtained, but the failure to digest the terminal restriction sites efficiently represented the major cause. This is because certain restriction enzymes digest sites located at the ends of DNA strands poorly; SalI, the choice of Libert et al., being one of these. Therefore, a method which has been employed by some, but with little success here, is to blunt-end the PCR products with a suitable polymerase and ligate the products into extensive concatamers. In this way, restriction sites become flanked by long stretches of DNA, permitting their unhindered digestion (ref. 32). Invitrogen market a novel cloning system for PCR products; the 'TA Cloning System'. The operation of this kit relies on the observation that Taq polymerase adds a supplementary adenosine base overhang to the 3' end of many of the DNA strands that it synthesizes. Thus, a vector possessing 3' thymidine base overhangs is able to anneal and be ligated to these products. Because of the low efficiency of this process, a super-competent preparation of bacteria is used in the transformation step. This system of cloning was used in the majority of PCR cloning experiments described in the following pages.
The first strategy involved the use of an amplification program very similar to that used by Libert et al. Twenty-five cycles of the program:

93°C	1.5 min
55°C	2 min
72°C	4 min

were performed. An aliquot of the reaction product was subjected to a second round of amplification and then electrophoresed on an agarose gel. The resulting 520 bp band (the expected size-range for products of family members varies from 500-1000 bp) was cloned into the TA kit vector (pCR II). Of 5 insert-containing clones obtained, only one showed an extended reading frame from the primer sequence which encoded key residues consistent with it being a member of the GPCR family.

Clone (expt. 1)	GPCR Product
11	α1b adrenergic receptor (ref. 33)

The second cloning attempt differed by the lowering of the annealing temperature to 50°C in order to increase the possibility of amplifying members of the family less homologous to the primer sequences, albeit at the risk of decreasing the specificity of the reaction. The reaction products were electrophoresed on a low melting point agarose gel so that products within the expected size-range could be cut out and purified. The purified DNA was subjected to a further round of amplification prior to cloning. As expected, the number of non-specific products was high, but two members of the GPCR family were identified.

Clone (expt. 2)	GPCR Product
4	Dopamine d1 receptor (ref. 34)
16	Cannabinoid receptor (ref. 35)

The third strategy involved the creation of a pre-selected cDNA template. By using the 3' PCR primer instead of an oligo-dT primer in the reverse transcription of hippocampus mRNA, any cDNA which did not contain that sequence would not be present as a template for the PCR reaction. The subsequent PCR amplification further selects for GPCR members as there is a requirement for a site on the cDNA complementary to the 5' primer. This strategy proved to be very efficient in removing much of the background amplification products such that, out of 19 clones, 9 were genuine GPCRs. These are listed below.

Clone (expt. 3)	GPCR Product
1	Rat testis GPCR (ref. 36)
2	Fusion product*
8, 12, 18	Adenosine a1 receptor (ref. 37)
9	α1b Adrenergic receptor
13, 16	Dopamine d1 receptor
14	β2 Adrenergic receptor (ref. 38)

*The fusion product contained in clone 2 was comprised of sequences from a GPCR, 'R334' (ref. 39) and from the dopamine d1 receptor. The fusion event probably arose in the insert-to-vector ligation step.

To extend the range of possible GPCRs amplifiable, a new set of primers were employed (see section 2.5). These were devised with a sequence bias that predisposed them to amplify GPCRs of the neuro- and bio-active peptide receptor subfamily such as the cholecystokinin and bombesin receptors: the activities of which may have important roles in the modification of synaptic transmission. At first, a lower stringency reaction (55°C annealing temperature, two rounds of 30 cycle amplifications) was performed which yielded only 2/10 positive GPCR clones.

Clone (expt. 4)	GPCR Product
3	Rat testis GPCR
8	Vasoactive intestinal peptide receptor (ref. 40)

When the annealing temperature was increased to 59°C, only one clone out of 20 proved to be a genuine GPCR.

Clone (expt. 5)	GPCR Product
12	Endothelin 1 receptor (ref. 41)

The final method used was 'nested PCR'. PCR products from a reaction, in which the the PCR primers used were the newly-designed pair, acted as a template for a secondary round of amplification using the same 3' primer coupled with the old 5' primer. Because the old 5' primer is complementary to sequences downstream of the new 5' primer, only those primary products which were genuine GPCRs should be able to act as template for the secondary amplification. The term 'nested' is applied because the second set of primers acts internally to the first set. One problem with this particular application is the fact that, due to there being a bias in the primers' affinities for subsets of GPCRs, the combination of such primers might result in an actual decrease in the scope of transcripts amplifiable. Out of 20 clones initially analysed, 14 were GPCRs as listed below.

Clone (expt. 6)	GPCR Product
1, 2, 5, 12	Endothelin 1 receptor
4, 6, 8, 9, 10	α 1b Adrenergic receptor
7	R334 receptor
13	Adenosine a3 receptor (ref. 42)
14, 15	Dopamine d1 receptor
17	β2 Adrenergic receptor

A further 520 colonies were gridded, transferred onto nitrocellulose filters, and probed with a mixed probe corresponding to the above clones. Those colonies which failed to hybridise (and were, therefore, none of the above) were sequenced but no new GPCRs emerged.

This brought to an end the GPCR amplification attempts on hippocampus cDNA. In all, 10 members of this family were isolated, none of which were either novel or restricted to the hippocampus in expression.

2.7: Introduction to metabotropic glutamate receptors

The second candidate gene family which underwent degenerate PCR analysis was the metabotropic glutamate receptor family (ref. 43). Pharmacological experiments originally defined three types of glutamate receptors on the basis of antagonist binding specificities (Chapter 1); N-methyl-D-aspartate (NMDA), quisqualate/α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (Q/AMPA), and kainic acid (KA) receptors. Evidence for a fourth came from both the effects of another agonist, 2-amino-4-phosphonobutyrate (L-AP4), and the realisation that glutamate could induce secondary messenger production (refs. 44-46). These data suggested that unlike the ion channel operation of the first three, the new type required the heterotrimeric G protein family to elicit electrophysiological changes; hence the name metabotropic glutamate receptors.

The introductory chapter described the work which has been carried out to investigate the role of these receptors in the functioning of the brain, specifically in the processes of memory creation: this will not be discussed in any further detail here.

In the search for the genes encoding these receptors, two groups screened clones in a Xenopus oocyte expression system and isolated identical clones which encoded a protein with the correct functional characteristics, subsequently named mGluR1 (refs. 47 and 48). Analysis of the nucleotide sequence of this cDNA revealed that the protein it encoded was topologically similar to other members of the GPCR superfamily, in that it possessed seven transmembrane domains, but had little in the way of direct sequence homology (fig. 2.2). The N-terminal extracellular domain is much larger that that of other GPCRs and contains weak homology at the amino acid level to the membrane-bound form of the sea-urchin guanylate cyclase and the GluR B receptor. The carboxy-terminus region contains proline/glutamine- and glutamate/aspartate-rich stretches similar to those found in adrenergic and muscarinic acetylcholine receptors.

The following years have seen the cloning of seven additional members of this family from brain tissue. mGluRs 2, 3 and 4 were cloned by using mGluR1 cDNA as a probe in a low stringency screen of a brain cDNA library (ref. 49). mGluR5 was cloned using PCR primers directed against conserved amino acid regions in TM domains I and V to amplify members of the family from hippocampus cDNA (ref. 50). mGluR6 was cloned by another low stringency library screen, this time using a rat retinal cDNA library (ref. 51). mGluR7 was cloned independently by two groups. Okamato et al. (ref. 52) used PCR primers based on TM domains III and VI to amplify from brain cDNA whereas Saugstad et al. (ref. 53) used PCR products amplified from olfactory bulb cDNA (using primers against TM II and intracellular loop III) to screen an olfactory bulb cDNA library. From this latter screen, another novel mGluR (8) was cloned but its sequence has not yet been published.

The table below summarises the agonist specificities and the signal transduction pathways used for each of the eight mGluR subtypes known.

mGluR1	Quisqualate sensitive	IP_3/Calcium signal pathway
mGluR5	"	"
mGluR2	trans-ACPD sensitive	cAMP production pathway
mGluR3	"	"
mGluR8	"	"
mGluR4	L-AP4 sensitive	"
mGluR6	"	"
mGluR7	"	"

2.8: Metabotropic glutamate receptor primer design

Among the five members of this family that were originally published, the amino acid sequences were relatively highly conserved which made primer design more straightforward. As before, the regions chosen corresponded to the transmembrane domains (fig. 2.1). The precise sequences of the primers are listed in table 2b.

2.9: Metabotropic glutamate receptor PCR results

Using the primers described, PCR amplification was performed both on rat hippocampus cDNA and an aliquot of the rat hippocampus cDNA library (created by

Table 2 b Table listing the degenerate PCR primers used to amplify members of the mGluR family and the conserved amino acid sequences from which they were derived. Conserved amino acid residues are shown above the degenerate nucleic acid sequence of the primer. For each primer an approximate annealing temperature can be calculated using the equation described in the Materials and Methods chapter. Because these primers are degenerate, there are a range of values. Primer 1; 49-63°C, and primer 2; 49-51°C.

Table 2b

Primer	Sequence								
	Amino Acid (N-C)		S	G/S	R	E	L	S/C	Y
1	Nucleic Acid	5'_	TC(A/C/G)	(G/A)G(T/C)	(C/A)G(G/A)	GA(G/A)	CT(C/T/G)	(T/A)GC	TA_3'
	Amino Acid (N-C)		T	M	Y	T	T	C	
2	Nucleic Acid	3'_	TGG	TAC	ATG	TG(G/A/T)	TGG	ACG_5'	

J. O. Mason and M. Richardson, Centre for Genome Research). The program used was based upon that used for the amplification of GPCR family members.

95°C	5 min
93°C	1.5 min
52°C	2 min
72°C	4 min 30 cycles

Products at the expected size of approximately 520bp were observed from both amplifications. In addition, the cDNA-derived products showed bands at other sizes which were not removed by increasing the annealing temperature to 55°C. Of those aberrantly sized products sequenced, none showed any homology to the metabotropic glutamate receptors.

The correctly sized products were pooled and cloned using the 'TA Cloning' strategy due to the absence of restriction sites at the 5' ends of the primers. Of the seven clones initially sequenced, one was mGluR1 (clone 1), one was mGluR5 (clone 4), and five were mGluR3 (clones 3, 5, 8, 11 and 12). The remaining 12 clones were 'T-tracked'; a process of sequencing clones with respect to just one base so that a quick identification can be made. Eight clones corresponded to mGluR3, the rest were resequenced in full. Only one of these four (clone 10) represented a genuine member of the family; mGluR4.

Because mGluR3 was represented most highly in the products, ways of increasing the abundance of other members were sought. One method attempted relied on the fact that only mGluR3 contained a Taq I restriction site within the PCR product sequence. Therefore, the PCR products were cut with this enzyme and then reamplified prior to cloning. No different mGluRs were identified using this approach.

2.10: Introduction to heterotrimeric G protein α subunits

The logical follow-up to the study of two G protein-coupled receptor families was the heterotrimeric guanosine triphosphate-binding proteins themselves (refs. 54-60). Additional to their described roles in neural functioning, there are also examples of members which exhibit restricted tissue distribution: these factors suggested that they would be a good candidate for a degenerate PCR approach. The members of the GTP-binding protein superfamily, of which the α subunits are a subclass, mediate a wide range of cellular processes. They share the ability to carry out their various functions

only when in the activated, GTP-bound, state. Hydrolysis of the GTP molecule into GDP, results in the cessation of this activity. Therefore, this family represents a molecular switch for a variety of cellular processes. The scope of their activities extends to signal transduction (heterotrimeric GP α subunits and *ras* family), vesicular trafficking (RABs), proliferation/differentiation control (*ras* family) and protein synthesis regulation (elongation factors). As might be expected from molecules sharing such a function, regions of structural and sequence homology are found in common between all members.

Region G1 (A or P) contains the amino acid motif:-

.....G X_4 G K (S/T).....

where X is any residue. It is thought to be involved in the binding of the α and β phosphates of the GTP molecule in the active form.

Region G2 (B) contains a threonine residue which might chelate magnesium ions required for the GTP hydrolysis step.

Region G3 (C or G') contains the amino acid motif:-

.....D X_2 G Q.....

and is involved in the binding of the γ phosphate of the GTP molecule.

Region G4 (G) is defined by the amino acid motif:-

.....Z_4 (N/T) (K/Q) X D.....

This region is known to bind the guanine ring.

Region G5 (I or G") is characterised by the amino acid motif:-

.....T C A t D T.....

It is thought to play a role in the hydrolysis of the GTP molecule.

The α subunit of the G proteins forms a heterotrimeric complex with the β and γ subunits when in the GDP-coupled state. This complex associates with a G protein-

coupled receptor (the precise type depending on the subunit composition; ref. 61). The α subunit is made competent to exchange the GDP molecule for GTP by the structural changes in the receptor brought about by ligand interaction. This activation results in the dissociation of α and β/γ from each other and the receptor. The GTP-bound α subunit is then free to carry out the effector functions for which it is specific. Because one stimulated receptor can activate numerous G proteins, and these do not immediately hydrolyse their GTP cofactors, the original 'signal strength' of the ligand-receptor interaction can be amplified many-fold by the repeated triggering of downstream events, such as the production of secondary messengers. It must also be pointed out that the β/γ subunits exist in multiple forms (at least four in the case of β, and six in the case of γ). These all have particular expression patterns and specific combinatorial preferences within the complex. Recently, it has become apparent that the dissociated β/γ complex can also function as a downstream effector (refs. 62-65).

Much work has been undertaken to clone all α subunits and to define their localisations and precise functions. Early work centred on the susceptibility of certain physiological systems to pertussis toxin. This indicated that a receptor-initiated G protein pathway was involved. With the advent of molecular cloning techniques, the first α subunit was cloned ($G_{\alpha}s$; refs. 66 and 67). Subsequent isolation of new forms has relied primarily on the use of degenerate PCR, with primers directed against conserved sequence motifs (fig. 2.3).

Cholera toxin affects the $G_{\alpha}s$ and $G_{\alpha}t$ subunits by causing an ADP-ribosylation modification of an arginine residue (R201 in $G_{\alpha}s$). This results in a decrease in the activity of the subunit and also, a decreased affinity for the β/γ subunits.

Pertussis toxin also acts via ADP-ribosylation, but does so on a conserved cysteine residue present at the carboxy-terminus of those α subunits with a susceptibility to its effects. The result, a failure to interact with the relevant receptor, aided the assignment of function to this region of the protein.

Other regions of the α subunit have been subjected to alterations to assess their roles. N-terminal cleavage causes a failure to interact with β/γ subunits. The ability of $G_{\alpha}s$ to stimulate adenylyl cyclase falls by 90% when the amino acids L and R (positions 282 and 283) are changed into F and T (the corresponding residues in the adenylyl cyclase-inhibiting α subunit, $G_{\alpha}i$). This is notable as there are almost no other effects observed on the function of the protein.

Figure 2.3 Diagram of the conserved regions within G protein α subunits. The five functional domains, G1-G5, are shown (see text), two of which were used to design the degenerate PCR primers. Also featured are the insertions present in the $G_{\alpha}s$ subunit.

Figure 2.4 a The catalytic domain of the protein tyrosine phosphatases shown in diagrammatic form. Conserved amino acid sequence regions are highlighted in black; three of which were used to design the degenerate PCR primers. **b** Diagram illustrating the structures of the five principal classes of transmembrane PTPs (left) and five typical cytosolic PTPs (right). The diagonally hatched rectangles represent catalytic domains. Strongly stippled squares represent fibronectin type III (FN III) domains, cross-hatched circles represent Immunoglobulin-like domains (Ig), triangles represent MAM domains (see text), and the ellipse represents a carbonic anhydrase (CAH) domain. PTP 1C possesses 2 SH2 domains. PTP Meg1 possesses a cytoskeleton-interacting domain. PTP PEST possesses a domain causing its rapid degradation. PTP 61F contains a nuclear-localisation signal. PTP 1B contains a domain which targets it to the endoplasmic reticulum.

Figure 2.3

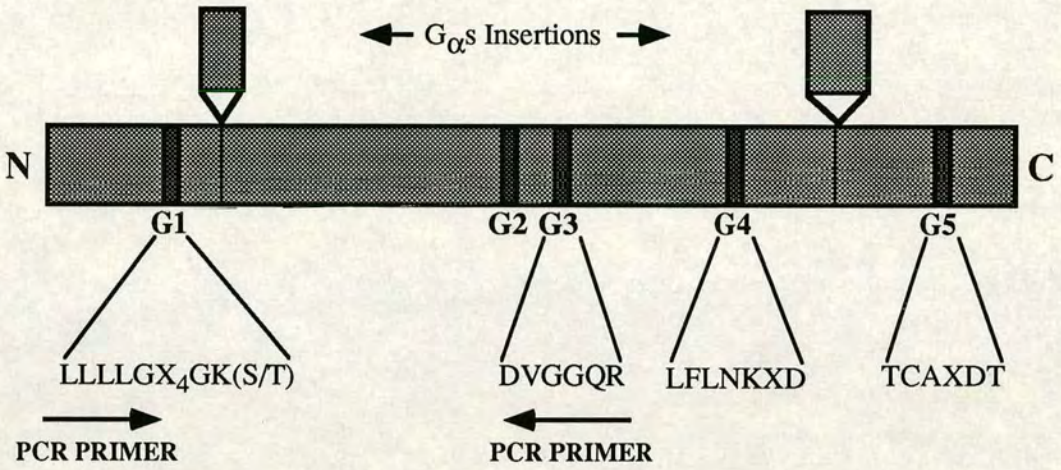


Figure 2.4a

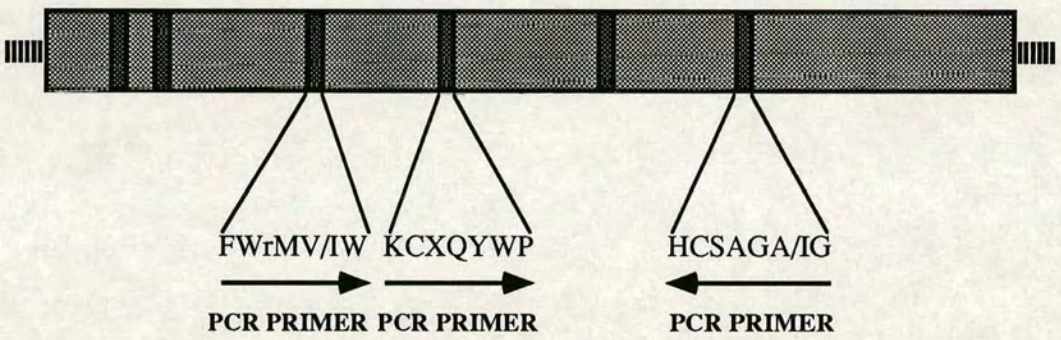
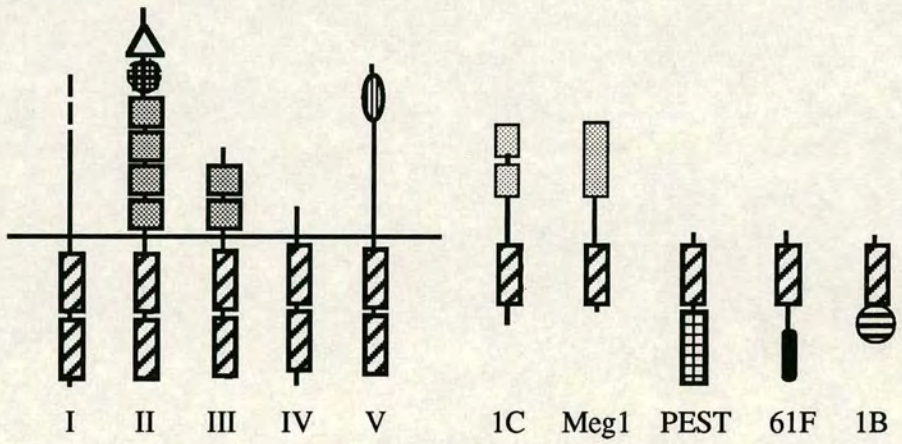


Figure 2.4b



To date, eighteen different α subunits have been cloned. Through sequence comparison, it has been possible to divide these into four groups. The following list arranges those published into their respective groups and also describes the roles that they play.

$G_{\alpha s}$ family

$G_{\alpha s}$ This α subunit typifies its group by coupling receptor activation to the stimulation of adenylyl cyclase (with a resultant rise in cAMP levels) and opening of certain calcium channels. The family is inhibited by cholera toxin, a process which is described below. There are at least four splice variants expressed in the brain (ref. 68).

$G_{\alpha olf}$ This closely related α subunit (88% amino acid identity) was originally identified as the olfactory tissue-specific G protein responsible for transducing odorant signals (see section 2.16; ref. 69).

$G_{\alpha i}$ family

$G_{\alpha i}$ 1, 2, and 3 are typified, like most members of this family, by their susceptibility to inhibition by pertussis toxin (refs. 70 and 71). They share 85% amino acid identity and are found at highest levels in the brain. Their downstream effects include inhibition of adenylyl cyclase, activation of phospholipases C and A, and the opening of potassium ion channels.

$G_{\alpha o}$ 1 and 2 also exist at high levels in neural tissue and have been seen to play a large role in growth cone functioning where they are activated (contrary to paradigm) by GAP-43 which is not a GPCR (ref. 72).

$G_{\alpha t}$ 1 and 2 are the 'transducins'; coupling the photo-activation of rhodopsin and cone opsin, respectively, to the activation of retinal phosphodiesterase (ref. 73).

$G_{\alpha z}$ (also known as $G_{\alpha x}$) is another mainly neuronal α subunit (refs. 74 and 75). Unlike to other members of this family, it is not affected by pertussis toxin. It possesses a non-canonical G1 domain with the 'GAGES' motif being replaced by 'GTSNS'.

Gustducin is a member of the $G_{\alpha i}$ group and is localised to the papillae of the tongue where it is thought to mediate at least part of the taste response (ref. 76).

$G_{\alpha q}$ family

$G_{\alpha}q$ and $G_{\alpha}11$, like the rest of the group, are insensitive to the effects of Pertussis toxin, and act by stimulating PLC (ref. 77). They contain the G1 motif 'GTGES'.
$G_{\alpha}14$ is expressed in a number of tissues but has not been well characterised (ref. 78).

$G_{\alpha}15$ and 16 are restricted to B- and T-cell haematopoietic lineages, respectively and contain the G1 motif 'GPGES' (ref. 80).

$G_{\alpha}12$ and 13 family

$G_{\alpha}12$ is expressed in the brain among other tissues whereas $G_{\alpha}13$ is entirely non-neurally located (ref. 81). They share a separate grouping by virtue of their sequence divergence. They are both pertussis toxin insensitive.

2.11: G protein α subunit primer design

Degenerate primers were created against conserved amino acid motifs in the G1 and G3 domains as illustrated in fig. 2.3. The primer sequences are listed in table 2c.

2.12: G protein α subunit cloning results

The G protein α subunit family of genes were easier to amplify than members of other gene families due to the highly conserved nature of the domains against which the primers were directed.
Hippocampus cDNA was taken through a PCR cycling program similar to those used before:-

94°C	30 sec	
49 °C	2.0 min	
72 °C	4.0 min	30 cycles

A clear doublet was seen when the PCR products were run out on an agarose gel. This agreed with the expected sizes of GP α products; approximately 550 bp for $G_{\alpha}s$-derived products (due to the presence of an additional intervening stretch of amino acids; fig. 2.3), and approximately 510 bp for other members of the family. The products were cloned using the TA vector system resulting in 37 colonies with insert-containing plasmids. Nine were sequenced, of which six encoded $G_{\alpha}q$ and three

Table 2 c Table listing the degenerate PCR primers used to amplify members of the α subunits of the G protein family and the conserved amino acid sequences from which they were derived. Conserved amino acid residues are shown above the degenerate nucleic acid sequence of the primer. Also shown (where included) are the 5' restriction sites which were intended to aid cloning of amplified products. For each primer an approximate annealing temperature can be calculated using the equation described in the Materials and Methods chapter. Because these primers are degenerate there are a range of values. Primer 1; 39-45°C, and primer 2; 52-57°C.

Table 2c

Primer	Sequence								
	Amino Acid (N-C)	XHO I	R/K	L	L	L	L	G	
1	Nucleic Acid	5'_GGGG CTCGAG	(A/C)A(G/C)	ITG	CTG	CTI	(C/T)T(G/T)	GG_3'	
	Amino Acid (N-C)		D	V	G	G	Q	R	XBA I
2	Nucleic Acid		CT(G/A)	CAI	CCI	CCG	GTC	(G/T)C(C/T)	AGATCT GGGG_5'

encoded $G_{\alpha}i1$. Subsequently, twenty-eight were 'T-tracked' (Materials and Methods Chapter) to identify further members that had been cloned. Of these, sixteen represented $G_{\alpha}q$ and four $G_{\alpha}i1$. The remaining eight, after sequencing in full, were shown to be $G_{\alpha}s$, $G_{\alpha}olf$ (twice; one clone truncated artifactually), and $G_{\alpha}i3$; the rest being non-specific amplification products.

A second successful amplification, using a new batch of cDNA, was cloned into both TA vector and 'pBluescript' (using the restriction sites in the primers), such that 140 colonies from the former and over 300 from the latter were obtained. These were gridded on plates, transferred onto filters, and hybridised with a mixed probe consisting of $G_{\alpha}q$, $G_{\alpha}s$, and $G_{\alpha}i3$ components. Non-hybridising colonies were sequenced but no novel members of the family were found.

The same technique of negative selection was applied to a third set of amplification products which had been amplified from cDNA synthesized with the 3' PCR primer as the reverse transcription primer. Although the non-specific background products were more abundant, all the family members were found in an initial sequencing test. 980 colonies were screened but, again, none of the non-hybridising colonies represented novel members: only non-specific products or G proteins which had failed to hybridise (probably due to the highly variable amount of DNA attached to the filters in a bacterial colony transfer) were sequenced.

2.13: Introduction to protein tyrosine phosphatases

The introductory chapter described the role of phosphorylation in the modulation of protein activity. Protein phosphorylation can occur on serine/threonine residues or on tyrosine residues; these two types mediated by separate families of kinases. Much work has been reported concerning the isolation and characterisation of these enzymes in numerous biological systems, including LTP/LTD; where the alteration of phosphorylation states is critical for the early stages of synaptic efficacy modification. Just as there are two types of kinases with specificities for the identity of the residue to be phosphorylated, so there are two types of 'phosphatase' enzymes. The family of phosphatases that remove phosphate moieties from tyrosine (as opposed to serine/threonine) residues are known as 'protein tyrosine phosphatases' (PTPs) and will be discussed in this chapter (refs. 82-88).

One important point is that PTPs should not be thought of as carrying out purely deactivation processes in the cell: several examples of PTPs eliciting/propagating stimulatory cascades have been described. CD45 is required for the T-cell activation response. It achieves this by dephosphorylating the carboxy termini of the non-

receptor tyrosine kinases *lck* and *fyn*, which results in a kinase-dependent cascade leading to the activation of the cell. Precisely how CD45 is triggered into the active state is not yet understood, but it probably arises from interactions with T cell surface markers (such as the T cell receptor or CD4) as they bind their respective ligands.

Purification of a protein tyrosine phosphatase activity (from human placenta) was first achieved by Charbonneau et al (ref. 89). Partial amino acid sequencing yielded a surprising homology to two cytoplasmic domains of the leukocyte common antigen (LCA or CD45; ref. 90). This was confirmed by full-length cloning of the cDNA (named PTP 1B; ref. 91). Subsequently, related domains were identified in leukocyte common antigen-related protein (LAR. ref. 92) and T Cell PTP (which was purposefully cloned on the basis of this homology; ref. 93).

PTP 1B and T Cell PTP are small cytoplasmic proteins which contain one copy each of the ~250 amino acid homology domain (74% identity between the two). CD45 and LAR are 4-5 times larger and are transmembrane proteins. They each contain two homology domains (I and II) which are both located in the cytoplasmic portion of the protein. Protein sequence information indicated that the extracellular regions are involved in some form of ligand binding. Thus, like the tyrosine kinase family, the PTP family could be divided into receptor-like and cytoplasmic forms.
It has been shown that the conserved domain is, in fact, the catalytic domain responsible for the phosphatase activity. Fig. 2.4a shows the catalytic domain in diagrammatic form with the conserved amino acid regions highlighted. A conserved cysteine residue in an eleven amino acid motif:-

I/V H C X A G X X R S/T G

seems to be critical for the phosphatase activity (ref. 94) by forming a thio-phosphate intermediate.

More than thirty members of this family have been cloned to date and it is likely that many more will follow. The classes of cytoplasmic and transmembrane PTPs can be further subdivided because of the accumulation, throughout evolution, of additional functional domains which specify the precise role or subcellular localisation of the phosphatase activity. Fig 2.4b illustrates examples of these different forms.

Receptor PTPs usually have two intracellular catalytic domains but hPTP β and D PTP10D have only one. A purpose for two catalytic domains has yet to be determined; it was originally thought that the second one was inactive but, at least in

the case of CD45, catalytic activity has been found in domain II as well as I; perhaps there is some auto-regulatory function in the second domain (ref. 95).

Five classes of receptor-like PTPs have been designated on the basis of functional analysis:-

I - includes CD45 and related members which have extensive splicing in the extracellular domains.

II - includes LAR/hPTP κ/hPTP μ and is distiguished by the presence of extracellular fibronectin type III (FN III) and immunoglobulin-like (IG) domains. This is an arrangement reminiscent of the cell adhesion molecule, NCAM, and thus these molecules are thought to act in a similar fashion.

III - includes D PTP10D and D PTP 99A. They possess extracellular FN III domains.

IV - includes PTPs α and ε made notable by the presence of only a very short extracellular domain which is heavily glycosylated.

V - includes h PTP ζ (a.k.a. β and 18) and h PTP γ. They are typified by the presence of an N-terminal carbonic anhydrase-related domain which is not catalytically active but probably plays a role in ligand-binding (section 2.16). A vaccinia virus protein (D8) has a related domain and is known to be a cell adhesion molecule.

PTPs μ and κ are two of the few receptor-PTPs with known ligands (the function of these two phosphatases will be discussed later in the chapter) and the search for other ligands is an area which demands further study. In particular, it would be interesting to discover if there is a triggering of catalytic function by ligand-binding induced receptor dimerisation similar to that observed with receptor tyrosine kinases.

Cytoplasmic PTPs show a wide variation in structures and resulting functions. PTP 1B contains a C-terminal amino acid motif which causes it to be transported to the endoplasmic reticulum where it carries out its functions. Nuclear localisation is specified by another C-terminal sequence in the *Drosophila* PTP, D PTP 61F (ref. 96), whereas a motif in the PTPs, PTP-PEST and PTP PEP, directs these proteins to be rapidly degraded (ref. 97). PTP MEG1 and PTP H1 contain a glycophorin-binding domain which is related to those in the proteins ezrin and talin (ref. 98). This presumably acts as a cytoskeleton-anchoring domain for these PTPs so that dephosphorylation activity can be focused on the proteins involved in the cytoarchitecture of the cell. PTP MEG2 contains a cellular retinaldehyde-binding protein (CRALBP) domain which may enable this PTP to interact with lipid moieties

(ref. 99). Again, reminiscent of the kinase cascade, there exist two PTPs (PTP 1C and SH PTP 2) containing SH2 domains (src homology domain 2), which are known to interact with phosphotyrosine moieties on other proteins (ref. 100). This may indicate that these proteins are involved in the deregulation of receptor protein tyrosine kinase activity.

2.14: Protein tyrosine phosphatase primer design

Three conserved regions in the catalytic domain were used to design degenerate PCR primers. These are shown in fig. 2.4a, and the precise sequences are presented in table 2d. As can be seen, a number of primer sequence variations were used in some cases. The pair of primers which resulted in the amplification of the PTPs was the third set.

2.15: Protein tyrosine phosphatase PCR results

The successful amplification required the adoption of an entirely different PCR amplification protocol which, together with a third set of PCR primers, was a gift of K. Lee (Centre for Genome Research). The full protocol is described in the Materials and Methods chapter, but the key differences probably lay in the use of random-primed cDNA synthesis (thus avoiding the requirement of oligo-dT-primed reverse transcriptase enzyme to pass through large 3' untranslated regions before entering PCR-amplifiable coding sequence) and the use of a different source of Taq polymerase. Agarose gel electrophoresis of the DNA products from the second attempt at the reaction revealed a strong signal corresponding to the expected product size-range. This material was cloned into the TA cloning vector and transformed into competent bacteria.

Of the first seventeen clones sequenced, nine different PTP catalytic domains were identified:-

Clone	Sequence Identification
1	PTP ζ (domain I)
3	PTP γ (domain I)
4	Novel sequence 1
6	PTP δ (domain I)
7	PTP H1 (domain I)
9	PTP α (domain II)
11	PTP γ (domain I)
12	T Cell PTP

Table 2 d Table listing the degenerate PCR primers used to amplify members of the PTP family and the conserved amino acid sequences from which they were derived. Conserved amino acid residues are shown above the degenerate nucleic acid sequence of the primer. Also shown (where included) are the 5' restriction sites which were intended to aid cloning of amplified products. For each primer an approximate annealing temperature can be calculated using the equation described in the Materials and Methods chapter. Because these primers are degenerate there are a range of values. Primer 1; 47-55°C, primer 2; 49-59°C, primer 3; 49-53°C, primer 4; 55-65°C, primer 5; 63-65°C, and primer 6; 47-55°C.

Table 2d

Primer	Sequence									
	Amino Acid (N-C)	SAL I or ECO RI	K	C	_	Q	Y	W	P	
1	Nucleic Acid	5'_C GTCGAC	AA(A/G)	TG(C/T) .	III	(C/G)A(A/G)	TA(C/T)	TGG	CC_3'	
2	Nucleic Acid	5'-GG GAATTC	AA(A/G)	TG(C/T)	G(C/A)I	CA(A/G)	TA(C/T)	TGG	CC_3'	
	Amino Acid (N-C)	XHO I	F	W	r	M	V/I	W	E/D/q	
3	Nucleic Acid	5'_GGGG CTCGAG	TT(C/T)	TGG	III	ATG	(G/A)T(G/C)	TGG	(C/G)A_3'	
	Amino Acid (N-C)		H	C	S	A	G	A/I	G	BAM HI or XBA I
4	Nucleic Acid	3'_	TG	ACG	TC(G/A)	CG(A/G/T)	CCI	(C/T)(A/G)I	CC(T/C/G)	CCTAGG C_5'
5	Nucleic Acid	3'_	GTG	ACG	TCA	CC(C/G/T)	CC(C/G)	III	CCG	AGATCT GGGG_5'
6	Nucleic Acid	3'_	GT(A/G)	AC(G/A)	TC(A/G)	CGI	CCI	(C/T)AC	-	CTAGG C_5'

22	PTP α (domain II)
23	PTP P19
24	Novel sequence 2
25	PTP γ (domain I)
26	PTP P19
27	Putative rat PTP H1 (domain I)
28	PTP α (domain II)
29	PTP ζ (domain I)
37	T-Cell PTP

A mixed probe corresponding to equal parts of the two novel PTP PCR products was used to screen duplicate filter transfers (plating and transfer carried out by G. Stapleton; Centre for Genome Research) of a rat hippocampus cDNA library (created by Dr J. O. Mason and M. Richardson; C.G.R.). This library consisted of 20 plates of 50,000 plaques each, which gave a representation of 10^6 cDNAs.

Six positive plaques were purified to homogeneity and analysed by sequencing (fig. 2.6b/c for analysis of clones). Two clones (13.1 and 16b.1) corresponded to the first novel PTP, three (10.1, 16a.1, and 18.1) to the second, and one clone represented a mitochondrial sequence which showed some cross-hybridisation to the second novel PTP probe sequence. This was not studied further. The two novel PTPs were also used as probes for Northern hybridisation in order to obtain information concerning the expression of the transcripts in the brain and other tissues (fig. 2.8).

2.16: Summary of the genes cloned by this approach

G Protein-Coupled Receptors
α1b Adrenergic
β2 Adrenergic
Dopamine d1
Adenosine a1
Adenosine a3
Cannabinoid
Endothelin 1
R334
Rat Testis GPCR
VIP (discredited)
Metabotropic Glutamate Receptors
1
3
4
5
G Protein α Subunits
i1
i3
q
s
Olf
Protein Tyrosine Phosphatases
P 19
T Cell
H 1
α
γ
ζ
δ
Novel PTP 4
Novel PTP 24

In this section of the chapter the relative successes of each of the cloning attempts made on the four families will be discussed. As stated earlier, the aim of this approach was to discover gene-family members which were restricted in expression to the hippocampus. This was examined by using the cloned PCR products as probes on Northern blots of brain regions or, in the case of those that had been cloned and published previously, the literature concerning expression was consulted.

The questions arising from these experiments are:-

1) Were the primers able to amplify some hippocampus-expressed members and not others?

2) What is the likelihood of there being other (unisolated) members of the gene family in the hippocampus?

3) Are there within the amplified/published members any that show the required expression pattern?

G protein coupled receptors

The attempts to clone members of this family by degenerate PCR raise all three of the above questions. At face value there seems to be a lack of the 'classical' GPCR neurotransmitter receptors that would be expected (and are known to be present) in the hippocampus. This is probably due to the combined effects of their low expression levels and the biasing of the primer design (especially the second set which were not aimed at the neurotransmitter receptor subset of GPCRs).

The ten identified members of this family can be subdivided into three functional groups.

The classical neurotransmitter receptors were represented by five genes; α1b adrenergic, β2 adrenergic, dopamine d1, adenosine a1, and adenosine a3 receptors.

The first two receptors in this list are present due to the many adrenergic afferents entering the hippocampus (for example those from the locus ceruleus and ventral tegmentum). Briefly, the α1b receptor is localised post-synaptically and is known to couple ligand binding to activation of calcium channels. The β2 receptor is found post-synaptically as well as on some glia. It is thought to act by the stimulation of cAMP production. In addition, because of its affinity for adrenalin, as well as nor-adrenalin, it is thought to play a role in the association of emotion/stress levels to learning and memory.

The dopamine d1 receptor is thought to play a role in diseases such as Parkinsonism and schizophrenia; with which the hippocampus is intimately linked. It is transcribed from an intronless gene and expression is seen in the cortex, thalamus, amygdala, septum, basal ganglia and low levels in the hippocampus. The adenylyl cyclase enzyme is stimulated in response to receptor activation.

The two cloned adenosine receptors are known to mediate various effects on brain physiology such as sedation, vasodilation, gluconeogenesis, lipolysis, increases in potassium flux, and decreases in calcium flux. Both a1 and a3 receptors act by inhibiting adenylyl cyclase whereas a2 (not cloned in this study) acts through the reverse process. The localisation of a1 is not well characterised with only brain and thyroid confirmed to be sites of expression. a3, however, has been observed in the cortex, striatum, and olfactory bulb as well as testis, lung, kidney, and heart (where it can control the rate and extent of contractility).

The cannabinoid and endothelin receptors are not classical neurotransmitter receptors and are probably localised outside of the synaptic cleft.

CNS depression and analgesia are two effects of the administration of drugs of the cannabinoid family. The receptor causes the inhibition of the adenylyl cyclase pathway. Expression has been shown in the hippocampus, cortex, amygdala and ventromedial hypothalamus but not every neuron expresses the receptor and so, in the granule cell layer of the dentate gyrus, for instance, punctate labelling is seen with *in situ* hybridisation. Recently, the endogenous ligand for the receptor has been identified as anandamide, a derivative of arachidonic acid (ref. 101).

There are three members of the endothelin peptide family and the receptor for endothelin 1 was cloned here (although published studies have shown that endothelin 2 can also stimulate this receptor to a small degree). A large N-terminal domain is found in these receptors; typical of peptide hormone receptors. Expression sites for this receptor subtype include the cortex and midbrain among other regions. The peptides themselves are some of the most potent vasocostrictors known and probably play a part in the regulation of blood flow in the brain.

The final classification of cloned GPCRs is the group that possess unidentified ligands. Three of these 'orphan receptors' were amplified from hippocampus cDNA.

R334 was originally cloned from a rat pituitary library and expression of a 7 kb transcript has been seen by Northern blot in the brain and testis. Within the brain the principal sites of expression are the piriform cortex and the lateral septal nuclei.

Comparison of its cDNA with other sequences seems to suggest that this is not a member of the classical neurotransmitter receptor subfamily.

The 'rat testis GPCR' is reported to be observable by Northern blot only in the testes of sexually mature rats although it was originally cloned from cerebellum cDNA and was amplified here from the hippocampus. Further expression studies on brain region Northern blots seem to confirm the observation that this transcript is expressed at a level in the brain below the sensitivity limit of Northern hybridisation (data not shown).

The third member of this group was originally published as the receptor for vasoactive intestinal peptide (VIP). VIP, as well as its long-established role in smooth muscle contraction, has been shown to have positive effects on the growth and survival of neurons in culture. The receptor is found principally in the presynapses of cholinergic neurons and acts by stimulating the activity of adenylyl cyclase. A transcript of 2.7 kb has been described in the brain, colon, heart, lung, kidney, spleen, and small intestine. However, a second group published a sequence which they claimed encoded the genuine VIP receptor (ref. 102). This gene showed substantial homology to the secretin/glucagon receptors which was expected, considering these peptides can act as VIP receptor agonists and antagonists. Secretin and glucagon were originally described as capable of binding to the first receptor, but this observation was not reproducible in other laboratories. Therefore, the true identity of the ligand for the receptor cloned here remains to be elucidated. Further analysis of the precise distribution of the mRNA for this receptor was performed by hybridising a brain region Northern blot with a probe derived from the PCR product (fig. 2.5a). The results indicate that this receptor is expressed at comparable levels in hippocampus, cerebellum, cortex, olfactory bulb, and the whole brain-minus-hippocampus. The size of the transcript matches that published in the original description of this gene.

The GPCR family appeared a promising source of region-specific genes because of the large number of members cloned to date. The low number of family members obtained by the degenerate PCR approach, in this particular case, was probably caused by the diverse nature of the sequences together with their low expression levels. Many more receptors that are known to be expressed in the hippocampus should have been cloned. Outside of the hippocampus, the same primer sets were capable of amplifying a serotonin receptor (B Davies, CGR; personal communication) thus leading to the assumption that the expression level is a critical factor determining their presence or absence in these PCR products.

Figure 2.5 a Messenger RNA distribution of discredited VIP receptor within the brain as obtained by Northern hybridisation. The transcript size of 2.7kb matches that presented in the original publication. **b** Messenger RNA distribution of $G_{olf}\,\alpha$ subunit within the brain and other tissues. Ribosomal protein S26 was used as loading control for the Northern filter. (Hc; hippocampus, Ce; cerebellum, Cx; cortex, Olf; olfactory bulb, RoB; rest of brain, Ht; heart, Mu; muscle, Te; testis, Ki; kidney, Lu; lung, and Sp; spleen.)

Fig. 2.5a

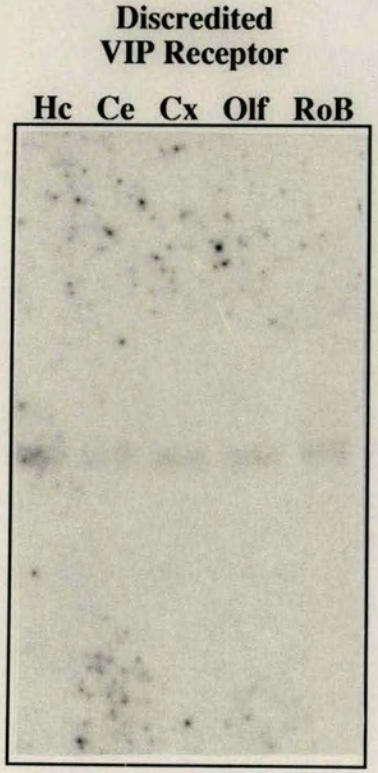
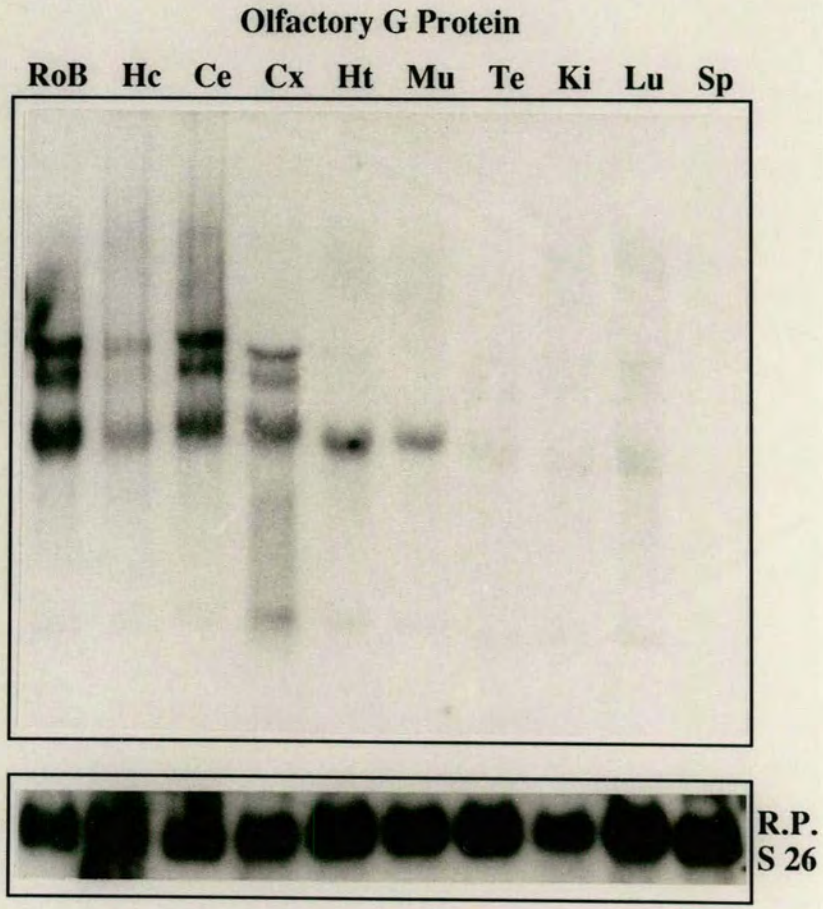


Fig. 2.5b



Metabotropic glutamate receptors

In total, four different members of this family were cloned. Seven members have been published and studied in detail with an eighth member as yet, unpublished. Expression data from these papers indicate that six out of seven show at least low-level expression within the hippocampus. It seems to be a general characteristic of the mGluR family (with the exception of mGluR 6) that the expression profiles seen are relatively widespread within the brain. Therefore, any particular region seems to express a mixture of these receptors; maybe due to the multitude of different cell-types within that region or because the individual receptor types are responsible for particular responses to the glutamate signal. For example, it is known that some mGluRs are found on the post-synaptic membrane, as expected for the normal transduction of the glutamate signal, while others are located in a presynaptic manner (autoreceptors) and cause negative feedback, limiting the amount of glutamate release. The table below illustrates the published expression profiles of the known mGluR transcripts. mGluRs 1, 3, 4, and 5 were cloned in a small, non-exhaustive search.

1-cortex, hypothalamus, olfactory bulb, cerebellum, hippocampus.
2-hippocampus, olfactory bulb, cerebellum, cortex, hypothalamus.
3-'similar to mGluR4'
4-olfactory bulb, cerebellum, low hippocampus.
5-hippocampus, cortex, olfactory bulb, striatum.
6-retina.
7-cortex, thalamus, hippocampus, striatum, olfactory bulb, etc.

From these data, it seems as if PCR products derived from mGluRs 2 and 7 should also have been isolated. Reasons for not cloning family members by degenerate PCR may include the low abundance of those forms in the hippocampus and possibly, divergence from the canonical sequence in the region used to design the PCR primers. With respect to mGluRs 2 and 7, only mGluR7 shows significant divergence; found at the 3' end of the downstream primer sequence. This may explain its absence from the PCR products (because of its relatively recent cloning, the sequence of mGluR7 was not used for primer design). An additional observation was that the low expression of mGluR4 in the hippocampus was reflected in the infrequency of its corresponding PCR product in the cloned and identified material.

It seems likely that the majority of the mGluR subtypes in the brain have been identified. Of course, a receptor with a sufficiently different sequence might escape PCR amplification but would probably have been isolated in the low stringency

library screening experiments described earlier. It is possible that a subtype expressed at a low level in a small brain region or one that is developmentally-/activity-regulated might have escaped detection, but that would not prove useful for our purposes by virtue of those characteristics.

Heterotrimeric G proteins; α subunits

Five members of this family were amplified from hippocampus cDNA (G_{α}i1, i3, q, s, and olf). Of these, the presence of the olfactory G protein α subunit was particularly surprising as this had been reported to be specifically expressed in the olfactory bulb, as explained in section 2.7. However, even though a specifically expressed 3' untranslated region exists, the coding region is widely expressed in the CNS. This was demonstrated here by using the PCR product as a probe on a Northern blot of various tissues (Fig. 2.5b). Prominent expression was observed in hippocampus, cerebellum, cortex, rest of brain (brain-minus-hippocampus), heart, and muscle. Brain expression of this gene existed in the form of three major transcripts of approximately 2, 3.5, and 4 kb size. Published Northern data on olfactory epithelium expression indicates an additional transcript if 4.7 kb size which may account for the erroneous assignment of olfactory-specific expression (ref. 103). It will be interesting to see if this subtype, with its relatively high expression in the brain, will be found to play an important role in general CNS function.

It was encouraging to note that of the members cloned, a representative was found from each of the α subunit subgroups ('s', 'i' and 'q') except for the '12' subgroup. However, within the subgroups, certain expected members (because of their reported expression in the nervous system) were absent. This could be explained by brain expression being limited to non-hippocampal tissue, or by the fact that the primers were not a good match for their sequences. For example, G_{α}o, G_{α}x, and G_{α}12 are all reported to be expressed in the brain. Analysis of the 3' end of the downstream primer of G_{α}x showed that it diverged slightly from the primer sequence; this may explain its absence.

Finally, it must be pointed out that G proteins could might not possess hippocampus-specificity through restricted expression pattern. This is because a 'specificity of function' within the hippocampus might be achieved by the unique combination of the multiple forms of the three subunits that make up the heterotrimeric complex, and not by the expression of a spatially restricted form. Countering this, is the reported restricted expression of the transducin and gustducin α subunits to their respective tissues. These, however, are located in primary sense-detection tissues, and thus may have evolved to carry out these specialised functions. Is the hippocampus sufficiently

different, or demanding in function, to require a specific α subunit? This question will be returned to in the discussion (Chapter VI).

Protein tyrosine phosphatases

Recently, a survey of PTP expression similar to the one described here was published (ref. 104). Using degenerate PCR primers to amplify PTPs from mouse brain, eight different members were identified. Of six published, only one was cloned in common with this attempt (PTP δ). This could indicate (ignoring differences in experimental conditions) that each brain region expresses a distinct subset of PTPs such that when the whole brain is analysed, only those PTPs which are widely expressed at a sufficiently high level will be evident (i.e. regional PTPs expressed at a low level will not be well represented in whole brain).

Due to the assignment of function to the non-catalytic domains of the phosphatases, it is possible to make an educated guess as to the cellular processes occurring in the hippocampus that might require particular subtypes. Interestingly, two-thirds of those cloned here belong to the receptor PTP class which seems to be largely involved in cell-cell interactions. This reflects the growing realisation (as discussed in the introduction) that cell-adhesion plays a large role in neuronal function, whether through development, maintenance of neuronal morphology, or activity-dependent changes of synaptic efficacy. However, the cytoplasmic PTPs that were cloned, and their putative roles in hippocampus activity, are discussed first.

PTP P19 was originally cloned from P19 embryonal carcinoma cells and was reported to be highly upregulated in cultures which had been taken through a differentiation protocol designed to create neuronal lineages (retinoic acid induction; ref. 105). Apart from this information, nothing is known about the expression of this PTP in the brain. Therefore, it was used as a probe on a Northern blot. Extremely low level expression was seen in the cortex, olfactory bulb, and brain-minus-hippocampus. No expression changes were observable after electro-convulsive stimulation (Chapter V).

T Cell PTP is named after its original site of cloning. Expression of this gene has also been observed in brain, spleen, thymus, and placental tissues. Like PTP P19, the absence of defined domains renders the function of this protein unknown.

PTP H1 has a domain in the N-terminal region of the protein which shows significant homology to regions in the proteins band 4.1, ezrin, and talin (ref. 106). In these proteins the domain is thought to act by localising them to the junctions of the cytoplasmic membrane and the cytoskeleton (such as focal adhesions). Members of

the *src* family of cytoplasmic tyrosine kinases are known to play a part in the functioning of focal adhesions, so it is interesting that a PTP might also be present; perhaps in a regulatory capacity. In the nervous system, such cytoskeletal-cytoplasmic junctions occur at the 'post-synaptic density' to localise receptors and signalling molecules in direct opposition to the presynaptic bouton. In the hippocampus, there are high levels of one particular tyrosine kinase, *fyn*, which plays an important role in the modification of synaptic efficacy during LTP (Chapter I). This kinase also activates a more specific kinase known as focal adhesion kinase (FAK) which is widely expressed in neurons (ref. 107). It would be interesting to discover if there was direct interplay between these two kinases and PTP H1 in the creation, or stabilisation, of LTP. The PTP H1 PCR products obtained from the rat hippocampus show an amino acid sequence homologous to that obtained from human HeLa cells. In all probability this represents the ortholog. Expression patterns for this PTP have not been published. Therefore, an analysis of the expression was performed on Northern blots of brain region RNAs. Transcripts were detected in all tissues assessed (data not shown) and did not appear to be affected by electro-convulsive stimulation (Chapter VI).

The following clones are derived from receptor PTPs. In the case of the two novel members this classification could only be made definitively for one of them (PTP 24) after the cloning of larger cDNAs as described. PTP 4 is also expected to belong to the receptor class by virtue of sequence homology and transcript size.

PTP α (ref. 108) is a transmembrane protein with only a very small extracellular domain which is highly glycosylated. Its published expression pattern is the brain, kidney, and liver with lower levels elsewhere.

PTP γ (ref. 109) is a candidate tumour suppressor gene located on human chromosome 3p14.2-p21. Transcript sizes of 8.5 and 5.5 kb are seen in brain, lung, kidney, liver, heart, muscle, spleen, and testis (where there also exists a 3 kb product). In the brain, the expression of PTP γ appears to be developmentally regulated. Postnatal expression is seen in the rat septal/thalamic nuclei, cortex and hippocampus but this becomes increasingly restricted to the hippocampus during maturation (albeit with low levels elsewhere in the brain). The protein is a member of the subset of PTPs which has one carbonic anhydrase-like (CAH) and several fibronectin type III (FN-III) domains at the N-terminal end of the extracellular region.

A second member of this subset is PTP ζ (aka RPTP β; ref. 110). Expression of this gene has been localised to the brain; more specifically, to the dentate gyrus, cerebellum and ventricular region. Compared to γ, there is a larger extracellular domain, but it still possesses the CAH and FN-III domains at the N-terminal. A recent paper (ref. 111) sheds new light on the function of the carbonic anhydrase-like domain. Using expression cloning techniques, a receptor for the CAH domain has been found and identified as 'contactin', a GPI-anchored neural cell-adhesion receptor which was previously known to play a role in CNS development by inducing axonal growth. Therefore, this represents one of the few proven examples of a PTP converting a signal (in this case, a ligand) into a defined cellular response.

PTP δ mRNA exists in multiple splice-forms that differ in their number of constituent extracellular domains (ref. 112). These domains are the immunoglobulin-like and fibronectin type III domains; the longest protein form consists of 3 Ig and 8 FN III domains. Overall, this receptor PTP is 62.7% identical to the LAR PTP described in the introduction. Expression is seen in the brain, B cells, kidney, and heart. Localisation of the PTP δ transcript within the brain shows high levels in the hippocampus, thalamus, and piriform cortex.

2.17: Further characterisation of two novel PTPs

The complete characterisation of the two novel members of the PTP family was not fully carried out. This was due to the early realisation that their expression patterns did not match our requirements. In addition, because of their large transcript sizes and low expression levels, full-length clones for either PTP were not obtained from a screen of the rat hippocampus cDNA library. Nevertheless, some attempt has been made to interpret the obtained sequence data in terms of the functioning of these molecules in the hippocampus (fig. 2.6a for alignments of the novel PTP PCR products with corresponding regions from a selection of common PTPs).

Sequencing of clone PTP 4 showed that it contained substantial homology to PTP μ (ref. 113). Soon after, another related PTP, κ, was published (ref. 114). PTP μ mRNA is found as a 5.7 kb transcript in the lung and, to a lesser extent, in the heart and brain. It has been localised to human chromosome 18 pter-q11 by analysis of somatic hybrids. In a C-terminus to N-terminus direction, it consists of two catalytic domains, an unusually large serine/threonine-rich spacer region of seventy amino acids, a transmembrane domain, four FN III domains, one Ig-like domain and a 'MAM' domain. The MAM (μ/A5/meprin) domain is an approximately 160 amino acid motif containing four cysteines which form disulphide bridges. It was originally described

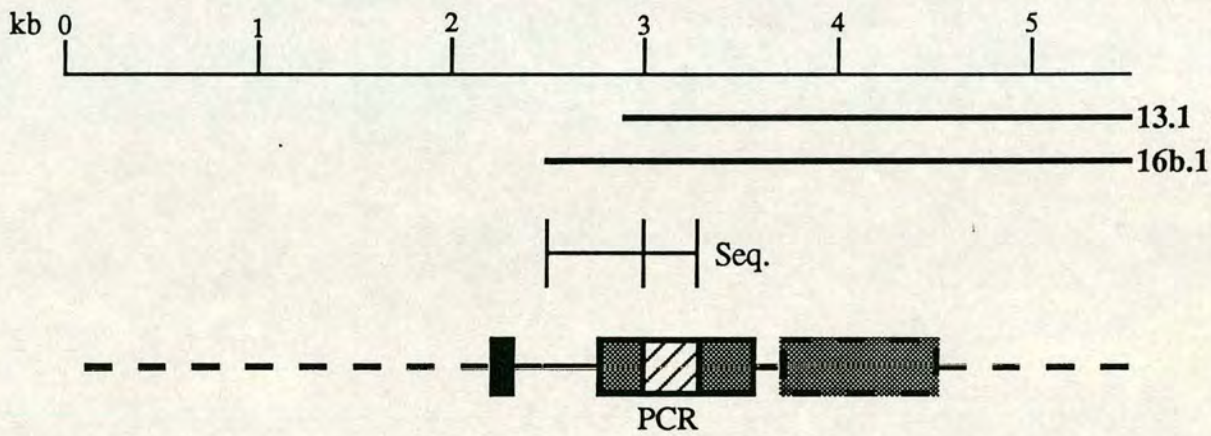
Figure 2.6 a Alignment of the amino acid sequences derived from the novel PTP PCR products (PTP 4 and 24) with a selection of corresponding regions from other PTP family members. 'Cat. I' indicates that the sequence is from the first catalytic domain. Boxed residues are those which match the consensus/majority. In this list, PTP 4 showed most similarity to LAR PTP (54%), whereas PTP 24 showed most similarity to hPTPβ (30%). **b** Diagram showing the analysis of two cDNAs corresponding to PTP 4. The two clones (13.1 and 16b.1) are shown below a scale indicating their size. Below this is an indication of the extent of sequence data. At the bottom of the figure is a cartoon showing the expected domain structure of PTP 4. This was deduced from sequence analysis (solid regions) and comparison with PTPs μ and κ (dashed regions). **c** A similar analysis was performed on the three cDNA clones (10.1, 16a.1, and 18.1) which corresponded to PTP 24. Clone 16a.1 has the site of the insertion indicated (see Appendix).

Fig. 2.6

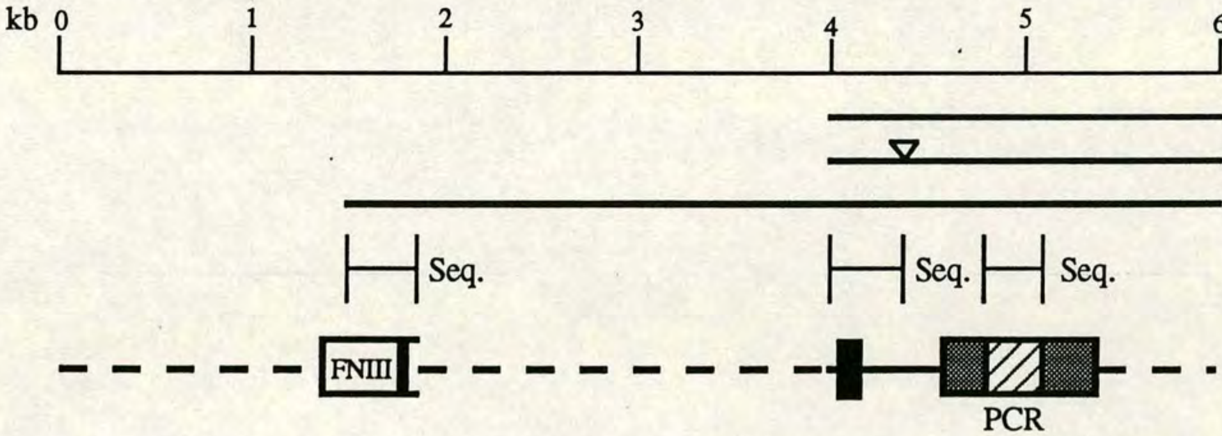
a

```
     A T D E D T - - Y G D - - I K V T L V S E D V L  Majority
                     10                  20
1    E - D S D M - - Y G D - - I K I T L V K T E T L  PTP 4 PCR Prod.
1    F T E E - P I A Y G D - - I T V E M V S E E E Q  PTP 24 PCR Prod.
1    A R G T E T - - Y G L - - I Q V T L V D T V E L  Rat LAR PTP Cat. I
1    - T D D Q E M L F K E T G F S V K L L S E D V K  Human T-Cell PTP Cat. I
1    D Q G C W T - - Y G N - - I R V S V E D V T V L  Human RPTP-Alpha
1    A - D Q D S L Y Y G D - - L I L Q M L S E S V L  H-PTP Beta Cat. I
1    Q K E E K E M I F E D T N L K L T L I S E D I K  Human PTP-1B Cat. I

     A D Y T V R T F A L - - - G E S E N R D E T R L  Majority
               30                  40
58   A E Y V V R T F A L E R R G Y S A R H E - - - -  PTP 4 PCR Prod.
64   E D W A S R H F R I N - - - - - - - Y A D E A Q D  PTP 24 PCR Prod.
61   A T Y T M R T F A L H K S G S S E K R E - - - -  Rat LAR PTP Cat. I
70   S Y Y T V H L L Q L - - - - E N I N S G E T R T  Human T-Cell PTP Cat. I
61   V D Y T V R K F C I Q Q V G D M T N R K P Q R L  Human RPTP-Alpha
64   P E W T I R E F K I - - - C G E E Q L D A H R L  H-PTP Beta Cat. I
73   S Y Y T V R Q L E L - - - - E N L T T Q E T R E  Human PTP-1B Cat. I

     I R H F H F T A W P D H G V P - - E S P A S L L  Majority
       50                  60                  70
118  V R Q F H F T A W P E H G V P - - Y H A T G L L  PTP 4 PCR Prod.
118  V M H F N N T A W P D H G V P P A N A A E S I L  PTP 24 PCR Prod.
121  L R Q F Q F M A W P D H G V P - - E Y P T P I L  Rat LAR PTP Cat. I
130  I S H F H Y T T W P D F G V P - - E S P A S F L  Human T-Cell PTP Cat. I
133  I T Q F H F T S W P D F G V P - - F T P I G M L  Human RPTP-Alpha
127  I R H F H Y T V W P D H G V P - - E T T Q S L I  H-PTP Beta Cat. I
133  I L H F H Y T T W P D F G V P - - E S P A S F L  Human PTP-1B Cat. I

     A F L R K V R A S G P L S A - - G P V V V        Majority
                   80                  90
184  A F I R H V K A S T P P D A - - G P V V I        PTP 4 PCR Prod.
190  Q F V Y T V R Q Q A T K S - - K G P M I I        PTP 24 PCR Prod.
187  A F L R R V K A C N P L D A - - G P M V V        Rat LAR PTP Cat. I
196  N F L F K V R E S G S L N P D H G P A V I        Human T-Cell PTP Cat. I
199  K F L K K V K A C N P Q Y A - - G A I V V        Human RPTP-Alpha
193  Q F V R T V R D Y I N R S P G A G P T V V        H-PTP Beta Cat. I
199  N F L F K V R E S G S L S P E H G P V V V        Human PTP-1B Cat. I
```

b PTP 4



c PTP 24



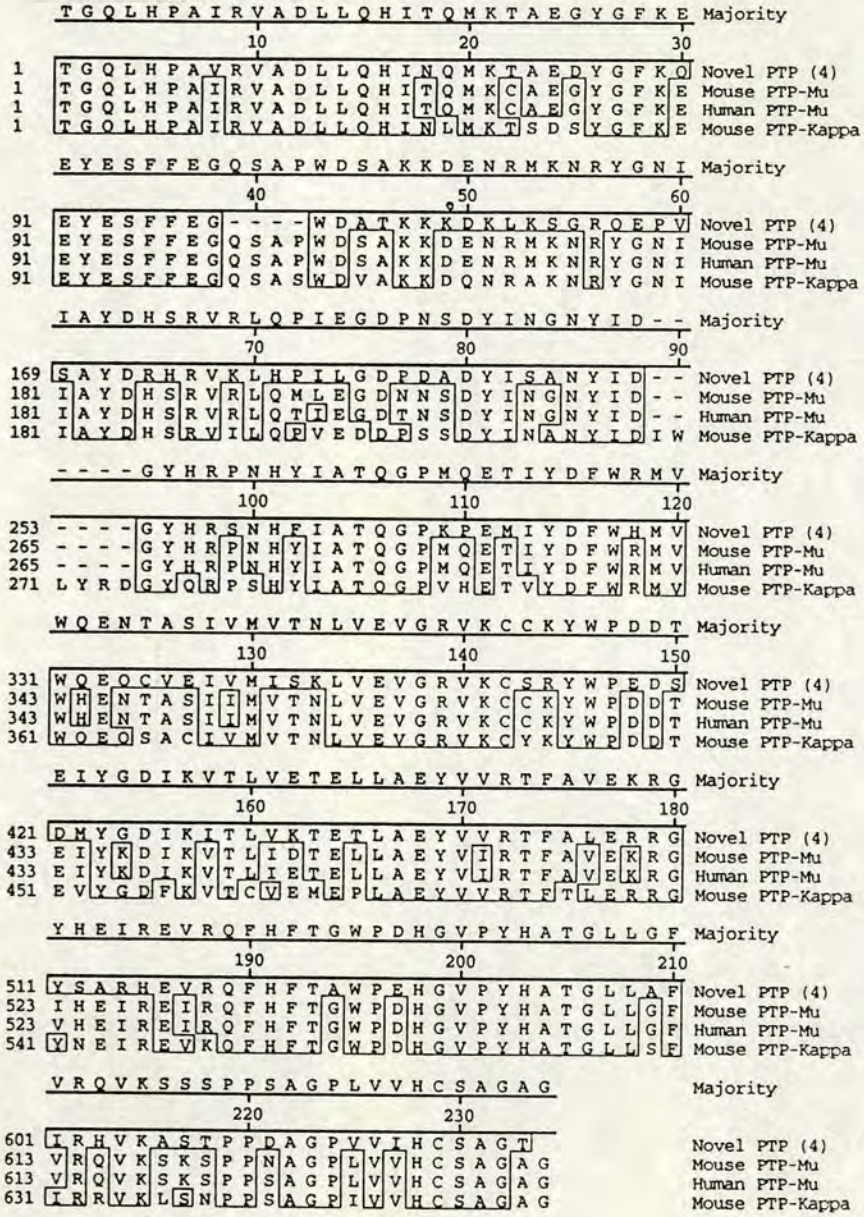
in the cell-surface proteins, A5 and meprin. There is some homology in the spacer region to the cell-adhesion molecule, cadherin. The corresponding region in that protein is responsible for interaction with the modulatory proteins, β-catenin and plakoglobin, although no positive evidence is available for a similar interplay in μ (or κ). PTP κ mRNA is found primarily as a 5.3 kb transcript in the kidney and liver with lower levels observed in the lung, heart, and brain. There does seem to be some modification of expression sites in the brain as maturation progresses, with an increase in olfactory bulb expression and concomitant decrease in cerebellar expression. Some evidence exists of cleavage of the extracellular portion (in the case of κ, at least) by a protease, furin, although the function of the soluble product is not clear. Key experiments (refs. 115 and 116) involving the expression of these two PTPs in cells, or the attachment of their extracellular domains to beads, have shown that they function as cell adhesion molecules. Moreover, they are homophilic receptors; a μ molecule will only bind to another μ, and a κ will only bind to another κ molecule. This adhesion is mediated by the extracellular domains, as might be expected, with the precise specificity of binding being dictated by the MAM domain. Hence, chimaeric PTPs with a particular MAM domain will only interact with a like MAM domain-containing PTP (ref. 117).

Two cDNAs which hybridised to PTP 4 were obtained from a screen of a rat hippocampus cDNA library (fig. 2.6b). The 5' ends of these terminate in the same region of the cDNA; that which corresponds to the N-terminal end of the first catalytic domain. The longer of the two clones was ~3 kb. This indicates that approximately 2.5 kb of the upstream coding sequence remains to be cloned. By comparison with the protein structure of PTP μ, there appears to be sufficient coding potential in the uncloned region of PTP 4 for it to possess a similar extracellular domain arrangement. An alignment of comparable amino acid sequences of μ, κ and PTP 4 is shown in fig. 2.7a.

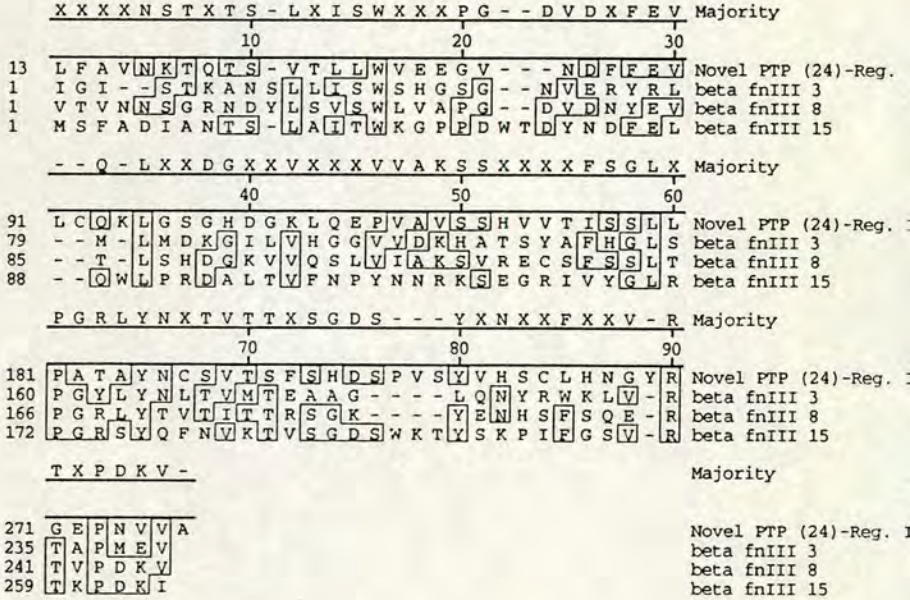
The second novel PTP (clone PTP 24) shows homology within the amplified catalytic domain to a number of PTPs, most convincingly with hPTP β (ref. 118). Three cDNAs corresponding to this gene were obtained from the rat hippocampus cDNA library (fig. 2.6c). Two of these contain sequences at the 5' end which correspond to the region of the PTP protein immediately internal to the membrane-spanning region. In fact, the amino acid sequence encoded by the longer of the two clones contains a stretch of hydrophobic amino acids which is likely to be this region. Puzzlingly, this clone also contains an additional 83 nucleotides as an internal insertion (Appendix). If it had been found at the ends of the clone, a cloning artifact might be an explanation, but the fact that it is present internally seems to support it being an

Figure 2.7 a Amino acid sequence deduced from the two PTP 4 cDNA clones aligned with those from PTPs μ and κ. Boxed residues are those which are identical to the consensus/majority. PTP 4 shows 64-65% sequence similarity with μ and κ whereas between PTPs μ and κ there is a similarity score of 76-77%. **b** Amino acid sequence deduced from PTP 24 cDNA clone 18.1 aligned with those from 3 fibronectin type III domains from hPTPβ. PTP 24 shows 13-15% similarity to these sequences (similar to the equivalent comparison of domains 3 and 15). Despite the low score, several key residues are conserved. **c** Greater similarity scores are seen in the alignment of amino acid sequence deduced from the 5' ends of PTP 24 cDNA clones 10.1 and 16a.1 with those from other PTPs. Similarity scores between PTP 24 and dPTP10D or hPTPβ (25-26%) are twice the amount of that between PTP 24 and LAR. Residues between positions 10-30 may constitute the transmembrane domain.

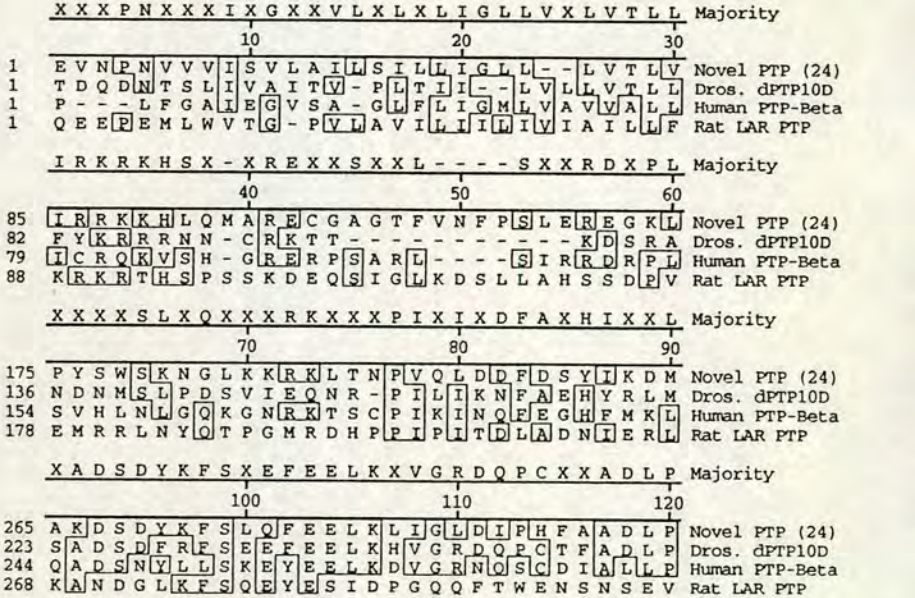
Fig. 2.7a


TGQLHPAIRVADLLQHITQMKTAEGYGFKE Majority
10 20 30
1 TGQLHPAVRVADLLQHINQMKTAEDYGFKQ Novel PTP (4)
1 TGQLHPAIRVADLLQHITQMKCAEGYGFKE Mouse PTP-Mu
1 TGQLHPAIRVADLLQHITQMKCAEGYGFKE Human PTP-Mu
1 TGQLHPAIRVADLLQHINLMKTSDSYGFKE Mouse PTP-Kappa
EYESFFEGQSAPWDSAKKDENRMKNRYGNI Majority
40 50 60
91 EYESFFEG----WDATKKKDKLKSGRQEPV Novel PTP (4)
91 EYESFFEGQSAPWDSAKKDENRMKNRYGNI Mouse PTP-Mu
91 EYESFFEGQSAPWDSAKKDENRMKNRYGNI Human PTP-Mu
91 EYESFFEGQSASWDVAKKDQNRAKNRYGNI Mouse PTP-Kappa
IAYDHSRVRLQPIEGDPNSDYINGNYID-- Majority
70 80 90
169 SAYDRHRVKLHPILGDPDADYISANYID-- Novel PTP (4)
181 IAYDHSRVRLQMLEGDNNSDYINGNYID-- Mouse PTP-Mu
181 IAYDHSRVRLQTIEGDTNSDYINGNYID-- Human PTP-Mu
181 IAYDHSRVILQPVEDDPSSDYINANYIDIW Mouse PTP-Kappa
----GYHRPNHYIATQGPMQETIYDFWRMV Majority
100 110 120
253 ----GYHRSNHFIATQGPKPEMIYDFWHMV Novel PTP (4)
265 ----GYHRPNHYIATQGPMQETIYDFWRMV Mouse PTP-Mu
265 ----GYHRPNHYIATQGPMQETIYDFWRMV Human PTP-Mu
271 LYRDGYQRPSHYIATQGPVHETVYDFWRMV Mouse PTP-Kappa
WQENTASIVMVTNLVEVGRVKCCKYWPDDT Majority
130 140 150
331 WQEQCVEIVMISKLVEVGRVKCSRYWPEDS Novel PTP (4)
343 WHENTASIIMVTNLVEVGRVKCCKYWPDDT Mouse PTP-Mu
343 WHENTASIIMVTNLVEVGRVKCCKYWPDDT Human PTP-Mu
361 WQEQSACIVMVTNLVEVGRVKCYKYWPDDT Mouse PTP-Kappa
EIYGDIKVTLVETELLAEYVVRTFAVEKRG Majority
160 170 180
421 DMYGDIKITLVKTETLAEYVVRTFALERRG Novel PTP (4)
433 EIYKDIKVTLIDTELLAEYVIRTFAVEKRG Mouse PTP-Mu
433 EIYKDIKVTLIETELLAEYVIRTFAVEKRG Human PTP-Mu
451 EVYGDFKVTCVEMEPLAEYVVRTFTLERRG Mouse PTP-Kappa
YHEIREVRQFHFTGWPDHGVPYHATGLLGF Majority
190 200 210
511 YSARHEVRQFHFTAWPEHGVPYHATGLLAF Novel PTP (4)
523 IHEIREIRQFHFTGWPDHGVPYHATGLLGF Mouse PTP-Mu
523 VHEIREIRQFHFTGWPDHGVPYHATGLLGF Human PTP-Mu
541 YNEIREVKQFHFTGWPDHGVPYHATGLLSF Mouse PTP-Kappa
VRQVKSSSPPSAGPLVVHCSAGAG Majority
220 230
601 IRHVKASTPPDAGPVVIHCSAGT Novel PTP (4)
613 VRQVKSKSPPNAGPLVVHCSAGAG Mouse PTP-Mu
613 VRQVKSKSPPSAGPLVVHCSAGAG Human PTP-Mu
631 IRRVKLSNPPSAGPIVVHCSAGAG Mouse PTP-Kappa

b


XXXXNSTXTS-LXISWXXXPG--DVDXFEV Majority
10 20 30
13 LFAVNKTQTS-VTLLWVEEGV---NDFFEV Novel PTP (24)-Reg.
1 IGI--STKANSLLISWSHGSG--NVERYRL beta fnIII 3
1 VTVNNSGRNDYLSVSWLVAPG--DVDNYEV beta fnIII 8
1 MSFADIANTS-LAITWKGPPDWTDYNDFEL beta fnIII 15
--Q-LXXDGXXVXXXVVAKSSXXXXFSGLX Majority
40 50 60
91 LCQKLGSGHDGKLQEPVAVSSHVVTISSLL Novel PTP (24)-Reg.
79 --M-LMDKGILVHGGVVDKHATSYAFHGLS beta fnIII 3
85 --T-LSHDGKVVQSLVIAKSVRECSFSSLT beta fnIII 8
88 --QWLPRDALTVFNPYNNRKSEGRIVYGLR beta fnIII 15
PGRLYNXTVTTXSGDS---YXNXXFXXV-R Majority
70 80 90
181 PATAYNCSVTSFSHDSPVSYVHSCLHNGYR Novel PTP (24)-Reg.
160 PGYLYNLTVMTEAAG----LQNYRWKLV-R beta fnIII 3
166 PGRLYTVTITTRSGK----YENHSFSQE-R beta fnIII 8
172 PGRSYQFNVKTVSGDSWKTYSKPIFGSV-R beta fnIII 15
TXPDKV- Majority
271 GEPNVVA Novel PTP (24)-Reg.
235 TAPMEV beta fnIII 3
241 TVPDKV beta fnIII 8
259 TKPDKI beta fnIII 15

c


XXXPNXXXIXGXXVLXLXLIGLLVXLVTLL Majority
10 20 30
1 EVNPNVVVISVLAILSILLIGLL--LVTLV Novel PTP (24)
1 TDQDNTSLIVAITV-PLTII--LVLLVTLL Dros. dPTP10D
1 P----LFGAIEGVSA-GLFLIGMLVAVVALL Human PTP-Beta
1 QEEPEMLWVTG-PVLAVILIIILIVIAILLF Rat LAR PTP
IRKRKHSX-XREXXSXXL----SXXRDXPL Majority
40 50 60
85 IRRKKHLQMARECGAGTFVNFPSLERERGKL Novel PTP (24)
82 FYKRRRNN-CRKTT----------KDSRA Dros. dPTP10D
79 ICRQKVSH-GREERPSARL----SIRRDRPL Human PTP-Beta
88 KRKRTHSPSSKDEQSIGLKDSLLAHSSDPV Rat LAR PTP
XXXXSLXQXXXRKXXXPIXIXDFAXHIXXL Majority
70 80 90
175 PYSWSKNGLKKRKLTNPVQLDDFDSYIKDM Novel PTP (24)
136 NDNMSLPDSVIEQNR-PILIKNFAEHYRLM Dros. dPTP10D
154 SVHLNLGQKGNRKTSCPIKINQFEGHFMKL Human PTP-Beta
178 EMRRLNYQTPGMRDHPPIPITDLADNIERL Rat LAR PTP
XADSDYKFSXEFEELKXVGRDQPCXXADLP Majority
100 110 120
265 AKDSDYKFSLQFEELKLIGLDIPHFAADLP Novel PTP (24)
223 SADSDFRFSEEFEELKHVGRDQPCTFADLP Dros. dPTP10D
244 QADSNYLLSKEYEELKDVGRNQSCDIALLP Human PTP-Beta
268 KANDGLKFSQEYESIDPGQQFTWENSNSEV Rat LAR PTP

alternative, or immature, spliceform. The presence of the insert affects the reading frame of the resultant protein such that it is shifted, causing a translation stop signal five codons after the insertion. Perhaps this represents a modification that is seen in some other receptors; the independent expression of the extracellular domain, which might act in a dominant negative fashion if direct coupling of receptor dimerisation to phophatase activity occurs. The third clone is substantially longer and would be expected to extend into extracellular domain-encoding sequence. Analysis of the 5' sequence from this clone showed it had significant homology to fibronectin III domains and thus it is probably part of the extracellular receptor domain. The presence of FN III domains in this gene also fits with the homology to hPTP β which possesses 16 such domains. hPTP β is also distinguished by the presence of only one catalytic domain. Whether PTP 24 possesses one domain remains to be directly elucidated, but the sequence information from the 2 kb clones seems to indicate that there would only be enough 3' coding information for one catalytic domain. In addition, approximately 4kb of sequence is known to exist upstream of the region encoding the transmembrane domain. If the average coding sequence for a fibronectin III domain is assumed to be 270 bp, then there is a potential capacity for at least 14 FN III domains in PTP 24.

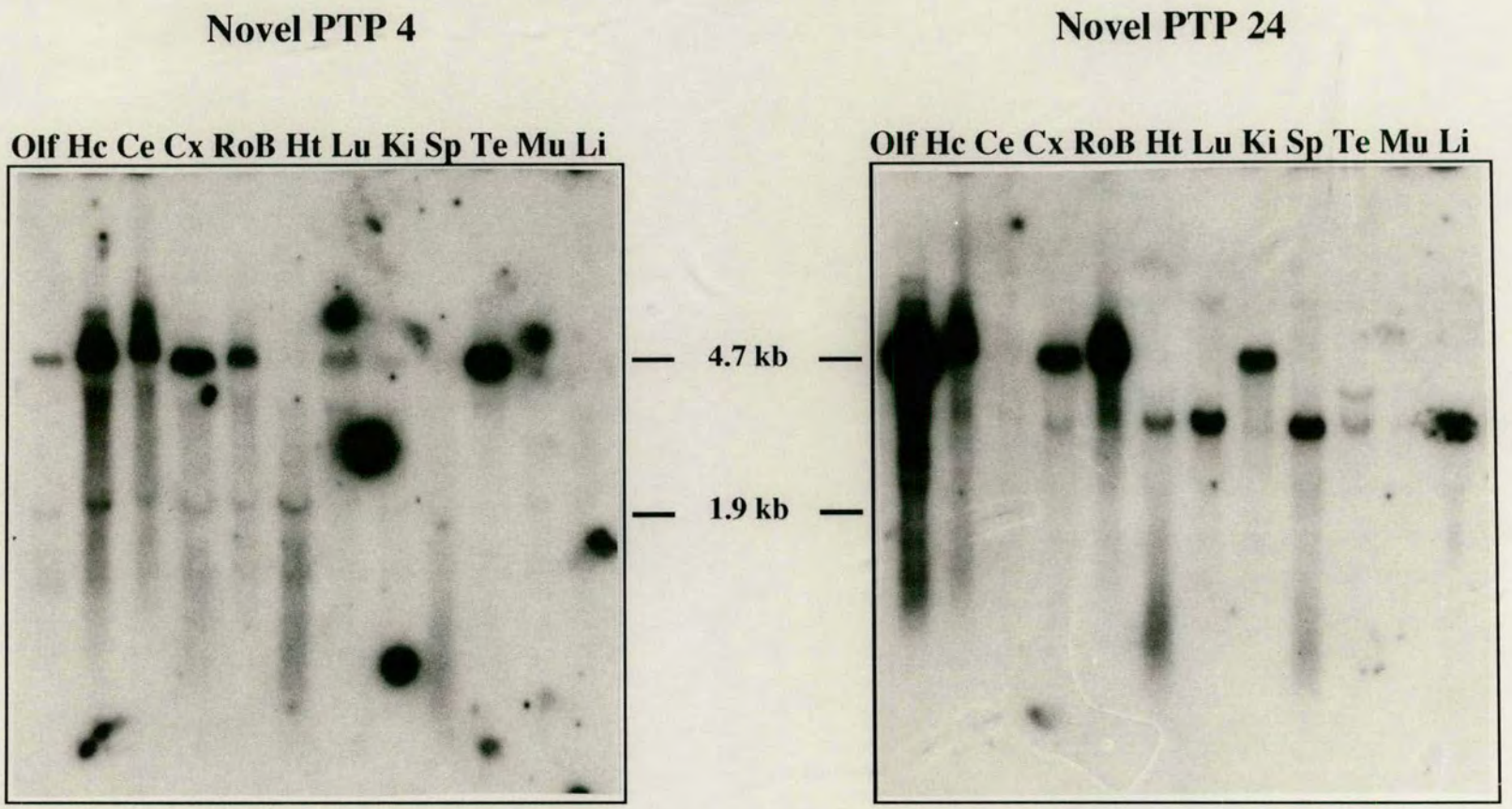
Expression data was collected for these two novel PTPs by using them as probes on Northern blots (fig. 2.8).

PTP 4 demonstrates a major transcript size of approximately 5.5kb (compare μ; 5.7 kb, and κ; 5.3 kb) and is principally localised to the CNS with peripheral expression seen in lung and testis. PTP 24 exhibits transcript sizes in the CNS (not cerebellum) of approximately 6kb, whereas in the rest of the tissues a transcript size of 4 kb or 5 kb is seen. Overall, PTP 24 is less restricted in expression than PTP 4.

No change in expression was seen in either PTP as a result of electro-convulsive stimulus (Chapter V for more information).

Figure 2.8 Northern expression analyses of the two novel members of the PTP family. Positions of the two major species of ribosomal RNA are marked approximately. (Olf; olfactory bulb, Hc; hippocampus, Ce; cerebellum, Cx; cortex, RoB; rest of brain, Ht; heart, Lu; lung, Ki; kidney, Sp; spleen, Te; testis, Mu; muscle, and Li; liver.)

Fig. 2.8


Novel PTP 4
Olf Hc Ce Cx RoB Ht Lu Ki Sp Te Mu Li
4.7 kb
1.9 kb
Novel PTP 24
Olf Hc Ce Cx RoB Ht Lu Ki Sp Te Mu Li

Chapter III

Chemical Cross-Linking Subtraction

3.1: Introduction to the methods available for directly identifying specific gene expression

Although degenerate PCR amplification has many advantages and has had notable successes with respect to the isolation of tissue specific genes, it suffers from the fact that, at best, the choice of gene family is an educated guess. Ideally, in order that a search is not unduly limited, tissue-specific genes should be isolated using techniques which operate purely on the basis of the presence or absence of a particular transcript in the tissue of interest without any prior subjective biasing in favour of certain genes. At present, there exist three techniques which attempt to fulfil this ideal; 'differential screening', 'subtractive hybridisation', and 'differential display'. Each has its own benefits and disadvantages and specific situations in which it would be the optimal choice. These are discussed below.

Furthermore, the study of brain region specificity presents difficulties in its own right which seem to push the operating capabilities of these techniques to the limit. These problems, which were also discussed in the introductory chapter, will be returned to later.

The following two techniques originated 15 years ago when it was realised that the intrinsic complementarity of nucleic acids, which had been utilised in Northern and Southern hybridisations and cDNA-mRNA reassociation kinetics, could be exploited in the comparison of transcript expression in different tissues.

Differential screening

One requirement for this approach is that a cDNA library of the tissue in question be created before any experimental step is taken; a relatively labour-intensive procedure. However, because any resulting genes may have to be characterised by cloning full-length transcripts, the initial work is rewarded at a later stage. If 'tissue X-specific' genes are required, a cDNA library is created from tissue X mRNA. This is plated out such that plaque density is relatively low compared to a conventional library screen.

Duplicate representations of each plate are made on nitrocellulose filters. A probe derived from a tissue X cDNA template is hybridised to one set of filters and, in parallel, a probe derived from a comparable tissue, 'Y', is hybridised to the second set of filters.

The relative strength of hybridisation to a given plaque when probed with X or Y gives an indication of the corresponding transcript's abundance in that tissue. Hence, if the filter-bound plaque DNA gives a positive signal by autoradiography in the X probing but not by the Y probing, it can be assumed that the expression of the transcript corresponding to the plaque is much higher in, or specific to, tissue X.

As with other techniques that will be described below, there are certain problems with differential screening which can affect its efficiency. Because the specific activity of a transcript species within the cDNA probe will be directly proportional to its abundance within that tissue, rare transcripts will probably fall below the level of efficient detection by this procedure. Therefore, tissue-specific genes of low abundance will be missed and transcripts might be erroneously assigned tissue X-specificity if expression in tissue Y is below the threshold. Technical problems centreing on the representability of the probe and the comparabilities of the duplicate screens often complicate interpretation of the final plaque differentials. However, differential screening has often been combined with subtractive hybridisation to increase sensitivity.

Subtractive hybridisation

This is, in effect, the inverse procedure to differential screening. The selection for expression in one tissue and not the other is achieved through solution hybridisation: specific/enriched genes that emerge from the procedure can be cloned and analysed directly. Typically, if tissue X-specific transcripts are required then X cDNAs (the 'tester' or 'tracer') are hybridised in solution to a 10- to 20-fold excess of tissue Y mRNA (the 'driver'). Depending on the choice of subtraction protocols, there will be a method of discarding excess mRNA together with the mRNA-cDNA hybrids, leaving just unassociated cDNA (which is cloned). Theoretically, the reassociation kinetics of the hybridisation step should ensure that the only X cDNA species to remain unassociated to Y mRNA are those which have no complementary counterpart in the Y mRNA; i.e. they are specific to, or highly enriched in, tissue X.

The precise protocols used for subtractive hybridisation have been modified extensively since its original description. The key steps of preparation of the nucleic acid pools, hybridisation, removal of hybrids, and analysis of the products have all been subject to alterations in an attempt to improve the efficiency of the technique. However, the

abundance of transcripts and the representabilty of the tracer and driver are still crucial factors in the subtraction process.

Differential display

This involves the comparison of electrophoresed subsets of PCR-amplified cDNA (refs. 1 and 2). RNA is extracted from different tissue types or one tissue in different states. This is reverse transcribed using a primer such as:-

$$5'\text{-[anchor]-}(T)_{12\text{-}15}\text{-XY-}3'$$

where 'anchor' is any defined sequence, 'X' is any of the bases A, C, or G and 'Y' is any of the bases A, C, G, or T. Unlike conventional reverse transcription, only a subset of transcripts can be initiated by any one such primer (1/12 of the total assuming equal base usage). A PCR reaction is performed on this material using an arbitrary 10-mer oligonucleotide together with a primer identical to the anchor sequence. To create an amplifiable product, there has to be complementarity between the arbitrary primer and a region on the cDNA near to the 3' end of the transcript; again, this selects a further subset of transcripts from the total. The resulting amplified and radiolabelled products are electrophoresed on a polyacrylamide gel so that the spectrum of PCR products derived from one tissue can be compared to that in another by looking for changes in band intensity at a equivalent sizes. The two factors determining the successful application of this technique are, first, the display spectrum from a sample should be reproducible and, second, the differences between two samples should represent genuine expression differences and not amplification biases/incomparable representation. By using different combinations of reverse transcription primers and arbitrary PCR primers a complete expression profile can be approached. Many variations have been applied by different groups (ref. 3) but the overall strategy remains the same.

The major limitation to this technique is that cDNA fragments corresponding to rare transcripts will not be visualised by autoradiography due to their low abundance. In many situations this is irrelevant (ref. 4), but in the context of brain region-specific expression it might prove a problem. An advantage is that because PCR is involved, the availability of large amounts of starting material is not a requirement.

3.2: Historical application of these techniques

Numerous applications of these three techniques to a wide variety of biological systems have been described over the past 15 years. Early approaches were straightforward

differential screens with the focus more on proving that transcriptional differences existed rather than identifying molecules. Shiosaka and Saunders (ref. 5) looked at transcription in leukaemic and normal lymphocytes and obtained several cDNA clones which appeared to be upregulated in the former. More recent attempts such as Anderson and Axel's screen for transcriptional differences in neural crest derivatives (ref. 6) and Oberdick's screen for cerebellar purkinje cell-specific cDNAs (ref. 7) resulted in the cloning of specific genes but, in all likelihood, these experiments were limited to the detection of medium abundance transcripts. Beadling et al. (refs. 8 and 9) have tried to circumvent this problem by artificially boosting transcriptional changes in IL-2-stimulated T cells by the inclusion of translational inhibitors; thus preventing feed-back deregulation of induced genes by their protein products.

Subtractive hybridisation by itself, or in tandem with differential screening, has been the most frequently used approach in recent years. One of the earliest examples of its use was Sargent and Dawid's analysis of expression in the Xenopus gastrula (ref. 10) which used hydroxyl-apatite columns to remove hybrids between gastrula cDNA and placental mRNA, allowing the cloning of gastrula-enriched cDNAs. The resulting library was differentially screened with cDNA obtained from the gastrula and other developmental stages to confirm that many were gastrula-specific. The cloning of the T cell receptor (ref. 11) and the myoblast determining factor, MyoD, (ref. 12) were achieved in an almost identical fashion by comparing expression in T and B cell lines and myoblasts and fibroblasts, respectively. Modifications since then have been primarily alterations in the precise details of the protocols either to increase sensitivity or reduce technically difficult/laborious steps.

Because subtraction requires large quantities of driver mRNA, it is impractical in some biological systems. Therefore, one approach (refs. 13-15) has been to convert tester and driver tissues into cDNA libraries, from which unlimited mRNA can be made by *in vitro* transcription from RNA polymerase initiation sites within the vector. *Drosophila* head-specific genes and a floor-plate-specific gene (F-Spondin) have been cloned in this manner. Another approach to this problem is to convert the two 'tissues' into pools of PCR-amplifiable cDNA; this has been used by Duguid et al. in the search for scrapie infection-responsive genes (refs. 16 and 17) and by Wang and Brown (see next chapter). The extent and rate of hybridisation can be increased in some cases by the use of the 'phenol emulsion reassociation technique' (PERT) by which, through a still-unexplained phase-boundary effect, hybrids form more readily. Travis et al. (ref. 18) applied this to the transcriptionally complex monkey brain for the isolation of cortex-enriched genes.

Separating hybrids from non-hybrids has been facilitated by a wide selection of techniques. Driver nucleic acid can be modified by photobiotinylation (refs. 19-21) or by the addition of magnetic beads (refs. 22 and 23) and the resulting hybrids and excess driver removed by streptavidin/phenol-chloroform or magnetism, respectively. Alternatively, hybrids can be enzymatically destroyed by double-stranded DNA-specific exonucleases (ref. 24).

As detailed in the introduction, with respect to gene expression, the brain is the most complex of all tissue types. Have subtraction techniques performed as well in the brain as in other systems, and have examples of brain region-specific expression been found?

Rhyner et al. (ref. 25) isolated several forebrain-enriched clones in a subtracted library screen using subtracted forebrain cDNA as a probe and claimed that some were present at an abundance level of 0.0005% (differential screening alone has a sensitivity down to an abundance of approximately 0.05%; ref. 26). However, the very fact that this probe has had abundant sequences removed by the subtraction procedure will increase to detectable levels the specific activity of cDNAs corresponding to rarer mRNA species: this does not mean that they are specific. To control for this, the subtracted library should also have been differentially screened with subtracted probes from other brain regions. The same group have also, more satisfactorily, examined changes in gene expression in the forebrain after sleep deprivation (ref. 27). Several clones showed altered expression levels, albeit by only a factor of two or less. The isolation of cerebral cortex-specific genes in the monkey (ref. 28) by the use of subtracted probes proved to be equally challenging with an initial isolation of 163/60,000 clones showing cortical enrichment that later proved less conclusive by Northern hybridisation assessment. A maximum of two-fold enrichment was seen over other brain regions. The protocol relied solely on cerebellum mRNA as the driver in the subtraction, hence, the probability that a cortex-specific gene would have been obtained was decreased. Much less was demanded of a subtraction carried out to enrich for brain-specific sequences (ref. 29). In the completed library 55% of the clones showed brain-specificity or high enrichment, but when it is considered that a random selection of brain clones carried out by Milner and Sutcliffe (see Chapter I) revealed that approximately 30% were restricted to the brain (admittedly, the search for other sites of expression was not exhaustive), the result is not so impressive. Kato (refs. 30 and 31) screened 950 rare cDNAs from a mouse cerebellar cDNA library. By *in situ* hybridisation, 130 appeared to show some restriction to brain regions and, of these, 5 were identified as being cerebellum-specific; 4 granule cell-specific and one purkinje cell-specific. Perhaps this work (in conjunction with that of Oberdick) reveals that the cerebellum is sufficiently different in structure and function to require specific transcription.

In comparison, more success has been observed when subtractive technologies have been employed in the search for genes induced by neuronal activity: a description of such experiments is to be found in Chapter V.

In conclusion, subtractive techniques have met a severe obstacle in the form of brain transcriptional complexity that has resulted in limited success in the search for region-specific expression.

3.3: Chemical cross-linking subtraction

The first subtraction method used to search for hippocampus-specific genes was based on a technique devised by Hampson, Pope, Cowling, and Dexter (ref. 32). In short, a subtracted probe is synthesized which can be used in a differential screen to select for tissue-specific gene expression.

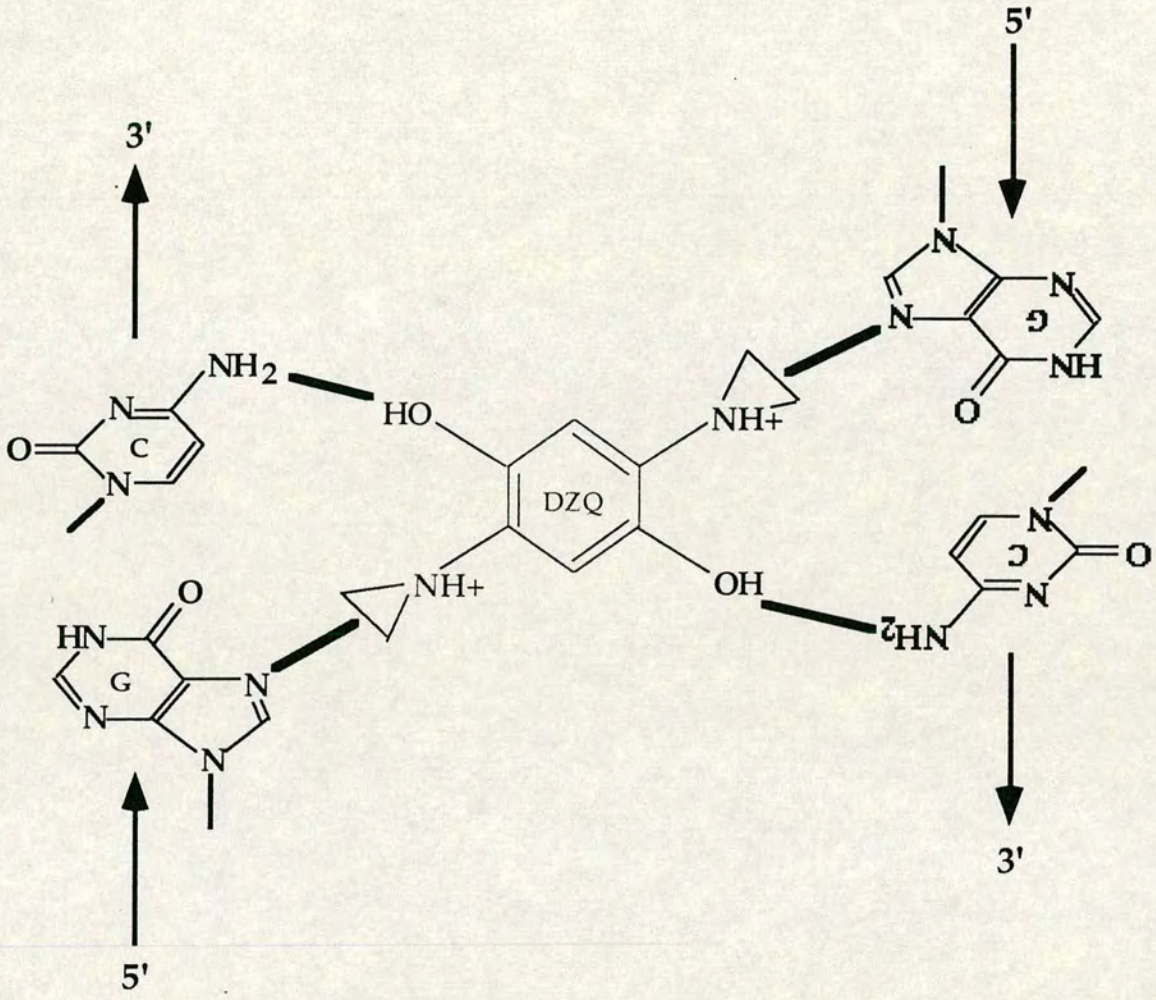
Potentially, the removal of excess driver and driver-tracer hybrids by the traditional methods described in the introduction can be inefficient or result in the loss of much tracer material required for cloning or probe synthesis. 'Chemical cross-linking subtraction' (CCLS) circumvents the need for physical separation of this material from unhybridised cDNA. This is made possible by use of the reagent diazirydinyl benzoquinine (DZQ) in reducing conditions to irreversibly cross-link -GC- dinucleotides in one strand of the duplex with opposing -GC- dinucleotides in the other strand (refs. 33 and 34).

Fig. 3.1 shows a representation of the reaction that occurs at a -GC-/-GC- dinucleotide site on the mRNA/cDNA hybrid when the the cross-linking agent is added in reducing conditions. The reactive groups on DZQ react with each of the four bases forming covalent bonds which prevent the separation of the two strands. This compound has been used as an antitumour drug, although the reaction pathways that cause its *in vivo* tumouricidal effects are probably different from the one described here.

The post-subtraction nucleic acid material is purified and used directly as template for a random-primed probe synthesis reaction. Only uncross-linked cDNA can act as a template for the 'Sequenase II' enzyme (product of United States Biochemicals) in the probe synthesis reaction; thus, the probe is enriched for sequences not found in the driver mRNA. This particular enzyme is employed because it cannot use RNA as a template, has no exonuclease activity, and will not displace a presynthesized strand from template cDNA. One advantage of such a technique is that if hybridisation between cDNA and RNA is only over a short length of the cDNA (for example, due to repeat elements, alternative splicing, or a tract of one particular nucleotide) then a probe

Figure 3.1 Cartoon illustration of the structure of the diaziridinylbenzoquinone (DZQ) cross-linking agent and the manner in which it irreversibly interacts with guanine (G) / cytosine (C) dinucleotides in complementary nucleic acid strands.

Fig. 3.1



can still be made from the unhybridised regions. A more traditional technique would remove such hybrids completely.

In the original paper describing the technique, an enrichment factor (defined as the abundance of a particular transcript's component in a probe after subtraction, divided by the same transcript's abundance before subtraction) of 240-300 fold was quoted for an amitozolomide-induced transcript from RJKO cells. Extrapolating from information concerning probe hybridisation sensitivity limits mentioned in the introduction to this chapter, a transcript originally present at an abundance of 0.00017-0.00021% would be detectable by this method.

CCLS has been recently used in the investigation of genes induced by the tumour suppressor p53 (ref. 35). A library created from p53-induced cDNA was screened with a subtracted probe (induced cDNA subtracted with normal mRNA). Of 99 clones that appeared to be differentially expressed, 45 were genuine. 28 of these encoded the same transcript; subsequently dubbed 'WAF1'. Although the authors state that the subtraction causes a one hundred-fold enrichment for WAF1, they also admit that the transcript constitutes 0.4% of the mRNA post-induction. Because this is such a high figure (coupled with the non-neuronal nature of the tissue), tthe subtraction procedure was not taxed by this experimental system.

3.4: Application to the identification of hippocampus-specific transcripts

Previous work in this laboratory has focused on the application of a differential screening technique to the identification of specifically expressed genes. This involved the probing of duplicate filter lifts of a rat hippocampus cDNA library (constructed by J. O. Mason and M. Richardson in the laboratory) with either hippocampus or 'rest of brain' (brain minus hippocampus) cDNA probes. Differentially hybridising plaques were selected and studied further as described (G. Stapleton; thesis, 1994 and ref. 36). Not all plaques were studied in depth, so a technique was required which would be able to select the best candidates for further analysis. A combined subtraction and differential screening approach was used as a means to this end.

A subtracted hippocampus probe for use in the differential screening process was created by the method of chemical cross-linking. The precise protocol is described in the Materials and Methods chapter, but briefly, 0.38 μg of hippocampus cDNA was mixed with 20 μg of non-hippocampus brain tissue mRNA and hybridised for 20 hrs. The resulting material was cross-linked and aliquots taken for probe synthesis reactions. In an attempt to quantify the success or otherwise of the subtraction process a 'spike' was added to the hippocampus cDNA. A ~270 bp fragment of the SV40

polyadenylation sequence was chosen as the spike because it is not found in brain mRNA. This was transcribed in vitro and then reverse transcribed such that single-stranded cDNA resulted. Enough of this material was added to the hippocampus cDNA such that it constituted one ten-thousandth of the total cDNA content. This figure was chosen because the quoted enrichment factor of this subtraction protocol would ensure that only its post-subtraction signal would be of suffient specific activity to generate detectable hybridisation. Another efficiency assay carried out was the monitoring of a ubiquitous transcript (actin, in this case) which should be reduced in specific activity after the subtraction procedure.

The probe was used on a 'mini-library' created by pooling the plug suspensions of the 436 remaining positive plaques obtained by the original differential screen. Because each plug consisted of multiple bacteriophage species (estimated at 10-20) due to particle diffusion and the relatively high plating density of the screen, the total library was made up of approximately 9000 cDNA species. This was plated onto 5 bottom agar plates such that there were in the order of 8000 plaques per dish; thus giving an approximate 4.6 times representation.

Duplicate transfers of this library were made on nitrocellulose filters which were probed with an aliquot of the subtracted hippocampus probe or with a cDNA probe derived from rest of brain mRNA. By comparison of the images of hybridised plaques on X-ray film, it was possible to select 44 plaques which appeared differential: they showed hybridisation to the subtracted probe but not with the rest of brain probe.

Filters spotted with SV40 polyadenylation sequence DNA and actin DNA were hybridised at the same time as the differential screen. It was evident from the lack of hybridisation signals that the subtracted probe had not been enriched to a degree whereby the SV40 spike was detectable above background hybridisation. The actin signal appeared to be slightly diminished (data not shown).

These plaques were individually replated at low density in order that a 'secondary screen' could be carried out to reveal well-isolated differential plaques that consisted of a single cDNA clone. Because of the failure to replicate some differential signals in this screen, the number fell to 36 cDNA clones.

Due to the nature of the bacteriophage vector used in the construction of the library ('Lambda Zap II'; Stratagene), it was possible to rescue the cDNA clone in the more manipulatable form of 'pBluescriptII' plasmid by '*in vivo* excision' from plaque plug suspensions using a 'helper' bacteriophage strain and a bacterial line; SOLR (see Materials and Methods chapter). In addition, the construction of the library had been

carried out in such a manner that the cDNAs were mostly cloned in one orientation in the vector. The initial sequencing of the clones was performed using the T7 primer because, as the vector was in the KS configuration, this resulted in sequence information being obtained from the inherently more informative 5' end of the cDNA clones. Table 3a lists the 36 clones with information concerning insert size (estimated from restriction digests of the clones) and sequence identification. The clone numbers are prefixed by 'sub' to indicate that they came from this screen. Those that gave polyA$^+$ or repeat motifs with T7 sequencing were subjected to T3-primed sequencing.

In all, 13 of the 36 clones were assigned positive identifications on the basis of their sequence data and these are discussed below.

Of these 13 clones, 10 different transcripts were represented (super-oxide dismutase-2 (SOD-2) appeared twice and synaptosomal-associated protein-25 (SNAP-25) three times). The clones can be split up into three categories as follows:-

1) Mitochondrial genes (cytochrome b, clone sub8, clone sub25, and cytochrome c oxidase III).

2) Metabolic genes (fructose-1,6-bisphosphate aldolase A, and glyoxalase-1).

3) Others (SNAP-25, a novel Zn-finger transcription factor family member, SOD-2, and inhibitory insulin-like growth factor binding protein 4).

The first group represents clones which obviously cannot be differentially expressed as every cell must possess a complete complement of these genes to be functional. The question then arises how they appeared as differentials. One possibility which might explain this anomaly is that if the concentration of a cDNA is over a certain threshold, it might escape complete subtractive removal despite the presence of driver sequences at 10-20 times concentration. This doesn't explain how the 'rest of brain' probe signal was under-represented causing a differential to exist. Localised differences in the efficiency of filter hybridisation could possibly be responsible for this.

The second group, the metabolic enzymes, might possibly be expressed at different levels in different regions of the brain in order to satisfy different biochemical requirements. Among organs, the brain is particularly metabolically active, and within it some regions may be more active than others; especially in certain situations. This has been demonstrated by PET scans of the brain performing specific tasks.

Table 3 a The analysis of all of the subtraction clones is summarised in this table. R>H indicates that the rest of brain (RoB) abundance was greater than the hippocampus (Hc) abundance as observed by Northern expression data. 'mito.' indicates that the sequence is mitochondrial in origin. A dash indicates that the expression was not examined.

Table 3a

Clone Number	Secondary Diff.	Insert Size (kb)	T7 Sequence	T3 Sequence	H/R Northern
1	2	3	tandem repeat elements	poly A	-
2	3	1	SNAP-25	-	-
3	3	0.5	line/B2 element	repeat	-
4	2	0.5	mito. gene (cytochr. B)	-	-
5	2	2	Aldolase	-	-
6	2	1	line element	-	-
7	2	0.1	line element	-	-
8	2	0.7	poly A	mito. gene	-
9	1	1	EST	-	equal
11	2	1.2	no homology	-	R>H
12	2	2	no homology	-	R>H
13	1	0.5	poly A	no homology	equal
14	2	1	line element	-	-
16	2	1	prob. B1 element	-	-
18	3	4.5	zinc finger homology	line element	equal
19	3	-	repeat	no homology	-
20	3	0.1	repeat	no homology	-
21	4	0.2	no homology	poly A	equal smear
22	2	0.8	SNAP-25	-	-
23	3	0.1	same as 3	-	-
24	3	0.5	mito. D-loop	poly A	equal
25	2	0.2	poly A	mito. gene	-
26	2	1	repeat	-	-
27	2	1	poly A	poly A	equal smear (Apa I)
28	5	0.2	SOD-2	poly A	-
29	2	1	glyoxalase I	-	-
30	2	2.3	poly A	poly A	equal smear (Apa I)
31	2	0.1	no homology	-	R>H
32	2	0.1	cytochr. ox. III	-	-
33	3	3	poly A	poly A	equal smear
35	2	1	SNAP-25	-	-
38	3	2	repeat	-	-
39	2	2	repeat	-	-
41	3	0.5	rat homol. inIGF-BP	-	-
42	3	3.5	no homology	-	equal smear
43	3	1.5	SOD-2	-	-

Aldolase A (ref. 37) is a constituent enzyme of the glycolytic pathway. Therefore, it is unlikely to be expressed in an absolutely polarised fashion in the brain, although it could have relatively higher expression in the hippocampus because of this brain region's high metabolic rate. Another factor which could explain its failure to be subtracted will be discussed in the analysis of this and subsequent subtractive experiments. It can be appreciated that by the synthesis of different splice-forms of a transcript (each with different stabilities, cellular localisations, or encoded functions), great diversity can be created from a small number of genes. Alternative transcripts are seen as a result of differing transcription start-sites, differing polyadenylation sites, and the incorporation of different exons. This presents an alternative possibility in the search for hippocampus-specific transcripts; perhaps the specificity might not reside in the absolute expression of a gene, but in its structural composition resulting from processing undergone by the immature transcript. Aldolase A

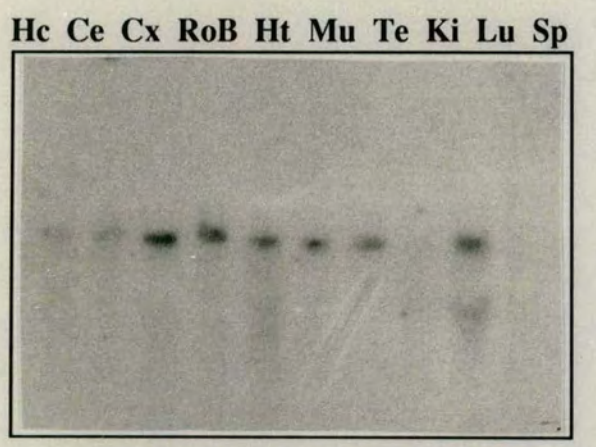
has been described as existing in three transcript forms which arise from alternative transcript initiation sites. Forms I, II, and III possess successively more upstream sequence as ascertained from primer extension experiments. The cDNA clone (sub5) that was obtained from this screen has a 5' end which corresponds to 5' untranslated sequence that could be derived from either mRNAs II or III. In an attempt to examine whether one of the Aldolase A transcripts is expressed preferentially in the hippocampus, a probe derived from sub5 was used on a Northern blot of brain regions (fig. 3.2). No obvious differences were seen in the expression levels or the transcript sizes caused by these splice-forms (admittedly, the small differences in size of the three forms of Aldolase A transcript might not be discernible by Northern blot).

The other metabolic enzyme cloned was the rat form of human glyoxalase-1 (~80% identity at the nucleic acid level: ref. 38). This is an enzyme which catalyses the transformation of methylglyoxal and glutathione into S-lactoylglutathione, a reaction whose significance is not entirely understood, but which probably plays a part in the detoxification of glyoxal derivatives as well as the metabolism of cellular α-ketoaldehydes. High expression is seen in tumours and in undifferentiated cells. Therefore, it seems that the expression of this gene can be regulated to some extent in different circumstances, but whether there is tissue-specific expression has not been examined elsewhere. The clone isolated from the screen (sub29) has a 5' end which starts at the beginning of the mRNA open reading frame and probably extends to the transcripts 3' end. This was used as a probe on a Northern blot (fig. 3.2) which revealed no tissue specificity within the brain.

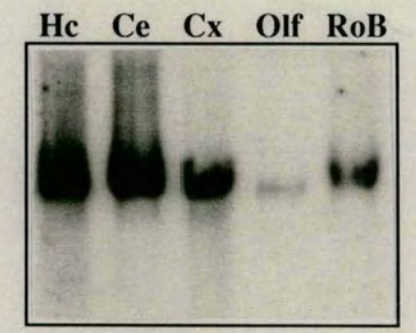
Figure 3.2 Tissue expression patterns of clones obtained from the subtraction. The panels on the left-hand side of the page show the virtually ubiquitous expression of sub18 (ethidium-stained gel below to indicate RNA loading comparability). The right-hand panels show the brain region distributions of other 'sub' clones (Aldolase A, Neuraxin, Glyoxalase-1, and Superoxide Dismutase-1). Note the smearing effect in some cases caused by the presence of repeat sequences within the probes.

Fig. 3.2

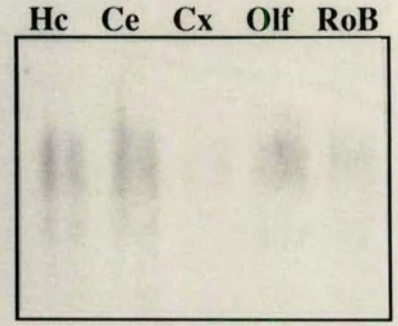
Sub 18: Novel Zinc-Finger Transcript



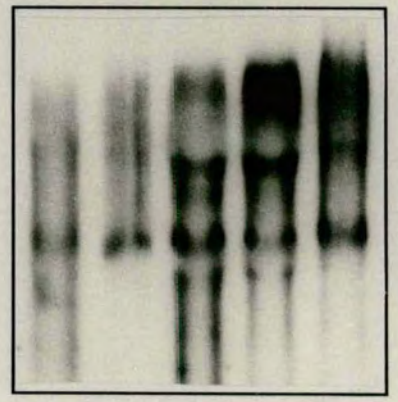
Aldolase A



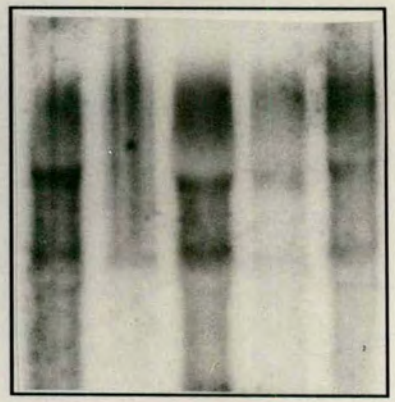
Neuraxin



Glyoxalase-1



S.O.D.-2



It was the final group which represented the type of transcript which, at face value, might be most likely to be differentially expressed.

SNAP-25 was identified (ref. 39) as a major protein component of synaptosomal preparations of hippocampus tissue. Its presynaptic localisation in selected brain regions has made it the focus of studies into the mechanisms responsible for neurotransmitter release. The 25kD protein that results from translation of the message does not seem to be membrane spanning but instead has an amino terminal helix that may permit association with the membrane. Its structure suggests that it may coordinately bind metal ions. Recent work has gone some way to defining the cellular functioning of SNAP-25. It appears to associate with another protein, syntaxin, such that together they mediate the docking and priming of synaptic vesicles at the presynaptic membrane by binding a vesicular protein, synaptobrevin (ref. 40). In terms of the relevance to these studies, the expression pattern is probably not suitable for further experimentation. High levels of expression are seen in the hippocampus (especially in the CA3 and dentate gyrus regions), but there is also expression in neocortex, piriform cortex, anterior thalamic nuclei, pontine nuclei and the granule cell layer of the cerebellum. Northern analysis does not show any evidence for different splice-forms. Of the three clones isolated, sub2 and sub35 are identical, commencing immediately downstream of the ORF start, whereas sub22 starts upstream but does not seem to be long enough to encode the full ~2 kb transcript. This is probably the result of internal EcoRI/NotI sites or cDNA fragmention prior to ligation to the lambda ZAPII vector. Coincidentally, this gene was the only one cloned in common with the initial differential screen (G. Stapleton; as above). The high levels of expression reported in the hippocampus might explain the failure to remove this cDNA during the subtraction process.

A novel zinc-finger protein (sub18) was isolated as a 4.5 kb clone. When sequenced from the 5' end, it appeared to correspond to an ORF containing the stereotypical cysteine and histidine residue pairs responsible for the coordinate binding of a zinc metal ion. The sequence shows some frame-shifting in this region which does not simplify the interpretation of the resulting amino acid sequence. This could be due to the cloning process, although it must not be ruled out that this clone is a pseudogene. However, the observation of a transcript on a Northern blot argues against this (fig. 3.2). At the nucleic acid level, sequence similarities of 52% and 62% are obtained between the novel transcript and the most closely related members of this family (h-plk and znf20x, respectively; ref. 41). Fig. 3.3 shows the best fit amino acid sequence of the novel clone (deduced by comparison with other members of the zinc-finger transcription family and therefore to be treated with caution) aligned with the related

zinc-finger genes (see Appendix for all sequence obtained). Homologies over this region at the amino acid level are 45% and 52% . The role of transcription factors is to regulate the expression of other genes. The cloning of a hippocampus-specific transcription factor would go some way in the comprehension of the mechanisms that dictate this brain region's identity and profile of gene expression. Unfortunately, the comprehensive analysis of the distribution of this transcript seems to suggest that it is widely expressed. Because the full-length sequence was not be used as a probe due to the presence of repeat sequences, hippocampus-specific regions of the transcript could possibly have been absent from the actual probe fragment. However, no alternative transcript sizes were observed on the Northern blot.

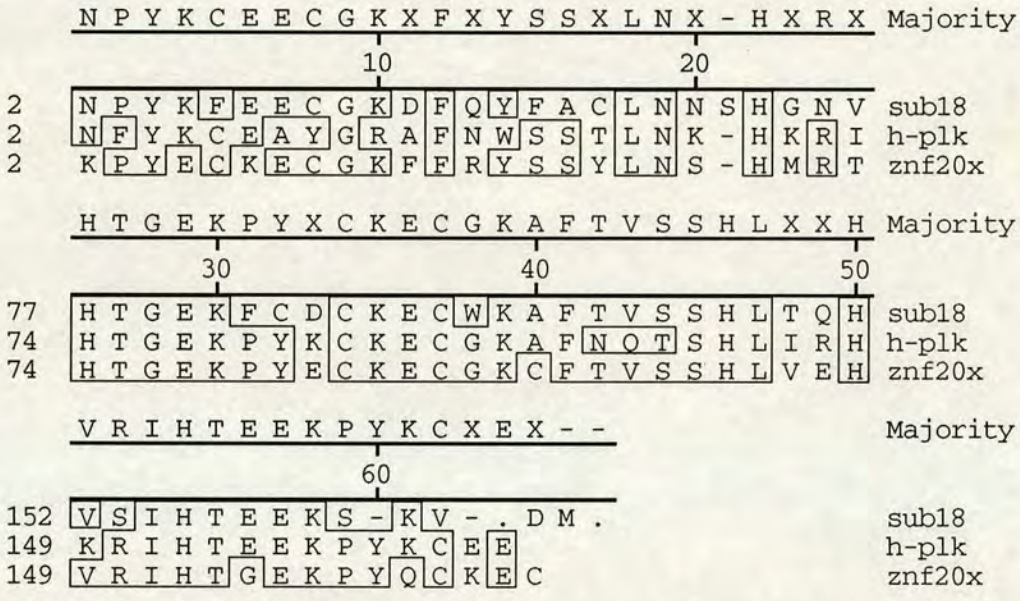


Figure 3.3 Alignment of 'best-fit' amino acid sequence of sub 18 with two closely related zinc-finger proteins. Note conserved cysteine and histidines.

Two separate clones were identified corresponding to the enzyme superoxide dismutase-2, which is a manganese-containing protein responsible for the deactivation of oxygen free radicals (ref. 42). These radicals are highly toxic and mutagenic to a cell and so must be removed with high efficiency (ref. 43). They occur as by-products of the action of cytochrome P-450s in the respiratory chain of mitochondria; the cellular compartment where this protein is found. Because the excitational activity of neurons results in those cells being subject to 'oxidative stress' efficient mechanisms have to be present to counteract the harmful nature of by-products such as superoxide and other free radicals. Left alone, these highly reactive species can mutate DNA (especially that in the mitochondrion, which can be 15 times more oxidised in aged humans) and damage cellular machinery. The originally published sequence for this gene identified 5

exons present in a region of genomic DNA almost 10 kb in length. Ref. 42 describes the presence of 5 differently-sized SOD-2 transcripts on a liver tissue Northern blot. To prove that the heterogeneity was due to the use of different polyadenylation signals, they used successively more downstream (3') genomic fragments as probes on these Northern blots. As expected, the increase in fragment distance from the translated region of the clone resulted in the hybridisation to fewer and fewer bands; those bands being of increasing size. Therefore, a SOD-2 transcript can be generated by the utilisation of any one of a number of polyadenylation sites culminating in differing transcript sizes. Two clones corresponding to this gene were identified here. Sub43 (~2 kb in size) commences at the 5' end of the final exon (5) and sub28 commences approximately 800 bp downstream of this exon. It was a possibility that one or both of these clones were selected because of the precise polyadenylation site used. For instance, the hippocampus, in comparison with other brain regions might use a distal site such that this region of the cDNA would only be found in this brain region. This hypothesis was tested by the use of clone sub43 as probe on a brain region Northern blot. The result was not entirely conclusive because of the presence of repeat sequences within the probe, but there seem to be only two transcripts which show virtually ubiquitous expression (fig. 3.2).

Inhibitory insulin-like growth factor binding protein-4 was the final clone to be identified on the basis of sequence homology (ref. 45). In general, this family of proteins binds to IGF, prolonging its cellular half-life. In cell culture, this binding can either be stimulatory or inhibitory to the growth-promoting effects of the IGFs. In the hippocampus there are numerous growth, neurotrophic, and survival factors that play roles in the development and function of this brain region. IGF-1 has recently been shown to stimulate oligodendroglial myelination of CNS neurons and may be used as a future therapeutic agent for demyelinating diseases such as multiple sclerosis (ref. 46). A model whereby IIGFBP modulates this activity in a positive or negative manner could be postulated. The paper describing the cloning of this particular gene showed Northern expression data demonstrating that its transcript was present in cortex and hypothalamus amongst other tissues and organs. The cDNA identified in the screen (sub41) has a size of approximately 500 bp and by sequence analysis of the clone's 5' end, seems to represent the 3' portion of the published transcript. It seems likely, therefore, that this does not represent an alternatively spliced form.

Most of the clones that were analysed by the database-searching program FASTA (GCG 7 and 8. Wisconsin DNA analysis package) did not show meaningful homologies to any known genes. In addition, a sizable fraction of the clones contained

repeat element sequences, repetitive sequences, or polyadenylation stretches at both ends. Such repeats included the typical B1/LINE elements (e.g. sub/6/14) as well as more unusual repeats (sub19/20/26 possessed $[CT]_n$ and $[AC]_n$ dinucleotide repeats and seemed to be identical). It is not certain whether the transcripts which contain these repeats were genuinely differentially expressed or merely isolated because such repeat domains are intrinsically refractory to subtraction.

The presence of repeat regions in these transcripts made their expression analysis difficult. In many cases the result of Northern hybridisation with a library-derived clone as probe was non-specific hybridisation at all molecular sizes in both hippocampus and rest of brain RNA lanes. In a largely unsuccessful attempt to alleviate this problem, restriction digests were carried out on a number of these clones in an attempt to obtain subfragments which would be more suitable for use as probes on Northern filters.

Table 3a lists those clones that were used as probes on these Northerns together with the expression patterns obtained. These experiments were carried out in many cases by using small strips of nitrocellulose filter onto which had been blotted hippocampus and rest of brain RNA (as described in the methods section) in denaturing conditions. It must be noted that these Northern strips were not intended to provide definitive expression data, but were a fast and efficient screen for those clones selected. In several cases, a more extensive survey of expression was performed (see fig. 3.2).

All clones for which expression data is available (table 3a) displayed either repeat-indicating smears or an expression pattern that indicates that the probe sequence was represented equally in all tissues (or, in three cases, more expression in the rest of brain than in the hippocampus).

3.4: Discussion of the results of this subtraction experiment

This subtraction did not yield hippocampus-specific cDNAs. There are three hypotheses that can be made as to the reasons for this failure which will be dealt with in turn.

Firstly, the subtractive enrichment might not have been accomplished to the required degree. The failure to detect the spiked cDNA by hybridisation indicated that this subtraction's efficiency was not as great as the one published. However, because the brain is transcriptionally complex and hippocampus-specific gene expression might only occur in rare transcripts, the assumption that the enrichment factor would be as high as that quoted in the original publication of this protocol was probably over-optimistic. The cloning of 'housekeeping genes' such as the metabolic enzymes is a

failure to some extent, but several groups trying differential screening-based approaches have reported that these high-abundance transcripts are never fully removed from a subtracted probe (R.S.P. Beddington, personal communication). RNA as a driver is always vulnerable to enzymatic degradation, especially in this example where the hybridisation time was 20 hours. Sufficient degradation of the driver pool would result in the failure to subtract adequately.

Secondly, in the introduction it was stressed that this subtraction would allow hippocampus-specific regions of a transcript (possibly due to alternative splicing) to contribute towards the final probe unlike traditional subtraction protocols. Because the rest of brain probe in the differential is not discriminatory, it will hybridise to the 'common' regions of a cDNA clone even if it has tissue-specific segments, thus, obscuring the differential nature of such a transcript.

Lastly, the mini-library used in this screen was made from positives obtained from a theoretically less sensitive procedure. Hence, flaws in the original selection and the failure to detect low abundance, yet specific, transcripts could not be corrected by the CCLS procedure.

It was with this possibility in mind that the decision was made to carry out a small-scale probing of the original unselected rat hippocampus cDNA library (one tenth of the library because of the lower plating density necessitated by a differential screening method). 92,000 plaques were transferred to duplicate filters and screened with a subtracted probe and 'rest of brain' probe as before. From this, 12 differentials were selected and subjected to sequence and Northern blot analysis. Six transcripts of unidentified sequence were used as probes on Northern blots (data not shown) but these did not show any hippocampus specificity. Two clones were positively identified from database searches as neuraxin (ref. 47) and the 110kD subunit of phosphatidylinositol-3-kinase. The clone for neuraxin was over 4 kb in size with a 5' end corresponding to the start of the 3' untranslated region of the published sequence. Because this published sequence did not amount to more than 600 bp in the 3' untranslated region, there existed the possibility that this might represent an alternative splicing or polyadenylation site use. This was tested by using the clone as a probe on a Northern blot (fig. 3.2). As can be seen, despite the presence of repetitive sequences within the probe which cause the streaky appearance, there are no hippocampus-specific transcripts visible.

The results of this limited experiment seem to suggest that the low efficiency of the subtractive enrichment is the key restriction to the application of this technique to the isolation of hippocampus-specific genes.

Chapter IV

Application of a Second Subtraction Technique to the Search for Hippocampus-Specific Genes

4.1: A novel subtraction technique

The chemical cross-linking protocol for subtraction described in the previous chapter has advantages over many approaches. These include the relative ease of the procedure, the ability to identify subtle changes in the arrangement of a transcript and the high enrichments reported to be obtainable. However, in the context of the search for transcripts restricted in expression to the hippocampus it did not prove effective.

The two fundamental criteria for the selection of an alternative approach were that it should result in a much greater extent of enrichment for appropriate transcripts and that the resulting subtracted material should be directly analysable (as opposed to being used as a probe to screen a cDNA library) so that, if there are particular regions of a transcript that are specific, only those will remain.

A 'Gene Expression Screen' technique published by Z. Wang and D.D. Brown (ref. 1) seemed to fulfil the above criteria. The technique they described was applied to the isolation of genes whose expression was altered in tadpole tail tissue after treatment with thyroid hormone; the physiological signal in maturing tadpoles for the resorption of the tail. 16 different up-regulated genes (including thyroid receptor β) and 4 down-regulated genes were obtained from this screen.

It is necessary to go into some of the technique's experimental details so that there is a framework for the interpretation of the results The full protocol is described in the Materials and Methods chapter and a flow chart of a modified form of the protocol (section 4.7) can be found in fig. 4.3 a and b. The seven features responsible for the increased performance of the subtraction are as follows:-

1) The two mRNA extracts from the tissues to be compared (whether they be different tissues, as in this case, or the same tissue before and after a particular treatment) are

converted into double-stranded cDNA, thus avoiding the problems accociated with the manipulation of RNA.

2) Each cDNA pool is divided in two. One half is digested with the restriction enzyme AluI and the other with AluI and RsaI combined. These enzymes, by virtue of their four base-pair recognition sequences, cleave DNA frequently; on average, at 256 base-pair intervals. This results in most cDNAs being split into several smaller fragments which can behave independently of each other in the subsequent subtraction. Wang and Brown describe these fragments as 'alleles' stressing the similarity of this technique to genetic screens. In those instances where alternative transcript regions may be preferentially expressed, corresponding cDNA fragments should behave independently of the ubiquitously expressed regions. This would also have the benefit, as in the case of the chemical cross-linking protocol, that the entire length of a specific transcript is not removed if a part of it contains repetetive sequence or sequences refractory to enrichment. In addition, if there is a transcript that is differentially expressed in the two pools then all cDNA fragments derived from it should be represented in the final subtracted material. This presents a way of verifying that a transcript is genuinely differentially expressed.

3) Oligonucleotide linkers were ligated onto the cDNA fragments from each pool in order that the cDNA material could be amplified using a PCR primer complementary to the linker. A potential drawback to this step is that, in order to be amplified efficiently and representatively, the cDNA fragments must be below a certain length. Thus, the frequency of AluI/RsaI restriction sites in a given transcript is critical. Wang and Brown estimate from an analysis of *Xenopus* cDNA sequences in a database that 75% of the nucleic acid material originally present would be represented in an amplified pool. However, because the two pools undergo comparable treatment, a fragment that does not amplify efficiently in one pool should behave similarly in the other pool. This means that, even if the PCR amplified pools are not a genuine representation of the mRNA expression from which they were derived, they should be equivalently 'flawed' such that the relative expression levels of a randomly selected cDNA fragment in the two pools should remain faithful to the relative levels of expression in the mRNA pools. This is vital if false positives/negatives are to be avoided.

4) The subtraction process itself is carried out in tandem. If the two tissue pools are, for example, 'X' and 'Y', one subtraction will operate to identify X-specific cDNAs and the other to identify Y-specific cDNAs. As in a typical subtraction, nucleic acid from one pool (the 'tester' or 'tracer') is hybridised for a set time period with an excess of nucleic acid from the other pool (the 'driver'). The driver material is treated

prior to hybridisation with photobiotin; a chemical that binds to the cDNA in the presence of light and which irreversibly associates with the protein streptavidin. Thus, the excess driver together with the tracer/driver hybrids are removed by streptavidin binding in conjunction with phenol-chloroform extractions, leaving only the unhybridised (subtracted) tracer cDNAs in the aqueous phase.

5) Six rounds of subtraction are carried out in this protocol which are divided into three sets of long and short hybridisations. Wang and Brown state that short hybridisations favour the removal of those cDNA species which are common to both pools and present at relatively high abundance. Such species are usually refractory to subtraction because of their quantity. Their removal here is due to the number and duration of hybridisations together with the nature of the driver (see below). The long hybridisation steps permit the subtraction of all species present in both pools, including those of low abundance.

6) As the subtraction procedes, the long hybridisation drivers are created from material from the equivalent subtraction stage of the other pool (e.g. stage 4 X tracer cDNA is hybridised to an excess of stage 4 Y driver cDNA and *vice versa*). Thus, successively more competent drivers are used to subtract away rare sequences. Using the starting pools throughout as drivers for the long hybridisations would result in the plateauing of the effectiveness of the subtraction procedure in later stages. The short hybridisations, however, always use the starting pools as drivers. It is those pools which contain the greatest percentage of the high abundance cDNAs; the target species of the short hybridisation steps.

7) In order to generate sufficient material for subsequent subtraction steps after each hybridisation, the tracer is subjected to PCR amplification using a primer complementary to the linker. If the driver sequences are not fully removed from the tracer during the post-hybridisation extractions they could go on to be amplified, negating the previously achieved enrichment. To avoid this possibility, the driver cDNA is subjected to complete digestion with the restriction enzyme EcoRI. There is an EcoRI site in the linker sequence. Hence, cleavage at this site removes the linker sequences from the driver cDNA resulting in material which is unamplifiable thereafter.

4.2: The application of this technique to study hippocampus-specific transcription

In the application of the protocol described below, the two PCR-amplifiable cDNA pools were synthesized from hippocampus and rest-of-brain (RoB; as defined previously) mRNAs, respectively. The resulting PCR pools seemed to be of

comparable quality with respect to the spectrum of product sizes as judged by the intensity of staining of the cDNA material resolved on an agarose gel. Sufficient starting material was subjected to procedures (described in the Materials and Methods chapter) resulting in the creation of drivers and tracers from both pools. The subtraction steps were carried out on both pools. General observations were that the average cDNA size of the pools diminished as the subtraction proceded (probably due to PCR bias) and certain cDNA species emerged as the subtraction proceded. These species became visible as discrete bands within the background of the other cDNA products when electrophoresed in an ethidium bromide stained agarose gel (see fig. 4.4a). The occurrence of such bands was to plague all attempts at subtraction using this technique. They do not seem to be merely due to the accumulation of pool-specific cDNAs as the subtraction procedes, but appear to be either refractory to the subtraction process or preferentially amplified. Attempts to explain this phenomenon will be made later in this chapter.

The amplified cDNAs from the 6 hippocampus subtraction stages and the 4 RoB subtraction stages were subjected to several tests to examine the efficiency of the subtractive process. All cDNA stages were electrophoresed in agarose gels and blotted onto filters. These filters were hybridised separately with three different probes. The first probe was synthesized from the sixth (final) hippocampus subtraction stage and was intended to show that there had been a steady enrichment for these sequences in the hippocampus pools and that these sequences were not present in the RoB pools at any stage (fig. 4.1a). The results seem to prove the former point in that there is very little hybridisation to the starting hippocampus pool but increasing amounts to the subsequent enrichment stages. However, there was a considerable degree of hybridisation to the final RoB subtraction stage. This can be interpreted as the result of contamination from hippocampus driver material from later subtraction stages which has been amplified together with the RoB tracer cDNA. It is also possible that the final RoB stage material contaminated the hippocampus tracer (stage 4). This suggests that the described precautions (driver cDNA digestion and multiple steptavidin binding/phenol-chloroform extractions) did not work as efficiently as expected in these particular subtraction steps. A counter-probing; to test if stage 4 RoB material was present in the hippocampus pools was also performed (data not shown). This gave a positive result demonstrating that cross-contamination had occurred. The final hybridisation was carried out using a probe derived from the starting RoB sequences (fig. 4.1a). As can be seen from the autoradiograph, higher abundance RoB sequences (as represented by this unsubtracted material) were effectively removed from the hippocampus material by stage 4. The RoB lanes do show diminishing hybridisation to this probe (as ubiquitous sequences are removed)

Figure 4.1 a The efficiency of the subtraction procedure as monitored by probing subtraction stages with enriched hippocampus (H^{+6}) or initial RoB (R^{-}) cDNA material. The results indicate that as the subtraction progressed RoB material was removed and hippocampus material enriched. Cross-contamination is revealed by the hybridisation of the H^{+6} probe to the R^{-4} cDNA material. **b** Bacterial colonies containing the subtracted hippocampus material were transferred to nitrocellulose filters. Shown here are three probe hybridisations (H^{+}, R^{-4}, and H^{+6}) to a region of one such filter. The purpose of the probings was to identify contaminating cDNAs and to select hippocampus-specific clones (see text).

Fig. 4.1a

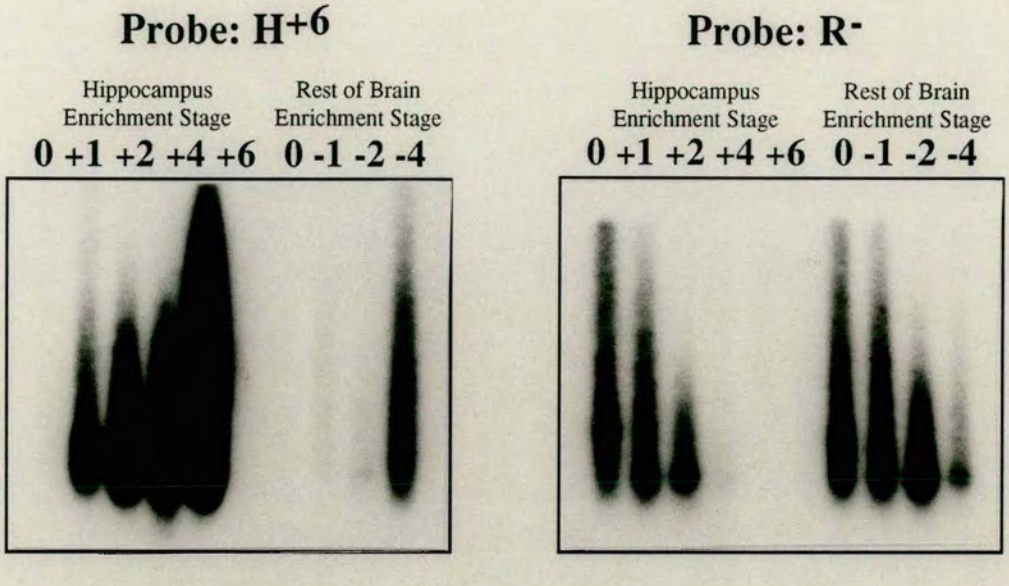
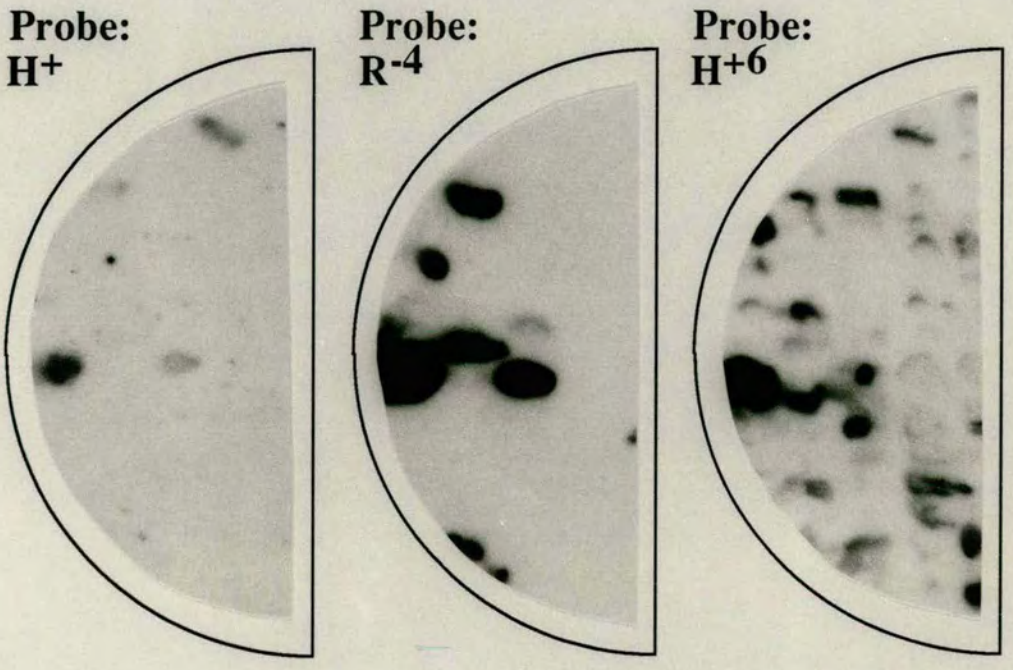


Fig. 4.1b



but to a lesser extent than the corresponding hippocampus stages; as might be expected from a probe which is derived from RoB cDNA.

4.3: Results from this subtraction

cDNA from the final subtracted hippocampus stage (H^{+6}) was cloned into the plasmid vector pBluescriptII (KS) and transformed into competent bacteria. The resulting colonies were gridded onto two plates and transferred onto filters for hybridisation. A probe derived from the final subtracted material was hybridised to these filters in order to select for those clones strongly represented in that material. Twenty such colonies were selected and plasmid DNA isolated for the purpose of sequencing. Table 4a lists these twenty clones and describes the results of the restriction, sequencing, and expression analyses performed on clones selected for their high level hybridisation to the probe.

Clone	Insert Size (bp)	Hyb. Intensity	Seq. Homology	cDNA Southern	Northern
3	800	***	None	-	All lanes
5	250	*	None	-	-
6	510	**	-	H. = R.	-
9	400	***	None	-	-
10	650	*	-	-	All lanes
13	250	***	-	-	All lanes
14	-	***	None	-	-
19	500	***	17-mer repeat	R.-specific	None
20	-	***	None	-	-

Table 4a First screen of first subtraction. See text for details. 'hyb. intensity' represents an approximate comparative guide to hybridisation strength on the filter. Dashes indicate that information was not obtained.

Expression of the clones was assayed in two ways. Firstly, by probing 'cDNA Southerns' which were made by electrophoresing and blotting the starting PCR-amplified pools onto filters. These give a representation of the distribution of the cloned cDNA as it was initially presented to the subtraction procedure. This technique is useful as a quick screen of clones because large amounts of nucleic acid can be electrophoresed in each gel lane, thus providing an abundant target for any probe. The second method was simply to use the cloned cDNA fragments as probes on brain region RNA Northern blots. These provide a more accurate analysis of brain expression, but require longer exposure times.

As can be seen from the table, this route to the selection of subtracted clones did not yield any that were hippocampus-specific. One clone (19) showed what seemed to be a specificity for the RoB cDNA pool when used as a probe on a cDNA Southern. When the same probe was used on a Northern blot, no discernible signal was observed. Sequencing of this clone revealed that it possessed six successive 17-mer nucleic acid repeat motifs which could be translated into three 17-mer amino acid repeats in one reading frame. Its unexpected presence in the final hippocampus subtraction stage can be explained only in terms of contamination of this cDNA material by RoB sequences; thus, it was present in the material cloned into the vector and in the screening probe used for the selection of candidates. Nevertheless, it does provide proof that some pool-specific enrichment and selection has occurred.

4.4: A second screen of the subtracted material

A more rigorous screen was performed on a larger number of colonies derived from the same material (560 colonies gridded onto 4 plates). In addition to the filters being probed separately with the first and final subtracted hippocampus stage material, duplicate filter lifts were probed separately with the second and fourth stages of the RoB subtraction (fig. 4.1b). Thus, in essence, a differential screen was performed on these colonies with the intention of discounting those clones which had come through the subtraction either as a result of a failure in the removal of ubiquitous sequences or by cross-contamination of the pools.

Probing with the final subtracted hippocampus stage not only permits a selection of those colonies which are recombinant, but also allows an estimate of the abundance level of each clone in the final subtracted material. Comparing this screen with that using the initial hippocampus cDNA material illustrates the extent to which sequences have been enriched during the subtraction process. Of the 560 colonies gridded out, only approximately 15 contained inserts which were of sufficient abundance in the initial pool to be detected when this material was used as a probe.

Probing the colonies with cDNAs from stages 2 and 4 of the RoB subtraction was performed in order that colonies might be screened out if they represented either clones which had escaped subtraction despite being present in both initial pools or clones that were the result of cross-contamination and amplification of the two cDNA pools as the subtraction progressed. Probing with stage 2 RoB cDNA was intended to examine the first of these possibilities as it had not been through sufficient subtraction steps to entirely remove ubiquitous sequences or to be contaminated with a cDNA

present at high abundance from the hippocampus stages. Of the 560 colonies, only 11 showed significant hybridisation to this probe showing that relatively few clones were derived from material that had escaped subtraction.

4.5: Cross-contamination of the cDNA pools occurred during the subtraction

It can be appreciated that cross-contamination of the pools as the subtraction process progresses would substantially decrease the likelihood of a successful outcome. A pool-specific cDNA species should be enriched as the subtraction procedes. If it contaminates the other tissue's cDNA pool then two problems arise. Firstly, the other pool now contains this sequence and so if a differential screen is performed then this will appear not as a specific clone but as a ubiquious cDNA which has escaped the subtraction process: probes derived from both pools will detect it. Secondly, contamination of a pool with a cDNA which is genuinely specific to the other pool will have the effect of negating the enrichment of that sequence in subsequent subtraction steps: the two pools average out the expression differences instead of accentuating them. This was the reason for the screen using stage 4 RoB as a probe on the cloned hippocampus stage 6 cDNA. 15 out of 560 clones were hybridised to this probe of which 4 were additional to those seen on the stage 2 RoB probing. Assuming that the majority of the cross-contamination occurred in the later stages (as the information presented in Fig. 4.1a seems to suggest) then these represent cross-contamination. Therefore, contamination is seen to a certain degree but the damage to the efficiency of the subtraction as a whole is not determinable from this approach. The exact direction of contamination is uncertain without further analysis of those clones as they could have arisen from RoB sequences contaminating the hippocampus material prior to cloning or from the hippocampus sequences contaminating the RoB material prior to its use as a probe; both events have the same outcome.

4.6: The clones obtained from this screen show contradictory expression analyses

Five colonies were selected on the basis of hybridisation to the hippocampus, but not RoB, probings and these are listed in table 4b. The striking feature of this subtraction, also seen in subsequent subtractions, is that there is a considerable discrepency between the expression profiles obtained by the cDNA Southern approach and those obtained by conventional Northern hybridisation (fig. 4.2a).

Figure 4.2 a These three clones (3a, 4a, and 5) display hippocampus-specificity at the level of the cDNA Southern (smaller panels: H; hippocampus pool, R; rest of brain pool) which disappears upon Northern expression analysis (larger panels). **b** An experiment to discover if the subtraction procedure is able to remove high abundance cDNA material. The lower panel shows the ethidium-stained DNA before (bef.) and after (aft.) subtraction at various size-marker spike concentrations. The top panel shows this DNA blotted onto a filter and probed with size-marker DNA. The results indicate that the subtraction is very efficient, even after one round.

Fig. 4.2a

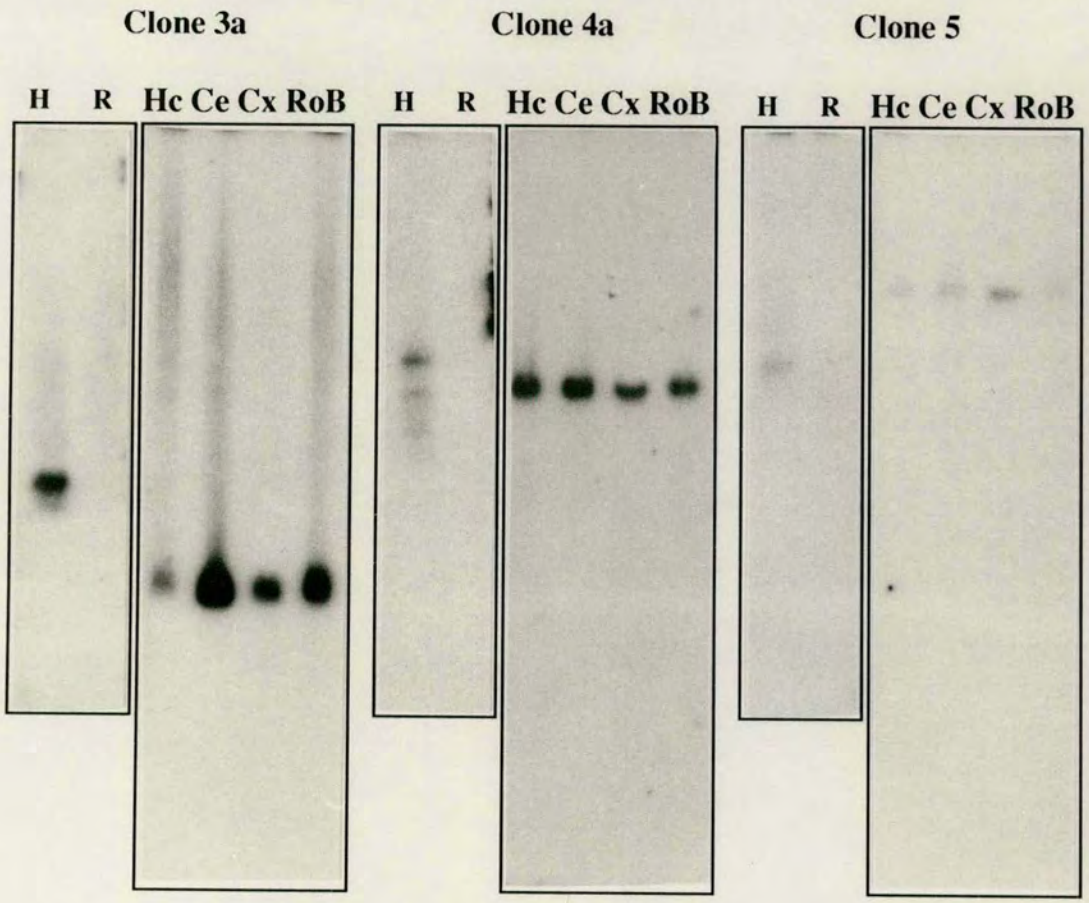
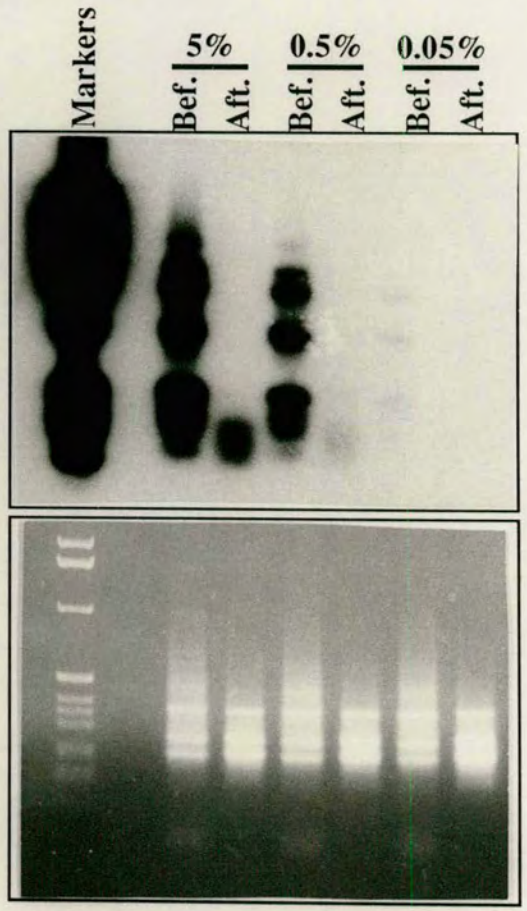


Fig. 4.2b



Clone	Insert Size (bp)	Hyb. Intensity	Seq. Homology	cDNA Southern	Northern
3a/5a	400	***	Rib. Prot. S25	H. Specific	All Lanes
4a	550	**	None	H. Specific	All Lanes
4b	380	**	None	H. and R.	-
5b	250	**	None	H. Specific	All Lanes

Table 4b Second screen of first subtraction.

Four clones (3a (identical to 5a), 4a, and 5b), together with one (5) from a screen not presented here, showed hippocampus-specificity when used as probes on the cDNA Southerns. However, when the same probes were used on Northern blots, the results indicated that they were ubiquitously expressed throughout the brain regions represented. What was the reason for this difference? cDNA Southerns are representations the two cDNA pools as they begin the subtraction. The five isolated clones only hybridised to the hippocampus pool and therefore can only have been present in that pool at the start of the subtraction. Hence, the conclusion that must be drawn from these contradictory results is that the initial synthesis of the PCR-amplifiable cDNA pools cannot have been truly representative of the actual mRNA distribution in the case of the RoB cDNA pool synthesis. Because there was no direct cloning and selection for those cDNA species which had been enriched in the RoB subtraction, it is not possible to tell if there was a similar failure in the representation of the hippocampus cDNA pool (with respect to a different subset of sequences). Typical of this failure was clone 3a which, upon sequencing, was identified as the ribosomal protein S25; not a gene that would be expressed in a brain region-specific manner.

Regardless of the failure to enrich for genuinely hippocampus-specific sequences, the results do show that when a cDNA species is present in one pool, and not the other, the subtraction process is sufficiently thorough to enrich for those sequences. In this example, the fault lies not with the efficiency of the subtractive process, but the failure to create truly representative PCR-amplifiable cDNA pools from the native mRNA. This representation failure has two features which may contribute to a better understanding of the processes that lead to it. Firstly, it was restricted to particular cDNA fragments, as not all probes exhibited specific expression on cDNA Southerns, and secondly, the difference in representation of these cDNA fragments on the cDNA Southerns was extreme. A typical observation was that there would be almost complete restriction of the cDNA fragment to the hippocampus cDNA pool, as analysed by cDNA Southern. If the failure to create the initial PCR-amplifiable cDNA fragment pools was of a stochastic nature, then it might be expected that there would be a two- or three-fold variation in the levels of any cDNA between the two

pools; this was not the case. Therefore, in the search for a root cause for the failure to achieve comparable RoB cDNA representation, features have to be found which make specific sequences refractory to cDNA synthesis or amplification with respect to one pool and not the other. The final section of the next chapter attempts to rationalise these problems in light of results from subsequent subtractions and experiments.

4.7: A solution to the problem of cross-contamination

In two subsequent cDNA subtraction experiments attempted; one to identify hippocampus-specific transcripts and the other to identify activity-related genes in the hippocampus (see next chapter), a modification to the subtraction protocol was made to prevent the occurrence of pool cross-contamination (fig. 4.3 a and b). This was prompted by the publication of a paper (ref. 2) which described similar problems to those above. The proposal made in this paper was that by creating each tissue's cDNA pool using a different linker set, they could be made to act independently of each other during PCR amplification. Thus, if tissue X cDNA tracer became contaminated with tissue Y driver in one round of subtraction, the subsequent PCR step would only amplify X cDNA material because of the specificity of the PCR primer used. The two sets of linkers and primers used in these subtractions are listed in the Materials and Methods chapter.

4.8: Certain cDNA species accumulate erroneously as the subtraction procedes

After each round of subtraction the amplified products from the two cDNA pools were electrophoresed on an agarose gel in order to monitor their quality and average size. As the subtraction progressed certain bands grew stronger in intensity until they represented a large percentage of the nucleic acid present in the pool (fig. 4.4a). Their abundance threatened to lower the efficiency of the subtraction because they reduced the concentration of genuinely enriching cDNAs. Also, the final subtracted material prepared for cloning primarily consisted of these species, hindering the cloning of specific sequences. It was presumed that these major species did not in themselves represent enriching cDNAs as they were often present in the same distribution in both tissue pools.

Several explanations were postulated as to their origin and failure to subtract. Based upon these, efforts were made to counteract their accumulation.

The first explanation was that these species accumulated as the subtraction progressed because they were intrinsically more amplifiable than the other cDNA species. In this

Figure 4.3 a The subtraction protocol: cDNA pool synthesis and subtraction mechanism. a) tissue X mRNA converted into double-stranded cDNA b) restriction digestion and ligation of linker sequences c) amplification and denaturation of tracer/tester d) hybridisation to excess of denatured and biotinylated driver cDNA. Separation of excess driver and tracer-driver hybrids from single-stranded tracer. This material is enriched for tissue X-specific sequences. **b** The subtraction protocol: repeated and reciprocal subtractions. The long hybridisations, short hybridisations, and PCR steps are indicated for each cDNA pool. The numbers by these steps indicate the name of the subtracted cDNA material at that point. Curved lines feeding into the hybridisation steps indicate the identity of the driver cDNA used. Only four subtraction stages are shown here, as was used in the later subtraction experiments.

Fig. 4.3a

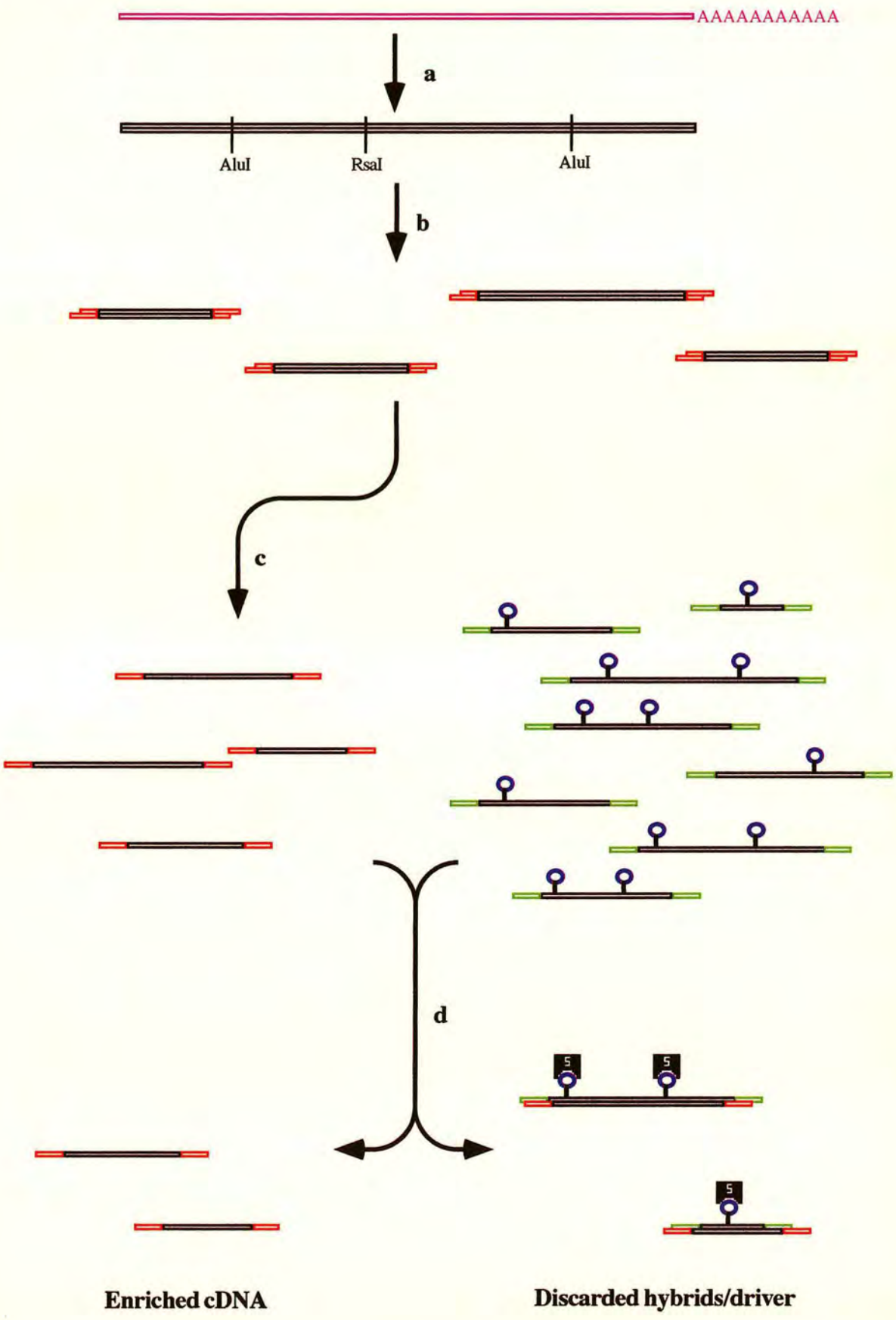
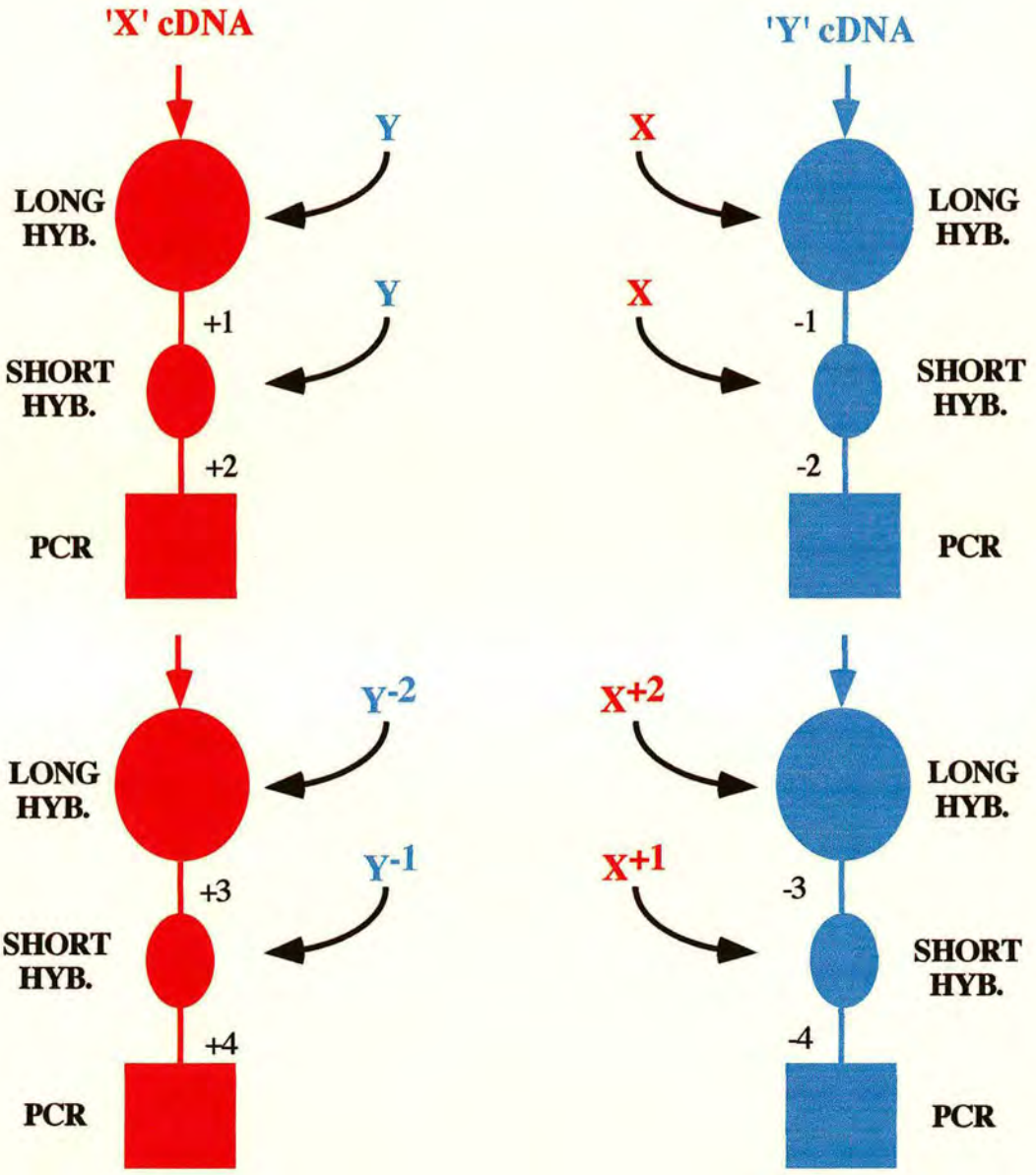

AAAAAAAAAAA
a
AluI
RsaI
AluI
b
c
d
Enriched cDNA
Discarded hybrids/driver

Fig. 4.3b


'X' cDNA
Y
LONG HYB.
Y
+1
SHORT HYB.
+2
PCR
'Y' cDNA
X
LONG HYB.
X
-1
SHORT HYB.
-2
PCR
Y-2
LONG HYB.
Y-1
+3
SHORT HYB.
+4
PCR
X+2
LONG HYB.
X+1
-3
SHORT HYB.
-4
PCR

situation, because of the large number of cycling steps between the cDNA synthesis and the cloning of the subtracted material (approximately 102), there exists ample opportunity for a hypothetically advantaged cDNA to increase its relative abundance through such a mechanism. There are a number of problems with this explanation. Firstly, the subtraction steps should actually reduce the relative abundance of these species as they are not specific to one pool. Any advantage that these cDNAs possessed with respect to amplification should have been countered by subtraction. Secondly, if an amplification advantage exists it would have to be marginal otherwise the amplification products would be swamped by these species after the initial 30 cycles. For example, if these hypothetical species have an initial abundance of 0.5% and possess a 5% amplification advantage over normal cDNAs, then after 102 cycles they will represent 72.5% of the cDNA (0.5×1.05^{102}). This is probably an underestimation of the actual abundances of these bands at the initial stages as some were visible before any subtraction took place. Thirdly, it is hard, on the basis of our knowledge of PCR, to come up with a mechanistic description of preferential amplification. Disadvantages to amplification are known, such as large cDNA size, skewed base composition or secondary structures. However, the abundant species do not seem to be excessively small: DNA fragments of 4kb have been amplified using these cycling conditions, so size does not seem to be a major factor. Nevertheless, the failure to find a precedent for preferential amplification does not discount this theory.

The second explanation was that these cDNAs were initially present at a concentration above a threshold for their effective and complete removal: even with an excess of driver the kinetic constraints would prevent complete removal of that cDNA from the tracer. This does not explain their accumulation over the course of the subtraction. This might happen if, through synthesis artifacts or natural distribution, the two initial populations contained unequal levels of these high abundance cDNAs. In that unbalanced situation, one pool might, by being above the threshold value, dominate over the other in subtracting that cDNA. In that case the pattern of accumulating bands would not be identical in the two pools when electrophoresed. It is hard to imagine a situation where the efficiency of mRNA into cDNA pools could be sufficiently different between the two pools; and with respect to just a few molecular species.

The third explanation centred on the fact that these species appeared to be resistant to subtraction. Because only a few cDNA species behaved in this way, any explanation must be based on the nature of the sequences of these particular cDNAs. The subtraction process itself relies on the complementarity of DNA strands; those that hybridise to the driver sequences will be removed from the pool. Therefore, it was

hypothesised that cDNAs that could not hybridise to complementary sequences in the driver, but could, nevertheless, be amplified by PCR would accumulate in the observed fashion. It was presumed that the failure to hybridise was due to self-complementarity within the cDNA strand such that cDNAs in both the tracer and the driver which contained such sequences would form internal secondary structures preventing the formation of double-stranded hybrids. Even though the formation of such secondary structures is a unimolecular process (and, therefore, occurs extremely rapidly in solution), there would still be an energetic advantage for such strands to form double-stranded hybrids in a bimolecular reaction (due to the increased extent of hydrogen bonding). The subtraction protocol requires the use of two different hybridisation time-periods; two hours or twenty hours. Even though kinetic equations have not been calculated, it may be the case that short hybridisation steps favour the unimolecular reaction but long steps permit the formation of the correct duplexes. Thus, the use of short hybridisation steps might favour the accumulation of such unsubtractable material.

4.9: Two experiments designed to test these hypotheses

If certain DNA species were present at a concentration that prevented their efficient removal then these species would accumulate throughout the subtractive process. An experiment was designed to test the efficiency of the subtractive hybridisation technique when confronted with cDNAs of a high percentage abundance. A 1 kb DNA marker ladder (Gibco BRL) used to estimate the size of DNA fragments electrophoresed on agarose gels was converted into a PCR amplifiable form by the addition of the linkers that were used to create the cDNA pools. Ladder fragments with the same linker type as used in the creation of the hippocampus cDNA pool were added at defined percentage abundances to a 'background' of the hippocampus cDNA pool. Therefore, in the three experiments the ladder constituted 5, 0.5, and 0.05% of the total DNA content of the respective pools. Similar ladder fragments possessing the rest-of-brain cDNA pool-type linkers where added to a rest-of-brain cDNA 'background' to the same final percentage abundances. The subtraction was performed so that hippocampus (plus set percentage of ladder) tracer was hybridised to rest-of-brain (plus equivalent set percentage of ladder) driver in a short (two hour) hybridisation period. The resulting subtracted tracer was analysed for the percentage of ladder DNA remaining.

The results suggested that the ladder was completely removed at all initial starting concentrations upto 5% of the starting material (fig 4.2b). Thus, the subtractive process works very efficiently, even during short hybridisation steps. In the light of

this experiment, it seems unlikely that the initial abundance of a given cDNA can affect its ability to be subtracted. There are two potential caveats to this experiment. Firstly, in each case the tracer and the driver contained equivalent percentage quantities of the ladder; one possibility mentioned above was that the initial starting quantities in the cDNA pools could be sufficiently imbalanced as to cause the failure to subtract in one direction. Secondly, the 5% maximum limit imposed on this experiment might be below the threshold at which a species fails to subtract efficiently. As can be seen in the figure, at 5% the bands of the ladder are just visible through the background of hippocampus cDNA so this percentage may be under that seen with the major bands.

One particular region of electrophoresed hippocampus cDNA material enriched in accumulating unsubtracted material was purified and cloned into pBluescriptII for sequence analysis. All eight clones sequenced (table 4c) were identified from the database. Their sequences were analysed for the existance of secondary structure using the 'Fold' program of the Wisconsin GCG8 DNA analysis package.

Clone	Identification
mb4	16S rRNA fragment (mito.)
mb6, mb15	Cytochrome c oxidase I (mito.)
mb9	Human EST (muscle)
mb10	Cytochrome b
mb11	Gαs (3' untranslated region)
mb12	28S rRNA fragment (cytoplasmic)
mb19	B1 repeat
mb20	Mouse EST (testes)

Table 4c Identities of eight clones obtained from cDNA material co-migrating with an unsubtracted band.

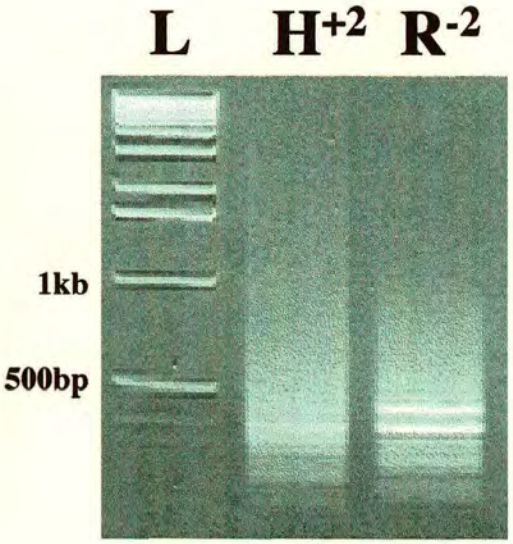
The sequences for cytochrome c oxidase I and cytoplasmic ribosomal RNA 28S showed the greatest degree of secondary structure (the others also possessed significant amounts). Diagrams showing the putative secondary structures of these two clones are shown in figs. 4.4b and c, respectively (their cDNA sequences are listed in the Appendix). The genuine secondary structures of these two clones may be very different, as these are only derived from a computer prediction. However, the extent to which these sequences possess regions of self-complementarity cannot be ignored.

Even though these clones were isolated from DNA co-migrating with the unsubtractable material, it is not certain that they were the unsubtractable cDNAs

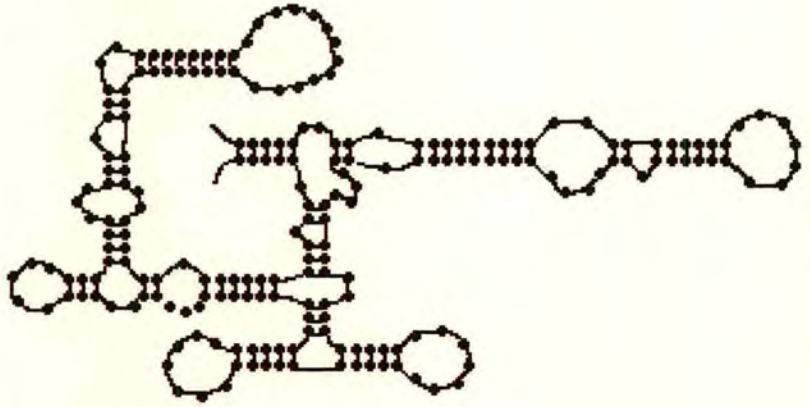
Figure 4.4 a Typical appearance of electrophoresed and ethidium-stained cDNA pool. Note the presence of the major cDNA species after two rounds of subtraction, especially in the R^{-2} lane. **b** Proposed secondary structure of a section of major band clone mb6 (cytochrome c oxidase) as determined using the 'Fold' program of the Wisconsin GCG7 DNA analysis package. **c** Proposed secondary structure of a section of major band clone mb12 (28S rRNA) determined as before. Full sequences of mbs 6 and 12 are to be found in the Appendix.

Fig. 4.4

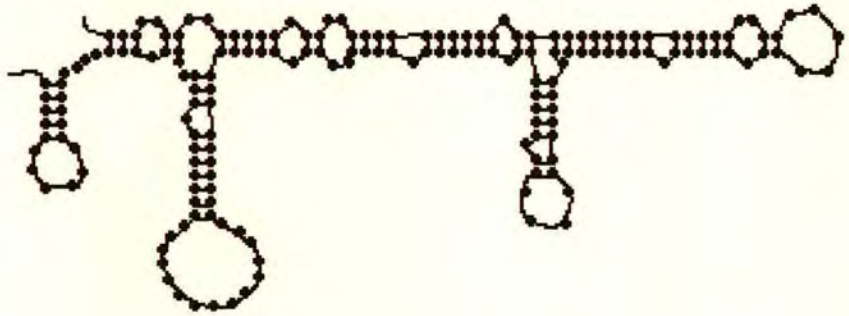
a



b



c



themselves. To confirm that these behave in the hypothesised manner they would have to be seeded into a cDNA pool and their accumulation monitored relative to control species: this experiment was not carried out. The secondary structure explanation for the accumulation of these species is still not definitively proven.

4.10: Attempts to remove unsubtractable material from the cDNA population

Based on the sequence analysis of the putatively unsubtractable material together with the physical characteristics that may give rise to their behaviour, two protocols were designed and applied with the aim of discriminating between cDNAs on the basis of their secondary structure. These are described below.

It is a property of nitrocellulose membrane that only single stranded nucleic acid is able to bind to its surface. Therefore, if suitable conditions are created, this could act as a selection device for the cDNA material. The cDNA pools (resuspended in TE buffer/100mM NaCl) were denatured by boiling so that all species were present in the single-stranded state. The cDNA solution was allowed to cool to room temperature slowly; thus permitting the formation of secondary structures where possible. The solution was then applied to a small section of membrane which was then subjected to gentle washing steps with the same buffer solution to remove unbound cDNA from the surface. The membrane was then boiled in a small volume of water to remove bound DNA. This was then ethanol precipitated and used as a template for the standard subtraction PCR reaction. Thoeretically, only single-stranded cDNA molecules could become reversibly bound to the membrane whereas those exhibiting a large degreee of secondary structure would be washed off. The amplified material, however, showed no improvement in quality with respect to the percentage of the putatively unsubtractable cDNAs. Perhaps the single-/double-stranded nucleic acid discrimination of nitrocellulose membrane is not as absolute as this technique requires.

A second protocol designed to separate the cDNAs on the basis of their secondary structure relied on the resistance of these stuctures to enzymatic activity. Again the cDNA pool was denatured by boiling and allowed to slowly cool to room temperature in order to permit the formation of secondary structures. The relevant primers and reaction constituents were then added to this material (at a temperature of 16^{o}C) such that a second-strand synthesis reaction could be carried out using the Klenow subunit as the polymerase enzyme. The rationale behind this reaction was that only cDNAs that were single-stranded along their entire length could be converted, in these conditions, to double-stranded DNA molecules. A region of secondary structure

within a cDNA would hinder the progress of the Klenow enzyme at this temperature and would not be converted into the double-stranded form. S1 Nuclease enzyme was added to the products in order to digest any single-stranded DNA present in a partially double-stranded molecule. Such a molecule would become unamplifiable after this digestion. The remaining double-stranded material was subjected to the normal subtraction PCR amplification. This protocol did not produce a substantial improvement in the quality of the cDNA pools. Explanations for this include the creation of double-stranded (re-annealed) forms of the unsubtractable material in the slow cooling step which would not be removed by the subsequent steps. The presence of such species is made all the more likely by the fact that they exist at a high concentration in the initial pool.

For the following two subtractions, the most sucessful (but not completely so) method of preventing the unwanted species from accumulating was the extra care taken to ensure that as much ribosomal RNA material was removed from the purified mRNA as possible. Hence, two rounds of mRNA selection on oligo-dT cellulose columns were performed. This had the effect of creating initial cDNA pools which appeared devoid of any discrete bands; previous preparations using a less stringent mRNA purification method had resulted in cDNA pools which contained high abundance bands (possibly derived from ribosomal RNA) in a background of the other cDNA species.

4.11: The revised subtractive hybridisation protocol used in an attempt to isolate hippocampus-specific transcripts

Using the more efficient mRNA purification protocol and different linker combinations for the two cDNA pools, a second attempt at the Wang and Brown subtraction system was carried out.

The tissue samples used to create the pools were derived from sham-treated brains (see next chapter for a fuller description). As before, cDNA pools corresponding to hippocampus and the rest-of-brain were synthesized and amplified. The subtractive hybridisation steps were performed as set out in the Materials and Methods chapter except that only four rounds of reciprocal subtraction, instead of the normal six, were completed prior to the cloning step. This was due to the appearance, once more, of unsubtractable material which manifested itself as prominent bands in the background of the cDNA pools when electrophoresed.

It was possible to clone the final stage (H^{+4}) subtracted hippocampus DNA into the bacteriophage vector, M13tg130, by the use of the XbaI restriction sites in the linkers of the cDNA fragments. Duplicate filter lifts of the resulting M13 plaques were taken through a differential screen for two purposes. Firstly, to gauge the extent of the expression specificity of a given clone and, secondly, as a negative selection against the unsubtractable cDNA-containing clones which were present in the fourth round hippocampus subtracted material. With this in mind the probes used in the screen were synthesized from fourth round subtracted hippocampus cDNA (H^{+4}) for the positive screen, and a mixture of new and old rest-of-brain cDNA pools (R^{-} and R^{-4}) for the negative screen. The differential filter probings were compared and only plaques which were H^{+4} positive and R^{-}/R^{-4} negative were selected for further analysis.

Fig. 4.5a shows a comparison of the differentially screened filters and table 4d describes the eighteen most studied clones obtained from this screen. As before, the clones were sequenced to find homology to the GenBank/EMBL database of DNA sequences. PCR-amplified cDNA inserts from the M13 clones were also used as probes for expression analysis. Both cDNA Southerns and Northern blots of brain regions were used for this purpose.

The same expression discrepencies between these two methods were seen, just as they had been for the previous attempt at hippocampus-specific cDNA subtraction. According to expression data from the cDNA Southerns, many of these clones represented transcripts which were very highly enriched in the hippocampus and, in two cases, appeared to be almost specific to this region (fig. 4.6). Northern analysis, however, contradicted these results in most cases (fig. 4.5b).

The sequences of four clones (H4M 10, 27, 33, and 37) showed homology to sequences in the database.

H4M 37 showed homology to a human expressed sequence tag (EST) originally described in the large-scale sequencing of brain cDNAs (ref. 3)

H4M 33 showed identity over its entire length to the rat 5-hydroxytryptamine 1a receptor (ref. 4). This receptor is coupled to the inhibition of adenylyl cyclase activity upon agonist stimulation. The cloning of this receptor is interesting in two ways: firstly, neurotransmitter receptors are generally low abundance transcripts (showing that the subtraction procedure is relatively sensitive) and, secondly, because this transcript shows a restricted distribution. The predominant sites of expression in the brain described in the paper include the hippocampus, septum, and thalamus with

Figure 4.5 a Final subtraction stage hippocampus cDNA cloned into M13 bacteriophage vector, transfected, plated, and transferred onto duplicate filters. One region of a set of duplicate filters is shown here to demonstrate the results of the differential screen. Only plaques which were exclusively hybridised by the H^{+4} probe were analysed further. **b** The brain expression distributions of two clones (H4M29; no homology and H4M10; hippocalcin) by Northern hybridisation. Ribosomal protein S26 is included as an RNA loading control.

Table 4 d Analysis of the clones that emerged from the second attempt at hippocampus subtraction. Insert size is given in base pairs. Hybridisation intensity is an approximate assessment of relative hybridisation strength. Dashes indicate that no expression experiments were carried out.

Fig. 4.5a

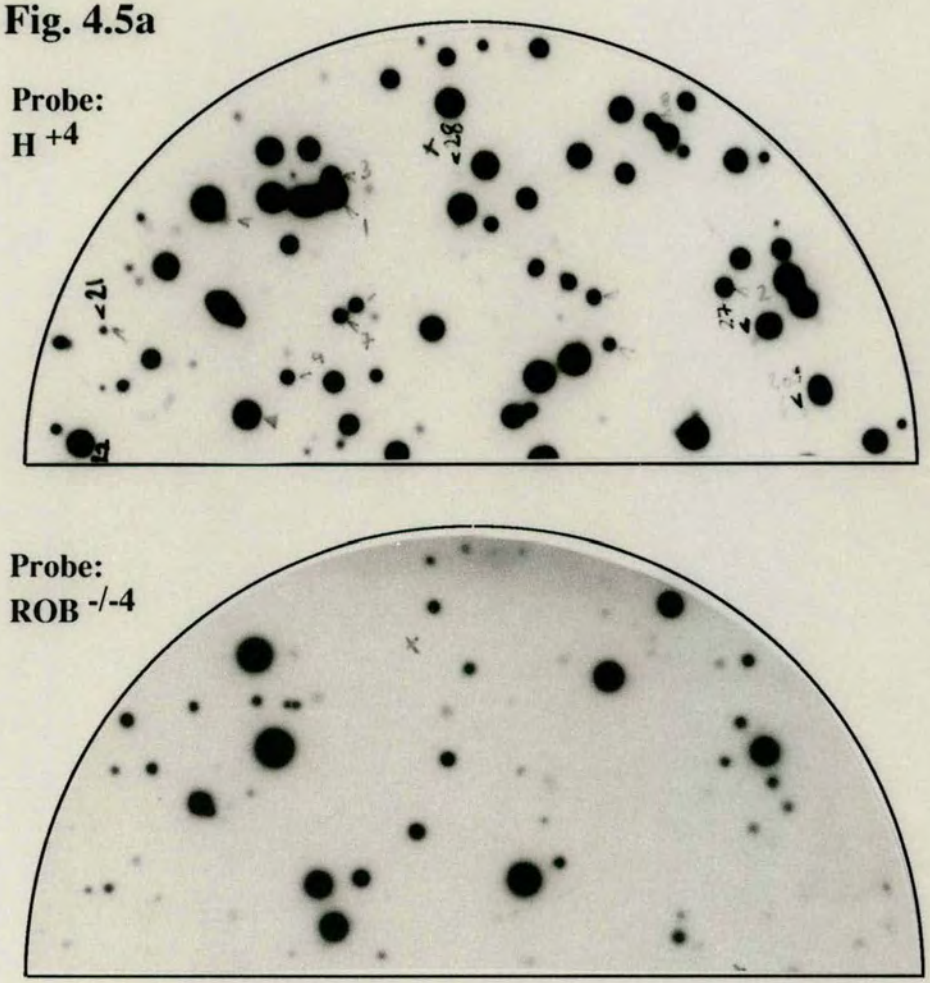


Fig. 4.5b

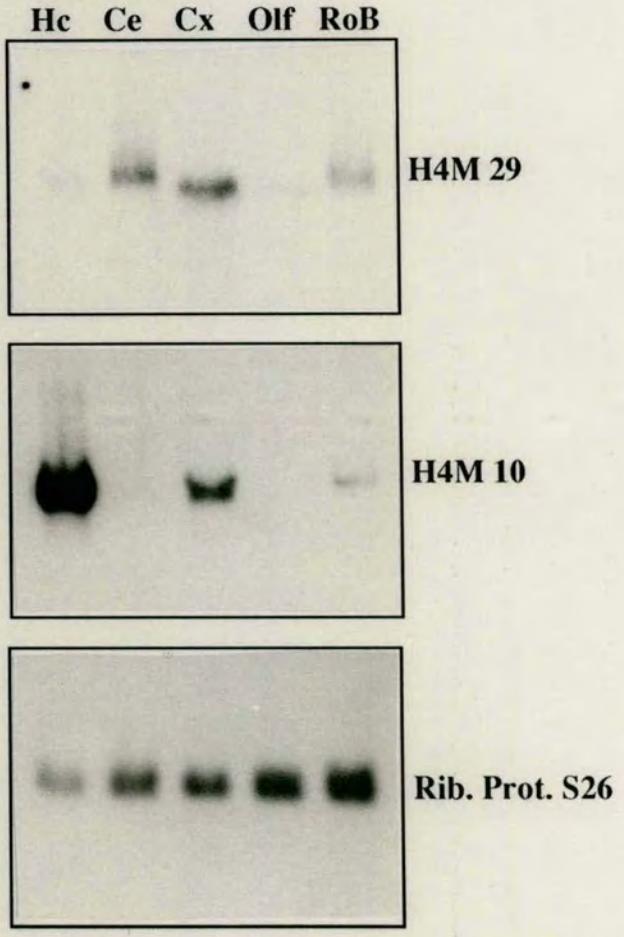


Table 4d

H+4 M13 Clone	Insert Size	Hyb. Intensity	Seq. Homology	cDNA Southern	Northern
6	300	**	No Homology	H.>>>R.	-
10	500	**	Hippocalcin	H. Specific	H.>>Cx.>R.>>Cer.
19	350	*	No Homology	H.>>R.	-
21	300	***	No Homology	H.>>R.	-
22	500	**	No Homology	H.>>R.	-
23	-	*	Linkers	-	-
24	250	***	Linkers	-	-
25	330	***	No Homology	H.>>R.	-
26	-	*	Linkers	-	-
27	220	*	NHE-1	-	-
28	250	*	No Homology	H.>R.	H.=Cx.>Else
29	300	*	No Homology	H.>>R.	Ce.=Cx.>H.
31	-	*	Linkers	-	-
33	300	*	5-HT1a Receptor	-	-
34	-	***	Linkers	-	-
35	150	*	No Homology	H. Specific	All Lanes Equal
36	100	*	Linkers	-	-
37	250	**	hEST T04934	H.>R.	-

lower levels of expression seen in some mid- and hind-brain structures. The implications for such a distribution on the sensitivity of the subtraction will be discussed later.

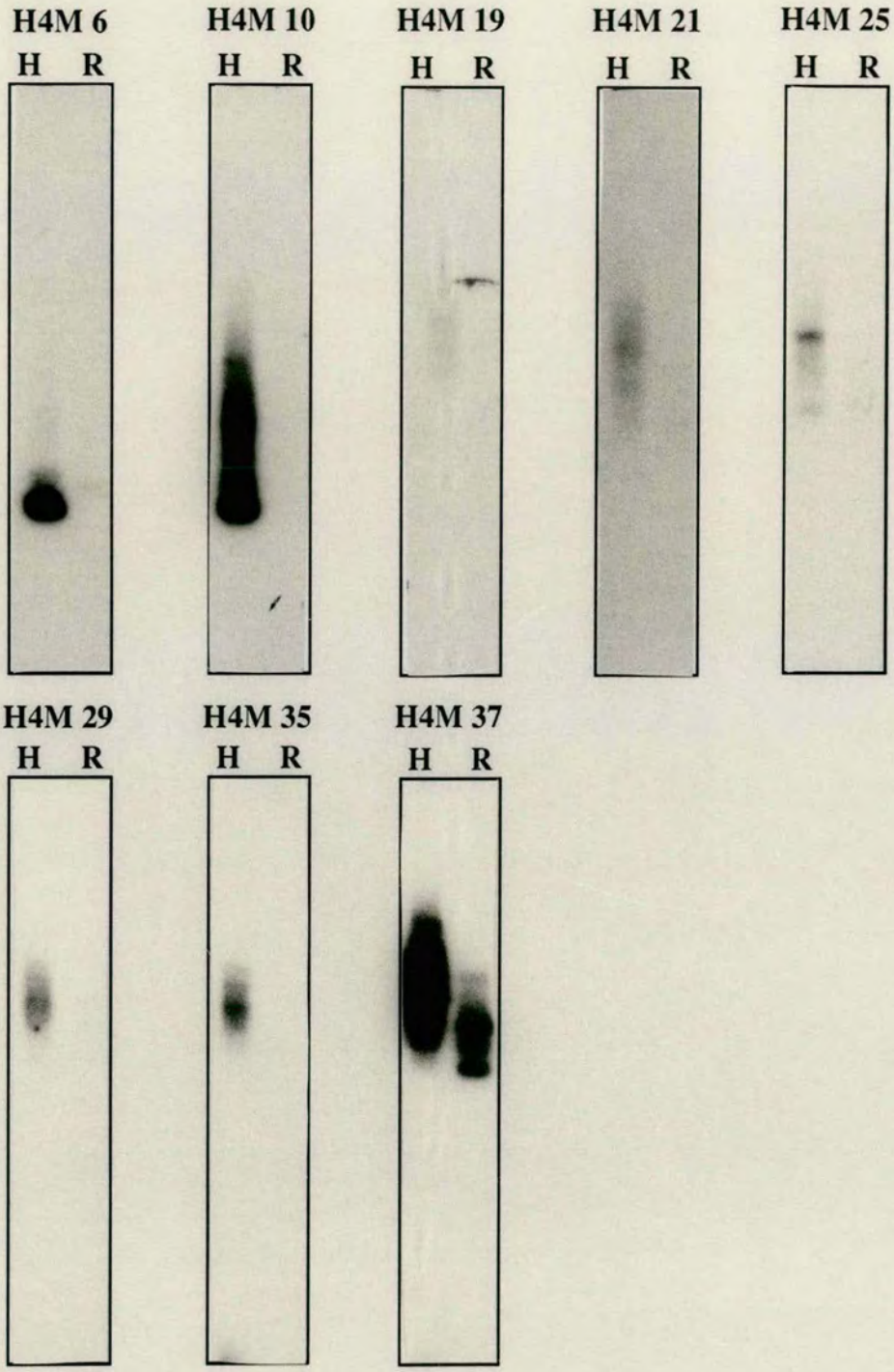
Clone H4M 27 showed identity to the sequence for the rat Na/H exchange-1 protein (ref. 5) which seems to be expressed in most tissues studied to date. Its function is to mediate the exchange of sodium and hydrogen ions across the cell membrane; a process which is important in a variety of ways, including pH balance and absorption efficiency. Its effect on pH balance has been studied in isolated hippocampal neurons but it is not known whether it exists in other brain regions also.

The fourth identified clone, H4M 10, is identical to a sequence, hippocalcin (ref. 6). This protein is a member of the recoverin family of calcium-binding proteins and is described as being "...exclusively expressed in (the) pyramidal layer of the hippocampus...". cDNA Southern analysis concurred with the hippocampal specificity (fig 4.6) but this was not so pronounced when the clone was used as a probe on Northern blot of brain regions; expression was also seen in lesser amounts in the cortex and cerebellum (fig. 4.5b).

4.12: Dilution of brain regions within the RoB pool might lead to false positive clones

The expression patterns of hippocalcin and the serotonin receptor point to an explanation for their enrichment in the subtraction. The subtraction is performed using cDNA pools derived from hippocampus mRNA and RoB mRNA. The second of these is a mixture of mRNAs obtained from all of the non-hippocampal brain regions. As such, there are two factors which dictate the representation of a given transcript within this pool; the abundance of that transcript and the percentage of the brain that expresses it. Hence, the representation of a cDNA in the remainder-of-brain pool is an average of its abundance throughout this region as a whole. For example, a transcript might be present at equivalent abundances in the hippocampus and the hypothalamus but when used a probe on a cDNA Southern of the two pools would look much higher in the hippocampus than remainder-of-brain because the hypothalamus makes only a small contribution to the latter pool. Hippocalcin is probably the best example of this; the Northern blot shows that the transcript is present in the cortex, but the non-hippocampal brain tissue lane shows very low expression as the cortex signal only represents a small part of it. This 'dilution effect' makes a subtraction of this kind more difficult to achieve but it cannot be used as an explanation for all of the false-positive clones obtained here. H4M 35 seems to be specific according to cDNA Southern but is ubiquitously expressed as analysed by

Fig. 4.6


H4M 6
H R
H4M 10
H R
H4M 19
H R
H4M 21
H R
H4M 25
H R
H4M 29
H R
H4M 35
H R
H4M 37
H R

Northern blot (data not shown). Other explanations have to be found to account for such discrepencies (see Chapters V and VI).

Although not described in any detail here, a second screening was attempted on a larger selection of clones. In this case the negative probings were native cDNA probes derived from cerebellum, cortex, anterior mid-brain, and posterior mid-brain. This was used to avoid both the problems associated with the dilution effect and failure to achieve complete representation of transcripts in the amplified cDNA pools. No specific clones were isolated.

Chapter V

Long-Term Activity-Dependent Modification of Gene Expression in the Hippocampus

5.1: Introduction

Functional distinctions for the hippocampus are plentiful and this raises the possibility that the hippocampus might only exhibit transcriptional individuality when carrying out its principal role; that of memory formation. The experiments described elsewhere in this thesis failed to distinguish this region from others at the level of transcription and were carried out using cDNA obtained from unstimulated tissue (or sham-treated tissue, in the later experiments described in Chapter IV) dissected from rats caged in a cognitively undemanding environment. As the hippocampus is the brain region required for the creation of certain types memories then perhaps specific gene expression might be induced in rats when they are subject to a situation demanding the increased activity of this brain region (such as swim maze training or introduction to a novel and complex environment). This might be one explanation why searches for hippocampus-specific genes up to now have been fruitless. The introductory chapter referred to experiments showing that rats housed in enriched environments seem to undergo structural changes in hippocampal synaptic morphology, although little work has been done to investigate the gene expression changes in such animals. The role of neuronal gene-induction has been extensively studied in lower organisms such as *Aplysia* and *Drosophila*. This has revealed that there are defined stages in the process that leads to the consolidation of modified behaviour. These phases are distinguished from each other by their susceptibilities to pharmacological disruption.

5.2: Long-lasting LTP requires de novo transcription and translation

Synaptic excitation of sufficient strength can result in *de novo* gene expression. The introductory chapter described the effects of transcription and translation inhibitors on the consolidation of STP into LTP. These experiments prove that the memory formation process is not simply the result of protein phosphorylation or the triggering of certain biochemical cascades, but requires the synthesis of new proteins.

5.3: Activity-dependent gene expression in the brain

How might newly-expressed gene products contribute to the potentiation state of a neuron? To answer this it has been necessary to clone genes whose expression changes after hippocampal stimulation procedures (ref. 1). Previous attempts to isolate activity-dependent transcripts have had two main features. First, much of the work has concentrated on a narrow time-window immediately following the particular stimulus paradigm used. Second, for reasons explained below, stimulus protocols have been chosen for maximal potentiation effect. The choice of the time post-stimulation when brain expression is monitored has generally limited the genes isolated to a particular functional group; the immediate early gene family (refs. 2-4). Such genes undoubtably play an important role in medium-term potentiation events (and in initiating the long-term events).

The following section briefly describes the results from published experiments designed to isolate transcripts which show responsiveness to neuronal activity.

High frequency stimulation of the perforant pathway to the dentate gyrus granule cells leads to a rapid induction of one particular IEG transcription factor, zif/268 (also known as egr-1, NGF1-A, and krox 24). Other IEG transcription factors such as c-fos, c-jun, and junB are induced to a lesser extent and not consistently. The activation characteristics of zif/268 are typical for IEGs in that its mRNA accumulates from 15 minutes after the stimulus, reaches a maximum before one hour, and then returns to a base-line level after approximately three hours. The protein product persists for eight hours after the stimulus. Two results point to a close relationship between zif/268 upregulation and the induction of LTP (ref. 5). First, upregulation of its mRNA seems to be critically dependent on whether the stimulatory signal was above a certain threshold and second, the induction is prevented by the NMDA receptor antagonists MK801 and CGS-19755. These characteristics closely match the induction requirements of LTP. Interestingly, maximal electroconvulsive shock stimulation to the brain induces the expression of this gene even in the presence of NMDA receptor inhibitors; implying that the effects on memory seen after this technique may be, in part, NMDA R-independent. However, recent work has shown that ketamine (a NMDA receptor blocker) can reduce the amnesic effects of electroconvulsive stimulus (see below) when administered prior to stimulus. The effect of volatile anaesthetics on LTP and zif/268 induction appears to be variable (ref. 6) but completely prevents the other IEG transcription factors from being upregulated. Thus, it seems likely that different mechanisms operate to cause the transcriptional activation of the different members of the IEG family. The activity-dependent entry

of calcium into the post-synaptic cell is the likely trigger for events that lead up to the induction of these genes, but the precise mechanisms have yet to be elucidated.

2D gel electrophoresis of the proteins produced in Aplysia neurons before and after the induction of long-term sensitization (or serotonin administration) revealed the presence of two products which remained at elevated levels for some time. These were identified as BiP (a heat shock-related protein involved in protein trafficking at the endoplasmic reticulum; ref. 7) and calreticulin (a calcium binding protein; ref. 8). The functional significance of their upregulation is not yet known, but expression of members of the heat shock protein family is often observed upon cellular stimulation in a wide range of biological processes, probably to aid the correct folding of nascent proteins.

Ever since the detection of increased protease activity in post-stimulation hippocampus perfusate, there has been great interest in the function of these enzymes in the brain. A differential screening experiment designed to isolate genes upregulated soon after metrazol-induced seizures produced five 'brain activity-dependent' (BAD) genes. One of these encoded an extracellular serine protease, tissue-plasminogen activator (tPA; ref. 9), which exhibits a classical NMDA receptor-dependent IEG expression profile under a variety of activation protocols. Metrazol-induced seizures caused widespread upregulation, kindling (experimental epileptogenesis) caused bilateral hippocampal upregulation, and perforant pathway stimulation only affected ipsilateral hippocampus. Thus, an estimate of the comparative strength and range of these stimuli can be made from these data. The role of protease activity in neural tissue has been proposed to be one of synaptic remodelling; by reducing the rigidity of the extracellular matrix and the adhesivity of cell adhesion molecules, synapses can alter their structure, divide, or form entirely new connections. A tPA null strain of mice has been generated by targeted disruption of the gene locus. These mice exhibit altered blood clotting response (ref. 10) and possible resistance to excitotoxicity (ref. 11). Unpublished information (abstract: IX International Neurobiological Symposium, Magdeburg, 1995 and personal communication, E. R. Kandel) on the hippocampal function in these mutant mice seems to show that they lack conventional LTP. This is the first 'knock-out' experiment which has targeted an activity-dependent gene in the brain and the putative phenotype appears to suggest that mutations of this type may be more informative than the disruption of constitutively-expressed genes. Recently, another activity-inducible serine protease gene has been discovered. Neuropsin (ref. 12) expression seems to be restricted to the limbic system (hippocampus and amygdala; see Chapter VI).

The proposed presynaptic glutamate receptor GR33 shows a degree of NMDA receptor-dependent upregulation in dentate gyrus granule cells for a few hours after high frequency stimulation (ref. 13). Because of the location of the encoded protein in the presynaptic (axonal) processes of these cells, an upregulation could have an effect on the post-synaptic CA3 neurons. In this way, activation of gene expression in one section of the hippocampal network could have a knock-on effect on the excitability of the next section (by acting as a negative feedback control on the release of glutamate neurotransmitter). This kind of process is not Hebbian (nor is the potentiation exhibited by synapses between dentate gyrus and CA3 neurons) as it does not require the simultaneous activity of the post-synaptic neuron to evoke a change in potentiation. It would be interesting to see if the same sort of upregulation was seen upon CA1 LTP stimulation; a region displaying Hebbian LTP.

Kindling has been shown to have an down-regulatory effect on ligatin (a membrane-bound protein) gene expression (ref. 14). This lasts for up to four months and represents one of the longest-lasting gene expression shifts observed in the brain to date, even though the change in the level of expression is small.

LTP induction in the CA1 region of the hippocampus has a positive effect on the transcription of two neurotrophins, brain-derived neurotrophic factor (BDNF) and, to a lesser extent, neurotrophin 3 (NT-3) (ref. 15). These are growth and survival factors and may play a part in the avoidance of excitotoxicity and the creation of new synaptic connections. This is especially evident in the dentate gyrus region which exhibits mossy-fibre sprouting in response to certain electrical stimuli.

Recent advances in molecular biological techniques have permitted the isolation and analysis of mRNA from a single neuron (ref. 16). In this way, it has been possible to examine individual cellular responses to stimulation. Stimulated CA1 neurons show significant but modest increases in α-CaMKII, zif/268, and GABA β1 expression and comparable size decreases in the levels of mRNAs encoding PKC β1, BDNF, TRK, and muscarinic acetylcholine receptor 3 mRNA; all sampled at 30 minutes to 3 hours after stimulation.

In another experiment, a subtractive hybridisation was performed to enrich for dentate gyrus genes upregulated six hours after intraperitoneal injection of kainate (ref. 17). The resulting enriched library was used in a differential screen to further select these genes. Of 1000 clones studied to date, 52 show some degree of upregulation. Many of the previously described IEGs and activity-regulated genes were represented in this screen, together with several novel cDNAs. Two of these, cpg-1 and cpg-2 (candidate plasticity genes), were described in more detail. Cpg-1 is possibly related to the

neurotrophin family and shows preferential expression in the dentate gyrus after perforant pathway stimulation. Unlike cpg-2, it is activated in an NMDA-dependent fashion. Sequence information on cpg-2 is not yet available.

Perhaps the most interesting experiment from the point of view of the work presented in this thesis was the isolation of pentylenetetrazole seizure-induced genes by differential screening. This was made technically easier by the co-administration of cycloheximide, a treatment known to attenuate the down-regulation of immediate early genes. The precise methodology of the differential screening process (the screening of duplicate lifts of an activated hippocampus cDNA library with control cortex and activated hippocampus cDNA probes) had the dual outcome of revealing both hippocampus-enriched and immediate early activity-dependent genes. Three clones were described in differing detail in this paper; c-fos, a 'clone predominantly expressed in the hippocampus' (see Chapter VI), and an activity-regulated gene (arg3.1; ref. 18). The last of these exhibits a typical IEG activation time-course within the granule cells of the dentate gyrus but, interestingly, its mRNA is localised to the soma and dendritic processes of these cells. Only a limited number of neuronal transcripts have been shown to undergo this sort of trafficking throughout the cell (refs. 19-21). These include MAP2, α CaMKII, and the inositol trisphosphate receptor.

Pulse labelling of activated fragmented synapses shows that between 8-10 mRNAs are capable of being rapidly translated (in a transcription-independent fashion) at the synapse. These may be the transcripts that are important in the consolidation of LTP and which are affected in the translation inhibitor experiments described above. The data indicate that these mRNAs may be stored in the synaptic region until required. This has important implications for one of the tenets of LTP theory; that of 'synapse specificity'. This states that only associatively activated synapses can become potentiated. Events at a subset of synapses have to be communicated to the nucleus for transcriptional changes to ensue. The problem of accommodating the concepts of 'synapse specificity' with activity-dependent gene expression comes when these transcription or translation products have to be returned to the relevant activated synapses where, presumably, they act to stabilise synaptic potentiation. A 'potentiating' synapse must somehow present an identity signal to the protein and mRNA trafficking systems that it is a suitable site for such modification. It is not clear at the moment whether there is a distinction between constitutively expressed dendritically localized transcripts, which might (upon activity-dependent translation) form the basis of this signal, and those transcripts which travel to the activated synapse later (such as arg3.1), which may be part of the consolidatory process. The

analysis of potential targeting signals of these transcripts would be a useful approach. Arg3.1 has an unconfirmed homology to the cytoskeletal protein spectrin, and may therefore, play a role in activity-related synaptic remodelling.

5.4: Stimulus paradigms employed for gene expression induction

Because techniques such as subtractive hybridisation and Northern blots have low sensitivity limits, non-physiological stimulation of the hippocampus is usually required to permit observation of activity-dependent changes in gene expression. It is a property of the *in vivo* hippocampus that when it is active (for instance when the animal is exploring a novel environment) fewer neurons fire than when at rest. This observation is known as 'sparse coding' (ref. 22). The functional implication of this highly restricted activity is a 'computational' benefit arising from the lack of processing interference; in fact, this has been adopted by neural network designers as an efficient model. However, the implications for the molecular biologist are that during hippocampal activity only a small number of neurons will exhibit transcriptional changes: these will be diluted by the uninduced majority. With this in mind, stimulus paradigms have therefore been adopted which maximise the percentage of transcriptionally active neurons. Inevitably, a compromise has to be struck between the proportion of the hippocampus driven into the expressing state and the artficiality of the stimulatory method. It has to be borne in mind that the genes whose expression is changed because of a particular inductive regime may not be those which participate in the normal memory creation process. Certainly the gross result of intraperitoneal injection of kainate (ref. 17) is violent seizures which can be fatal: this would be expected to induce gene expression changes in its own right which may be in response to the drug's toxicity rather than the induction of potentiation. Nevertheless, much of the investigation of LTP from a pharmacological or electrophysiological stand-point has faced similar concerns over the abilty to infer the mechanisms of the natural process from experimental intervention.

There is a danger that a gene can be ascribed a hippocampus-specific expression pattern (and hence, a role in learning in memory) purely because the stimulus paradigm used selectively activates that part of the brain; for example, perforant pathway stimulation. The brain expression pattern of induced tPA is very different depending on the precise stimulus used. Therefore, the assessment of induced expression should be performed after a wide variety of stimuli (including, if possible, a 'natural context' behavioural activity such as swim maze navigation) before the induced expression of a gene can be described as both restricted to the hippocampus and linked to its function.

5.5: Electroconvulsive stimulus (ECS) : depression treatment, LTP model, and stimulatory paradigm

The aim of the subtraction described in this chapter was to isolate genes which are up- and down-regulated in response to electrical activity in the hippocampus. The choice of the stimulus protocol was electroconvulsive stimulation (ECS) which has characteristics that make it suitable for this experiment (ref. 23). These include the longevity of the induced potentiation changes and the lack of spontaneous epileptic episodes in the rats (in contrast to kindling).

Electroconvulsive therapy (ECT) is a treatment for chronic depressive illness in humans which can be used as an alternative or complement to drug treatment. Patients who receive this therapy sometimes suffer from anterograde amnesia for a few weeks after the end of the course of treatment. A similar effect has been seen in rats which have undergone the analogous procedure, ECS. *In vivo* electrophysiology has permitted the greater understanding of these side-effects. It appears that after a defined number of ECS shocks have been administered there is a semi-permanent increase in the general potentiation level of neurons in the hippocampus (amongst other regions). This results in the inability to induce additional LTP (by artificial means) to the same extent as can be induced in control animals (ref. 23). It is observed that this effect attenuates after 30-40 days in these animals: a time-scale similar to that of the human side-effects. Perhaps this can explain the amnesic side-effects reported by the patients; hippocampal synapses are uniformly potentiated to such an extent (saturated) that genuine memory-forming potentiation cannot occur.

ECS has been used in an attempt to tighten the link between LTP and spatial learning. Several studies have described efforts to induce total LTP saturation in the hippocampus by means of bilateral perforant pathway stimulation along the entire length of the hippocampus. Rats treated in this way have then been assayed for hippocampal function in a variety of behavioural tests; the aim being to prove that hippocampal LTP saturation prohibits subsequent learning. It seems likely that the highly variable results published from such experiments can be explained in terms of the failure to elicit ubiquitous and maximal stimulation within the hippocampus (ref. 24) Moreover, network models of hippocampal function predict that the amount of memory deficit observed would not follow a linear relationship with the extent of LTP saturation. The relationship is likely to be sigmoidal in that a threshold saturation level has to be attained before the onset of any hippocampal deficit, but when this is achieved the effects of any further saturation are substantial (ref. 25). In this way, the

adoption of electroconvulsive stimulation as a means to ensure complete saturation has led to the reproducibility of behavioural deficits.

Very little is known about the precise mechanisms responsible for the anti-depressive effect of the treatment. One feature of note is that approximately 5-6 ECS shocks are required to elicit the maximum induction of potention in rats: this matches the number of ECT treatments necessary to produce the most beneficial and sustained anti-depressant effects in human patients. An understanding of the gene expression changes that accompany ECS in rats may go some way to explaining how this particular treatment acts and possibilities for novel treatment approaches.

5.6: Molecular studies of ECS illustrate its similarity to conventional LTP-inducing procedures

Several groups have begun to investigate the molecular biology of ECS, generally examining the transcriptional changes of candidate genes that might be involved in the etiology of depression. One of the very first attempts (ref. 26) was published in 1979 and concerned the immunoelectrophoretic detection of several neural markers (synaptin, D1, D2, D3, and 14-3-2). After a course of EC stimuli, increased levels of the markers synaptin and 14-3-2 (which was tentatively suggested to be enolase enzyme) were detected in the frontal and occipital cortices. The advent of more sophisticated molecular biological techniques has revealed expression changes at the level of transcription. In a recent paper (ref. 27), levels of CRH mRNA in the paraventricular nucleus and levels of TH mRNA in the locus coeruleus were assayed after 1, 3, 7, and 14 EC stimuli. The results show that TH is upregulated after one stimulus whereas CRH requires 7 to induce maximum transcriptional change. The raised levels of these two mRNAs persist for three weeks in the case of CRH and eight weeks for TH. Such long-term changes in gene expression have been rarely documented in the literature concerning brain gene expression.

It may be that ECS represents an efficient tool to investigate expression changes that follow LTP because of the large-scale and widespread potentiation that occurs. Admittedly, the relationship between the anti-depressive actions, the potentiation saturation effects, and the LTP induction properties of ECS is not yet understood. Nevertheless, the ECS-induced gene expression changes that have been reported are similar to more standard excitation protocols. For example, the immediate early gene transcription factors have been shown to be upregulated within the first few hours after an EC stimulus. A subtractive and differential screening approach (ref. 28) was used for the identification of early response genes induced by maximum

electroconvulsive shock (MECS). One gene identified was mitogen-inducible cyclo-oxygenase (COX-2/prostaglandin synthase 2) which is a constituent enzyme in the synthesis pathway responsible for the production of thromboxane and prostaglandin; two modulatory compounds thought to have wide-ranging effects on neurons. Again the activation profile is typical of the IEG family with an expression peak at 30 minutes and a return to base-line at 8 hours. This gene is upregulated developmentally, in the post-natal brain, and in response to stress. Recently, the targeted disruption of this gene has been reported (ref. 29). The phenotype shown by mutant mice includes the presence of kidney abnormalities and a susceptibility to peritonitis. No attempts were made to address possible cognitive deficits. A second gene isolated from this screen has been identified as a member of the small GTP-binding protein family (ref. 30). *Rheb* is most closely related to *ras*1 and *rap*2 at the sequence level. Its transcriptional induction following maximal electroconvulsive stimulus is approximately 2-5-fold above basal levels. The time-course of upregulation follows the typical immediate early gene pattern, with the maximal increase observed 4 hours after stimulation. In addition, a novel member of the EGR/NGF transcription factor family, egr3, has been cloned from subtracted ECS-treated brain cDNA by the same group (ref. 31). As with all of these IE genes, a return to baseline expression levels is seen within 24 hrs. Other genes shown to be upregulated by ECS include neuropeptide Y(ref. 32), nerve growth factor, basic growth factor, α 1b (but not 1a) adrenergic receptor, 5HT1a, and 5HT2a receptors (ref. 33).

5.7: The study of long-term gene expression changes

The study of activity-dependent gene expression changes described in this chapter was performed using mRNA extracted 72 hours after the final stimulus in a series, as opposed to the majority of experiments which are concerned with relatively immediate changes in gene expression. Our aim was to obtain genes whose expression levels in the hippocampus remain altered for long periods of time in response to ECS. Such genes might be responsible for the consolidation or the maintenance of synaptic efficacy changes. However, it is important to note that though the effects of ECS extend far beyond the 72 hour time-point, it is not certain that these effects are mediated by a continued change in gene expression (even though some genes are upregulated for this period, as mentioned in section 5.6). The potentiation/saturation at these later time-points could be mediated by early synthesised proteins that persist in their roles of synaptic restructuring or signal transmission modification.

Section 5.8: The ECS regime and the subtraction process

All experiments involving ECS were carried out in collaboration with Drs. Ian Reid and Caroline Stewart (then based at the Biomedical Sciences Division, Aberdeen University, and now at the University of Dundee). They were responsible for carrying out the full ECS regime on the experimental animals.

To maintain comparability, only male Fisher rats of approximately four months of age were used. The electroconvulsive stimulation procedure involved the rats being divided into two groups; ECS and Sham. Both groups were subjected to light halothane anaesthesia prior to the attachment of electrodes to the ears. Only rats in the ECS group received the EC stimulus (see Materials and Methods section) which induced tonic/clonic seizures lasting approximately 15 sec Sham rats were subjected to the same degree of handling but were not stimulated. This protocol was repeated on alternate days such that, by the end of the program, the ECS rats had received 10 shocks each (6 shocks are normally considered adequate to elicit maximal electroconvulsive stimulation). 72 hrs after the final stimulation, the brains of these rats were removed and dissected into subregions.

cDNA derived from hippocampus mRNA was used to create two PCR-amplifiable cDNA pools (from Sham and ECS rats) as described in the Materials and Methods section. These formed the starting material for the subtractive hybridisation process as well as DNA material for use in cDNA Southern hybridisation experiments.

The subtraction was limited to four reciprocal rounds of hybridisation for the reasons described in the previous chapter. Stage four ECS (E^{+4}) and Sham (S^{-4}) cDNA pools were cloned into a bacteriophage M13 vector (tg131) in order to create small libraries of the final subtracted products. These libraries were plated, transferred to nitrocellulose filters and taken through a differential screening process using particular cDNA stages as templates for the various probes. E^{+4} filters were screened with probes derived from stages E^{+4} and S^{-}/S^{-4}. S^{-4} filters were screened with probes derived from stages S^{-4} and E^{+}/E^{+4}. Differentially hybridising plaques were picked and purified to isolation.

Plaques only hybridised by E^{+4} probe were designated ECS up-regulated clones (ECU clones) whereas plaques only hybridised by S^{+4} probe were designated ECS down-regulated clones (ECD clones). The cDNA inserts of the ECU and ECD plaques were isolated and studied by sequence and expression analysis as described below.

5.9: Sequence analysis of clones

The majority of ECU and ECD clones were subjected to sequence analysis and used to search the GenBank/EMBL databases for homology using the FASTA program of the GCG8 Wisconsin DNA analysis package (tables 5a and 5b).

Most clones lacked database matches, although the number might be reduced upon more extensive sequencing of the clones or examination of corresponding full-length cDNAs. It was also notable that very few of the putative down-regulated clones were identified by sequence homology. This might be in part due to the lower average abundance of these clones (see section 5.10): lower abundance transcripts are less well represented in any database.

Three sequences showed a high degree of homology to human expressed sequence tags (ESTs) which are short regions of sequence obtained from the automated analysis of cDNA libraries. Because brain cDNA libraries have been frequently subjected to this kind of analysis, it was likely that at least some clones would exhibit homology to EST sequences.

The other identified clones are described below.

Aconitase

Clone ECU 6, while being only 40 base pairs in length, possessed 100% identity, when hypothetically translated, to pig heart aconitase (ref. 34). Aconitase is unusual in that the protein it encodes has two very different functions. First, it is a mitochondrially located enzyme which catalyses one stage in the citric acid cycle. As such it is an enzyme containing a [4Fe-S] cluster cofactor which is responsible for the redox conversion of its substrate, citrate, into isocitrate. The iron-sulphur part of this enzyme is itself very sensitive to disruption by free radicals that accumulate during hyperoxic shock or NO release. Second, the aconitase enzyme with a deactivated and dissociated iron-sulphur core behaves as an entirely different protein (dubbed the iron regulatory protein, IRP) which acts by binding mRNAs encoding iron metabolism enzymes (ref. 35).

NF-YB

Clone ECU 9 corresponds, at the 5' end, to the published sequence of NF-YB (ref. 36). NF-YB protein product, when complexed to that of NF-YA, forms a transcription factor (NF-Y/CP-1/CBF). This factor, along with other factors such as CP-2 and NF-1

Table 5 a ECS up-regulated (ECU) clones including sequence homology information and expression pattern by cDNA Southern and Northern hybridisation. **b** ECS down-regulated (ECD) clones including sequence homology information and expression pattern by cDNA Southern and Northern hybridisation.

Table 5a

ECU Clone	cDNA Southern	Sequence Homology	Northern Results
2	E>>>>>Sh	No Homology	All lanes
6	E>>>>>Sh	Aconitase	-
8	E>>>>>Sh	No Homology (=14/24)	-
9	E>>>>Sh	NF-YB Tr. Fact.	All lanes
10	-	Phosducin-Like Protein (1)	-
11	-	No Homology	Smear/band in all lanes
12	E>>>>>>Sh	Phosducin-Like Protein (2)	All lanes
13	E>>Sh	B-Catenin	-
14	-	No Homology (=8/24)	-
15	E>>Sh	Hu.EST T50042	All lanes
16	-	No Homology	-
17	-	No Homology	All lanes
18	-	B-Catenin	-
19	-	No Homology	-
20	-	No Homology	-
21	E>>>>>Sh	No Homology (=26/35)	-
22	-	No Homology	-
23	-	No Homology	-
24	-	No Homology (=8/14)	-
25	-	No Homology	-
26	-	No Homology (=21/35)	All lanes
27	-	No Homology	-
28	-	No Homology	-
29	-	No Homology	-
30	-	B-Catenin	-
31	-	Hu.EST Z40081	-
33	-	No Homology	-
34	-	No Homology	-
35	-	No Homology (=21/26)	All lanes
36	E>>>Sh	Guanine-Nucleotide Rel. Fact.	All lanes
37	-	No Homology	All lanes?
38	-	No Homology (fusion prod.)	No signal
39	-	No Homology	-
40	-	B-Catenin	-

Table 5b

ECD Clone	cDNA Southern	Sequence Homology	Northern Results
1	Sh>>E	-	-
2	Sh>>>E	No Homology	-
6	Sh>>>E	No Homology	No signal
7	Sh>>>E	No Homology	-
9	Sh>>>E	-	-
10	-	No Homology	-
12	-	hEST F07937	-
14	Sh>>>E	No Homology	-
24	Sh>>>E	-	No signal
25	-	No Homology	No signal
26	-	No Homology	All lanes: not baseline
28	-	No Homology	-
35	-	No Homology	-
38	-	No Homology	-
40	-	No Homology	-
50	-	No Homology	-
54	-	No Homology	-
60	-	No Homology	-

bind to the DNA sequence CCAAT; a motif present in the promoters of many eukaryotic genes, and studied in detail in the promoters of the major histocompatability class II genes (ref. 37). Other promoter examples include herpes simplex virus thymidine kinase, mouse MHC I, mouse albumin, interleukin-4, thrombospondin 1, tryptophan hydroxylase, rat aldolase B, and mouse α and β globins. When the two constituent genes were originally cloned and sequenced, it was postulated that together they possess a zinc finger-like structure with each member protein containing amino acid motifs (CXXC in NF-YB and HXXH in NF-YB) which may act together as a zinc ion chelator, as in other examples of this family. In non-CNS mouse tissue NF-YB transcripts of 1.2 and 3.4 kb have been described. Differences at the levels of splicing and polyadenylation sites were proposed to explain the different forms but only the cDNA which corresponds to the smaller species has been obtained and sequenced to date. Clone ECU 9 was used on Northerns created from both base-line and Sham/ECS brain RNA. Only one transcript was observed at a position in agreement with it being of over 5 kb in length. ECU 9 is approximately 600 bp in length and when sequenced from the 5' end shows identity with a region of the published sequence commencing 280 bp upstream of the polyadenylation site. Near to the published polyadenylation site this homology abruptly ends (fig. 5.3). The remainder of the ECU9 sequence (300 bp) possesses three potential polyadenylation signal sequences, of which the third one is utilised. Hence, this gene could exist in at least four polyadenylation forms (two published, one used here, and one other putatively defined by its polyadenylation motif; see Appendix for sequence analysis of ECU9). To confirm that this clone does not represent an artifactual fusion event which might have occurred during cDNA pool synthesis or subtracted material cloning, a 180 bp PCR product encompassing the 3' end of the sequence was used as a probe on another Northern; exactly the same transcript size was observed. Even though ECU9 possesses a slightly longer 3' untranslated region, an additional 4 kb must be located in the 5' untranslated region to account for the larger transcript size. It is possible that this transcript size is brain specific as it was not detected in other regions in the original publication. To function as a transcription factor, both subunits have to be present. The presence of NF-YA protein in the brain has been demonstrated. With respect to NF-YB, expression has not been described in the brain (ref. 38).

Phosducin-like protein

Two clones, ECU 10 and ECU 12, emerged from the subtraction as the most highly expressed and upregulated genes at the level of cDNA Southern analysis. Sequencing of these clones revealed that they were homologous to the same gene, 'phosducin-like

protein' (PhLP; ref. 39), but were derived from differently spliced transcripts. Signalling pathways downstream of G protein-coupled receptors rely on the participation of both the α and the β/γ components of the heterotrimeric G Proteins (see Chapter II for examples). In addition to the actions of the α subunit, these seem to be mediated by the interactions of the β/γ complex with proteins containing a 'pleckstrin homology' (PH) domain (refs. 40 and 41). Members of the growing family which contain this domain include β-spectrin, *ras*-GRF (also isolated as an upregulated clone), β adrenergic receptor kinase (see Chapter II), protein kinase C μ, phospholipase C γ, Btk tyrosine kinase, and the syntrophins (a protein family associated with dystrophin). However, it must be noted that not all β/γ complex-binding proteins contain this domain (e.g. the potassium channel GIRK1 and adenylyl cyclase II).

In a screen for genes upregulated by alcohol administration to cells in culture, a clone was obtained which exhibited sequence homology to phosducin, especially at the C-terminal end (65% overall at the amino acid level), which was therefore dubbed 'phosducin-like protein' (PhLP). Its expression is increased by a factor of two upon 24 hour exposure to 100 mM ethanol. Phosducin, itself, was first isolated from retinal cells by virtue of its transducin β/γ complex-binding function and it has been shown to play a role in the negative modulation of that signalling pathway. Sequence homology to β-ARK demonstrated that the β/γ-interacting region of phosducin was localised to the N-terminal region of the protein (refs. 42 and 43). This was confirmed in a binding assay. Interestingly, this N-terminal region contains residues with significant homology to the C-terminal portion of the canonical pleckstrin homology domain. Thus this protein possesses a '1/2 PH domain' which has been shown elsewhere to be sufficient for the protein interaction.

During the initial characterisation of the PhLP cDNA clones, the authors saw considerable heterogeneity of sequence at the 5' end of the transcript. This was due to the existance of at least four 5' exon combinations as illustrated in fig. 5.3. One of these splice variants creates a transcript which, when translated, produces a longer protein product than the others. Moreover, the additional amino acid sequence of this extended protein contains the typical residues of the 1/2 PH domain, making it able to bind to β/γ complexes. Thus, by alternate splicing, the cell is able to regulate the β/γ complex-binding potential of the resulting protein. Spatial restrictions on the expression of some of the transcript types has been reported. It would also be interesting to discover if the splicing choice was activity dependent.

The two clones isolated by this subtraction corresponded to the heterologous 5' region of the PhLP cDNA (see fig. 5.3). ECU 10 was derived from a transcript containing

exons 4 and 5 that would give rise to the longer (PH-containing) protein product, whereas ECU 12 was derived from exons 3 and 5

β-Catenin

Four ECU clones showed sequence identity to β-catenin at its 3' end. Three clones (ECUs 13, 18, and 30) were of 120 bp length, whereas a fourth (ECU 40) consisted of a sequence that overlapped with the others but extended an additional 370 bp further in the 5' direction.

Adhesion between cells is a fundamental process in many biological systems. The ways in which neurons (specifically, their synapses) are attached to each other has been the subject of much recent research; especially in the light of data that suggest a role for cell adhesion in plasticity events that underlie memory formation (see introductory chapter). Cell adhesion is mediated by a large number of diverse protein families. One such family, the E-cadherins, forms homotypic cell-cell interactions in the presence of calcium ions. In epithelial cells, these adhesion molecules are restricted in localisation to the zonula adherens on the lateral surface of the cell where they are associated with two main proteins (ref. 44). These proteins, α-catenin and β -catenin, seem to function in the regulation of cadherin-mediated cell adhesion. β-catenin, in particular, is regulated by the signalling pathways of two proto-oncogenes, *wnt* and *src* (ref. 45). *Src* non-receptor tyrosine kinase activation brings about a decrease in cellular adhesion whereas *wnt*-1 activity has the reverse effect. β catenin (and γ catenin/plakoglobin) show a high sequence conservation at the amino acid level to a *Drosophila* protein, armadillo, which has been shown, in this organism, to participate in both of these signalling cascades (ref. 46).

ras-GRF

The final clone identified by sequence homology (ECU 36) was shown to be the guanine-nucleotide-releasing factor for the *ras* monomeric G protein (*ras*-GRF; ref. 47). The product of this gene is responsible for the conversion of the inactive guanine diphosphate-bound form of *ras* into unbound *ras*. This can then bind guanine triphosphate in a reaction catalyzed by the *ras* activation protein, *ras*-GAP. This process is, in turn, regulated by *ras*-GAP's activation by a variety of cell-type specific stimuli, usually emanating from receptor tyrosine kinase ligand binding. The importance of the *ras*-GRF function is that it is responsible for the rate-limiting step in the *ras* activation cycle and therefore dictates the intensity of the *ras* response to stimulation.

5.10: Expression analysis by 'cDNA Southerns'

Many of the clones that were isolated as differentials from the subtraction were used as probes on cDNA Southerns (the initial, pre-subtraction cDNA pools electrophoresed and blotted onto filters) as a quick assay for the efficiency of the subtraction. In comparison to the other subtractions that had used this technique, it was clear that the majority of clones that had emerged from the differential screening step exhibited a striking bias of distribution according to this assay. As this is a test of how well the subtractive process had performed with respect to the ability to select cDNAs which had a skewed starting distribution, the subtraction seemed to work more efficiently than before. Figures 5.1 and 5.2 list the results of a selection of cDNA Southerns.

Several observations concerning the nature of the hybridisations were made. Firstly, from the results of the screen, there seem to be fewer cDNA sequences which appear down-regulated. Moreover, the general abundance of these species together with their degree of down-regulation was less than that for the up-regulated genes. Secondly, some of the expression differences are extremely pronounced such as those for PhLP, the 8/21 family of clones, and NF-YB, whereas others are less so. Thus, the subtraction appears to able to isolate differentially expressed genes present at a variety of abundances. Thirdly, in the case of the up-regulated clones, several repetitions of cloning were observed (for example, β-catenin, PhLP, and the 8/21 family) and while the expression level by cDNA Southern did not always correspond to the number of times a perticular sequence was cloned, there was some agreement. The fact that some clones were appearing multiple times can be used to argue that the total number of up-regulated clones enriched in this subtraction is not far in excess of those which were picked in this study. Fourthly, the precise pattern of hybridisation to the cDNA Southern was notable in a few examples. In the creation of the cDNA pools, the three factors which should determine the sizes of fragments derived from a parent transcript are; the distribution of the restriction sites used to digest the cDNA, the 5' extension by the reverse transcriptase enzyme, and the length of fragment that can be efficiently amplified by PCR. If a clone that emerges from the subtraction is used as a probe on the cDNA Southerns two patterns of hybridisation (or combinations thereof) would be expected; either discrete bands in the case of defined restriction fragment sizes, or smears in the case of 5' fragments which terminate at a random spread of positions. The predominance of the latter may indicate that 5' ends were more extended in one pool than in the other leading to false positives (see below section for a fuller explanation and counter-argument). The presence of incomplete digestion of the pre-amplification cDNA has been observed at the level of sequencing

Figure 5.1 cDNA Southerns probed with ECS up-regulated (ECU) clones obtained from the subtraction. S represents the sham cDNA pool and E, the ECS cDNA pool. Also shown is a cDNA Southern probed with a 700 bp fragment of the 8/21 family (see section 5.14).

Figure 5.2 cDNA Southerns probed with ECS down-regulated (ECD) clones obtained from the subtraction. S represents the sham cDNA pool and E, the ECS cDNA pool. Also shown are cDNA Southerns probed with a candidate plasticity gene (cpg-1) and a loading/representation control, ribosomal protein S26.

Figure 5.3 a Cartoon representation of the transcript sizes observed for NF-YB. 1.2 kb and 3.4 kb transcripts have been described in the literature and a 5.5 kb transcript was observed in brain Northerns carried out here. Directly underneath is a representation of the structure of the 1.2 kb transcript (solid lines, ORF; open reading frame) and the additional mRNA required to create the larger transcripts (dashed lines). Clone ECU 9 contains an additional novel 3' untranslated region of NF-YB. **b** Possible exon combinations of a phosducin-like protein transcript. Under each is shown the resultant open reading frame for translation. Regions corresponding to the sequences of ECU 10 and 12 are indicated. Note that the exon combination -4-5- will give rise to a longer protein product upon translation.

Fig. 5.1

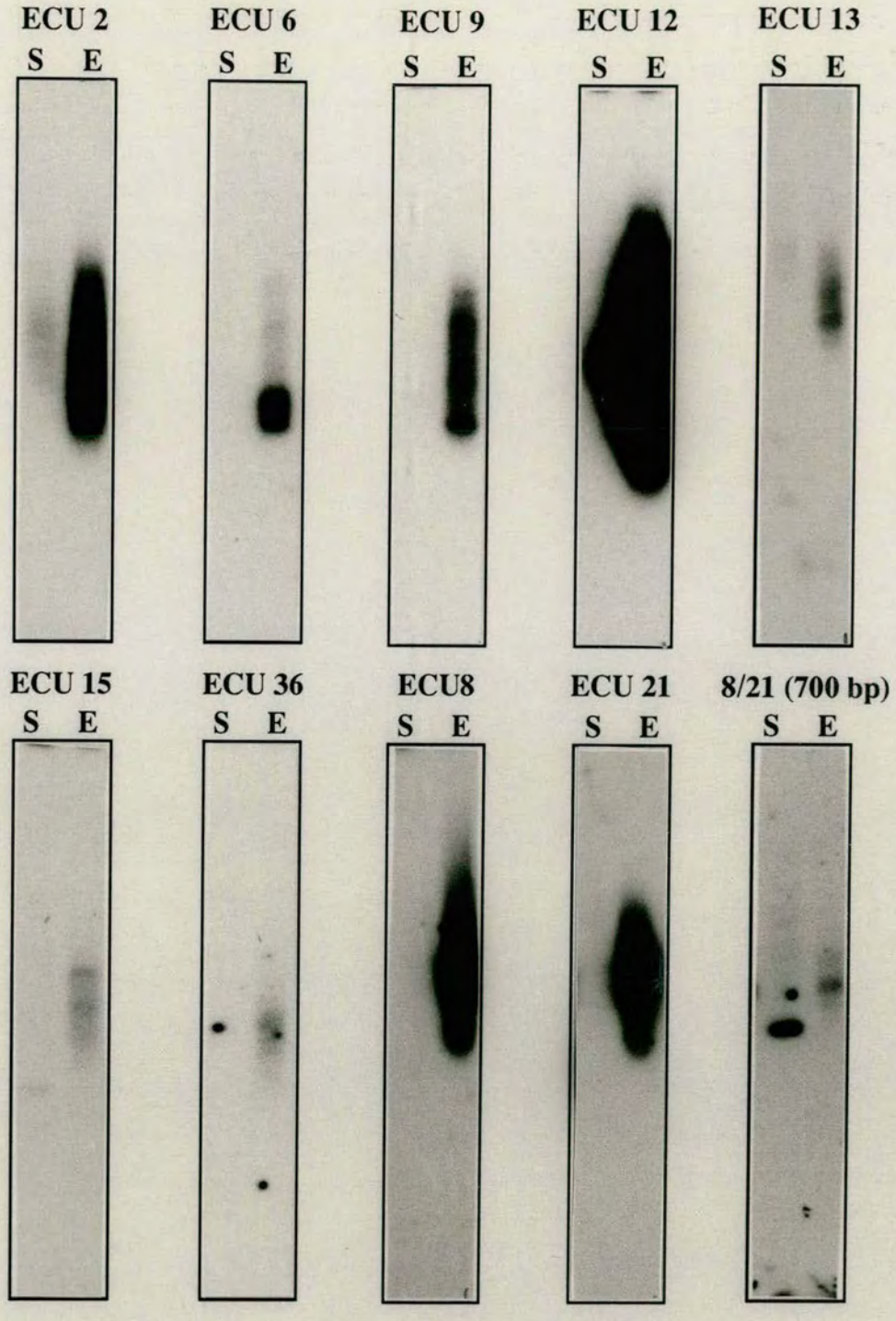

ECU 2
S E
ECU 6
S E
ECU 9
S E
ECU 12
S E
ECU 13
S E
ECU 15
S E
ECU 36
S E
ECU8
S E
ECU 21
S E
8/21 (700 bp)
S E

Fig. 5.2

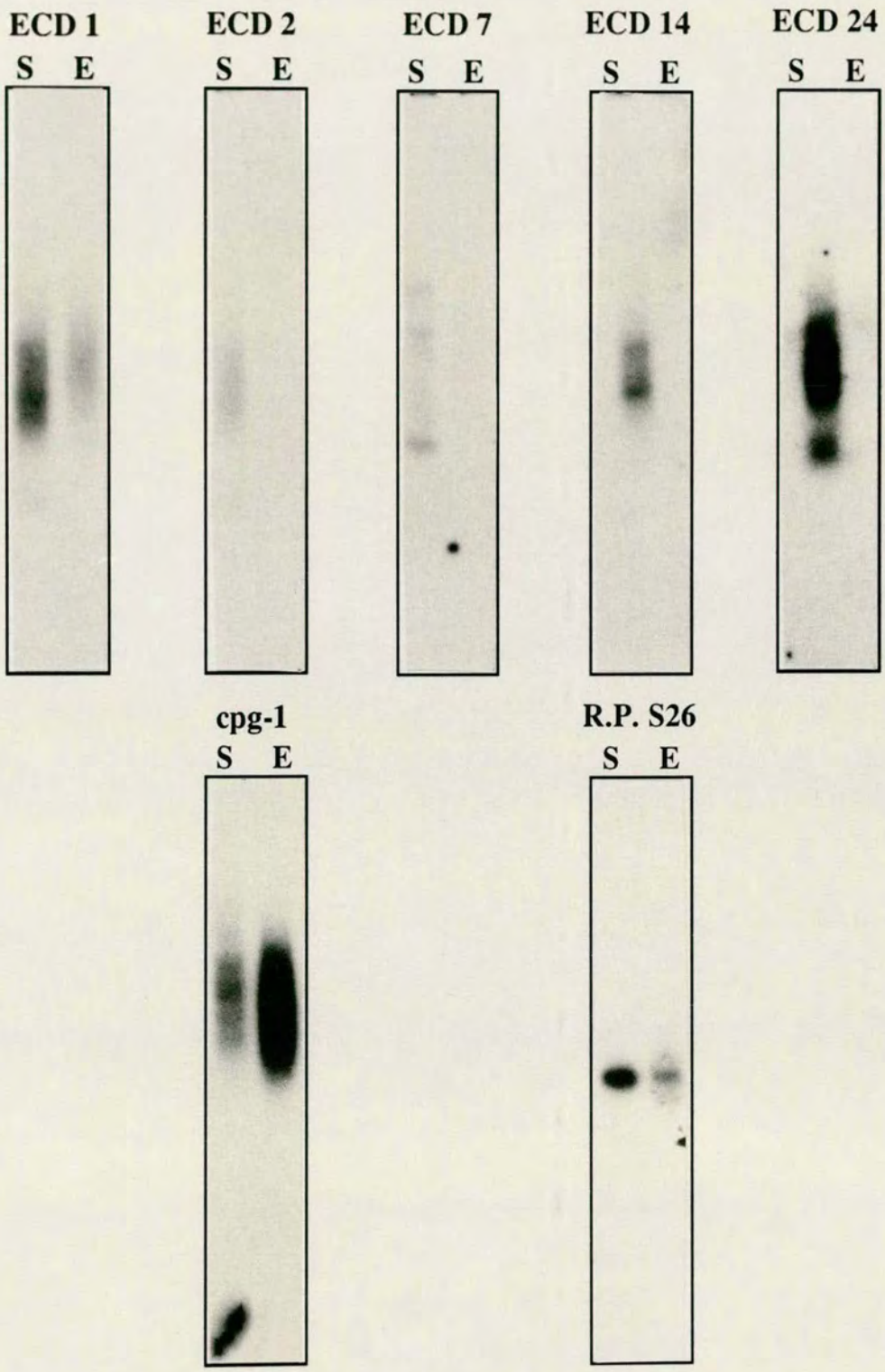


Fig. 5.3a

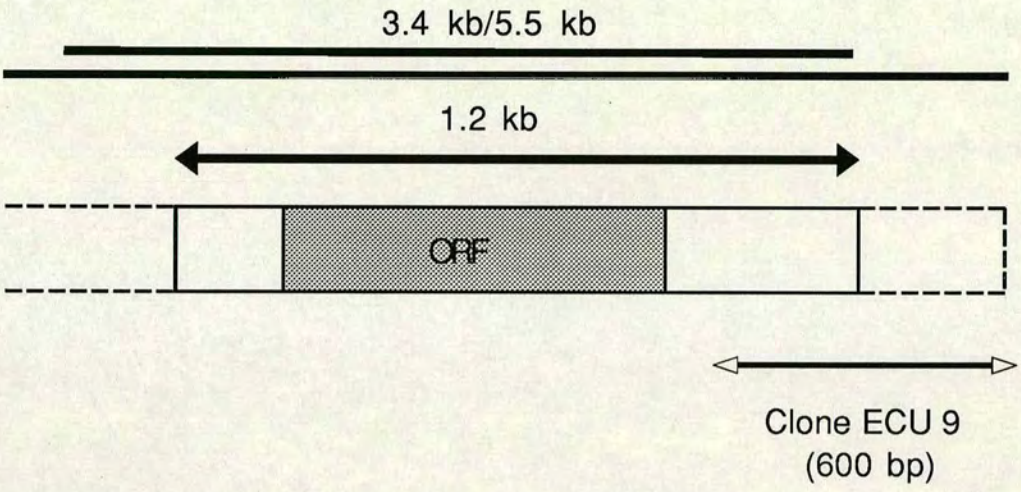
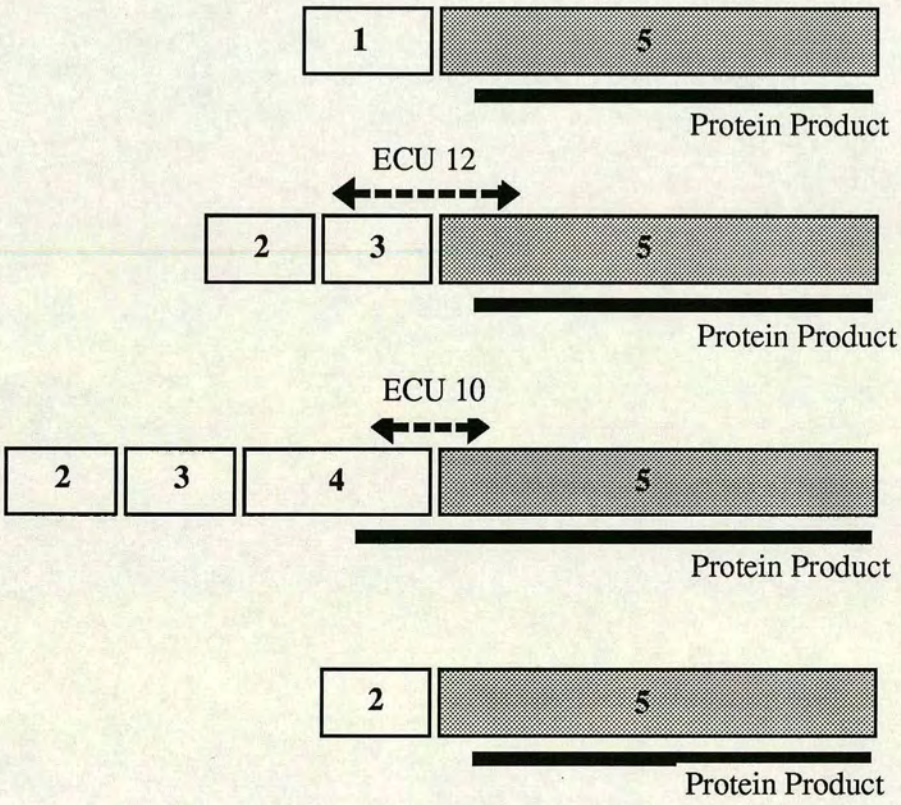


Fig. 5.3b



and may cause a complication in the hybridisation pattern analysis of these clones. Fifthly, closer inspection of some of the cDNA Southerns shows that the expression is not entirely restricted to one pool. In some cases, the hybridisation pattern between the two pools differed with respect to the size of the cDNA fragments which are being hybridised (e.g. in the case of ECU 13; β catenin). This may indicate that the differentials are not genuine but the result of different extents of restriction digestion during the two cDNA pool syntheses.

5.11: Expression analysis by Northern hybridisation

Total RNA corresponding to selected brain regions was obtained from a second batch of rats taken through the Sham/ECS procedure. Northern blots were created from this material in order to obtain a more accurate assessment of the expression level changes of the subtraction clones after ECS.

Fig. 5.4 shows Northern filters hybridised by a representative selection of the subtraction clones. The striking feature of these is that the differences between Sham- and ECS-treated tissues, as seen on cDNA Southerns, are not maintained at the Northern hybridisation level. For a complete list of the Northern results see tables 5a and 5b.

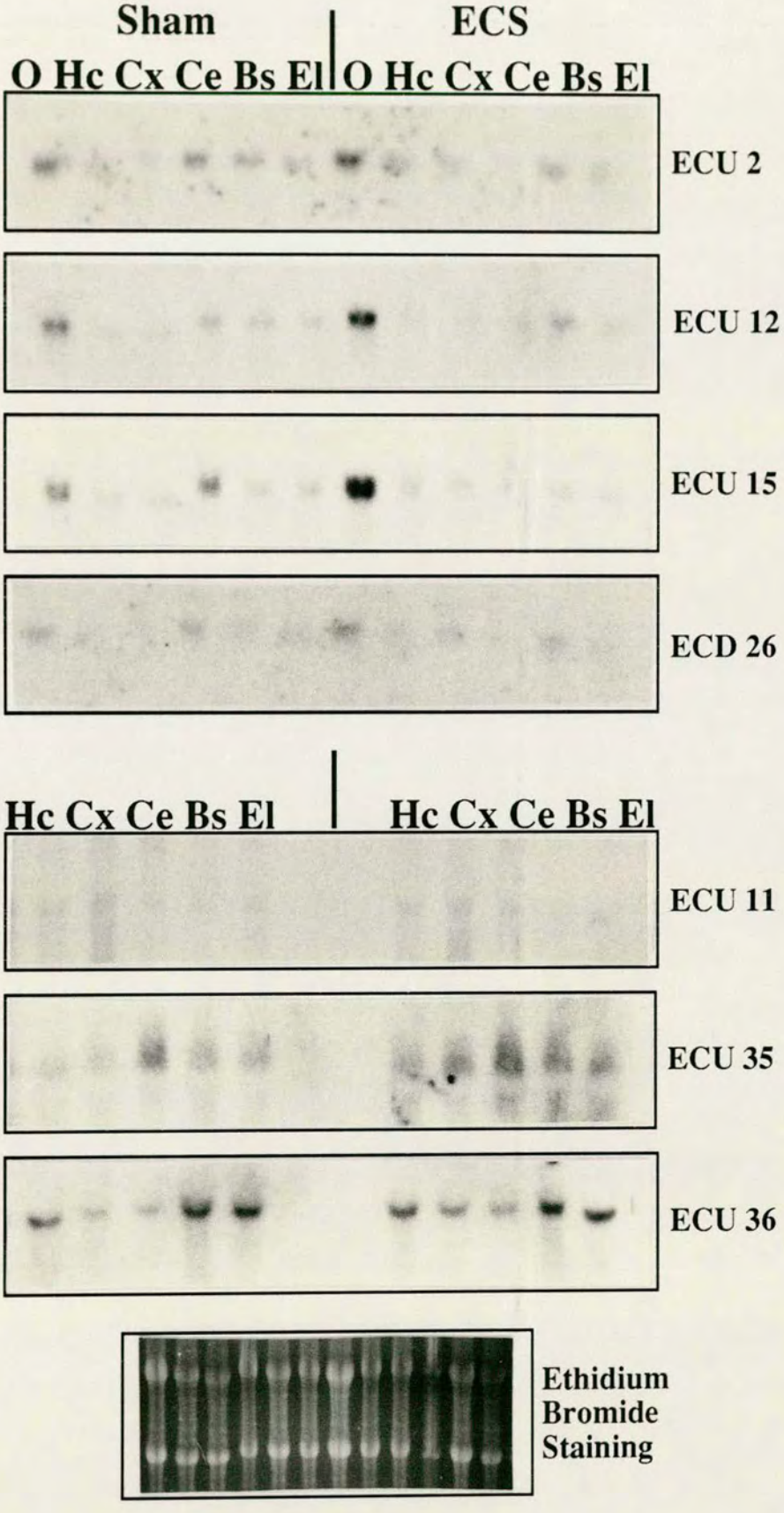
Several general observations can be made about the Northern hybridisation results. First, only a few ECD clones showed detectable expression by this method which suggests that these clones are, on average, expressed at a lower level than ECS clones. Second, although not a quantitative observation, the relative hybridisation strength of any given EC clone on a cDNA Southern did not always correlate with its expression level by Northern. The most extreme example of this discrepency was ECU 9, which gave one of the strongest signals by cDNA Southern, but displayed very weak expression on a Northern blot. One potential explanation for such a result is that preferential amplification (see Chapter IV) has actually occurred in the case of ECU 9, leading to a falsely high abundance level in the original and subtracted ECS cDNA pools.

5.12: Hypotheses for the discrepencies seen between cDNA Southern and Northern expression analyses

The failure to corroborate the expression patterns of the clones on cDNA Southerns with expression on Northern blots was a feature of the previous subtraction, described in Chapter IV (carried out simultaneously). From a naive stand-point, the results from

Figure 5.4 Northern expression analysis of a selection of up-and down-regulated clones fromn the subtraction. Tissue RNA lane abbreviations are O; olfactory bulb, Hc; hipoocampus, Cx; cortex, Ce; cerebellum, Bs; pons/medulla/brainstem, and El; else (brain minus O/Hc/Cx/Ce/Bs i.e. thalamus/striatum etc.). Expression comparisons between Sham and ECS-treated rat brains are shown for the clones listed on the right-hand side of the page. The bottom of the page shows a typical ethidium-stained Northern gel to show comparable RNA loading: it can be seen that the olfactory bulb lane is slightly overloaded in every case.

Fig. 5.4


Sham
ECS
O Hc Cx Ce Bs El
O Hc Cx Ce Bs El
ECU 2
ECU 12
ECU 15
ECD 26
Hc Cx Ce Bs El
Hc Cx Ce Bs El
ECU 11
ECU 35
ECU 36
Ethidium
Bromide
Staining

sequencing of the clones appear to conflict with the notion that they are merely artifacts. The genes that have been identified do not seem to be the result of inefficiency in the subtraction process: if that were the case then it might be expected that highly expressed, mitochondrial, housekeeping, or ribosomal transcripts would have been cloned in greater numbers (as they were in the experiment described in Chapter III). Moreover, the cloning of several independent cDNA fragments from the same gene (for example, β-catenin, PhLP, and, possibly, the ECU8/21 family of clones) seems to suggest that they are genuine. However, several possible explanations for the failure are listed below. Section 5.13 describes the results from experiments which were designed to test these theories.

1) The mRNA which was used for the creation of the cDNA pools prior to the subtraction, and the RNA extraction which formed the basis for the Northerns, were derived fron different batches of rats; taken through the ECS procedure at different times. All possible efforts were made to ensure that these rats underwent identical protocols to ensure their comparability but, because of their different origins, there may have been inconsistencies between them at the level of transcription.

2) During the various steps that were carried out, there could have been a stochastic failure with respect to one pool to convert some mRNAs into PCR-amplifiable DNA strands. Thus those sequences would appear to be enriched in the other pool. Stochastic processes would present more of a problem if the amount of nucleic acid material present in the pool synthesis steps was very low; approaching single representation with respect to some transcripts. The PCR-amplified material would not be a good representation of the true expression profile in such a case.

3) The polyadenylation extent of a transcript is known to play an important role in its stability. In addition, specific examples of polyadenylation changing in response to a stimulus have been well-documented and assayed (for example, tPA; ref. 48). If the polyadenylation of a given transcript fell below a certain threshold length then it might not be purifiable using an oligo-dT cellulose column (see Materials and Methods chapter; mRNA extraction). Reverse transcription was performed using oligo-dT primers which would also affect the efficiency of conversion to cDNA for such a transcript. Hence, it could be postulated that up-regulated clones are derived from transcripts which are raised above a threshold polyadenylation state by ECS and down-regulated clones by the reverse process. The Northern expression data would not easily reflect this difference as total RNA (no selection for polyA$^+$ RNA) was used.

4) Differential splicing is a process which has been shown to be altered in a tissue-specific and developmental stage-specific manner (ref. 49). If ECS causes a change in the splicing arrangement of any transcript then this could result in the conflicting expression seen. If a gene had the restriction sites necessary to create cDNA fragments of amplifiable size present in one transcript splice-form (present as a result of ECS) but not in the other, then it could be represented in one pool and not in the other. This bias in the pools would be absolute, and sequence-specific. Out of all the genes expressed in the hippocampus, only a few might exhibit this splicing and fewer still the correct arrangement of restriction sites. This selectivity matches that seen in the probable number of clones that came out of this procedure. Features of this hypothesis include the presence of other regions of the transcript in both pools; those which are not constrained by the specific splicing/digestion/amplification parameters. In addition, if the subtracted clone corresponded to a region of the transcript where the upregulated exon was spliced to a constitutive exon, then its use as a probe on a Northern blot would result in hybridisation to both normal and upregulated transcripts, masking the upregulated nature of the splice-form from which it was derived.

5) This kind of argument can be extended into more general situations. For example, if the average length of reverse-transcribed cDNA is sufficiently different between the two pools, then additional 5' sequences will be present in one pool compared to the other; especially if extension of the cDNA synthesis in a 5' direction exposes relevant restriction sites. The size of the transcript would also play a part in the bias as long mRNAs would be especially susceptible to incomplete reverse transcription according to this model.

Alternatively, it became clear upon sequencing many of the clones that the restriction digests used to divide transcripts up into small amplifiable fragments had not gone to completion. If one pool had been digested to a greater extent (i.e. consisted of smaller cDNA fragments) than the other then it is likely that it would have a better representation than the other pool because of the size constraint on PCR. Because both of these processes are stochastic, the differences between one pool and the other would very likely not be absolute.

6) A variant on this theme is based on the experimentally-created differences between the two cDNA pools; that of the linker sequences ligated onto the ends of the cDNAs. These were chosen for the purpose of distinguishing cDNA members of one pool from the other in order that cross-contamination would not occur. However, the use of separate primers for the amplification of the pools raises the question of whether these

amplifications can be considered equivalent. An example of a situation that would expose these primer-based differences would be if a certain cDNA which was too large to be amplified by the PCR contained within it a sequence complementary to the 3' end of one primer but not the other. PCR of this cDNA using one primer set might result in a product that was within the size range of the amplification process. Because the corresponding cDNA in the other pool is amplified with a different primer sequence, no PCR product would result. Only the final six or seven bases of the primer need to be complementary to the cDNA sequence for PCR priming to occur. This process is sequence-specific and absolute; as required for an explanation of the observed results.

Although harder to explain mechanistically, it may simply be the case that the presence of one linker set on a particular cDNA fragment renders it more or less amenable to the PCR amplification process.

5.13: Testing the explanations for the expression discrepency

Different origins of the tissue samples.

The first explanation raised the possibility that the subtraction results were correct but there was a failure to replicate the stimulatory or preparative conditions in the second batch of rat RNA used in Northern analysis. This possibility was tested by the use of RNA samples obtained from a third set of rats. In addition, this batch contained RNA samples derived from rats which had not been subjected to the Sham or ECS regimes; denoted 'baseline'. These were included to confirm that expression levels in the Sham hippocampi truly represented a control level. A probe derived from ECU10 was used as a probe on a Northern blot created from this material. The results were identical to those obtained from the second batch of RNA (data not shown). Moreover, no differences were observed between baseline and sham hybridisation signals.

Brain sections obtained from the original set of rats were used for *in situ* hybridisation experiments. Expression changes seen in these sections should be directly comparable to that of the cDNA pools as they were derived from the same batch of rats. The individual results will described in a section 5.14 but the overall result was similar to the Northern data in that no expression changes were observed.

Stochastic failures

The observed patterns of hybridisation of the clones in question exhibit certain features which seem to suggest that stochastic failure is not a viable explanation. Not all sequences exhibit expression differences between the Sham and the ECS pools; for

example, SNAP-25 (data not shown) and ribosomal protein S26 show virtually equivalent distributions in the two pools. In addition, clones have been isolated which are virtually exclusive to one pool or the other. Together, this suggests that neither pool was vastly inferior (with respect to representational quality) to the other. It might be expected that an inefficiency of cDNA synthesis/conversion into amplifiable sequences would be reflected in the loss of rare cDNAs. In fact, some of the expression discrepencies occur in relatively highly expressed cDNA species: a stochastic failure could cause relative abundance differences between the two pools but would be unlikely to result in the absolute differences that were seen in some of these cases. If we can then assume that those clones displaying the cDNA Southern/Northern inconsistances represent only a small fraction of the initial cDNAs then it is necessary to find a transcript-specific or sequence-specific explanations.

Nevertheless, an experiment was carried out to determine if the expression differences were due to the cDNA pools used in the subtraction being of poor quality. Because of the excess ribosomal RNA material present in early mRNA preparations (see previous chapter), Sham and ECS hippocampus cDNA pools had been created at an earlier time-point but had not been successfully used in subtraction experiments. However, these two pools were synthesized entirely independently of those used in the actual subtraction and could, therefore, be used to verify the expression differences of the isolated clones. Two points must be noted; first, these two sets of cDNA pools were created from material obtained from the same batch of rats, and second, this experiment should expose stochastic/operator flaws in the synthesis of the pools. A selection of PCR products corresponding to ECS and ECD clones were electrophoresed on an agarose gel and capillary blotted onto a nitrocellulose filter. This filter was subjected to four separate hybridisations using probes derived from both sets of ECU and Sham cDNA starting pools (old E^+ and S^- and used E^+ and S^-). In effect, the representation of the clones within the cDNA pools was verified with respect to changes brought about by ECS treatment together with changes brought about by cDNA pool batch variability.

The results are shown in fig. 5.7a and 5.7b. Overall, ECU expression differences are maintained too a good degree (fig. 5.7a); only a few clones lost their specificity upon reprobing with the other set of pools. The results for the ECD clones were not so reproducible, as the old ECS probe hybridised to many of the ECD clones. Perhaps this represents a slight failure to achieve good representation in the ECS pool used in the subtraction (as it should have removed these ECD cDNA species). Some of the ECU cDNA clones whose expression appeared to be verified by the second probing were used on Northern blots. No differential expression was seen. In the light of these

results, it is necessary to find a systemic or sequence-specific (rather than stochastic) explanation to the artifactual representation of the pools.

Polyadenylation

The differences in the length of the polyA$^+$ tail would have to be quite extreme to permit the failure or otherwise of oligo dT-cellulose purification or oligo dT-primed reverse transcription. One factor that argues against this hypothesis is that if false-positive transcripts exhibited polyadenylation differences after ECS treatment then this should be evident on total RNA Northern blots as a shift in the transcript size. No cases of such migratory shifts were observed. In addition, an explanation such as this would require all parts of the transcript to be affected in the same manner. An example of different regions of a transcipt being represented differently in the two pools is described in section 5.14.

Splicing

The differential splicing hypothesis states that some parts of a cDNA are only amplifiable (through size constraints) when the correct arrangement of splicing is present and that this is an activity-dependent process. Thus, one splice-form is found in Sham transcripts and another after ECS. Because constitutively expressed portions of the transcript may also be present in the cDNA clone, analysis by Northern will not pick up the subtle rearrangement of transcripts. Clones corresponding to the genes phosducin-like protein, NF-YB, and the ECU 8/21 family seemed to be the most likely candidates for such a process. In the analysis of these genes and the corresponding clones that emerged from the subtraction, it is difficult to know precisely which region is up-regulated because multiple forms exist. It was with this in mind that a PCR-based approach was applied to phosducin-like protein transcripts (detailed in the next section).

Efficiency of 5' extension by reverse transcriptase/restriction digestion extent

One flaw in these hypotheses is that they would produce false-positives in one direction only. For example, if the ECS pool was poorly represented, through whatever failure in the synthesis process, only Sham false-positives would have been apparent. It would not be possible to have false-positives in both pools (as were seen) unless there were a combination of different failings occurring simultaneously.

Different linker/PCR primer sequences

The fact that a number of the false-positive clones were derived from genes which have published sequences allowed the study of their end-points. We wished to know if the individual amplified cDNAs had arisen by the addition of linkers to digested cDNA fragments (as expected) or had arisen through priming off a region of complementary sequence within an otherwise unamplifiable stretch (as the hypothesis suggests). No instance of genes displaying primer-complementary sequences corresponding to the ends of the subtracted clones were found. Therefore, with respect to these clones, this hypothesis has been disproved.

In addition, an attempt was made to PCR amplifiy the synthesized pools (pre-amplification) using a mixture of the primers; in this way it was hoped, the pool-specificity (caused by primer artefacts) of some of the EC clones would disappear. However, the presence of a mixture of primers proved inhibitory to the amplification process and, thus, no firm conclusions could be drawn.

Contrasting representation in cDNA and cDNA pools

In order to demonstrate that the false-positive subtracted clones were not differentially expressed in unamplified/undigested cDNA (derived from mRNA from the third batch of rats), the ECU and ECD clones were blotted onto a nitrocellulose filter as described at the beginning of this section. This filter was probed with a Sham cDNA probe and, separately, with an ECS cDNA probe. No significant differential signals were observed. This experiment confirms the Northern and *in situ* results.

5.14: Further analysis of selected clones

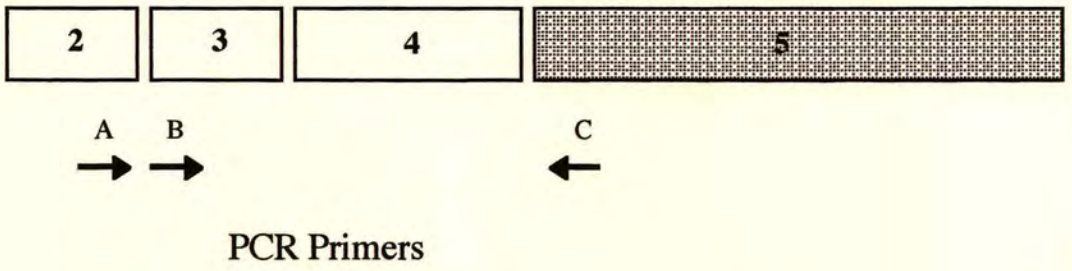
Phosducin-like protein

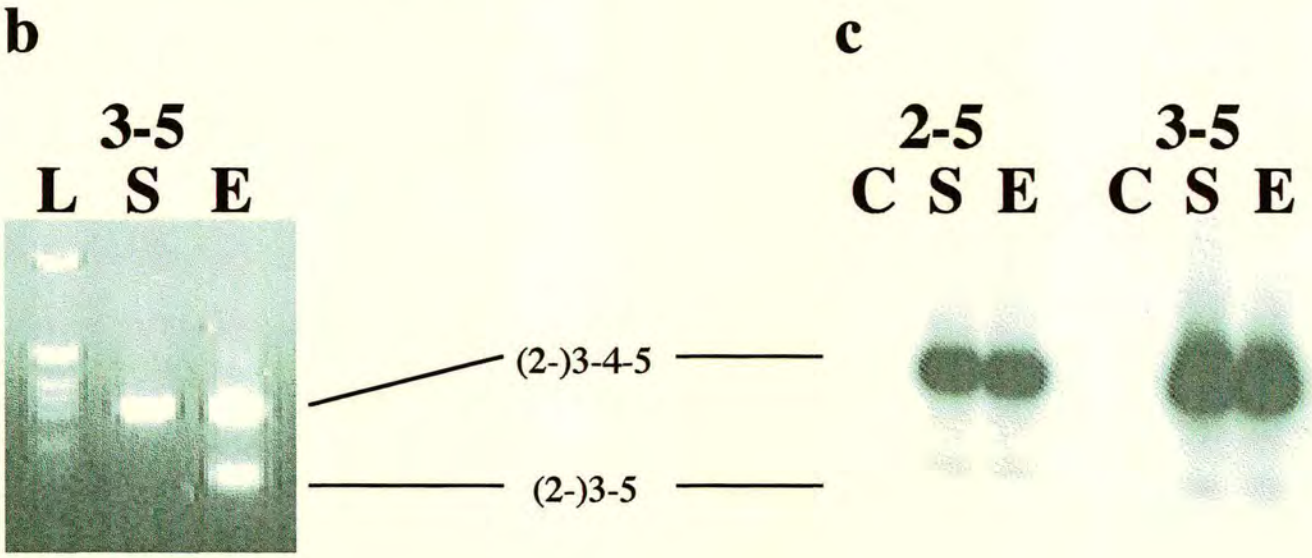
Fig. 5.3b shows that the two clones corresponding to this gene identified from the subtraction contain different exon combinations. PCR primers were directed against sequences found in particular exons in the 5' portion of the gene; the region from which the subtraction clones originate (fig. 5.5a). The aim of the experiments described below was to amplify the various exon combinations from Sham and ECS cDNA material in an attempt to see if any were found preferentially after ECS. It must be pointed out that these experiments were designed only to give qualitative rather than quantitative expression data.

A PCR reaction using the primer combination corresponding to exons 3 and 5 was carried out on the cDNA pools (fig. 5.5b). The amplification products (verified by cloning and sequencing) indicated that the Sham cDNA pool only possessed

Figure 5.5 a Cartoon representation of the arrangement of the exons in a PhLP transcript. Underneath are indicated the regions to which PCR primers were directed. **b** PCR carried out on sham and ECS cDNA pools using primers B and C (exons 3 and 5). Products of this reaction were electrophoresed and photographed. It can be seen that both pools possess the exon combination 3-4-5, but only the ECS pool possesses the exon combination 3-5. Lane 'L' is a size marker. **c** A similar experiment was carried out using primer combinations A and C (exons 2 and 5) or B and C (exons 3 and 5). The substrate for the PCR reaction was newly synthesized cDNA derived from hippocampus mRNA from the third set of rat dissections. Lanes 'C' are no-DNA controls. The PCR products were electrophoresed, blotted onto nitrocellulose paper and probed with exons 3-4. It can be seen that there are no exon combinations specific to, or up-regulated in, sham or ECS cDNA. PCR primers: exon 2 (ccactgcagcttttctc), exon 3 (gggaatttcggctttcggttacttctct), and exon 5 (cggcgccagtcattgatc). Cycling parameters: 94°C; 1 min, 53°C; 1 min, and 72°C; 1 min (30 cycles).

Fig. 5.5a





amplifiable cDNA fragments with the exon combination 3-4-5 whereas the ECS cDNA pool possessed fragments with the 3-4-5 and the 3-5 arrangement. Hence, the 3-5 arrangement of exons appeared specific to the ECS-treated tissue. This form would result in a protein that lacked the 1/2 pleckstrin homology domain.

This experiment was repeated on untreated cDNA material obtained from the third set of rats (fig. 5.5c). In addition, amplification was performed with a set of primers directed at exons 2 and 5. With both sets of primers no difference was seen between the Sham and ECS cDNA samples. Exon combination (2-)3-4-5 was amplified to a greater extent than the (2-)3-5 arrangement, but this may not necessarily reflect the true relative abundance of these two forms.

8/21 Family

Two groups consisting of three identical upregulated clones (ECUs 8/14/24 and ECUs 21/26/35) were identified from the subtraction. When representatives of these were used as probes on cDNA Southerns, similar intensities of hybridisation and banding patterns were observed. Upon Northerns, they hybridised with the same intensity to transcripts of the same molecular weight.

A novel technique named 'cDNA capture' was applied to both these groups with the aim of obtaining longer regions of the parent transcript from which both seemed to be derived. This technique is described in greater detail in the Materials and Methods chapter but, in essence, the protocol uses the identified clones as immobilised 'DNA hooks' in non-radioactive hybridisation experiments to capture longer complementary cDNA fragments from the initially created cDNA pools. Representatives from both of the clone groups isolated a cDNA fragment corresponding to the size of the cDNA species to which they had hybridised on the cDNA Southern experiments. This 1.1 kb cDNA was cloned, EcoRI digested and subcloned to permit sequencing in full. The results of this sequencing show no homology to any sequence in the database and due to lack of a convincing open reading frame, no estimation can be made as to the nature of the protein product. There is a region of sequence match between 8/14/24 and the 1.1 kb clone. This does not extend the full length of 8/14/24 (see fig. 5.6). It was thought that the point at which these two sequences diverge might represent a splice junction but no consensus splice sites could be found in this region. Of interest, was the fact that this clone did not possess any sequence homology to the 21/26/35 group of clones which had also isolated it.

A 700 bp EcoR1 fragment of the 1.1 kb cDNA clone was used as a probe on a cDNA Southern. The originally isolated cDNA clones of the ECU 8/21 family exhibited ECS pool-specific expression, whereas the longer probe detected not only the same

ECS band as before, but also a band in the Sham cDNA pool (fig. 5.1). This second band was of a different size, suggesting that the extent of restriction digestion might play a role in the failure to achieve complete representation in the cDNA pools with respect to this clone. Hence, different regions of the same transcript were represented differently in the two cDNA pools; a result contrary the polyadenylation extent hypothesis discussed earlier.

Another cDNA capture experiment (using regions of the 1.1 kb clone and ECU 21 as the 'hooks'), isolated a fragment of approximately 700bp which contained a region of identity to the entire length of the ECU 21/26/35 group. This clone was present in the database as a number of human expressed sequence tags. A possible reading frame is present which shows no significant database homologies.

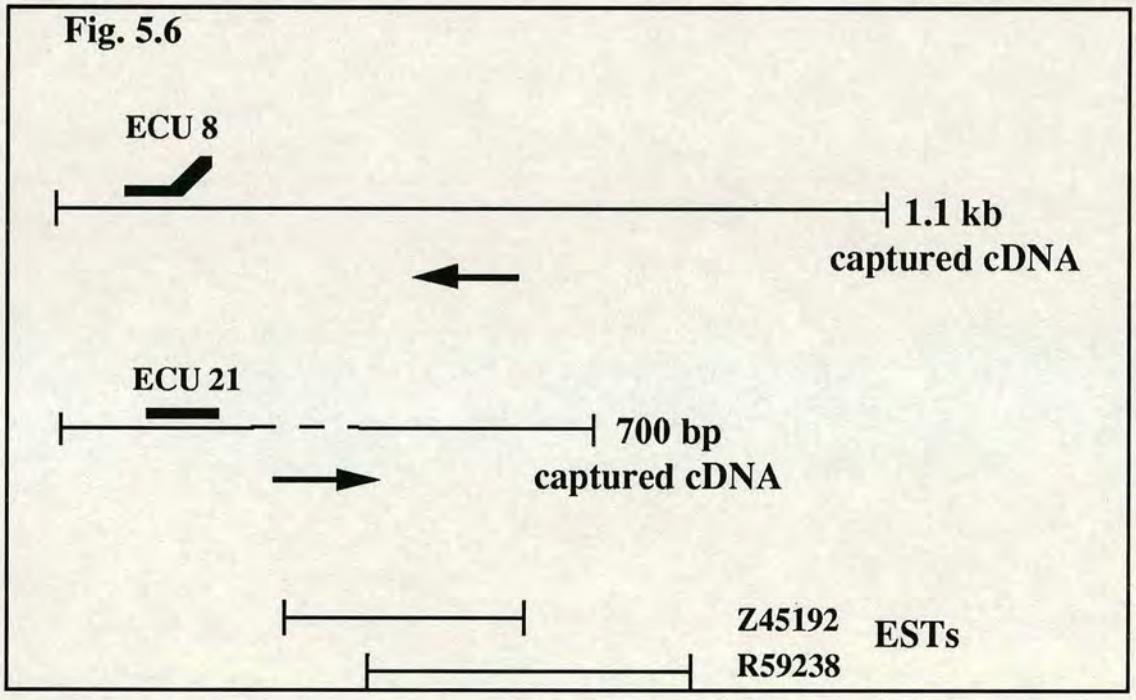


Figure 5.6 Two larger fragments of the putative 8/21 family were obtained by cDNA capture. A 1.1 kb fragment contained a region which was identical to one end of ECU 8. A 700 bp fragment (not completely sequenced; as denoted by dashed central portion) contained a region of identity with the entire length of ECU 21. It also contained an ORF at the 3' end. Two human ESTs have been isolated which show homology with this end of the cDNA. Arrows under the two captured cDNAs indicate the probable 5'-3' direction of their parent transcript(s) as deduced by *in situ* sense/antisense hybridisation (1.1 kb fragment) or ORF direction (700 bp fragment). See Appendix for all 8/21 family sequences.

In conclusion, the exact relationship of the two groups (8/14/24 and 21/26/35) has not yet been conclusively determined: there is circumstantial evidence for their common origin in a parent transcript, but no conclusive sequence evidence.

In situ experiments

In situ experiments were carried out with the assistance from Dr. Muriel Steel (Centre for Genome Research) who was responsible for mounting the brain sections on microscope slides and for initial instruction on the technique. Sham and ECS rat brain (first batch) coronal sections were hybridised (as described in the Materials and Methods chapter) with sense and antisense ribonucleotide probes derived from *in vitro* transcription of templates corresponding to the subtraction clones. In the four probings listed below (table 5c), the sense probings showed no more than background hybridisation signals. As mentioned in section 5.13, no expression differences were seen between Sham and ECS sections (fig. 5.8).

Probe Identity	Clone Region	Expression Pattern
Phosducin-like protein	Exons 3 and 4	Ubiquitous but strong expression in hippocampus, habenula, lateral ventricle walls and anterior third ventricle walls.
8/21 family	340bp EcoRI fragment of 1.1kb clone	Widespread but strongest expression in cortex, caudate putamen, hippocampus (CA2 esp.) and habenula.
NF-YB	Whole ECU9	Ubiquitous/low level.
Aconitase	~400bp (cDNA captured)	Ubiquitous.

Table 4c *In situ* hybridisation patterns of four ECU clones. No differences were seen between Sham and ECS brain sections.

The expression pattern of 8/21 was of special interest because it showed clear expression in the CA2 region of the hippocampus. This is one of the few instances described in the literature (neurotrophin-3 being another; ref. 50) where a gene has higher level expression in CA2 than other regions of the hippocampus.

5.15: Summary of the subtraction described in this chapter

The experiments in this chapter concerned the application of a subtraction technique to isolate hippocampus transcipts which are subject to long-lasting up- or down-regulation in response to ECS. The subtraction technique suffered not from a failure to enrich specific genes, but from a failure to create starting cDNA pools which were

Figure 5.7 a PCR-amplified ECU clone inserts were electrophoresed and blotted onto a nitrocellulose filter. This was then hybridised by four different cDNA pool-derived probes as indicated by the labels under each panel. An asterisk indicates that the cDNA used in the probe synthesis was from an early independent batch not used in the subtraction procedure (see section 5.13). **b** The same approach applied to the ECD clone inserts.

Figure 5.8 *In situ* hybridisation experiments were performed on sham/ECS coronal rat brain sections using the ECU/ECD clones as templates for the synthesis of riboprobes. ECU 8/21 on a sham section (**a**) and an ECS section (**b**). ECU 10 on an ECS brain section (**c**). Hybridisation patterns are described in section 5.14. Sense probes gave no detectable hybridisation signals. X-ray film images were scanned using Canon CLC 10, magnified, and printed (hence low resolution).

Fig. 5.7a

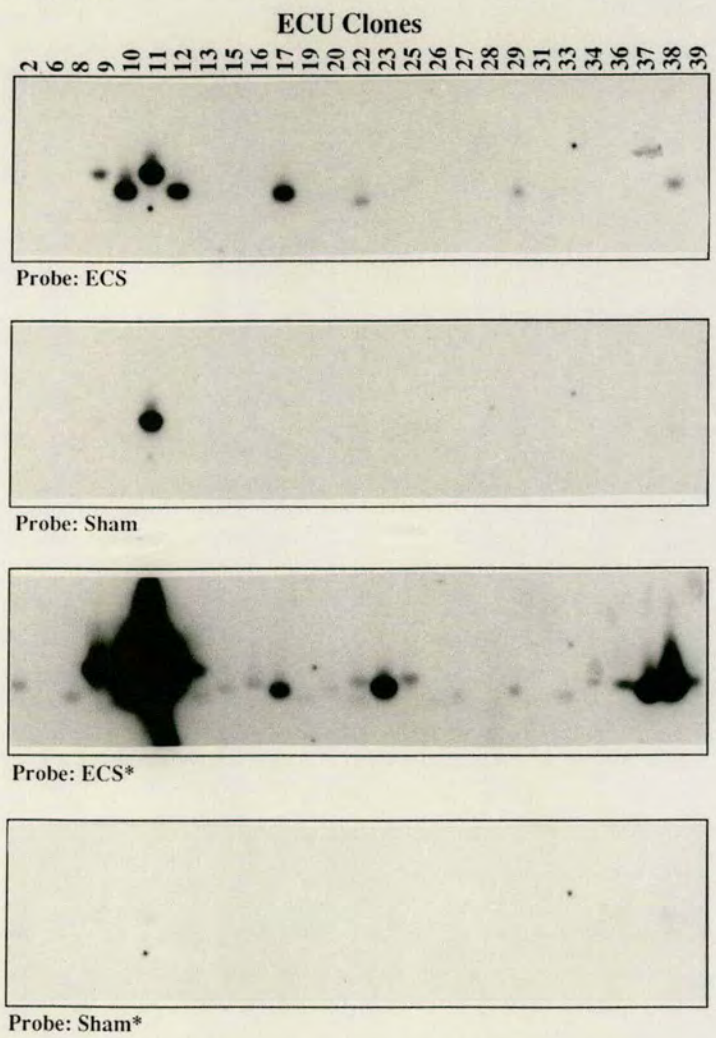


Fig. 5.7b

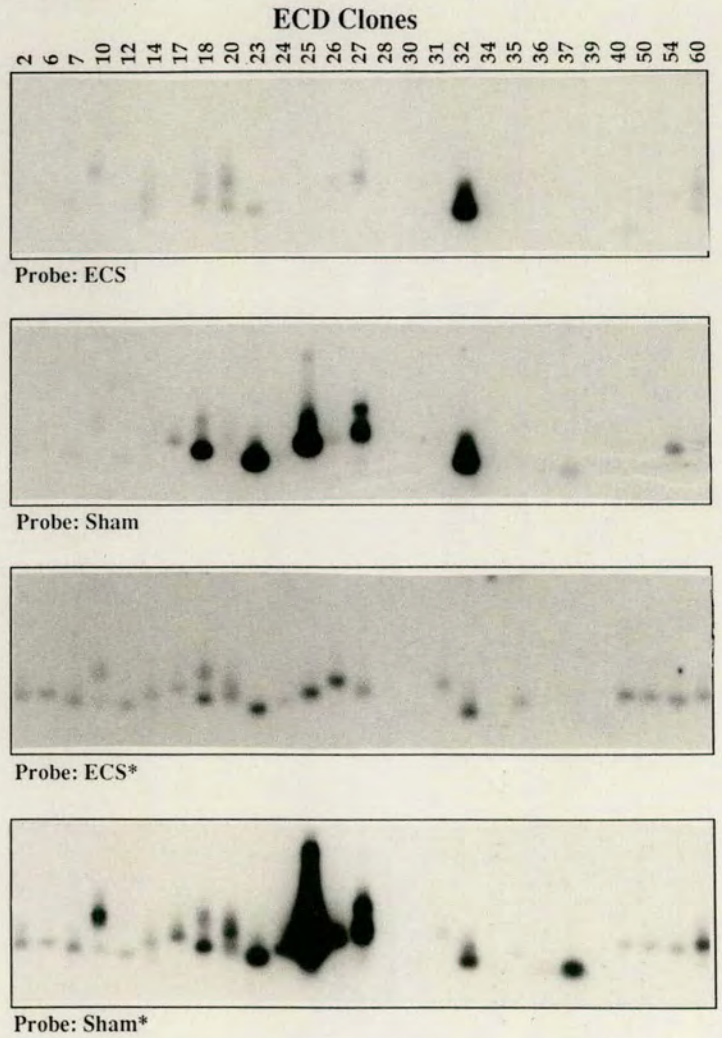
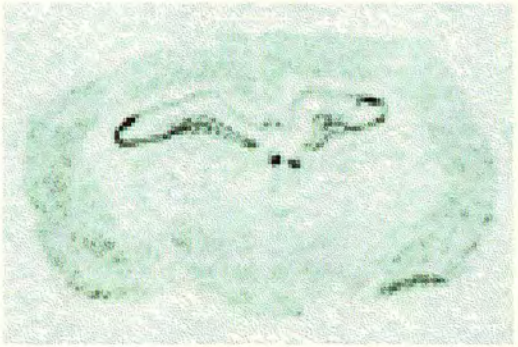


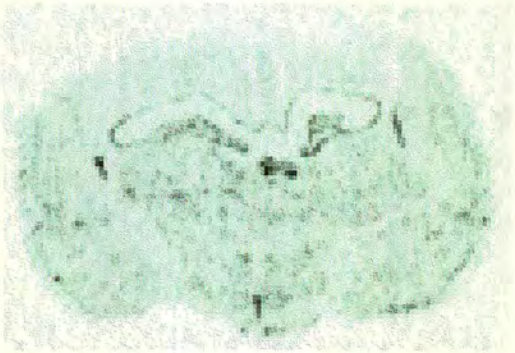
Fig. 5.8

a



b

c



representative of the true mRNA expression profile of the hippocampus. Several explanations for this failure were put forward and tested.

As this and other subtractions (Chapter IV) have shown, the enrichment process has selected those cDNAs which were artifactually specific to one pool and increased their proportion. Assuming that genuinely ECS-induced/-downregulated genes were present in the cDNA pools, it could be inferred that their expression levels and fold enrichment were not equal to those false positives that were obtained in this screen. It is possible that the 72 hr time-point represents a phase of hippocampal synaptic modification that is independent of transcriptional change. It is with this possibility in mind that experiments may be undertaken in the future to examine expression changes at an earlier time-point (24hrs).

The following chapter assesses the usefulness of this subtraction technique and examines ways in which its performance could be improved.

Chapter VI

Summary and Discussion

'People nowadays tend to over-interpret their findings. Neurobiology is a fascinating field, but it has become a bit like Disneyland. There is a lot of self-advertising, which is no good for science....Let's just say the field is a bit overheated right now.'

Bert Sakmann, as quoted in Thomas A. Bass's *Reinventing the Future: Conversations with the World's Leading Scientists* (Addison Wesley)

6.1: The aims of the work described in this thesis

An important direction of biological research concerns underlying mechanisms of cognition. Within this, the study of memory has advanced quickly due to the relative ease with which it may be assayed and because a discrete brain region, the hippocampus, seems to have a key role in its formation. To make further insights into the cellular mechanisms of the hippocampus responsible for memory, it will become necessary to use specific transgenic manipulation. Hippocampus-specific perturbation of cellular function by transgenesis requires the use of appropriate regulatory elements.

To obtain such genetic elements, a search was undertaken to identify genes whose expression is restricted to this region. In addition, it was hypothesised that some transcripts might be restricted to the hippocampus, but only expressed in response to neuronal activity. Hence, later experimentation attempted to identify such gene expression changes.

Hippocampus-specificity within the brain was defined, for our purposes, as restricted expression at the levels of sensitivity of Northern and *in situ* hybridisation. Because of the intention to create hippocampus-specific transgenes, there are certain characteristics which an obtained hippocampus-specific gene would ideally possess. These include moderate to high levels of expression in the adult and little or none during development.

6.2: Summary of techniques applied to the search for hippocampus-specific genes

The techniques applied to the search for hippocampus-specific gene expression included degenerate PCR of multi-gene families (Chapter II), differential screening of a hippocampus cDNA library using a subtracted probe (Chapter III), and a PCR-based subtraction strategy (Chapter IV and V).

Degenerate PCR

The use of degenerate PCR directed against large multi-gene families (outlined in Chapter II) yielded sequences from a total of 28 genes present at varying abundances in the hippocampus. All but two of these genes had been cloned and described in the literature previously. With respect to hippocampus specificity, a protein tyrosine phosphatase (PTP γ) showed the greatest restriction of expression to this brain region. However, because the distribution of this gene is widespread before brain maturation, its use in transgenic experiments would be subject to the interpretational problems described previously. If one of the inducible transgenic techniques recently described (such as the tetracycline system) were combined with the restricted distribution, specific manipulation of hippocampus function at the molecular level might be possible.

Two novel protein tyrosine phosphatases cloned been characterised to a limited extent. While their expression distributions limit their usefulness for transgenic experiments, they, nevertheless, possess features which make them interesting in their own right. Both appear to be transmembrane receptors with anticipated roles in cell-cell interactions and adhesion. With such processes generating interest in studies of development and actvity-dependent changes in the CNS, the precise functions of these molecules deserve to be investigated further.

Differential screening/subtraction

The techniques subsequently employed had the advantage of objectivity, in that the selection process was made on the basis of actual hippocampal expression rather than the assumption that some members of a gene family might be expressed specifically. This objectivity is not without problems itself. Without the selectivity of the PCR approach, the success of these techniques depends on the transcriptional complexity of the tissue. Hence, ubiquitous, higher abundance transcript expression has to be discarded before relevant genes can be obtained. In this respect, the brain is the most testing tissue for these techniques.

A number of difficulties were encountered with the combined subtractive probe enrichment and differential screen outlined in Chapter III. These can be summarised as a lack of efficiency of the subtraction process and technical inconsistencies occurring in the cDNA library differential screening. Among the clones obtained were those corresponding to genes which were clearly ubiquitous but had escaped subtraction because of their high expression levels. Others exhibited a small degree of enrichment in the hippocampus, such as SNAP-25. Most genes obtained by this method were restricted to the high abundance class of transcripts. Thus the sensitivity of this technique was seriously limited.

Chapter IV described the application of a second subtraction protocol based on repeated subtractions of PCR-amplifiable cDNA pools created from hippocampus and 'rest of brain' mRNA. Two variations on the approach were applied to the search for hippocampus-specific gene expression; the major difference of the second was the use of separate linker sequences for the creation of the hippocampus and rest of brain cDNA pools. This was to ensure that sequences in one pool did not contaminate those in the other as was the case in the first subtraction described in Chapter IV.

The second subtraction described in Chapter IV resulted in the cloning of a number of genes which are enriched in the hippocampus. In addition, their expression levels were generally lower than the clones described in Chapter III, indicating that the subtraction process was progressively removing non-specific, high abundance transcripts. A feature of subtractions using this technique was that the enrichment process seemed to be very efficient: cDNA fragments that were specific to one cDNA pool (present at a wide range of initial abundances) were present at high abundance in the final subtracted material. Hence, as a general method to enrich for such sequences, this particular technique is extremely efficient and represents a significant improvement over the single-step subtraction experiments detailed in the introduction to Chapter III.

However, because the starting cDNA pools were not a true representation of the genuine tissue mRNA populations, the full potential of this technique could not be realised. If the representational failures had been equivalent in both pools, this would have been acceptable because the comparative abundance of a sequence in the two pools would have remained equivalent to that in the original mRNA populations. The results of 'cDNA Southern' and Northern analyses were indicative of pool- and sequence-specific representation failures in the two pools. In Chapter V, several

hypotheses were discussed as to how these inconcistencies could have arisen. In summary, plausible explanations were the use of separate linkers for the two pools (leading to non-comparable amplification) or inefficiencies in the stages of the cDNA pool synthesis process (for example, the failure to achieve complete restriction digestion of the cDNAs).

One other factor hindered the isolation of hippocampus-specific genes. Because the rest of brain cDNA pool represents the average expression levels of genes in the non-hippocampal brain, contributions of high-level gene expression restricted to one particular region, such as the striatum, would be diluted out. Thus, driver derived from RoB would not remove these sequences from tracer, even though they were not hippocampus-specific. This most likely led to the selection of false-positives such as hippocalcin and the 5-HT1a receptor.

6.3: Activity-dependent gene expression in the hippocampus

The subtraction described in Chapter V had two main goals; the identification of genes whose expression levels were subject to long-term alterations resulting from electroconvulsive stimulation (ECS), and the characterisation of those genes to determine if any were hippocampus-specific. The latter goal was based on the assumption that some genes might only be expressed as a consequence of hippocampal activity. If the function of the hippocampus is the formation of memory, and this is dependent on the synthesis of new mRNA and protein, then it is possible that region-specific transcript expression might be induced by hippocampus neuronal activity. Certainly, in the large number of reports concerning activity-dependent immediate early gene induction, expression is often restricted to the hippocampus; particularly the dentate gyrus.

The precise nature of the stimulus can affect the location of expression. Ideally, the expression of transcripts would be examined after behavioural induction rather than by artificial means. However, the quantity of neurons expressing these transcripts has to be over a threshold before they are detectable by current techniques. This has necessitated the utilisation of more powerful stimulation techniques, as described in section 5.4. ECS was chosen as the stimulatory method in experiments described here as this is known to have widespread and long-lasting effects on synaptic plasticity.

Subtractive hybridisations were performed such that ECS up- and down-regulated genes would be obtained. In comparison to previous subtractions using this approach, a large number of clones were isolated which were found in one cDNA pool but not the other. This showed that the subtractive steps were operating efficiently. However, as before, these results were not corroborated by Northern studies of gene expression. Selected clones were studied in greater detail, but none revealed a more subtle activity-dependent enrichment.

Several other problems may have contributed to the subtraction failure. First, in order to avoid the cloning of immediate early genes, analysis of gene expression was carried out at a late post-stimulus time-point (72 hours). While the activity-dependent expression changes in some neuronal genes have been seen to persist for weeks, the degree of expression change is only moderate. This contrasts with immediate early gene expression changes which are very large, if only transient. The difference in these two profiles of induction may mirror their different roles; immediate early genes are required to bring about rapid changes in the synapse, whereas those genes that show small expression changes which persist for days or weeks may be responsible for the long-term alteration of synaptic efficacy or the maintenance of those changes. It may be the case that a subtractive technique is not suitable for the isolation of genes exhibiting small expression changes. Second, the use of halothane anaesthesia has been shown to affect LTP induction and, in some cases, prevent the expression of immediate early gene family members. It might be the case that long-term gene expression changes are not so pronounced after such anaesthesia. However, the negative effects of halothane are probably superseded by the potency of ECS stimulation.

In addition, activity-related changes in synaptic transmission efficacy are seen in regions outside of the hippocampus, such as the cortex and cerebellum. It is not unreasonable to assume that the molecular mechanisms underlying these processes would be similar to those occurring in the hippocampus; even to the extent of the expression of a similar set of genes. This may add to the difficulty in isolating activity-dependent hippocampus-specific gene expression.

6.4: Can the subtraction technique be improved?

The weakness in the subtraction technique applied in Chapters IV and V lies not in the enrichment process itself, but in the creation of PCR-amplifiable cDNA pools

which suitably reflect the expression of transcripts in the mRNA populations from which they were derived. Several modifications to the protocol which may result in the lessening of the observed problems are listed below.

First, each cDNA pool taken through the subtraction process should be derived from the summation of several independent cDNA pool syntheses so that a more representational collection of cDNA fragments would result.

Second, in preparation for for the initial PCR amplification, the two pools should be created using identical linker oilgonucleotides. This would ensure that the primary pools are amplified in a more comparable fashion; size being the major remaining factor affecting cDNA fragment amplification. Following this amplification competence step, different linker sequences could be ligated onto the cDNA pools to prevent cross-contamination during the subsequent subtraction process. Parallel subtractions, reversing the linker assignments to each pool, would eliminate sequence-dependent cDNA amplification effects.

Third, in order to solve the problem associated with the dilution of an individual brain region's contribution to the rest of brain cDNA pool, separate subtractions of hippocampus cDNA pools against other brain sub-regions (such as cortex, cerebellum, thalamus, and brainstem) could be carried out. cDNA fragments enriched in all of the separate subtractions would most likely represent genuine hippocampus-specific transcripts.

6.5: The existence of hippocampus-specific transcripts

A question that arises at this point is whether the functions of a particular sub-region of the brain are defined by the wiring pattern/connectivity of the constituent neurons or by the intrinsic nature of those neurons. To put this question in another form, is it the developmental patterning or the adult capabilities of the neurons that dictate the operation of a brain region? The answer has important implications for the molecular biological techniques described in this thesis because, if the former is true, then adult brain regions would not be distinguishable at the transcriptional level. However, to have created these sub-regions during development, the primordial cells would have been subject to instructions specifying cell-fate, migration and association rules; instructions likely to be transcriptional in nature. It is less certain that these transcriptional identities are maintained in the mature brain. Anatomical characterisation of neurons, descriptions of pharmacological properties of neuron

types, and the analysis of patterns of gene expression in the brain all show that regional distinctions do exist, but the limitation of one particular feature to just one particular brain region is a much rarer occurrence. Rather, a gene that is highly expressed in the hippocampus, for example, is usually also expressed in another region of the brain such as the cortex or the cerebellum. Many experiments have defined the specialised role of the hippocampus in the brain, but the question still remains whether its function is sufficiently distinct from other brain regions to merit specific gene expression.

The penultimate section of Chapter I made reference to a study which attempted to categorise transcripts into expression distribution and abundance groups. The results implied an inverse correlation between the expression level of a gene and its degree of brain specificity. Putative hippocampus-specific genes may thus be expressed at a level below that of the sensitivity of the differential screening/subtraction techniques applied in this thesis. In addition, these results have implications for the use of the promoter/enhancer elements of such a gene for the control of transgene expression.

6.6: Have hippocampus-specific genes been described elsewhere?

Few groups have published any data concerning the existence of genes restricted in expression to the hippocampus. The only case of such a transcript being cloned in a screen for hippocampal-specificity is that from Dietmar Kühl's laboratory (personal communication, D. Kühl and ref. 1). In the screen which isolated the activity-regulated gene, arg3.1 (see 5.3), another clone was found on the basis of its failure to hybridise to a cortex-derived probe. This has since been identified as neuropsin, a serine protease gene which may be regulated in an activity-dependent fashion. This gene has been cloned independently by degenerate PCR amplification in a Japanese laboratory (see section 5.3 also) and in this laboratory (B. Davies, personal communication). It appears to be expressed at a low abundance (perhaps explaining the failure to isolate the gene in the experiments described in this thesis) principally in the limbic regions of the brain including the hippocampus and amygdala. The applicability of this gene to transgenic intervention has not yet been investigated.

Other cloned genes show expression patterns which are highly enriched, but not specific, to the hippocampus. Brain-derived neurotrophic factor (BDNF) was originally isolated from the hippocampus. Expression is very high in this brain region. All splice variants of this gene show significant induction by kainate administration (ref. 2). Interestingly, one particular exon of this transcript is

expressed virtually exclusively to the dentate gyrus of the hippocampus upon kainate treatment.

A degenerate PCR approach directed at the G protein-coupled receptor class of serotonin receptors has identified two novel members (ref. 3). One of these, MR22, has an expression pattern limited to the hippocampus (CA1 pyramidal cells), medial and lateral habenular nuclei, and raphe nuclei. Its expression level in these regions is extremely low, requiring 10μg of polyA$^+$ mRNA on a Northern blot to generate a detectable hybridisation signal.

A member of the *eph/elk* receptor tyrosine kinase family (embryo brain kinase, *Ebk*; ref. 4) has also been cloned using degenerate PCR. Its expression in mouse is widespread throughout the developing brain but becomes progressively more restricted during maturation (reminiscent of PTP γ; see section 6.2). Adult expression is described as being entirely restricted to the hippocampus (CA1 especially, with lower levels in the other CA regions and the dentate gyrus) except for trace levels of expression in the subicular region. Perhaps this represents the gene most closely matching the requirements for hippocampal specificity. The abundance of this transcript is not fully described but it is detectable by Northern hybridisation. A probe corresponding to this gene has been used on a rat hippocampus/rest of brain cDNA Southern which suggested a lack of specificity (data not shown) but this has yet to be confirmed by Northern hybridisation. Admittedly, the expression in the mouse (relevant for the transgenic approach) appears convincing even though the developmental expression pattern leaves it subject to the interpretational problems of transgenic use discussed previously.

In this laboratory, other techniques have been employed in the attempt to isolate hippocampus-specific genes. A differential screen mentioned in Chapter III (ref. 5) identified a gene, hct-1, which encodes a cytochrome P-450. This gene's expression seems to be largely restricted to the hippocampus in rat but is widespread in mouse brain. Therefore, this limits its usefulness in transgenic experiments. It may, however, be possible to reproduce the rat expression pattern in mouse transgenics by using rat regulatory elements as a promoter. Another line of research concerns the investigation of several gene-trap lines (ref. 6) which show expression in the mouse hippocampus (M. Steel, personal communication). One of these, *Kin*, appears to be largely restricted to the hippocampus and may prove useful for transgenic experimentation. By engineering a transgene into the gene-trap locus a similar pattern of expression may be adopted.

In conclusion, despite intensive screening, both in this laboratory and elswhere, no absolutely hippocampus-specific gene has yet been purposefully isolated.

6.5: Alternatives to hippocampus-specificity

It may be possible to achieve specific transgenic intervention in the hippocampus even without possession of a hippocampus-specific gene. Several experimental approaches describing alternatives to hippocampus specificity are set out below.

Promoter manipulation

It might be possible to alter the promoter of a gene which is expressed predominantly in the hippocampus so that it becomes exclusively expressed there. This approach presupposes there to be discrete promoter/enhancer domains within gene promoters that define site of expression. The reality may not be so clear-cut, with disparate genetic elements each contributing to the final expression distribution. However, evidence for the subtlety of promoter/enhancer-directed expression comes from a study of the expression patterns of several promoter deletion constructs of a cerebellum specific gene, L7 (ref. 7). LacZ was used as a marker for the cerebellum expression pattern of each of the constructs. Surprisingly, the promoter of this gene revealed itself to be governed by a number of positive and negative control elements such that several deletion constructs directed expression to restricted domains within the cerebellum.

If a minimal hippocampus-specific promoter could be isolated, this would provide a simple means for the transgenic manipulation of hippocampus-dependent processes.

Combinatorial expression

Expression levels of putative hippocampus-specific genes may be unsuitable for transgenic experimentation. It may be possible to apply the genetic elements which direct higher abundance expression in a combinatorial fashion. The method requires two genes; one (A) expressed in the hippocampus and another brain region(s), and a second (B) expressed in the hippocampus and a different set of other brain regions. The key point is that A and B share a site of expression only in the hippocampus. If the transgene could be made to function only in the common region of expression then its activity would be hippocampus-specific. Moreover, it might be possible to select promoters which overlap only within defined domains of the hippocampus, allowing even more selective experimentation.

One experiment might involve the expression of the tTA gene product (see section 1.19) from promoter A and the expression of cre recombinase from B. Mice containing both of these transgenes would act as the 'background' strain to which mice containing the experimental transgene would be mated. The experimental transgene would consist of a construct expressing a transcript under the control of the tTA gene product but this would produce no functional protein upon translation. If, however, the construct was located in cells expressing cre recombinase then it would be altered to produce translationally competent transcripts (possibly by the deletion of a 5' exon containing stop signals in all frames). The features of such a system would include hippocampus specificity and inducible action; tetracycline could either be administered to the mother to prevent developmental expression of the transgene or administered to the adult mouse to observe a return to the wild-type phenotype.

Naturally, these experiments are critically dependent on the availability of suitable 'A' and 'B' promoters. Candidates might include the promoters of BDNF, the neurotransmitter receptors, *Ebk*, PTP γ, neuropsin etc.

6.6: Future research into hippocampal function

Transgenic techniques, if applied in a specific manner, represent a very important tool for the analysis of hippocampus function. Two questions deserve to be early subjects of such experimentation.

First, with functional disruption limited to the hippocampus, it will become possible to categorise the exact cognitive processes with which the hippocampus is associated. It is to be expected that the spatial learning role of this brain region will be joined by many others requiring the use of its particular attributes. As such, it is becoming increasingly necessary to devise more precise behavioural assays with a view to examining the role of the hippocampus in these cognitive systems.

Second, at the molecular level, activity-dependent changes in gene expression need to be investigated more thoroughly. Constitutive expression of genes described in section 5.3 might be possible using hippocampus-specific promoters. In some cases it would be expected that permanently potentiated synapses would result; causing similar saturation effects to those described in the context of ECS (section 5.5). Studies of the electrophysiology, synaptic morphology, and gene expression perturbation in such animals would go some way to revealing the functions of these genes in long-term synaptic plasticity.

It is to be hoped that the knowledge and experience gained from the study of the hippocampus will be soon be applied to the study of the neocortex. It is widely held that the hippocampus acts only as a temporary repository for memories before they are transferred to the neocortex. Does synaptic plasticity in the neocortex operate according to the same rules as that in the hippocampus? Some evidence exists for gene induction in defined cortical regions after specific behavioural activities. A truly informative approach to the study of memory would arise if it were possible to detect activity-dependent gene expression changes (by marker genes under the control of activity-dependent regulatory elements) in defined sub-regions of the hippocampus and the cortex in response to specific behavioural tasks. In this way a 'molecular phrenological' map could be envisaged, matching specific behaviours to specific gene expression in sub-regions of the hippocampus and neocortex.

Materials and Methods

Materials:-

For most materials and methods, the following manual was used:-
'Molecular Cloning - a Laboratory Manual' (1989) Sambrook, J., Fritsch, E.F., and Maniatis, T., Second Edition, Cold Spring Harbor Laboratory Press.

Ma.1: Suppliers of reagents and enzymes

Enzymes

Superscript II Reverse Transcriptase	GIBCO BRL
Taq Polymerase	Boehringer Mannheim
Taq Polymerase	Promega
Restriction Endonucleases	Boehringer Mannheim
DNA Ligase	Boehringer Mannheim
DNA Ligase	New England Biolabs
Alkaline Phosphatase	Boehringer Mannheim
Polynucleotide Kinase	Boehringer Mannheim
T4 DNA Polymerase	Boehringer Mannheim
Klenow Polymerase	Boehringer Mannheim
E.coli DNA Polymerase	Boehringer Mannheim
E.coli DNA Ligase	Boehringer Mannheim
E.coli RNase H	Boehringer Mannheim
T7/T3 RNA Polymerase	Boehringer Mannheim
'Sequenase II'	United States Biochemicals

Reagents

Most salts/Solvents/Acids	BDH (AnalaR)
SDS/Guanidine tc./Mineral oil	Sigma
Tris base	Boehringer Mannheim
NaCl/EDTA/Solvents/Phosphates	Fisons
Bacterial media constituents	DIFCO
Phenol	GIBCO BRL

32P dCTP*/35S dATP*/Hybond-N filters	Amersham
32P CTP/UTP*	NEN

Equipment

Flowgen; sequencing apparatus

Pharmacia; electrophoresis tanks

GIBCO BRL; electrophoresis tanks

Techne; heating blocks, hybridisation ovens.

Sorval; centrifuges

Cecil; spectrophotometer

Gilson, Bibby; pipettes

Hybaid; PCR cycling machines

Ma.2: Standard solutions used for molecular biology

Ampicillin

50 mg/ml in water

Used at 60 µg/ml

Denhardt's Reagent (50X)

5 g Ficoll (Type 400, Pharmacia)

5 g Polyvinylpyrrolidone

5 g Bovine Serum Albumin (Fraction V, Sigma)

Water to 500 ml.

EDTA (Ethylene Diamine Tetra Acetate)

0.5 M mixture made to dissolve by adjusting pH to 8.0 with NaOH.

Gel Loading Buffer

0.25% Orange G dye

30% Glycerol in water

IPTG (IsoPropylThio-β-D-Galactoside)

20% solution made in water.

Phenol/Chloroform

20 ml phenol

20 ml chloroform
10 ml TE buffer, pH 7.5.
Vigourously mixed and allowed to separate into organic and aqueous phases overnight at 4°C.

PBS (Phosphate Buffered Saline)
0.8% NaCl
0.02% KCl
0.144% Na_2HPO_4
0.024% KH_2PO_4
pH adjusted to 7.4 with HCl

SDS (Sodium Dodecyl Sulphate)
10% solution

20X SSC
175.3 g NaCl
88.2 g NaCitrate
Adjust pH to 7.0 with NaOH
Made up to one litre with water.

STE (TEN)
0.1 M NaCl
10 mM Tris-HCl, pH 8.0
1 mM EDTA

TAE (tris acetate)
50X
242.2 g Tris base
57.1 ml glacial Acetic acid
100 ml 0.5 M EDTA
Water to a litre

TBE (tris borate)
10X
108 g Tris base
55 g Boric acid

40 ml 0.5 M EDTA
Water to a litre

TE Buffer
10 mM Tris-HCl, pH as appropriate with HCl
1 mM EDTA

Tetracycline
5 mg/ml in ethanol
Used at 50 μg/ml

Tris Buffer (one molar)
121.1 g per litre
Adjust to required pH with HCl

X-gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside)
20 mg/ml solution made up by dissolving powder in dimethylformamide.
Stored at -20°C in light-tight container.

Ma.3: Solutions for bacterial culture etc.

Maltose
20% in water (filtered)

SM Buffer
5.8 g NaCl
2 g MgSO4.7H2O
50 ml 1m Tris-HCl, pH 7.5
5 ml 2% Gelatin
Water to one litre

LB Medium (Luria-Bertani)
10 g Bacto-Tryptone
5 g Bacto-Yeast Extract
10 g NaCl
Water to one litre
pH adjusted to 7.0 with NaOH

Autoclaved

SOB/SOC medium
20 g Bacto-Tryptone
5 g Bacto-Yeast Extract
0.5 g NaCl
10 ml of 250 mM KCl
Adjust pH to 7.0 with NaOH
Water to one litre
Autoclave
Before use, add 0.5% of sterile 2M $MgCl_2$
In the case of SOC, 2% of 1 M Glucose is also added.

2X TY Medium
16 g Bacto-Tryptone
10 g Bacto-Yeast Extract
5 g NaCl
Water to one litre
pH adjusted to 7.0 with NaOH
Autoclaved

Agar Plates
Bacto-Agar added to LB medium in the following quantities:-
Bottom 15 g/l
Top 7 g/l
For top agarose (colony transfer experiments) use 7 g/l agarose.

Methods:-

Me.1: Brain/hippocampus dissection

Rats were sacrificed by carbon dioxide asphyxiation and subsequent cervical dislocation.
After decapitation, the top of the skull was exposed and the constituent plates of bone overlyimg the brain were removed in a posterior to anterior direction such that the whole brain (including olfactory bulbs) could be removed in an intact state.

Access to the hippocampi was achieved by peeling back the two neocortical hemispheres from the midline to the sides.
Corpus striatum tissue was scraped off the hippocampi and then incisions were made rostrally and caudally to sever the attachments of the hippocampi to the rest of the brain.
The two hippocampi could then be removed as a distinct 'sausage-shaped' structures 1 cm in length and 0.5 cm in diameter (size varying according to age and sex).
Other tissue regions obtainable as discrete dissectable units included olfactory bulbs, cerebellum, cortex, brain-stem, and 'rest of brain' which was the entire brain after removal of the hippocampi.
All tissue samples were immediately frozen in liquid nitrogen or on dry ice and then stored prior to use in a -70°C freezer.

Me.2: Total RNA extraction

After Chomczynski et al., Analytical Biochem. 162:156-59 (1987)

Solution D

4 M Guanidinium Thiocyanate	100 g
25 mM Na Citrate pH 7.0	7 ml of 0.75 M
0.5% Sarcosyl	10.5 ml of 10%
	then 117 ml H_2O

The mixture was heated to 65°C to dissolve and then filtered through 0.45 μm filter.
0.36 ml β-mercaptoethanol was added per 50 ml D prior to use.

One gram (or 1 cubic cm) of was tissue was homogenized in 9 ml of solution D for 30 sec in a 50 ml Corning tube.
The following solutions were added sequentially with inversion of the tube after each addition.

1 ml 2 M Na Acetate pH 4.0
10 ml water-saturated phenol
2 ml Chloroform:Isoamyl alcohol (25:1 ratio mixture)

This mixture was shaken for 10 sec and then transferred to a 35 ml Corex centrifuge where it was left for 15 min on ice.
The tubes were spun at 10,000 x g for 20 min at 4°C.

The aqueous layer (containing the RNA) was removed from the organic layer and interface (containing DNA and proteins) and mixed with an equal volume of isopropanol and left for 1 hr at -20°C.
The tubes were spun at 10,000 x g for 20 min at 4°C.
The resulting pellet was resuspended in 3 ml of solution D.
An equal volume of Isopropanol was added and then stored at -20°C for 1 hr.
The tubes were spun at 10,000 x g for 10 min at 4°C.
The pellet was washed once in cold 75% Ethanol.
The tubes were spun at 10,000 x g for 5 min at 4°C.
0.5 ml of 0.5% S.D.S. was added and the pellet resuspended at 65°C for 10 min.

The absorbance of a one hundred-fold diluted aliquot of this RNA was measured at 260 nm and 280 nm in a Cecil 4400 spectrophotometer.
An absorbance of 1 at 260 nm indicated a concentration of 40 μg/ml RNA.
A ratio of absorbances at 260 nm/280 nm above 1.8 indicated that the RNA was largely free of protein contamination.

Me.3: Poly A$^+$ purification

A 'Fast Track' mRNA isolation kit Version 3.5 (Invitrogen Corporation) was used for the preparation of mRNA for use in the degenerate PCR work.

Alternatively, for the subtractions described in Chapters IV and V, total RNA from section me.2 was resuspended in 'Binding Buffer';

0.5 M NaCl
10 mM Tris-HCl, pH 7.4
1 mM EDTA

This was then heat denatured (65°C for 5 min) and applied to a column packed with oligo dT-cellulose which had previously been equilibriated with the binding buffer solution. The flow-through was re-applied to the column and allowed to run out. Several washes were then performed using the same solution.

Bound mRNA was eluted from the oligo dT-cellulose column using 0.5 ml aliquots of 'Elution Buffer':-

10 mM Tris-HCl, pH 7.4
0.1 mM EDTA

Those aliquots which contained the eluted RNA were pooled and subjected to a second round of polyA$^+$ selection as before.
Absorbance readings were performed as described in previous sections.
Generally, approximately 1 µg of PolyA$^+$ mRNA was obtained from one hippocampus (two per brain).

Me.4: Reverse transcription / cDNA synthesis procedures

(Adapted from Gibco BRL Life Technologies Inc. protocol for use in conjunction with their RNase H$^-$ Reverse Transcriptase enzyme).

mRNA (max. 5 µg) and 0.5 µg/µg oligo dT primer were mixed in a total volume of 9 µl.
This was incubated at 70°C for 10 min (to remove secondary structure from the mRNA) and then rapidly cooled on ice for 3 min.
The following reagents were added (on ice) to create a total volume of 20 µl;

1 µl RNA Guard
4 µl 5x Reaction Buffer;
250 mM Tris-HCl pH 8.3
375 mM KCl
15 mM $MgCl_2$
2 µl 0.1 M D.T.T.
1 µl mixed dNTP stock (10mM each)
1 µl (200 U)/ 1 µg mRNA Reverse Transcriptase enzyme (Superscript II).

3 µl were taken and added to 0.5 µl (5 µCi) $\alpha-{}^{32}P$ dCTP for the purpose of determining the size distribution and efficiency of first strand cDNA synthesis.

Reverse transcription reactions were typically carried out at 42°C to reduce secondary structure interference in the full readthrough of a transcript. Incubation times of 1-2 hr were used.

Assaying the quality of the first strand synthesis reaction

The cDNA synthesis tracer reaction was made upto 100 µl with water and 90 µl used for size analysis, with the remainder used for analysis of the synthesis efficiency.

Size analysis was achieved by Phenol/Chloroform extraction of the tracer aliquot followed by precipitation using 0.4 volumes of 5 M Ammonium Acetate and 2.5 volumes of ethanol. The resultant pellet was devoid of protein and unincorporated isotope. After resuspension in water and addition of 0.1 volume of 1 M NaOH it was incubated at 37°C for 5 min to denature the cDNA from its complementary mRNA. This mixture was run on a normal agarose gel in Tris-Borate running buffer together with a radio-labelled DNA marker ladder (Gibco BRL).

The gel was then dried on Whatman 3MM paper and exposed to autoradiography film.

A normal disribution of cDNA lengths exhibited an average size of between 1 and 2 kilobases.

cDNA synthesis efficiency analysis

The 10 µl aliquot was divided into two 5 µl aliquots and one added to;

0.6 µl 0.2 M NaPyrophosphate
50 µl 5 mg/ml B.S.A. (carrier)
0.15 ml 50% Trichloroacetic Acid (TCA)

After 10-20 min on ice, the solution was pipetted onto a Whatman GF/C filter, washed with 5% TCA, and left to dry.

The second aliquot of tracer was added directly to another Whatman filter. This represents the total amount of isotopically-labelled dCTP in the reaction whereas the precipitated and washed aliquot represents the label incorporated into the cDNA.

The filters were subjected to Cerenkov radiation counting and the following equations used to ascertain the efficiency of the synthesis:

$$\frac{\text{incorporated cpm}}{\text{total cpm}} = \text{ratio of incorporation}$$

dNTP quantity in reaction (nmoles) x ratio incorporation x 330 ng/nmole= ng synthesized

$$\frac{\text{ng cDNA synthesized}}{\text{ng mRNA in reaction}} = \%\ \text{conversion}$$

typical values for conversion were 5-20%

Second strand synthesis

For some applications, such as the Biotin/Streptavidin subtraction, it was necessary to have the cDNA in double-stranded form.

The protocol is based on the RNase H-mediated nicking of the mRNA/DNA hybrid followed by the RNA-primed DNA Polymerase I extension of the second strand. The multiple second strand fragments are joined together by the action of the DNA Ligase enzyme.

To achieve this the following reagents were added sequentially on ice to the first strand synthesis reaction:

94.8 µl D.E.P.C.-treated water

32 µl 5x Second strand synthesis buffer:

- 94 mM Tris-HCl, pH 6.9
- 453 mM KCl
- 23 mM $MgCl_2$
- 750 µM β-N.A.D.
- 50 mM $(NH_4)_2SO_4$

3 µl mixed dNTPs (10mM each)

6 µl 0.1 M D.T.T.

2 µl E. coli DNA Ligase (7.5 Units/µl)

4 µl E. coli DNA Polymerase I (10 Units/µl)

0.7 µl E. coli RNase H (2 Units/µl)

This was incubated at 16°C for 2-4 hr and then for a final 5-15 min after the addition of 10 Units/µg first strand cDNA T4 DNA Polymerase.

To stop the reaction 10 µl of 0.5 M EDTA were added.

The product was now double-stranded, blunt-ended cDNA.

Me.5: Polymerase chain reaction (PCR)

Taq polymerases from Boehringer Mannheim and Promega were used during the course of the work described in this thesis.

The reaction buffer (10X) from B. M. was as follows:-

100 mM Tris-HCl, pH 8.3
15 mM $MgCl_2$
500 mM KCl

The reaction buffer (10X) from Promega was as follows:-

separate addition of $MgCl_2$ (final concentration usually 1.5 mM)

100 mM Tris-HCl, pH 9
500 mM KCl
1% Triton X-100

General description of the PCR technique
There are four key points to the amplification process:-

1) The DNA template is denatured.

2) The two PCR primers anneal to their complementary sequences on the + and - template strands at an appropriate temperature.

3) A heat-stable DNA polymerase (usually 'Taq' polymerase) utilises the primers and reaction constituents to commence synthesis of DNA strands complementary to the template.

4) The resulting duplicated DNA strands act as templates in subsequent cycles of these steps.

To allow the reaction to work efficiently, a balance has to be struck between PCR product quantity and quality (specificity). Achieving this requires the optimisation of the reaction protocol for each individual application. The following list highlights some of the factors that were adjusted in the PCR reactions described later.

Cycling conditions
Efficient PCR requires the complete denaturation of the template prior to primer annealing. For short cDNA molecules, a denaturation step of 30 sec to 1 min at

94°C is sufficient. For genomic DNA template, sonication followed by ten minutes of boiling before addition to the PCR reaction is often performed to ensure single-stranded template.

Because the sequences of the oligonucleotides designed for the PCR are known, the equation:-

$$T_m = 4x(\text{No. of G/C nucleotides}) + 2x(\text{No. of A/T nucleotides})$$

can be applied to estimate the melting temperature (in centigrade) of a short (10-22 bp) duplex in 1 M NaCl (Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B. (1981) Use of Synthetic Oligodeoxyribonucleotides for the Isolation of Specific Cloned DNA Sequences. ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. XXIII, pp. 985-691, Academic Press.). A suitable annealing temperature is taken as five degrees below the melting temperature. Choosing the correct annealing temperature is vital if non-specific/mismatched priming is to be avoided. For this reason, a variant on the standard PCR reaction is sometimes employed. 'Hotstart' PCR delays the addition of the DNA polymerase to the reaction mixture until after the first denaturation stage; this prevents premature/incorrect priming at lower temperatures before the reaction has commenced.

It also should be appreciated that the annealing temperature changes during the course of the reaction due to a primer concentration factor which is not taken into account in the equation. This results in the T_m actually being lower at the start of the reaction because of the large excess of primers present.

The temperature at which the complementary strand is synthesized is 72°C; the enzyme has maximal polymerase activity at this temperature as well as being relatively thermostable. The extension time required is varied according to the predicted size of the PCR product, with an extension rate of 1000 nucleotides per minute assumed.

The number of program cycles required to amplify a template to the required degree is normally in the range of 25 to 40 but depends largely on the application and the target template concentration.

Reaction constituents

Several factors have to be considered in the creation of suitable reaction conditions for PCR amplification.

The concentration of dNTPs can affect the yield, specificity, and fidelity (copying accuracy) of the reaction and so must be optimised to create a balance between all three factors. A working concentration of 20-200 μM is generally recommended although much of the PCR work that is described in this thesis used a concentration of 250 μM to no detrimental effect.

The concentration of magnesium ions has important effects on primer annealing, melting temperatures, enzyme activity, and reaction fidelity. The standard 'window' for magnesium ion concentration (in the form of magnesium chloride) is between 0.5 and 2.5 μM with 1.5 μM being the most widely used figure.

Primer concentrations of between 0.1 and 0.5 μM are the norm; again a balance has to be struck between the amount and the specificity of the of the product synthesized, with higher concentrations of primers leading to a greater number of mis-priming events.

Further points

An ideal PCR reaction would yield the maximum amount of product for the cycles used; that product comprising of material with a high signal-to-noise ratio (correctly primed product : spuriously amplified product).

The flanking sequences, against which the the primers will be designed, are chosen to ensure good amplification. Because the rate of complementary strand synthesis is finite, the distance between the primers should be small enough to ensure efficient amplification; typically between 100 and 1000 base pairs. High fidelity, long-range polymerase enzymes are now available with processivities which permit the amplification of products in the 1-20 kb range, but these were not used here. The extension time is also a critical factor when it is considered that after a certain number of program cycles the amplification of the products falls below the exponential rate as reaction constituents and enzyme activity decrease. If the extension time is too brief or the number of cycles too great, then there will be an increased percentage of incomplete products synthesized. For that reason, a final extension period of 15 min at 72^{o}C is often included to ensure that all products are double-stranded.

Primer design

Typically, primers contain 18 or more nucleotides complementary to the template DNA, 17 nucleotides being the statistically-derived length at which any given stretch of DNA is likely to be found uniquely in a mammalian genome. With two primers required for amplification of a product, the probabilities of non-specific PCR decrease still further. To aid the subsequent cloning of the PCR products, some primers are synthesized with appropriate restriction sequences at their 5' ends. It is apparent, however, that some restriction enzymes do not readily cleave at positions near the termini of a DNA molecule and so extra nucleotides are included on the primer's 5' end to provide a '5-clamped' restriction site which acts as a superior enzyme substrate.

Selection of the sequences for these primers is subject to three main criteria:-

1) The G/C content must be suitable to create adequately high annealing temperatures to prevent spurious priming events. Also, G's and C's at the 3' end of a primer should be avoided as these increase the probability of mis-priming.

2) Effort should be made to ensure that the primers do not contain regions of secondary structure (self-complementarity) which might interfere with the template annealing step.

3) The two primers should not have any regions of complementarity between each other, especially at the 3' ends, as this may cause the preferential production of 'primer-dimers' instead of genuine products.

Amplification programs

PCR amplifications were carried out according to programs described in the text.
The successful amplification of the protein tyrosine phosphatase family required the application of the RT-PCR technique described below.

Reverse transcription step:

mRNA (1 µg)	3.5 µl
RNA Guard	0.5 µl
dNTP mix (2.5 mM)	10 µl
Promega PCR buffer	2 µl

Random hexamers (100 pmol)	1 µl
$MgCl_2$ (25 mM)	2 µl
Reverse transcriptase (Gibco BRL)	1 µl

37°C	30 min
95°C	5 min
52°C	hold while adding PCR constituents

PCR reaction:-

The following reagents were incubated at 94°C for 4 min and then added to the RT products held at 52°C.

5' PCR primer	1 µl
3' PCR primer	1 µl
PCR buffer	8 µl
$MgCl_2$ (25 mM)	8 µl
Water	61.5 µl

0.5 µl of Taq polymerase (2.5 units activity) was added to the final mix and then the PCR amplification program was initiated.

72°C	45 s
94°C	30 s
52°C	30 s

35 cycles were carried out and then a final extension of 72°C for 5 min. The expected size of product was approximately 250 bp.

Me.6: Chemical cross-linking subtraction

Chapter III describes the application of this technique for the search for hippocampus-specific genes. It was based on a protocol published by Hampson et al. in Nucleic Acids Research, Vol. 20, No. 11, p. 2899, 1992.
First strand cDNA was synthesized (as described previously) from approximately 5 µg of hippocampus mRNA. The cDNA was purified away from the RNA template by alkaline hydrolysis (0.5 M NaOH at 55°C for 15 min) followed by spin-column

fractionation (see probe synthesis). 500 ng of this cDNA was hybridised to 10-20 μg of mRNA prepared from brain tissue with the hippocampus removed. The hybridisation took place in a volume of 10 μl of:-

0.5 M NaCl
25 mM HEPES buffer (pH 7.5)
5 mM EDTA
1% SDS

After 20 hr of incubation at 68°C, the solution was diluted 5-fold with distilled water and precipitated with ethanol. The resulting pellet was dissolved in 50 μl of:-

25 mM Tris-HCl, pH 7.5
1 mM EDTA
5% D.M.S.O.
2 mM Ascorbic Acid

This was incubated at 68°C for five minutes (to remove secondary structure) and then mixed with D.Z.Q. (to a final concentration of 200 μM. The DZQ was a generous gift from Ian Hampson, Paterson Institute for Cancer Research, University of Manchester, England) and left to incubate at 45°C for 20 min. This material was then ethanol precipitated oncemore.
Depending on the experiment, this cross-linked nucleic acid was either used directly as a template for probe synthesis or was subjected to another round of subtraction.
Probe synthesis used one half of the material prepared and was performed as normal except that 6 units of 'Sequenase' enzyme were used instead of Klenow enzyme (for the reasons described in Chapter III) and the isotope quantity was increased to 100 μCi instead of the conventional 30 or 40 μCi.

Me.7: Streptavidin/biotin subtraction technique

Creation of representative cDNA pools for amplification
Double-stranded cDNA was made from the two poly A^+ RNA pools (either hippocampus and rest of brain or E.C.S. hippocampus and Sham hippocampus) as previously described.
The resultant cDNA from each tissue was divided up into two aliquots; one of which was digested with Alu I restriction enzyme, while the second was doubly digested

with Alu I and Rsa I. The two aliquots were then taken through phenol/chloroform purification and ethanol precipitation and finally recombined.

This material was ligated to 10 μg of phosphorylated linker. The phosphorylated linker was made by the mixing of equimolar amounts of the two oligonucleotide constituents in standard high-salt restriction digest reaction solution. After the addition of 10 mM ATP and 1 μl polynucleotide kinase, this mixture was incubated at 37°C for 20 min. This was followed by 45°C incubation for 45 min to permit oligonucleotide annealing.

The sequences of the oligonucleotides are shown below;

```
5'      ctcttgcttgaattcggacta  3'  linker
3'  acacgagaacgaacttaagcctgat  5'

5'  tgtgctcttgcttgaatt  3'     PCR primer
```

The 3' overhang on one strand ensured the directional ligation of the linker onto the cDNA fragments and prevented the build-up of concatameric linkers on the ends of each cDNA fragment. Ligation products were run briefly (5-10 min) on a low melting temperature agarose gel and a region corresponding to DNA lengths upwards of 200 bps was cut out.

Using 1-2 μl of melted agarose per 100 μl reaction, the cDNA fragments were amplified by the P.C.R. technique (using the primer complemetary to the linker and 0.7 μl of Taq enzyme) such that a stock of the cDNA fragments could be built up for each tissue sample.

The P.C.R. program was designed to avoid amplification bias that might favour smaller cDNA fragments.

94°C	1 min
50°C	1 min
72°C	2 min, with a 25 sec increment with each passing cycle.

30 cycles overall (reduced to 25 for amplifications after each subtraction step).

Reaction constituents:-

Agarose/cDNA	1-2 µl
Primer	2 µl
Buffer (10X)	10 µl
$MgCl_2$ (25 mM)	6 µl (1.5 mM)
dNTPs (5 mM)	5 µl (0.25 mM)
Water	75.3 µl
Taq Polymerase	0.7 µl

The P.C.R. products were chloroform extracted to remove the oil overlay and then phenol/chloroform extracted followed by ethanol precipitation. All products from one tissue origin were pooled in 50 µl water and assayed for concentration by absorption at 600 nm.

Correct representation within these initial pools was assayed by cDNA Southern analysis (that is, amplified material from the two sources was run on a gel, denatured and blotted onto 'Hybond-N' filters) Ubiquitously expressed genes were used as probes to ensure correct representation in both pools.

Preparation of the driver cDNA

100 µg of the required cDNA was digested to completion using 1500 units of EcoRI restriction enzyme to remove the linker regions from the fragment in order to prevent subsequent amplification of residual driver.

Digestion products were phenol/chloroform extracted, put through a Sephadex G-50 spin column (to remove linker ends) and then ethanol precipitated.

The precipitated cDNA was resuspended in 1µg/µl photobiotinylation buffer.

5 mM HEPES buffer, pH 7.5
1 mM EDTA

The cDNA was then mixed with an equal volume of an equal concentration of photobiotin acetate and incubated on ice under two 150 W bulbs for one hour.

An equal volume of 0.1 M Tris-HCl, pH 9 was then added.

Excess unincorporated photobiotin was removed by 3 successive extractions with water-saturated butanol. The top organic phase was discarded each time.

After ethanol precipitation the photobiotinylation procedure was repeated once more.

Hybridisation of the tracer and driver

5 μg of the tracer cDNA was added to the 100 μg of the biotinylated driver and resuspended in a volume of 20 μl of water.

This was boiled for three minutes and then 20 μl of 2X Hybridisation buffer (see below) were added to bring the final volume to 40 μl.

1.5 M NaCl (0.75 M)
50 mM HEPES buffer, pH 7.6 (25 mM)
10 mM EDTA (5 mM)
0.2% S.D.S. (0.1%)

After covering the liquid with a layer of oil the solution was boiled for another 3 minutes and then incubated at 68°C for 20 hours (2 hr for the short hybridisation step).

260 μl of :-

10 mM HEPES, pH 7.6
1 mM EDTA

were then added to bring the final NaCl concentration to 0.1 M. The mixture was incubated for 5 minutes at 55°C (this is the equivalent to washing a filter after hybridisation).

20 μl of streptavidin (2 μg/μl dissolved in 0.15 M NaCl, 10 mM HEPES, pH 7.6, 1 mM EDTA) was added to the mixture. It was left to stand for 20 minutes at room temperature to allow the streptavidin/biotin complexes to form.

A phenol/chloroform extraction was carried out such that the organic phase together with the Streptavidin/Biotinylated cDNA interface were discarded. Four repeats of the streptavidin addition and phenol/chloroform extraction were carried out to ensure maximum removal of excess driver/driver hybrids.

The resultant material was ethanol precipitated and either subjected to P.C.R. amplification or a second, short, hybridisation with 50 μg of appropriate biotinylated driver.

A full scheme of the subtraction protocol is presented below.

Subsequent modification of the technique

To prevent the cDNA driver of one tissue contaminating the cDNA tracer of the other tissue a modification was made to the protocol. This was based on a suggestion made in a paper written by Balzer and Baumlein (see Chapter IV) that the two cDNA pools derived from the two tissues should be made independently amplifiable. This was achieved by the use of different linker sequences for each pool. Thus, the primer used to amplify tracer cDNA after a subtraction step would not be complementary to any contaminating driver cDNA (and, hence, would not permit its amplification)

In the subsequent subtraction steps, one cDNA pool had the previously described linker sequences added, whereas the other pool had the following linker added:-

```
5'      agttacacgtctagaatggct  3'
3' atagtcaatgtgcagatcttaccga  5'
```

Because there is no possibility of cross-amplification, there wass no need for any digestion step to remove the ends of the linker from each cDNA. Also, for the same reason, there is no need for the purchase of a separate PCR primer for the amplification of these sequences as the upper oligonucleotide suffices for this purpose. This new linker contains an internal Xba I restriction site which can be used for the cloning of the subtracted material.

Analysis of subtracted material

At the end of the subtraction procedure, the material obtained was used in a variety of ways, as described in the text of Chapters IV and V. To directly clon and analyse the material, the final subtracted material was made fully double-stranded. 3 μg of the material was subjected to one round of amplification to achieve this. This was then digested with the appropriate restriction enzyme (EcoRI or XbaI), purified, and then ligated into a vector cut with the same restriction enzyme (and phosphatased). The two vectors used were the plasmid pBluescriptII, KS configuration, and the M13 filamentous bacteriophage vector, tg130 or 131.

The ligation products were transformed (generally by electroporation) into the appropriate host bacterial strain as described elsewhere.

Me.8: Creation of competent cells

Conventional competent cells

A single fresh colony of desired strain (XL1 Blue or DH5α were commonly used) was grown for 2 hours in 5 ml of TYM-broth, shaking at 37°C.

2% Bacto-Tryptone
0.5% Bacto Yeast Extract
0.1 M NaCl
0.01 M $MgCl_2$ or $MgSO_4$

This was transferred to 100 ml of fresh TYM-broth and incubated for a further 2-3 hr, or until the ABS_{600nm} was 0.5.
The cells were spun at 2500 r.p.m. for 5 min at 4°C in two 50 ml 'Corning' tubes.
The cell pellet was resuspended in 20-40 ml of 'Tfb I' per 100 ml of starting culture.

30 mM KOAc
50 mM $MnCl_2$
100 mM KCl
10 mM $CaCl_2$
15% by volume of glycerol
pH 5.8 (using dilute acetic acid), filtered.

The resuspended cells were left on ice for 5 min-3 hr, depending on the strain used.
The cells were spun at 2000 r.p.m. for 5 min at 4°C.
The pellet was resuspended in 4 ml of 'Tfb II' per 100 ml of starting culture.

10 mM Na-MOPS pH 7.0
75 mM $CaCl_2$
10 mM KCl
15% by volume of glycerol

The cells were aliquotted into conveniant volumes for storage at -70°C.

Electrocompetent cells

2X 50ml volumes of LB medium were each inoculated with 1 ml of overnight bacterial culture (XL1 Blue bacterial strain). When the ABS_{600} reached a value of 0.5-1.0 the cells were taken through the preparative procedure.

1) The cells were chilled on ice for 15-30 min.
2) In 50 ml Corning tubes the cells were centrifuged at 4000 xg for 15 min. at 4°C.
3) The cells were resuspended in 100ml of ice-cold distilled water.
4) The cells were centrifuged as in 2.
5) The cells were resuspended in 50 ml of ice-cold distilled water.
6) The cells were centrifuged as in 2
7) The cells were resuspended in 2 ml of ice-cold 10% glycerol solution.
8) After a final spin as in 2, the cells were finally resuspended in 250 µl of 10% glycerol.
9) This mix was divided into 40 µl aliquots and stored at -70°C.

Me.9: Transformation of bacterial cells

Conventional cell preparations

1-10µl of the ligation was added to 200µl of thawed competent cells and left on ice for 30 min.

The cells were then subjected to a 90 sec. heat-shock at 42°C.

After 3 min recovery on ice, 400µl of LB medium was added and the mixture was placed in a shaking incubator at 37°C for 30 min.

The cell suspension was then plated on appropriately prepared agar-filled petri dishes and left to grow overnight at 37°C.

Electro-transformation

For every ligation to be transformed, one 40 µl aliquot of electro-competent cells was used. 1 µl of the ligation mix was mixed well into the thawed cells and allowed to sit on ice for one minute.

This mixture was then added to the bottom of an electroporation cuvette. The cuvette was placed in a 'Gene Pulser' apparatus set at 25µF, 2.4kV, and 200Ω.

After one pulse, the cuvette was immediately removed from the apparatus and 1ml of SOC medium added to the cells. The cells were transferred to a Falcon tube and placed in a 37°C shaking incubator for a period of 1 hour.

The cell suspension was then plated on appropriately prepared agar-filled petri dishes and left to grow overnight at 37°C.

Me.10: Preparation of plasmid DNA - plasmid mini-preps

The E. coli colony containing the plasmid of interest was grown overnight (15-16 hr) in 1.5 ml of LBM medium in a 37°C shaker.
The culture was decanted into a 1.5 ml Eppendorf tube and spun for 2 min at 13,000 r.p.m.
The pellet was resuspended in 100 µl of 'Lysis Buffer':

25 mM Tris-HCl, pH 8.0
10 mM EDTA
50 mM Glucose
2 mg/ml lysozyme

200 µl of 0.2 M NaOH/1% SDS was added, followed by 10 min of tube inversion.
Then 150 µl of 3 M KOAc/2 M Acetic acid were added and the solution left on ice for 15 min.
The cloudy precipitate was removed by a 10 min bench-top centrifugation and the 450 µl of supernatant transferred to a clean Eppendorf tube.
The DNA was phenol/chloroform extracted and then precipitated by the addition of 1ml of Ethanol.
After 30 min at -70°C the DNA was pelleted by a 15 min spin.
The pellet was resuspended in 50 µl of :-

10 mM Tris-HCl, pH 8.0
1 mM EDTA
5 mg/ml RNAse A

After a 20 min incubation at 37°C the solution was analysed for the presence of inserted DNA by appropriate restriction digestion.

For some applications, such as high quality sequencing, the DNA was subjected to a further round of purification involving PEG precipitation. This involved the addition to the 50 µl plasmid preparation of 30 µl of 20% PEG in 2.5 M NaCl. This mixture was left on ice for 1 hour (or overnight) and then spun at 13,000 r.p.m. for 15 min. The pellet was washed several times with 70% ethanol to ensure complete removal of PEG solution from the DNA.

Me.11: Radiolabelled DNA probe synthesis

The template DNA required for the synthesis of the probe usually consisted of 2-3 µl of mini-prep plasmid DNA digested with the relevant restriction enzymes and electrophoresed on a low melting temperature agarose gel. The appropriately sized band was cut out of the gel and the DNA purified by the use of the 'Gene-Clean II' or 'Quiaex' kits.

Purified DNA was resuspended in 18.4 µl of water and boiled in a screw-topped Eppendorf tube for 5 min followed by quenching in ice for three min.

The following reagents were added in the order given:

6 µl OLB (Oligo-Labelling Buffer)
- 20 µl Solution A
  - 1.21 M Tris-HCL pH 8.0
  - 0.121 M $MgCl_2$
  - 1.74% β-Mercaptoethanol
  - 0.484 mM each dATP/dTTP/dGTP
- 50 µl Solution B
  - 2M HEPES buffer, pH 6.6 with NaOH
- 30 µl Solution C
  - Hexadeoxyribonucleotides dissolved to
  - 90 O.D. units/ml in 3 mM Tris-HCl, pH 7
  - and 0.2 mM EDTA

1.2 µl of 10 mg/ml enzyme-grade Bovine Serum Albumin

40 µCuries of radiolabelled dCTP (4µl)

0.4 µl Klenow fragment polymerase

The reaction mix was left at room temperature/37°C for 1-3 hr.

The reaction mixture was then made up to 100 µl with STE/TEN buffer and spun through a Sephadex G-50 spin column (1ml volume equilibriated with STE/TEN buffer). This removed unincorporated nucleotides.

After scintillation counting, the probe was denatured by boiling for 5 min and then quenching on ice.

The probe was then added to the hybridisation vessel.

Me.12: Probe hybridisation conditions for Northerns, Southerns and library screens

Ref: Church and Gilbert, (1984) P.N.A.S., Vol. 81, p.1991.

Standard hybridisation procedures required the filter(s) to be pre-hybridised for over one hour at the desired temperature (usually 65° or 68°C).

(pre)Hybridisation Solution:-

7% S.D.S.
0.5 M $NaHPO_4$ (pH 7.2)
per litre of one molar (pH 7.2):
71 g anhydrous Na_2HPO_4
4 ml H_3PO_4 (85%)
1 mM EDTA
1% Bovine Serum Albumin

After the addition of the denatured probe, the filter was left to hybridise for 16 hours or longer.
Removal of non-specifically hybridising probe was done by three successive washes with wash solution pre-equilibriated to the hybridisation temperature.

Wash Solution (per 2.3 litres):-

92 ml of	1 M (40 mM) $NaHPO_4$ (pH 7.2)
4.6 ml of	0.5 M (1 mM) EDTA (pH 8.0)
23 g of	S.D.S.
	water to 2.3 litres.

After the final wash the filter(s) were wrapped in Saran Wrap and exposed to X-Ray film for the required period of time.

N.B. Depending on the size and number of filters, the hybridisations were done either in glass hybridisation cylinders or in heat-sealed plastic bags.

Me.13: Northern blotting and hybridisation

RNA samples to be blotted (1-5 µg poly A^+, 15-25 µg total RNA) were precipitated in ethanol and resuspended in 12 µl of sample buffer:-

25 µl 10X MOPS
- 0.4 M MOPS
- 100 mM NaAcetate
- 10 mM EDTA

125 µl Formamide
44.5 µl Formaldehyde
55.5 µl D.E.P.C. Water

The RNA in solution was heated to 60°C for 10 minutes and then quenched on ice to ensure loss of secondary structures.
After addition of loading dye (containing Ethidium Bromide) the samples were loaded onto a 1% agarose gel:-

1.2 g Agarose
12 ml 10X MOPS
86.4 ml D.E.P.C. Water

This was melted then cooled prior to the addition of 21.6 ml Formaldehyde.
The solution was poured into 120 ml gel tray and allowed to set.
The set gel was covered with running buffer in the electrophoresis tank:-

100 ml 10X MOPS
720 ml D.D.Water
180 ml Fomaldehyde
per litre

The gel was electrophoresed at approx. 90V until the Bromophenol Blue dye had progressed one third of the way through the gel.
After a brief rinse in water the gel was photographed under U.V. light and then blotted overnight in the same way as a Southern blot (see below).

Me.14: Southern blotting

DNA samples were electrophoresed in appropriate conditions/agarose percentage. The DNA in the gel was then subjected to denaturation for 15-30 min in 1.5 M NaCl, 0.5 M NaOH:-

4 Litre Stock
350.64 g NaCl
50 g NaOH
Water

This was followed by neutralization of the gel in 1.5 M NaCl, 1 M Tris-HCl, pH 7.4:-

4 Litre Stock
484.4 g Tris
350.64 g NaCl
272 ml HCl
Water

A final rinsing step was carried out using 20X SSC.

The capillary-blotting procedure was carried out as follows:-
1) Whatman 3MM paper was used as base for the stack and as a wick because its ends were in contact with a bath of 20X SSC.
2) Upon this was placed the inverted gel.
3) Hybond-N paper was cut to size and placed on the top of the gel.
4) Four sheets of Whatman 3MM paper were placed on this followed by a stack of hand-towels to a depth of 5-8cm.
5) A weight (approx. 1kg) was placed on top of the stack and then the apparatus was left overnight.
6) The stack was then dismantled and the Hybond-N filter paper baked at 80°C for 2 hours prior to prehybridisation.

Me.15: In situ hybridisation

Riboprobe synthesis

The cDNA to used as a probe was cloned into pBluescript II (KS form) plasmid vector. One µg per probe of the clone was digested with an appropriate restriction enzyme in order to create a linear DNA template which will produce a probe of the correct complementarity: antisense or sense control. This was resuspended in 2 µl of water. To this was added the following reagents:-

0.75 µl	200mM DTT
0.75 µl	2mg/ml BSA
2.25 µl	3.3 mM each of the ribonucleotides not present in radioactive ribonucleotide.
0.5 µl	RNA Guard RNase inhibitor
6.25 µl	Radioactive ribonucleotide
1.5 µl	10X Transcription buffer
1 µl	T7/SP6/T3 RNA Polymerase

This was incubated at 37°C for 1 hr.

DNase (1 µl) was added and incubated at 37°C for 20 min.

The reaction was made up to 85 µl with water and then Phenol/Chloroform extracted.

The probe was purified from excess ribonucleotides by Sephadex G50 spin column centrifugation.

4 µl of 1 M DTT was added to prevent oxidative degradation.

Scintillation counting of 1% of the probe should give approximately 300,000-1,000,000 counts per minute.

Pretreatment of sections prior to probe hybridisation

Coronal brain sections mounted on glass microscope slides were dewaxed in xylene and then ethanol dehydrated.

They were then post-fixed in paraformaldehyde in PBS for 20 min.

Two PBS washes were carried out on the sections.

20 µg/ml Proteinase K in 50 mM Tris-HCl, 5 mM EDTA was used for 20 min at room temperature to deproteinate the tissue.

After one PBS wash the sections were re-fixed in paraformaldehyde as before.

After a distilled water rinse the sections were placed in a bath with 0.1 M triethanolamine. Acetic anhydride was added to a final concentration of 2.5 ml/l. This was stirred slowly until fully dissolved and then the sections were left for 10 min.
After 5 min PBS and saline washes the sections were dehydrated through an ethanol concentration series and left to air dry.

Prehybridisation and hybridisation conditions
The hybridisation mixture consists of:-

50% deionised formamide
0.3 M NaCl
20 mM Tris-HCl, pH 8.0
5 mM EDTA
10 mM NaPO4, pH 8.0
10% Dextran sulphate
1x Denhardt's solution
0.5 mg/ml yeast RNA
prior to use the mixture was de-gassed in a vacuum drier.

400 µl of this was placed over the sections and then Parafilm cover-slips were put over the sections to prevent evaporation. The pre-hybridisation was carried out in a perspex box with a tissue soaked in 50% formamide/5X SSC included to maintain a humid environment.
This was incubated at 55°C for 2 hours.

The probe was prepared by dilution in hybridisation solution such that the specific activity was 10^4-10^5 counts per minute/µl. DTT was added to a final concentration of 10mM. Just before use the probe solution was denatured at 80°C for 3 min.

The coverslips were removed and 200 µl of the denatured probe added to the top of the sections. New coverslips were added and trapped air bubbles removed.
Incubation was carried out as before at 55°C overnight.

Washing of hybridised sections

Coverslips were removed by placing the slides vertically along their long edge in a bath of 5X SSC and 10 mM DTT at 55°C. After 15-30 min the slides were removed and taken to the washing steps.

High stringency wash solution:-

50% Formamide
2X SSC
0.1 M DTT

The first wash was carried out at 65°C for 20-30 min.
Three washes with:

0.5 M NaCl
10 mM Tris-HCl
5 mM EDTA

were carried out at 37°C for 10 min each.
RNase (to a concentration of 20 µg/ml) was added to this solution and left to incubate at 37°C for 30 min.
A final wash with this solution was carried out prior to the second high stringency wash (as before).
Two final washes, one 2X SSC and one 0.1X SSC, were performed followed by ethanol series dehydration.
After air drying, the slides were placed against BioMax autoradiography film and allowed to expose for 24 hr-1 week.

Me.16: DNA sequencing

Both double-stranded plasmid preparations and single-stranded M13 preparations were sequenced by dideoxy sequencing. The sequencing of plasmid DNA generally gave poorer results.

Sequencing reaction

Approximately 12 µl out a 50 µl plasmid prep was sufficient for an individual sequencing reaction. This was precipitated with ethanol and resuspended in 18 µl of TE buffer. To this was added 1 µl of 20 ng/µl of oligonucleotide primer (T3/T7/SP6)

together with 1 µl of 4 M NaOH. This mixture was left at room temperature for 5 min. to permit the complete denaturation of the double-stranded plasmid DNA. After ethanol precipitation and 70% ethanol washing, the pellet was resuspended in 7 µl of water. To this was added 1 µl Di-methyl Sulphoxide (DMSO) and 2 µl of (5X) sequencing buffer:-

0.2 M Tris-HCl buffer, pH 7.5
0.1 M $MgCl_2$
0.25 M NaCl

A stock reaction solution was made up such that for every plasmid sample to be sequenced an additional :-

1.1 µl 0.1 M DTT
1.76 µl water
1.95 µl TE buffer
0.44 µl labelling mix (1.5 µM each of dGTP, dTTP, and dCTP)
0.5 µl ^{35}S-dATP (=5 µCi)
0.28 µl Sequenase Enzyme

was added.
From this reaction solution stock 5.25 µl was transferred to each of the plasmid samples and then left for 5 min at room temperature. This step causes the newly forming strand to be labelled with isotope to a high specific activity.
Four 3.5 µl aliquots from each sequencing reaction were placed in individual wells of a 96-well plate. To each of the four aliquots for any one plasmid sequencing reaction 2.5 µl of one particular dideoxy termination solution was added such that termination reactions at all four bases were represented for each plasmid. The termination mixtures consisted of:-

8 µM of the relevant dideoxynucleotide
80 µM each of the other three deoxynucleotides
10% DMSO
50 mM NaCl

After 5 min of incubation at 37°C this final reaction step was stopped by the addition of 4 µl of:-

95% Formamide
20 mM EDTA
0.05% Bromophenol Blue dye
0.05% Xylene Cyanol FF dye

Sequencing gel

The gel was poured between glass plates, one of which had been silicon treated, and a square-teeth comb was used to create the wells *in situ.*

The gel was made using solutions obtained from the 'Sequagel' kit of National Diagnostics. For a 50 ml gel the following quantites were used:-

14.4 ml Concentrate
39.6 ml Diluent
6 ml Buffer
0.48 ml 10% Ammonium Persulphate
50 µl TEMED (N, N, N', N'-Tetramethyl-Ethylene-Diamine)

The sequenced DNA samples were denatured at 80°C for 10 min and then 2 µl of each was loaded into the wells in a defined order (T-C-G-A was used here). The gel was subjected to electrophoresis (30-40 W, 1200 V) for 3 hours or more.

The gel, while still attached to one of the glass plates, was fixed for 15 min in the following solution:-

10% Acetic Acid
10% Methanol
Water

The gel was then transferred to two sheets of Whatman 3MM paper and covered with Saran wrap. This was then vacuum-dried at 80°C using an ice-trap to condense the evaporating fix solution.

The dried gel was then placed against Kodak BioMax autoradiography film in a cassette and left to expose overnight. By assigning base composition and order of the electrophoresed bands it was possible to deduce the sequence of the DNA.

Me.17: Methods associated with bacteriophage λ use

Library plating: plating bacteria

1 ml of an overnight (tetracycline selected) culture was added to 25 ml of LB medium supplemented with 0.2% maltose.

This was shaken in a 37°C incubator until the ABS_{600} was approximately 1.

The cells were pelleted by 10 minutes centrifugation at 4000 xg and then resuspended in 25 ml of 10 mM $MgSO_4$

Library plating: plating

The bacteriophage was diluted to the correct concentration in SM solution such that the correct number of plaque forming units per plate was contained in a few microlitres. This quantity was placed inside a Falcon tube into which 1.32 ml of plating cells had been pipetted. The cells were briefly mixed with the 'phage and then left motionless at 37°C for 20 min.

Meanwhile, 15 cm diameter petri dishes were filled to a depth of 1 cm with bottom agar, dried so that the surface was just furrowed, and then left at 37°C to equilibriate to that temperature. Top agarose was melted and then placed in 13 ml aliquots in Falcon tubes and left at 48°C.

The top agarose contents of one tube were gently poured into the tube containing the cells so that the two mixed without the creation of bubbles. This was then quickly poured over the bottom agar to form a level covering of top agarose before it setted.

Each poured plate was left to stand at room temperature and then inverted and placed in a 37°C incubator.

After 4-5 hr the bacterial lawn was apparent and several hours after that the 'phage plaques began to appear. Plaque sizes of 1-2 mm were chosen as an indication that the plates were ready for storage at 4°C.

Library plating: plaque lifts

After an hour-overnight at 4°C the plaques were transferred to nitrocellulose filters.

Plates were placed on a light box and a correctly-sized filter slowly placed onto the agarose surface. This was left in place for 1 minute, during which time three holes were punched through the filter and into the agarose using a syringe needle. Successive filter-lifts were taken using the needle holes to orient the positions for subsequent filter marks. For each successive lift, the time in contact with the plate was doubled.

All filters were treated as follows:-

1) 2 minutes on top of 3MM paper soaked with denaturation solution (see Southern section).
2) 5 minutes on top of 3MM paper soaked with neutralisation solution (see Southern section).
3) 2-3 minutes on top of 3MM paper soaked with 2XSSC solution.
4) After air-drying, the filters were baked in a 80°C oven for 2 hours.
5) The filters were subjected to the relevant hybridisation experiments.

Library plating: picking colonies

Hybridisation signals on the X-ray film were aligned to corresponding plaques on the plates using the needle holes as a guide. These plaques were isolated by the use of 'pastettes' with the tips cut off so that the diameter of the exposed end was approximately 0.5 cm. These were used to suck up the plaque and the surrounding region. The plaque plug was expelled into 1 ml of SM solution and left overnight for the bacteriophage particles to diffuse out into the solution. Secondary and tertiary screens were then performed at lower plating densities using aliquots of the plug suspensions to obtain pure isolates of the plaques.

Library plating: in vivo excision

Because the lambda strain used to create the principle hippocampus cDNA library was λZAPII (Stratgene), *in vivo* excision could be carried out to obtain a bacterial plasmid containing the correct cDNA insert which enables its easier preparation and manipulation.

Approximately 10^5 plaques in 100 µl of SM buffer were added to 200 µl of XL1 Blue plating cells and 1 µl of 'ExAssist' helper phage. This was mixed briefly and left to incubate for 15 min at 37°C.

To this mixture was added 3 ml of 2X TY medium and then the cells were left for 3 hours in a shaking 37°C incubator.

The cells were lysed in their tubes by 20 min incubation in a 70°C water bath. Cellular debris was removed from the lysate by centrifugation for 5 min at 4000Xg. The supernatant was transferred to a sterile tube. 5 µl of this was added to 200 µl of SOLR cells and the mixture was incubated for 15 min at 37°C.

Various amounts of the product were plated out on 8 cm ampicillin-containing agar plates and left to incubate overnight at 37°C. Colonies should contain the excised cDNA insert within 'pBluescriptII KS' plasmid vector.

Me.18: Bacteriophage M13 methods

M13 bacteriophage plating

The procedures required for plating M13 bacteriophage are very similar to those for λ. The following list describes the major differences:-

1) cDNA inserts ligated into M13 bacteriophage vector (tg131; Kieny, M.P., Lathe, R., and Lecocq, J.P. (1983) Gene, Vol. 26, pp. 91-99) have to be transfected into bacterial cells (XL1 Blue was used as a suitable host strain). This was achieved either by conventional transfection or electro-transfection as described elsewhere.

2) Transfected cells were added to the top agarose as before except that IPTG (10 µl) and X-GAL (100 µl) were also included (if the conventional transfection technique was used then extra plating cells (200 µl) derived from an ABS_{600}=1 incubation were added).

3) Plates were left overnight and the white plaques obtained represented insert-containing vectors.

4) Filter lifts were performed as before except that no denaturation or neutralisation steps had to be carried out on the filters because the M13 plaques contain single-stranded DNA.

5) Plaque plugs were taken as before (and stored in LB medium) or plaques were stabbed with a sterile toothpick which was then used to streak another plate to obtain isolated plaques.

M13 Bacteriophage single-stranded DNA preparations

Single-stranded bacteriophage vector DNA can be used for direct sequencing or as a template for PCR amplification of the cDNA insert.

10 µl of the plaque suspension was added to 25 µl of an XL1 Blue overnight bacterial culture and 2 ml of LB medium. This was left to shake at 37°C for 4-5 hours.

This was centrifuged in a microfuge at 8000rpm for 15 min. 1 ml of the supernatant was removed.

To this was added 200 µl of 20% PEG (polyethylene glycol) in 2.5 M NaCl. This was left at room temperature for 30 min.

This was centrifuged as before and all the supernatant was discarded. The pellet was resuspended in 100 µl of 1.1 M NaOAc, pH 7 and incubated at 65°C for 10 min.
After phenol/chloroform and chloroform extractions the aqueous phase was precipitated with ethanol.
After a half hour chill at -20° or -70°C the precipitate was pelleted by a 20 minute microfugation. The pellet was washed in 70% ethanol, dried, and resuspended in 20 µl TE buffer.

For sequencing purposes, 5 µl was incubated with 20 ng of '-40' primer at 55°C prior to the sequencing reaction.
PCR amplification of the cDNA insert was carried out using the -40 primer and a primer designed to be complementary to a sequence on the bacteriophage vector distal to the insert. The amplification program was 30 cycles of:-

94°C	1 min
54°C	1 min
72°C	1 min

PCR products were used as probe templates or cloned into pBluescriptII plasmid vector for more extensive sequencing/*in situ* template creation etc.

Me.19: Electroconvulsive stimulation (ECS) protocol.

Male hooded Lister rats were used.
Light halothane anaesthesia was used prior to the shock or by itself in the case of the sham rats.
The ECS shock consisted of a 200 V (sine wave) pulse, 40-60 mA for 2 seconds.
Tonic-clonic seizures were observed.
ECS treatments were separated by 48 hr.
A total of 10 stimulations were applied and then the rats were sacrificed at 72 hr post-stimulation.

Me.20: cDNA Capture

In order to obtain longer fragments of cDNAs cloned in the subtraction process a novel technique was devised.

The clone of interest (1/4 of a single-strand M13 bacteriophage prep. was sufficient) was denatured in a volume of 20 µl of water and applied to a 1 cm by 2 cm piece of Hybond nitrocellulose. This was baked for 2 hr at 80°C.

The filter was then placed in a 15 ml Corning tube and 6 ml of Church and Gilbert hybridisation solution added (see me.11). Prehybridisation of the filter was carried out by inserting the tube into a hybridisation cylinder filled with paper towels and placing this into a normal hybridisation oven for 2-4 hr at 65°C.

5 µl of a typical PCR amplification of the initial cDNA pool was denatured in a final volume of 25 µl and then added to the 15 ml Corning tube. Hybridisation was carried out overnight as before.

Washes were carried out as described in me.11. The washed filter was added to 100 µl water in an eppendorf and boiled for 5 min.

The water was removed and ethanol precipitated with a glycogen carrier. The resulting pellet was resuspended in 10 µl of water from which 1 µl was used in a PCR reaction using the appropriate cDNA pool primer and standard amplification program.

PCR products were electrophoresed on a 1.2% agarose gel. Often discrete bands were visible which could be cloned directly. Alternatively, the captured material could be compared to the original cDNA pool by Southern blotting and probiong with the original cDNA fragment. Much greater hybridisation to the captured material should be seen.

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Chapter III: Chemical Cross-Linking Subtraction

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Appendix

Chapter II

PTP 4 PCR product nucleic acid sequence

gaggactcagacatgtatggggacatcaagatcacgctggtaaagacagagacactggc
tgagtacgtggtgcgcacctttgccctggagcggagaggttactcagcccggcatgaggt
ccgccagttccacttcacagcgtggccagagcatggtgttccctaccatgccacngggct
gctggccttcatccggcncgtgaaggcttccactccacctgatgctgggcctgttgtcat
t

PTP 24 PCR product nucleic acid sequence

ttcacggaagaacccattgcttatggagacatcaccgtggagatggtctctgaggaagaa
caggaggactgggccagcagacacttccggatcaattatgctgacgaggcccaggatgt
gatgcactttaacnacacggcctggccagaccatggcgtgcctccagcaaatgctgctga
gagcatcctgcagttcgtgtacactgtccggcagcaagccaccaagagcaaaggaccca
tgatcatc

PTP 4 5' nucleic acid sequence from cDNA clones

acagggcagcttcaccctgcagtacgagtggctgacctgctgcagcacatcaaccagat
gaagacagccgaggnctacggcttcaagcaagagtacgagagtttctttgaaggctggga
tgccaccaagaagaaagacaagctgaagagcggccggcaggagccagtgtctgcctatg
accgacaccgagtgaagctgcacccgatnctgggagaccctgatgccgactacatctct
gccaactacatagacggctaccacaggtcaaaccacttcatagccactcaaggnccaaa
gcctgagatgatctacgacttctggcncatggtgtggcaggagcagtgtgtagagatcgt
catgatcagcaagctggtagaggtgggcagggtaaagtgctctcgatactggcccgagga
ctcagacatgtatggggacatcaagatcacgctggtaaagacagagacactggctgagta
cgtggtgcgcacctttgccctggagcggagaggttactcagcccggcatgaggtccgcca
gttccacttcacagcgtggccagagcatggtgttccctaccatgccacngggctgctggc
cttcatccggcncgtgaaggcttccactccacctgatgctgggcctgttgtcattcactg
cagtgcaggcact

PTP 24 5' nucleic acid sequence from cDNA clone 18.1

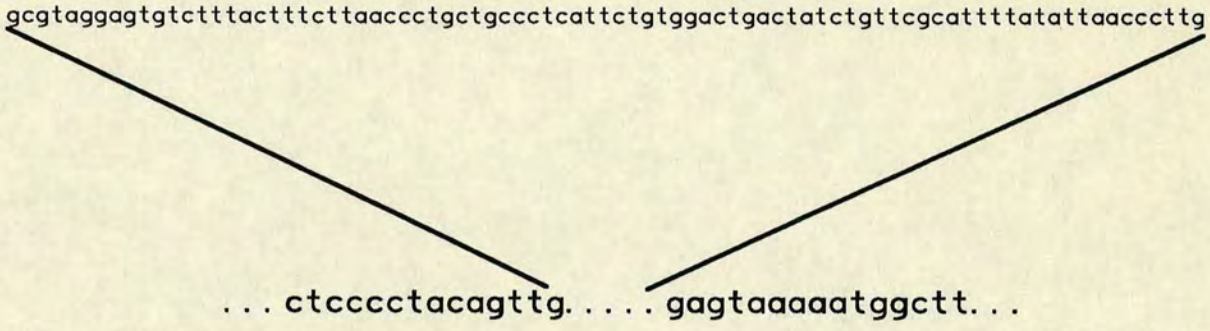
cctccaaaatcactcttcgcagtgaacaaaacgcagacatcggtcaccctgctttgggtt
gaggagggcgtanntgatttctttgaagtcctctgtcagaagctcgggtctggccacgat
ggcaaactccaggaaccggtagctgtttcctcccacgtggtgaccatctccagcctcctc
ccagccactgcctacaactgcagtgtcaccagcttcagccacgacagtccagtgtcctac
gttcatagctgtctccacaatggttacagaggtgaacctaatgtggtgg

PTP 24 5' nucleic acid sequence from cDNA clone 10.1/16a.1

gaggtgaaccctaatgtggtggtgatctcggtgctggccatcctcagcatacttttaattg
gactgctgttagtgacccttgtcattcggagaaagaagcacctgcagatggccagggagt
gtggggctggcacgtttgtcaattttccatccttggagagggaagggaaactcccctaca
gtt**gg**agtaaaaatggcttaaagaagaggaaactgacgaaccctgttcagctggacgatt
ttgattcttacatcaaggacatggccaaggactctgactataaattctctcttcagtttga
ggagttgaagttgattggactggatattccacactttgctgcagatcttccgct

'gg' highlighted by bold print indicates the point at which the insertion was found in cDNA clone 16a.1 (see below).

PTP 24 83 base pair insertion present in cDNA clone 16a.1



Chapter III

Sub 18 nucleic acid sequence

aaacccctacaaatttgaggaatgtggaaaggactttcagtattttgcatgccttaataat
tcccatgggaatgtgcacactggagaaaaattctgtgactgcaaggaatgttggaaagcc
ttcacggtttcctcacacctaactcagcatgtatcgattcacactgaagaaaaatcaaag
gtgtaagatatgt

Chapter IV

Nucleic acid sequences of putatively unsubtractable clones

mb6/15

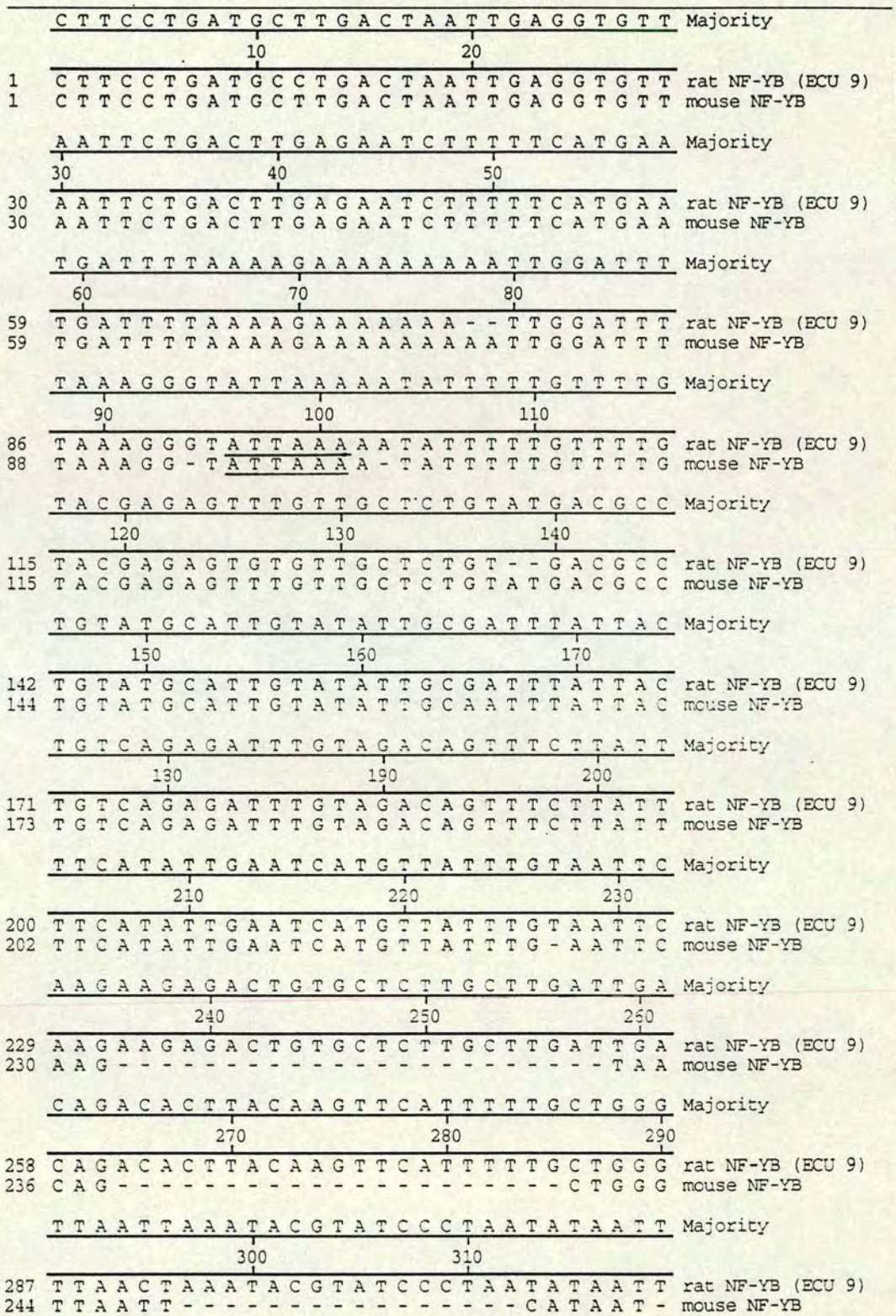
acatcttaattcttccagggtttggaattatttcacatgtagttacctattactctggaaa
aaaagaacccttcggatatataggtatggtatgagccataatatctattggcttcctagga
tttattgtatgagcacatcacatatcacagtaggcctagatgtagacaccgagcctactt
acatctgccactatatatcgcaatcctacaggcgtaaagatttcag

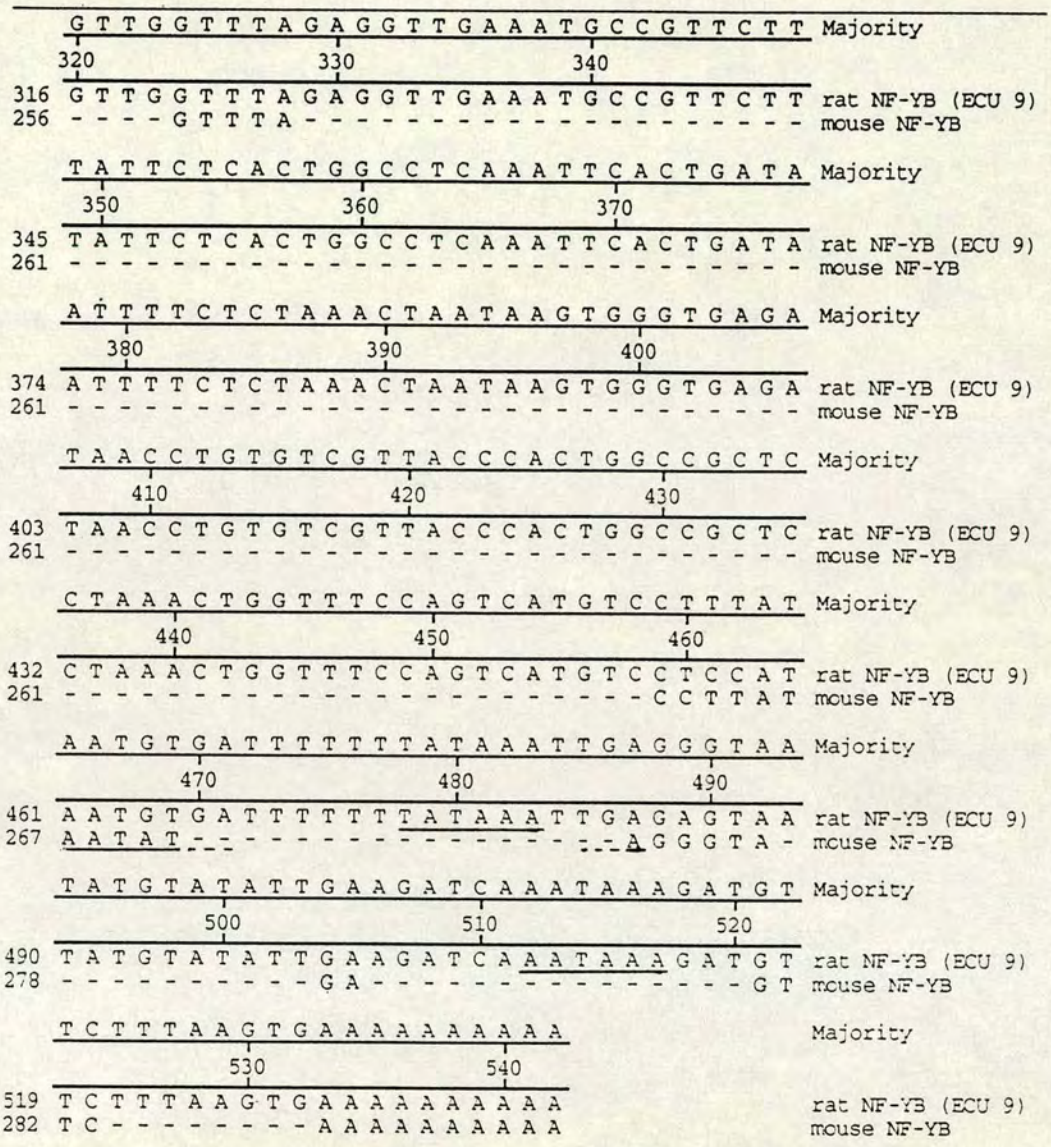
mb12

acgaatacagaccgtgaaagcggggcctcacgatccttctgacctttgggttttaagca
ggaggtgtcagaaaagttaccacagggataactggcttgtggcggccaagcgttcatagc
gacgtcgctttttgatccctttcgatgtcggctcttcctatcattgtgaagcagaattcac
caagc

Chapter V

NF-YB (ECU 9) rat nucleic acid sequence aligned with 3' end of published mouse cDNA





Motifs which may act as polyadenylation signals are underlined.

A selection of EC clones which were used as probes on Northern blots. Not all represent full sequences of the clones.

ECU 2 nucleic acid sequence

cacagtggtgaaggacctggcacagaactcataagcgtctcctcgcaaagggctttccac
acctcgcctggtcacactcgatgcctatttctactgaccaagttgaagaacaccctctac
tgaccttagaaacacacgcactctcatacagacacatgaacctttgga

ECU 11 nucleic acid sequence

ggactacaattcgcggccgctttttttttgaagcagctggttctttgtattccgtcacta
aaaacacaagagctctacaggcacagcagcttactcacacactcgaactgcaccaga
ctgtagcaaatggagaaagatggatgccttcaagttcactgggtggaactatgaaaagta
gaccttgggaatctcttg

ECU 12 nucleic acid sequence

cggttacttctatttctctaagcaagcgttctttgttggtgaccggagttgctagtccaga
aggaacagactgagtgagcgaggtccaaaaggggtgatcaatgactggccccgcttcaa
gcagttggagacagagcagagggaggagcagtgccgggagatggagcgctgatcaaaag
ctgtctatgagctgccaggtcccatctg

ECU 15 nucleic acid sequence

cttatggacataaggacacaggggcatcatcaaaggagggaaaagagaaaaaagagaag
cataagacgaagacgccccaacagattgccaaggacatggagcggtgggcccgagtctc
aacaagcaaaagaaacttcaaaacgcttccagcctattagt

ECU 35 nucleic acid sequence

tctatatattcttggctgcctttgaattcactttgtagacgaggctgacctcgaactcaga
acctctggaagagcttaacacat

ECD 26 nucleic acid sequence

tttgtttgtttaaagatcctaacagaacatagcctaacagtactggtcttccagttttatt
catttcaattatgtgtagtgcgctgagcagaaccactgtgtgttacttctttaaagagctg
ctacactttaaatgttcatagctagatggccagcccttcagtgtgtacttgaataatatat

ECU 8 nucleic acid sequence

ctccaatagagacaccagaagcccaaaatagaggctttctcgagggactatttg
agtaaacaaatcgcctaacatgtcctgaatattatcacacatttcttggctatatgttaac
cgttgaattc

Region highlighted by bold print is homologous to that in the captured cDNA fragment below (1.1 kb).

ECU 21 nucleic acid sequence

tgatgtgtctcttgataaagccctcgatgagctgatggatggtgatatcatcgtgtttcag
aaggatgacccggaaaactataacagtgagtagtccgaattc

1.1 kb 'captured' nucleic acid sequence containing region of homology to ECU 8

atgaaggacaggcattataccttgtctccccaactgaaattcaacggttaacatatagcc
aagaaatgtgtgataatattcaggacatgttaggcgatttgtttac**tccaacaatagag**
acaccagaagcccaaaatagaggctttctcaagggactatttggtggaagtggac
agacatttgacagagaagaactctttggggaagcctcagcaggaaaagcatctcgcagc
ctcgcacagcacattcctggaccgggcagcatagaagggatgaagggcgctgctggcgg
ggtgatgggagagctaacccgagcccggattgcacttatgagagagggcagcgtctgggt
gagctggaggagaagactgcaggcatgatgaccagtgcagaagcatttgccaagcacgc
acacgagctaatgctgaaatataaggacaagaaatggtaccagttctaacttttaccttc
taaagaagctgtggtgctctgagaaccagtattcagggaaaccaaattgattcccagcat
cacgattcaactgctagaaccagtttcttctacaagatgtcccatatagtccatctgaagt
tctcgtaaaggagacttctccaaggcagaagcactgcacaacaaaaggctggaccctgg
ttaaaatcccaagagatggccgaacctgtcttttccccacgaggaatgaatattatcatc
ttggcatgtaacttgttaagaattcatgtttggaaactatggggagtccttttgatttgaat
ttcctgtacaaacaaaagcattaatgcgcttaatgaaaattctttgcatgataaattaaca
tcttaagaggaaagaaacaaagcatgttataacagaaggaaaggatttatggtaaactac
tttcacgaattgggccacacccaacaattttaaaatctgcgttagaagatatgagtgcatt
tcaagtgtatttgccctacccaaccgaaggaatcaaaacatacccagattttcttctcat
ctgaaactctccttatatactagaacatactgaattcttacatcaagaatccgattaatgt
ttctctctctctcgtatttcacatcacttacag

Region highlighted by bold print is that which is found in the first half of ECU 8.

700 bp 'captured' nucleic acid sequence containing region of homology to ECU 21.

From 5' end

actgtggacacatctacacacccatatctgtaaaatacgtgacttgctcccggttatgtgt
gacagagcaggatttatccaggatactagccttatctctatgaggaagttgagccgaatt
taacagagagaattcaggacta**tgatgtgtctcttgataaagccctcgatgagct**
gatggatggtgacatcatcgtgtttcagaaggatgacccggaaaacgataac
agtgag

Region highlighted by bold print is that which is identical to ECU 21.

From 3'end

cggactactccacggctttttacattcttctaacaggtcccggacacagccatgcttctc
tgggtatagtgttatttcctcttccctaaattggctgtttaaccatatacacttaaaacttc
tcctgttctcaaagtccgtgattttcatcttaagctgctgatagtaaagtttcttaggttgt
cttggcttgaagaattgtagaagatctcttaaagtaccttcgtaattatgcctaaggggat
taccggggccgtccctataaccttgagacttgaaaaactgaagcaacatggggtcgtgtt
gagcctc