FUNCTIONAL ANALYSIS OF THE ID4 PROTEIN BY CONDITIONAL OVEREXPRESSION

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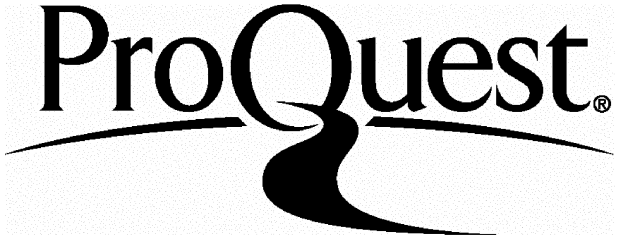

Abstract

The Id4 protein is a member of a subclass of helix-loop-helix (HLH) proteins, which are involved in the regulation of transcription as well as cell growth and differentiation. It has been shown that during mouse development the Id4 is exclusively expressed to the developing nervous system. In adult tissues, Id4 expression is more widespread and most abundant in brain, kidney and testis. The Cre/loxP recombination system is a powerful tool that allows *in vivo* modifications of genes in a tissue/ time restricted way. This thesis will describe the employment of the Cre/loxP system, in order to assess the importance of the Id4 protein in the development and cell lineage specification of the central nervous system *in vivo*. In addition, the same system was used to address the question of functional redundancy of the Id genes by ectopically expressing Id4 in thymocytes *in vivo*

First, mice carrying a dormant Id4 transgene were characterised. In these mice, expression of the Id4 transgene is blocked by transcriptional and translation stop signals (TSS), situated between the promoter and the Id4 gene. The TSS are flanked by loxP sites and can be deleted through Cre/loxP mediated recombination.

To activate the dormant Id4 gene specifically in neuronal cells, mice were generated by Dr Elisa Cinato. These mice express the Cre recombinase in the developing nervous system and adult brain. Mating of the two transgenic lines resulted in mice, which overexpressed the Id4 protein specifically in the nervous system. In these mice, accumulation of neurons and reduction in glial cells in the cerebral cortex was observed. The shift in the neural cells numbers was accompanied by proliferation and apoptosis. These results establish the importance of Id4 protein in neural cell fate determination and differentiation.

Simultaneously, the Id4 transgenic mice were mated with mice expressing the Cre recombinase in thymocyte specific way. The mice generated from these matings overexpressed Id4 in thymocytes. This expression resulted in disrupted thymocyte development and resembled the effect of overexpression of other Id proteins in thymocytes. Since the Id4 is not normally expressed in thymocyte, our results support the idea of functional redundancy of the Id genes.

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ABBREVIATIONS

A	Amber
bp	Base pair
BrdU	5'-Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	Complementary DNA
CNPase	2',3' cyclic nucleotide-3'-phosphodiesterase
DAB	Diaminobenzidine-4-HCl
DEPC	Diethyl pyrocarbonate
ddH_20	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DTT	Dithiothreitol
EDTA	Ethylene-dia mine-tetra-acetic acid
EGTA	Ethylene-glycol-tetra acetic acid
ES cells	Embryonic stem cells
EtOH	Ethanol
FACS	Flow cytometric analysis
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
g	gram
HLH	Helix loop Helix
HRP	Horseradish peroxidase
Id	Inhibitors of differentiation and DNA binding
Ig	Immunoglobulin
IMDM	Iscove's Modified Dulbecco's medium
IPTG	β-D-thiogalactopyranoside
LB	Luria Bertani medium
lck	Leukocyte kinase
lt	Litter
m	Mili-
ì	Micro-
M	Molarity
NSE	Neuron specific enolase
OD	Absorbance (Optic Density)
O/N	Overnight
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFH	Parafolmadehyde
RNase	Ribonuclease
rpm	Rounds per minute
RT-RCP	Reverse transcription followed by PCR
RT	Room temperature

SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SV40	Simian virus 40
Taq DNA Polymerase	DNA Polymerase from *Thermus aquaticus*
TBE	Tris-Borate buffer
TBS	Tris-buffered saline
TCR	T cell receptor
TE	Tris-EDTA buffer
TK	Thymidine kinase
v/v	Volume per volume
w/v	Weight per volume
X-gal	5'-bromo-4'-chloro-3'-indolyl-β-D-galactoside

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I would like to thank my supervisors Professor Fred Sablitzky and Dr Hazel Smith, for all the ideas, support and encouragement, throughout my thesis.

I would also like to express my gratitude to Dr. Elisa Cinato for initiating the project and Dr Owen Williams for all his help and guidance with the FACS analysis. My gratitude also to people in the Windeyer Institute (UCL) and the Institute of Genetics in the University of Nottingham, who are too numerous to mention, for all the help they afforded me during my PhD.

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CHAPTER 1

INTRODUCTION

THE Id PROTEINS

(THE Id4 PROTEIN)

1.1 INTRODUCTION

The Id4 protein is a member of the Id sub-group of the helix-loop-helix (HLH) protein family (Murre, Bain et al. 1994; Massari and Murre 2000). The HLH family contains transcription factors that are involved in regulation of expression of genes implicated in cell growth and differentiation (Norton, Deed et al. 1998 and reference therein). These proteins are characterised by the existence of an HLH domain in their structure.

1.2 MODE OF FUNCTION OF THE Id PROTEINS

Besides the HLH domain, most of the HLH proteins contain an N-terminal conserved region of basic amino acids (bHLH subgroup) (Murre, Bain et al. 1994; Massari and Murre 2000). Based on their expression pattern, these proteins are further divided in the ubiquitously expressed class A and the tissue restricted class B, (Appendix A). Class A proteins (E12, E47, E2-2, and HEB), also known as E proteins, are expressed in many tissues and are capable of forming either homo- or heterodimers (Murre, McCaw et al. 1989). The tissue restricted class B proteins (i.e. MyoD, NeuroD, etc.) are, with few exceptions, incapable of homodimerisation and preferentially heterodimerise with the Class A proteins (Murre, McCaw et al. 1989). The basic regions of the class A/class B heterodimer recognise and bind specific regulatory elements for the transcription of genes involved in differentiation (E box or N box) (Figure 1.1), (Murre, McCaw et al. 1989). Thus, heterodimerisation and DNA binding are essential for the bHLH proteins to exercise their function in the regulation of gene expression.

In contrast to the other HLH proteins, the Id proteins (Ids) lack the DNA binding basic region (Benezra, Davis et al. 1990; Benezra, Davis et al. 1990). They function primarily as dominant negative regulators of the bHLH proteins by preventing them from binding to DNA (Figure1.1). This is accomplished through the formation of non-functional heterodimers with bHLH proteins, mainly of the class A type (Sun,

Copeland et al. 1991). Such heterodimers are incapable of binding DNA and at the same time prevent the functional heterodimerisation of Class A and B proteins. Thus the name Id, which stands for inhibitors of DNA binding and inhibitors of differentiation (Benezra, Davis et al. 1990).

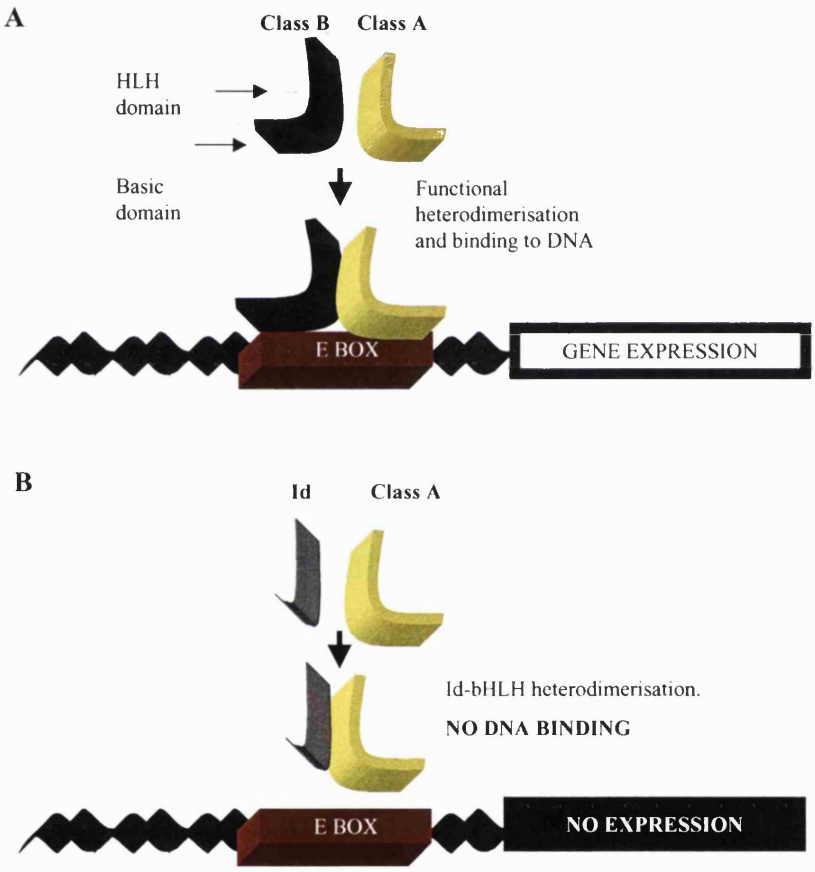

Figure 1. 1: A) Mode of function of bHLH proteins. B) Inhibition of DNA binding by the Id proteins. See text for details.

Although all Ids are capable of binding with all class A proteins, recent data support the idea that individual members may interact preferentially with one or other class A protein partners (Langlands, Yin et al. 1997; Deed, Jasiok et al. 1998). For instance, a specially favoured interaction of Id3 protein with the HEB class A factor was indicated by experiments with HEB deficient mice. Crossing of these mice with Id3, but not Id1, knockout mice could rescue the HEB -/- lethal embryonic phenotype (Barndt, Dai et al. 2000).

Besides the bHLH proteins, a number of other proteins have been identified, as partners of the Ids. The pocket proteins pRB, p107 and p130 for instance, have been found to interact with the Id2 HLH domain (Iavarone, Garg et al. 1994; Lasorella, Iavarone et al. 1996). In contrast, Id1 and Id3 do not exhibit the same interaction. Proteins of the ETS family and the MIDA 1 transcription like factor interact with Id1 (Inoue, Shoji et al. 1999; Yates, Atherton et al. 1999). The Id3 protein has been also shown to interact with proteins of the ETS family (Yates, Atherton et al. 1999).The functional significance of these interactions is still under investigation.

1.3 STRUCTURE OF Id GENES

Besides the Id4, three other mammalian members have been identified so far: Id1, Id2 and Id3. The Id1 was the first to be discovered (Benezra, Davis et al. 1990).

The chromosomal localisation of the Id4 gene in human is in chromosomal location 6p21.3-22 and in mouse in chromosome 13 (Pagliuca, Bartoli et al. 1995; Mantani, Hernandez et al. 1998; van Cruchten, Cinato et al. 1998). The chromosomal localisation of the other Id members in human and mouse are depicted in Appendix A. In addition, homologues in other species, such as *Drosophila melanogaster,* (Ellis, Spann et al. 1990), *Xenopus laevis*, (Wilson and Mohun 1995), *Cynops pyrrhogaster*, (Shimizu-Nishikawa, Tazawa et al. 1999), zebrafish (Sawai and Campos-Ortega 1997) and trout (Rescan 1997), have been documented.

All four mammalian genes share a similar genomic organisation (three exons and similar exon/intron structure), indicative of evolution from a common ancestor (Deed, Hirose et al. 1994). It has been suggested that the common ancestor of the Id gene had a DNA binding region, which was lost during evolution (Atchley and Fitch 1997). Alternative reading frames encoding for proteins of different sizes have been identified for genes Id1 and Id3 (Hara, Yamaguchi et al. 1994; Deed, Jasiok et al. 1996; Nehlin, Hara et al. 1997). These products appear to have attenuated heterodimerisation properties (Deed, Jasiok et al. 1996). Moreover, a possible product from a human Id2 pseudogene has also been reported (Kurabayashi, Jeyaseelan et al. 1993).

The Id4 gene gives rise to four transcripts of different sizes, due to differential

polyadenylation (Riechmann, van Crüchten et al. 1994; van Cruchten, Cinato et al. 1998). These transcripts seem to encode a single protein (van Cruchten, Cinato et al. 1998). Transcription of the Id4 gene is initiated in a region 300 bp up-steam of the start ATG codon (van Cruchten, Cinato et al. 1998). Multiple transcriptional start sites are used probably due to the lack of TATA box and the presence of GC rich regions (van Cruchten, Cinato et al. 1998). The Id4 gene regulatory elements also contain putative binding sites for the SP1 and Erg-1 proteins (van Cruchten, Cinato et al. 1998). In line to this, Pagliuca and co-workers, (1998) have shown that the human Id4 promoter is negatively regulated by proteins of the SP family (Pagliuca, Cannada-Bartoli et al. 1998). These data suggest a functional role for these proteins in Id4 gene regulation.

1.4 STRUCTURE OF THE Id PROTEINS

The Id proteins have relatively a small size of 13-20 KDa. The Id4 protein size is 16 KDa. Apart from the highly conserved HLH domain, they share five other areas of homology (Figure 1.2), (Norton, Deed et al. 1998). These may possibly represent areas of additional functional significance.

The HLH domain consists of two amphipathic helices separated by an intervening loop of variable size. From analysis of a three-dimensional homology model, constructed for the HLH domain of human Id3, it was deduced that Id proteins are able to dimerize without DNA stabilisation (Wibley, Deed et al. 1996). This prediction was also confirmed by *in vitro* protein-binding experiments (Wibley, Deed et al. 1996). In addition, a similar model of the Id1 protein revealed a possible site of charge-charge repulsion in the hypothetical Id1 homodimer. That might explain the preference of Id proteins to form heterodimers rather than homodimers (Wibley, Deed et al. 1996).

The necessity of the HLH domain for dimerisation was illustrated in experiments by Pesce et al (1993). These investigators also established that two aminoacids at the end of the loop are essential for its activity, most likely by defining the heterodimerisation partners (Pesce and Benezra 1993). More recently, the HLH domain was additionally found to be required for the trans-activation activity, observed when Ids were fused to the GAL4 DNA-binding domain (Bounpheng,

Melnikova et al. 1999). The phenomenon was enhanced at the presence of Class A proteins.

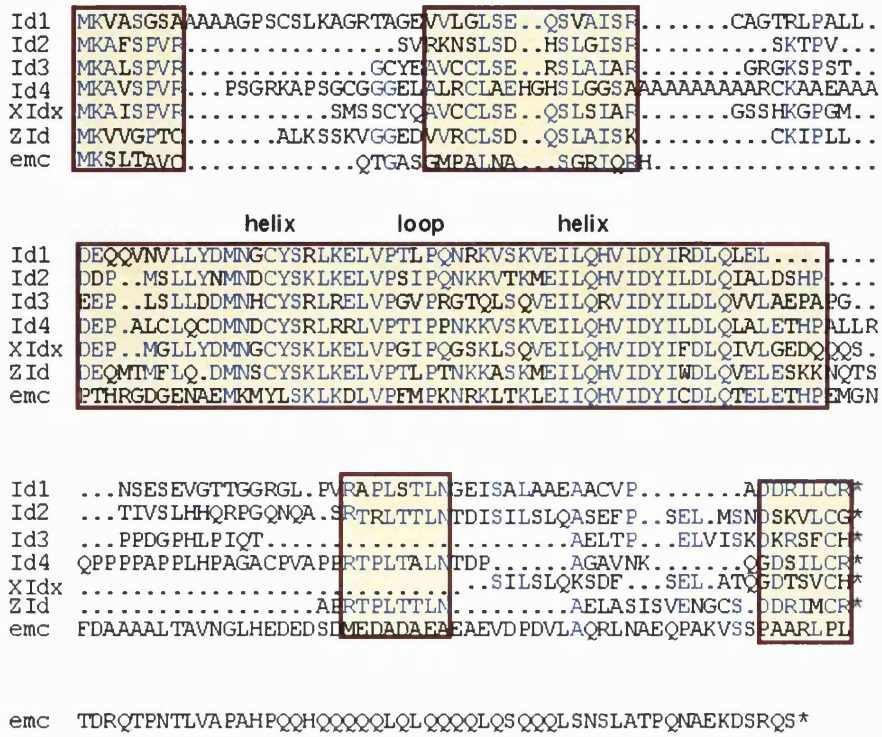

Figure 1.2: Structure of Id proteins. Alignment of predicted aminoacid sequence alignment of Id1, Id2, Id3, Id4, Xenopus (*Xldx*), zebrafish (*ZId*) and *Drosophila* (*emc*) proteins. Identical aminoacids in more than two sequences are shown blue. Areas of similarity are boxed. Photo reproduced from Norton, Deed et al. 1998.

The Id proteins half-life is reported to be between 20 min-1 h (Bounpheng, Dimas et al. 1999). Their intracellular levels, with the exception of the Id4 protein, seem to be regulated by the ubiquitin-proteasome degradation pathway (Bounpheng, Dimas et al. 1999). The half-life is extended by heterodimerisation with bHLH partners (Bounpheng, Dimas et al. 1999).

Potential phosphorylation sites for the cyclin-A-CDK2 and/ or cyclin–E have been identified in position Serine 5 in Id2 and Id3, but not Id1. Phosphorylation at this site for Id2 and Id3 has been reported during the transition of cells from the G1 to S phase, during the cell cycle (Deed, Hara et al. 1997; Hara, Hall et al. 1997)

Finally, in vitro experiments using a truncation version of the Id3 protein revealed a functional significance in the C terminal region for stable expression and Id3 function (Chen, Han et al. 1997).

1.5 EXPRESSION PROFILE OF Id PROTEINS

The Id proteins are widely expressed in a variety of tissues and cells lineages. In general, they are primarily found in proliferating cells, while their expression is down regulated in cells that are undergoing differentiation (reviewed in Norton, Deed et al, 1998). During mouse embryogenesis, in complete contrast to the other Id genes, Id4 is predominantly expressed in the mouse nervous system (Riechmann and Sablitzky 1995; Jen, Manova et al. 1996). In adult mice, the Id4 gene is highly expressed in the brain, kidney and testis (Riechmann, van Crüchten et al. 1994; van Cruchten, Cinato et al. 1998) (Figure 1.3).

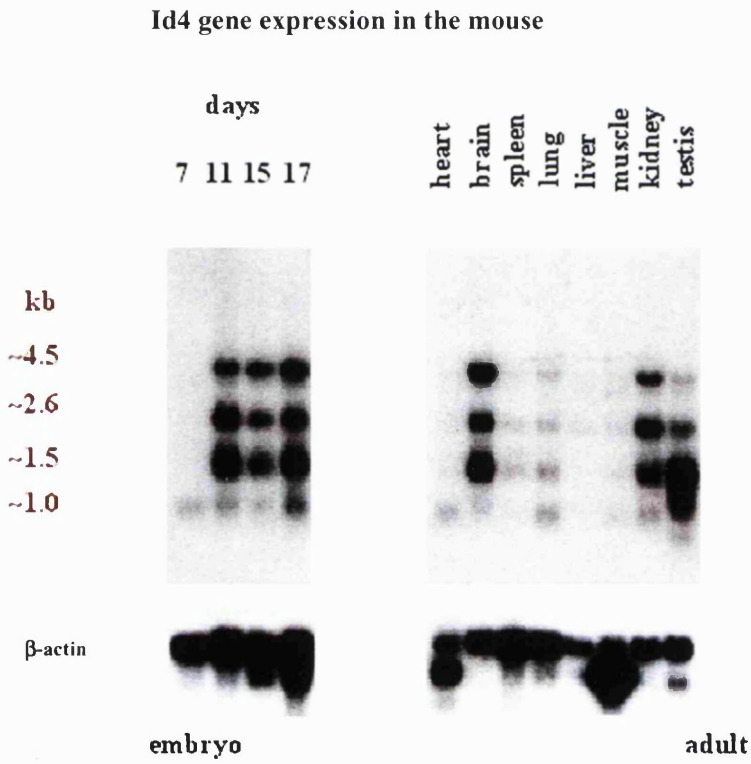

Figure 1.3: Id4 gene expression. A) Expression at different developmental stages, during mouse embryogenesis. B) Expression in murine adult tissues. Four different transcripts were identified by Northern analysis (4.5kb, 2.6kb, 1.5kb and 1kb respectively). The levels of the transcripts were variable between different tissues. PCR with β-actin specific primers was used as control for the quality and the quantity of RNA. Modified photo, from van Cruchten et al. 1998.

In other tissues, Id4 is present at very low levels or absent. Interestingly, no expression of Id4 has been reported in the mouse haematopoietic and lymphoid systems, where the other Id genes are predominately expressed in a stage specific manner (Cooper, Brady et al. 1997). In human, however, medium to low levels of expression have been observed in tissues, such as the spleen and lymph nodes, where mature haematopoietic cells reside (van Cruchten, Cinato et al. 1998).

1.5.1 Expression in the central nervous system

All Id proteins are expressed in the nervous system during mouse development. Each of these has a unique pattern of expression, but as a rule, they are all found in proliferating neural precursors or presumptive neurons (a detailed account of their sites of expression is provided in Table 1.1). Most of the data are based on RNA detection studies, such as Northern and *in situ* RNA hybridisation.

Weak Id4 transcripts appear first around day 9.5 days post coitus, (dpc). In the following days, expression is upregulated and at 12.5 dpc is distinctive of some areas of the brain (An account of the areas of the developing and adult brain is presented in Appendix A). More specifically, Id4 is found in the parietal and frontal ventricular zone of the lateral ventricle, the epithalamus and thalamus, serotonergic nuclear complex, pre-, supra- and post-optic areas and optic recess. Lower levels of expression are also detected in the superior cerebellar penduncle, the pontine flexure, the pyramidal tract and the medulla. Id4 positive signals are also found in the spinal cord, but not in the mesencephalon or the metencephalon (Riechmann, van Crüchten et al. 1994; Riechmann and Sablitzky 1995; Jen, Manova et al. 1996; Jen, Manova et al. 1997).

Later, at day 14.5 the expression pattern is similar to the one at 12.5 dpc. In addition, Id4 expression is now detected in the mesencephalon and metencephalon. The spinal root ganglia and the fifth cranial nerves also show some weak expression.

Table 1.1: Expression pattern of the Id members in the developing and adult mouse central nervous system.

	8.5dpc	12.5dpc	14.5dpc	16.5dpc	P1-P6	Adult
Id1	Neural folds	VZ of forebrain, midbrain, hindbrain and spinal cord. Dorsal root ganglia and trige minal ganglion.	Medial telencephalic wall. Chorioid plexus.	Hippocampus.Chorioid plexus	CA areas of hippocampus. Corpus callosum. Ventricular, subventricular zone.	No expression.
Id2	Neural folds	Neocortex. Septal primordia, infundibulum, Olfactory bulbs, supra-optic area. Diencephalon. Allar and roof plate. Isthmic areas and cerebellar nuclei.	Mantle zone of neocortex. High olfactory bulb. Primordia of cerebellar nuclei and Purkinje cells. Postmitotic neurons in the midbrain and hindbrain.	Cortical plates. Olfactory bulb. Diencephalon. Epithalamus. Purkinje cells.	Distinct layers of the neocortex.	Layers II-III neocortex. Pyramidal cells. Olfactory bulb. Purkinje cells
Id3	Neural folds. Neural groove.	Telencephalic vesicles. Roof and floor plate. Pons. Chorioid plexus. Dorsal thalamus and tectum.	Hippocampal region of forebrain. Floor of midbrain. Metencephalon. Myelencephalon. Neocortex.	No expression.	No expression.	No expression.
Id4	No expression.	Frontal and parietal cortex of tencephalon. Epithalamus. Optic areas. Cerebellar pendulle. Pontine flexure. Pyramidal tract. Medulla and spinal cord.	VZ and cortical plate of the telencephalon. Thalamus. Postmitotic cells in midbrain and hindbrain.	VZ and cortical plate of the telencephalon Thalamus. Epithalamus Brainstem.	Ependyma. Thalamus. Neocortex. Trigeminal ganglion. Purkinje cells of cerebellum.	Cortex Purkinje cells.

Interestingly, Id4 expression is detected not only in proliferating cells, but also in postmitotic nuclei that have initiated differentiation. The expression pattern is similar at later stages (Riechmann, van Crüchten et al. 1994; Riechmann and Sablitzky 1995; Jen, Manova et al. 1996; Jen, Manova et al. 1997).

After birth, at post-natal (P) day 0.5 strong Id4 specific signals are detected in the thalamus, ependyma and trigeminal ganglion. Some expression is also found in the Purkinje cells of the cerebellum and the cortex. In adult brain Purkinje cells of the cerebellum and few cells in the neocortex still express Id4, but the hybridisation signal seems to be lost form the other areas (Andres-Barquin, Hernandez et al. 2000).

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It appears that the Id4 and Id3 genes have mutually exclusive patterns of expression during embryogenesis (Riechmann and Sablitzky 1995). For instance, at stage 12.5 dpc of development Id4, but not Id3, is expressed in the serotonergic nuclear complex, optic areas, cerebellar pendulle, pontine flexure and pyramidal tracts. Conversely, Id3 is expressed in the ventricular zone of the 4^{rth} ventricle, chorioid plexus, the developing pons and tectum, where Id4 is undetectable. Id3 and Id1 are only expressed in proliferating neural precursors. In contrast, Id4 along with Id2 are also expressed in postmitotic presumptive neurons that are differentiating (Jen, Manova et al. 1996). Moreover, Id2 and Id4 are the only proteins still, present in the adult brain (Andres-Barquin, Hernandez et al. 2000).

Recent studies have documented the expression of Id genes in glial cells. Analysis in cultured Schwann cells (glial cell type found in the peripheral nervous system) showed expression of all four Id genes in precursors and mature cells. In the

precursors, Id2 and Id4 were localised in the nuclei, whereas Id1 and Id3 in the cytoplasm. In contrast, in mature cells, only Id4 had nuclear localisation (Stewart, Zoidl et al. 1997; Thatikunta, Qin et al. 1999).

Similarly, Id1-Id4 were all detected in cultured astrocytes (Tzeng and de Vellis 1997). In primary astrocyte cultures derived from different areas of the mouse brain, Id genes are expressed at variable levels. Id4 was highly expressed in cultures generated from forebrain, cerebellum and quadrigeminal bodies (Andres-Barquin, Hernandez et al. 1998). Finally, Id2 and Id4 are expressed in oligodendrocyte precursors. Moreover, Kondo and co-workers, (2000) showed that Id4 expression is present in rat oligodendrocyte precursors, but decreases with cell differentiation (Kondo and Raff 2000).

1.6 FUNCTIONAL ROLE OF Id PROTEINS

1.6.1 Id proteins in development

The first evidence for the role of Id proteins in differentiation came from studies in *Drosophila*. Multiple analysis with gain and loss of function mutations of the *Drosophila* Id homologue, *emc*, revealed its role as a dominant negative regulator of neurogenesis and sex determination (reviewed in Jan et al, 1993; Lee et al, 1997). More precisely, in the sensory organs of *Drosophila*, a very sensitive balance is established between the bHLH proteins encoded by the ubiquitous expressed *daughterless* gene, the specific gene complex *achaete-scute* and the *emc*, for normal neurogenesis to proceed. Low levels of *emc* lead to inappropriate appearance of sensory organs. Overexpression of *emc* on the contrary, prevents interaction between the bHLH proteins and prevents organ formation. A similar situation is involved in sexual differentiation (Murre, Bain et al. 1994).

In mammals, the function of the Id proteins, as inhibitors of differentiation, has been established in a variety of *in vitro* experiments, in which overexpression of any of Id1-Id3 proteins in cultured cells inhibited their differentiation (reviewed in Norton, Deed et al, 1998). Indirect evidence was also derived by experiments with bHLH proteins (Appendix A). E2A deficient mice for example, die before birth, due to a variety of developmental defects (Bain, Engel et al. 1997; Yan, Young et al.

1997). However in E2A/Id1 double knockout mice, there is a rescue in phenotype, suggesting that a partial reason for the defects is increased levels of Id1 protein.

Analysis of mice deficient in any of the Id proteins did not reveal any major developmental defect. This is probably due to functional redundancy between the different Id proteins. Nevertheless, in double knockout mice the situation is different. Id1/Id3 deficient mice are not viable and die at 12.5 dpc of embryogenesis with severe brain haemorrhages (Lyden, Young et al. 1999). These mice show brain developmental defects (discussed more in detail below) and additional problems in vascular formation (angiogenesis). Interestingly, mice bearing only one Id1 allele and none of the Id3 alleles functional ($Id1^{+/-}/Id3^{-/-}$) appeared to have increased endurance in tumour xenografts due to inefficient tumour vascularisation (Lyden, Young et al. 1999).

1.6.2 Id proteins in cell cycle and apoptosis

1.6.2.1 Id proteins in cell cycle

Given the strong relationship between cell proliferation and cell growth, a positive role for Id proteins in cell cycle progression has also been identified (for an overview see Figure 1.4 (Norton and Atherton 1998)). Initial *in vitro* experiments showed a role for Id1, Id2 and Id3 in enhancing progression of cells from G1 to S phase (Barone, Pepperkok et al. 1994; Iavarone, Garg et al. 1994). The Id genes' expression pattern, after mitogen stimulation of cells *in vitro*, indicates an immediate response. Yates et al, 1999, have recently documented that Id proteins are capable of interacting with ternary complex factors (TCFs). This interaction inhibits upregulation by the TCFs of early response genes such as c-fos and erg-1 (Yates, Atherton et al. 1999).

In addition, Id2 *in vitro* expression was able to reverse the inhibition of cellular proliferation and the block in cell cycle progression mediated by the product of the retinoblastoma tumour suppresser gene pRB (Iavarone, Garg et al. 1994). These experiments suggested that the block happened through an interaction between the pocket domain or RB and the HLH domain of Id2. Interactions between Id2 and other pocket proteins (p107 and p130) have also been documented (Lasorella, Iavarone et

al. 1996). Contrary to this, Id1 and Id3 do not seem to interact with any of these proteins (Lasorella, Iavarone et al. 1996).

In parallel, Id1 overexpression in NIH3T3 cells accelerated cell growth by inhibiting E2A regulated expression of p21 (Prabhu, Ignatova et al. 1997). The protein p21 is involved in negative regulation of pRB phosphorylation, which is essential for activation of genes required for progression into S phase.

It seems that phosphorylation of Id proteins, is also involved in this mechanism. Id2 and Id3 are all phosphorylated during late G1/early S phase (Deed, Hara et al. 1997; Hara, Hall et al. 1997). Moreover, phosphorylation mutants of Id3 protein show altered properties for cell cycle progression (Deed, Hara et al. 1997).

In addition, interaction with a novel cell growth promoting protein MIDA1 has been discovered for Id1. It is speculated that MIDA1 may act as a mediator of the growth-promoting function of Id1 (Inoue, Shoji et al. 1999).

Finally, very recent evidence has been provided for the role of Id4 protein as a cellular timer involved in the regulation of timing of cell cycle withdrawal and initiation of differentiation (Kondo and Raff, 2000, see more details below).

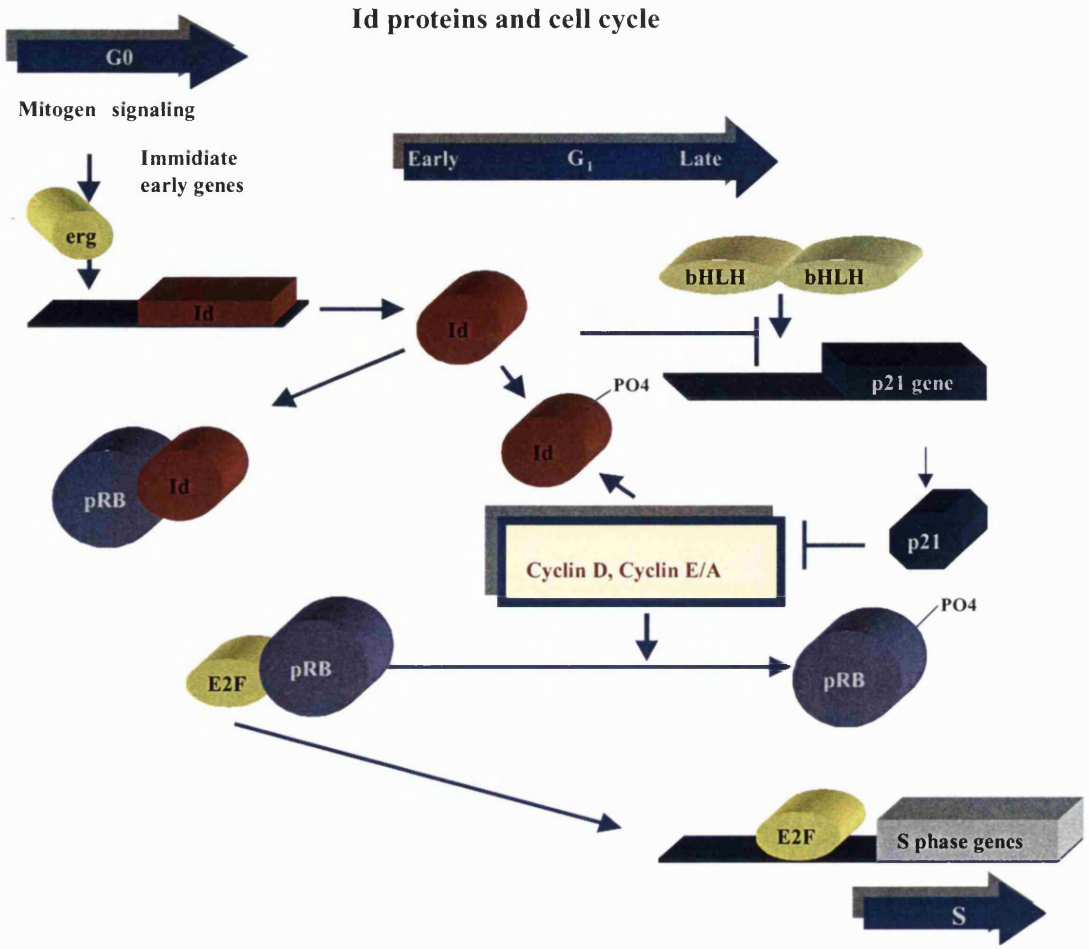

Figure 1. 4: An overview of the putative role of Id proteins in cell cycle control. After mitogenic signalling, early response genes are expressed immediately. These genes positively regulate Id genes expression. The Id proteins, through inhibition of their bHLH partners, prevent expression of other genes, such as the p21 gene. This protein inhibits phosphorylation by cyclin-dependent kinases. Inhibition of p21 results in phosphorylation of the Ids, but also in phosphorylation of the pRB. This results in release of the E2F transcription factor from the E2F/pRB complex and expression of genes that allow progression to the S phase. Co-ordination of this process is also performed in another level by formation of an unphosphorylated pRB and Id complex. Phosphorylation of Id proteins also provides another switch that adds further control in the interaction of Id with bHLH or pocket proteins (i.e. pRB). An overview of genes involved in cell cycle progression is presented in appendix A.

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1.6.2.2 Id proteins and apoptosis

Many independent studies suggest a pro-apoptotic role for the Id proteins (reviewed in Norton, Deed et al, 1998). Atherton et al, 1998 were the first to demonstrate a positive effect of the Id3 protein in cell cycle progression. In particular they showed that ectopic expression of the Id3 gene in cultured C2C12 myoblasts resulted in an enhanced proliferative capacity associated with a delay in exit from the cell cycle (Atherton, Travers et al. 1996). In rat 3Y1 derivative cell lines for instance, enforced expression of any of Id1, Id2 or Id3 also resulted in accumulation of cells in S phase and apoptosis before entrance into G2 phase (Nakajima, Yageta et al. 1998). Increased apoptosis was also observed in neonatal cardiac myocytes after infection with Id1 expressing vector (Tanaka, Pracyk et al. 1998). In contrast, expression of Id1 in endothelial cells, vascular smooth muscle cells, or fibroblasts did not affect the viability of these cells (Tanaka, Pracyk et al. 1998). In addition, thymocyte specific expression of the Id1 protein in mice increased levels of apoptotic cells during T cell development (Kim, Peng et al. 1999). More recently, treatment with trichostatin A (TSA), an inhibitor of histone deacetylases in lung adenocarcinoma cells showed upregulation of mainly Id2 but also Id1 and Id3 genes. The upregulation was associated with increased susceptibility of these cells to programmed cell death (apoptosis) (Eickhoff, Ruller et al. 2000). Interestingly, Id2 pro-apoptotic function has been assigned in an area outside the HLH domain. This area resides in the N-terminal region and is involved in upregulation of pro-apoptotic proteins (Florio, Hernandez et al. 1998).

1.6.3 Id proteins in tumorigenesis

The Id proteins are also involved in tumour formation. Expression of Id genes is highly upregulated in a variety of human cancers, (reviewed in Israel, 1999). Enforced expression of Id genes is capable of immortalising cell lines or inducing them to exhibit tumourigenic phenotype (Alani, Hasskarl et al. 1999; Janatpour, McMaster et al. 2000).

It has already been mentioned that mice deficient for Id3 and with only one functional allele for Id1 exhibit reduced capacity for tumour formation, because of

deficiencies in tumour angiogenesis (Lyden, Young et al. 1999). In line to this, a role was assigned to Id1 in human breast cancer cells (Desprez, Lin et al. 1998; Lin, Parrinello et al. 1999). In these studies, it was observed that aggressive and metastatic breast cancer cells have deregulated Id1 levels. Enforced expression of Id1 enhanced the aggressive phenotype, while treatment with Id1 antisense oligonucleotides (reduction of Id1 levels) reduced the ability of estrogen to stimulate cell proliferation (Lin, Singh et al. 2000). These results strongly suggest a role, for Id1 at least, in tumour progression and development. An association between the Id1, Id2 and Id3 and Bone morphogenic protein 2 (BMP-2) gene expression in the breast cancer cell line MCF-7 has also been reported recently (Clement, Marr et al. 2000).

1.7 SUMMARY

In brief, Id proteins are negative regulators of transcription factors and they are involved in cell growth and differentiation in multiple ways. The Id1, Id2 and Id3 proteins have been studied extensively. However little analysis has been conducted on the Id4. Given its intriguing unique expression in the developing nervous system, it would be of interest to address its role in this system. In addition, it would be constructive to establish if Id4 is also involved in functions where the other Id proteins have been well known to contribute, such as the lymphoid system.

BIOLOGY OF THE NERVOUS AND THE LYMPHOID SYSTEM

1.8 THE NERVOUS SYSTEM

1.8.1 Cell types in the nervous system

The nervous system consists of cells, whose main functional property is the transfer of information between the body and the processing centre: the brain. The specificity in the function of neural cells directs the specificity in their morphology and structure. There are two main types of neural cells; the neurons and the glia. The neurons constitute the basic functional units of the nervous system. They transfer information from and to the brain. The neurons are morphologically a very diverse population.

The glial cells represent the majority of cells in the nervous system (8-10X more than neurons) and their primary function is to provide support and nourishment in the neurons (Van Wynsberghe, Noback et al. 1995). They are further divided in three categories the microglia, the oligodendrocytes and the astrocytes (Compston, Zajicek et al. 1997).

1.8.2 Cell lineage and histogenesis of the nervous system

The origins of most of the cells found in the nervous system can be traced in the multipotential stem cells (Figure 1.5). These cells proliferate very actively and gradually give rise to the neural precursors. These precursors have the capacity to develop in either neuronal or glial progenitor cells (Walsh and Cepko 1990).

The neuronal precursors maturate to give neuroblasts (cells undergoing mitosis, which begin to produce cell processes that will ultimately become axons and dendrites). The earlier neuroblasts are bipolar (contain two processes at the opposite direction). Eventually, they develop many processes (multipolar), which contain accumulations of masses of rough endoplasmatic reticulum in their cytoplasm. At this point, the cells start to make connections with other neurons or organs.

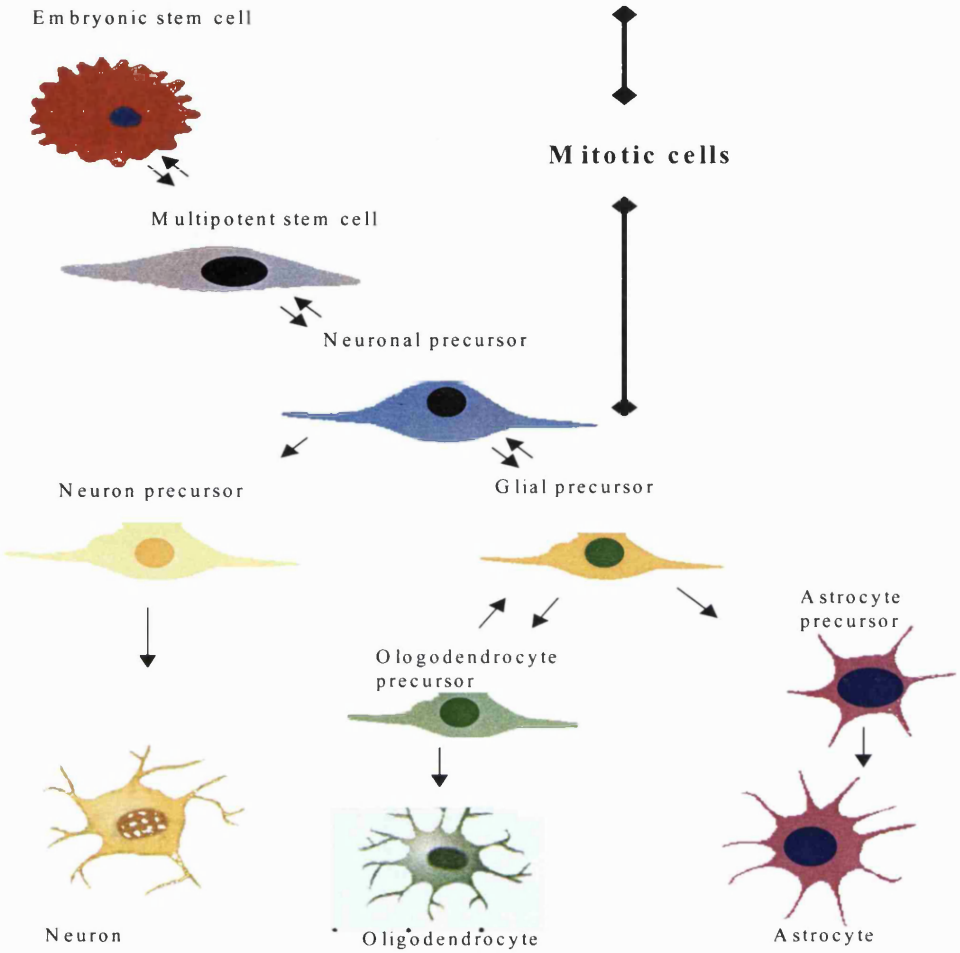


Figure 1.5: Neural cell fate determination. The central nervous system consists mainly of neurons (main functional unit of nervous system) and glial cells (supporting cells with some functional activities). Early precursors cells are proliferating and gradually they cease proliferation and differentiate to become mature neurons or glial cells. In more detail, embryonic stem cells (cells that have the ability to give rise in many lineages) gradually become committed to the neural cell fate (neural precursors). These cells are progressively committed to become either neurons (neuron precursors) or glial cell (glial precursors). The latter, subsequently, give rise to oligodendrocytes and astrocyte precursors, which finally give rise to mature oligodendrocytes and astrocytes. The process is reversible and is heavily influenced by extracellular signals.

The glial progenitors also undergo mitosis and their progeny splits in the oligodendrocytes, the astrocytes and the radial glial precursors (Debbage 1986; Noble, Fok-Seang et al. 1990). Each of these precursors gradually gives rise to oligodendrocytes, astrocytes and special glial cells (e.g Bergmann cells, Muller cells) respectively (Van Wynsberghe, Noback et al. 1995). The microglia do not originate from those glial progenitors, but they are mesodermally derived cells that migrate to the brain through the blood vessels.

1.8.3 Id proteins and the nervous system

Given the interesting expression pattern of Id proteins in the nervous system, many efforts have focused on the elucidation of their functional significance in nervous system development and physiology (reviewed in Andres-Barquin et al, 2000). Most of these studies have focused on the regulation of Id expression in neuronal cell lines. Id2 expression analysis in a variety of neuroblastoma cell lines showed divergent results. For example, Id2 expression was downregulated upon differentiation in NG108 neuroblastoma-glioma cells, whereas no change was detectable in the N18 neuroblastoma or PCC7 teratocarcinoma cells (Neuman, Keen et al. 1993).

In rat PC12 cells, upon induction of differentiation with nerve growth factor (NGF) treatment, all three Id1, Id2 and Id3 gene transcripts initially increased and then decreased (Nagata and Todokoro 1994). When NGF was blocked with the DNA methyltransferase inhibitor 5-azacytidine, no decrease in the Id levels was observed (Persengiev and Kilpatrick 1997). These results strongly suggest not only that Id proteins are downstream targets of NGF during neuronal differentiation, but also that DNA methylation is involved in the process. A role for phosphorylation has also been suggested for modulation of Id function during neuronal differentiation (Nagata, Shoji et al. 1995).

More extensive analysis has been conducted for the Id4 protein's role in glial cell cultures. In differentiating astrocyte cultures, activation of the cAMP-dependent pathway, (a pathway involved in astrocyte differentiation) inhibited expression of the Id4, but not the other Id genes (Andres-Barquin, Hernandez et al. 1999). This strongly

proposes a role for Id4 in astrocyte differentiation. In an astrocyte cell line, overexpression of Id4 resulted in apoptosis (Andres-Barquin, Hernandez et al. 1998). Moreover, levels of Id4 were decreased after astrogliosis by mechanical injury in cultured forebrain astrocytes (Andres-Barquin, Hernandez et al. 1998). In contrast, the other Id levels remained the same.

Recently, light was shed on the role of Id4 in oligodendrocyte differentiation. Levels of Id4 decreased as cells differentiated in culture. In addition, induction of premature differentiation by withdrawal of PDGF (Platelet Derived Growth Factor) resulted in premature decrease in the Id4 levels. Accordingly, overexpression of Id4 inhibited differentiation and promoted cell proliferation. These results indicate that the decrease in Id4 expression is part of a timing mechanism, which determines when the cell will withdraw from cell cycle and start differentiating. (Kondo and Raff 2000).

Despite the extensive analysis of the Id proteins *in vitro*, limited data from *in vivo* models are available. In the avian system, enforced expression of Id2 in ectodermal precursors, (cells that have the potential to give rise epidermal or neural tissues), resulted in increased proliferation. In addition, it acted as switch in ectodermal precursors towards neurogenic fate, rather than becoming epidermal tissue (Martinsen and Bronner-Fraser 1998). Premature neurogenesis was also observed in the Id1/Id3 double knockout mice. In these mice, reduced proliferation of the neural precursors and premature appearance of neuron markers was observed (Lyden, Young et al. 1999).

1.9 THE LYMPHOID SYSTEM

1.9.1 The T cell compartment of the immune system.

The immune system's main role is to protect the organism from pathogens (any organism or component that can cause disease). The immune system responds to the pathogen through a variety of cells. These cells are divided in the lymphoid lineage and the myeloid lineage. Cells of the first category recognise antigens (specific molecules on the pathogens), whereas cells of the latter internalise the pathogens and degrade them. The cells of the lymphoid lineage appear as two major types: the B

cells and the T cells.

The B cells encode cell surface receptors that recognise specific antigens. Once the B cells encounter the antigens, they are activated and start to secrete these receptor molecules (antibodies). The antibodies bind to the antigen and initiate an immune reaction (Janeway and Travers 1996)

The T cells are subdivided in various groups (Janeway and Travers 1996). One group for instance interacts with the B cells and helps them to initiate the immunological reaction (T helper cells). Another group interacts with cells of the myeloid system and assists in the destruction of the pathogens (Janeway and Travers 1996). In addition, a group of T cells (T cytotoxic cells) protects the organism from viruses by destroying the cells that host these pathogens. In all groups, the recognition of the antigen is important for the T cell function. This is achieved by a specific T cell receptor, which associates with surface cell markers on host cells (Class I MHC or Class II MHC cell markers) (Janeway and Travers 1996).

In mammals, T cells maturate in the thymus and then they migrate in organs in the periphery, such as the spleen and the lymph nodes.

1.9.2 Lymphoid system organs

1.9.2.1 Thymus

The thymus is an organ, which consists of two lobes. Each lobe is further divided in smaller lobules, which are separated by connective tissue. Each lobule is divided in the outer cortex and the inner medulla. The cortex is the area, where the early thymocytes precursors reside, whereas the medulla is the area where more mature T cells are found (Janeway and Travers 1996; Roit, Brostoff et al. 1996).

1.9.2.2 Spleen

The spleen consists of distinct areas: the red pulp, where blood cells, macrophages and other cells of myeloid origin lie and the white pulp, which consists of lymphoid cells (Martihn A. Nolte 2000). The white pulp is divided in the germinal follicles, where B cells are found and a central artery that is surrounded mostly by T

cells (periarteriolar sheaths). The whole area is surrounded by a marginal zone, where a mixture of T cells and other types of cells are observed (Martihn A. Nolte 2000).

1.9.3 Thymocyte development

Foetal haematopoietic precursors colonise the foetal thymus between 10.5-11.5 dpc of mouse development (Zuniga-Pflucker and Lenardo 1996). These precursors originate from the foetal liver or of the first wave of multipotential stem cells, which emerge at gestation day 9.5 from the aorta-gonad mesonephros region. For the rest of gestation, the liver is the source of precursors, until around 16.5 dpc when the bone marrow is established as the primary site of haematopoietic activity. From then on and for the rest of adult life all the precursors that colonise the thymus are derived from the bone marrow. At 11.5 dpc in the foetal thymus, the most immature precursors resemble phenotypically and functionally in the Haematopoietic stem cells (HSCs-cells that have the potential to become either myeloid or lymphoid cells). The potential for the myeloid lineage is lost and not detectable by day 12.5-13.5 of gestation. An overview of the lymphoid lineage development is depicted in Figure 1.6.

The multipotential precursor cells are becoming committed to specific cell fate gradually. This process can be monitored by the detection of cell surface proteins that are specifically expressed at certain stages of development (Rothenberg 2000). The early precursors express the CD44 and CD25 markers and are negative for both the CD4 and CD8 markers (DN stage). At the next stage, the cells become double positive for both the CD4 and CD8 (DP stage).

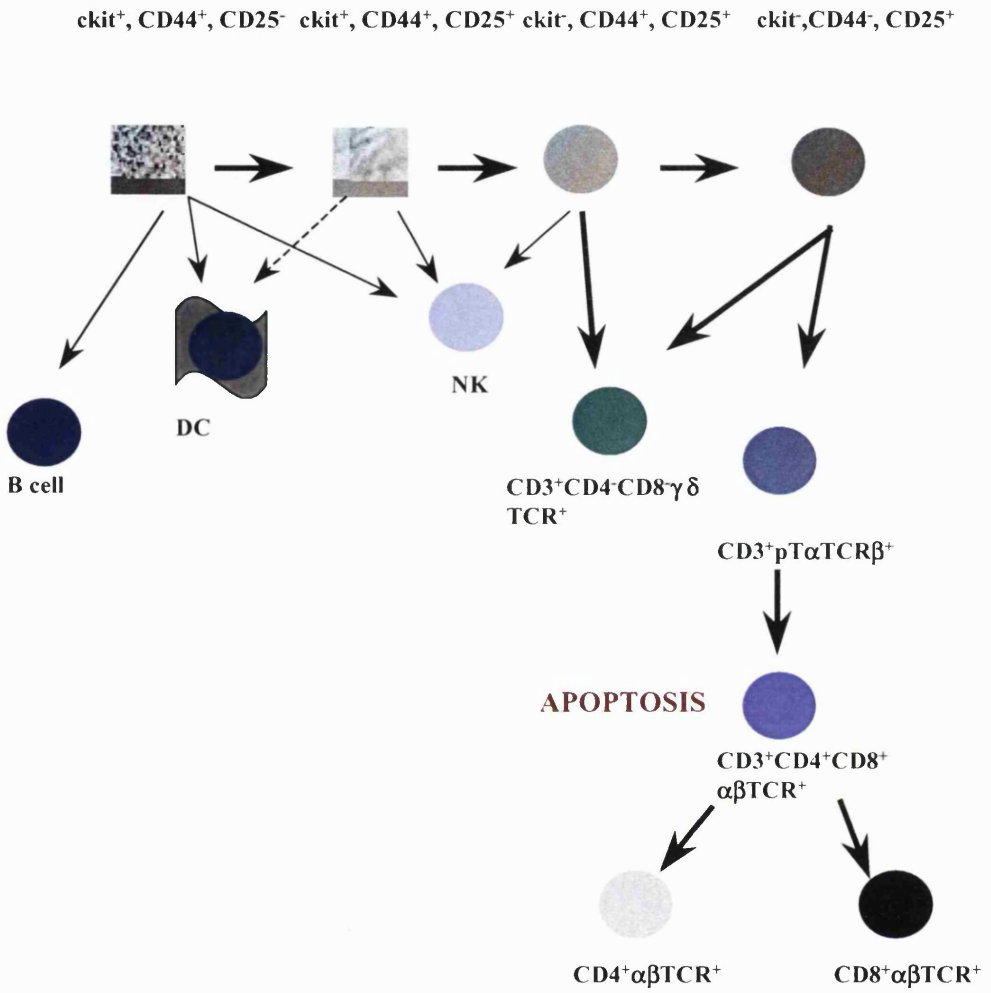


Figure 1.6: Thymocyte development. For details see text.

The productive rearrangement and expression of the T cell receptor (TCR) chains are essential for further maturation and differentiation of these committed precursors. The mature T cells are expressing either the αβ TCR or the γδ TCR markers (MacDonald and Wilson 1998).

In mice, the γδ TCR^+ cells are detected at day 14.5 of development. The majority of γδ TCR^+ cells are DN, except a small subset that is $CD8^+$. The ãä version of the TCR arise as follows: Allelic exclusion occurs for both chains, so that mice expressing rearranged ã and ä transgenes do not rearrange any further the

corresponding gene segments (MacDonald and Wilson 1998). Presumably progenitor cells split into two lineages, depending on whether the γ silencer is switched on or not. In mouse unlike the human the γδ TCR^+ cells predominate in association with epithelial tissue (Roit, Brostoff et al. 1996). The first wave of foetal γδ TCR^+ cells expresses the same V genes and colonises the skin. The second wave uses the same δ gene combination but different γ V-J pair and they seed the female reproductive organs. In the adult, there is more receptor diversity and they colonise the intestine, spleen, lymph nodes and blood (Roit, Brostoff et al. 1996).

The αβ TCR^+ cells begin to appear just before birth (18.5 dpc). For the αβ receptor, the development occurs as follows: The Vβ is first rearranged in the DN cells and associates as β homodimer with the CD3 cell marker. These cells are positively being selected in contrary with the unrearranged prothymocytes. Then the cells are driven to the double positive stage, and another round of selection takes place, until finally the mature αβ TCR^+ arise (MacDonald and Wilson 1998).

1.9.4 Id proteins and lymphoid lineage specification

An extensive analysis of the Id1, Id2 and Id3 proteins role in thymocyte and B cell development has been conducted.

Mice overexpressing Id1 specifically in the B cell compartment appear to have a developmental stop at an early stage (Sun 1994). In human B cells, overexpression of Id3, using a retroviral vector system in foetal liver precursors, inhibited the precursors to become B cells, but not myeloid cells (Jaleco, Stegmann et al. 1999). Moreover, Id3 expressing precursors exhibited a tendency to become NK cells when cultured in appropriate conditions (Jaleco, Stegmann et al. 1999).

B cell deficiency was also observed in the Id3 knockout mice. These mice showed impaired humoral immune responses, mainly due to reduced proliferation and limited activation of B cells after stimulation (Pan, Sato et al. 1999).

The Id proteins are heavily implicated in T cell differentiation and lineage specification. Overexpression of Id3 in bipotential T/NK human precursors for instance, influenced their lineage specification capacity (Heemskerk, Blom et al.

1997). As a rule, these cells primarily become T cells in the human thymus. However, overexpression of Id3 blocked their development into T cells and favoured development into NK cells. In addition, overexpression of Id3 at later stages of development influenced the development of the αβ TCR^+ cells (Blom, Heemskerk et al. 1999).

Mice overexpressing the Id1 protein specifically in thymocytes as well appeared to have reduced numbers of mature thymocytes, due to massive apoptosis during development (Kim, Peng et al. 1999). Despite the apoptosis, these mice developed T cell lymphomas later in life (Kim, Peng et al. 1999). Lymphomas also appeared in mice that overexpressed Id2 specifically in the thymocytes (Morrow, Mayer et al. 1999). An early developmental block at stage $CD4^-CD8^+TCR^-$ was observed in these mice (Morrow, Mayer et al. 1999). Id2 is also implicated in NK cells development. Id2 deficient mice had reduced numbers of NK cells (Yokota, Mansouri et al. 1999). This is probably due to a role of Id2 in NK precursor cells differentiation (Yokota, Mansouri et al. 1999).

The above data are also supported by analysis of the Id major partners, bHLH gene products, in B and T cells. For example, mice lacking the E47 bHLH protein had more mature $CD4^+$ and $CD8^+$ T cells (Bain, Quong et al. 1999). A similar result was obtained by overexpression of its inhibitor, Id3. (Bain, Quong et al. 1999).

All these findings collectively, establish a role for Id1, Id2, and Id3 in lymphoid system development and lineage commitment. It is speculated that the three have a partially overlapping but complementary role during lymphopoiesis. Whether Id4 has a similar role remains to be tested.

CRE-LOXP RECOMBINATION SYSTEM

1.10 INTRODUCTION

The potential to effectively manipulate the mouse genome, by introducing loss or gain of function mutations, is the basis of current efforts to gain insight into the function of mammalian genes. In the past, assignment of gene function was based on defective phenotypes and random discovery of mutations (phenotype-based: phenotype- discovery of gene and mutation- assignment of gene function). For at least a decade however, gene targeting and transgenesis technology transformed this process by permitting the production of irreversible changes in almost any gene desired. Nowadays, the favoured approach for functional gene analysis is gene based (identification of gene- introduction of mutation- phenotype- assignment of gene function). This technology together with other advancements, such as the Mouse Genome Project, provide a powerful tool for better understanding of the mammalian genetic make up and function.

Indeed, there are many examples where gene targeting and transgenesis have assisted in elucidating the role of genes involved in a variety of developmental and physiological processes (Mullins, Morley et al. 1996; Mayford, Mansuy et al. 1997; Gao, Kemper et al. 1999; Muller 1999). Despite their importance however, these approaches are not ideal yet. One drawback for example, is that the introduced mutations occur throughout ontogeny (formation of an organism) and in every tissue. This denotes that compensation with other genes during development may hide the gene's effect and lead to an unaltered phenotype. Alternatively, changes in the regulation of other genes are likely to give a misleading or too complex phenotype. Mutation of a gene throughout development may as well cause embryonic lethality and thus analysis of the effect of the mutation at later stages of development can not be performed.

It has been made clear through the years therefore, that a more strict temporal and/ or spatial control over the introduction of mutations is necessary for an in depth analysis of gene function. One possibility for further manipulation of the introduced DNA is by including targets for site specific recombinases in the experimental strategy. So far, two recombinases have been used successfully for conditional

mutagenesis of genes. These are the FLP recombinase from the yeast *Saccaromyces cerevisiae* and the Cre recombinase from the bacteriophage P1 (Abremski and Hoess 1984). Cre recombinase is more efficient than FLP *in vivo*, consequently is more widely used for genetic manipulation in animal models.

1.11 CRE RECOMBINASE

1.11.1 Properties and molecular structure

Cre recombinase is a 35-38 kDa bacteriophage P1 encoded protein (Abremski, Hoess et al. 1983; Abremski and Hoess 1984). It belongs to the integrase protein family, which consists of site-specific recombinases, involved in the integration and excision of the bacteriophage genome during its life cycle. In particular, Cre recombinases natural function is to disengage bacteriophage dimmers following replication and thus to maintain the phage genome as a monomeric plasmid during the lysogenic state (Sternberg, Hamilton et al. 1981; Abremski, Hoess et al. 1983).

The Cre recombinase protein contains two primarily helical domains (Guo, Gopaul et al. 1997). The N-terminal domain consists of five helical segments connected by a short loop. On the other hand, the C terminal domain forms a β sheet packed against an area of nine helical domains (Guo, Gopaul et al. 1997).

1.11.2 Recombination mechanism

The Cre recombinase recognises repeats of a 34 bp DNA sequence, called loxP sites (Hoess, Ziese et al. 1982). Each loxP site consists of two 13 bp palindromic sequences separated by a core spacer sequence of 8 bp (Figure 1.7 C). The inverted repeats comprise the recombinase binding sites, whereas the spacer region is where cleavage and ligation occur (Hoess, Ziese et al. 1982; Hoess and Abremski 1984). An important feature of the loxP sites is that they are relatively small (thus easy to manipulate) and at the same time long enough to be unique. The possibility of random occurrence of 34bp specific repeats in the eukaryotic genome is very unlikely (10^{-18}). Some cryptic loxP sites that recombine with low efficiency have been reported to exist in the yeast and human genome (Sauer 1992; Thyagarajan, Guimaraes et al. 2000), but no such sites have been found in the mouse. This ensures that Cre mediated

recombination occurs only, if the appropriate recognition loxP sequences are present.

The asymmetry of the spacer region confers directionality in the loxP sites. This results in a variety of recombinant products (Figure 1.7 A and B). If a DNA fragment is flanked *in cis*, by two loxP sites in the same orientation, Cre excises that segment of DNA, leaving a single loxP site behind (Hamilton and Abremski 1984). The excised DNA is degraded. In the reverse case, where the DNA is flanked by two loxP sites in the opposite orientation, Cre recombination causes inversion of the flanked DNA. Intramolecular interactions resulting in chromosome loss, duplications, or translocations are also possible, when the loxP sites are located on different chromosomes.

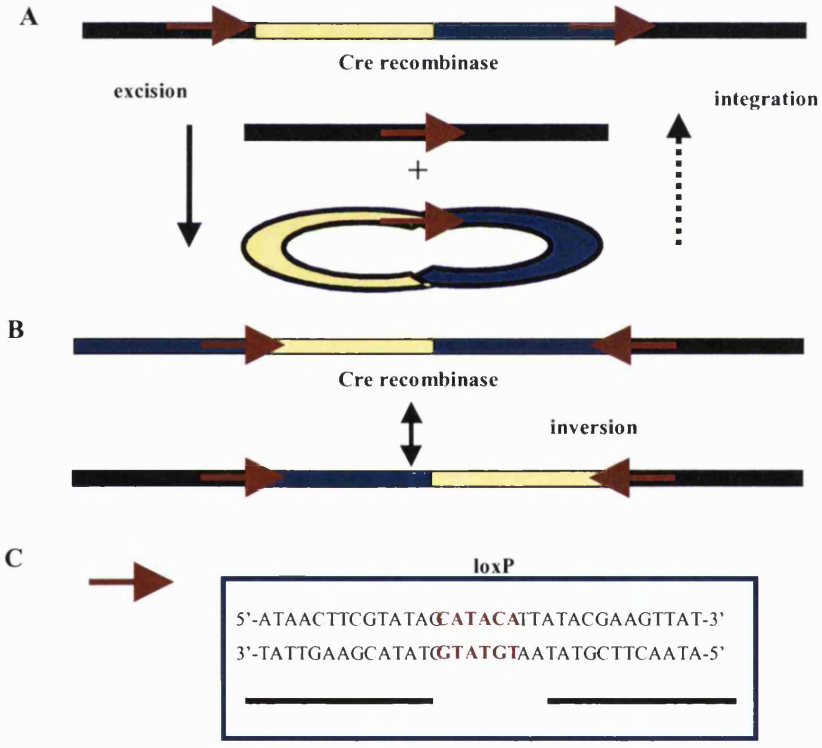


Figure 1.7: Cre mediated recombination. A) Excision and integration. B) Inversion. C) LoxP site sequence. The Cre protein binding sites are underlined. The 6bp-crossover region is indicated by the red bold font. The loxP sites are represented by the red arrows.

In all cases, the reaction takes place with no ATP or topoisomerase activity, or any other additional cofactors or sequence elements (Abremski et al, 1984). In addition, the reaction is independent of the mitotic stage of the cells, since Cre mediated recombination has been shown to occur in highly differentiated postmitotic cells (Adams, Bliska et al. 1992; Agah, Frenkel et al. 1997).

Recently, new information has been obtained about the molecular mechanism of the Cre mediated recombination reaction (Figure 1.8). According to the proposed model, Cre recombinase binds to the recognition sites by forming a C shaped clamp around the DNA duplex (Gopaul and Duyne 1999). Two molecules of Cre protein are bound per loxP site (each of them contacts 13 bp the palindromic sequence and 2 bp of the core region) (Mack, Sauer et al. 1992). Both the N terminal and the C terminal are involved in the binding, while there is supportive evidence that the DNA substrate also contributes to the mechanistic course of the reaction (Guo, Gopaul et al. 1999) (Lee and Saito 1998).

These Cre/DNA arm complexes form a pseudo-fourfold symmetric tetramer, through an extensive network of protein-protein interactions. The site-specific recombination takes place by stepwise cleavage and exchange of each strand in the DNA substrate. At first, conserved tyrosine residues from the protein, cleave the DNA by forming transient 3' phospho-tyrosine linkages and creating free 5' hydroxyl groups. Intermolecular attack from these 5' hydroxyl groups to the phospho-tyrosine bonds results in the transient formation of a Holliday Junction and the exchange of the first pair of DNA strands. Another round of the same process generated the complete recombinant products (Gopaul, Guo et al. 1998; Gopaul and Duyne 1999).

Further support for this mechanism is provided by studies in other integrases (Guo, Gopaul et al. 1999). However, some aspects of the model have not yet been fully elucidated. For example, it is not yet well established, whether the Cre molecule that donates the aminoacid is bound immediately adjacent to the site of cleavage (*cis*) or the tyrosine donor lies somewhere else in the synaptic complex (*trans*).

So far, evidence from two independent investigators supports both models (Guo, Gopaul et al. 1997; Shaikh and Sadowski 1997).

Extensive analysis by *in vitro* and *in vivo* studies has shown that Cre can cause recombination in a variety of cell types, such as granulocytes (Clausen, Burkhardt et al. 1999), mammary gland (Selbert, Bentley et al. 1998), neuronal cells (Feltri, D'Antonio et al. 1999), suggesting that probably all cell types can support Cre recombination. In addition, several animal models have proved the effectiveness of Cre mediated recombination *in vivo* (Lakso, Sauer et al. 1992; Orban, Chui et al. 1992; Rossant and Nagy 1995; Kuhn 1997; Drago, Padungchaichot et al. 1998; Jerecic, Single et al. 1999; Rossant and McMahon 1999; Liu, Yakar et al. 2000; Nagy 2000). These results, along with the simplicity of the Cre loxP recombination system have led to its wide use in experimental strategies calling for controlled genetic manipulations.

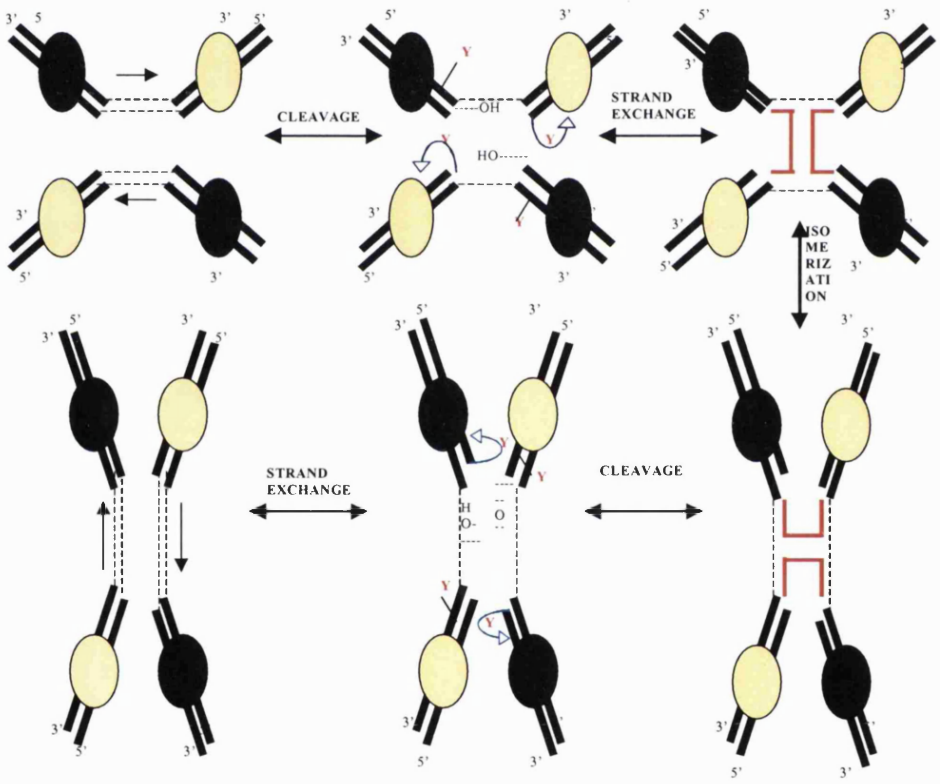


Figure 1.8: Cre loxP-site recombination mechanism. Four recombinases are bound in the loxP sites. Conserved tyrosine residues from two recombinase molecules cleave the DNA, releasing 5'-hydroxyl ends. These are subjected to nucleophilic attacks from the tyrosine residues and one pair of strands is being exchanged. The same round of events with the other two Cre units results in the recombination event

1.12 CRE RECOMBINATION SYSTEM APPLICATIONS

1.12.1 Gene targeting

One of the most widespread uses of the site-specific recombination system is in gene targeting strategies. Using homologous recombination specific, genes are targeted in embryonic stem (ES) cells. These cells can later be used for the generation of mice lacking the gene targeted (gene knockout) (Muller 1999).

In addition to the alteration introduced in the genome, it is essential for a selectable marker gene to be co-introduced. The product of the marker gene is used for the identification of the targeted ES cells. However, this marker gene can and often does interfere with gene expression of the targeted gene, as well as with neighbouring genes (Fiering, Epner et al. 1995; Fiering, Bender et al. 1999). It has been reported for instance, that introduction of a neomycin resistance gene (neo), as a selection marker in a knockout strategy, reduced the expression of the targeted gene at variable levels (Nagy, Moens et al. 1998). This usually means that hypomorphic alleles of the targeted gene are created, which do not abolish its expression completely, as required. Cre recombinase can help to overcome this problem. All that is required is the incorporation of loxP sites flaking the selectable marker in the targeting vector. After incorporation of the vector in ES cells and selection of the targeted clones, transient expression of Cre protein removes the marker leaving a loxP site behind (Figure 1.9 A). This loxP site does not seem to have any effect in the expression of the gene of interest or its neighbours.

Simultaneously, this method permits the use of the hypomorphic alleles, generated by the presence of the marker, in a constructive way. Using different breeding schemes and expression of the Cre recombinase, mice bearing a combination of such an hypomorphic allele with null or wild type allele can give rise to lines with variable levels of the targeted expression. The importance of such an analysis is exemplified in the case of N-myc gene (Nagy, Moens et al. 1998). Null mutations of this gene are embryonic lethal. By employing the above strategy, Nagy and co-workers managed to generate mice with reduced levels of expression of N-myc. These mice were viable and their analysis revealed a role of N–myc in the development of a variety of tissues, such as the lung, heart and skeleton. Furthermore, in mice carrying

the hypomorphic alleles Cre expression could excise the neo marker, reconstituting normal levels of gene expression. This conversion of the mutant phenotype to the wild type holds further promise for use in a Cre mediated gene repair strategy, where a gene is mutated and corrected *in situ*. Such an approach would expand the potentials for the analysis of complex multiple gene functions.

Still, the most promising use of the Cre/loxP system at present lies in the generation of conditional knockout mice (Rajewsky, Gu et al. 1996; Rossant and McMahon 1999). These are mice that lack a certain gene, only in a specific tissue or cell line and at certain time during development or adult life. The strategy relies on the concept of flanking the gene of interest or essential part of it with loxP sites (Figure 1.9 B). The integration of loxP sites should not influence gene expression and mice carrying the floxed (flanked by loxP sites) allele should appear to be normal. Expression of the Cre recombinase in a site and time restricted way results in the inactivation of the gene at a site and temporal controlled way.

In a refined development of this approach, both the gene and the selection marker gene are flanked by loxP sites. Cre expression and selection of the appropriate recombination event gives rise to the proper mutation. Although this idea appears to be simple, there are many complications especially, in regard to achieving the appropriate Cre recombination events. Recently, along with the advances in the FLP recombinase (Buchholz, Angrand et al. 1998; Buchholz, Ringrose et al. 1996), the favoured strategy is to use a combination of FLP and Cre recombinase. More precisely, FLP recombinase can be used for the removal of the selection marker and Cre recombinase targeted to a specific tissue for the generation of the conditional mutation (Rodriguez, Bucholz et al. 2000). Such a complex strategy was applied successfully for the analysis of the FGF8 (Meyers, Lewandoski et al. 1998). A transgenic line was created (allogenic) carrying an hypomorphic allele, which could give rise to the null mutation, by crossing with Cre transgenic animals. At the same time crossing with FLP expressing mice removed the selection marker and converted the hypomorphic allele to wild type.

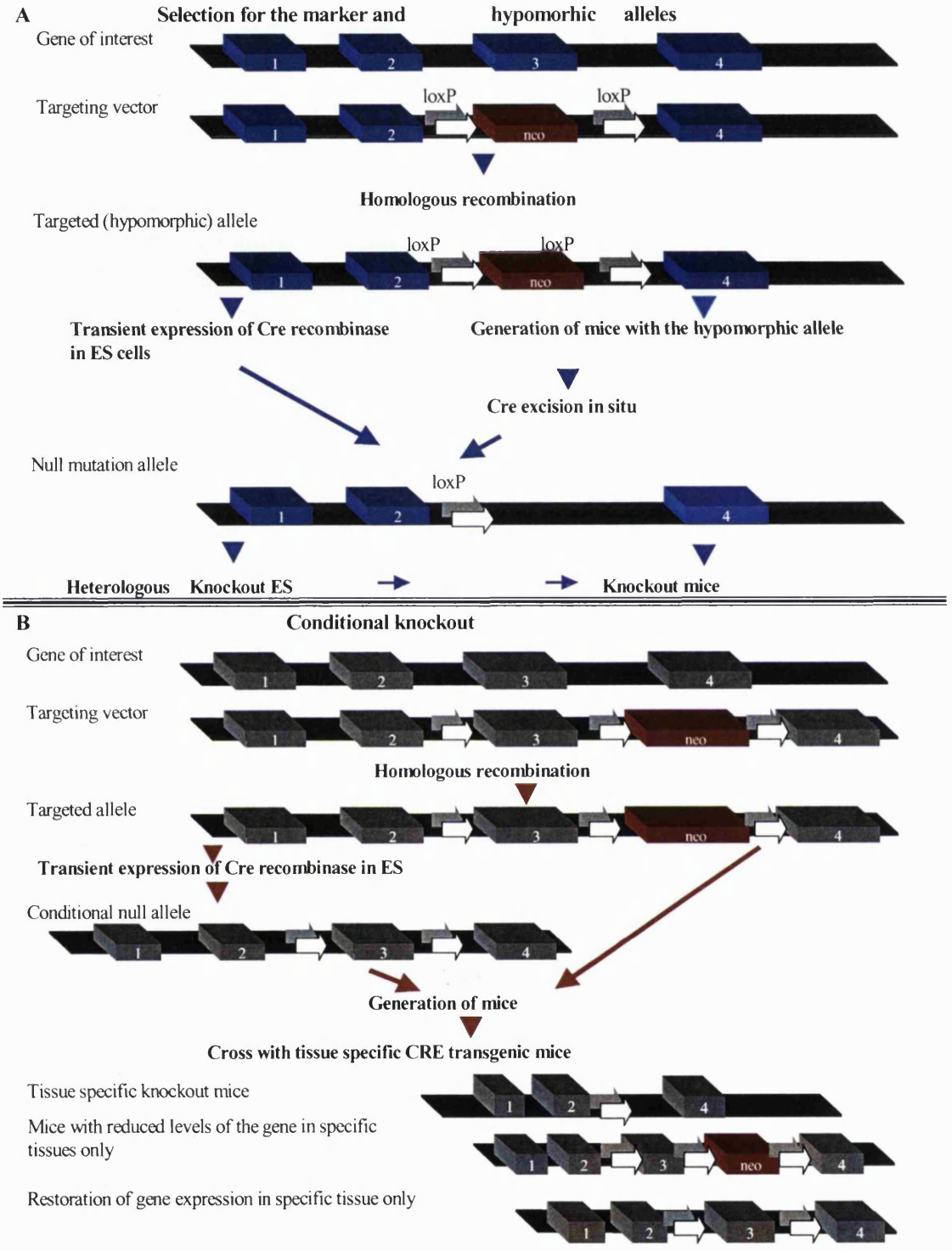


Figure 1.9: A) Strategy for excision of the selectable marker and/or generation of hypomorphic alleles. B) Strategy for conditional gene targeting. See text for details.

1.12.2 Chromosomal alterations.

Cre mediated recombination has also been successfully employed for the generation of large chromosomal deletions and inversions (Metzger and Feil 1999; Ramirez-Solis, Liu et al. 1995). Since the frequency of the recombination is reduced in this case, selection for the recombinant product is essential. The loxP sites are usually introduced in Hrpt deficient lines and restoration of the Hrpt expression can be achieved by Cre expression and the generation of the appropriate recombination event (Figure 1.10 A). In a similar approach, Cre mediated inversion was used to assess the process of mature B cell selection (Lam and Rajewsky 1998). Using a targeting vector and Cre recombinase, B cells were engineered to switch on/off two different types of B antigen receptors. Depending on the receptor expressed the cells were committed to different B cell sublineages.

The Cre loxP system can also be used for the generation of duplications and deletions by trans-allelic chromosomal recombination (Figure 1.10 B). In this case, the loxP sites are located *in trans* within the same genetic locus (Medberry, Dale et al. 1995);(Herault, Rassoulzadegan et al. 1998). Chromosome pairing and expression of Cre recombinase during meiosis will generate two novel alleles; one carrying a duplication and the other a deletion of the gene. This strategy (named TAMERE for trans-allelic targeted meiotic recombination) is exemplified in the analysis of Hox12 locus by Yann Herault and co-workers (Herault, Rassoulzadegan et al. 1998).

Another area where Cre recombinase is employed is the site-specific integration of genes. Intermolecular recombination between a circular DNA carrying a loxP site and a loxP site bearing chromosome, can result in the integration of DNA at this site (Feng, Seibler et al. 1999). This integration is not very stable due to persistent Cre expression, which results in the excision of DNA. The development and use though, of different mutant loxP sites has proved to eliminate this possibility (Araki 1997) (Figure 1.10 C). Bearing also in mind that this integration can be restricted through Cre expression in specific tissues/cell lineages, it seems that this approach holds a lot of promise for the future.

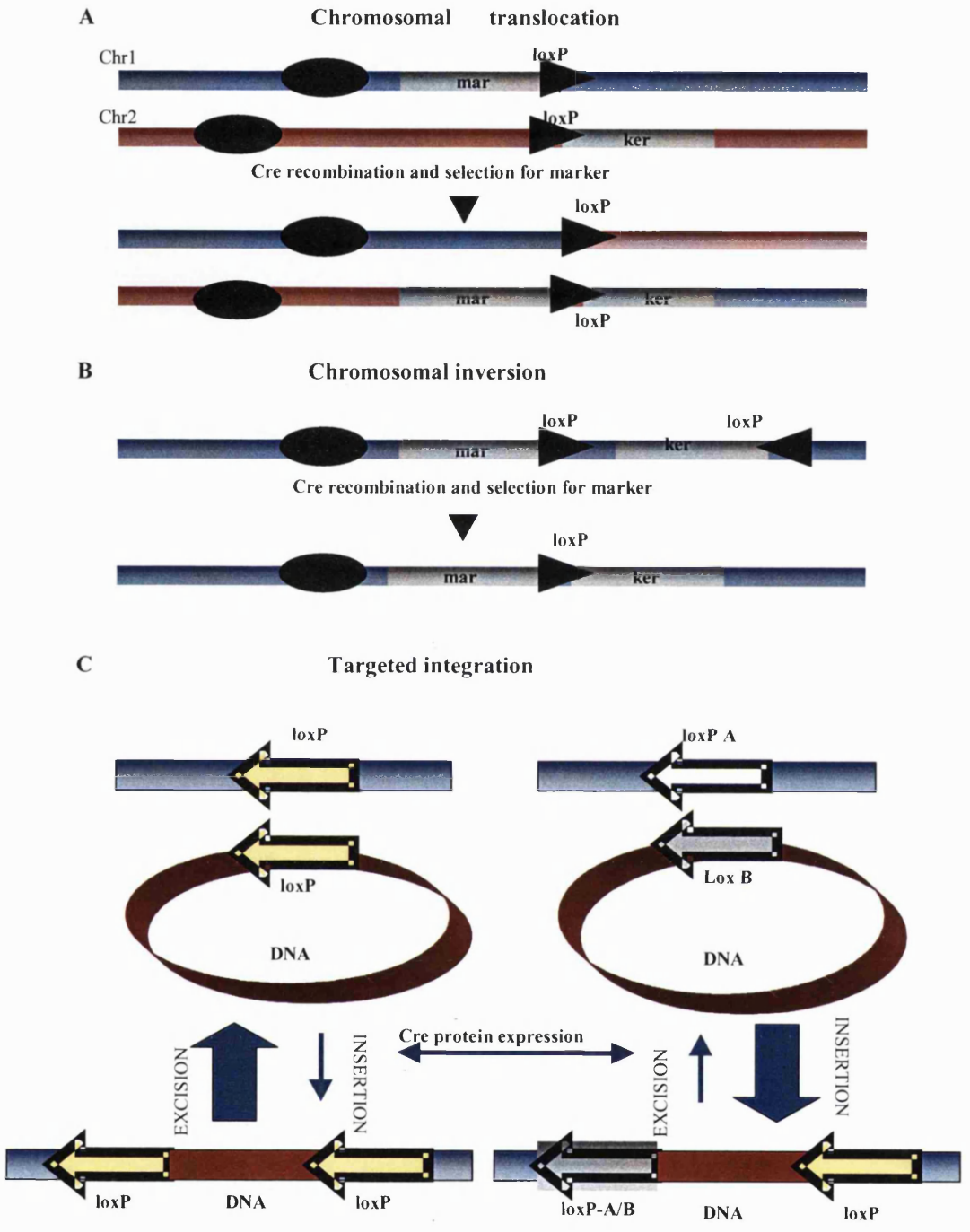


Figure 1.10: Schematic of the chromosomal modifications possible by employment of the Cre/loxP system. A) Translocation. B) Inversion. C) Integration.

1.12.3 Conditional transgenesis

Transgenesis is another area where the Cre recombinase has proved to be very useful. Traditional transgenesis, through the insertion of a gene early in the zygote by microinjection, has the drawback that limited control can be exercised over when and in which cells the exogenous gene is expressed. Conditional expression of transgenes using the Cre/loxP system is based on the idea of transgene expression being dependent on Cre mediated recombination. If Cre recombinase expression is under spatial and temporal control, then the same control is acted upon transgene activation. The binary systems of transgenes required for such a strategy is shown in Figure 1.11.

A promoter drives expression of the gene of interest, only after Cre mediated deletion has excised a transcription and/or translation STOP cassette. If there is no Cre expression, the transgene is not expressed (dormant state of the transgene). Usually Cre recombinase is under the control of a lineage cell specific or inducible promoter. The transgene can be under the control of a specific or ubiquitous promoter.

The latter option has the advantage that by crossing mice with the dormant transgene with a panel of different tissue restricted Cre transgenes, extensive analysis of the gene of interest in many tissues can be performed. The first example of such a strategy is exemplified in the experiments of Lasko and colleagues, (Lakso, Sauer et al. 1992), which achieved overexpression of an oncogene specifically in the mouse lens. Since then, other studies have also shown the effectiveness of this approach (Isaka, Ishibashi et al. 1999; Scott, Marples et al. 2000). For instance, this method has been efficiently used to address questions about cell lineage connections and cell fate determination. By Cre/loxP mediated excision of a STOP cassette, Yamauchi et al, (1999), expressed a β-galactosidase gene in neural crest cells (multipotential cell precursors). Cells that express this gene were stained blue, when treated with X-gal solution. In this way, cell populations, which had been derived from the marked neural crest cells, were identified at later stages of development (Yamauchi, Abe et al. 1999).

Furthermore, this approach has led to the generation of transgenic mice that express a marker gene, such as the β-galactosidase or the green fluorescent protein,

after Cre mediated recombination, (reporter strains) (Kawamoto, Niwa et al. 2000); (Lobe, Koop et al. 1999; Mao, Fujiwara et al. 1999). These reporter mice, apart from cell specification experiments, facilitate the quick and efficient analysis of the Cre transgenic mice expression patterns.

The Cre recombinase lines available to date cover a wide spectrum of tissues/ cell lineages offering a powerful means for controlled transgenesis. Further improvements of the system are still required however.

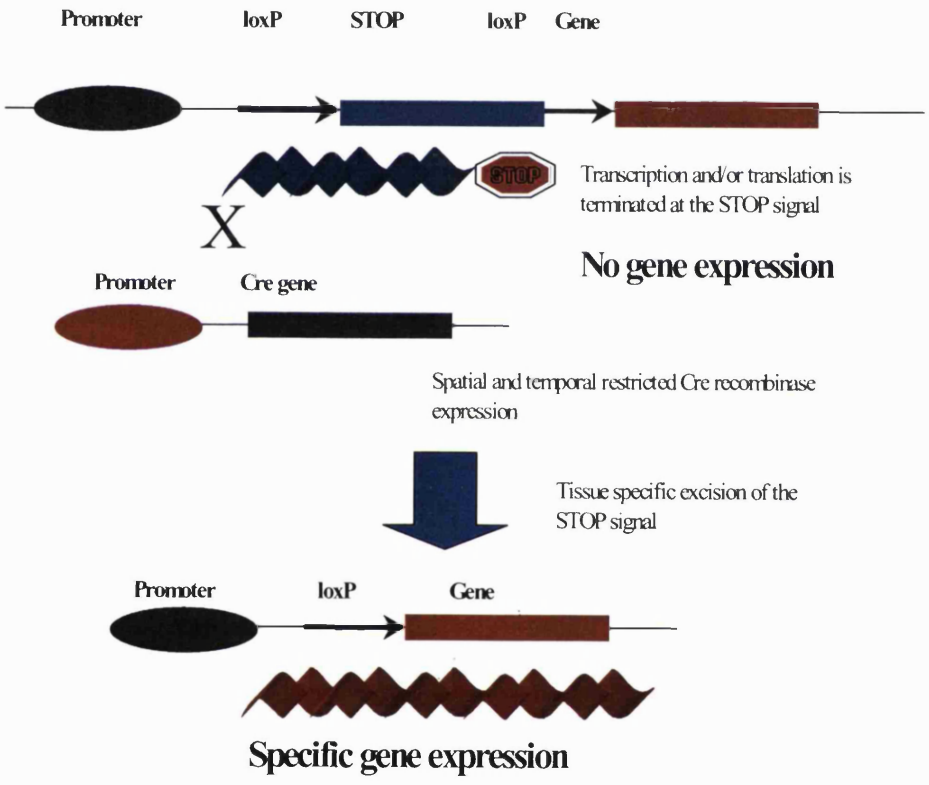


Figure 1.11: Conditional transgenesis. Expression of a dormant transgene under a ubiquitous promoter can be specifically activated upon crossing with mice, which show targeted expression of the Cre recombinase in a certain tissue/cell lineage. Cre specificity can be achieved by using transcription elements, which restrict expression in the area of interest.

1.13 Future directions

Depending on the strategy and the specific requirements of an experiment, there are many ways of introducing Cre recombinase into cells (in vitro electroporation or calcium precipitation, in vivo transgenic line expressing Cre, pronuclear expression of Cre expression vector, injection of Cre RNA and protein. retrovirus or adenovirus delivery systems). Each of these methods has advantages and disadvantages. However, in most of the cases the establishment and use of transgenic lines with tissue specific Cre recombinase expression is preferred.

The advantage of this approach is that once the mice are generated, they provide a permanent and reproducible tool for controlled Cre recombinase expression. Obviously, for this to materialise, the transgenic lines need to fulfil some basic requirements, such as reliable expression, sufficient levels of Cre protein, high fidelity (reproducible) and specificity. These difficulties reflect the general problem of transgenic expression. The Cre transgenic lines, presently available, only partially fulfil the above requirements. The situation, although better than the past, still needs a lot of improvement.

A limiting factor in using Cre recombinase is that recombination function involves nuclear access to the cells. This problem has been solved by incorporation of a specific sequence in the Cre gene. These include Kozac translation elements for better transgene expression and nuclear localisation signals (Gu, Zou et al. 1993). These sequences allow the transport of the Cre recombinase protein in the nucleus, where it accesses the chromatin and thus recombines loxP flanked DNA segments.

One other problem is that the levels of Cre mediated excision are not always 100%. This, in most of the cases, is due to insufficient Cre protein levels or in inaccessibility of the loxP a site to the protein. The partial excision constitutes a great problem in conditional knockout strategies, where complete deletion of a gene in all cells of a specific tissue or lineage is required. Yet, in other strategies it can be proved to be useful, since mutant cells can be analysed in parallel with normal ones. Another feature that needs to be taken into consideration, especially in conditional transgenesis experiments, is the loxP sites copies. If there are too many copies of a transgene

bearing loxP sites, there is the possibility that Cre mediated, recombination will result in chromosome loss (Lewandoski and Martin 1997).

In addition, the kinetics of the Cre transgene expression itself may cause problems. Cre protein expression is a stochastic event, i.e. there is a time lapse between the onset of Cre expression (as dictated by the specific transgene promoter) and the time when Cre protein reaches sufficient levels to induce recombination. With the same logic, there is also a discrepancy between the time when Cre stops being expressed and when the recombination events cease to occur. In some experiments however, it is very important for a co-ordination of the onset of the Cre recombinase expression and the actual biological effects of this expression.

One of the major problems that need to be resolved as well, is tissue specificity. Although some promoter elements are well characterised to confer tissue/cell lineage specific expression *in vitro*, when they are used to drive expression of transgenes the resulting expression pattern is not the same. Some of the problems that arise are mosaic or ectopic, variegated and not reproducible expression (Robertson, Garrick et al. 1995; Martin and Whitelaw 1996; Garrick, Fiering et al. 1998; Henikoff 1998). The integration sites of the transgene as well, have been reported to influence levels and tissue specificity (Baubonis and Sauer 1993). An answer to these complications can only be achieved by more precise characterisation of promoter elements and better understanding of transgene expression mechanisms. For this, it is essential more transgenic lines to be established and to be analysed.

To add further control in the timing of the Cre expression, systems for inducible expression of the Cre protein have also been established. The idea is based on fusing the Cre recombinase with regulatory elements that activate/suppress expression in the presence/absence of an exogenous molecule. Presently, the most promising inducible systems are the tetracycline and the RU-486 (a synthetic steroid ligand). For example, Kellendonk and co-workers, (1999), made a fusion protein between the Cre recombinase and a truncated ligand-binding domain of the progesterone receptor (Kellendonk, Troche et al. 1999). This fusion protein could only be activated by the synthetic steroid RU486, but not by its physiological hormone progesterone analogue and its expression was targeted to the brain. By using the calcium-calmodulin-dependent kinase II alpha or the Thy-1 genes promoter Cre expression was restricted

to the adult brain. Administration of RU486 to mice resulted in Cre-mediated recombination activation of a LacZ reporter transgene specifically in the brain.

1.14 SUMMARY

The Cre loxP system provides a powerful tool for *in vivo* gene functional analysis. To date, despite the existing problems, it is widely applied in many aspects of genome alterations. Further developments of the system and the generation of new Cre recombinase transgenic lines will add significantly to potential. In addition, the generation of a panel of transgenic mice expressing the Cre recombinase in a time and tissue restricted way holds promise for unravelling issues in developmental biology and physiology.

AIM OF THE PROJECT

In vivo analysis of the role of Id4 in central nervous system development and lymphopoiesis.

Employment of the Cre/loxP recombination system to overexpress Id4 specifically in neuronal cells or thymocytes.

Characterisation of mice expressing the Cre recombinase in a neuronal specific way.

Characterisation of mice carrying an Id4 dormant transgene.

Employment of these mice to overexpress and study the role of Id4 protein, specifically in neuronal cells.

Utilisation of the Id4 transgenic mice and a currently available transgenic line that express Cre in thymocyte specific way to overexpress Id4 in thymocytes and analyse the effect in thymocyte development.

The experimental strategy is depicted in Figure 1.12 .

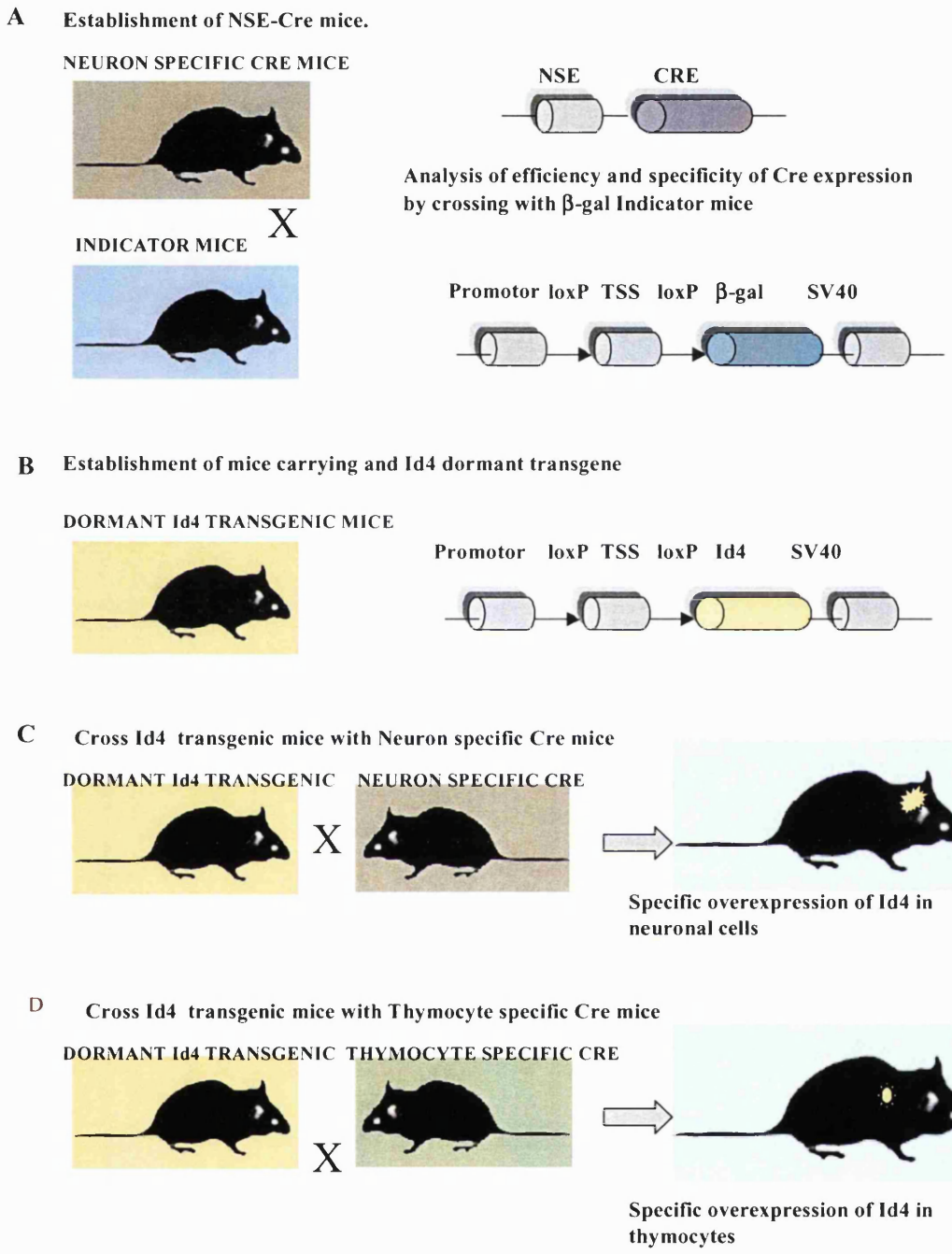


Figure 1.12: Experimental strategy. Establishment of transgenic mice, which express the Cre recombinase in a neuronal specific way. Analysis of these mice by mating with a β-galactosidase reporter strain. Establishment of the dormant Id4 transgenic line and mating of this line with the neuronal specific or the thymocyte specific Cre line. Analysis of the effect of Id4 expression in the neural lineage determination and the thymocyte development.

CHAPTER 2

MATERIAL AND METHODS

MATERIALS AND METHODS

General laboratory reagents were of analytical grade and purchased from various companies. A list of the reagents and the reagent suppliers, used for the experiments described below, is presented at the end of the chapter in Table 2.3.

2.1 TRANSGENIC LINES

The nucleotide sequences of the transgenic constructs generated in our lab are provided in Appendix B.

2.1.1 β-galactosidase reporter transgenic lines

For the analysis of the Cre transgenic lines, two different reporter transgenic lines were used. The loxP-LacZ indicator mice, (pAMA) (Ayral et al, 1998), and the plnlacZ13 indicator mice (Thorey, Muth et al. 1998). The pAMA mice carry a single transgene, in which expression of the E.*coli* β-galactosidase gene is driven by the polyoma enhancer and thymidine kinase (POE/TK) promoter. Between the promoter and the gene, there have been placed transcriptional and translational stop signals (TSS) that are flanked by loxP sites (Figure 2.1). Therefore, mice carrying the transgene do not express the LacZ gene. Cre recombinase protein expression however, induces excision of the loxP flanked stop signal and subsequently expression of the LacZ gene. In a similar way, the plnlacZ13 indicator mice contain a single copy of a transgene, in which the pgk promoter drives very strong expression of the LacZ gene after Cre mediated deletion of a loxP flanked neo gene.

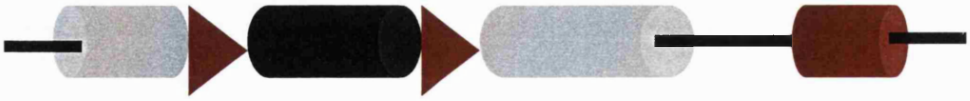


Figure 2.1:The loxP-LacZ transgene. The polyoma enhancer and thymidine kinase (POE/TK) promoter drives expression of the β-galactosidase gene (β-gal), after Cre mediated deletion of the TSS. Figure is modified from original paper (Ayral et al 1998). (not drawn in scale).

2.1.2 Thymocyte specific Cre recombinase expressing mice

Mice that express Cre recombinase specifically in the thymocytes were already available and were obtained by Dr J Marth.

These mice carry the Cre recombinase gene under the control of the mouse distal lck promoter. The lck-Cre transgenic mice are well characterised and they have been found to confer high level (80%–95%) of Cre mediated deletion of loxP sites only in thymocytes (Orban, Chui et al. 1992; Gu, Marth et al. 1994; Ayral 1998). The lck promoter drives expression of the transgene early in development and ceases being active, when the thymocytes maturate and leave the thymus to migrate to the periphery. This does not represent a problem for our strategy, because once the stop signal is deleted, the Id4 transgene stays active, even if Cre recombinase is not present anymore.

2.1.3 Neuron specific Cre transgenic mice

2.1.3.1 Neuron specific enolase promoter (NSE)

Mice were generated (by Dr Elisa Cinato in our lab), that express the Cre recombinase under the rat neuron specific enolase (NSE) promoter (Figure 2.2). The choice of the promoter was based on previous reports, where it has been shown to confer neuron specific expression (Forss-Petter, Danielson et al. 1990; Andersen,

Garber et al. 1992; Race, Priola et al. 1995). More precisely, the neuron specific enolase (NSE, also known as γ-enolase) is a neuron specific subunit of the dimeric glycolytic enzyme enolase (Schmechel, Marangos et al. 1978). The onset of NSE expression in the neurons coincides with their functional maturation during synaptogenesis (Marangos, Schmechel et al. 1980; Schmechel, Brightman et al. 1980). During development, the level of transcription is low and only slowly attains adult levels, after postnatal day 10 (Yoshida, Sakimura et al. 1983). Moreover, NSE levels rise at a slower rate in brain areas known to develop over a more extended period (forebrain, cerebellum), compared to areas that develop more rapidly (brain stem) (Marangos, Schmechel et al. 1980). Neuron specific enolase was first believed to be present exclusively in central neurons. More recently however, it has been found in peripheral autonomic nerves and in a number of endocrine cells (Bishop, Polak et al. 1982). It has also been reported that some NSE expression is detected in differentiating oligodendrocytes and proliferating Shwann cells, but it is fully repressed in adult mature glial cells (Vinores, Herman et al. 1987; Deloulme, Lucas et al. 1996).

In agreement with the above, when the proximal 1.8 kb 5' flanking sequence, including the first exon, of the rat NSE promoter was used to drive the expression of a reporter LacZ gene in transgenic mice, reporter gene expression was targeted in terminally differentiated, postmitotic and postmigrating neurons and endocrine cells (Forss-Petter, Danielson et al. 1990). In other studies as well, fusion of genes with the NSE promoter resulted in targeted expression in neurons of certain areas of the adult brain, such as the striatum, cerebellum, hippocampus and the cerebral cortex (Race, Priola et al. 1995) and (Chen, Kelz et al. 1998). Thus, there are sequences in the proximal 5' flanking region of the NSE gene that are sufficient to confer neuron specific expression. Recently, Twyman and Jones (1997) have restricted these elements in the area within the proximal 255 bp of the NSE promoter (Twyman and Jones 1997).

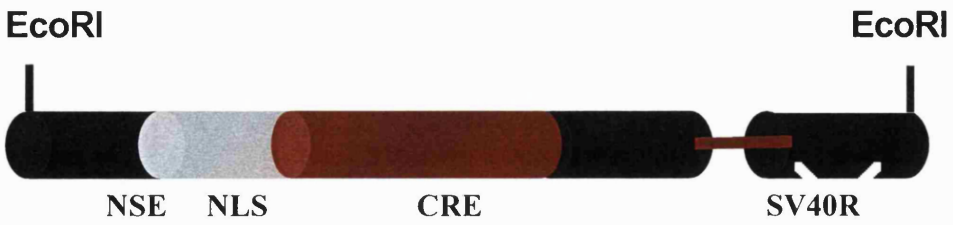


Figure 2.2: The NSE-Cre transgene. A 3.9 kb EcoRI-EcoRI fragment containing the NSE promoter, the Cre gene fused to nuclear localisation signals (NLS) and SV40 intron-exon and polyadelylation signal were used to establish NSE-Cre transgenic mice. Upon transgene expression, the NLS would transport the Cre protein into the nucleus. (Not drawn in scale).

2.1.3.2 Transgenic construct and generation of mice

The NSE- Cre transgene was produced as follows: A 31.1kb Hind III-NdeI fragment containing the Cre gene fused to nuclear localisation signals (NLS) (Gu, Marth et al. 1994) was cloned into Hind III- XhoI sites within a Ubi-junB plasmid , essentially replacing the Jun B (Schorpp, Jäger et al. 1996). The Ubi promoter was then excised with BglII and HindIII and replaced with a 1.8 kb BamHI-HindIII fragment containing the rat proximal NSE promoter (Forss-Petter, Danielson et al. 1990). A 3.9 kb fragment was excised from the pNSE-Cre plasmid, purified and microinjected in fertilised oocytes from the C57/BL6 X CBA strain of mice (see Appendix D for information), (Hogan, Bennington et al. 1994). Founder mice were backcrossed to C57/BL6 X CBA mice to generate lines heterozygous for the transgene.

2.1.4 Dormant Id4 transgenic mice

In order to activate the Id4 gene, by employing the Cre/loxP site- recombination system, specifically in tissues of interest, it was essential to establish transgenic mice carrying a dormant Id4 gene. In the transgenic construct generated for this purpose, expression of Id4 is blocked by TSS situated between the promoter and the Id4 gene. The TSS are flanked by loxP sites and can be deleted through Cre/loxP mediated recombination (Figure 2.3). The promoter used is the human Ubiquitin C promoter

and in previous studies, it had been reported to drive high ubiquitous expression of transgenes. In some tissues, particularly, it could confer 10-20X fold increase in the gene expression (Schorpp, Jäger et al. 1996).

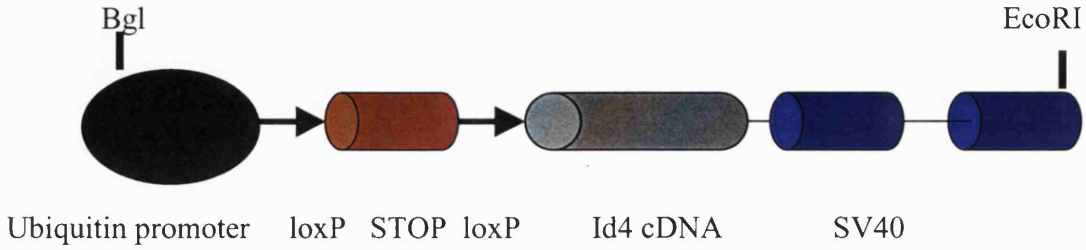


Figure 2.3: The Id4 transgene. The Ubiquitin C promoter drives expression of the Id4 cDNA only after Cre mediated deletion has removed the STOP signals that are flanked by loxP sites.

2.1.4.1 Transgenic construct and generation of mice.

In the dormant Id4 transgene construct, the Ubiquitin C promoter drives expression of a modified Id4 cDNA and an SV40 expression cassette (Figure 2.3). The SV40 cassette contributes intron- exons splice sites and poly-A sites for termination of transcription. The translational (STOP codon) and transcriptional (poly- A sites) stop signals are derived from the 3' end of the neomycin resistance gene cassette. To prevent any expression of the Id4 cDNA from potential read-through transcripts, the ATG start codon was mutated. The 5' loxP site was modified by adding Kozak sequences at its 5' end. In addition, a translation initiation codon ATG was also added in frame of the open reading frame of the loxP site (Ayral et al, 1998). The 3' loxP site was fused in frame with the Id4 cDNA. Cre mediated deletion therefore, will initiate translation at the modified ATG codon, 5' from the loxP site resulting in a slightly modified protein containing the whole Id4 codon sequence plus 16 aminoacids from the remaining loxP site. Generation of mice was achieved again by microinjection in fertilised oocytes from C57/BL6 X CBA mice (Hogan, Bennington et al. 1994). These constructs and mice were also generated by Dr. Elisa Cinato.

2.2 MOLECULAR BIOLOGY

2.2.1 Gel electrophoresis

2.2.1.1 DNA agarose gel electrophoresis

Visualisation of the DNA was performed by electrophoresis in agarose gels. Because the DNA is a negatively charged molecule, if current is applied, the DNA molecules are repelled from the negative electrode towards the positive one. During the electrophoresis, the DNA molecules are fractionated by size through a porous agarose gel.

The agarose gels were prepared as follows: 0.8 -1.5 % (w/v) of agarose was added in 1X TBE buffer. The mixture was heated, until melting of the agarose. Then, it was left to cool down (less than 65°C) and Ethidium Bromide, at a final concentration of 0.5 μg/ml, was added in. The mixture was poured into an electrophoresis gel tank and the agarose was left to solidify. The Ethidium Bromide binds the DNA molecules and stains them bright under UV illumination. To assist the loading of the DNA to the agarose gel, 1X DNA Loading Buffer was added to the DNA samples. A marker with defined sizes of DNA was used (GIBCO, UK). Gels were run at 20-60 mA. When low melting point agarose was used, the gels were run at less than 40 mA.

2.2.1.2 RNA denaturing agarose gel electrophoresis

A 1.2% (w/v) Formaldehyde agarose (FA) gel was prepared. For electrophoresis four volumes of RNA was mixed with one volume of 5X RNA Loading Buffer. The sample was incubated for 5 min at 65°C and then was loaded in the gel. The gel was run at 90V for 15-30 min in 1X FA Gel Running Buffer and then pictures were taken. For Northern analysis, a medium size gel was prepared, which was run until the dye was about 2 cm before the end. As indication of the RNA integrity, the ribosomal bands at 26S and 23S had to be clear and double the amount of the ribosomal bands at 16S and 18S.

2.2.2 DNA extraction techniques

2.2.2.1 Tail genomic DNA

Tail genomic DNA used for Southern analysis was extracted with standard phenol/chloroform extraction (Sambrook, Fritsch et al. 1989), according to the following procedure:

Tail snips of 1 cm length were being incubated O/N at 55°C in 700 µl Tail Genomic Extraction Buffer +0.5 mg/ml Proteinase K (Sigma #P6556). Next day, 26 µl of 10 µg/ µl RNase A was added and the mixture was incubated at 37°C for 1 h. Then, 700 µl of Phenol under 0.1 M Tris pH 8.0 was added and the mixture was whirlmixed and centrifuged for 5 min at 10000 rpm. The supernatant was collected and mixed with 700 µl of Phenol/Chloroform 1:1 (v/v). Again, the mixture was whirlmixed and centrifuged for 5 min at 10000 rpm. The supernatant was collected again and 700 µl chloroform was added followed by centrifugation at 10000 rpm for 5 min. These steps were performed in order to remove the protein debris from the sample (proteins gather at the layer containing Phenol).

Afterwards, the supernatant was kept and mixed with 700 µl Isopropanol. It was then subjected in whirlmixing for 30 min at RT, to precipitate the DNA. If the precipitation did not occur, Sodium Acetate, pH 5.4, at a final concentration 2.8 mM, was added and centrifugation for 5 min at 10000 rpm was employed. The white precipitate was washed with 70% EtOH, by centrifugation at 8000 rpm for 3 min and finally the pellet was resuspended in 100-200 µl TE or ddH_2O after drying for 15 min at RT.

For analysis by PCR, the DNA was extracted according to the Simplified Mammalian DNA Isolation procedure (Laird 1991). In brief, tail snips were added in 0.5 ml Lysis Buffer followed by O/N incubation at 55°C. Next day, the lysate was subjected to centrifugation at 10000 rpm for 15 min and the supernatant was mixed with one volume of Isopropanol and whirlmixed for 1 h. The mixture was subjected to 10 min centrifugation to precipitate the DNA, the pellet was washed with 70% EtOH, left to dry at RT and resuspended in 20-500 µl TE or sterile ddH_2O. Complete dissolution of the DNA sometimes required several hours of agitation at 37°C.

2.2.2.2 Tissue genomic DNA

For extraction of DNA from tissues, a DNA extraction kit (Boehringer, UK) was used according to the company's instructions. Alternatively, a modified Simplified Mammalian DNA Isolation procedure was applied. Depending on the tissue, 500-2000 µl of Lysis Buffer was used. The procedure was the same as above (2.2.2.1), but the volume of Isopropanol, used for the precipitation, was adapted to the initial volume of the Lysis Buffer.

2.2.2.3 Plasmid DNA

All midi and maxi plasmid DNA preparations were performed using the Plasmid DNA Purification kit (Qiagen # 2143, #12162) in accord with the protocol provided by the company. For mini-preps, the alkaline lysis method was used (Sambrook, Fritsch et al. 1989). The definitions maxi, midi and mini refer to the quantity of plasmid DNA extracted from the preparation (maxi: up to 500 µg, midi: 50-100 µg and mini: 2-5 µg)

For mini-preps, bacteria cells hosting plasmid DNA were inoculated O/N in 2 ml of LB+Ampicillin. Since most plasmids have been engineered to contain an ampicillin resistance gene, the ampicillin was used to ensure the growth of bacteria containing the plasmid of interest only. The inoculate was centrifuged briefly, until a cell pellet was formed. The supernatant was removed and the pellet was subjected to centrifugation to remove any remaining LB medium. The bacterial cell pellet was then resuspended in 100 µl cold Buffer I. For the cell lysis, 200 µl of Lysis Buffer II was added in the resuspension. The tubes were mixed by inversion and kept at RT for 2 min. After that, 150 µl cold neutralisation Buffer III was included in the reaction to stop the lysis and the tubes were inverted again and left on ice for 5 min.

Next, the samples were centrifuged at 4°C for 10 min at 10000 rpm. To acquire RNA-free DNA, 100 µg/ml RNaseA was added to the supernatant and the mixture was incubated at 65°C for 15 min. After that, one volume of Phenol/Isoamyl alcohol pH 8.0 was included and the mixture was centrifuged at 10000 rpm for 5 min at RT. The supernatant was collected and the DNA was precipitated with 1 ml Isopropanol, washed with 100% EtOH and 70% EtOH and resuspended in ddH_2O.

2.2.2.4 DNA purification from agarose gels

For purification of DNA from agarose gels, the Hybaid DNACleanTM (#DC-15) was used. The concept of this kit lies in that the DNA is removed from the gel and binds preferably a filter. It is then purified by washes with EtOH containing buffer and finally is eluted from the filter to a clean tube. In brief, the procedure was as follows: a Binding Buffer was put in a spin filter and the gel slice was added (300 mg of gel per filter containing 3-5 μg of DNA approximately). The mixture was heated at 55°C for 5 min to melt the agarose. Then, it was spun for 30 seconds to transfer the liquid in a catch tube. The Wash Solution was added in the filter and the spinning was repeated. The catch tube was emptied and the filter was spun again for 1 min to dry the pellet. Finally, the filter was transferred in a new catch tube and addition of 20 μl of Elution Buffer and spinning for 1 min to transfer the eluted DNA in the catch tube followed. The DNA solution was stored at –20°C.

2.2.2.5 Precipitation of DNA

To the aqueous DNA sample, 0.1 volumes of 3 M Sodium Acetate and 2 volumes of cold 100% EtOH were added. The mixture was whirlmixed and centrifuged for 10 min at 12000 rpm at RT. The pellet was resuspended in five volumes of 70% EtOH and centrifugation was repeated for 2 min. Finally, the pellet was left to dry at RT and resuspended in ddH_2O or TE.

2.2.2.6 Quantification of DNA

The approximate DNA quantity was estimated on an agarose gel stained with ethidium Bromide using a DNA ladder of known concentration per band. For a more accurate estimation, spectrophotometric measurement at wavelengths of 260nm and 280nm was used. The samples were considered pure if the ratio between OD260/280 was between 1.7-2. The concentration was determined based on the following equation:

OD (260)=1⇒ 50 μg/ml of double stranded DNA

2.2.3 RNA extraction techniques

2.2.3.1 Total RNA isolation

For RNA extraction, the RNasy kit (Qiagen # 74104) was used according to the instructions of the manufacturer. After isolation, the RNA was subjected to DNase I treatment for 15 min at 37°C at a final concentration of DNase I (RNase free, Boehringer Mannheim #776758) 1-2 U/μg of total RNA. The DNase was inactivated by 15 min incubation at 70°C.

2.2.3.2 RNA precipitation

If needed the RNA was precipitated according to the following process: Three volumes of DEPC treated ddH_2O and one volume of Phenol/Isoamyl alcohol pH 7.0 were added. The mixture was centrifuged for 5 min at 4°C at full speed (14.000 rpm). The supernatant was recovered and an additional one volume of Chloroform was added, followed by centrifugation for 5 min at full speed at 4°C. The supernant was recovered again and a mixed with 0.1 volumes of 2 M NaCl and 2.6 volumes of 100% EtOH. The mixture was being left from 20 min to O/N at –20°C and after that, it was centrifuged for 10 min at full speed and the pellet was washed with 80% EtOH. After drying for 15 min at RT, the pellet was dissolved in 10-20μl DEPC treated ddH_2O.

2.2.3.3 RNA concentration estimation

The concentration and the purity of RNA were measured either by spectrophotometry or by agarose gel electrophoresis. For the spectrophotometric analysis, the absorbance was measured at OD 260 and 280. An absorbance value of one corresponds to 40 μg/ml of RNA concentration. The RNA was considered pure, if the ratio between the absorbance values at 260 and 280 was between 1.7-2. The integrity of the RNA was checked by denaturing agarose gel electrophoresis and staining with Ethidium Bromide.

2.2.4 Restriction enzyme digestion

2.2.4.1 Genomic DNA digestion

The digestion of DNA with restriction nucleases (enzymes that recognise specific sites in the DNA and cleave it) was performed at 30 µl final volume and followed the general scheme:

Genomic DNA digestion reaction:
10 µg genomic DNA
1X enzyme buffer suggested by the manufacturer
80 U restriction enzyme
1 mg/ml BSA

The reaction was incubated at 37°C in a waterbath for at least O/N and the products of the digestion were then visualised by electrophoresis in 0.8% (w/v) agarose gels.

2.2.4.2 Plasmid DNA digestion

The DNA digestion was performed at 20 µl final volume following the general scheme:

Plasmid DNA digestion reaction:
0.5 -5 µg plasmid DNA
1X enzyme buffer suggested by the manufacturer
1 mg/ml BSA
10 U restriction enzyme per µg of DNA

The reaction was incubated at 37° C in a water bath for at least 1 h (up to 3 h) and

the products of digestion were analysed in an agarose gel (0.8% -1.5%). If necessary, the digested DNA was purified and precipitated with 1/10 volume of Sodium Acetate (3 M, pH 4.8) and 2.5 volumes EtOH by incubation at -70°C for 30 min. The DNA was collected by centrifugation at 10.000 rpm at 4°C for 15 min, washed with 70% EtOH, air-dried for 10 min and resuspended in the appropriate volume of ddH_2O.

2.2.5 Southern blot of genomic DNA

In this analysis, the DNA is subjected to restriction enzyme digestion, agarose gel electrophoresis, denaturation and transfer to a membrane. Specific sequences in this DNA are then identified by molecular hybridisation with the use of specific probes. A probe is a fragment of DNA or RNA, which is labelled in some way and is used to identify the presence of DNA sequences that closely related to its sequence. The molecular hybridisation involves mixing of the DNA molecules, separation of their double strands by denaturation and reassociation of the strands. The probe therefore, can bind complementary sites in the DNA. This binding can be easily visualised because the probe is labelled.

2.2.5.1 Probe preparation

For the Southern blot analysis, fragments of DNA were radiolabelled with α-[^{32}P]-dCTP. Plasmid DNA was digested and run on a 1 -1.5% (w/v) low melting point agarose gel. Then, the DNA fragment was purified from the gel as described above (2.2.2.4). At various experiments the following DNA probes were used:

Probes
CRE PROBE: **Hind III-BamHI fragment from pNSE-Cre plasmid, 350bp. Identifies the 3.9 kb and other band representative of the NSE Cre transgene**
Id4 PROBE: **NotI fragment from the pUbi-loxP-Id4 plasmid, 370 bp. Identifies both the endogenous Id4 gene and the bands representative of the Id4 transgene.**

pAMA LACZ PROBE: **ClaI-EcoRV fragment 289 bp or from pAMA plasmid. Identifies both the undeleted (5-6 kb) and the deleted (1.4 kb) versions of the pAMA transgene.**

2.2.5.1.1 Labelling of DNA

The random primed DNA labelling method (Feinberg and Vogelstein 1983) was employed. In this method, a DNA molecule is denatured and then left to cool down gradually. This allows a pool of different oligonucleotides to identify and bind complementary sequences within the DNA molecule. The bound oligonucleotides form the primers for the synthesis of new DNA strands. The reaction is catalysed by the Klenow subunit of the Polymerase I and the presence of dNTPs, one of which is labelled. For this reaction, a random primed labelling kit (Prime -It II KIT from STRATAGENE #300285) was employed. Between 25 ng and 40 ng of DNA substrate was used for labelling. This DNA was mixed with H_2O and 10 ì l of random 9-mer oligodeoxyribonucleotides (27 OD units/ml) at a final volume of 34 ì l. Boiling at 95°C for 5 min denatured the mixture. The following substances were then added:

DNA labelling reaction mixture
10 ì l 5X dCTP buffer containing dATP, dGTP, dTTP 0.1 mM each
1 ì l Exo (¯) Klenow 5U/µl
5 ì l Radioisotope [α-^{32}P] dCTP 3000Ci/mmol

The reaction was incubated at 37°C for 1 h and a drop of 0.5 M EDTA was added to end it., Following 5 min of denaturation at 95°C, the whole reaction mixture was finally added in the hybridisation mixture.

2.2.5.2 DNA electrophoresis and blotting

Genomic DNA was prepared and digested as described above (2.2.2 and 2.2.4.1) then, loading buffer was added and the samples were electrophoresed O/N in 0.8%

(w/v) agarose gel in 1X TBE buffer at 80 mA. Next day, the gel was soaked in 0.5 mg/ml Ethidium Bromide for 15 min, washed in 1X TBE +0.1 M $MgCl_2$ for 5-15 min and marking of the ladder bands (by radioactive ink) was performed. After that, the gel was placed in Depurination Buffer with agitation, until the dyes changed colour (overall about 15 min). Subsequently, the gel was rinsed with H_2O and placed in Denaturation Buffer with gentle agitation for 30 min. The capillary blot was set up as described elsewhere, (Sambrook, Fritsch et al. 1989), and the DNA was transferred in the membrane (HybondTM N^+ Amersham #RPN 203B) by O/N incubation in transfer buffer at RT.

The next day, the transfer apparatus was dismantled, the membrane was marked with pencil and left to air dry O/N or baked at 80°C for 2 h. The membrane was then kept at 4°C or used for the hybridisation after rinsing with 2X SSC.

In case of southern analysis of PCR products, the procedure was the same, apart from the fact that the depurination step was not applied. In addition, most of the PCR products were run in 1.2% (w/v) agarose gel at 45 mA for 2-5 h.

2.2.5.3 Southern hybridisation

All hybridisation solutions were done according to (Church and Gilbert 1984; Church and Gilbert 1985). Filters were prehybridised at least for 30 min at 65°C in prehybridisation solution. Then, the solution was thrown away and a mixture of fresh hybridisation solution pre-warmed at 65°C containing denatured salmon DNA, at a final concentration 100 μg/ml, and the denatured probe (see above) was added. The hybridisation was carried out O/N at 65°C. Next day, the filters were washed four times for 30 min each at 65°C with wash solution.

2.2.5.4 Autoradiographic detection of the hybridisation

Autoradiography is a method based on monitoring a radiolabelled compound in a solid sample by the production of an image. The radiolabelled probe binds the DNA, which is immobilised on the membrane. If this membrane, is placed in direct contact with an X-ray film the radioactive emission from the sample will generated dark areas on the developed film.

For the Southern analysis the, filters were placed on a Kodak MS-1 film (Anachem ltd.#8222648) inside an intensifying screen and exposed at -70^0C in various times (for 30 min to a few days). After exposure, the filters were washed for 30 min in boiling 1% SDS, then left for 30 min in 0.4 N NaOH at RT and finally rinsed in 0.2 M Tris-HCl pH 7.0 and 2X SSC. After that, the filters were checked again by autoradiography and if clean, stored at RT or 4°C.

A quantification of the signal was performed by using the Adobe PhotoShop software (Microsoft). The intensity of each band was estimated by the median value of light intensity after subtraction of the background signal.

2.2.6 Northern hybridisation

The Northern hybridisation is a modification of the Southern hybridisation. The main difference is that the target nuclei acid is RNA instead of DNA.

Approximately 10-20 μg of total RNA was run on a denaturing agarose gel, as described before (2.2.1.2), and was fixed onto a nylon membrane, using alkali blotting (2.2.5.2). After permanent transfer of the RNA to the membrane, the membrane was wet in 6X SSC and placed in a hybridisation tube. 1 ml of formamide solution per 10 cm^2 of membrane was added and the tube was placed in a hybridisation oven rotating for 1 h minimum at 42°C. The DNA probe was prepared, as described above, (2.2.5.1), and was added to the hybridisation medium after 5 minutes of denaturing. The probe was prepared to be at a final concentration of 10 ng/ml. Fresh pre-warmed formamide solution was used for hybridisation. The tube was left rotating O/N at 42°C. Next day the hybridisation solution was poured off and the blot was washed with 2X SSC/0.1% SDS for 3 min at RT twice. The wash solution was then changed to pre-warmed 0.2X SSC/0.1% SDS and the tube was incubated with rotation at 68°C for 15 min. This step was repeated once and then, the membrane was rinsed in 2X SSC, covered with a plastic wrap and subjected to standard autoradiographic detection (2.2.5.4) of the specific signals.

2.2.7 PCR analysis

The PCR is a rapid method for the selective amplification of certain DNA

sequences *in vitro*. Two oligonucleotide primer sequences complementary to the target DNA are required. These primers are designed so that in the presence of DNA Polymerase and dNTPs they can initiate DNA synthesis, which results in amplification of DNA sequences flanked by these two primers.

Approximately 500 ng of tissue genomic or tail DNA extracted according to the fast mammalian protocol (2.2.2) were used for PCR analysis. All the PCRs were performed in the following conditions:

PCR reaction	
primers 0.2 μM each,	Tween 0.05%,
dNTPs 100 μM each,	$MgCl_2$ 1.5 mM,
BSA 1 mg/ml	DMSO 0-9%
Taq Polymerase	1.25 -2.5 U.

In the PCR using the pair of primers, Ubi1-SV40R, Q solution from Qiagen at 1X final concentration was used instead of DMSO. All the PCRs were performed at a final volume of 20 μl. The primer pairs and the PCR Programmes are depicted in Table 2.1. The sequences of the primers and their position in the genomic substrates are presented in Appendix B.

Table 2.1: PCR programmes.

primers	Denature °C	Anneal °C	Polymerisation °C	cycles	product size bp
BACTIN	95-1 min	69-1 min	72-1 min	24-30	Genomic: 422, cDNA:248
TK-LACZ3	95-1 min	68-1 min	72-2 min	30	**pAMA transgene** Undeleted: 1048, Deleted 674
CRE2B-CRE2A	95-1 min	62-1 min	72-1 min	30	**NSE-Cre transgene**: 819
Id4S-R1	95-1 min	59-1 min	72-1 min	30	**Endogenous Id4 and Id4 transgene** Genomic: 618, cDNA 270
UBI1-R2	95-1 min	64-1 min	72-1 min	30	**Id4 transgene**: Undeleted: 1072, Deleted: 697
UBI2-NEOR	95-1 min	62-1 min	72-1 min	30	375
ID4S-SV40R	95-1 min	59-1 min	72-2 min	30-35	Genomic: 1180, cDNA: 1100, 860
UBI2-SV40R	95-1 min	62-2 min	72-3 min	30-40	Genomic undeleted: 1971, Genomic deleted: 1891, cDNA undeleted: 1620, 1450 , cDNA deleted: 1540, 1389

2.2.8 RT PCR analysis

A version of the PCR is the RT-PCR. This method is designed to achieve amplification of certain transcripts *in vitro* For this the RNA is converted to cDNA, which is then used as template for the PCR.

The first strand cDNA synthesis was performed using Gibco Superscript II (SUPERSCRIPT II RNase H- Reverse transcriptase Gibco BRL #18064) and the accompanying buffers according to the procedure described below:

2 -5μg of total RNA were mixed with OligodT primer (final concentration 200 nM) and ddH2O up to 12 μl final volume. The mixture was incubated at 70°C for 10 min and then transferred on ice for 1 min. Then, the following mixture was added, to reach a final volume of 19 μl.

RT- PCR reaction	
Buffer-Gibco	1X
$MgCl_2$	0 -2.5 mM
DTT	10 mM
dNTPs mix	500 mM

Following incubation at 42°C for 1 min, 200 U of SUPERSCRIPT II enzyme were added to the reaction, which was then left for further incubation, at the same temperature for 40 min. To inactivate the enzyme, a further incubation at 70°C for 10 min followed. Finally, 2-4 U of RNaseH (Promega) was added and the mixture was left at 37°C for 30 min. After that, a 1-10% of the reaction was used for PCR. The rest was stored at -20°C

2.2.9 Competent cells and bacterial transformations

2.2.9.1 Preparation of electrocompetent cells

Electrocompetent cells, are cells whose plasma membrane has been made permeable by short electric pulses and thus they are capable of taking up foreign DNA.

0.5 L of LB with 1/100 dilution (v/v) of a fresh O/N bacterial cell culture was incubated at 37°C with vigorous shaking to reach an OD600 of 0.5 -0.8.

The flask was chilled for 15 min at 4°C and the solution was separated in10 tubes of 50 ml, which were centrifuged at 6000 rpm for 15 min. The pellets were resuspended in 50 ml cold ddH_2O for each tube and the centrifugation was repeated. Then the pellets were resuspended in 25 ml for each tube and centrifugation was employed once more.

After that, the cell pellets were resuspended in 1 ml 10% glycerol in ddH_2O (v/v), they were pooled together in two 15 ml tubes and centrifugation was performed. The pellets were again resuspended in 10% glycerol in ddH_2O (v/v), so as the final concentration was at least 3X 10^{10} cells/ml. The bacterial cell suspension was aliquoted in 50 μl and stored at –80°C for up to 6 months.

2.2.9.2 Electroporation

An aliquot of 50 μl of electrocompetent bacteria was incubated with 50-300 ng DNA on ice for 10 min in 0.2 cm chilled electroporation cuvettes. Then, pulse was applied using the Gene Pulser apparatus (Biorad) set at 25Mf, 2.5KV and 100 Ω. Immediately after the pulse, 0.5 ml of LB medium was added and the bacteria were resuspended with Pasteur pipette. Then, they were transferred in a 5 ml tube and shaken for 1h at 37°C. Afterwards, they were plated on LB-antibiotic plates (plus X-gal and IPTG when the vector allowed a blue–white colour selection of the recombinant clones), which were incubated O/N at 37°C.

2.3 CELL CULTURE TECHNIQUES

2.3.1 NIH 3T3 cells

The NIH 3T3 is a widely used permanent cell line derived from embryonic murine tissue. It grows as a monolayer and has fibroblast like morphology. For their culture, DMEM medium (Gibco BRL), supplemented with FCS and the appropriate aminoacids and antibiotics (Dulbecco and Freeman 1959), was used. Cells were cultured until 70-80% confluence (coverage of the petri dish surface area) and then they were passaged to new culture dishes.

2.3.2 Passage of cells

When the cells were reaching 70% -80% confluence, they were transferred into new petri dishes. The medium was aspirated from the old dishes and the cells were washed briefly in PBS. Then, 2 ml, 5 ml or 8 ml (for 60 mm, 100 mm and 150 mm petri dishes respectively) of trypsin 0.25% Trypsin+0.53 mM in 1X $PBS^{-Mg+2,Ca+2}$ were added and the cells were incubated for 2 –5 min at 37°C, until they started to come off the plates. Next, equivalent volumes of DMEM were added and the cells were pipetted up and down to break any cell clumps. The cell suspensions were then centrifuged at 2000 rpm for 2 min and the pellets were resuspended in 1 -10 ml DMEM. The cells were then counted and seeded in the appropriate densities ($0.8X10^6$, $2.2X10^6$ and $5X10^6$ for the 60 mm, 100 mm and 150 mm petri dishes respectively), or they were frozen down.

2.3.3 Long term freezing of cells

Cells were trypsinised from a 150 mm petri dish. They were counted and then they were collected by centrifugation at 2000 rpm for 2 min. Next, they were resuspended in DMEM in a concentration of 10^7 cells/ml and aliquots of 0.5 ml were added in cryogenic vials on ice. Another 0.5 ml of a mixture, containing 2X Freezing medium (DMEM + 50% FCS + 20% DMSO) was added in each vial. The tubes were shaken and quickly transferred in a pre-cooled Styrofoam box in a 70°C freezer. After 24 h, the cells were transferred on dry ice into liquid nitrogen, where they were kept permanently.

2.3.4 Counting of cells

The Trypan blue exclusion method of counting viable cells is based on the fact that dead cells are stained blue, because the disruption in their membranes allows the dye to penetrate them.

A solution containing four parts of 0.2% Trypan blue and one part of 5X PBS was prepared the day of the experiment. Then, one part of cell suspension in dilution 1:2 or/and 1:5 was mixed with one part of the Trypan blue solution. The mix was left for three minutes on ice. Afterwards a haematocytometer chamber was loaded with the cell suspension+Trypan Blue mix and 8-10 squares (>200 cells) were counted under the microscope. Both types of cells; blue or unstained, were counted. Finally, the concentration of cells in the initial suspension was estimated according to the following equitation:

Viable cell/ml = average number of unstained cells per square X 10^4 X dilution factor.

2.3.5 Transfection of cells by electroporation

For transfection, cells were trypsinised and resuspended in Transfection Medium (DMEM+10 mM Hepes pH 7.0) at a concentration of 10^7 cells/ml. 0.5 X 10^7 cells was transferred to a 0.4 cm transfection cuvette, followed by electroporation at 625 V/cm, 500 Mf capacitance. Immediately after the transfection, 0.5 ml of Transfection Medium was added and the cuvettes were left at RT for 10 -20 min. After that, the cells were collected, diluted with the appropriate volume of DMEM and seeded in 10 cm plates. Plates with untrasfected cells were also prepared as controls. Plasmids, used for transfection, included the undeleted Id4 transgenic construct (pUbiloxPId4) (see 2.1.4), a plasmid containing a version of the Id4 transgene without the STOP signal, (delta pUbiloxPId4) and a plasmid carrying the Cre recombinase gene and a puromycin resistance gene (pCre-pac). Approximately 15-20 μg of plasmid DNA was transfected at each time. In the case of pUbi-loxP-Id4 and pCre-pac co-transfection, the cells were left for 24-48 h in culture and then selection with puromycin was applied for 24h-48h. The pUbi-loxP-Id4 was linearised, to achieve permanent integration, whereas transient transfection was achieved with circular pCre-pac

plasmid DNA. Cells that survived the selection were cultured further in normal culture medium, until they were enough for DNA and RNA extraction. An optimisation of the puromycin selection was performed by transfecting cells, with only the pCre-pac plasmid, and applying different concentrations of puromycin for various time points. The experiment was based on the idea that in cells, where both dormant Id4 and Cre recombinase existed, Cre mediated recombination of the STOP signal would give rise to cells ectopically expressing Id4. Selection with puromycin would facilitate the isolation and expansion of those cells

2.3.6 DNA and RNA extraction from cells

DNA and RNA extractions were prepared as in 2.2.2.1and 2.2.3.1. The only difference was that for the DNA extraction, the incubation in Lysis Buffer was performed at 37°C.

2.4 ANALYSIS OF TRANGENIC MICE TECHNIQUES

2.4.1 Sectioning

2.4.1.1 Paraffin sections

Fresh tissues were isolated washed in PBS and left in 4% PFH for 3h. Then, the tissue was cut into pieces of approximately 2mm in thickness and up to 2cm in length and width and transferred in fresh 4% PFH, (at least 10 volumes), where they were left O/N at 4°C. Dehydration of tissues was performed at RT as follows:

70% EtOH 1 h

95% EtOH 1 h

90 % EtOH 1 h

100% EtOH 1 h

100% EtOH 1 h

After that, the tissues were immersed in two changes of at least 10 volumes of

Xylene for 1 h each at RT and one change of 70% melted paraffin in Xylene for 1 h at 65°C. Next, they were infiltrated in paraffin at 65°C by 3 changes for 1 h each. Finally, they were left O/N immersed into melted paraffin and next day they were placed in a plastic mould containing paraffin at RT, until the paraffin solidified. The moulds were stored at RT or 4°C.

Paraffin sections were cut using a Leica microtome at 5-10 μm. Immediately, they were placed on a drop of water in poly-L-lysine coated slides (Sigma #PO425) heated on a heat blot at 37°C-45°C. The sections were left to dry O/N at 37°C. Next day, they were placed for 5 min at 60°C, then they were used for histochemistry.

2.4.1.2 Cryostat sections

Fresh tissues were isolated, washed in PBS and left O/N in 4% PFH at 4°C in dark. Next day, the tissues were transferred in 30 % sucrose solution in PBS 1X and left there at 4°C for 24h. Then they were embedded by placing into OTC in a plastic mould, which was slowly frozen, by immersing in isopentane containing beaker, surrounded by liquid nitrogen. After that, the samples were stored in – 80°C or they were processed straight away, by cutting 10 -30 μm sections in a cryostat at – 20°C. Following cutting, the sections were transferred onto poly-L-lysine coated slide and left to dry at RT O/N.

2.4.2 Haematoxylin and eosin staining for paraffin sections

For Haematoxylin (blue nuclear) and Eosin (dark pink cytoplasmic) staining of sections, the following standard protocol was used.

The sections were dewaxed by washing in 100% Xylene for 3 min at RT. Subsequent dipping of the sections in decreasing volumes of EtOH as depicted below, performed their dehydration:

100% EtOH 3 min at RT

90% EtOH 3 min at RT

70% EtOH 3 min at RT

ddH_20 3 min at RT

Staining in Haematoxylin (Sigma), was performed by dipping the sections in it for 5 min at RT, followed by washing in running tap water, until the sections became blue. This was followed by immersion in 1% hydrochloric acid in 70% EtOH for 5 sec and washing again in running tap water, until the sections became blue again. After that, sections were immersed for 3-5 min in Eosin (Sigma) and then they were dehydrated by passing through increasing volumes of EtOH as follows:

ddH_2O 50 sec at RT

70 % EtOH 10 sec at RT

90% EtOH 10 sec at RT

100 % EtOH 10 sec at RT

Finally, the sections were cleared by 2 min immersion in Xylene and mounting in DPX.

2.4.3 Neutral red staining

For Neutral Red staining, sections were incubated for 3 -5 min in 0.1% Neutral Red +30% of 95% EtOH in PBS (v/v). Then, they were briefly rinsed in PBS, dehydrated by passing through increasing volumes of EtOH, washed in Xylene and mounted in DPX medium.

2.4.4 Nissl staining

For Nissl staining, sections were left for 20 min in 1% Cresyl Violate. This chromogen stains the accumulation of endoplasmatic reticulum in the neurons' cell bodies. After the 20 min, the sections were washed briefly in ddH_2O. Then, they were left in 70% EtOH, until the stain reached the desirable intensity, and subsequently the sections were passed through increasing volumes of EtOH and mounted in DPX.

2.4.5 X-gal staining

2.4.5.1 Whole mount X-gal staining

For X-gal staining, the embryos were dissected in PBS and immediately transferred into fixative, where they were kept at 4°C in dark for 2 h to O/N (embryos older than 12.3dpc were punctured). Then the embryos were rinsed 3 times for 15 min at RT with Rinse Buffer and stained for 2h to O/N in Staining Buffer at 37°C. After that, they were washed once with PBS and were dehydrated depending on size and stage according to the following scheme:

1h - O/N	70% EtOH
1h - O/N	96% EtOH
1h - O/N	100% EtOH

Subsequently, the embryos were transferred into fresh clearing solution for about 15 min to 1 h, until they were transparent and then they were observed under microscope and pictures were taken using a Zeiss digital camera.

2.4.5.2 X-gal staining of sections

For X-gal staining of cryostat sections a similar procedure as above was applied. In detail, the sections were post-fixed by immersing into cold acetone for 5-15 min at 4°C. Then, they were rinsed in $PBS^{-Mg+2,Ca+2}$ twice, for 5 min at 4°C. At the end, they were stained O/N in X-gal staining solution. Next day, they were immersed briefly in PBS, counterstained in Eosin (2.4.2) and mounted in DPX.

2.4.6 Immunocytochemistry

An overview of the concept of immunohistochemical detection is depicted in Figure 2.4.

Strategies for immunohistological detection of proteins.

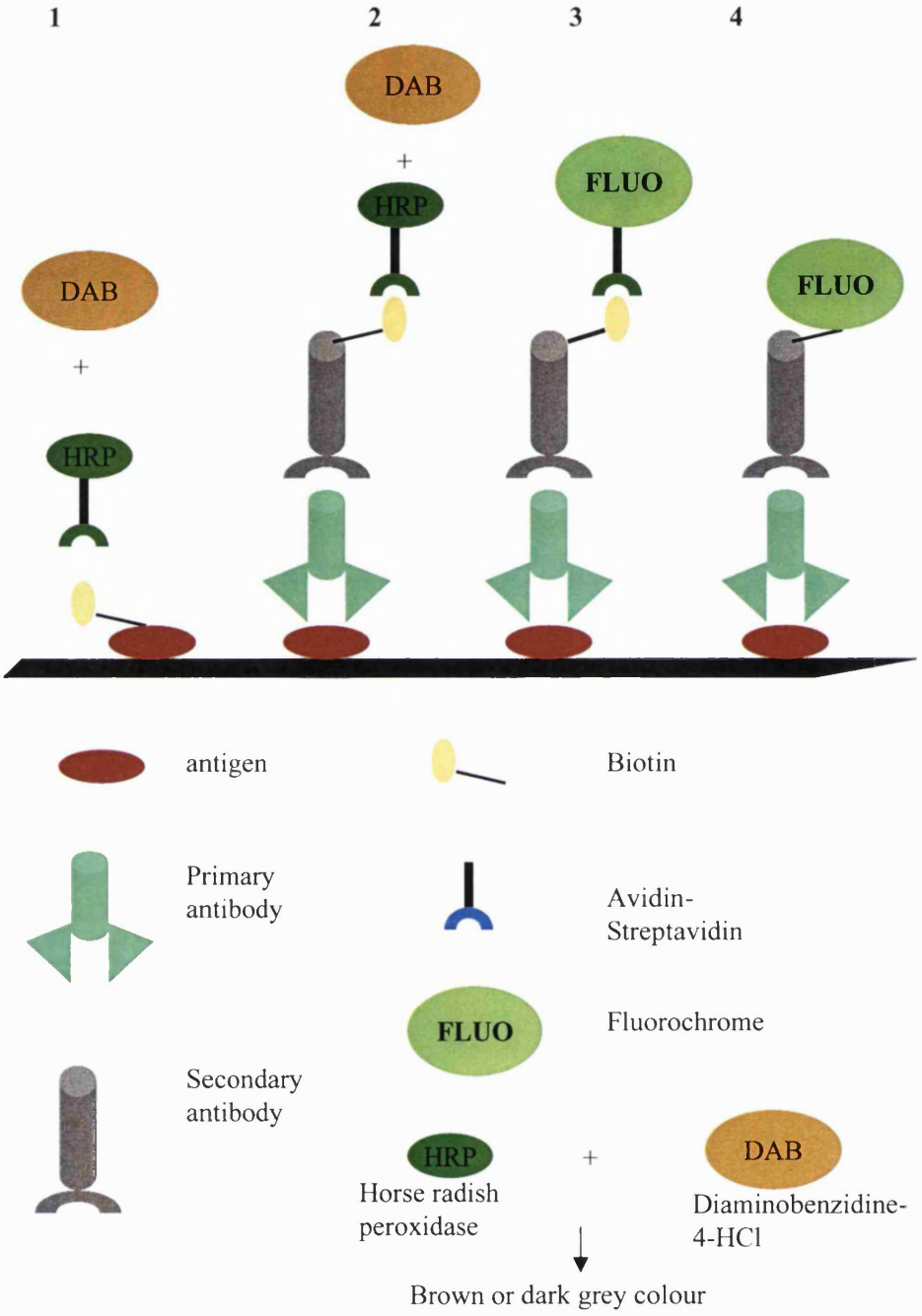


Figure 2.4: Strategies for immunohistological detection of proteins.

2.4.6.1 Immunohistochemistry with the Cre recombinase antibody

Brain and kidney from adult mice were fixed for two days in formalin, then cut into pieces and embedded in paraffin waxing. Sections of 10 ì m were made in a microtome. After drying O/N at 37°C the sections were dewaxed and rehydrated, incubated for 10 min with 3% Hydrogen Peroxide (for blocking of the endogenous peroxidase) and then treated with Dako pH 6.0 Target Retrieval Solution. (Dako #S1699) for unmasking of the epitopes. Normal Swine Serum (Dako #X0901) was used as blocking solution. Incubation with CRE biotinylated antibody (Dabco #106L) 1:100 in TBS was implemented O/N at RT. Then, the sections were washed in TBS+0.05% Tween $_{20}$ and ExtrAvidin conjugated to HRP (Extravidin is a complex of Streptavidin and Avidin, which binds Biotin with high efficiency. Sigma #E2886) was applied for 30 min in 1:15 dilution. The Peroxidase was visualised by incubation in 1 mg/ml DAB (Biostat Ltd #N4170). Sections were counterstained with Haematoxylin.

2.4.6.2 Immunohistochemistry with the Id4 antibody

2.4.6.2.1 Triple Id4 specific staining with DAB Chromophore

Paraffin sections from brain, kidney, spleen and thymus were dewaxed and then left for 2 min in TBS buffer. Because of the fixing procedures, sometimes the epitopes are masked, due to the acquisition of different conformations. To retrieve the epitopes, the sections were subjected to 20 min 800W microwaving in Citrate buffer pH 6.0. Following this, they were cooled in running tap water and left in TBS buffer for 1 min. To quench any endogenous Peroxidase, the sections were left for 10 min in 3% H_2O_2 diluted in 20% methanol (v/v) in H_2O. Then, they were washed for 2 min in TBS-T and subsequently blocking solution of 10%, Normal Swine Serum (v/v in TBS-T) was applied for 10 -20 min at RT. After the incubation, the blocking solution was tipped off and primary Rabbit polyclonal Id4 antibody (SantaCruz) was applied, in 1:200-1:1000 (v/v) dilution in TBS-T, for O/N incubation at 4°C. Next day, the sections were washed in three times for 3 min each (3X 3 min) in TBS-T buffer. Secondary biotinylated Swine anti-Rabbit or Goat anti-Rabbit antibody in dilutions 1:400 and 1:15 (v/v) respectively were applied then, followed by incubation for 1h at RT. The antibodies were all diluted in TBS-T. Then, the sections were washed in 3X

3 min in TBS-T buffer and Extravidin-HRP was applied in 1:15 -1:25 dilution in TBS-T (v/v) for 30 min at RT. The section were washed again for 3X 3 min at RT and DAB solution was applied for 5 -10 min. Finally, washing in running tap water and standard staining with Haematoxylin was performed

2.4.6.2.2 Triple Immunofluorescence Id4 specific staining

For immunofluorescence, a similar procedure was followed, with the following exceptions:

- There was no incubation in H_2O_2 solution
- All the washes were performed in TBS buffer for 3 X 5 min
- The antibodies were diluted in TBS+5% Normal Swine Serum
- Instead of Extravidin, Streptavidin (Amersham #RPN 1232) conjugated to a green fluorochrome was used in dilution 1:100.
- Not counterstaining was performed.

After staining, sections were incubated in 0.5% Sudan black stain diluted in 70% EtOH (w/v) (to quench the autofluorescence), washed in 70% EtOH and mounted using glycerol based mounting medium (Sigma #10004).

2.4.6.3 Neuronal markers

For the detection of neurons, oligodendrocytes and astrocytes the anti-neurofilament 200 (anti-N200), anti-2',3' cyclic nucleotide-3'-phosphodiesterase (anti -CNPase) and anti- Glial fibrillary acidic protein (anti-GFAP) monoclonal antibodies respectively were used (Sigma #N0142, #C5922, #G3893). The sections were dewaxed, rinsed with TBS + 0.1% Triton X and incubated for 10 min with 50% Normal Goat Serum (Dako #X0907) in TBS (v/v) at RT. Then, each of the above primary antibodies was applied for 1 h at RT in 1:400 dilution in TBS. After the incubation, the sections were washed for 3X 2 min in TBS + 0.1% Triton X and then incubated in secondary antibody (goat anti-mouse Alexa 546 from Molecular Probes #A-11003) 1:100 or 1:1000 dilution in TBS (v/v) for 1 h at RT. Finally, the sections

were washed 3X 2 min in TBS + 0.1% Triton X and once in TBS and were mounted in glycerol based mounting medium. For observation of the slides and preparation of the photos, a Zeiss microscope and the Axiovision software were used. In order to inhibit any autofluorescence from the tissues, prior to incubation with the antibody, the sections were incubated for 30 min at RT in 1% Sodium Borohybridide in PBS (w/v).

2.4.6.4 Lymphoid markers

B cell (CDD45R/B220), T cell (CD3ε) and erythrocyte (TER-119) specific mouse monoclonal biotinylated antibodies from Pharmigen were used. The CDD45R/B220 antibody reacts with the CD45 glycoprotein, which is expressed on B lymphocyte at all stages of their development (Johnson and Maiti 1997). The CD3ε antibody reacts with the T cell antigen receptor (Weiss and Stobo 1984). It binds the mature and after prolonged incubation, the immature T cells. In the procedures described in this study, the CD3ε antibody was used to identify mature T cells. The Ter-119 antibody identifies the TER 119 antigen, which is expressed in most immature and mature erythroid cells (Ikuta, Kina et al. 1990). The staining procedure described below, was the same for all the three antibodies:

Paraffin section of spleen and/or thymus were dewaxed and left in TBS for 2 min. Microwaving for retrieval of the antigens was performed in Target Retrieval Solution pH 6.0 (Dako) for 20 min. The sections were cooled down in running tap water and then incubated in 0.03 M Periodic acid in TBS for 20-30 min. This step was employed in order to quench the endogenous Peroxidase, which is at high levels, especially in spleen. Then, the section were washed in TBS-T buffer, incubated for 10 min in 20% Normal Goat Serum and the primary antibody diluted 1:100 in TBS-T+5% normal Goat Serum (v/v) was applied for O/N at 4°C. Next day, the section were washed in TBS-T buffer for 3X 3 min and Extravidin-HRP was applied in 1:50 dilution in TBS-T (v/v) for 30 min at RT. The sections were then washed 3X 3 min in TBS-T and DAB stain was applied for 5-10 min. Finally, they were washed in running tap water and mounted in DPX.

2.4.6.5 Proliferation analysis

2.4.6.5.1 BrdU labelling

For the assessment of BrdU incorporation in the brain cortex, 1 mg/ml of BrdU in PBS/g of body weight, was injected in mice overexpressing the Id4 protein and littermates. The littermates were used, as control, for the normal levels of BrdU incorporation in the adult mouse brain. Approximately 1 -2 h after the injection, the mice were sacrificed and the brains were collected. Brains from untreated mice were also collected as negative control. The brain samples were afterwards prepared as usual for embedding in paraffin and sectioning. Visualisation of the BrdU incorporation was performed by antibody against the BrdU and green fluorescence detection. The procedure was the following: The sections were dewaxed as usual and immersed in 2 N HCl for 1 h at RT. Samples also included brain sections from untreated animals as negative control. The sections were then removed and rinsed once in H_2O and TBS-T for 10 min each. The liquid excess was dried with paper and blocking solution consisting of 5%, Normal Goat Serum in TBS-T (v/v) was applied for 30 min. After that, the excess blocking serum was tipped off and primary Mouse anti-BrdU antibody (Dako #M0744) in 1:10 dilution in TBS-T+5% Normal Goat Serum (v/v) was applied for O/N at 4°C. Control slides without primary antibody, were also included. Next day, the samples were washed in TBS-T 3X 5 min and secondary Goat-anti-Mouse IgG biotinylated antibody (Dako #E 0433) was applied in 1:200 in TBS-T+5% Normal Goat Serum (v/v) for 1 h at RT. Controls, without secondary antibody, were also included. The sections were then washed 4X 5 min in TBS and Streptavidin conjugated to green fluorochrome (FITC) was applied in 1:100 dilution in TBS for 40 min -1 h. Finally, the sections were dipped in Sudan Black 1% in 70% EtOH (w/v), washed with 70% EtOH and mounted in glycerol-based mounting medium. The sections were then observed under Zeiss microscope and pictures were taken using the a Zeiss digital camera and the Axion vision software.

2.4.6.5.2 Immunohistochemistry with the Ki-67 antibody.

The Ki-67 protein is expressed in the nuclei of cells undergoing proliferation (Boehringer Mannheim #1742345 and product information). For staining with the anti-Ki67 antibody sections were dewaxed, as usually and then incubated for 30 min

in 0.1% Trypsin in TBS (w/v). They were then washed in TBS and subjected to microwaving in Citrate buffer, for 20 min at full power. After incubation for 10 min with 25% Normal Goat Serum in TBS (v/v), primary Mouse anti-Ki-67 antibody in 1:10 dilution in TBS-T+5% Normal Goat Serum was applied O/N at 4°C. Next day, the sections were washed 3X 5 min in TBS-T and secondary Goat-anti-Mouse IgG biotinylated antibody was applied in 1:200 in TBS-T+5% Normal Goat Serum for 1 h at RT. The sections were then washed 3X 5 min in TBS-T and Extravidin conjugated to HRP was applied in 1:50 dilution for 30 min. After washing with TBS 3X 5 min, the specific binding in the sections was visualised with DAB chromogen. Sections were counterstained with Nissl staining 2.4.4.

2.4.6.6 Tunel assay

For the TUNEL assay, the Apoptosis fluorescein detection system from Promega (#G3250) was used according to the protocol provided by the manufacturer. This system measures the fragmented DNA of apoptotic cells by incorporating fluorescein-12-dUTP at 3′-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). This enzyme forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay

According to this procedure, nuclei from cells undergoing apoptosis are stained green. Counter-staining with Propidium Iodine stains the cell nuclei red. Co-localisation of the two fluorochromes gives a yellow colour indicative of apoptosing cells.

2.4.7 FACS analysis-multicolour immunofluorescence

Thymus and/or spleen were dissected, washed in PBS and transferred to cold IMDM AB (Gibco BRL). The organs were mashed and resuspended in 5 ml of IMDM AB medium. In case of the spleen, prior to this 5 ml of GEYS, solution was added and the suspension was incubated for 10 min at 37°C. The cells were precipitated by mild centrifugation for 7 min at 1200 rpm at 4°C and finally resuspended in the 5 ml of IMDM AB. They were then counted by the Trypan blue exclusion method and $0.5X10^6$ cells of each type (thymocytes or splenocytes) were transferred in a V round bottom micro-dish. The cells were then centrifuged from 5

min, 1200 rpm at 4°C and 100 ì l of the first layers (usually CD4 marker conjugated to PE and CD8 marker conjugated to FITC) diluted in PBS+1% BSA+0.1 mM Azide was added to the cell pellet. Next, the cells were left for 30 on ice protected from light. After that, they were washed with PBS+0.1 mM Azide and they were suspected to another round of centrifugation. A second wash was followed by adding 100 ì l of the second layer of staining (Biotinylated antibody) again diluted in PBS+1% BSA+ 0.1 mM Azide and 30 min incubation on ice. The cells were washed again, and Streptavidin RED670 in dilution 1:300 was applied for another 30 min on ice. Finally the cells were washed and resuspended in PBS+0.1 mM Azide and transferred to small FACS tubes for analysis. In some cases, staining with 7-Aminoactonomycin D (7AAD) at a dilution of 250 ì g/ml in PBS was used. This red fluorochrome stain binds single stranded DNA in the nucleus. It is a useful marker for apoptosis and cell cycling. A list of the antibodies used in our analysis is depicted in Table 2.2.

Table 2.2: Immunological cell surface markers.

Antibody	Clone	Company	Dilution	Marker
CD8 FITC	YT5169.4	Pharmigen	250 μg/ml	CD8
CD4 PE	H129.19	Pharmigen	250 μg/ml	CD44
H57biotinylated	H57-597	Pharmigen	250 μg/ml	αβTCR
CD25 biotinylated	7D4	Pharmigen	250 μg/ml	CD25
CD44 biotinylated	IM7	Pharmigen	250 μg/ml	CD44
CD69 biotinylated	H1.2F3	Pharmigen	250 μg/ml	TCR activation
DX5 biotinylated	DX5	Pharmigen	250 μg/ml	NK
Thy1 biotinylated	53-2-1	Pharmigen	250 μg/ml	Early thymocyte
GL3 biotinylated	GL3	Pharmigen	250 μg/ml	γδTCR

2.4.7.1 Instrument calibration/standardisation procedures

When performing multi-colour immunofluorescence analysis using a flow cytometer, an intrinsic spectral overlap of the different fluorochromes used is common. If appropriate adjustments are not made, misinterpretation of data from false positive populations is highly probable. Compensation is the process of correcting for this overlap. It is described, as the subtraction of unwanted signal to remove the effects of spectral overlap. Using appropriate single and double stained control samples, compensation for spectral overlap can be successfully achieved.

Fluorochromes, such as fluorescein isothiocyanate (FITC), R-phychoerythrin (R-PE), and Red 670, were used in this analysis. FITC emission is measured as a green signal (530 nm peak fluorescence) by the FL1 detector, R-PE is measured as an

orange signal (575 nm peak fluorescence) by the FL2 detector, and RED670 is measured as a violet signal (peak fluorescence 670 nm) by the FL3 detector. Frequently however, some orange fluorescence is present in the FITC emission and vice-versa some green fluorescence is present in the R-PE emission. By compensation, the measurement of fluorescence in a cell sample stained with one fluorochrome is set to be identical to that of the unstained cells. as far as the other types of staining are concerned.

To achieve compensation the FACS analysis software was adjusted as follows:

An unstained (autofluorescence control) cell sample was run. The FSC (forward scatter-cell granularity) and SSC (side scatter-cell size) detectors were adjusted so the cells of interest were displayed on scale. While gating on the cells of interest, the fluorochrome FL1, FL2, and FL3 detector were set so that autofluorescence background was diminished. Stained cells were then compared to the autofluorescence control (unstained cells), to confirm that the stained cells are on scale for each parameter.

The compensation was then set, based on running cells stained with each antibody-fluorochrome conjugate individually (single stain). In other words, for a PE-conjugated antibody stained cell population for example, the FL1-% FL2, on the FL2 vs. FL1 dot plot was set so that the FL2 positive population is vertically aligned with the FL2 negative population. Then, with the FITC-conjugated antibody stained cell population, the FL2-% FL1 on the FL2 vs. FL1 dot plot was adjusted so that the FL1 population is horizontally aligned with the FL1 negative population. The same was performed for the third colour, adjusting both FL2-% FL3 and FL3-% FL2. Next the procedure was repeated by running 2-color control cell samples stained with 1) FITC and PE antibodies and 2) PE and Streptavidin RED 670 antibodies.

For better understanding of the results at later chapters, an indicative FACS analysis of a spleen sample is explained in Figure 2.5 and the statistical software programme Excel (Microsoft) was used.

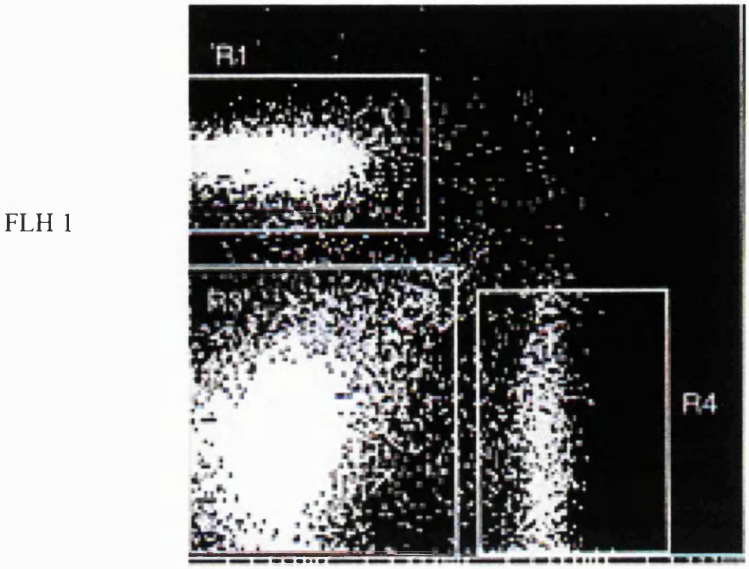
2.4.7.2 Foetal thymocyte organic culture (FTOC)

The FTOC was executed by Dr. Owen Williams. This method is based on the

idea of culturing immature thymocytes in an artificial environment that allows their development (Heemskerk, Blom et al. 1997). This environment simulates the conditions in the thymus and is cteated by culturing the thymocytes in drops in the surface of isolated thymus lobes. Foetal thymuses were obtained from mouse embryos at stage 15.5 dpc. The lobes were treated with Deoxyguanoside for 5 days and then thymocytes isolated from 15.5 dpc embryos were placed upon them in a hanging drop culture for two weeks. To analyse their differentiation, the cells were collected and subjected to FACS analysis, after two weeks.

2.4.7.3 Statistical analysis of the FACS analysis results.

To assess whether the differences in the results of FACS analysis, between the samples and the controls, were due to normal sample variance or not, the t-test (two tailed distribution) and the F-Test for the analysis of variance were applied. The purpose of the F-test analysis is to determine, whether a given factor (in our experiments the overexpression of Id4 in thymocytes) is influencing the results. By estimation of a statistical value termed the variance ratio F, it is possible to estimate if the variance is due to normal variability of the samples or is a result of the specific factor analysed. The t-test is used to establish if the mean values in the two samples under investigation are varying significantly or not. For this type of analysis the significance levels was taken at 90-95% and the statistical software programme Excel (Microsoft) was used.



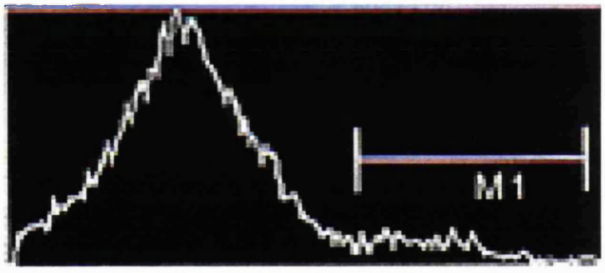


Figure 2.5: Indicative FACS analysis is spleen sample. The samples were stained with three different markers 1, 2 and 3, each one emitting a signal in the different channels FLH1, FLH2 and FHL3 respectively. The cells are first plotted for the FLH1 and FLH2 channels. Then the populations are divided according to their emission in four categories R1: positive for marker 1, R2: positive for both markers 1 and 2, R3: negative for both markers and R4: positive only for marker 2. To find out what is the expression of the 3rd marker at each of these four categories (R1, R2, R3 and R4) an histogram is generated, which for example shows the expression of marker 3 in a cell population positive only for marker 1, as it is shown here. M1: Represents cells that are both positive for marker 1 and 3. The estimations are given as % of total cell population.

Table 2.3: Solutions and reagent suppliers

Solution			Comments
ELECTROPHORESIS			
TBE	5X	54 g/lt Tris base 27.5 g/lt Boric Acid 20 ml/lt 0.5M EDTA	pH 8.0
DNA Loading Buffer	1X	1X TBE 50% v/v Glycerol 0.025% Bromophenol blue	
FA Agarose gel	1.2%	1.2 g agarose RNase free 10 ml FA gel Running Buffer up to 100 ml with DEPC treated ddH_2O 1.8 ml Formaldehyde 37% 1 µl Ethidium Bromide 10 mg/ml	The agarose mixed with the FA gel Running Buffer and the water was heated upon melting. Then the mixture was left to cool down and the rest of the ingredients were added. Prior to running the gel was equilibrated in 1X FA gel Running Buffer for 30 min
FA Buffer	10X	200 mM MOPS 50 mM Sodium acetate 10 mM EDTA	pH 7.0 with NaOH For 1 lt of 1X: 100 ml FA gel Running Buffer 10X 20 ml Formaldehyde
RNA Loading Buffer	5X	16 µl saturated Bromophenol blue 80 µl 0.5 M EDTA pH 8.0 720 µl Formaldehyde 37% 4 ml 50% Glycerol 1.084 ml Formamide 4 ml 10X FA gel Running Buffer DEPC treated H_20 up to 10 ml	Keep at 4°C for 1 month
DNA EXTRACTION			
Tail Genomic Extraction Buffer		50 mM Tris-HCl pH 8.0 100 mM EDTA 100 mM NaCl 1% SDS	
Simplified Mammalian Lysis Buffer		100 mM Tris-HCl pH 8.5 5 mM EDTA 0.2% SDS 200 mM NaCl 100µg Proteinase K/ml	
QIAGEN MIDI AND MAXI PREPS			
P1 (Resuspension Buffer)		50 mM Tris pH 8.0 10 mM EDTA 100 µg/ml RNase	Store at 4°C after addition of RNase A

P2 (Lysis Buffer)		0.2M NaOH 1% SDS	RT
P3 (Neutralisation Buffer)		3 M Potassium acetate pH 5.5	RT or 4^0C
QBT(Equilibration Buffer)		0.75 M NaCl 50 mM MOPS pH7.0 15% Isopropanol 0.15% Triton X-100	RT
QC (Wash Buffer)		1 M NaCl 50 mM MOPS pH 7.0 15% Isopropanol	RT
QF (Elution Buffer)		1.25 M NaCl 50 mM Tris-HCl pH 8.5 15% Isopropanol	
PLASMID DNA MINI PREP			
Buffer I		50 mM Tris pH 8.0 10 mM EDTA	Fresh
Buffer II		0.1 N NaOH 1% SDS	Fresh
Buffer III		3M Potassium acetate pH 5.5	Fresh
SOUTHERN ANALYSIS			
Depurination Buffer		0.25 M HCl in H_2O	Prepare fresh each time
Denaturation Buffer		0.4 N NaOH in H_20	
Transfer Buffer		0.4 N NaOH in H_2O	
Hybridisation solution		0.25 M Na_2HPO_4 pH 7.2 1 mM EDTA 1% BSA FRACTION V 7% SDS	
Wash Solution		0.2 M Na_2HPO_4 pH 7.2 1 mM EDTA 1% SDS	
NORTHERN ANALYSIS			
Prehybrydisation/Hybridisation Solution		50% (v/v) Formamide 5X SSC 5X Denhardt's Solution 1% (w/v) SDS	Add 100µg/ml denatured salmon sperm DNA just before use.
SDS	10%	100 g/lt SDS	Dissolved at 65°C pH 7.2
SSC	20X	175.3 g/lt NaCl 88.2 g/lt Sodium Citrate	pH 7.0 sterilised by autoclaving
BACTERIAL CELL CULTURE			
LB medium		10 g Tryptone 5 g Yeast Extract 5 g NaCl. Add H_2O to 1 lt	Adjust pH 7.5 with NaOH 10 N. Sterilise by autoclaving. Cool down before adding any antibiotics
LB Agar		15 g Agar in 1 lt LB medium	Autoclave. Cool down before adding any antibiotics.
SOC		20 g/lt Bactotryptone 5 g/lt Yeast Extract 0.5 g/l NaCl 200 mM sucrose	

Antibiotics	STOCK	Ampicillin 100 mg/ml in H_2O Tetracyclin 10 mg/ml in 80% EtOH Store at -20^0C	Working concentartions: Amplicillin 100 µg/ml Tetracyclin less than 10 µg/ml When tetracyclin has been added the plates should kept in dark
IPTG	0.1 M	1.2 g IPTG in 50 ml H_2O	Sterilise by filtration. Aliquot and keep at 4^oC
X-GAL STAINING			
Fixative	4%	4 g PFH 2 mM $MgCl_2$ 1.25 mM EGTA 100 ml 1X PBS	pH 7.2-7.4. Store at 4^oC in the dark.
Rinse Buffer		5 mM EGTA 0.01% Deoxycholate 0.02% NP_{40} 2 mM $MgCl_2$ in 1X PBS	
Staining Buffer		5 mM $K_3FE(CN)_6$ 5 mM $K_4Fe\ (CN)_6$ 5 mM EGTA 0.01% Deoxycholate 0.02% NP_{40} 2 mM $MgCl_2$ 1 mg/ml Xgal	Keep at 4^oC or RT protected by light
Xgal	40 mg/ml	Diluted in Dimethyformamide	
IMMUNOHISTOCHEMISTRY			
Citrate Buffer	10 lt	29.4 g Sodium Citrate 54 ml 1 M HCl H_2O up to 10 lt	Adjust pH to 6.0 with 1 M HCl
TBS	1X	80g NaCl 6.05 g Tris Base 44 ml 1 M HCl ddH2O up to 1lt adjust pH to 7.4 with 1 M HCl	
TBS-T	1X	TBS+0.05%Triton X	
CELL CULTURE			
Fibroblast Culture Medium	1X	430 ml DMEM 50 ml FCS 5 ml 10X Monoaminoacids 5 ml Glutamine 1 ml β -Mercaptoethanol 5 ml Sodium Pyruvate 5 ml Penicillin, Streptavidin	All solution were the standard culture media from GIBCO®
GENERALLY USED SOLUTIONS			
$PBS^{-Ca+2, Mg+2}$	10X	80 g NaCl 2 g KCl 14.4 g Na_2HPO_4 2.4 g KH_2PO_4 ddH_2O to 1lt	Adjust pH to 7.4 (7.2 if used for tissue culture). Autoclave.
PBS	1X	$1X\ PBS^{-Ca+2, mg+2}$	Autoclave.

		2 mM $MgCl_2$	
EDTA	0.5 M		pH 8.0 sterilise by autoclaving
TE		10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0	

REAGENT SUPPLIERS

Amersham
Anachem Ltd
BDH
Biological Laboratories Europe Ltd
Boeringer Mannhein
Daco
Dabco
Gibco BRL
Molecular probes
Pharmigen
Promega UK
Santacruz
Sigma Aldrich
Qiagen

CHAPTER 3

RESULTS

BRAIN SPECIFIC CRE RECOMBINASE TRANSGENIC MICE

3.1 ESTABLISHMENT OF NEURON SPECIFIC ENOLASE CRE TRANSGENIC MICE

In order to overexpress the Id4 protein specifically in the nervous system, it was essential to establish transgenic mice that express the Cre recombinase in a neural restricted way. For this purpose, mice were created that express the Cre recombinase under the control of the rat neuron specific enolase promoter (NSE) (Sakimura, Kushiya et al. 1995).

The NSE-Cre construct was microinjected into fertilised oocytes from C57BL/6 X DBA wild type mice. In this way, mice were born, which potentially had integrated the NSE-Cre construct in their genome (transgenic founders). DNA extracted from the tail of putative NSE-Cre transgenic founders was tested for the presence of the transgene, by Southern hybridisation, using Cre specific probe. Nine founders were established (#5, #6, #8, #16, #20, #25, #26, #28 and #30, data not shown) and consequently, each of them was backcrossed with C57BL/6 X DBA mice, in order to create lines of mice, carrying the transgene. Tail genomic DNA from the offspring of the resulting F_1 generations was tested for the presence of the transgene by Southern analysis (Figure 3.1).

Usually, microinjected DNA integrates into a single genomic locus as a multi-copy tandem repeats (head to tail orientation). Nevertheless multiple integration sites or different orientations (head to head or tail to tail) are possible (Keating and Sanguinetti 1996). Southern analysis of genomic DNA, digested with an enzyme that cuts once in the transgene, yields a band with the same size as the injected fragment. Junction fragments of novel lengths from the two ends of the array might also be detectable, if head to tail array integration has occurred. When other types of integration have taken place, the pattern of bands is more complex. This analysis in the F_1 progeny also provides information about the pattern of inheritance for the transgene, number of sites of integration and transgene copy number.

As it is illustrated in Figure 3.1, eleven independent NSE-Cre transgenic lines were generated by Dr Elisa Cinato. All lines transmitted the transgene in a Mendelian fashion, apart from transgenic line #25, which showed reduced numbers of transgenic offspring. Founder NSE-Cre#8 did not transmit the transgene (data not shown). Three lines, (#5, #6, and #16), appeared to have two different integration sites of the transgene, while in transgenic line #28 only partial incorporation of the construct had happened (Figure 3.1). The number of copies of the transgene incorporated, varied between the different lines with highest copy number in line #30. A summary of these results is presented in Table 3.1.

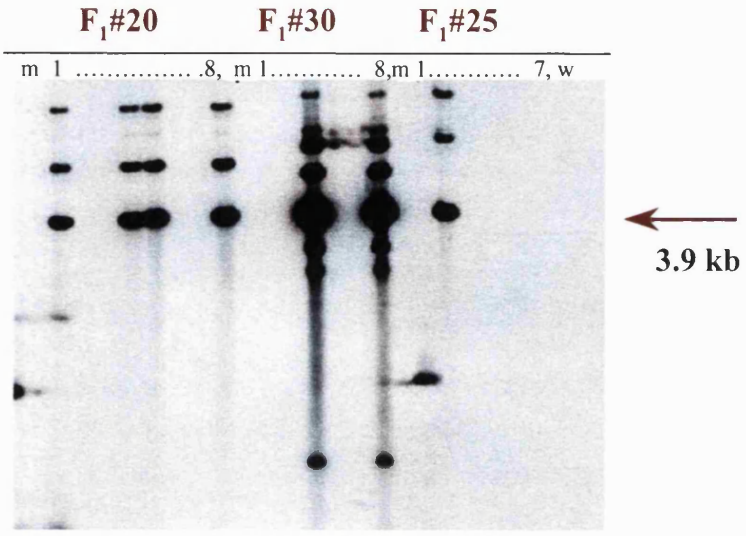
Cre recombinase protein activity in these eleven transgenic lines was determined, by crossing each of them with loxP-LacZ indicator mice (pAMA) (Ayral et al., 1998). In double NSE-Cre#/pAMA transgenic mice, tissues where functional Cre recombinase protein is present, express the β-galactosidase (LacZ) gene, due to Cre mediated deletion of the STOP cassette. In this dual system of expression, Cre mice confer the specificity, whereas indicator mice the intensity of expression.

Tail and tissue genomic DNA, from the crosses of each of the NSE-Cre lines with the pAMA indicator, was assessed by PCR and /or Southern analysis for the presence or absence of Cre and pAMA transgenes (Figure 3.2 and data not shown). Simultaneously, it was determined whether the loxP-flanked TSS fragment was deleted (Figure 3.2 B and C). For the detection of the deletion, pAMA specific primers, (TK and LacZ3) were designed that flank the STOP signals. In case of deletion, a product of 674 bp was generated. The undeleted version of the transgene on the other hand, gave rise to a 1048 bp PCR product. Similarly, Southern analysis using a β-gal gene specific probe detected two different bands of ~5-6 kb and 1.472 kb, representing the deleted and the undeleted version of the transgene respectively (data not shown). The summary of this analysis from all the NSE-Cre transgenic lines is shown in Table 3.1. The results are expressed as the deletion index (intensity of band representing deleted allele / intensity of both bands X 100). This type of estimation is possible, because the pAMA mice carry a single copy of the LacZ transgene. This means consequently, that the levels of deletion are proportionally analogous to the intensity of the DNA band representing the deleted version of the transgene.

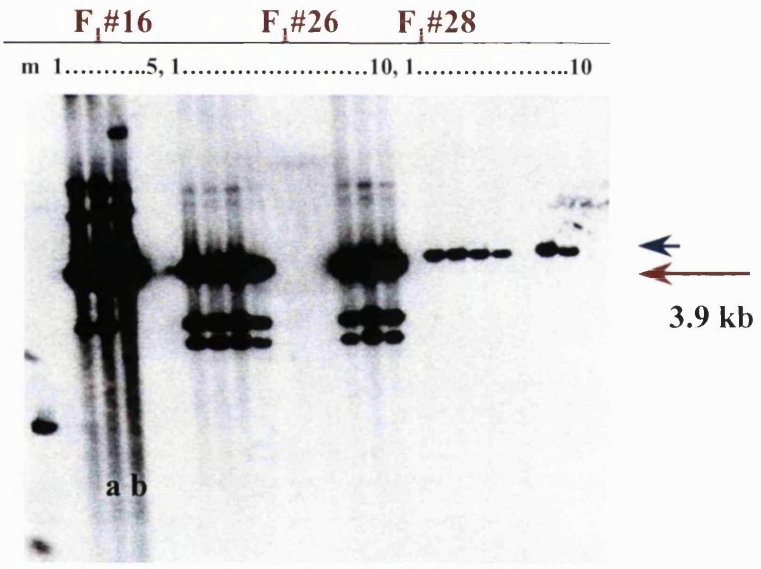
Figure 3.1: Southern analysis in NSE-Cre transgenic founders F_1 progeny for the detection of the NSE-Cre construct.

Tail genomic DNA from the F_1 progeny for each of the founders (F_1#20, F_1#30, F_1#25, F_1#16, F_1#26, F_1#28, F_1#6, F_1#5) was subjected to restrictive digestion with the EcoRV enzyme, (cuts once in the transgene construct), and transferred into a nylon membrane. Southern analysis using Cre specific probe revealed the mice that had inherited the NSE-Cre transgene (3.9 kb indicated by the red arrow). Founders #5, #6, and #16 transmitted two sites of transgene integration, as it was indicated by the different patterns of the transgenic specific bands (compare: F_1#16a and b, F_1#6a and b, F_1#5a and b). Founder 8 showed only aberrant integration of the transgene. This is visualised by the single aberrant band (blue arrow). Finally, eleven transgenic lines were identified (5a, 5b, 6a, 6b, 16a, 16b, 20, 25, 26, 28, 30). 1,2,3…: number of mouse; m : DNA ladder; +: digested plasmid DNA from pNSE-Cre plasmid; w: water as negative control.

A


F1#20
F1#30
F1#25
m 1 ..... .8, m 1..... 8,m 1..... 7, w
3.9 kb

B


F1#16
F1#26
F1#28
m 1.....5, 1.....10, 1.....10
a b
3.9 kb

C

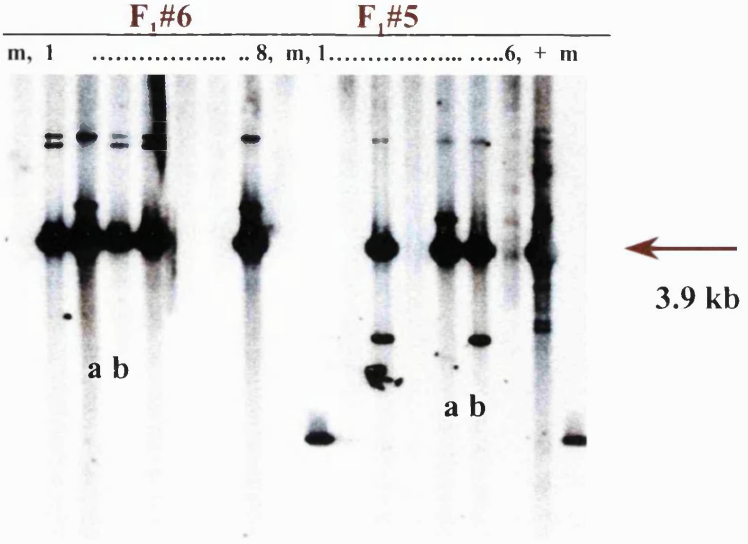
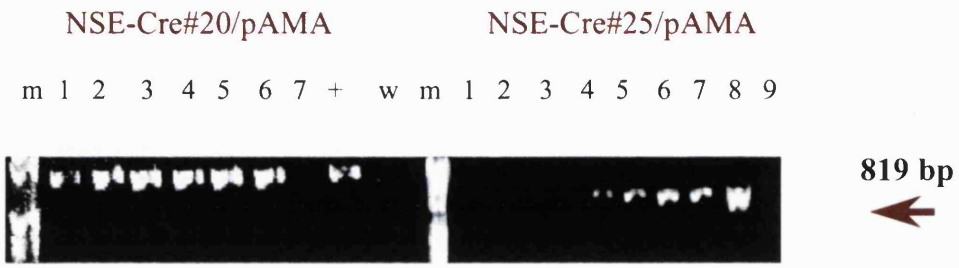

F1#6
F1#5
m, 1 .... 8, m, 1..... .....6, + m
a b
a b
3.9 kb

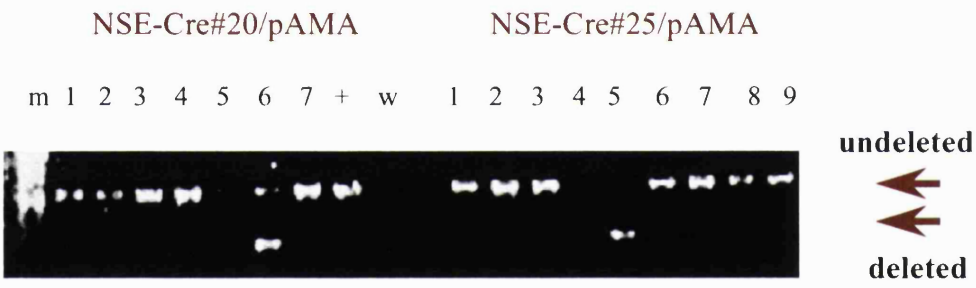
Figure 3.2: Indicative PCR analysis for the detection of the Cre mediated deletion in tail and tissue genomic DNA from of NSE-Cre#/pAMA progeny.

A) PCR in tail genomic DNA for the detection of the presence of the NSE-Cre transgene in the NSE-Cre#20/pAMA and NSE-Cre#25/pAMA mice. All mice are heterozygous for the pAMA transgene. B) PCR detection of the Cre mediated deletion in tail genomic DNA from NSE-Cre#20/pAMA and NSE-Cre#25/pAMA mice. Specific TK and lacZ3 primers can distinguish, between the undeleted (1048 bp) and the deleted (674bp) version of the pAMA transgene. Deletion was detected in the tail of some mice (note NSE-Cre#20/pAMA$^{\#6}$ and NSE-Cre#25/pAMA$^{\#5}$) C) PCR detection of the Cre mediated deletion in tissue genomic DNA from NSE-Cre#20/pAMA$^{\#6}$ and NSE-Cre#25/pAMA$^{\#5}$ double transgenic mice. In some cases, (#20) levels of deletion varied between different tissues of the same mouse (compare b_6 with l_6). D) Outline of the PCR analysis, for the detection of the deletion of the STOP signal. 1, 2, 3... number of mouse. b : brain; l : liver; m: DNA ladder. The examples illustrated here, are representative of the analysis conducted in all NSE-Cre transgenic lines. Similar results were also provided by Southern analysis (data not shown).

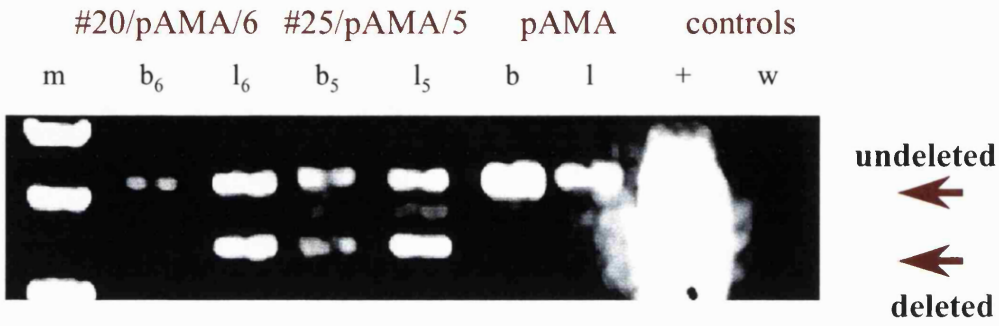
A **Cre PCR in tail genomic DNA**



B **TK- LACZ3 PCR in tail genomic DNA**



C **TK-LACZ3 PCR in tissue genomic DNA**



D

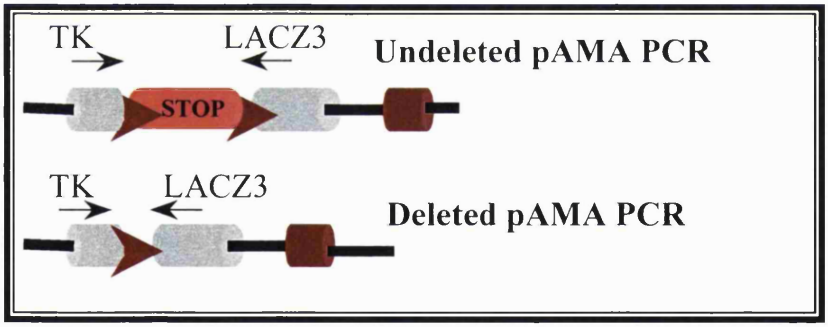


Table 3.1: Summary of the analysis in the NSE-Cre#/pAMA transgenic lines. Deletion level of the TSS, (δ=deletion), estimated as intensity of band representing deleted allele/ (intensity undeleted allele+intensity undeleted allele) X 100. Tissues tested include liver, kidney and in some cases spleen or testis. nt: not tested. *In case of line NSE-Cre#26 multiple tissues were tested (see below for details).

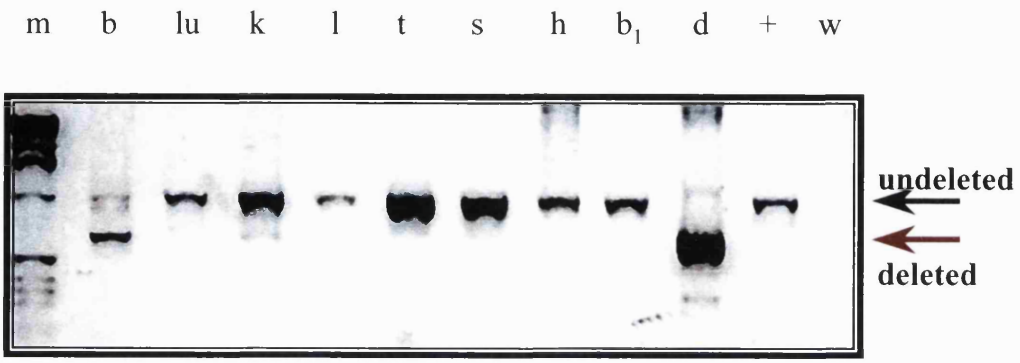
Founder	line	No of Copies	No of mice tested	NSE-Cre/ pAMA Deletion index %		
				tail	brain	Other tissue
5	5a	4	1	10%	10%	10%
	5b	4	1	no δ	no δ	no δ
6	6a	6	1	50%	no δ	no δ
			2	70%	nt	Nt
	6b	6	4	70%	no δ	no δ
			1	100%	100%	100%
			1	no δ	no δ	no δ
16	16a	3	1	100%	no δ	no δ
			1	10%	nt	Nt
	16b	3	1	50%	nt	n t
20		17	1	60%	60%	60%
			6	no δ	nt	Nt
			1	no δ	no δ	no δ
25		5	1	30%	30%	30%
			1	100%	nt	Nt
			7	no δ	no δ	no δ
26		10	13	no δ	δ	no δ*
28		aberrant	6	no δ	no δ	no δ
30		70	2	100%	nt	Nt
			2	80%	80%	80%

As it is demonstrated above, line #28 had no deletion in any of the tissues tested and presumably did not express Cre recombinase. This is in accord to the observation that only partial integration of the NSE-Cre transgene had occurred in this line (Figure 3.1). On the other hand, transgenic line NSE-Cre #30 showed high levels of deletion (80%-100%) in every tissue tested. This widespread and strong levels of Cre mediated deletion suggests that this line has the potential to act a deleter strain. A deleter strain is a Cre transgenic line that excises loxP sites flanked DNA at high levels and in every tissue of the embryo and /or adult mouse. In the majority of all the other lines, (#5, #6, #16, #20, #25) moderate deletion or no deletion at all, were detected in all organs examined. The index of deletion was variable not only among the different lines, but also among littermates. Moreover, variegated deletion was observed in different tissues of the same mouse. Only in the case of line #26, deletion of the loxP-flanked gene occurred in a CNS specific way (Figure 3.3).

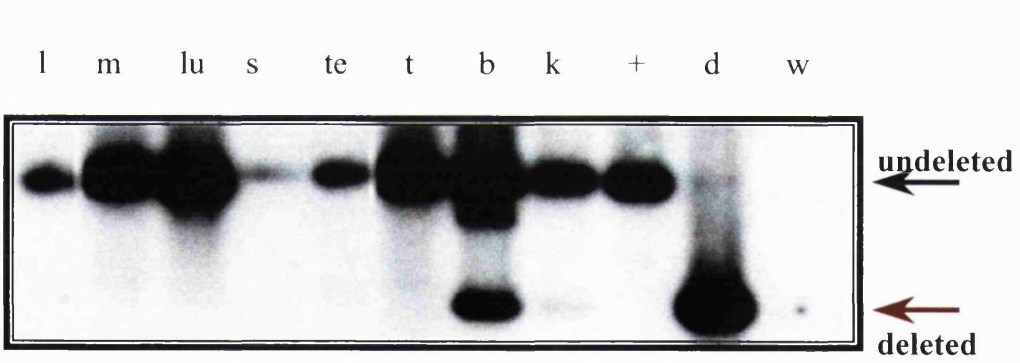
Figure 3.3: Cre mediated deletion is restricted to the central nervous system in NSE-Cre#26 transgenic mice.

A and B: tissue DNA from two different NSE-Cre#26/pAMA double transgenic mice. Amplification of the undeleted and the deleted version of the transgene gave rise to fragments of 1048 bp, (black arrow), and 674 bp, (red arrow), respectively. High levels of deletion were detected in the brain (b), but no other tissue, with the exception of kidney (k) and upper parts of spinal cord (data not shown). The DNA was analysed for Cre mediated deletion of the TSS by PCR. A is an inverted image of PCR analysis. B is Southern analysis of the PCR with LacZ specific probe. m: DNA ladder, lu: lung; l: liver; t: thymus; s: spleen; h: heart, muscle; te: testis; b_1: brain from pAMA mouse, as control for the undeleted version of the transgene; d: genomic tail DNA control from delta-pAMA (mice carrying the deleted version of the transgene); +: tail genomic DNA from NSE-Cre#26/pAMA mouse; w: water.

A


m b lu k l t s h b_1 d + w
undeleted
deleted

B


l m lu s te t b k + d w
undeleted
deleted

3.2 ANALYSIS OF THE NEURON SPECIFIC ENOLASE-CRE#26 MICE

Further analysis in the NSE-Cre#26 line, confirmed the brain specific Cre expression, with also some very low activity in the kidney and the spinal cord. The results of the analysis of the NSE-Cre#26/pAMA mice are summarised in Table 3.2. A few mice (around 15% of all mice tested) exhibited low levels of Cre mediated deletion in the tail (data not shown).

Table 3.2: Analysis of NSE/Cre#26/pAMA double transgenic mice. b: brain, l: liver, s: spleen, t: thymus, k: kidney, h: heart, lu: lung, mu: muscle, sp: spinal cord, te: testis; nt: not tested; + : deletion, -: no deletion; F: female; M: male, nt: not tested.

Mice #	sex	B	l	s	t	k	h	lu	mu	Sp	Te	tail
1	F	+	-	-	nt	nt	nt	nt	nt	nt	nt	-
2	F	+	-	-	nt	nt	nt	nt	nt	nt	nt	-
3	F	+	-	-	nt	nt	nt	nt	nt	nt	nt	-
4	F	+	-	-	nt	nt	nt	nt	nt	nt	nt	-
8	M	+	-	-	-	+	-	-	-	+	-	-
9	M	+	-	-	-	+	-	-	nt	+	-	-
10	M	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+
11	F	+	-	-	-	+	-	-	nt	nt	nt	-
13	F	+	-	-	-	+	-	-	nt	nt	nt	-
19	F	+	-	-	-	+	-	-	-	+	-	-
23	F	+	-	+	-	+	-	nt	nt	nt	nt	-
24	F	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+
26	M	+	nt	nt	nt	+	nt	nt	nt	nt	-	-

The level of Cre mediated deletion in the brain varied among different mice and extended from very low, up to 50% (Figure 3.4). Moreover, PCR analysis of various parts of the adult brain indicated that Cre-mediated TSS deletion occurred throughout the brain, albeit to various extent. Highest Cre activity was observed in the cortex and olfactory bulb, whereas brainstem and cerebellum showed only low level of TSS deletion (Figure 3.5).

Furthermore, whole mount X-gal staining of adult brain from NSE-Cre#26 crossed with another indicator line; the plnLacZ13 (2.1.1), confirmed these results. The X-gal staining method is based on visualisation of the β galactosidase expression in whole tissues or sections by staining with its chromogenic substrate, X-gal. When this reaction takes place, cells showing β-galactosidase expression are stained blue. Brains from double transgenic NSE-Cre#26/plnLacZ13 mice exhibited strong blue staining in cerebral cortex, olfactory bulb and a specific area within the temporal lobe (Figure 3.6). In addition, X-gal staining of sections of adult brain, revealed high level of LacZ expression in defined areas, such as the hippocampus, subiculum, the cortex, and the subventricular zone of the anterior horn of the lateral ventricle (Figure 3.7). LacZ expression was also observed, although to a lesser extent, in the medial and lateral septal nucleus, amygdaloid area and the striatum (Figure 3.7).

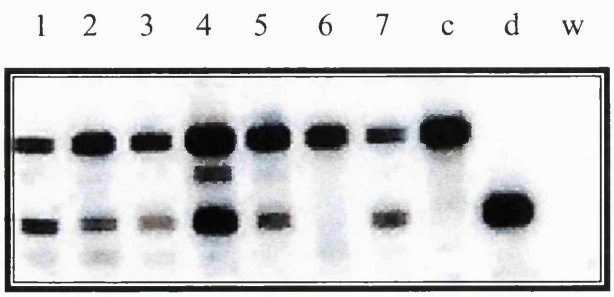
In the cortex, blue stained cells were found in distinct areas, such as the perirhinal, the piriform and auditory cortex. As it is illustrated in Figure 3.7, the levels of expression were variable among different mice, however the expression pattern was consistent in all of them. LacZ expression was undetectable in the cerebellum, an area where PCR had indicated low levels of deletion. This is due to the incapacity of adult plnlacZ13 mice to express the LacZ transgene in this area, as it was revealed by whole mount X-gal staining in adult brains from crosses of plnLacZ13 mice with the deleter strain NSE-Cre #30 (Figure 3.6 E).

Figure 3.4: Detection of Cre mediated deletion in DNA from the brain of double NSE-Cre#26/pAMA mice.

The levels of deletion were variable (table), even between littermates. Lanes 1-7; lanes 1-4 and 5-7 represent littermates. c: DNA from pAMA mouse brain; d: tail genomic DNA from delta pAMA mouse (mouse with the deleted version of the pAMA transgene); w: no template. Arrows indicate the bands derived from the undeleted and the deleted transgene.

Figure 3.5: Cre mediated deletion of the TSS is widespread in brain.

Southern analysis of PCR for the detection of the deletion in different areas of the adult brain from NSE-Cre#26/pAMA mouse (#5 in Figure 3.4). Deletion could be detected in every area tested, although the levels variegated. Cortex and olfactory bulb seemed to have the highest levels of deletion, as estimated by semiquantitative analysis of the bands signals (data not shown). th: thalamus; c: cortex; cer: cerebellum; fb: frontal area of the brain; ob: olfactory bulb; bs: brainstem; +: genomic DNA from pAMA mice; w: water. The deleted and undeleted specific bands are indicated by arrows.



#	% deletion
1	40%
2	26%
3	6%
4	45%
5	18%
6	6%
7	55%

FIG 3.4

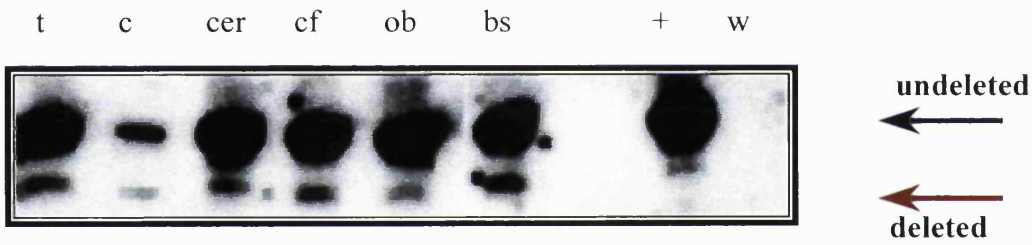
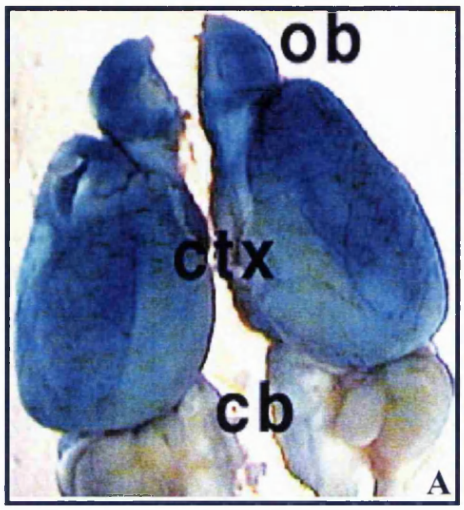


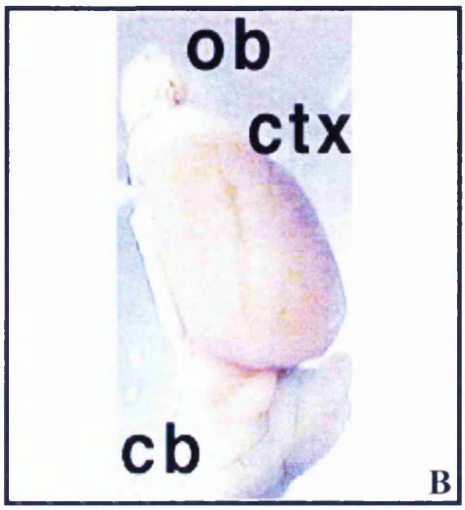
FIG 3.5

Figure 3.6: Strong and widespread Cre mediated LacZ expression in adult brain.

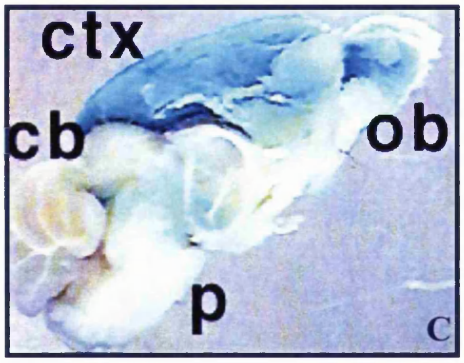
Whole mount X-gal staining of adult brain from double transgenic NSE-Cre#26/plnlacZ13 (A, C) and plnlacZ13 single (B, D) mice. Strong X-gal staining was observed in olfactory bulb (ob) and cortex (cxt). cb (cerebellum), p (pons). A, C: dorsal view; B, D: lateral view. E: whole mount X-gal staining in brain from adult NSE-Cre#30/pAMA mouse as positive control. Cerebellum and medulla did not stain blue.

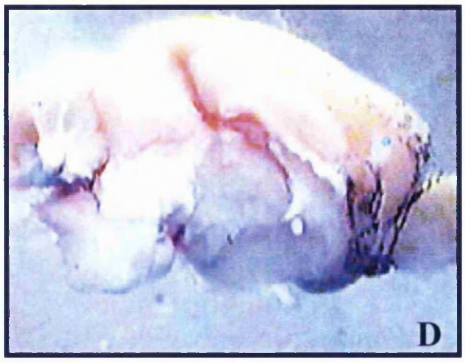
NSE-Cre#26/plnlacZ13 plnlacZ13





Dorsal view





Lateral view

NSE-
Cre#30/plnlacZ13

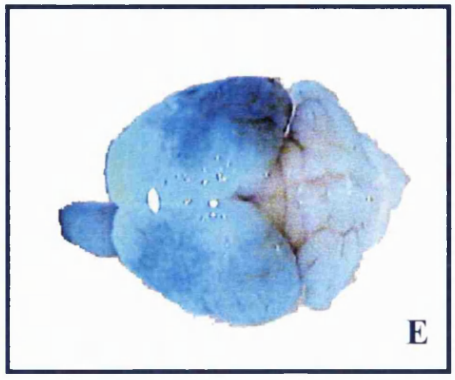
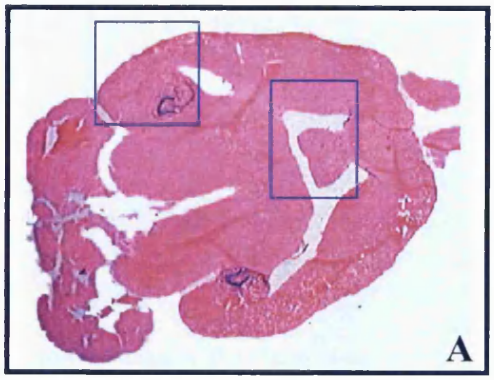
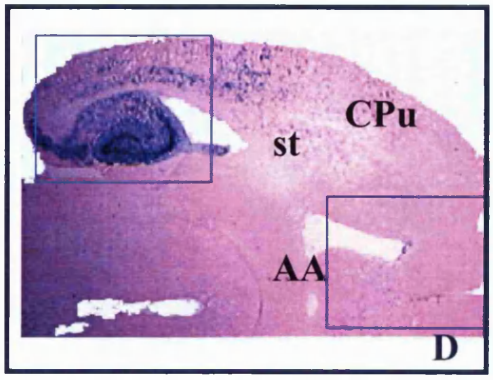
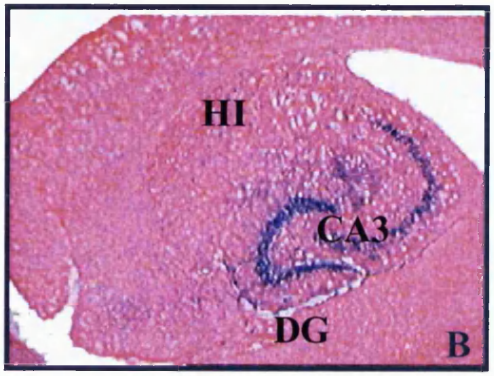


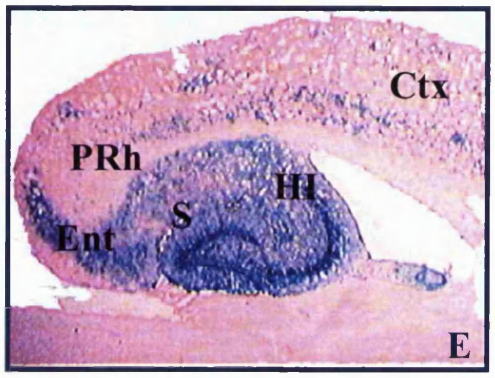
Figure 3.7: High level Cre mediated LacZ expression in hippocampus, cortex and anterior horn of the lateral ventricle.

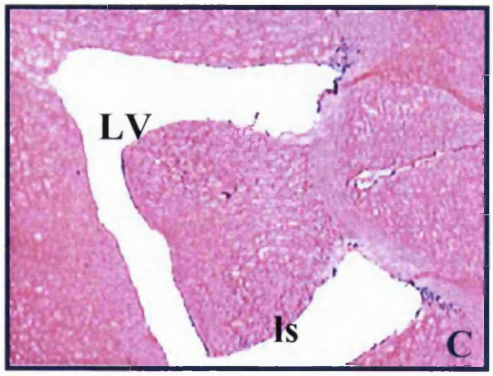
Horizontal brain sections of NSE-Cre #26 /plnlacZ13 double transgenic mice (A-F) and control single transgenic (G). High level of X-gal staining was found in hippocampus, (HI), cortex (cxt) and the anterior horn of the lateral ventricle. Weak expression was also found in the medial (ms), triangular (ts) and lateral (ls) septal nuclei, as well as the caudate putamen (CPu). Some positive cells were detected in the amygdaloid area (AA) and the striatum (st). LV: lateral ventricle; SVZ: subventricular zone; CA3: hippocampus region 3; DG; dentate gyrus, PRh: perirhinal cortex, Ent: entorhinal cortex. B and C as well as E and F are enlargements of A and D as indicated. Sections were counterstained with Eosin.

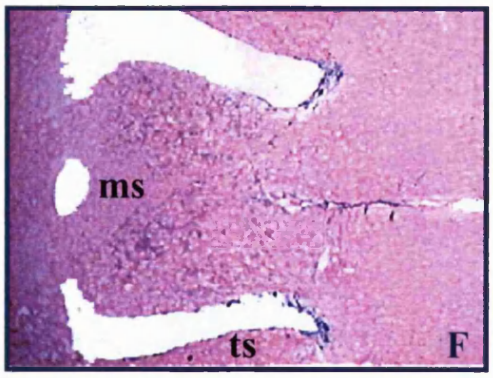

A


CPu
st
AA
D


HI
CA3
DG
B


Ctx
PRh
HI
S
Ent
E


LV
ls
C


ms
ts
F


G

3.2.1 EXPRESSION OF CRE RECOMBINASE DURING MOUSE EMBRYOGENESIS

Once the specificity of the Cre recombinase mediated deletion was established, it was most important to define when and where the Cre recombinase is actually being expressed. The NSE promoter has been reported to confer neuron specific expression as early as 10.5 dpc (Yoshida, Sakimura et al. 1983).

Hence, X-gal whole mount staining of double transgenic NSE-Cre#26/plnlacZ13 embryos at different stages of development, was performed. Littermate plnlacZ13 embryos were used as negative controls. In addition, embryos from crosses of plnlacZ13 mice with the deleter NSE-Cre#30 line were used, as control for the ability of the β-galactosidase transgene to be expressed in every tissue of the embryo, after excision of the STOP signals. This analysis revealed Cre-mediated LacZ activation, as early as 9.5 dpc, coinciding with the onset of neurogenesis, during mouse development (Figure 3.8 I). Moreover, Cre expression was restricted to the developing mouse brain. At stage 9.5 dpc, Cre-mediated LacZ expression was restricted to forebrain, hindbrain and a region along the midline flexure. Similarly, LacZ expression in later stages of embryogenesis was specific to telencephalon, metencephalon, subthalamus, and inferior colliculus and to a region around the pre-optic recess (Figure 3.8 II). Some expression was also being observed in the metamesonephric ridges of the 12.5 dpc embryo after extensive staining; an observation, which could explain the low level of Cre mediated deletion observed in the adult kidney (data not shown and Figure 3.3).

Figure 3.8: Cre mediated LacZ expression is restricted to the developing brain.

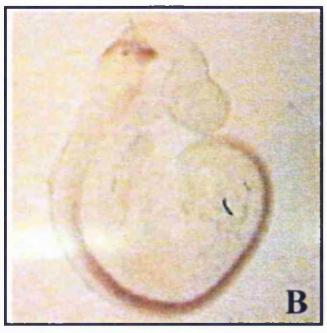
I) Whole mount X-gal staining of embryos at different developmental stages. A, C and E: Double transgenic NSE-Cre#26/plnlacZ13 embryos at 9.5 (A), 11.5 (C) and 13.5 (E) days post coitus. B, D, and F: Littermates control single plnlacZ13 embryos. High levels of transgene expression were detected in specific areas of the developing brain of double transgenic mice. Note the variegation in expression. G: NSE-Cre#30/plnlacZ13 embryo used as positive control for the X-gal staining. Embryos were genotyped for the presence of the NSE-Cre transgene by Cre specific PCR in DNA from yolk sac (data not shown).

II) A and B: Double transgenic NSE-Cre#26/plnlacZ13 embryos at 13.5 days post coitus. B was stained O/N. C and D Littermates control single plnlacZ13 embryos. At stage 13.5 of development strong X-gal staining was found in specific areas of the brain such as telencephalon (tel), inferior colliculus (ic) and metencephalon (met) (A, C). Some staining also appeared in the subthalamus (SUT) and preoptic recess (POR) after extensive staining (B). Some background staining was only found after extensive O/N staining with X-gal (D). AQ: Aqueduct of Silvius; LV: IV: fourth ventricle.

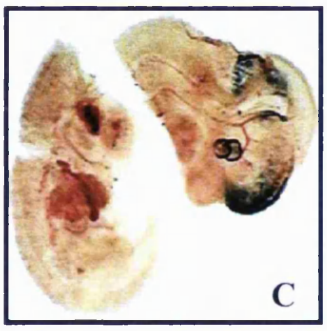
I)

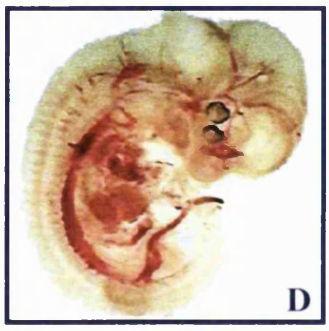
NSE-Cre#26/plnlacZ13

plnlacZ13

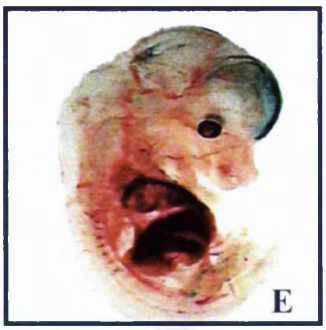


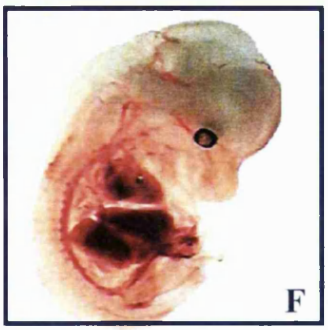
9.5 dpc





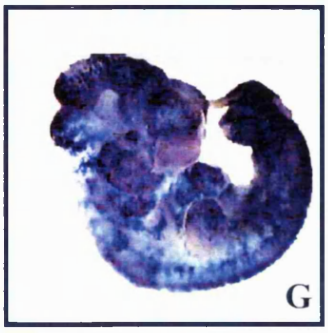
11.5 dpc





13.5 dpc

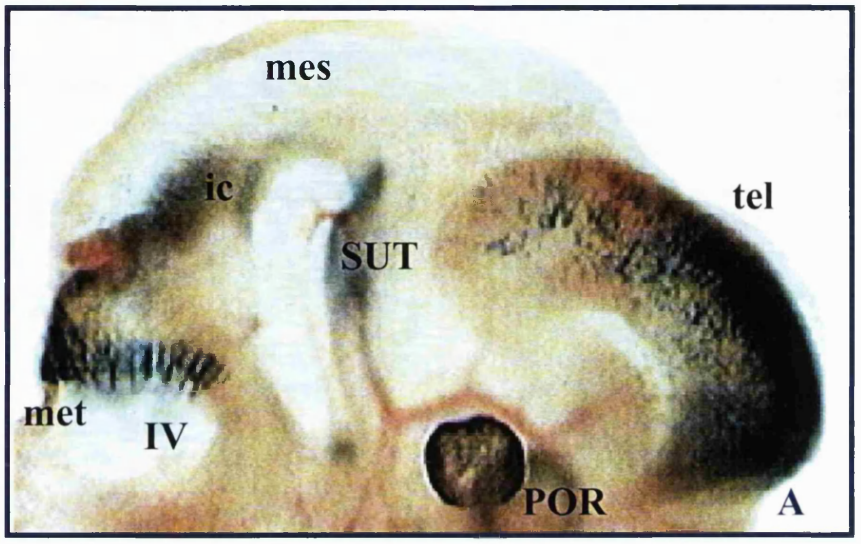
NSE-Cre#30/plnlacZ13

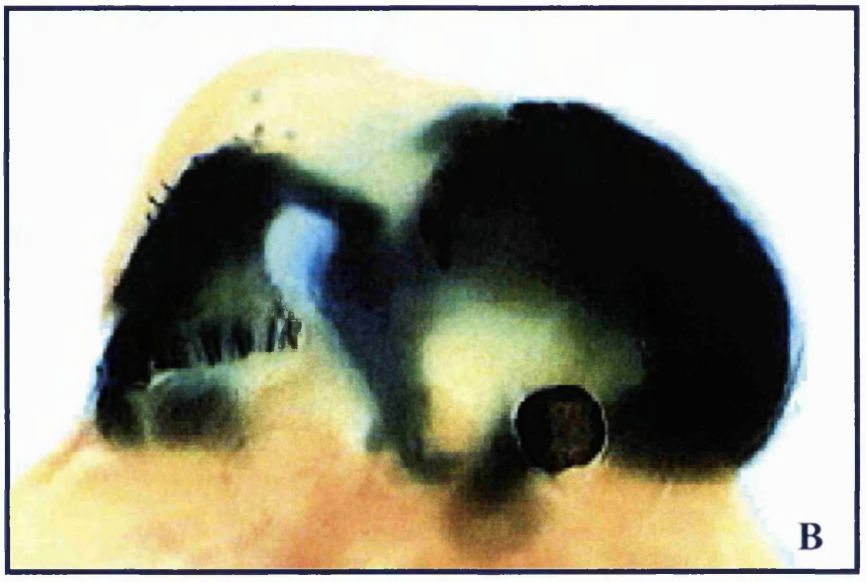


9.5 dpc

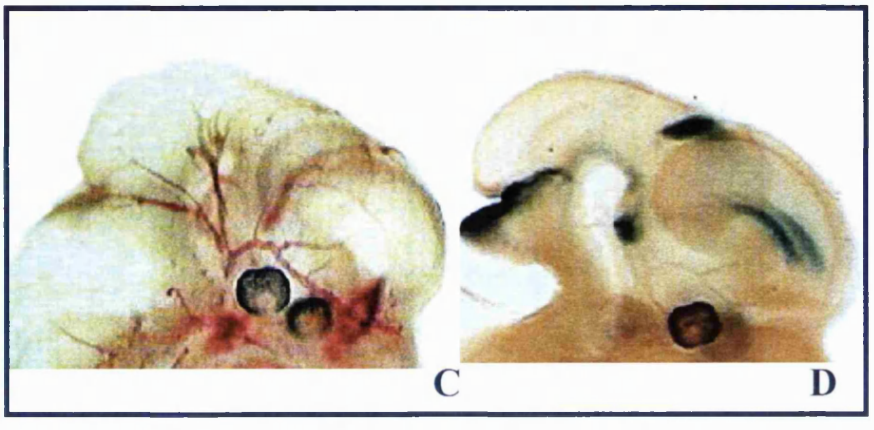
II)

NSE-Cre#plnlacZ13





plnlacZ13



3.2.2 EXPRESSION OF THE CRE RECOMBINASE IN ADULT BRAIN

The NSE promoter has also been reported to continue being active during adulthood (see 2.1.3.1 for details). It was intriguing to find out whether the Cre recombinase is still active in adult brain from NSE-Cre#26 mice. The significance of this is that, if Cre recombinase expression persists during adulthood, it will lead to an accumulation of cells with Cre mediated deletion. If this deletion results in activation of a potential transgene, there will be an enhancement of the levels and possibly the outcome of this overexpression.

RT-PCR analysis of RNA extracted from the brain from different NSE-Cre#26/pAMA double transgenic mice (Figure 3.9) confirmed expression of the Cre recombinase in adult brain. However, as revealed by semiquantitative analysis of the Cre specific signals, the levels of expression varied among different mice (Figure 3.9 and data not presented).

In addition, immunohistochemical analysis with an anti-Cre antibody revealed specific expression of Cre recombinase protein expression in cortex and the cerebellum of the brain of adult transgenic NSE-Cre#26 mice (Figure 3.10). In the cerebellum, Cre was expressed in the Purkinje cells, the neurons adjacent to them and in cells in the granular layer. Some low levels of protein were also detected in the dentate gyrus. These results indicate that the expression pattern of Cre recombinase during adulthood is similar to that during embryogenesis, but somewhat restricted.

Figure 3.9: The NSE transgene is expressed in adult brain of NSE-Cre#26 mice.

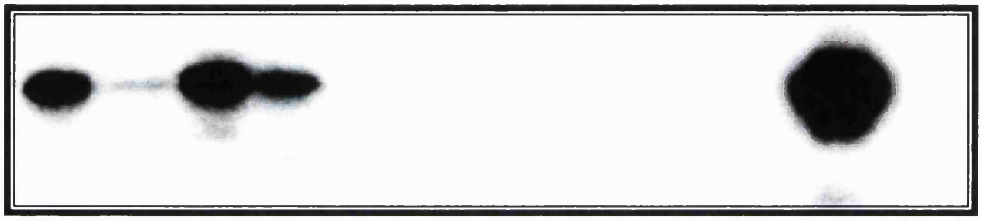
A) Cre recombinase specific RT-PCR (Reverse transcriptase PCR), using brain cDNA from four different transgenic mice (lanes 1- 4). cDNA from nontransgenic brain was used as a control (c). Amplification was performed either in the presence of reverse transcriptase or without. +: Genomic DNA from NSE-Cre#26/pAMA mouse; w: water. B) B actin specific RT-PCR in the cDNA used above, as control for the input of template. Comparison of the signals revealed that the transgene is expressed at variable levels (data not shown).

A

Cre specific amplification

Brain cDNA no Reverse Transcriptase

1 2 3 4 c 1 2 3 4 c + w



B

B-actin specific amplification

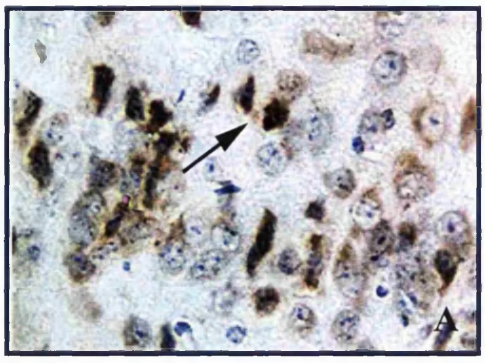
Brain cDNA no Reverse Transcriptase

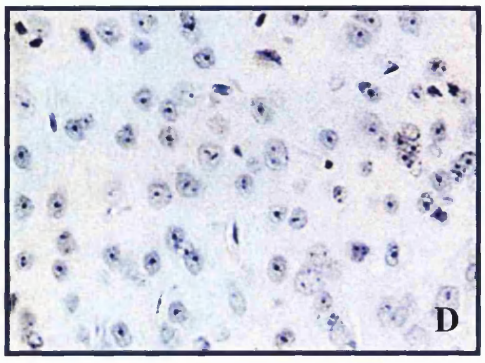
m 1 2 3 4 c 1 2 3 4 c w -

Figure 3.10: Cre recombinase expression in the adult brain.

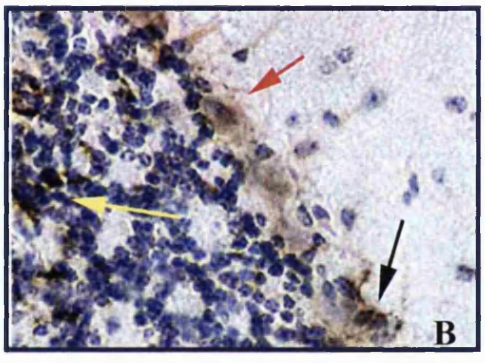
Coronal sections from Cre transgenic (A, B and C) and control littermates wild type mice (D, E and F) were stained with anti-Cre antibody. A, D: cortex. B, E: cerebellum. C, F: Dentate gyrus. Black arrows indicate Cre specific brown staining. In cerebellum, Cre was expressed in the Purkinje cells (red arrow), neurons adjacent to them (black arrow) and cells in the granular layer (yellow arrow). G is a control for the immunocytochemistry; no primary antibody used. A, B and C: X 40 /0.75. D, E, F, and G: X 20 /1.25.

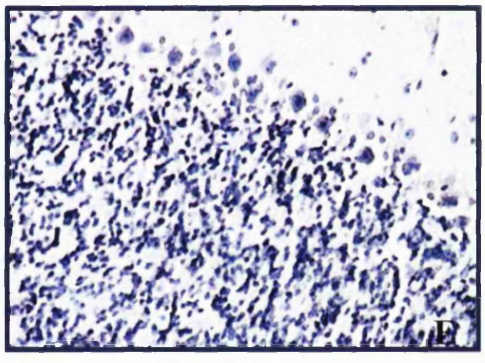
Cortex



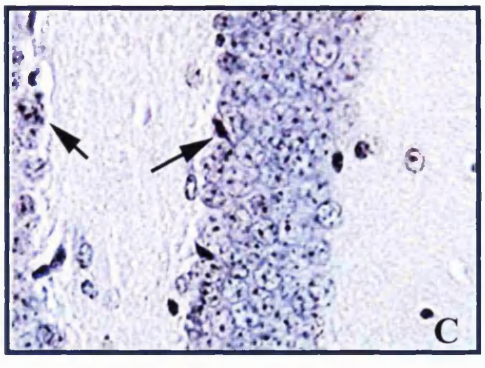


Cerebellum

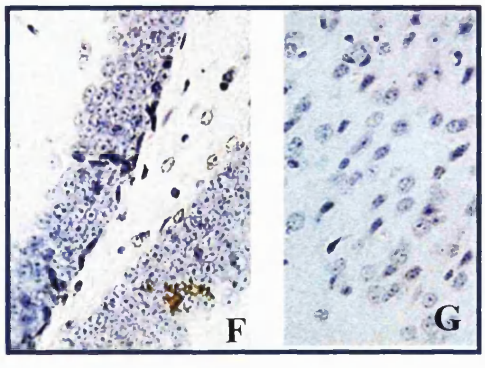




Dentate gyrus



peptide



3.3 SUMMARY

To summarise, from 9 independent founders 11 transgenic mouse lines carrying the NSE-Cre transgene were generated by Dr Elisa Cinato. Surprisingly, characterisation of these mice showed Cre mediated TSS deletion was apparent in tail DNA of double transgenic mice derived from most of the NSE-Cre lines. Further analysis of genomic DNA isolated from multiple tissues of double transgenic mice indicated widespread Cre-activity. These observations suggested that the NSE promoter is not always, as tightly regulated as previously shown, (Forss-Petter, Danielson et al. 1990). This is in line with the results presented by Aluani et al, (1993), who showed that NSE promoter was active in ES cells and preimplantation embryos. (Alouani, Ketchum et al. 1993). An expression window of the NSE promoter early in mouse development would explain our findings of widespread Cre-mediated deletion in the majority of NSE-Cre#/indicator double transgenic lines. In addition, Cre mediated deletion was variable not only in different lines, but also among littermates and tissues of the same mouse. This variegation in the expression is a common phenomenon in transgenic lines and is probably due to *cis* and *trans* elements in the genome, influencing the transgene expression (Robertson, Garrick et al. 1995; Martin and Whitelaw 1996; Garrick, Fiering et al. 1998; Henikoff 1998; Henikoff 2000). In some cases as well, such as in the plnLacZ13 transgenic line, the age of the mouse is important for the transgene expression (Robertson, Garrick 1996).

One transgenic line generated, the NSE-Cre#30, could confer high level Cre mediated deletion throughout the mouse embryo early in development. This line has the potential to act as complete deleter of loxP sites. This can be useful in experiments, where complete overexpression or knocking out of genes is desired.

Most importantly however, of the 11 NSE-Cre transgenic lines tested, one (NSE-Cre#26) exhibited neuron-specific Cre-activity. In these mice, the Cre recombinase started being expressed as early as 9.5 dpc, coinciding with the onset of neurogenesis during mouse development and continued during adulthood, though in a more restricted pattern. At an early stage, Cre-mediated deletion of loxP sites was restricted to forebrain, hindbrain and a region along the midline flexure. At later stages, Cre-mediated LacZ expression was specific to telencephalon, metencephalon,

subthalamus, inferior colliculus and to a region around the pre-optic recess.

In adult mice, with the exception of kidney, which exhibited some very low Cre-activity, the Cre-mediated TSS deletion was restricted to the central nervous system. PCR analysis of various parts of the adult brain indicated that Cre-mediated TSS deletion occurred through out the brain, albeit to various extent. Highest deletion levels were observed in the cortex and olfactory bulb, whereas brainstem and cerebellum showed only low level of TSS deletion. In addition Cre, mediated deletion was detected by X-gal staining in specific structures of the limbic system, such as the hippocampus and the septum, amygdaloid area and perirhinal cortex.

The medium levels of Cre mediated deletion, detected by PCR analysis reflect two possibilities: Either low levels of Cre expression in individual cells or restriction of high Cre expression in specific cells populations. The restricted pattern of Cre mediated deletion, along with the localisation of Cre expression in specific cell population, indicate that Cre protein is expressed in certain areas and cells of the brain. Therefore the medium levels of Cre mediated deletion detected in DNA from whole brain extracts are more likely due to specificity of expression in certain cells types, rather than inefficient Cre protein expression.

In brief, we established transgenic mice that express Cre recombinase in specific areas of the developing and adult mouse brain. Above all, these mice were essential to us to achieve overexpression of the Id4 protein restricted to the brain and thus addressing the question of the role of this protein in the nervous system development and physiology.

Id4 TRANSGENIC MICE

3.4 Id4 TRANSGENIC CONSTRUCT AND ANALYSIS IN CELL LINES

In order to activate the Id4 gene, by employing the Cre/loxP site-recombination system specifically in tissues of interest, it was essential to establish transgenic mice carrying a dormant Id4 gene. In the transgenic construct, generated for this purpose, expression of Id4, is driven by a strong and ubiquitous promoter, which is blocked by TSS situated between the promoter and the Id4 cDNA. The TSS are flanked by loxP sites and can be deleted through Cre/loxP mediated recombination (Figure 3.11).

Initially, the capability of the construct to express the Id4 transcript after Cre mediated recombination, was tested by transfection in a fibroblast-like cell line, (NIH3T3 cells). A plasmid carrying the Ubi-loxP-Id4 transgene (pUbiloxPId4) was cotransfected along with a plasmid carrying the Cre recombinase and the puromycin resistance genes (pCre-pac) in NIH3T3 cells by electroporation. For a positive control, cells were transfected with the deleted version of the Ubi-loxP-Id4 transgene plasmid (pUbi-loxP-Id4 delta). Untrasfected NIH3T3 cells, as well as cells transfected only with the pUbi-loxP-Id4 were used as negative controls. The pUbi-loxP-Id4 construct was linearised, to achieve permanent integration, whereas circular pCre-pac DNA was transfected transiently. In cells, which had incorporated both plasmids (pUbi-loxP-Id4 + pCre-pac) transient expression of the Cre recombinase would result in excision of the Id4 construct's STOP signals, resulting in Id4 transgene expression. DNA and RNA analysis would identify the cells, expressing the Id4 construct after Cre mediated deletion. PCR analysis of the genomic DNA extracted from these cells confirmed deletion, albeit in medium levels (data not shown). In parallel, extracted RNA was subjected to cDNA synthesis. The cDNA was used for PCR analysis with Ubi-loxP-Id4 specific primers that can identify the Id4 transgene specific products and can distinguish between genomic and cDNA templates (Figure 3.11).

Based on the Id4 transgene design, amplification with primers Id4S and SV40R would give rise to two specific transcripts of 1100 bp and 860 bp respectively. As it is illustrated in Figure 12 A, these two transcripts were apparent in the NIH3T3 cells transfected with either the pUbi-loxP-Id4 + pCre-pac or with the pUbi-loxP-Id4 delta,

indicating that the promoter is efficient for driving expression of the Id4. However, the fact that these transcripts also appeared in cells transfected with pUbi-loxP-Id4 plasmid only, suggests that the transcription stop signals are not efficient and allow transcription from the undeleted version of the transgene. Since the deletion of the STOP signal in the pUbi-loxP-Id4 + pCre-pac transfected cells, was not complete, it was not clear, whether the transcripts that appeared, were derived from the expression of the transgene after Cre mediated deletion. To clarify this, PCR analysis with two different primers that flank the loxP sites was employed. This PCR amplification can distinguish not only genomic and cDNA templates, but also the undeleted and the deleted versions of the transgene. Despite the low transcription levels, at least one of the two transcripts expected after Cre mediated deletion was apparent in the cells cotransfected with pUbi-loxP-Id4 + pCre-pac (Figure 3.12 B). One possible explanation of the fact that only one of the transcripts was detected, can be preferential amplification during PCR, or preferential expression of one of these transcripts in NIH3T3 cells.

Figure 3.11: The Ubi-loxP-Id4 transgenic construct.

The human Ubiquitin C promoter was used to drive expression of the Id4 cDNA. However, between the promoter and the cDNA there have been placed TSS that are flanked by loxP sites. In addition, at the end of the construct the splice donor (SD) and splice acceptor (SA) sites of an intron plus polyadelylation signals from the SV40 virus are used to enhance the transcription levels of the transgene. In mice carrying only the Ubi-loxp-Id4 transgene, only a small transcript (#1) should be generated. Still, in case where the transcription stop signal does not work efficiently, two other read-through transcripts are possible (#2 and #3). The first, is the result in splicing using an endogenous SD site from the Id4 and the second if the SD and SA of the SV40 intron are used. Cre mediated deletion results in removal of the TSS leaving a loxP site behind. Transcription after Cre mediated deletion generates two different Id4 transcripts. (#4 and #5). The red arrows indicate the site of transgene specific primers used for PCR amplification. Ubi, neoR, Id4, Id4R, SV40R are identification names for the primers.

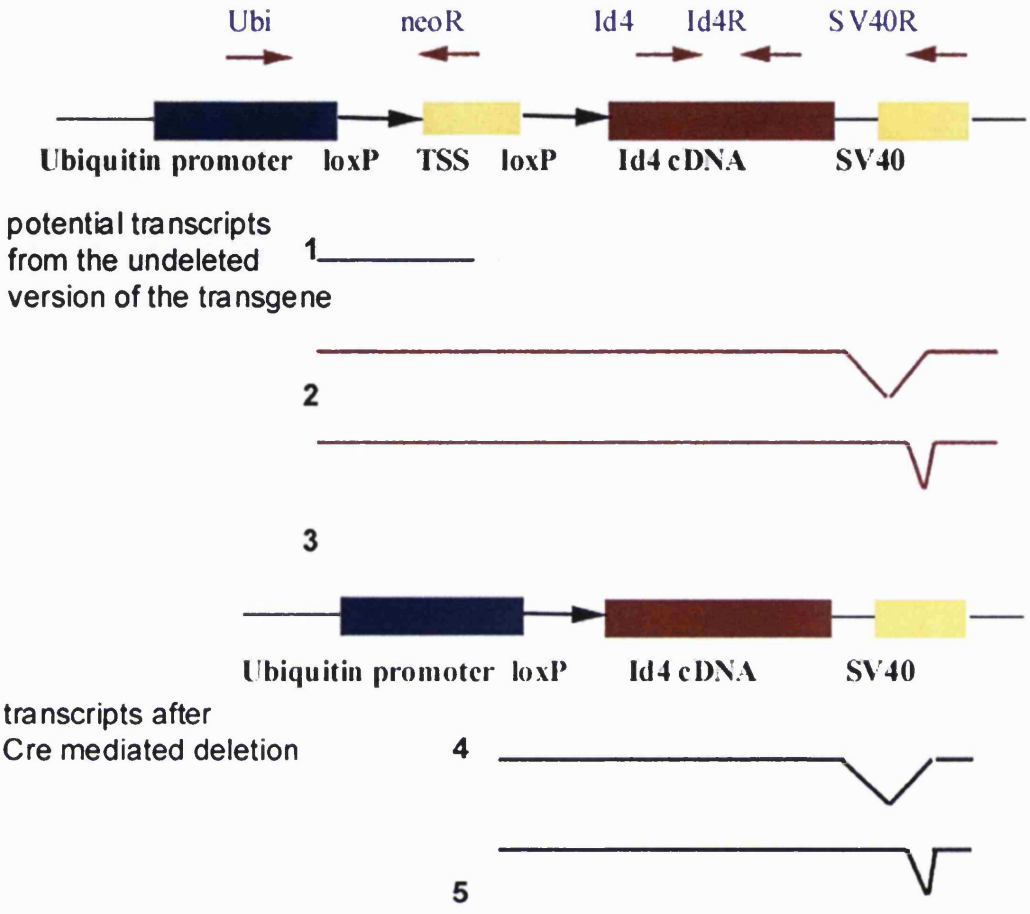
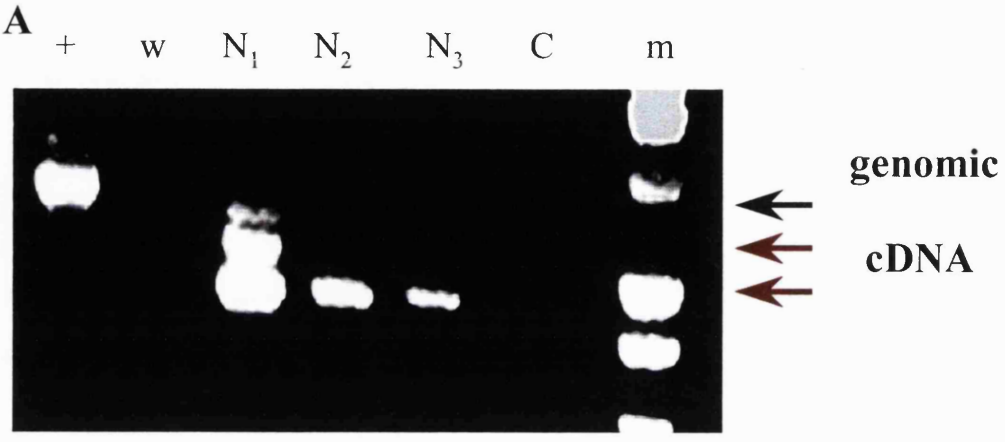

Ubi
neoR
Id4
Id4R
SV40R
Ubiquitin promoter
loxP
TSS
loxP
Id4 cDNA
SV40
potential transcripts from the undeleted version of the transgene
1
2
3
Ubiquitin promoter
loxP
Id4 cDNA
SV40
transcripts after Cre mediated deletion
4
5

Figure 3.12: Transcription of the Id4 transgene in the NIH3T3 cell line.

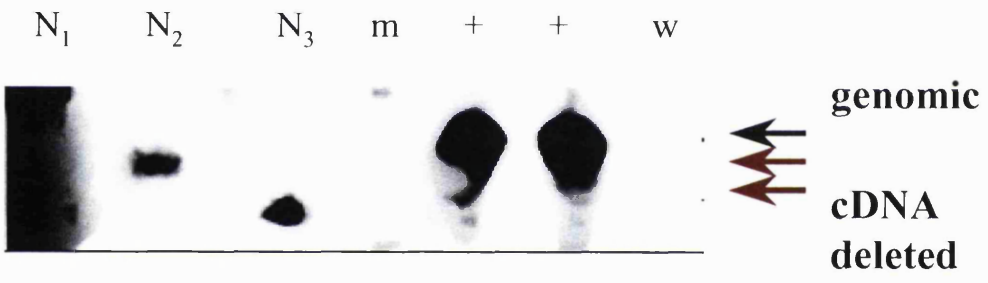
A) NIH3T3 cells were transfected with pUbiloxPId4, pUbi-loxP-Id4 + pCre-pac and pUbiloxPId4delta plasmids (N_1, N_2, N_3 lanes respectively). The pUbiloxPId4 and pUbiloxPId4delta were linearised to achieve permanent transfection whereas the pCre-pac plasmid was transfected transiently. The ratios of plasmids for the pUbi-loxP-Id4 + pCre-pac co-transfection were 1:1.After culture and appropriate selection, RNA was extracted and subjected to RT-PCR analysis using transgene specific primers (Id4- SV40R). Amplification with these primers gave rise to bands of 1180 bp for the genomic DNA template, 1100 bp, and 860 bp for cDNA template (red arrows). As expected cells transfected with pUbi-loxP-Id4 + pCre-pac or pUbiloxPId4delta appeared to have both transcripts. Yet, the smaller transcript was also found in pUbi-loxP-Id4 transfected cell, indicating that readthough transcription of the undeleted version of the Id4 transgene. B) To confirm that the transcripts appearing in A, were because of Cre mediated deletion, cDNA from the cells was subjected to PCR analysis with primers that flank the loxP sites. The outcome of this analysis was run in an Ethidium Bromide gel, which was then blotted. Finally, Southern analysis was performed with Id4 specific probe. This PCR amplification can distinguish not only genomic and cDNA templates, but also the undeleted and the deleted versions of the transgene. Undeleted genomic DNA (+) gave rise to a band of 1.9 kb, while amplifications of transcripts after Cre mediated deletion resulted in two bands of 1.5 and 1.3 kb (black arrows). Both these bands appeared in lanes N_2 and N_3. C) B actin PCR analysis in cDNA generated from the NIH3T3 cells, as control for the cDNA synthesis and genomic contamination. C: Non-transfected cells used as negative control; m: DNA marker.

A



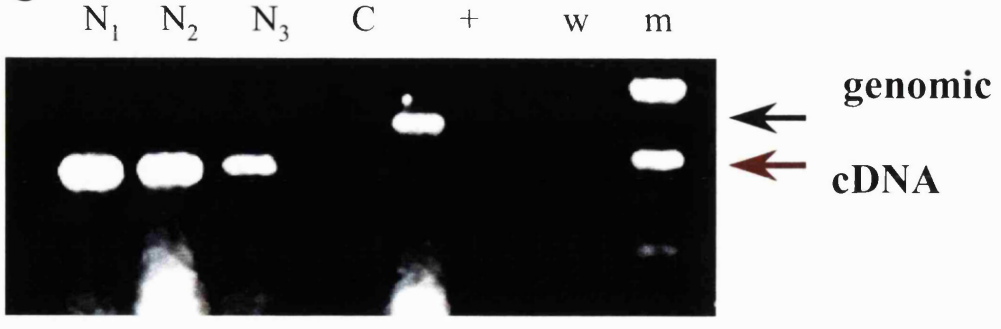
Id4S-SV40R

B



Ubi1-SV40R

C



- actin

3.5 ESTABLISHMENT OF DORMANT Id4 TRANSGENIC LINES

Once it was confirmed that the Id4 construct is capable of conferring efficient expression of exogenous Id4 transcripts in mammalian cells, transgenic mice were generated by oocyte microinjection, the way it has been already described. Two founders were established by this means (Ubi#10 and Ubi#37, data not shown) and consequently, each of them was backcrossed with wt (C57BL/6 X DBA) mice to establish transgenic line hemizygous for the Id4 transgene. Again, these mice were generated by Dr. Elisa Cinato. Tail genomic DNA from the offspring of the F1 generations, resulted from these crosses, was tested for the presence of the transgene by southern analysis and/or PCR. Two Ubi-loxP-Id4 transgenic lines were established, which transmitted the transgene in a Mendelian fashion, without rearrangements of the integration site (Figure 3.13). The estimated transgene copy number was 10-20 copies for both transgenic lines (data not shown).

3.6 ANALYSIS OF THE Id4 TRANSGENIC LINES

In transgenic mice, transcription silencing of the transgene is a common phenomenon attributed to a number of factors. These include, transgene copy number, local chromatin configuration, hypermethylation, site of integration, lineage, as well as cis acting elements within the transgene and the site of integration (Garrick, Fiering et al. 1998; Henikoff 1998). It was essential therefore, to test whether the transgene had been integrated in a site where it could be expressed in every tissue. According to the design of the transgenic construct, in Ubi-loxP-Id4 mice expression of the transgene is initiated, but interrupted at the STOP signal, resulting in the generation of a small size transcript (Figure 3.11).

RT-PCR analysis in different tissues, from F_1 generation Ubi-loxP-Id4 positive mice from both founders, using specific primers confirmed the existence of these small size transcripts in every tissue tested (Figure 3.14). This established that in both founders the transgene had been integrated in a 'permissible' for its transcription genomic region. The Ubi-loxP-Id4 mice hence, have the potential to overexpress the Id4 in any tissue desired.

Figure 3.13: Establishment of two hemizygous Ubi-loxP-Id4 transgenic lines.

Each of the founders Ubi#10 and Ubi#37 was crossed with C57BL/6 X DBA wt mice. Southern analysis with Id4 specific probe revealed that the transgene was transmitted normally. Red arrows indicate the endogenous Id4, while the blue ones refer to 3.2-kb transgene specific band. The other bands observed, are results of the different types of transgene integration. 1, 2… number of mice; +: DNA from wt mouse as control for the endogenous band; m: DNA ladder.

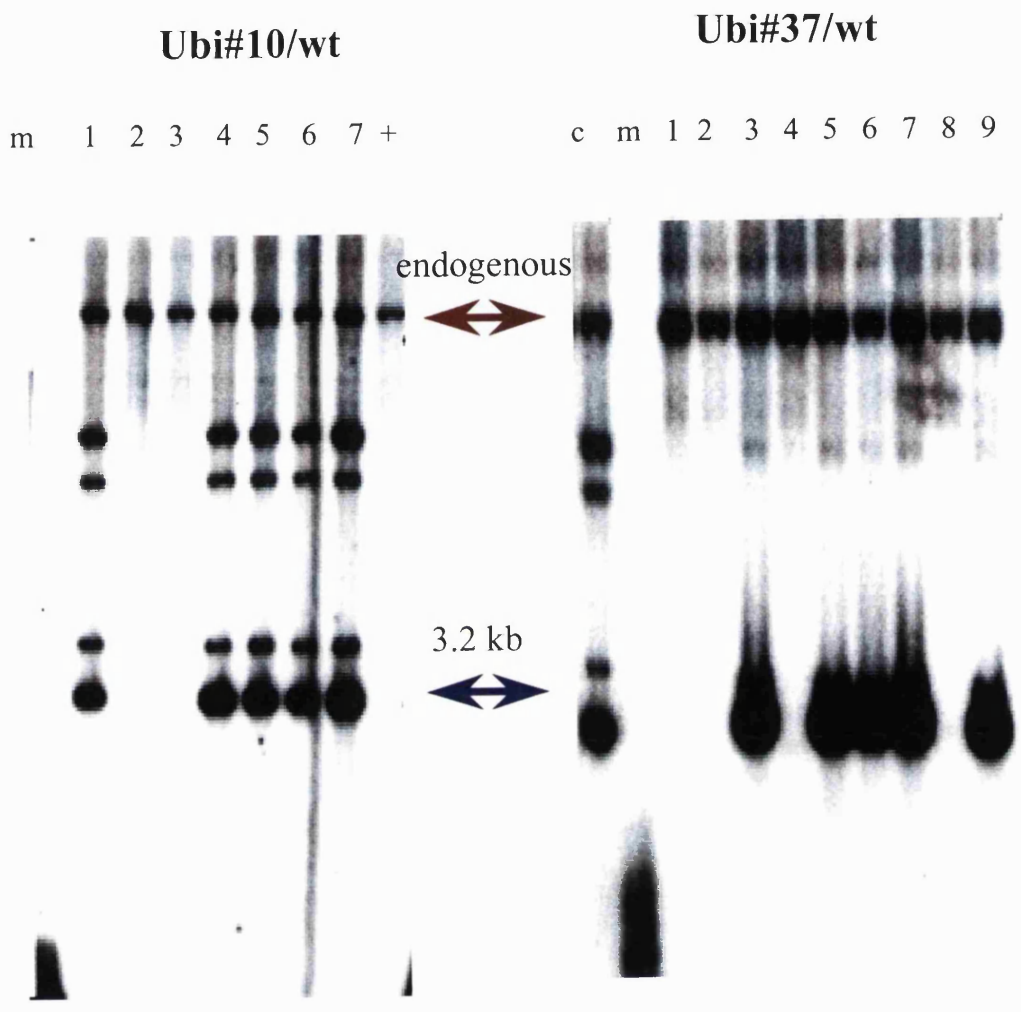
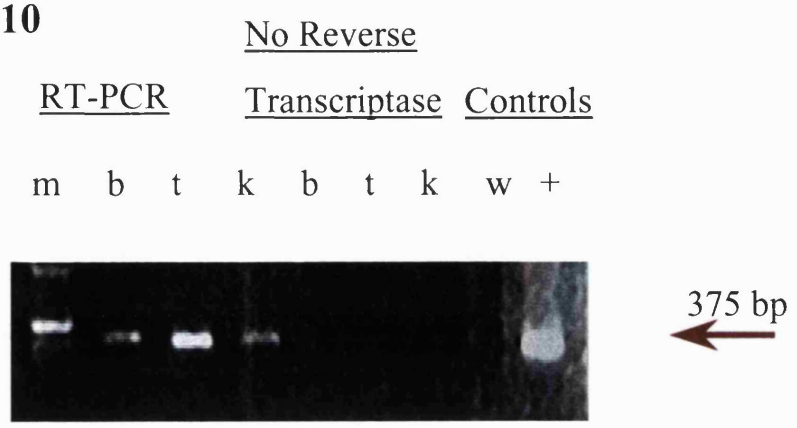

Ubi#10/wt
Ubi#37/wt
m 1 2 3 4 5 6 7 +
c m 1 2 3 4 5 6 7 8 9
endogenous
3.2 kb

Figure 3.14: The Id4 transgene has the potential to be expressed in transgenic mice.

RT-PCR analysis on RNA from different tissues of Ubi#10 (A) and Ubi#37 (B) transgenic mice. Ubi and neoR primers were used for the amplification, (375 bp band). B is Southern analysis in DNA generated from the Ubi-neoR PCR. B-actin PCR (inverted image from Ethidium Bromide stained gel), was used as a control for genomic contamination of the cDNA used in B (data not shown). C is an overview of the transgene and the primers used for the PCR analysis. b: brain; t: thymus, k: kidney, l: liver, h: heart, s: spleen, + genomic DNA, w: water as negative control; No Reverse Transcriptase: cDNA synthesis without reverse transcriptase as a negative control, m: DNA ladder.

A

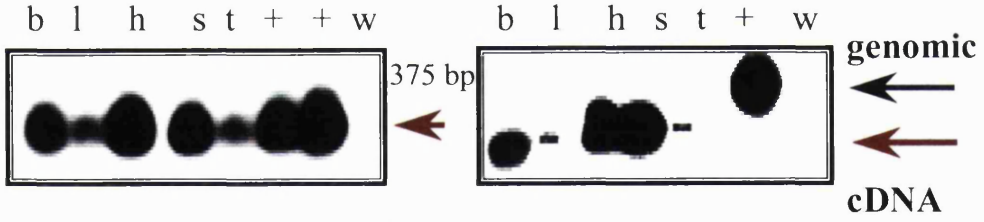
Ubi#10



Ubi2- neoR

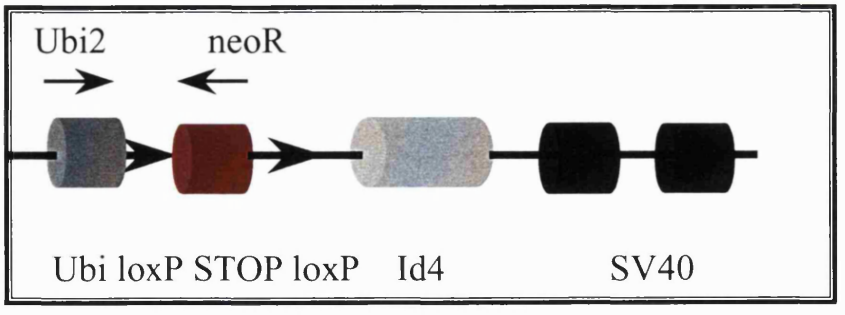
B

Ubi#37



Ubi2- neoR **-actin**

C



To avoid any complications in the interpretation of the results at later stages of our experimental strategy, it was important to ascertain that the Ubi-loxP-Id4 transgenic mice can express the Id4 transgene after Cre mediated deletion. It has been reported that Cre protein recombination can lead to elimination of transgenes carrying loxP sites in inverted orientation (Lewandoski and Martin 1997). Thus, it was essential to instate that recombination does not influence the ability of the Id4 transgene to be expressed. For this purpose, each of the Ubi-loxP-Id4 lines was crossed with NSE-Cre#30 transgenic mice. These mice start expression of the Cre recombinase early in development in a very widespread manner (see 3.1). This means, they can act as total deleter line that potentially can excise the loxP flanked TSS of the Id4 transgene in every tissue, resulting in complete overexpression of Id4 protein in double transgenic Ubi-loxP-Id4#/NSE-Cre#30 mice.

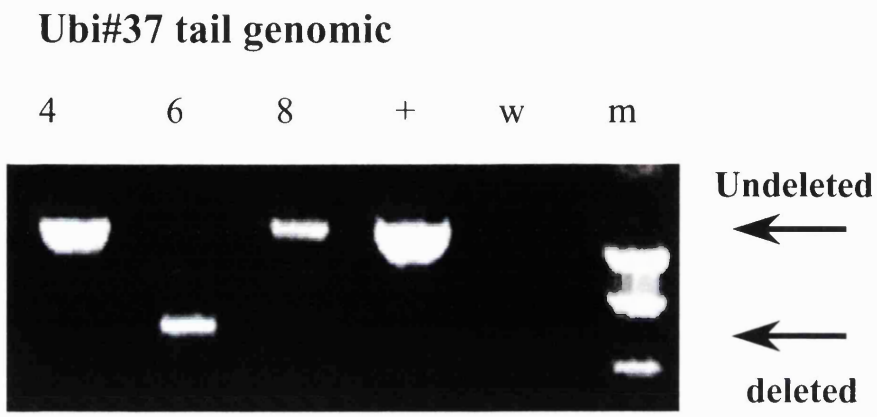
Despite the fact that generating double transgenic UbiloxPId4/NSE-Cre#30 mice was very difficult, finally 3 mice were obtained. These mice were established from the founder #37. Crosses of the founder#10 did not give any offspring of double transgenic Ubi#10/NSE-Cre#30 phenotype. The level of the Cre mediated deletion in the mice obtained, was variable, as it was estimated by PCR in tail genomic DNA (Figure 3.15 A). Further analysis in tissue genomic DNA from the mouse, which appeared to have the higher levels of deletion in tail (#6), confirmed that Cre mediated deletion of the Ubi-lox-PId4 transgene, was almost complete in every tissue tested (Figure 3.15 B).

RT PCR analysis in some of these tissues revealed expression of the transgene specific transcripts (Figure 3.16). Since the level of deletion was almost 100%, it was most likely that these transcripts were generated from deleted version of the Ubi-loxP-Id4 transgene. For this to be established undoubtedly, RNA from some of the tissues (testis and spleen) was subjected to further analysis. PCR with primers that can distinguish between potential transcripts, derived from the undeleted and the deleted version of the transgene, revealed the two specific bands (around 1.3 kb and 1.6kb respectively) (Figure 3.16 C). These bands were unique for the deleted version of the transgene. The specificity of the PCR products was additionally confirmed by restriction digestion (digestion with a variety of restriction enzymes gave rise to products of size as expected from the transgene design-data no shown).

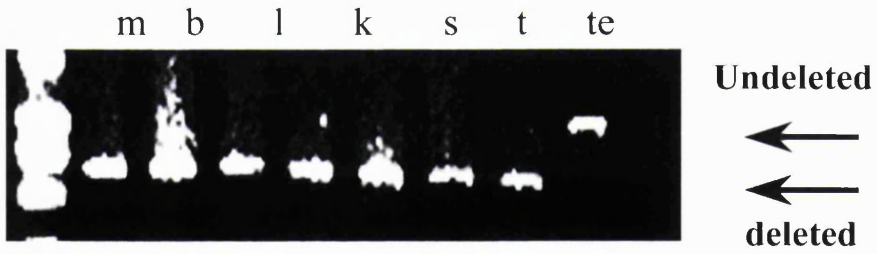
Figure 3.15: Complete Cre mediated deletion of the TSS site in Ubi-loxP-Id4/NSE-Cre#30 double transgenic mice.

A) PCR for the detection of the deletion of TSS (Ubi-Id4R primers) in tail genomic DNA from three Ubi#37/NSE-Cre#30 transgenic mice (# 4, #6, #8). The level of deletion was variable and in case of mouse #6 was complete. B) PCR for the detection of the deletion in tissue genomic DNA from mouse #6. Almost total deletion was observed in every tissue tested. b: brain; l: liver; k; kidney; s: spleen; t : thymus; te: testis; + control genomic DNA from Ubi#37 mouse; w: water; m; DNA ladder; 1072 bp: undeleted; 697: deleted. C) An overview of the PCR analysis design.

A


Ubi#37 tail genomic
4
6
8
+
w
m
Undeleted
deleted

B


Ubi#37/6 tissue genomic
m
b
l
k
s
t
te
Undeleted
deleted

C

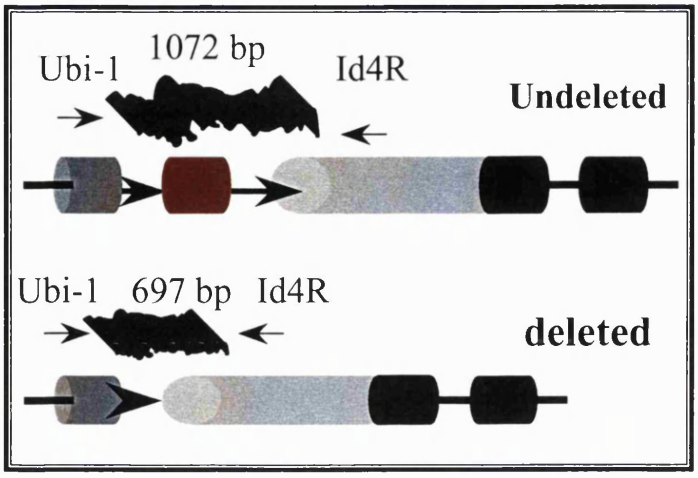
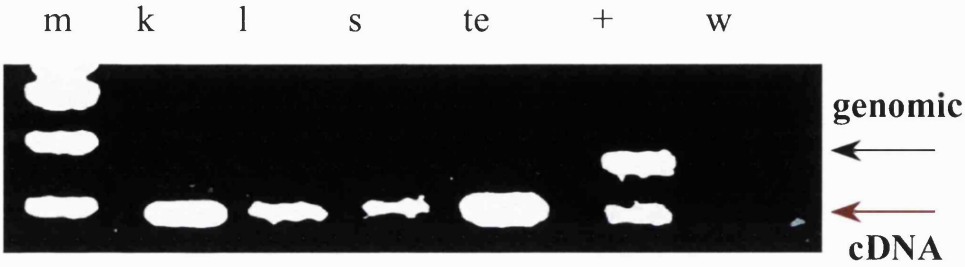

Ubi-1
1072 bp
Id4R
Undeleted
Ubi-1
697 bp
Id4R
deleted

Figure 3.16: Transcription of the Id4 transgene after Cre mediated deletion of the TSS in Ubi-loxP-Id4/NSE-Cre#30 mice.

A) β actin specific RT-PCR in cDNA from adult tissues, as control for genomic contamination. B) Id4-SV40R specific RT-PCR for detection of transgene expression. The transgene was expressed in every tissue tested. However, the levels of the two specific transcripts (1100 bp and 860 bp) were different among different tissues. In kidney (k) and testis (te), there was also some amplification from genomic contamination (1180 bp). C) RT-PCR analysis for the detection of transgene transcripts, after Cre mediated deletion of the stop cassette. Amplification with Ubi-SV40R specific primers resulted in bands of 1540 bp and 1289 bp from the deleted version of the Id4 transgene. Genomic template from tail from Ubi#37 mouse (+) gave rise to a band of 1971 bp. C is a Southern analysis from Ubi-SV40R PCR. m: DNA ladder, l: liver; s: spleen; w: water as negative control.

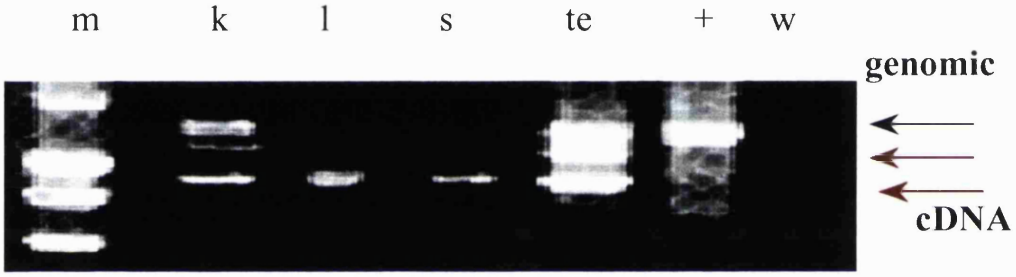
A

B actin



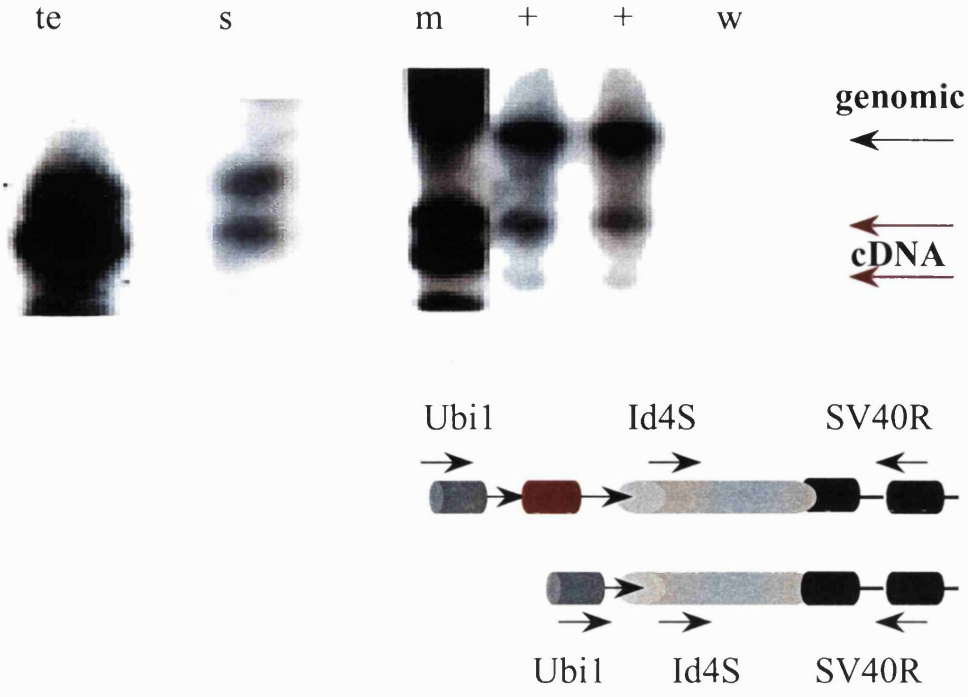
B

Id4S-SV40R



C

Ubi1-SV40R



3.7 SUMMARY

In brief, transgenic mice were established that have the potential to overexpress Id4 upon Cre mediated recombination in any tissue desired. Initially, the transgenic construct was tested *in vitro* for the ability to drive expression in mammalian cells. Upon confirmation that the transgene can be expressed, two Id4 transgenic lines were generated by Dr Elisa Cinato. Each of them was crossed with NSE-Cre#30 mice, which act as complete deleter of the stop cassette. Double transgenic mice Ubi#37/NSE-Cre#30 appeared to have complete deletion of the transgene in every tissue tested. Consequent analysis confirmed expression of the Id4 transgene upon mediated deletion in these tissues.

Despite the fact that Ubi#37/NSE-Cre#30 overexpressing the Id4 gene were obtained, this was something uncommon and in most of the cases no mice were born. This was more apparent in the case of crosses of the Ubi#10 line with NSE-Cre#30 mice, where no double transgenic were born. Further analysis of these mice may reveal a possible significant role of the Id4 protein in embryonic development.

To recapitulate, so far there have been established the appropriate neuron specific Cre recombinase and Id4 transgenic lines that were essential for the overexpression of the Id4 protein specifically in the nervous system. What was next it was the generation and analysis of double transgenic Ubi-loxP-Id4#/NSE-Cre#26 mice.

OVEREXPRESSION OF THE Id4 PROTEIN IN THE BRAIN

3.8 EXPRESSION OF THE Id4 PROTEIN IN ADULT BRAIN

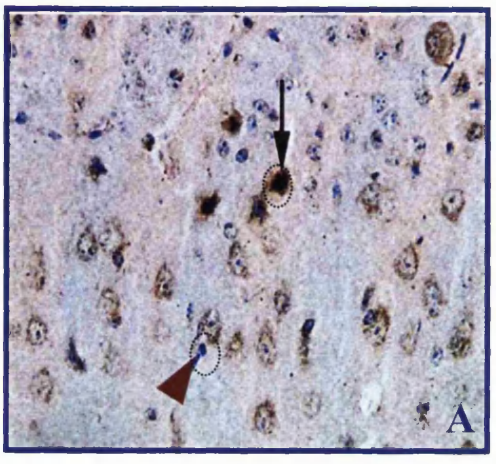
By crossing the Ubi-loxP-Id4 with the NSE-Cre#26 transgenic line, double transgenic mice would be obtained that would overexpress the Id4 protein during development and adulthood specifically in the brain. It has been reported that Id4 transcripts are found during development in presumptive neurons in the telencephalon, thalamus, presumptive cerebellum and spinal cord (Riechmann and Sablitzky 1995; Jen, Manova et al. 1996). Recent data are also providing evidence of Id4 gene expression in glial precursors (Stewart, Zoidl et al. 1997; Andres-Barquin, Hernandez et al. 1999; Kondo and Raff 2000). Finally, *in situ* hybridisation analysis has indicated expression of the Id4 in the cortex and cerebellum of adult mice (Andres-Barquin, Hernandez et al. 2000).

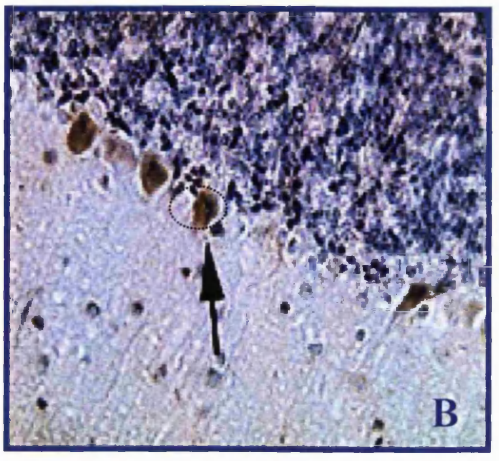
To establish that the Id4 protein is present in these areas, brain sections of wild type adult mouse brain were stained with the Id4 antibody. Binding of the antibody was visualised by DAB chromogenic reaction. By this method, cells expressing the Id protein are stained brown. In line to the results from Andres-Barkin et al., (Andres-Barquin, Hernandez et al. 2000), Id4 protein was found in neurons in the cortex and in the Purkinje cells in the cerebellum (Figure 3.17 A and B). Other areas of expression included the thalamus and the inferior colliculus (data not shown). In the hippocampus CA1-CA2 region and the dentate gyrus, Id4 protein was only detected in a few isolated cells (Figure 3.17 C and D). The staining was exclusively nuclear or peri-nuclear (Figure 3.17 C and D). No Id4 protein was detected in mature glial cells (Figure 3.17 A).

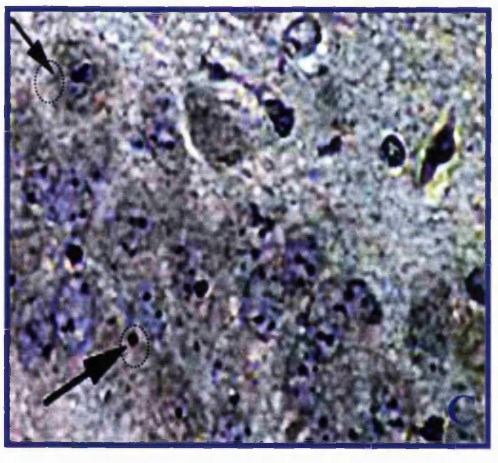
Figure 3.17: Expression of the Id4 protein in the brain of wild type mice.

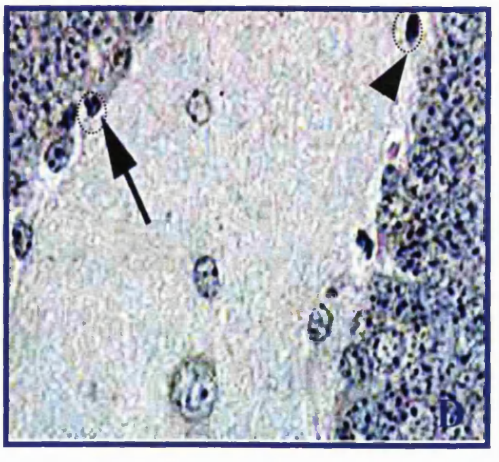
Immunocytochemistry with the anti- Id4 antibody revealed strong expression of the protein in neurons in the cortex (A, arrows) and the Purkinje cells in the cerebellum (B, arrows), as indicated by the brown DAB staining. Some expression, though at lower levels, was detected in the CA1 region of the hippocampus (C, arrows) and the dentate gyrus (D, arrows). Glial cells did not express the Id4 protein (red arrow in A). E: controls for the immunocytochemistry: incubation without primary antibody. F: preincubation with Id4 peptide, as control for crossreaction with other proteins.

NSE-Cre#26

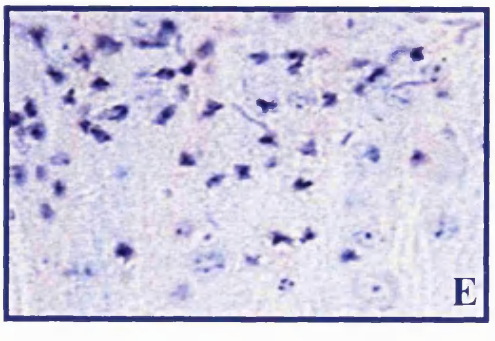


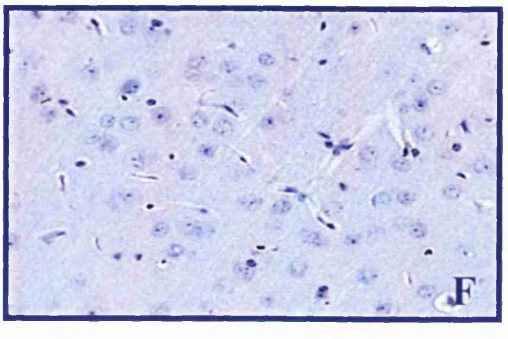






Controls





3.9 OVEREXPRESSION OF Id4 PROTEIN IN ADULT BRAIN

From the expression pattern of the NSE-Cre#26 mice, Cre recombinase was expected to drive high level deletion of the stop cassette of the Id4 transgene only in the brain, resulting in upregulation of the Id4 protein, exactly in these areas. PCR detection of the deleted form of the Id4 transgene in double transgenic Ubi-loxP-Id4/NSE-Cre#26 mice showed high levels of deletion only in the brain and some low levels in the kidney (Figure 3.18).

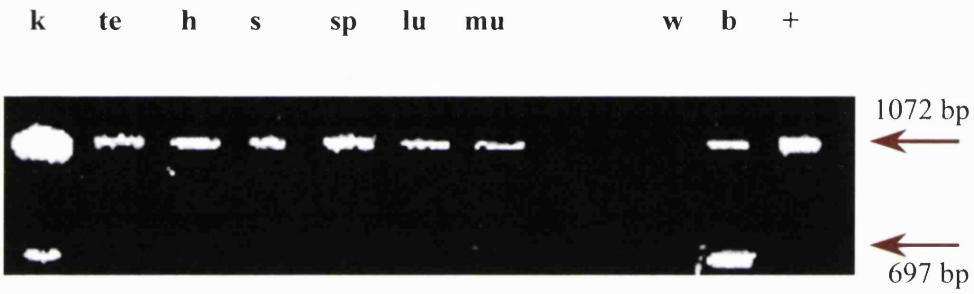


Figure 3.18: PCR detection of the deleted version of the Id4 transgene in double transgenic Ubi-loxP-Id4#/NSE-Cre#26 mice. Amplification with primers Ubi-Id4 gave rise to a band of 697 bp, representing the deletion of the TSS, exclusively in brain (b). Some low levels of deletion were also observed in the kidney (k). te: testis; h: heart; s: spleen; sp: spinal cord; lu: lung; mu: muscle; w: water as negative control; +: tail genomic DNA as positive control for the undeleted version of the transgene (1072 bp); m: DNA ladder.

Due to shortage in Ubi#37/NSE-Cre#26 mice at the time when this study was being conducted, all the subsequent analysis was performed with Ubi#10/NSE-Cre#26 double transgenic mice.

In correspondence to the Cre recombinase expression pattern, upregulation of the Id4 protein was observed in the cortex, hippocampus and cerebellum of the brain of double transgenic Ubi#10/NSE-Cre#26 mice (Figure 3.19 and Figure 3.20). Due to the design of our transgene, immunocytochemistry in Ubi-loxP-Id4 single transgenic

mice was conducted, in order to establish that no Id4 protein was generated, due to transcription through the STOP signal. Analysis of the Ubi-loxP-Id4 only, brain sections confirmed that Id4 protein in expressed in these mice in the levels identical to wild type mice (data not shown).

In the cortex, Id4 expressing neurons were spread out in all layers. Surprisingly, apart from the neurons, ectopically expressed Id4 protein was also detected in cells, which morphologically were identified as glials (Figure 3.19 A and B and Figure 3.20 A). A broader spectrum of Id4 protein expression was also observed in the cerebellum. There, apart from the Purkinje cells, (which showed stronger levels of expression), Id4 protein was also found in the interneurons between them, as well as in cells in the granular layer. Enhanced Id4 levels were also found in the amygdaloid areas (data not shown). In the hippocampus and dentate gyrus, levels of Id4 were higher than normal, but the upregulation was not as strong as in the other areas.

Figure 3.19: Overexpression of the Id4 protein in the hippocampus and cerebellum.

Immunocytochemistry with anti-Id4 antibody in coronal sections of adult brain. Visualisation of specific binding was performed by DAB brown staining. A and C: Dentate gyrus and CA1-CA2 region of the hippocampus respectively, from Ubi-loxP-Id4#10/NSE-Cre#26 brain. B and D: Control CA1-CA2 (B) and dentate gyrus (D) from single transgenic NSE-Cre#26 mice. In the dentate gyrus, Id4 positive cells were scattered along the granular and the polymorph layer (arrow). Red arrow in A indicates glial cell stained with the antibody. E, F: Cerebellum from double transgenic mice Ubi-loxP-Id4#10/NSE-Cre#26 mice. G: Cerebellum from NSE-Cre#26 mice as control. Note the strong brown staining in the Purkinje cells (black arrow), as well as n the interneurons adjacent to them. Some cells in the granular layer also appeared to express the Id4 protein (blue arrow). Controls for the immunocytochemistry procedure are the same, as in Figure 3.17 (data not shown).

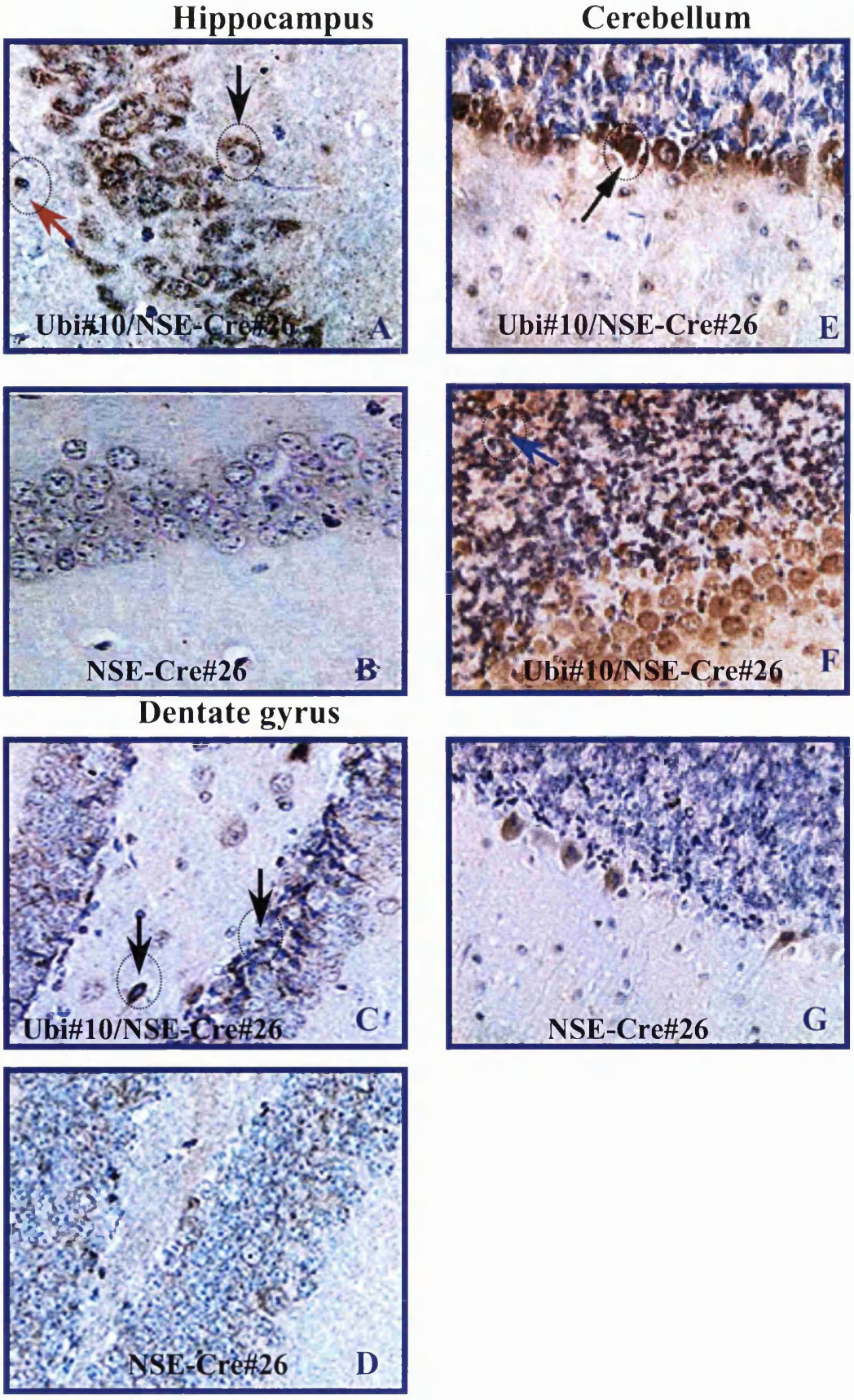
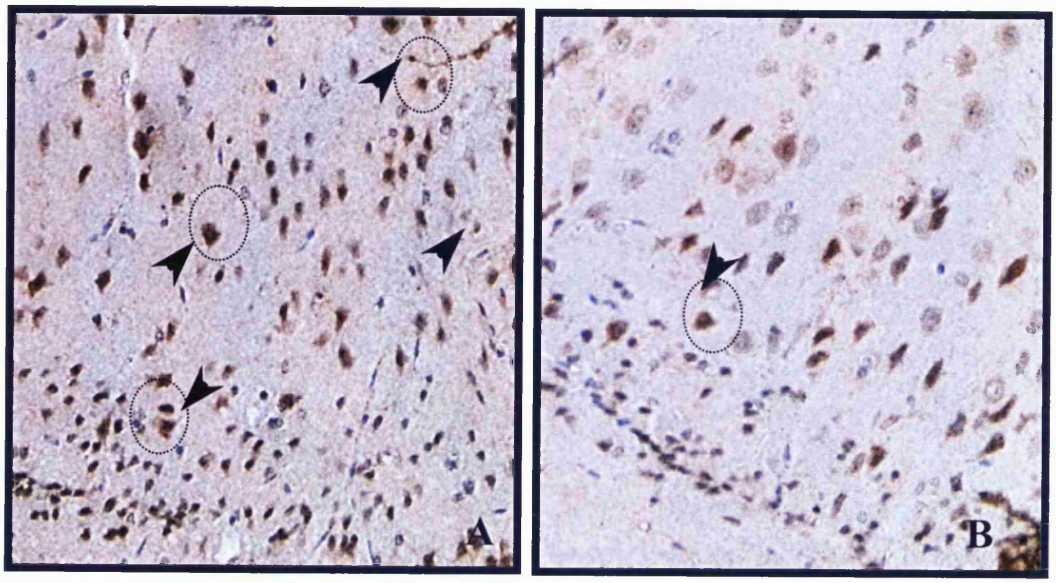

Hippocampus
Cerebellum
Ubi#10/NSE-Cre#26
A
Ubi#10/NSE-Cre#26
E
NSE-Cre#26
B
Ubi#10/NSE-Cre#26
F
Dentate gyrus
Ubi#10/NSE-Cre#26
C
NSE-Cre#26
G
NSE-Cre#26
D

Figure 3.20: Overexpression of the Id4 protein in the cortex of double transgenic Ubi-loxP-Id4/NSE/Cre#26.

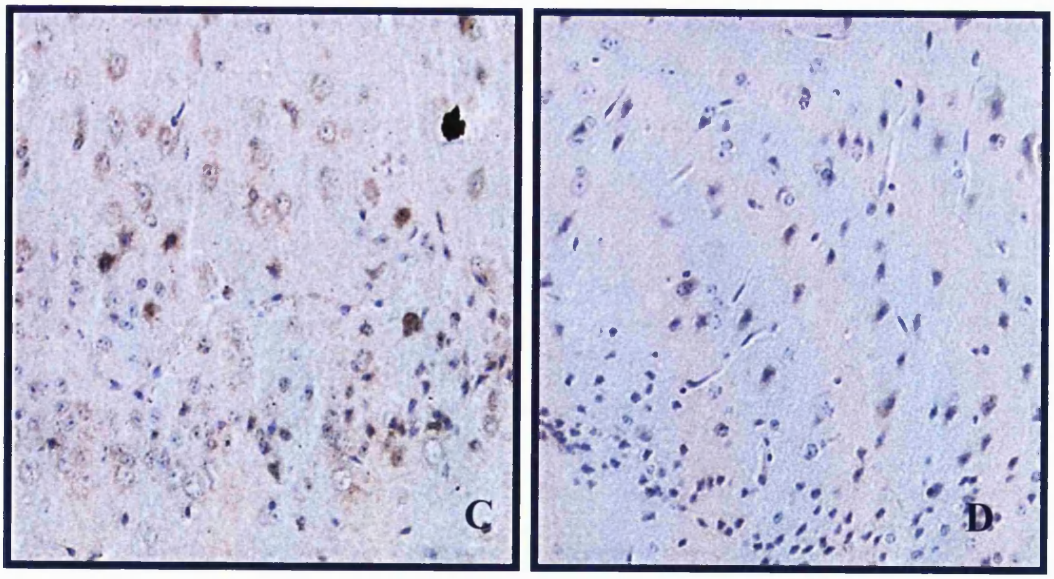
Staining with the Id4 antibody in coronal sections of adult brain. A and B: Cerebral cortex of the brain from transgenic mice Ubi#10/NSE-Cre#26.C: Cortex from single NSE-Cre#26 mice as control. As indicated by the brown staining, cells in the cortex of double transgenic mice upregulated the Id4 protein, in contrast to the control.. Black arrows point to Id4 expressing neurons, whereas the blue one indicates Id4 expressing glial cells. Control for the immunocytochemistry included no primary antibody (D) and preincubation with peptide (data not shown).

Ubi#10/NSE-Cre#26



NSE-Cre#26

No primary



3.10 ANALYSIS OF THE EFFECT OF OVEREXPRESSION OF Id4 IN THE CORTEX

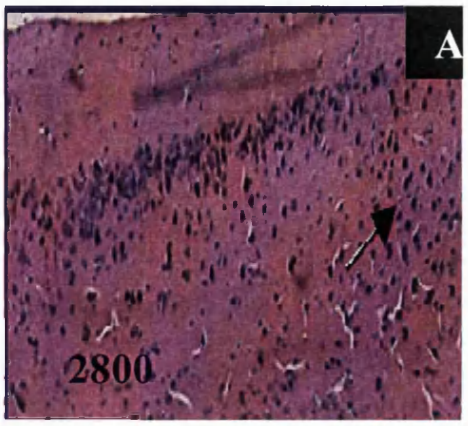
Evidently, once it was established that the Id4 protein is overexpressed in the cortex of double transgenic mice, we wanted to analyse the effect of this upregulation.

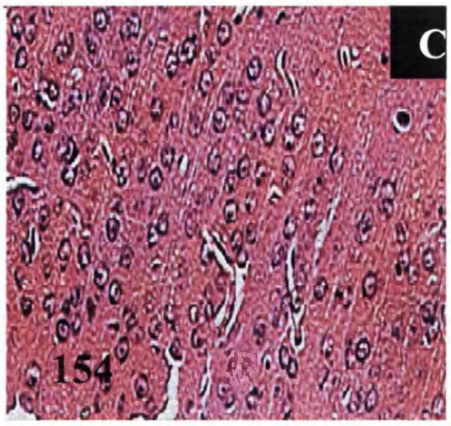
Initial analysis by Haematoxylin/Eosin (HE) staining indicated an accumulation of cells in the cortex of double transgenic mice (Figure 3.21). Counting of the cells in sections from two Ubi#10/NSE-Cre#26 mice and their NSE-Cre#26 littermate controls suggested that the expansion was at the range of 2X. These cells were found in the same areas and appeared morphologically to be the same with the cells expressing the Id4.

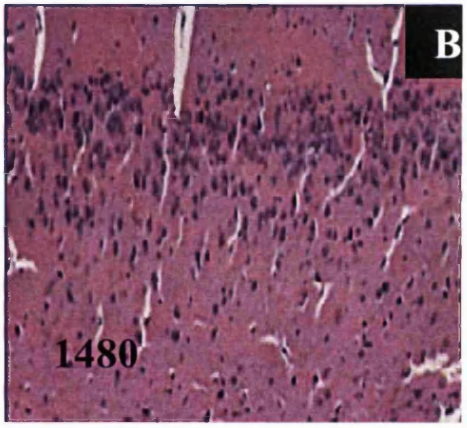
To find out the identity of these accumulating cells, immunofluorescence with neuron specific and glial specific markers was performed. Cells that are positive for any of the markers appear red under fluorescence microscope. Sections from two different double transgenic mice and two Ubi#10 only transgenic mice were analysed in replicates. The differences in the measurements in the mean intensities of light in red channel were measured using the Adobe PhotoShop. This analysis showed consistently an increase of 3-4X in the numbers of neurons in the cortex of mice overexpressing Id4 (Figure 3.22). In contrast, the numbers of oligodendrocytes and astrocytes tended to be lower, but not at the same scale (1.5-2X) (Figure 3.23). Expression of Id4 protein in these cells was confirmed by double immunofluorescence detection with anti-Id4 antibody and anti-neuronal markers (Figure 3.22 and data not shown).

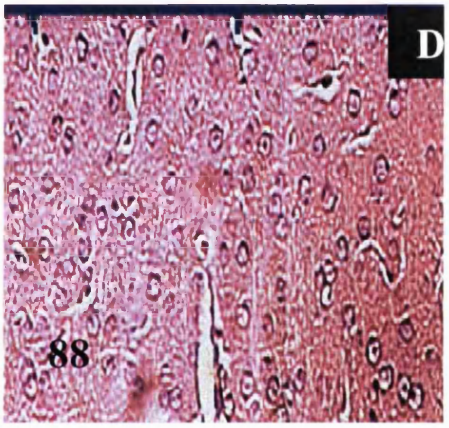
Figure 3.21: Accumulation of cells in the cerebral cortex of mice overexpressing the Id4 protein.

HE staining in coronal sections of brain revealed high numbers of cells in the cortex of Ubi-#10/NSE-Cre#26 mice (A and C) compared to the control cortex from NSE-Cre#26 (B and D). The number of cells counted in each section are indicated. The black arrow points to cells accumulated in the cortex of double transgenic mice. E: Low magnification photo of coronal section of wt mouse brain. The area framed by the red block represents the areas shown in A-D. A-D: Photos X20.


A
2800


C
154


B
1480


D
88

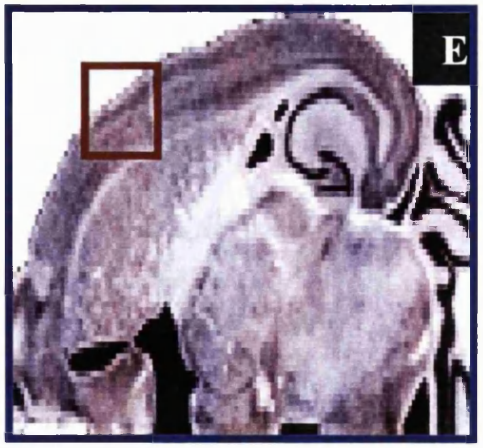

E

Figure 3.22: Accumulation of neurons in the cortex of mice overexpressing the Id4 protein.

Sagital sections of adult brain showing layers V-VI. Immunofluorescence with anti-N200, neurofilament specific antibody revealed increase in the neurons of double transgenic Ubi#10/NSE-Cre#26 mice (C), compared to the control Ubi-loxP-Id4 mice (B). The increase was estimated as the mean of intensity of the light in the red channel (numbers are indicated). A) Control for the immunocytochemistry: incubation without primary antibody. The experiment was conducted in two different samples of each type with three replications. D) Phase light photo of C indicating layers V-VI of the cortex. E: Higher magnification photo from B. F: Higher magnification photo from C showing neuron cell (red) stained with the anti-Id4 antibody. A-D: X20, E: X40, F:40X1.75.

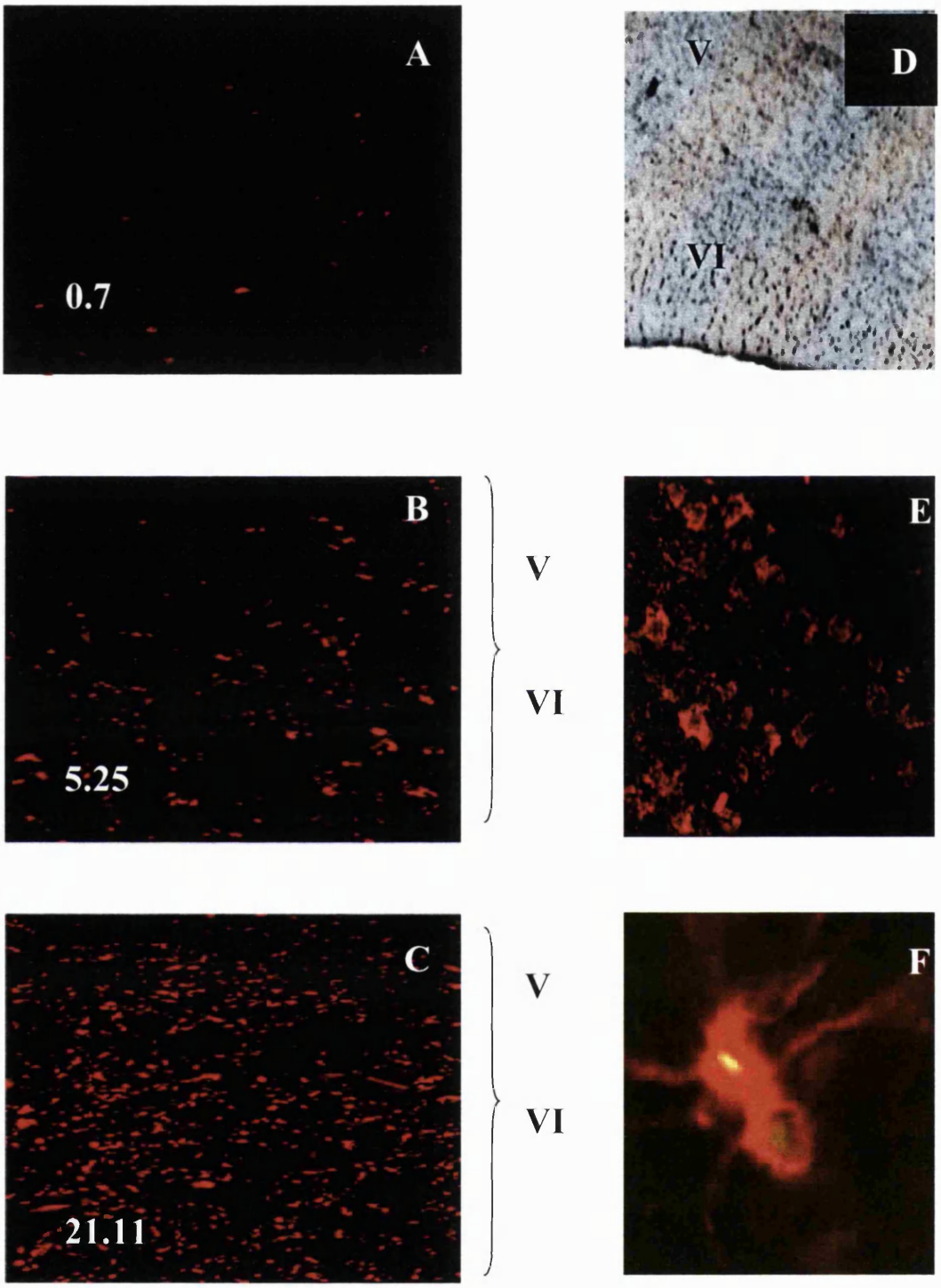
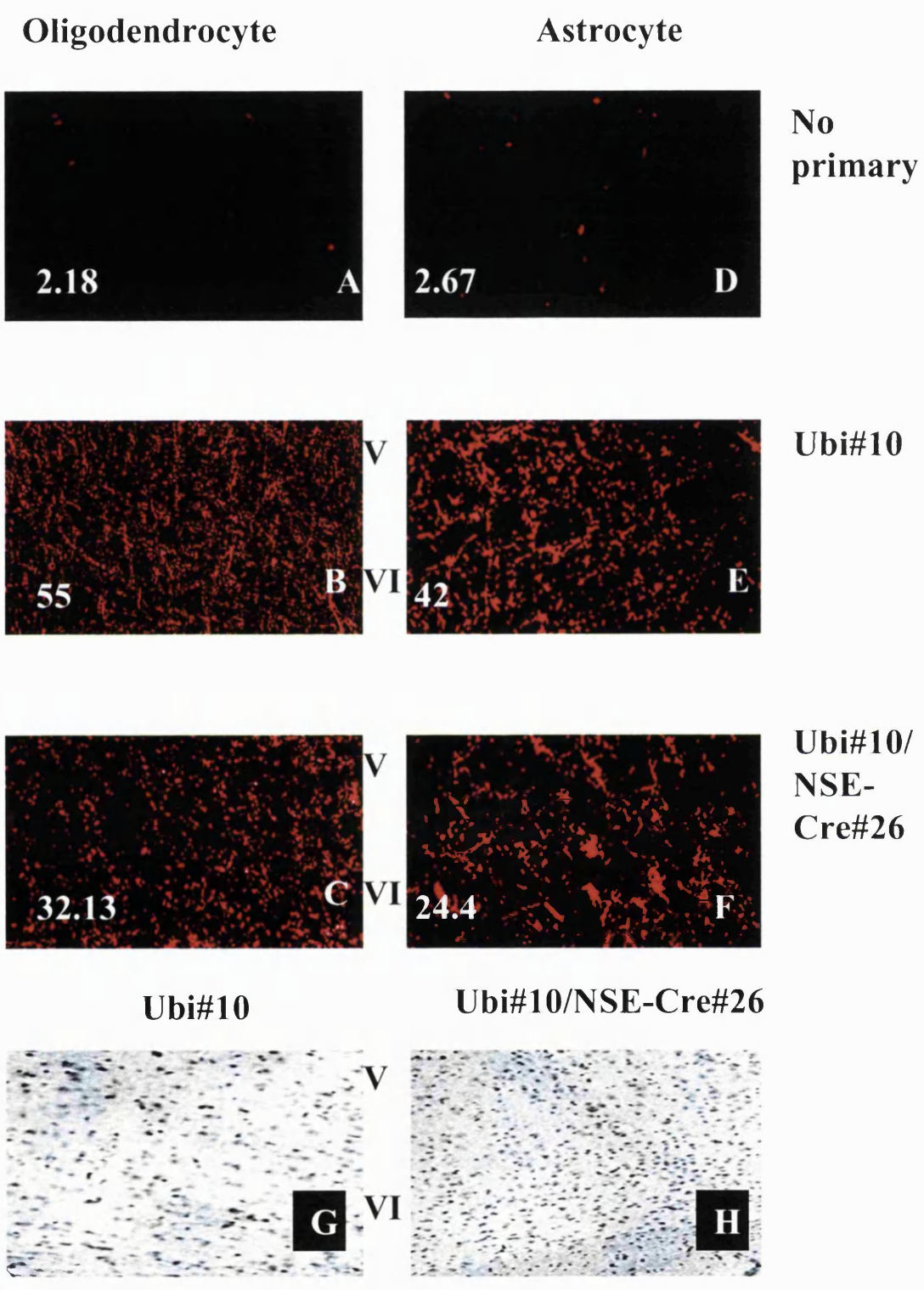

A
0.7
V
D
VI
B
5.25
V
VI
E
C
21.11
V
VI
F

Figure 3.23: Reduction in the glial populations in the cortex of mice overexpressing the Id4 protein.

Sagital sections of adult brain showing layers V-VI. Immunocytochemistry with anti-CNP (oligodendrocyte specific- A-C) and anti-GFAP (astrocyte specific- D-F) antibodies indicated reduction in the numbers of glial cells in the cortex of double transgenic Ubi#10/NSE-Cre#26 mice (C and F). The reduction was estimated as difference in the mean of intensity of light in the red channel (numbers indicated). B, E) Cortex from single transgenic mice Ubi-loxP-Id4, as control for the normal distribution of glial cells. A, C) Incubation without primary antibody, as control for the immunocytochemistry procedure. G and H: Light microscopy of photos B and C respectively. Photos taken at X20. Each experiment was repeated twice using two samples of each type (Ubi#10 and Ubi#10/NSE-Cre#26).


Oligodendrocyte
Astrocyte
No primary
2.18
A
2.67
D
Ubi#10
V
55
B
VI
42
E
Ubi#10/ NSE-Cre#26
V
32.13
C
VI
24.4
F
Ubi#10
Ubi#10/NSE-Cre#26
V
G
VI
H

In order to further understand the mechanism by which upregulation of Id4 resulted in this shift of cell numbers, analysis of the proliferative state of cells in the cortex of double transgenic mice was pursued. It has been widely reported that Id proteins are involved in cell cycle progression and apoptosis (For review see Norton, Deed, 1998).

Indeed, TUNEL assay, an apoptosis detection method, revealed increased cell death in the cortex of mice overexpressing the Id4 (Figure 3.24). DNA fragmentation is a hallmark of programmed cell death and is detectable long before other features of apoptosis become apparent. In this assay, fragmented DNA is visualised by green fluorescence, while the cell nuclei are counterstained red with propidium iodine. The result is that cells undergoing apoptosis appear to be yellow, under a fluorescence microscope. As it is shown in the table in Figure 3.24 the number of yellow stained cells in brain section from Ubi#10-NSE/Cre#26 mice 3-4X more than the control Ubi#10 only transgenic mice.

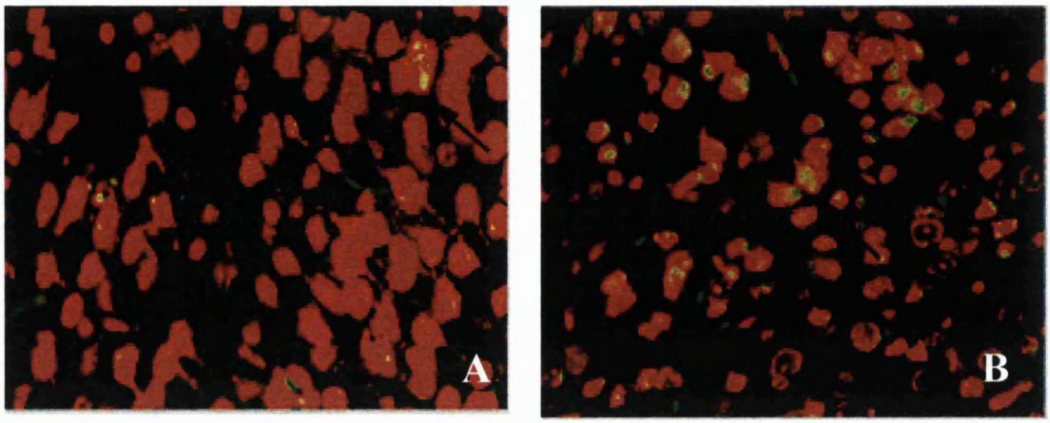
It has been reported that overexpression of Id1 protein in cultured fibroblasts had led to overproliferation and then apoptosis. On this basis, proliferation analysis in the cortex of mice overexpressing the Id4 protein was carried out. This type of analysis, by two independent ways, revealed proliferation in the cortex of double transgenic mice, in complete contrast to the control, where no proliferation (or very low) was observed (Figure 3.25 and Figure 3.26). In the first case, mice were injected with BrdU, after 1-2 h, they were sacrificed, and tissues were collected. Visualisation of BrdU incorporated in the DNA of proliferating cells was performed with anti-BrdU specific antibody binding and green fluorochrome detection of the binding. In adult mouse brain, cells do not usually proliferate. In certain areas, such as the dentate gyrus and the ependyma however, a few cells undergoing proliferation are observed. Intriguingly, Id4 overexpressing mice showed elevated levels of proliferation in the adult cortex by BrdU incorporation analysis (Figure). The same results were provided by using an antibody against the Ki-67 cell cycle specific marker (Figure 3.26). This marker is found only in cells that are proliferating and is absent from resting cells. Morphologically the cells stained positive with the anti-Ki-67 antibody were identified as neurons.

Figure 3.24: Increased apoptosis in the cortex of mice overexpressing the Id4 protein.

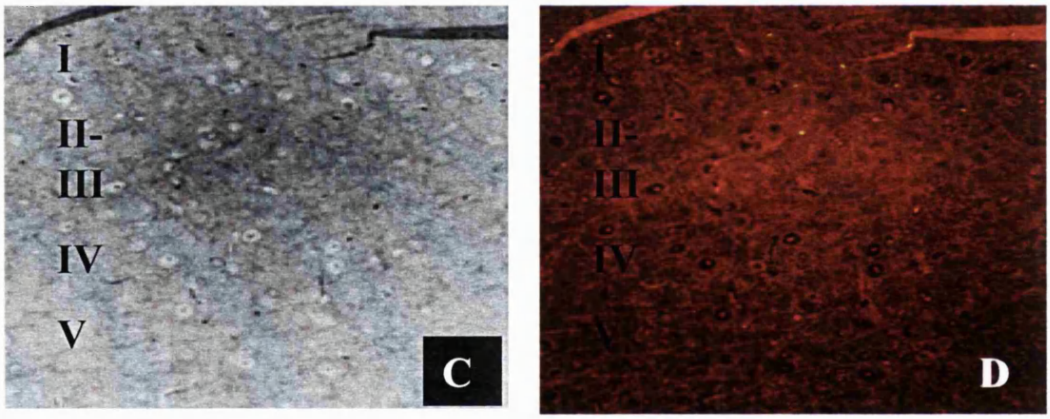
TUNNEL assay in sagital section of brain from mice overexpressing the Id4 showed elevated levels of programmed cell death (B). Some apoptosis was also observed in the cortex of control Ubi-loxP-Id4 mice (A). Yellow staining is indicative of DNA fragmentation in the nucleus. Apoptosis was more profound in layer V of cortex (area shown). Sections were counterstained with propidium iodine (red nuclear staining). Two samples of each type were analysed two times. C is phase light photo of A at X20 and D is X20 photo of A. A and B magnifications of X40. The table indicates the cells that were stained yellow after counting three different areas in each sample Ubi#10 and Ubi#10/NSE-Cre#26.

Id4#10 **Id4#10/NSE-Cre#26**

Area V



Id4#10/NSE-Cre#26 **Id4#10/NSE-Cre#26**

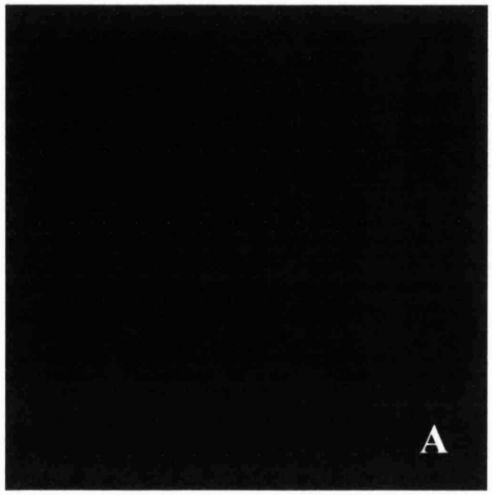


SAMPLE 1	**Ubi#10/NSE-Cre#26**	**Ubi#10**
Area 1	23	5
Area 2	10	2
Area 3	17	6
Mean	16.6	74.33
Standard Deviation	6.5	2.08
SAMPLE 2		
Area 1	12	4
Area 2	19	8
Area 3	22	6
Mean	17.66	6
Standard Deviation	5.13	2

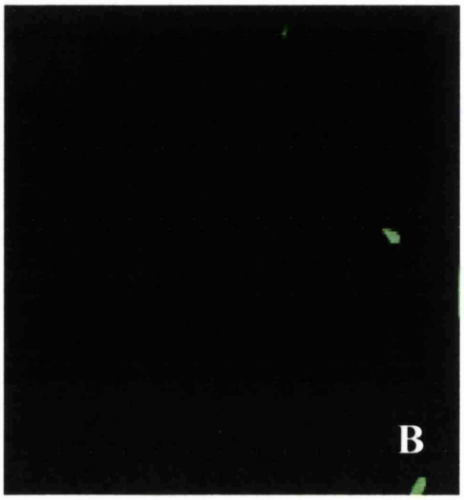
Figure 3.25: Proliferation in the cortex of adult mice overexpressing the Id4 protein.

BrdU incorporation and detection of the incorporated BrdU with specific antibody in the cortex of adult mice showed proliferation in the cortex of double transgenic Ubi-loxP-Id4/NSE-Cre#26 mice (D, arrow). This phenomenon was especially observed to the inner layers of the cortex (the layers V-VI shown here), as well in other distinct areas, such as the piriform cortex (not shown). No proliferation or very low proliferation was observed in the cortex from control mice (C). A and B are controls for the immunocytochemistry. A-D: Photos X40X1.6. E is a phase photo from Ubi#10/NSE-Cre mouse cortex taken at magnification X20. Layers V and VI are indicated at E. A table with the number of stained cell at layers V-VI from three repetitions of the BrdU staining experiment is shown. Two samples of each type were analysed in each experiment.

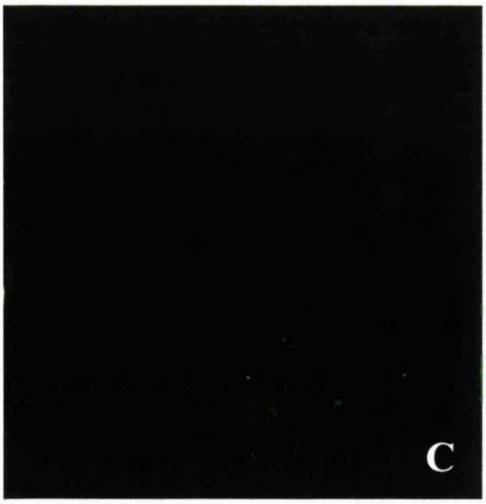
No primary antibody



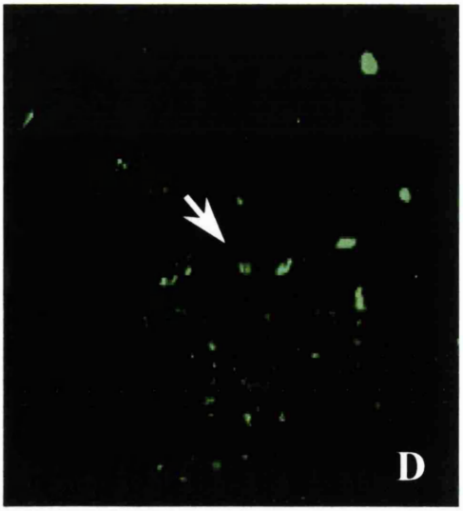
No streptavidin

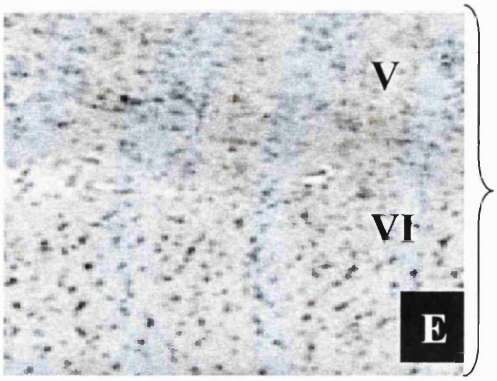


Ubi#10



Ubi#10/NSE-Cre#26



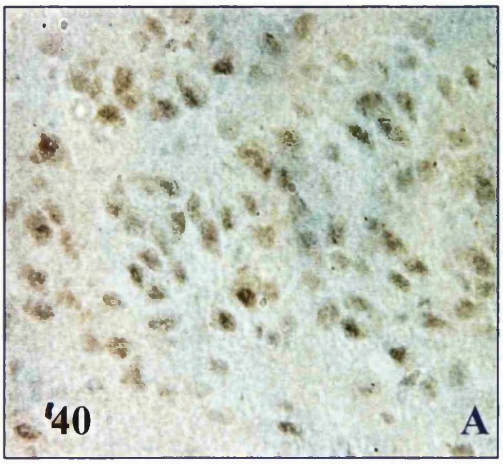


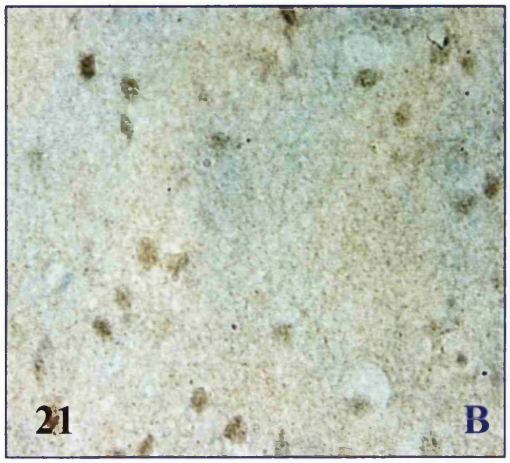
	Ubi#10/NSE-Cre#26	Ubi#10
Section 1	28.89	4
Section 2	38.095	3
Section 3	70	2
Mean	42.99	3

Figure 3.26: Cell cycling in the cortex of mice overexpressing the Id4 protein.

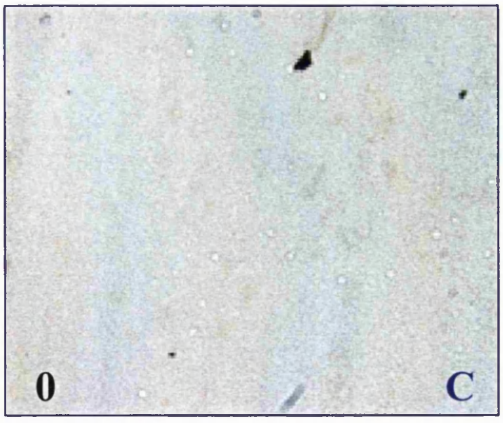
Immunocytochemistry with the anti-Ki-67 antibody. Brown specific signal of cycling cells was only detected in the cortex of mice overexpressing the Id4 (A and B). A: Piriform cortex. B: layers III-V of cortex. No staining was detected in cortex from control mice (B), or in sections incubated without no primary antibody (A). The number of stained cells is indicated. A, B, C and D are a magnification from photos of X40/0.75. Two samples of each genotype (Ubi#10 and Ubi#10/NSE-Cre#26) were analysed.

Ubi#10/NSE-Cre#26

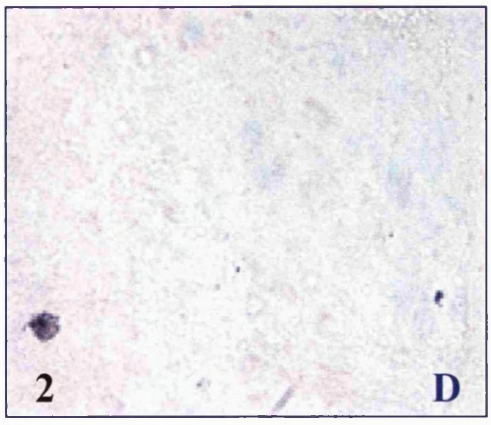




No primary



Ubi#10



3.11 SUMMARY

To recapitulate, mice were created that overexpress the Id4 protein specifically in the brain.

In accord with RNA analysis from previous studies, endogenous Id4 protein was detected in certain areas of the adult brain, such as the neocortex and the Purkinje cells in the cerebellum. Some expression could also be found in the thalamus and the inferior culiculus. Cells in the hippocampus did not express (or expressed at very low levels) the Id4 protein, at least within the limits of our analysis.

In double Ubi#10/NSE-Cre#26 transgenic mice, higher Id4 protein levels were detected in neurons in all of these areas. Moreover, ectopically expressed Id4 protein was found in glial cells, in cells adjacent to Purkinje cells in the cerebellum, the granular layer in the cerebellum and in the CA1 region in the hippocampus. In the dentate gyrus, an area where Cre recombinase is strongly expressed, limited levels of Id4 protein were observed.

Further analysis of the effect of Id4 overexpression in the adult cortex showed a tendency for accumulation of cells in this area, especially. Immunohistochemistry with various neuronal markers revealed an increase in neuron cells. On the contrary, numbers of oligodendrocytes and astrocytes cells showed slight decrease or no difference.

Since Id proteins are intensely involved in cell cycle progression and /or apoptosis, there was the possibility that the shift in the cell number was due to these events. TUNEL assay and two independent assays for the proliferation state of the cells, revealed both increase in apoptosis and cell proliferation in the cortex. Extended analysis to assess the significance of these changes and the identity of cells undergoing apoptosis or proliferation is required for further interpretation of these results.

ANALYSIS OF THYMOCYTE SPECIFIC OVEREXRESSION OF THE Id4 PROTEIN

3.12 OVEREXRESSION OF THE Id4 PROTEIN IN THYMOCYTES

The flexibility of the Cre loxP recombination system and the design of the Id4 transgenic mice enabled us to address the question of the role of the Id4 protein in thymocyte development. Id4 specific transcripts are normally present at very low levels in adult mouse thymus and spleen (Riechmann, van Crüchten et al. 1994; van Cruchten, Cinato et al. 1998). In an attempt to determine, if similar to the other Id proteins (see 1.9.4), overexpression of Id4 would have an effect in lymphoid system development, the Id4 transgenic mice were crossed with mice exhibiting thymocyte specific Cre recombinase expression (lck-Cre transgenic mice) (Orban, Chui et al. 1992).

For better understanding of the analysis of these mice an overview of the cell surface markers expressed during thymocyte development is presented in Figure 3.27.

The lck-Cre transgenic mice are well characterised and they have been found to confer high level (almost 80% –95%) of Cre mediated deletion of loxP sites, specifically in thymocytes (Orban, Chui et al. 1992; Gu, Marth et al. 1994; Ayral et al. 1998). To confirm that in the double Ubi-loxP-Id4#/lck-Cre transgenic mice, the TSS deletion is thymocyte specific, genomic DNA from different tissues and from isolated thymocytes was subjected to PCR analysis with specific, Ubi1 and Id4R, primers that flank the loxP sites. As it was anticipated, the deletion was specific to the thymus (Figure 3.28). Some deletion was also detected in adult spleen (data not shown); an observation that can be explained by the fact that around 35%-45% of the splenic cell population are thymocytes (Martihn A. Nolte 2000).

Taking into consideration that there are 10-20 copies of the transgene (see 3.5), and that effective Cre mediated deletion would leave a single copy of the transgene in the cells in which recombination takes place, the levels of the deletion in the thymus of the majority of Ubi-loxP-Id4/lck-Cre mice, were roughly estimated to be between 75%-95%. However, there were a few mice, which showed lower levels of deletion,

and in extreme cases no deletion at all (Figure 3.28).

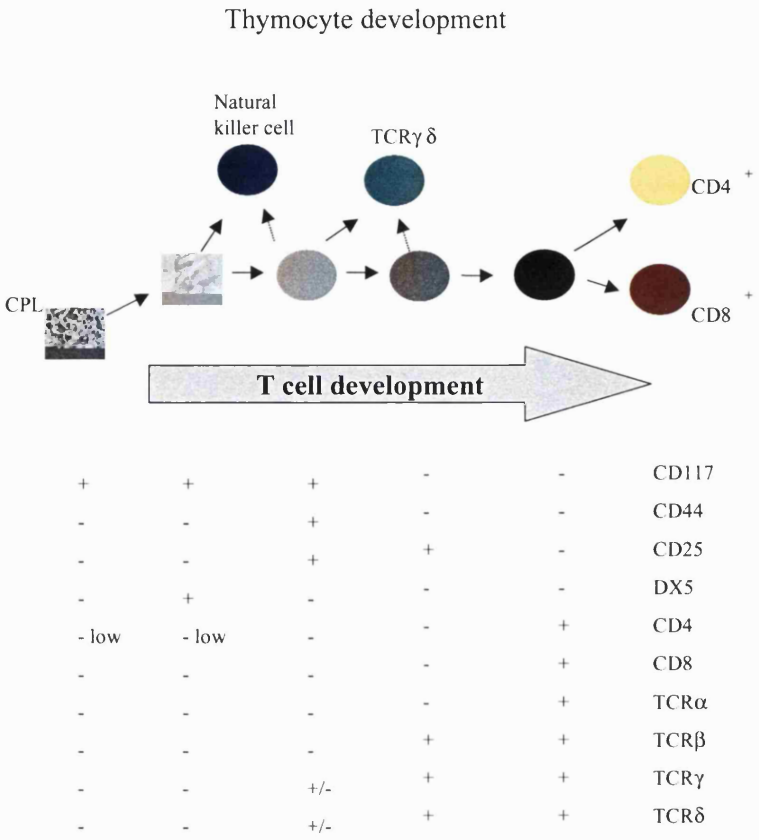
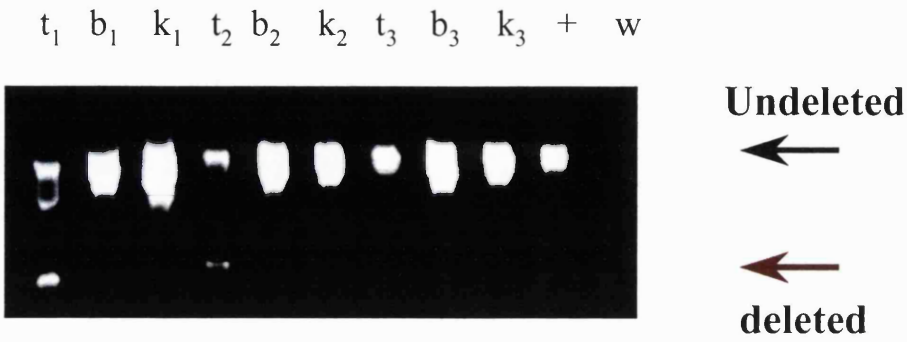


Figure 3.27: T cell development. Pluripotent common lymphoid precursors (CLP) migrate to the thymus and progressively become committed to the T cell lineage. Upon maturation, the T cells migrate to the periphery (blood, spleen, lymph nodes, liver, etc.). The cell surface markers at different stages of development are depicted. Cells positive for CD4 and CD8 are described, as double positive (DP). Cells negative, for both CD4 and CD8, are called double negative (DN). Photo is modified from Rothenberg, 2000.

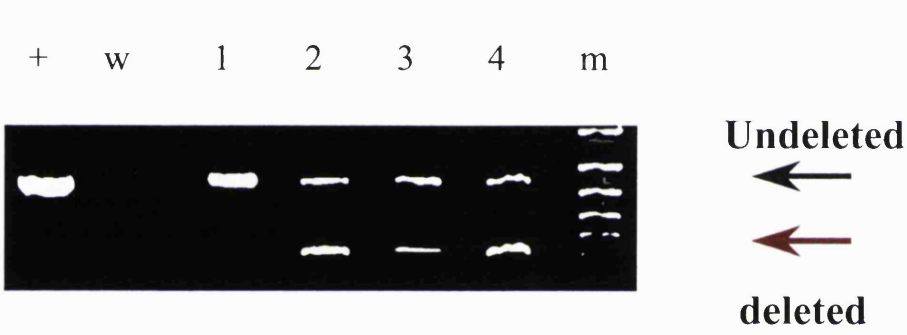
Figure 3.28: Thymocyte specific deletion of the TSS in double transgenic Ubi-loxP-Id4#/lck-Cre mice.

Cre mediated deletion of the TSS in genomic DNA was detected by PCR with primers, Ubi1 and Id4R, that flank the loxP sites. (A) PCR in tissue, genomic DNA from double transgenic Ubi#37/lck-Cre mice. Deletion of the STOP signals was specific in thymus and spleen (data not shown). The same results were obtained for Ubi#10/lck-Cre mice (data not shown). (B) DNA from purified thymocytes from double, transgenic mice from both founders (Ubi#37:1, 2 and Ubi#10: 3 and 4) was subjected to the same type of PCR analysis, as in (A). C) Table with the estimated deletion index for B. The estimation of the deletion levels was carried out by employing the Adobe PhotoShop Software, taking into consideration the DNA input and the number of copies of the Id4 transgene (10-30). The levels of deletion fluctuated mainly between 75%-95%, with exception a few cases, where lower levels of deletion (A: t_2, B: #3) or no deletion (A: t_3, B: #1) was detected. b: brain; k: kidney; t: thymus; +: tail genomic DNA from Ubi-loxP-Id4# /lck-Cre; w: water; m: marker. The arrows indicate the undeleted (1072bp) and the deleted (697bp) versions of the transgene. The band that appears in the middle is an artefact of the PCR procedure.

A



B



C

#	Genotype	% deletion
1	Ubi#37	0
2	Ubi#37/lck-Cre	83
3	Ubi#10/lck-Cre	25
4	Ubi#10/lck-Cre	82

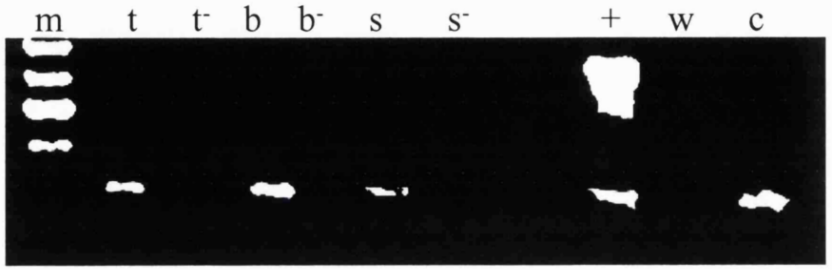
In order to establish that the Id4 transgene is actually expressed in the thymus and spleen from Ubi-loxP-Id4 /lck-Cre mice, RNAs from these tissues were subjected to RT–PCR (Figure 3.29). Brain RNA was also used, as control for the endogenous levels of Id4 transcripts. The cDNA generated by the reverse transcription was amplified with specific primers Id4-Id4R that can recognise both endogenous and transgene Id4 expression. Taken into consideration the cDNA concentration, (estimated by semiquantitative β actin RT-PCR), this analysis showed that the levels of the Id4 transcripts in thymus and spleen of double transgenic Ubi-loxP-Id4 /lck-Cre mice were similar or higher, than the ones that appeared in the brain (Figure 3.29 A and C). Since the endogenous levels of Id4 gene expression in mouse spleen and thymus are significantly lower than the ones in the brain (Figure 1.3), the above result established the increase in the Id4 expression in these tissues. To confirm that this upregulation was due to activation of the Id4 transgene, the same cDNA was amplified with primers Ubi2 and neoR, which flank the TSS. These primers give rise to a product of 375 bp, only in the presence the STOP cassette. As it is shown in Figure 3.29 B, in thymus and spleen from double transgenic Ubi-loxP-Id4/lck-Cre mice Cre mediated deletion removed the STOP signal and therefore no 375-bp transcript was generated. This fragment could still be detected in the brain, a tissue where Cre recombination does not take place (Figure 3.28 B). The collective outcome of this RT-PCR analysis clearly demonstrates an enhancement in the Id4 levels in spleen and thymus of Ubi-loxP-Id4/lck-Cre mice, due to Cre mediated activation of the transgene. This upregulation was approximately 3-4X fold, as it was estimated by comparison of the Id4 specific signals in Northern analysis of thymus RNA from Ubi-loxP-Id4/lck-Cre and wild type mice (data not shown).

Figure 3.29: Upregulation of the Id4 transcript is due to the deletion of the transgene TSS in spleen and thymus of Ubi-loxP-Id4/lck-Cre mice.

A) RT- PCR with specific primers Id4-Id4R that can recognise both endogenous and transgene Id4 expression (270 bp band from cDNA amplification) in cDNA from Ubi#37/lck-Cre mice. B) RT- PCR with primers Ubi and neoR, which flank the TSS, reveals the loss of the 375 bp neoR specific transcript in thymus and spleen, confirming the Cre mediated deletion of the STOP cassette. C) β-Actin RT-PCR, as control for the cDNA concentration and genomic contamination. The levels of the Id4 transcripts in thymus and spleen of double transgenic mice were similar or higher than the ones that appeared in the brain. This conclusion was reached, by taking into account the cDNA load (β actin specific signal) and the levels Id4 specific transcripts in each tissue. This upregulation was not due to read-through transcripts that might have occurred from the Id4 transgene, but was derived by expression of the deleted version of it (note that the Ubi2-neoR PCR product is not generated in thymus and spleen from double transgenic mice, due to Cre mediated loss of the STOP signals). b, t, s: brain, thymus and spleen from Ubi#37/lck-Cre mice; b^-, t^-, s^-: RT^-counterparts of the above as controls of genomic contamination; +: tail genomic DNA from Ubi-loxP-Id4# 37 mouse, c: Brain cDNA from wt mouse, w: water as negative control. β-Actin amplification gives rise to bands of 422 bp and 248 bp for genomic and cDNA templates respectively. The second band that appears in the genomic control is a result of overloading of DNA template. Similar results were obtained from analysis of Ubi#10/lck-Cre mice (data not shown).

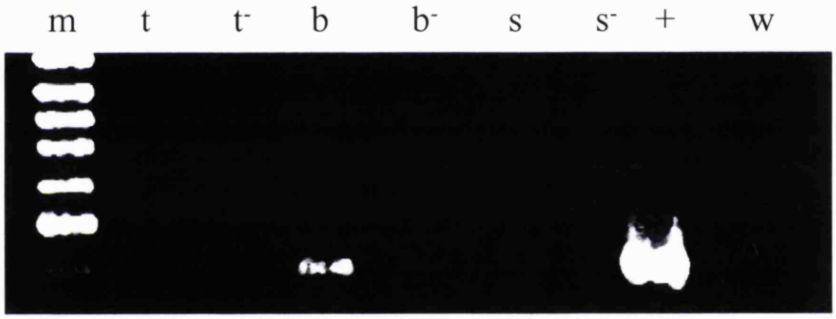
A

Id4-Id4R



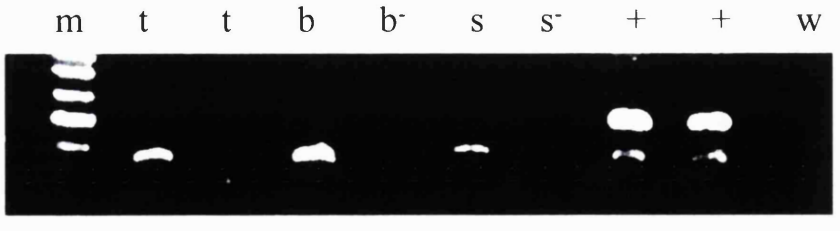
B

Ubi2-neoR



C

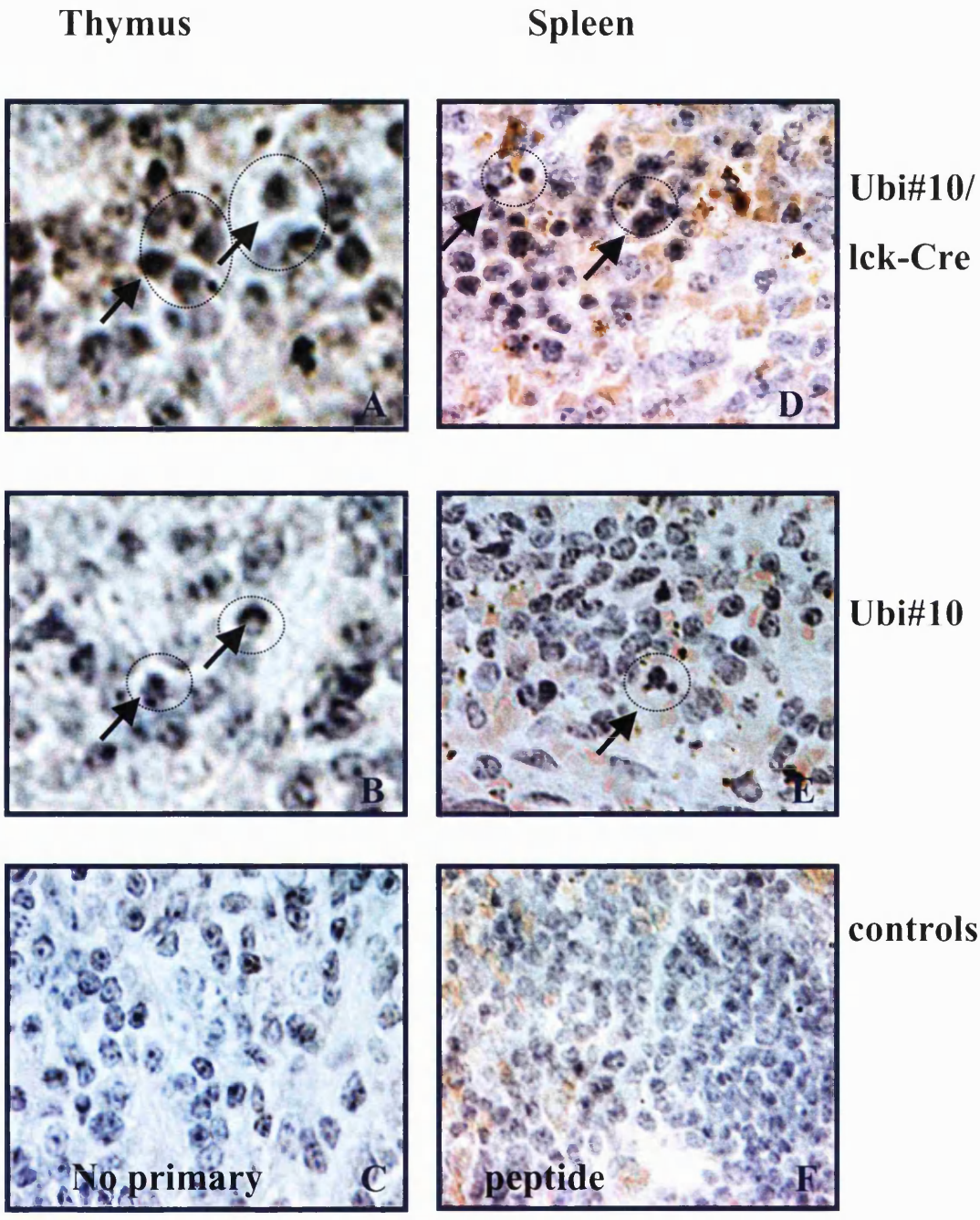
-actin



At the protein level, upregulation of the Id4 in spleen and thymus of Ubi-loxP-Id4/lck-Cre mice was tested by immunocytochemistry with an anti-Id4 specific antibody. As it is illustrated in Figure 3.30, increased Id4 protein levels were detected in both tissues. In the thymus Id4 expressing cells could be found, both in the cortex and the medulla. In the spleen, Id4 expression was located in the red pulp, the marginal zone and the periarteriolar sheaths. Controls for the immunocytochemistry included spleen and thymus sections from littermate single transgenic lck-Cre mice, preincubation of the primary antibody with Id4 peptide (to eliminate the possibility of crossreaction of the antibody with other antigens) and omission of the primary antibody.

Figure 3.30: Overexpression of the Id4 protein in adult mouse thymus and spleen.

Immunohistochemistry with anti-Id4 antibody revealed high expression of the Id4 protein in thymus (A) and spleen (D) sections from adult double transgenic Ubi#10/lck mice. In the thymus, Id4 protein was detected in both the cortex and the medulla (shown here). In the spleen, areas of expression included the periarteriolar sheaths, the red pulp and the marginal zone (shown here). B and E sections from thymus and spleen respectively from lck-Cre mice. C and F. Controls for immunocytochemistry included no primary antibody (C) and preincubation with the Id4 peptide (F). Visualisation of the binding was achieved with brown DAB staining and counter-staining with Haematoxylin. Some background was also observed especially in the spleen (yellow unspecific staining), but was clearly distinguished from the specific brown signal. A, B: magnifications from 40X/0.75, C, D, E and F: 40X/0.75. The same result was given from double transgenic mice established from the #37 transgenic line (data not shown). Black arrows indicate thymocytes stained for the Id4 protein.


Thymus
Spleen
A
D
Ubi#10/
lck-Cre
B
E
Ubi#10
No primary
C
peptide
F
controls

3.13 EFFECT OF Id4 OVEREXPRESSION IN THYMOCYTES

To analyse the impact of the ectopic expression of Id4 in thymocytes, immunohistological and FACS assays using a panel of immunological markers were performed. The FACS analysis was conducted in collaboration with Dr Owen Williams in the laboratory of Prof. Dimitris Kiousis, in the National Institute of Medical Research, UK. The analysis of thymi and spleens, from double transgenic Ubi-loxP-Id4/lck-Cre mice, derived from crosses of each of the two different Id4 transgenic lines (Ubi#37 and Ubi#10) with the lck-Cre mice, is presented separately. A detailed summary of this study is presented in Appendix C.

3.13.1 Analysis of the Id4 overexpression in thymus and spleen from Ubi#37/lck-Cre mice

According to the design of the transgenes, in double transgenic Ubi-loxP-Id4/lck-Cre mice, the lck promoter initiates expression of the Cre protein and therefore activation of the Id4 transgene in thymocytes, as early as 12.5 dpc. At these early stages of development, the thymus consists mainly of precursor thymocytes undergoing differentiation (see 1.9 for detail). The process is continued during adult life, though at a lesser extend. To assess the influence of Id4 overexpression in thymocyte differentiation, thymocytes derived from 15.5 dpc Ubi#37/lck-Cre and littermate lck-Cre control embryos were subjected to FACS analysis, with a panel of mature and immature T cell specific markers (CD44, CD25, CD4, CD8, αβ TCR and γδ TCR). Given the established potential of the early thymocyte precursors to become NK cells (reviewed in Carlyle, 1998), a specific marker for these cells, was also included.

This study did not show any differences in the T cell population in the thymus from mice overexpressing the Id4 protein. The representation of $CD4^+$, $CD8^+$ and DP cells was similar to the control lck-Cre thymocytes. However, a trend for increase was observed in the numbers of NK cells. These cells also expressed the CD8 marker, but from our analysis is not clear if they represent early CD8 expressing precursors or mature CD8 positive T cells (Table 3.3).

When analogous analysis was performed in thymocytes from adult mice, an

effect in the NK cell population could not be detected. This was probably due to the low levels of NK cells present in the adult thymus. Nevertheless, in the adult thymus of Ubi#37/lck-cre mice, an increase was observed in the $CD8^+$compartment (2-4X) (Table 3.3). Further FACS analysis with the same markers in splenic thymocytes from Ubi#37/lck-Cre mice replicated the effect observed in the thymus. In splenocytes from young (3 weeks old) Ubi#37/lck-Cre mice, higher numbers of NK and mature $CD8^+$ γδ TCR^+ cells were detected (Figure 3.31).

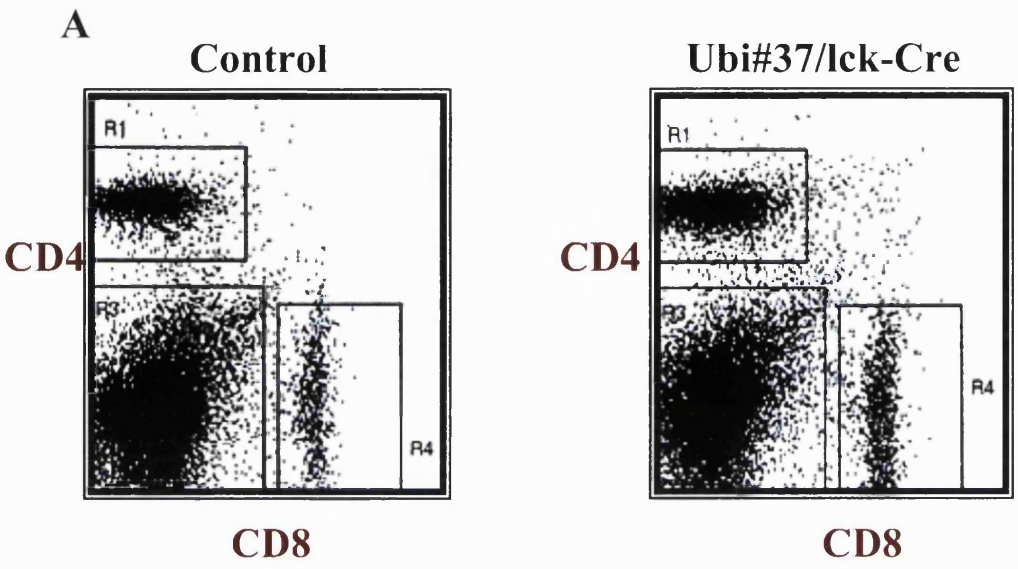
Table 3.3: Examples from the FACS analysis in thymi from Ubi#37/lck-Cre mice. The analysis for the CD8 and the NK markers is presented. The results are presented as percentage of total cell population. Thymi from lck-Cre embryos and adult mice were used as control. The significance level is set at α=0.1

Mice	**Thymocytes from adult Ubi#37/lck-Cre mice % of total**	**Thymocytes from Ubi#37/lck-Cre embryos (15.5 dpc) % of total**	
#	**$CD8^+$**	**$Cd8+NK^+$**	**DN NK^+**
Ubi#37/lck-Cre			
1	10.9	0.07	4.3
2	16.7	0.03	5.2
3	5.5	0.03	5.4
	-	0.03	1.5
Mean	11.3	0.04	4.1
Standard deviation	5.6	0.02	1.7
Control lck			
1	3.9	0.02	3.5
2	6.4	0.01	3.8
3	4.6	0.01	3.6
4	3.3		
Mean	4.5	0.013	3.6
Standard deviation	1.37	0.005	0.13
t test	0.06	0.07	0.7

Figure 3.31: Increase in the numbers of NK and γδ TCR^+ cells in the spleen of Ubi#37/lck-Cre mice (A and B).

Numbers are given as percentage of total population. Control spleen is from single, littermate lck-Cre mouse. Staining with antibodies, specific for the CD4, CD8, γδ TCR and NK markers (anti-CD8 FITC, anti-CD4 PE, GL3 bio and DX5 bio respectively) was performed. The different panels showing the cells counts are not drawn in scale. The table presents the number of mice analysed, the percentages of the different populations and the statistical analysis (t test-two tailed distribution, significance level α=0.05).

A



R1=CD4: 10.6%

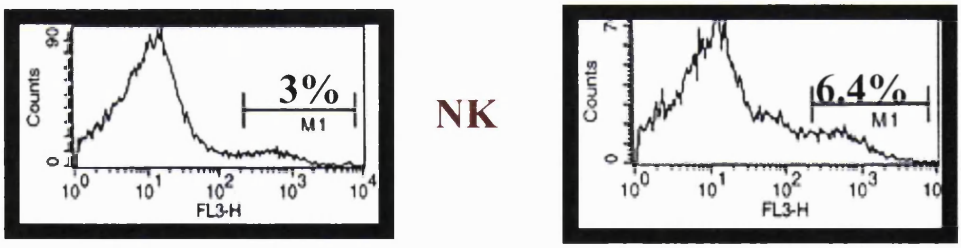
R3=DN: 65.5%

R4=CD8: 3%

R1=CD4: 16.7%

R3=DN: 61%

R4=CD8: 6%

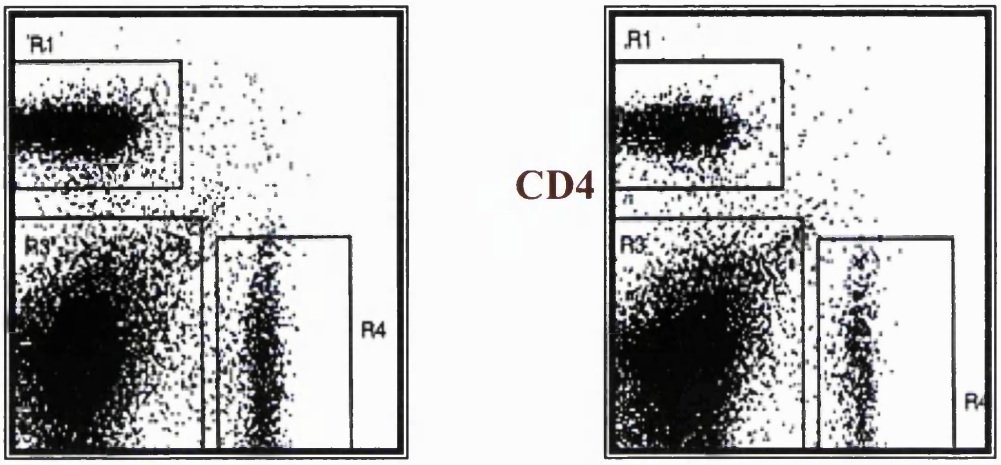


	CD4	CD8	DN	NK	gdTCR
Ubi37/lck-Cre					
1	13.37	3.9	81.7	5.88	1.44
2	19.6	7.4	71.5	5.9	1.1
3	28.6	7.6	62.2	5.8	1.1
Mean	20.52333	6.3	71.8	5.86	1.213333
standard deviation	6.251807	1.699019	7.963667	0.043205	0.160278
lck-Cre					
1	16.3	4.4	78.4	3.2	0.6
2	13.2	2.9	81.8	2.9	0.7
3	21.7	6.5	70.7	4.2	0.4
4	22.6	7.5	68.8	5.6	1.1
Mean	18.45	5.325	74.925	3.975	0.7
standard deviation	3.872015	1.79217	5.355079	1.054455	0.254951
t test	0.667534	0.565151	0.622731	0.047371	0.048919

B

Control

Ubi#37/lck-Cre



R1=CD4: 14%

R3=DN: 60%

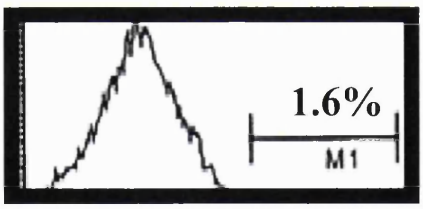
R4=CD8: 4%

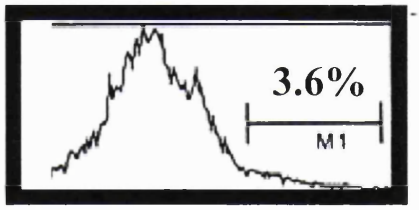
R1=CD4: 11%

R3=DN: 63%

R4=CD8: 4%

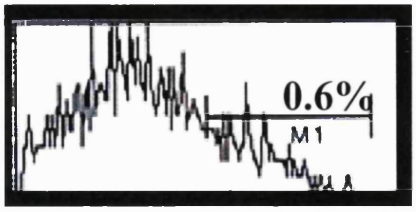
DN TCR+

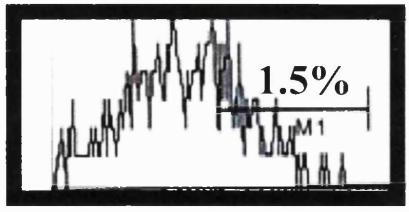




CD8 +

TCR+





3.13.2 Effect of the Id4 overexpression in thymus and spleen from Ubi#10/lck-Cre mice

In order to assess, if the Id4 overexpression had any effect in thymocytes during embryogenesis, thymocytes derived from 14.5 dpc Ubi#10/lck-Cre and littermate lck-Cre control embryos were subjected to FACS analysis with the CD4 and CD8 markers. At this stage, the majority of the cells in the thymus belong to the DN stage (Zuniga-Pflucker and Lenardo 1996). This analysis showed no difference between the Id4 expressing and the control thymocytes; most of the cells belonged to the DN stage (data not shown). However, when part of these thymocytes were left to differentiate *in vitro*, by culturing for 2 weeks in a FTOC system (experiment conducted by Dr. Owen Williams), FACS analysis with the same markers, revealed a variable, but consistent reduction of the numbers of double positive DP cells (2-7X fold) (Table 3.4). A similar effect was observed for the DP positive cells that have initiated α and β chains TCR rearrangements of α and β chains (αβ TCR^+ DP). The numbers of earlier precursors (Thy^+DN) or mature $CD4^+$ and $CD8^+$ cells did not very significantly (Appendix C). To address the question about the cause of this reduction in the DP positive cell numbers, detection of apoptosis by FACS with AAD staining was performed. Indeed, this study exposed a tendency for increased apoptosis in this specific cell population (3-6X fold).

When similar apoptosis detection analysis was applied in thymocytes from adult thymus, once more the levels of apoptosis in the DP population in the Ubi#10lck-Cre thymocytes were increased, compared to the control lck-Cre thymocytes. In addition, enhanced apoptosis was observed in the DN and $CD8^+$ (1.5-4X fold increase), but not in the $CD4^+$ compartment (Table 3.4).

Despite the apoptosis, detailed FACS analysis with mature (CD4, CD8, αβ TCR and γδ TCR) and precursor (CD44 and CD25) T cell markers in adult thymus showed a consistent tendency for increased numbers in the $CD44^+$, $CD25^+$, DP and $CD8^+$ cell populations. The most significant increase was observed in the $CD8^+$ and $CD4^+$ cell population (Table 3.4 and Appendix C). In addition, the numbers of αβ TCR $CD4^+$ and activated $CD4^+$ cells were all increased (2-4X fold) (Table 3.4). The increase in the mature T cell was also confirmed by immunohistological analysis in thymus

sections from Ubi#10/lck-Cre mice with a T cell specific antibody (anti-CD3). This assay indicated an increase of T cells numbers in the thymus medulla (Figure 3.32).

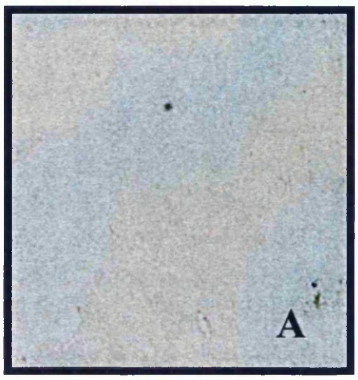
Table 3.4: FACS analysis in thymocytes from Ubi#10/lck-Cre mice. Two examples, of samples and controls respectively, are presented. DP: $CD4^+CD8^+$, DN: $CD4^-CD8^-$, CD69: marker for activated T cells; AAD: staining for the detection of apoptosis. The results are presented either as absolute numbers of cell, or as percentages of the total cell population. The t test for two tailed distribution at significance levels of 0.05 or 0.01 was applied. The values, which reached the significance level of 0.05, are presented in red.

	Apoptosis in adult thymus of Ubi#10/lck-Cre mice $10X^7$ cells			**$CD4^+$ cells in adult thymus of Ubi#10/lck-Cre mice cells $10X^7$ cells**			**FTOC of Ubi#10/lck-Cre thymocytes % of total**	
#	**DP^+ AAD**	**DN^+ AAD**	**$CD8^+$ AAD**	**$CD4^+$**	**$CD4^+$ TCR $\alpha\beta^+$**	**$CD4^+$ $CD69^+$**	**DP**	**DPAAD**
Ubi#10/lck-Cre								
1	11.1	13.2	0.1	90.47	79.6	52	5.3	1.7
2	12.6	15.4	0.3	102.3	90.6	60	1.5	3.7
3	1.8	4.4	0.09	82.6	73	25.6	5.7	2.4
4	2.4	4.4	0.05	57.3	51.2	23.7	1.3	6.3
5	11.9	15.3	0.1	89.5	79.9	23.2		
6				101.7	91.4			
7				85.6	76.8			
8				54	51.9			
9				64.4	62.7			
12				28.7	25.2			
13				29.5	25.7			
Mean	7.5	10.5	0.13	71.4	64.3	36.9	3.45	3.45
Standard eviation	5.9	5.7	0.09	26.5	23.4	17.6	2.37	2.01
Control lck-Cre								
1	1.6	4	0.03	37.4	27.2	17.3	14.5	0.75
2	2.4	4.5	0.03	19.7	19	23.6	8.7	1.1
3	4.6	3.4	0.01	24.3	23.6	26.7	7.3	1.2
4	1.2	7.4	0.05	63.9	55.4	30.2		
5				36.9	28.8	21		
Mean	2.45	4.8	0.03	36.4	30.8	23.7	10.1	1.01
Standard deviation	1.5	1.77	0.016	19.8	16.4	5.47	3.8	0.23
t test	0.09	0.09	0.09	**0.03**	**0.02**	**0.02**	**0.03**	0.09

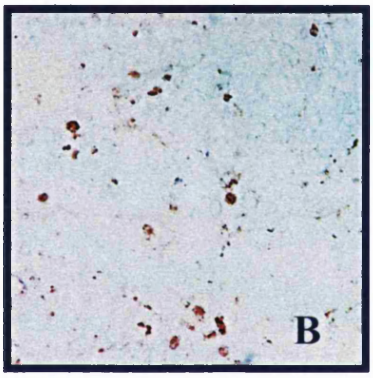
Figure 3.32: Increased numbers of mature T cells in the medulla of adult thymi from mice overexpressing the Id4 protein.

A) No primary antibody as control for the immunocytochemistry, B) horizontal sections from Ubi #10/lck-Cre thymus and C: horizontal sections from lck-Cre thymus. The tissues were processed before embedding in paraffin and sections were cut at 5ì . The binding of the mature T cell specific antibody (anti-CD3) was visualised with DAB brown staining. No counterstaining has been applied. Two animals were analysed (one of the Ubi#10/lck-Cre genotype and a lck-Cre single transgenic littermate). The table presents the numbers of cells and cells stained with the T cell specific antibody counted in three different areas of the medulla. The t-test was applied (two tailed distribution) at a significance level of α=0.1.

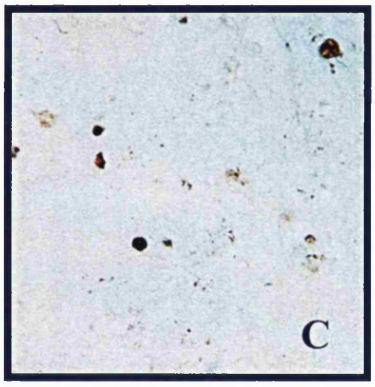
No primary



Ubi#10/lck-Cre



lck-Cre



	total number of cell counted	T cells	% of T cells
	Ubi#10/lck-Cre		
Area 1	100	70	0.7
Area 2	150	85	0.566666667
Area 3	150	83	0.553333333
Mean	133.3333333	79.33333	0.606666667
standard deviation	28.86751346	8.144528	0.0811035
	lck-Cre		
Area 1	100	37	0.37
Area 2	100	52	0.52
Area 3	100	25	0.25
Mean	100	38	0.38
standard deviation	0	13.52775	0.135277493
t test	0.116116523	0.010546	**0.067549406**

Initial stereoscopic examination in spleens from Ubi#10/lck-Cre mice and lck-Cre mice revealed profound splenomegaly in mice overexpressing the Id4 protein. The effect was more intense in older mice (10-12 months of age) (Figure 3.33). Further histological analysis by HE staining inferred a marked accumulation of nucleated cells in certain areas, such as the red pulp, the marginal zone and the periarteriolar sheaths (Figure 3.34). Moreover, cell counting in splenocytes from Ubi#10/lck-Cre mice indicated a massive increase in the splenic population (3-7X more cells than the control-data not shown).

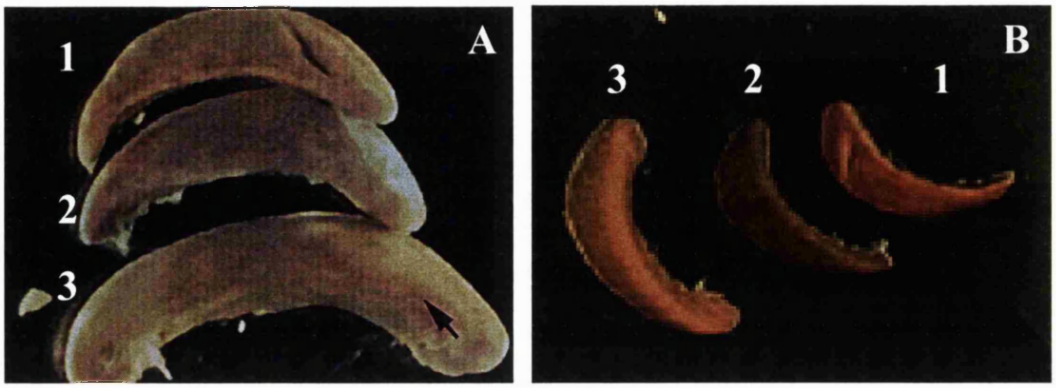
Taking into account the expansion of T cells in the thymus, in order to address the issue of this cell expansion, specific sorting of the splenic thymocytes by CD4, CD8 and αβ TCR markers was conducted. This study showed clearly elevated numbers of $CD4^+\alpha\beta$ TCR^+ mature cells and $CD8^+\alpha\beta$ TCR^+ mature cells (3-5X fold) in the spleen from Ubi#10/lck-Cre mice (Figure 3.35). The expansion in these cell populations was consistent, but more dramatic, than in the thymus. Additional analysis by immunostaining of spleen sections with the T cell specific antibody (anti-CD3) confirmed these results (Figure 3.36). Immunostaining with markers for other types of cells found in the spleen, such as erythrocytes and B cells excluded any possibility of any increase, due to boost in these cell populations (Figure 3.37 and Figure 3.38). However consistently with the expansion of the red pulp area, the distribution of the lymphoid follicles (areas where B cell reside) was constrained (Figure 3.38).

Figure 3.33: Splenomegaly in mice overexpressing the Id4 protein.

1 and 2: Double transgenic Ubi#10/lck-Cre spleens. 3: littermate single transgenic lck-Cre spleen. Note the difference, not only the difference in size, but also the difference in thickness (arrow). The mice were sacrificed at the age of 12 months. Figures A and B show the same samples photographed from a different plane.

Figure 3.34: Accumulation of cells in the red pulp of spleen from mice overexpressing the Id4 protein.

A and C: spleen horizontal sections from control single transgenic mouse. B and D: spleen horizontal sections from mouse overexpressing the Id4 protein. Note the increase in the red pulp area (compare A and B) and the number of nucleated cells (frame in D) appearing in the Ubi#10/lck-Cre mice. The areas where the accumulation is observed are the same ones where Id4 is overexpressed; red pulp, marginal zone of white pulp and periarteriolar sheaths. rp: red pulp; wp: white pulp. The tissues were dehydrated before embedding in paraffin and sections were cut at 5 μ. A and B: X20/0.45, C and D: Magnification of X40/0.75 photos.


1
2
3
A
3
2
1
B

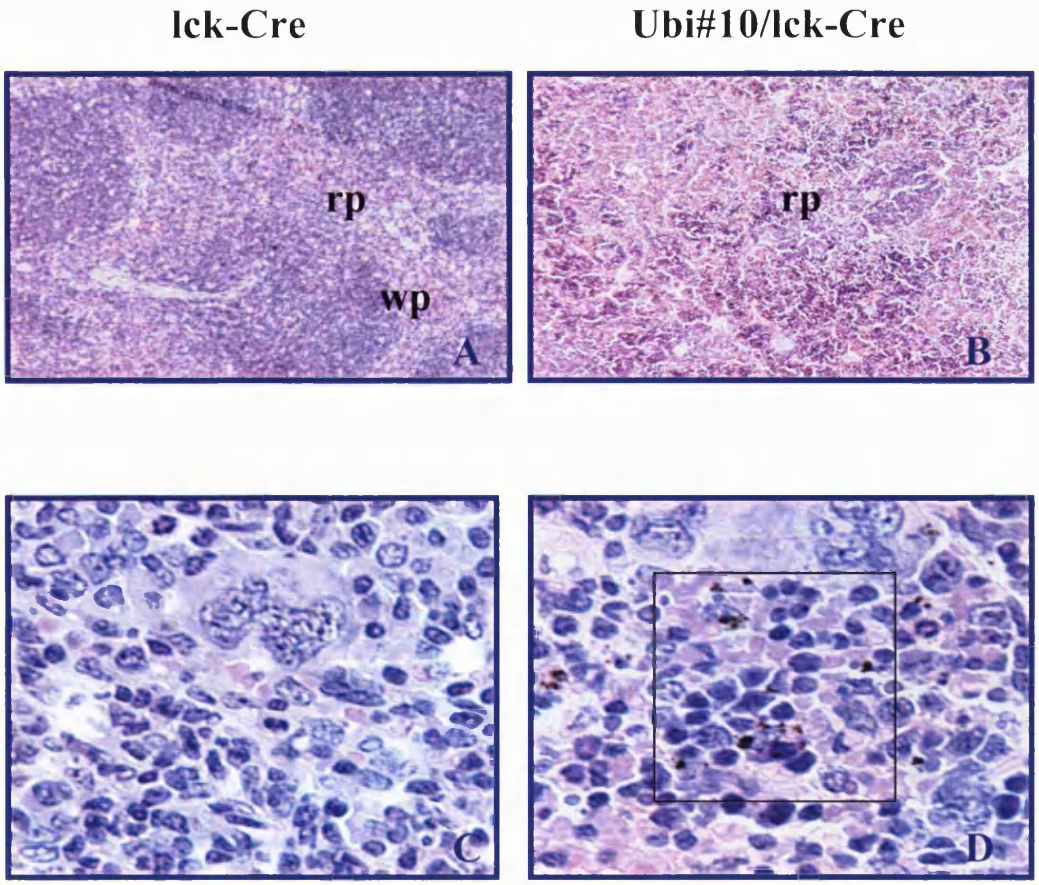
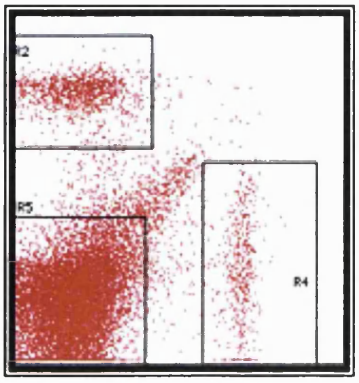

lck-Cre
Ubi#10/lck-Cre
rp
wp
A
rp
B
C
D

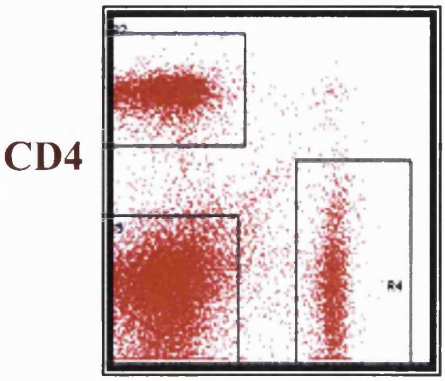
Figure 3.35: Increase in the numbers of mature $CD4^+$ and $CD8^+$ cells in spleen from Ubi#10/lck-Cre mice.

Numbers are given as % of total cells. DP refers to cells positive for both the $CD4^+$ and $CD8^+$ markers. Note the increase in the numbers of $CD4^+\alpha\beta TCR^+$ and $CD8^+$ $\alpha\beta TCR^+$ cell population (4-5X more than the spleen from the control single transgenic littermate). The anti-CD8 FITC, anti-CD4PE and anti-H57 bio ($\alpha\beta$ TCR specific) antibodies were used fort he analysis in dilution 1:100. (The different panels showing the cell counts are not drawn in scale). The table presents the measurements taken for 5 samples. The t-test (two tailed distribution) was applied at a significance level of α=0.05

Control

Ubi#10/lck-Cre





R1=CD4: 5%

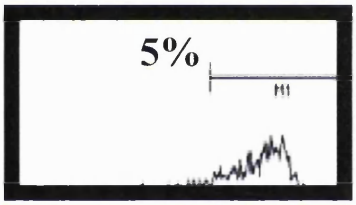
R3=DN: 83%

R4=CD8: 2%

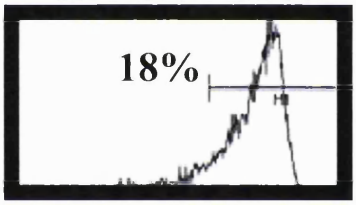
R1=CD4: 20%

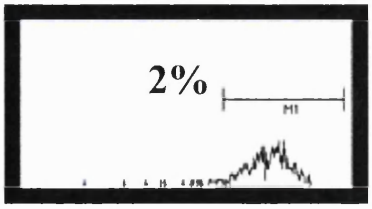
R3=DN: 67%

R4=CD8: 10.5%

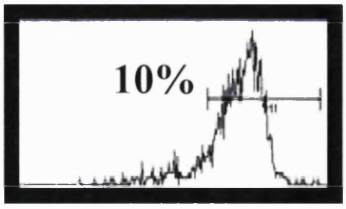


CD4+ TCR +





CD8 + TCR +



	CD4	CD8	abTCRCD4	ab TCRCD8	
Ubi#10/lck-Cre					
1	19.75	10.6	17.9	9.9	
2	15	8.2	14.3	7.4	
3	12.3	6.53	11.7	5.8	
Mean	15.68333	8.443333	14.63333333	7.7	
standard deviation	3.079592	1.670456	2.542090129	1.687207	
lck-Cre					
1	5.48	2.1	4.6	1.8	
2	7.4	2.85	5.7	1.95	
mean	6.44	2.475	5.15	1.875	
standard deviation	0.96	0.375	0.55	0.075	
t test	0.04985	0.030945	0.027568066	0.032455	

Figure 3.36: Increased numbers of mature T cells in the adult spleen from mice overexpressing the Id4 protein.

A: control lck-Cre horizontal spleen sections. B: Ubi#10/lck-Cre horizontal spleen sections. T cell specific binding was achieved with anti-CD3 antibody. Controls included incubation without primary antibody (not shown). The binding was visualised with DAB dark brown staining. The tissues were dehydrated before embedding in paraffin and sections were cut at 5 μ. No counterstaining was performed. Two animals were analysed (one of the Ubi#10/lck-Cre genotype and a lck-Cre single transgenic littermate). The table presents the numbers of cells and cells stained with the T cell specific antibody counted in three different areas of the spleen. The t-test was applied (two tailed distribution) at a significance level of α=0.05.

lck-Cre **Ubi#10/lck-Cre**

	total number of cell counted	**T cells**	**% of T cells**
	Ubi#10/lck-Cre		
Area 1	480	71	0.147916667
Area 2	520	82	0.157692308
Area 3	403	65	0.161290323
Mean	467.6666667	72.66667	0.155633099
standard deviation	59.46707773	8.621678	0.006920544
	lck-Cre		
Area 1	360	46	0.46
Area 2	370	59	0.59
Area 3	290	27	0.27
Mean	340	44	0.44
standard deviation	43.58898944	16.09348	0.160934769
t test	0.039979025	0.053011	**0.037744785**

Figure 3.37: Normal expression of erythrocytes in the red pulp of mice overexpressing the Id4 protein.

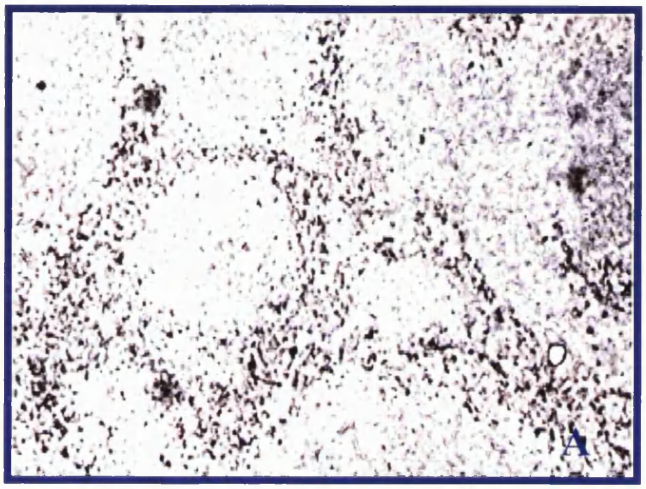
A and B spleen from control and UbiId4#10/lck-Cre mice respectively. The dark brown DAB staining indicated specific binding. Some background is also detected, but it is clearly insignificant (C and D). C, D: Controls for the immunocytochemistry. Sections were counterstained with Neutral red.

Figure 3.38: Restricted distribution of B cells in the spleen of mice overexpressing the Id4 protein.

Immunocytochemistry with B cells specific antibody in spleen from Ubi#10/lck-Cre mouse (B) revealed expression of B cells marker similar to the control (A). However, there was a perturbation in the distribution of B cells in the lymphoid follicles. The distribution of B cells was more restricted in the spleen from mice overexpressing the Id4. C: High magnification of A. B cells stained with the antibody, as indicated by the dark grey staining. D: control for the immunocytochemistry; no primary antibody. Sections were counterstained with Eosin.

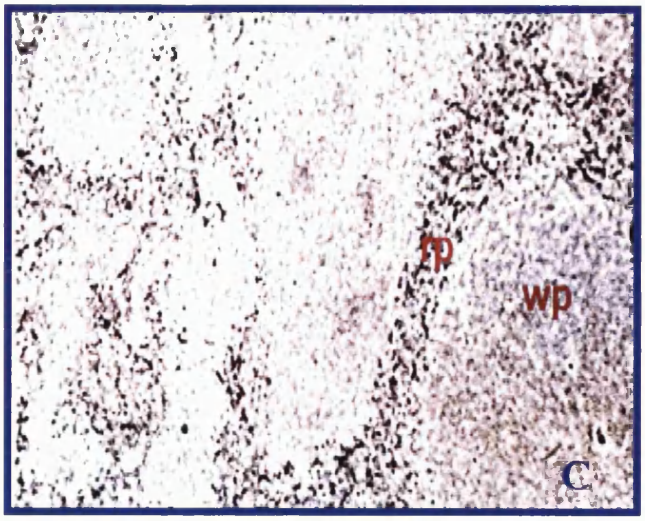
Erythrocyte specific staining

control



no primary

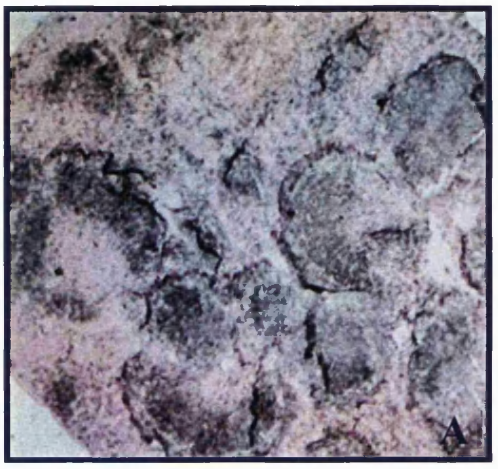
Ubi#10/lck



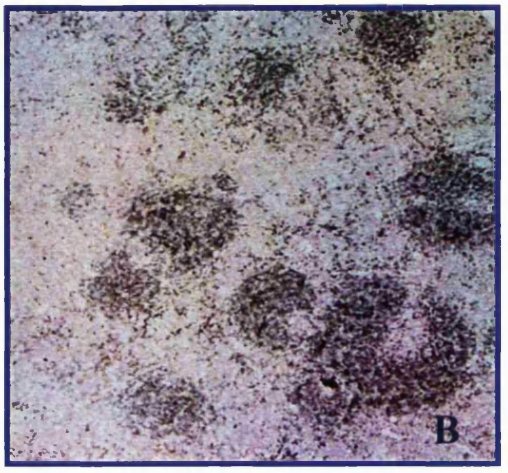
no streptavidin

B cell specific staining

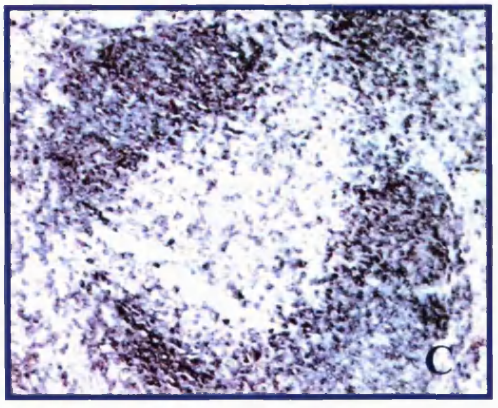
lck-Cre



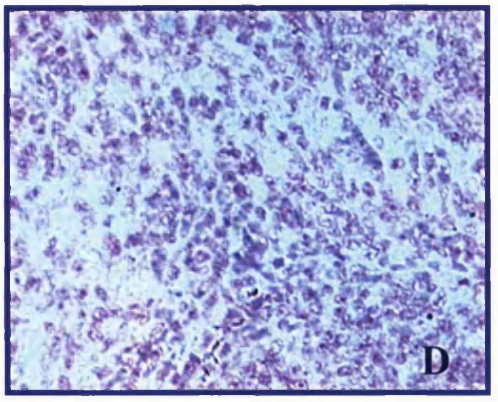
Ubi#10/lck-Cre



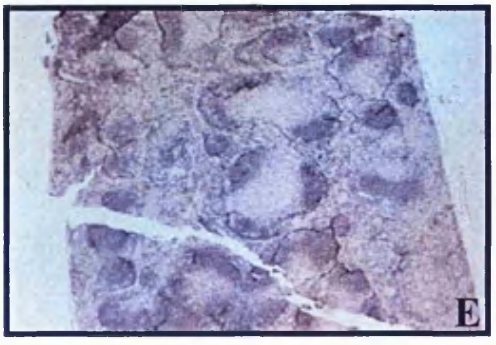
lck-Cre



No primary lck-Cre



lck-Cre



Ubi#10/lck-Cre

3.14 SUMMARY

Mice were generated that overexpress the Id4 protein in thymocytes. Id4 is not normally expressed in mouse thymocytes (Riechmann, van Crüchten et al. 1994; van Cruchten, Cinato et al. 1998). Thus, it was intriguing to find out, if any difference in the level of expression, would have an effect in T cell differentiation, similar to the other Id proteins (Heemskerk, Blom et al. 1997; Blom, Heemskerk et al. 1999; Kim, Peng et al. 1999; Morrow, Mayer et al. 1999).

The Ubi-loxP-Id4#/ck-Cre mice did show ectopic expression of Id4 in the adult thymus and spleen, albeit at low levels. This upregulation was more evident in the spleen, probably because the numbers of mature thymocytes are significantly higher in the adult spleen. Approximately ~35%-45% of the splenic population is mature thymocytes in contrast to the thymus, where the majority of thymocytes belong to the DP compartment and only 13% represent mature thymocytes (Zuniga-Pflucker and Lenardo 1996; Martihn A. Nolte 2000). Another possible explanation is the design of our experiment. Even if the levels of transgene expression were low in the thymus, once the STOP signal had been deleted, the transgene stayed on in mature thymocytes, resulting in a progressive accumulation of Id4 protein in thymocytes. Hence, the higher levels of Id4 in spleen.

The analysis was complicated further, by the variegation in the transgene expression levels. Nevertheless, analysis of the thymi from Ubi-loxP-Id4/lck-Cre transgenic mice revealed a reproducible effect in thymocyte development.

First, in the thymus from Ubi#10/lck-Cre mice, a trend for increased apoptosis could be detected at most stages of differentiation, in the DP and DN $CD25^+$, $DNCD44^+$, $CD25^+CD8^+$, $CD44^+CD8^+$ and $CD8^+$ compartment. Despite the apoptosis, the $CD4^+$ and $CD8^+$ populations in the adult thymus were increased. This increase was more notable in spleen, where the numbers of mature $CD4^+\alpha\beta TCR^+$ and $CD4^+\alpha\beta$ TCR^+ cells were significantly higher, than the control. The upregulation of mature thymocytes was also confirmed by immunohistological analysis with a T cell specific antibody.

In the Ubi#37/lck-Cre line, there was an increase in the NK^+ cells in both the

thymus and the spleen. In addition, an increase in the $CD8^+$ thymocytes was observed. This is slightly different from the Ubi#10/lck-Cre mice, in which an expansion in $CD4^+$ was also exhibited. This phenomenon of transgenic mice established from different founders behaving differently is something common and has been reported in other cases (Morrow, Mayer et al. 1999). Further analysis for addressing the question of apoptosis in the Ubi#37/lck-Cre thymocytes is still required.

Another intriguing feature of the spleen of the Ubi#10/lck-Cre was the observed splenomegaly and the disrupted histology. In all cases of Id overexpression, development of lymphomas at later stages was one of the common effects. Splenomegaly, vascularisation and accumulation of cells are associated with splenic lymphoma. However, further clinical analysis is required to establish this case with confidence.

In brief, the data presented here support the Id4 influence in thymocyte development and lineage commitment. The phenotype observed by the ectopic expression resembles in some cases the reported phenotypes from the overexpression of other Id proteins. Since the Id4 protein is not normally present in thymocytes, it is logical to assume that this phenotype is partially due to homology of the HLH domain. In other words, Id4 can compensate partially for other Id proteins.

CHAPTER 4

DISCUSSION

DISCUSSION

4.1 INTRODUCTION

The Id4 protein is the less studied from the group of Id proteins. The aim of this project was to employ the Cre/loxP system to assess the function of Id4 *in vivo*. Mice expressing the Cre recombinase specifically in the central nervous system were successfully generated. In the addition, mice that carry an Id4 dormant transgene that can be activated after Cre mediated deletion in a tissue-restricted way were also created. These lines along with the already established thymocyte specific Cre transgenic line enabled us to address the question of the role of Id4 in neurogenesis and lymphopoiesis.

4.2 NEURON SPECIFIC CRE MICE

From 11 transgenic mouse lines carrying the NSE-Cre transgene generated, only one expressed the Cre recombinase in a brain specific way. This indicates that the NSE promoter has the potential to drive neuron specific expression of transgenes, however its regulation is highly dependent on other factors, such as cis and trans regulatory elements in its vicinity.

More interestingly, when the NSE Cre transgenic mice were crossed with the Id4 transgene line, overexpression of the Id4 protein was detected not only in neurons, but also in glial cells. The NSE promoter was believed, until recently, to drive expression, only in mature neuron cells. This conclusion is partially based on other transgenic lines, in which the NSE was used to drive expression of transgenes. The published data from the analysis of these mice was mainly restricted to the areas of NSE driven expression and not in the specific cell populations. Our data strongly suggest NSE promoter activation in the glial cell lineage and are in good agreement with more recent reports, where NSE activity has been shown in glial precursors, but not in mature glial cells (Deloulme, Lucas et al. 1996).

Nonetheless, the NSE-Cre mice express the Cre recombinase in brain specific way. Cre protein expression is initiated early in development and in specific areas of the brain, such as forebrain, hindbrain and a region along the midline flexure. Due to

this early expression, Cre mediated deletion was widespread in the adult brain, albeit in variegated levels. The highest levels of Cre mediated deletion were detected in the cortex and hippocampus. Other areas of Cre protein activation included the septum, caudate putamen, cerebellum, striatum and the amygdaloid areas. The actual Cre expression in the adult brain however, was more restricted. Cortex and cerebellum expressed Cre recombinase, whereas in hippocampus the levels of Cre protein were very low. This pattern of expression is similar to the one observed when the NSE promoter was used to drive expression of luciferase or CREB (cAMP-response element binding protein) genes in an inducible system in adult mouse brain (Chen, Kelz et al. 1998).

For the purpose of this study, the expression pattern of the NSE-Cre#26 mice enabled us to overexpress Id4 early in development, during neuronal cells differentiation and to assess the importance of this expression at later stages of development. This can be also applied for functional analysis of other genes. The early activation of Cre recombinase in the NSE-Cre#26 mice constitutes a powerful tool for the assessment of developmental mechanisms involved in neurogenesis.

Lately, much interest has been shown for the application of conditional mutagenesis strategies in the study of complex neuro-physiological functions, such as learning and memory (Mayford, Abel et al. 1995; Mayford, Mansuy et al. 1997; Mayford and Kandel 1999). Recent anatomical and physiological studies indicate that there are strong similarities between the organisation of the human brain and that of other mammals, such as monkeys, rats and mice (Burwell and Jones 1996). In a pioneering experiment, Tsien et al, (1998), used Cre transgenic mice with targeted expression of the Cre recombinase in the CA1 pyramidal cells of the hippocampus to inactivate the NMDA (N-Methyl-D-Aspartate) receptor gene (Tsien, Chen et al. 1996). The outcome of this study was that mice had lost the capacity of long term potentiation (LTP) in the hippocampus. Long term potentiation is a mechanism involved in explicit memory, (concerns collection and retaining of information about facts, places, objects and people). These mice also showed spatial learning difficulties, as assessed by specific behavioural tests. Some of the areas where Cre recombinase activation was observed, in the NSE-Cre#26 mice, such as the hippocampus, septum, amygdaloid areas and perirhinal and entorhinal cortex are part

of a network known as the limbic system. It has been speculated that this network is heavily involved in explicit memory storage. Studies of learning and memory therefore, can be evidently benefited by the use of the NSE-Cre#26 transgenic line.

Finally, the NSE-Cre mice can contribute to the panel of neuron specific Cre mice already established. Each of these mice has a unique time point and pattern of Cre expression in certain areas of the central nervous system. For example, the transgenic line established by Isaka et co-workers, express the Cre recombinase under the nestin promoter and enhancer in a widespread manner during development; including most parts of the developing brain and spinal cord (Isaka, Ishibashi et al. 1999). On the other side, when the myelin basic protein (MBP) promoter was used to drive expression of the Cre recombinase, active Cre protein was found only in myelinating cells of the central nervous system. The NSE-Cre#26 transgenic mice therefore, can complement other neuron specific Cre lines for targeting mutations in specific areas the brain. This enables a more refined and controlled analysis of the role of genes in brain development, anatomy and physiology.

4.3 THE Id4 TRASNGENIC MICE

The Id4 transgenic mice have the potential to overexpress Id4 upon Cre mediated recombination in any tissue desired. This line provides a powerful tool for further analysis of the Id4 protein. It can be employed to achieve ectopic expression of the Id4 in any tissue of interest, upon crossing with the appropriate Cre transgenic line. For example, Id4 protein has been found to be expressed in testis in type A1 spermatogonia and at specific stages of differentiation of postmeiotic spermatids (Sablitzky, Moore et al. 1998). Recently, transgenic mice that express Cre recombinase in primary spermatocytes have been developed (Vidal, Sage et al. 1998). A possible cross of these mice with the Id4 transgenic line would aid to assess the significance of the Id4 protein in spermatogenesis.

Furthermore, the dormant Id4 transgenic mice can be used for rescue of the phenotype in Id4 knockout mice in a widespread or a tissue specific way. They can also be used for similar experiments with mice deficient for any of the other Id proteins, or their partners the Class A bHLH. Such a strategy would shed light in the interactions between the different members of the Id subgroup, as well as their

relation to the bHLH proteins.

4.4 ROLE OF Id4 IN THE LYMHPOID SYSTEM

By ectopically overexpressing Id4 in a tissue where it is not normally expressed, it was feasible to address the question about Id genes' redundancy. Initial analysis of knockout mice has indicated that the Id proteins can compensate for each other. On the other hand, *in vitro* analysis by overexpression of Id in cultured myocytes clearly showed that Id4 had no influence on their development (Melnikova, Bounpheng et al. 1999). On the contrary, Id1, Id2 and Id3 have been shown to negatively regulate myogenesis. In other studies as well, Id4 has been shown to behave differently, than the rest of the Id proteins (Andres-Barquin, Hernandez et al. 1998; Andres-Barquin, Hernandez et al. 1999). It was intriguing therefore, to assess if overexpression of Id4 would have an effect in thymocyte development.

Despite the low levels of Id4 overexpression in thymocytes, our results indicate an influence of Id4 in thymocyte development. Interestingly double transgenic mice established from both founders Ubi#10 and Ubi#37 behaved differently. This is something common and has been reported in other cases (Morrow, Mayer et al. 1999). A possible explanation of this difference in phenotype could be differential access of the transgene in the Cre recombinase. The two lines Ubi#10 and Ubi#37 have integrated the transgene at different sites in the genome. It is likely that *cis* and/ or *trans* elements in the vicinity of the transgene influence its conformation. Thus, there is variation in the time and the level of two transgenes' accessibility to the Cre recombinase. A different timing in Cre mediated recombination would result in a different effect of Id4 in thymocyte development, as it already has been stated for the Id3 protein (Heemskerk, Blom et al. 1997); (Blom, Verschuren et al. 1999). In addition, the different sites of integration influence the levels of transgene expression. This is translated in variation of the effect of the Id4 overexpression in the thymocytes development.

First, in the Ubi#37/lck-Cre line, there was an increase in the $CD8^+$ compartment in the thymus, similar to the increase presented by overexpression of the Id2 protein in thymocytes (Morrow, Mayer et al. 1999). In addition, these mice also appeared to have increased numbers of NK marker expressing cells both in thymus and in spleen.

It has been documented that enforced overexpression of Id3 protein in human thymocyte precursors resulted in enhancement of NK development probably, due to a shift from an T cell fate to the NK lineage (Heemskerk, Blom et al. 1997). In the thymus these cells also expressed the CD8 marker, but from our analysis it isnot clear if they represent early precursors or mature $CD8^+$ cells.

In thymus and spleen from Ubi#10/lck-Cre mice the phenotype was distinctly different and more profound. Apoptosis could be detected at most stages of differentiation and the mature $CD8^+$ compartment. In contrast, an increase of $CD4^+$ cells was observed. This increase was more significant in spleen, where in particular the numbers of $CD4^+\alpha\beta TCR^+$ cells were significantly higher than the control. This phenotype resembles the effect of knocking out the Id1 protein in thymocytes, where a more severe apoptosis level was detected in most stages of T cell differentiation (Kim, Peng et al. 1999). In contrast to the Id1 case though, where reduced numbers of thymocytes were observed in the adult thymus, in the Ubi#10/lck-Cre mice the numbers of CD4 positive mature thymocytes were actually increased 4-5X fold. Our results indicate that in these mice the Id4 may possibly act on the late checkpoint during development, when is determined whether the T cells will become $CD4^+$ or $CD8^+$ cells. It seems that Id4 expression partially favours cells to acquire the CD4 marker and/or inhibits them from becoming $CD8^+$. This resembles the situation in thymocytes overexpressing the Id3 protein, which demonstrated that the Id3 protein acted in an earlier check-point involved in the mechanism for TCR$\alpha\beta/\gamma\delta$ lineage commitment (Blom, Heemskerk et al. 1999). Constitutive expression of Id4 after this point may result in expansion of the mature T cells, as indicated by the accumulation of the mature T cells in the spleen. At present, more mice are analysed in order to establish with confidence the truth of the suggestions mentioned above.

Another intriguing feature of the spleen of the Ubi#10/lck-Cre is the observed splenomegaly and the disrupted histology. In all cases of Id overexpression, development of lymhomas at later stages of life was a common effect (see 1.9.4). However, further clinical analysis is required to establish this case for the Ubi-loxP-Id4/lck-Cre mice with confidence.

In brief, the data presented here support the idea of an Id4 influence in thymocyte development and lineage commitment. The phenotype observed by the ectopic

expression resembles in some cases the reported phenotypes from the overexpression of other Id proteins. Since the Id4 protein has not been detected in murine thymocytes, we assume that this phenotype is partially due to homology of the HLH domain. In other words, Id4 can compensate partially for other Id proteins.

4.5 ROLE OF Id4 IN THE NERVOUS SYSTEM

In the Ubi#10/NSE-Cre#26 mice, overexpression of Id4 in the cortex resulted in an accumulation of neuron cells, whereas glial cells showed slight reduction or no change at all. At the same time increased levels of apoptosis and proliferation were detected.

Although further analysis of the Ubi#10/NSE-Cre#26 mice is required to define more precisely the role of Id4 in neuronal development, our results clearly show that Id4 is involved in neuronal cell growth and differentiation. Most of the cells found in the mature nervous system originate from multipotential stem cells. These cells proliferate and at some point they become bipotential neural stem cell precursors, which give rise to neuron or to glial precursors cells. These precursors finally give rise to the mature neuron and glial cell populations (see 1.8.3 for details). This process is heavily influenced by extracellular signals and in some case it can be reversed. Kondo et al (2000) for example, have showed recently that oligodendrocyte precursors treated with FCS and BMPs (astrocyte differentiation inducing signals), resumed their multipotential neural cell properties. These findings strongly suggest that the establishment of neural cells fate is more complex, than previous thought.

Overexpression of Id4 in glial, precursor cell cultures has been both correlated with apoptosis (astrocytes) and increased proliferation (oligodendrocytes) (Andres-Barquin, Hernandez et al. 1999; Kondo and Raff 2000). Furthermore, it has been speculated that Id4 acts, as a time regulator, which determines, when oligodendrocytes will withdraw from cell cycle and start proliferation (Kondo and Raff 2000). In the Ubi#10/NSE-Cre#26 mice, both proliferation and apoptosis, were observed. Initial analysis in our lab indicated that the proliferation is detected in neuron-like cells, some of which express mature neuron markers (data not shown). Increased numbers of neurons in the cortex of these mice may be explained by this increased proliferation. In parallel, the increased proliferation in cells that do not

express mature neuron markers raise the possibility, that Id4 interacts with other factors, such as BMPs for instance, resulting in a reversion of the cells to multipotential state.

On the other side, the reduction in the glial cells, along with the increased levels of apoptosis, indicates that programmed cell death, at some point of development, may account for this reduction. Alternatively, overexpression of Id4 in early precursors cells may result in a shift in their fate from glial to neuron cells. It may be that Id4 is prohibiting differentiation towards glial cells, whereas it promotes cells to become neurons. Hence, increased numbers of neurons and reduction of glial cells. Proliferation may further account for the increase in the neuron cells. At the present, it is not clear from our analysis if these neurons are functional or if they undergo apoptosis.

Further analysis will help the elucidation of these questions and will state a role of the Id4 protein in neurogenesis. The current working model for the role of Id4 protein in neurogenesis is presented in Figure 4.1. If this models is proved to be true then the Id4 protein arises as an important factor in specification of neuronal cell fate and in addition raises questions about its potential to be used for brain self repair manipulations.

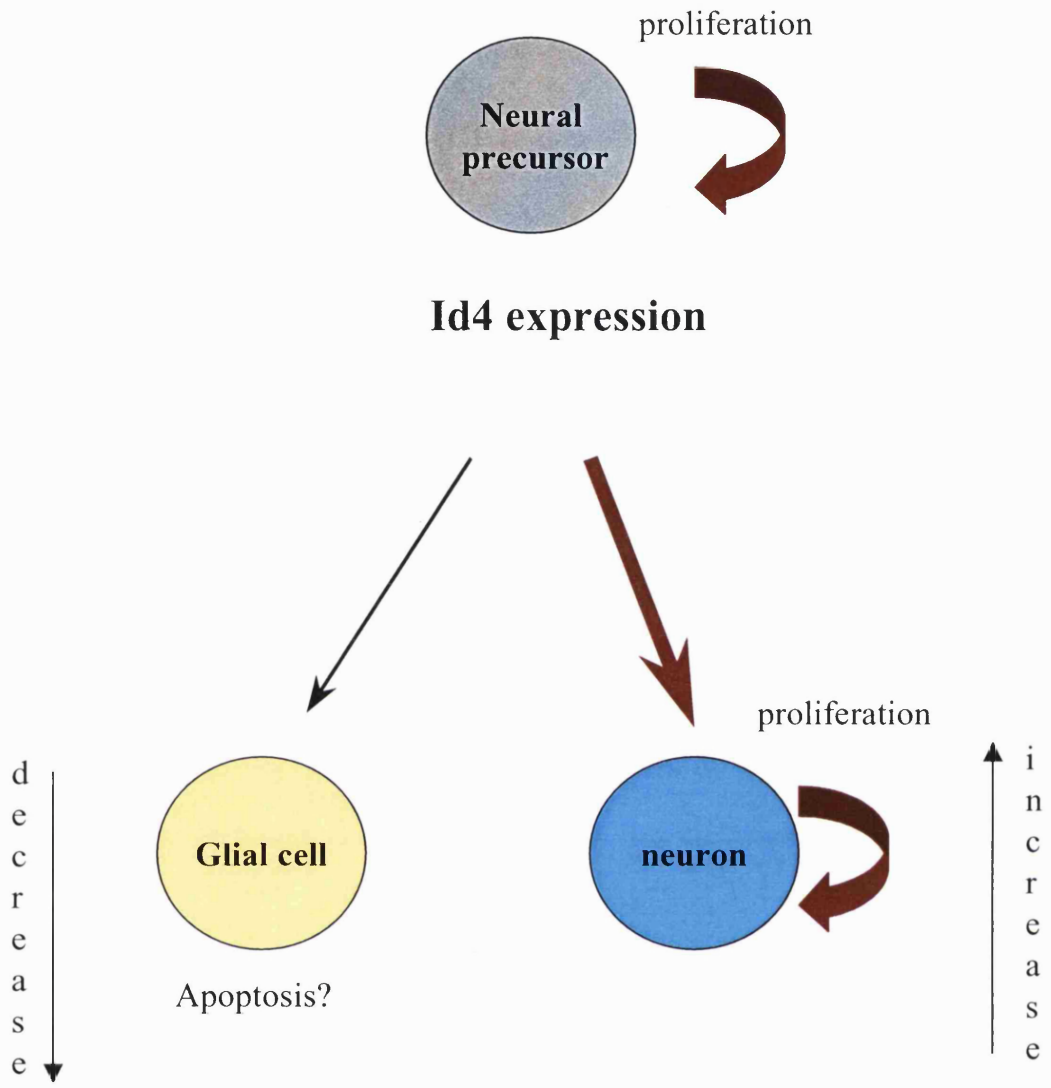


Figure 4.1: Working model for the role of the Id4 protein in the neuronal development. The overexpression of Id4 protein results in increase in the neuron and decrease in the glial cells. The phenomenon is accompanied by increased proliferation and apoptosis. Persistent expression of Id4 in neural precursors enhances the potential of these cells to become neurons (red arrow), instead of glial cells (black arrow). Overproliferation of neurons and apoptosis of glial cells are also probably involved in the shift in the cell numbers.

4.5.1 Future prospects

From the above, it clear that localisation of the proliferation and/ or apoptosis in neurons and/ or glial is required. Furthermore, analysis of neuronal precursors at earlier stages of development will give a clearer picture of the role of Id4 in determining their fate towards neurons or glial cells. In addition, analysis in more animals will determine more precisely the significance of these effects.

In case of the double knockout Id1/Id3 mice one of the phenotypes was immature differentiation (Lyden, Young et al. 1999). It would be useful as well to check the accumulating neuron cells in the Ubi-loxP-Id4/NSE-Cre#26 mice with various markers to check their differentiation state.

At the molecular level, further analysis should be conducted in regard to the other Id genes. It would be of interest to find out if Id4 overexpression has an effect on their levels of expression.

Apart from the cortex, in the Ubi-loxP-Id4/NSE-Cre#26 mice, Id4 is overexpressed in other areas such as the cerebellum and the hippocampus. Analysis of the outcome of Id4 protein overexpression in these areas should also be conducted.

Finally, these mice should be subjected in behavioural and physiological tests to consider any influence of Id4 expression in brain physiology. For the cerebellum for example it is speculated that is involved in control of locomotion and spatial understanding. A variety of behaviour tests could be applied in the Ubi-loxP-Id4/NSE-Cre#26 mice to assess if these mice have any locomotion problems.

4.6 CONCLUSION

This analysis clearly demonstrates the efficiency of the Cre/loxP system for targeted mutagenesis in specific tissues. The application of this system in functional analysis of Id4 protein established for first time that Id4 is implicated *in vivo*, in neuronal cell growth, apoptosis and differentiation.

APPENDIX

APPENDIX A

Chromosomal localisation of the Id genes

Appendix A, Table I: Chromosomal allocation of the Id genes

gene	chromosome
human	
Id1	20q11
Id2	2p25
Id3	1p36
Id4	6p21.3-22
mouse	
Id1	2
Id2	12
Id3	4
Id4	13

bHLH proteins

Appendix A, Table II: bHLH proteins

bHLH protein	Biological function
Class A:	
E12,E47, E2-5,	E proteins. B cell differentiation
E2-2, HEB	Also E proteins. B cell and T cell differentiation
Class B:	
Myogenic: MyoD, Myf-5, MRF4, myogenin	Initiation of myogenic differentiation when expressed ectopically in a variety of cell types. Knock out mouse models have problems with myoblasts and muscles, suggesting a role in myogenesis.
Neurogenic: NeuroD, Neurogenin	Implicated in terminal neuronal differentiation
Mash 1	Involved in neuronal development during embryogenesis
NSCL1, 2	Early neuronal; differentiation
Haematopoietic SCL	Involved in haematopoiesis

Cell cycle

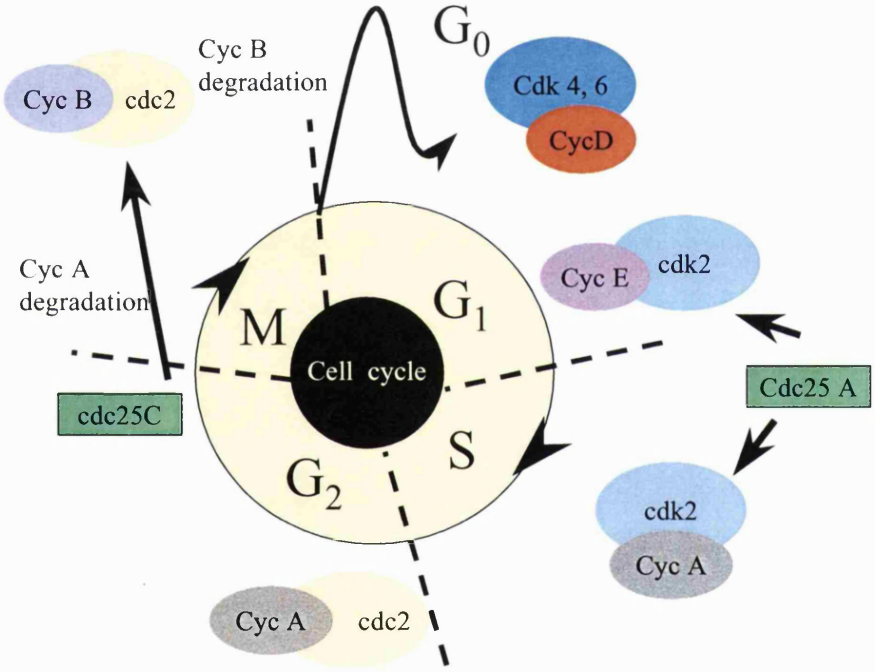
G_0: The cell are resting

G_1: The cell prepares to synthesize DNA

G_2: The cell prepares to undergo division and checks it's replication using DNA enzymes.

S: The cell undergoes DNA synthesis

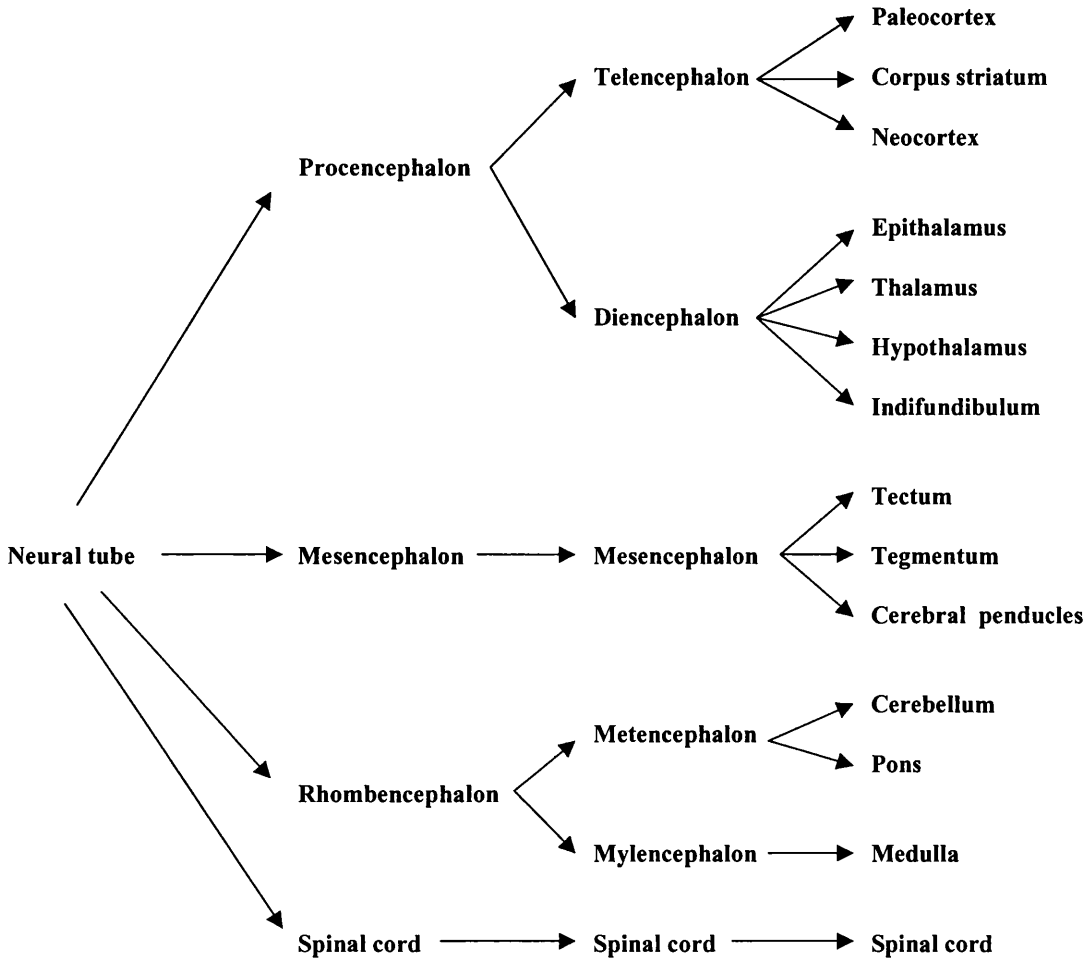
M: The cell undegoes division by mitosis or meiosis



Overview of the cell cycle.The different stages M, G_0, G_1, G_2, and S are depicted. Progression through the cycle is controlled by a group of kinases termed cyclin dependent kinases (cdk's). These kinases can interact with various molecules such as the pRB gene that are responsible for cell cycle progression. The kinases are activated by interacting with proteins whose levels of expression is altered at different stages of the cell cycle. These are the cyclins (Cyc). Once associated with cyclins the cdk's are activated by phosphorylation via CDK- activating kinases or dephosphorylation via CDC25.

APPENDIX A

Central nervous system development



APPENDIX B

Length of Ubi-loxP-Id4-SV40-pUC18

linear: 5661 bp.

Simple Restriction from: 1 to: 5661.

TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG

>< Bgl II

>< Nde I >< Pst I

Human Ubiquitin C promoter

CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGAG ATCTCTGCAG

NNNNNNNNN NNNNNNNNNNNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNN NN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNN NNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NN NNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNN NN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNN NNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNN NNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNNNNNNNNN NNNNNNN NN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNN NNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNNN NNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNN NNNNNNNNNNNNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNN N NNNNNNNNN NNNNNNNNNNNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNN NNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNNNNNNNNN NNNNNNNNN NNNNN NNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNNNNNNNNNNN NNN NNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN NNNNNNNNN

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNN

NNN NNNNNNNNNNNNNNNNNNN NNNNNNNNNN NNNNNNNNNG

Ubi 1

GAGTTGGCGA GTGTGTTTTG TGAAGTTTTT AGGCACCTTT

>< Spe I

TGAAATGTAA TCATTTGGGT CAATATGTAA TTTTCAGTGT TAGACTAGTA AATTGTCCGC

Ubi 2

TAAATTCTGG CCGTTTTTGG CTTTTTTGTT

>< Hind III >< Pst I

>< Sph I >< Sal I >< Pst I

AAGCTTGCAT GCCTGCAGGT CGACCTGCAG GCCGCCACCA TGGGAAAT

3' neo cassette

5' loxP site >< Not I >< >< Eco RV

AT AACTTCGTAT AATGTATGCT ATACGAAGTT ATTAGGCGGC CGCACTAGTG ATATCTCGT

C GTGACCCATG

GCGATGCCTG CTTGCCGAAT ATCATGGTGG AAAATGGCCG CTTTTCTGGA TTCATCGACT G

TGGCCGGCT GGGTGTGGCG GACCGCTATC AGGACATAGC GTTGGCTACC CGTGATATTG C

TGAAGAGCT TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CC

Neo R

GATTCGCA GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GGGGATCGGC

AATAAAAAGA CAGAATAAAA CGCACGGGTG TTGGGTCGTT

polyA

3' loxP site

TGTTCGGATC ATCCCGCGGA ATATAACTTC GTATAATGTA TGCTATACGA AGTTATTAGG T

CCC

Id4 cDNA

ATCAAG

GCGGTGAGCC CGGTGCGCCC CTCGGGCCGC AAGGCGCCGT CGGGCTGCGG CGGCGGGGAG

CTGGCGCTAC GCTGCCTGGC GGAGCACGGC CACAGCCTGG GTGGCTCGGC AGCCGCCGCC

>< Not I

GCCGCTGCGG CGGCCGCGCG CTGCAAGGCG GCCGAGGCGG CGGCCGATGA GCCGGCGCTG

Id4S →

TGCCTGCAGT GCGATATGAA CGACTGCTAC AGTCGCCTGC GGAGGCTCGT GCCTACCATC C

CGCCCAACA AGAAAGTCAG CAAAGTGGAG ATCCTGCAGCACGTTATCGA CTACATCCTG GA

CCTGCAGC TGGCGCTGGA GACTCACCCT GCTTTGCTGA GACAGCCGCC ACCGCCCGCG CCA

CCTCTCC ACCCGGCCGG GGCTTGTCCG GTCGCGCCGC CGCGGACCCC ACTCACCGCG CTCA

Id4R1 ← **Id4R2** ←

ACACTG ACCCGGCCGG CGCCGTGAAC AAGCAGGGTG ACAGCATTCT CTGCCGCTGA GCTGC

GATGG

Splice donor ↓

>< Not I>< Xho I

Fragment from 3' untranslated Jun B gene

ATGGCCAGGT GTGCGGCCGC CTCGAGNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNN

N NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNN

NNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNN

SV40 poly A site

NNNGATC TTTGTGAAGG AACCTTACTT CTGTGGTGTG **Splice donor** ↓

>< Dra I

ACATAATTGG ACAAACTACC TACAGAGATT TAAAGCTCTA AGGTAAATAT AAAATTTTTA A

GTGTATAAT **Splice acceptor** ↓

GTGTTAAACT ACTGATTCTA ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACTGATG AA

TGGGAGCA GTGGTGGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC CAT

CTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA AAAAAGAAGA GAAA

GGTAGA AGACCCCAAG GACTTTCCTT CAGAATTGCT AAGTTTTTTG AGTCATGCTG TGTTTA

GTAA TAGAACTCTT GCTTGCTTTG CTATTTACAC CACAAAGGAA AAAGCTGCAC TGCTATA

CAA GAAAATTATG GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT ATAATCAT

AA CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA ACTATGCTCA

AAAATTGTGT ACCTTTAGCT TTTTAATTTG TAAAGGGGTT AATAAGGAAT ATTTGATGTA

TAGTGCCTTG ACTAGAGATC ATAATCAGCC ATACCACATT TGTAGAGGTT TTACTTGCTT

>< Dra I **SV40 R** ←

TAAAAAACCT CCCACACCTC CCCCTGAACC TGAAACATAA AATGAATGCA ATTGTTGTTG T

TAACTTGTT TATTGCAGCT **Poly A**

TATAATGGTT ACAAATAAAG CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA C

TGCATTCTA >< Bam HI

>< Sma I

GTTGTGGTTT GTCCAAACTC ATCAATGTAT CTTATCATGT CTGGATCCCC GGGTACCGAG

Eco RI ><

>< Sac I **PUC 18 vector**

TCGAATTCG TAATCATGGT CATAGCTGTT TCCTGTGTGA AATTGTTATC CGCTCACAAT TCC

ACACAAC ATACGAGCCG ……..

AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG CCCTTTCGTC C

LENGNTH OF NLS -CRE SQUENCE (PstI – TAG fragment)

Linear size: 1062 bp

CTGCAGACCA TGCCCAAGAA GAAGAGGAAG GTGTCCAATT TACTGACCGT ACACCAAAAT

Cre2B →

TTGCCTGCAT TACCGGTCGA TGCAACGAGT GATGAGGTTC GCAAGAACCT GATGGACATG

Cre2A ←

TTCAGGGATC GCCAGGCGTT TTCTGAGCAT ACCTGGAAAA TGCTTCTGTC CGTTTGCCGG
TCGTGGGCGG CATGGTGCAA GTTGAATAAC CGGAAATGGT TTCCCGCAGA ACCTGAAGAT
GTTCGCGATT ATCTTCTATA TCTTCAGGCG CGCGGTCTGG CAGTAAAAAC TATCCAGCAA
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AATGCTGTTT CACTGGTTAT GCGGCGGATC CGAAAAGAAA ACGTTGATGC CGGTGAACGT
GCAAAACAGG CTCTAGCGTT CGAACGCACT GATTTCGACC AGGTTCGTTC ACTCATGGAA
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GGGAGAATGT TAATCCATAT TGGCAGAACG AAAACGCTGG TTAGCACCGC AGGTGTAGAG
AAGGCACTTA GCCTGGGGGT AACTAAACTG GTCGAGCGAT GGATTTCCGT CTCTGGTGTA
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TCTGCCACCA GCCAGCTATC AACTCGCGCC CTGGAAGGGA TTTTTGAAGC AACTCATCGA
TTGATTTACG GCGCTAAGGA TGACTCTGGT CAGAGATACC TGGCCTGGTC TGGACACAGT
GCCCGTGTCG GAGCCGCGCG AGATATGGCC CGCGCTGGAG TTTCAATACC GGAGATCATG
CAAGCTGGTG GCTGGACCAA TGTAAATATT GTCATGAACT ATATCCGTAA CCTGGATAGT
GAAACAGGGG CAATGGTGCG CCTGCTGGAA GATGGCGATT AG

PAMA Transgene

TK promoter

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CTGGAATGTTTCCACCCAATGTCGAGCAGTGTGGTTTTGCAAGAGGAAGCAAAAAGCCTC
TCCACCCAGGCCTGGAATGTTTCCACCCAATGTCGAGCAAACCCCGCCCAGCGTCTTGTC
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TK

ATATTAAGGTGACGCGTGTGGCCTCGAACACCGAGCGACCCTGCAGGCCGCCACCATGGG

5' loxP site

AAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGCGGCCGCACTAGTGATATCTC

3' end of neo gene-STOP cassette

GTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGA
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AATAAAAAGACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCGGATCATCCCGCGGAA

3' loxP site

TATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTCCCGGGGATCCC

B-gal gene

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LacZ3

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GCAAAAAGCCTCTCCACCCAGGCCTGGAATGTTTCCACCCAATGTCGAGCAAACCCCGCC
CAGCGTCTTGTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCCA
GGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAACACCGAGCGACCCTG

Poly A sites from the neo gene

CAGCCAATATGGGATCGGCCATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGG
TGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGT
TCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGA
ATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAG
CTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGC
AGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCG
GCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGA
GCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCA
GGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCT
CGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGG
ATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCG
TGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGC

CGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGGGGATCGG
CAATAAAAAGACAGAATAAAACGCACGGGTGTTGGGTCGTTTGTTCGGATCGGCCGCACTAGT
GATATCCCGCGGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCC

APPENDIX C

FACS ANALYSIS

Appendix C, Table I: FACS analysis in thymus of Ubi-loxP-Id4#10/lck-cre adult mice. Average cells numbers ($X10^7$) of each category of thymocytes is given. 5-10 mice of each type were used for the analysis. The F- test and t-test were applied for the significance of variance and change between the two samples at a level of 90%-95%.

Ubi-loxP-Id4#10/lck-Cre			
positive	$x10^7$	control	$x10^7$
CD4	62.65±31	CD4	36.5±15
DP	506.6	DP	350.7
DN	23.6	DN	32.7
CD8	11.5	CD8	6.9
αβCD4	54.65±27	αβCD4	30.8±12
αβDP	155.95	αβDP	149.9
αβDN	5.15	αβDN	4.1
αβCD8	7.85	αβCD8	5.3
CD25CD4	16.55	CD25CD4	23.8
CD25DP	167.75	CD25DP	302.2
CD25DN	16.71±16	CD25DN	22.7±2
CD25CD8	3.6±3	CD25CD8	5.2±0.7
CD44CD4	54.55	CD44CD4	28.5
CD44DP	435.1	CD44DP	315.9
CD44DN	17.9±17	CD44DN	23.9±4
CD44CD8	10.5±4.5	CD44CD8	5.6±1.2
CD69CD4	35.2±17	CD69CD4	24.6±4.6
CD69DP	175.8	CD69DP	125.6
CD69DN	21.4	CD69DN	12.9
CD69CD8	6.2	CD69CD8	4.3
AAD CD4	0.29	AAD CD4	0.22
AAD DP	5.5±5	AAD DP	2.7±1.3
AAD DN	8.3±5.5	AAD DN	5.9±4.9
AAD CD8	0.09±0.08	AADCD8	0.04±0.03
AAD CD4-4H	3.2	AAD CD4-4H	2.4

AAD DP-4H	53.4	AAD DP-4H	41.9
AAD DN-4H	7	AAD DN-4H	4.3
AAD CD8-4H	0.88±0.8	AAD CD8-4H	0.44±0.2

Appendix C, Table II: FACS analysis in cultured thymocytes from Ubi-loxP-Id4#10/lck-cre embryos. Average percentages of total thymocytes are given. 4 mice of each type were used for the analysis. The F- test and t-test were applied for the significance of variance and change between the two samples at a level of 90%-95%.

FTOC			
positive		negative	
THY+DN	55.2	THY+DN	52.2
THY+DP	7.8±3.5	THY+DP	15.9±6
APOPTOSIS HYDN	1.6	APOPTOSIS THYDN	1.6
APOTOSIS THYDP	3.5±2	AOPTOSIS THYDP	1±0.2
CD4	2.6	CD4	2.7
DP	4.2±2.3	DP	10.2±4
DN	85.6	DN	75.3
CD8	8.5	CD8	10.7
αβD4	2.7	ABD4	2.7
αβDP	3.5±2	αβDP	10.2±4
αβDN	84.8	αβDN	76.3
αβCD8	8.4	αβCD8	10.8

Appendix C, Table III: FACS analysis in thymus of Ubi-loxP-Id4#37/lck-cre adult mice. Average percentages of total thymocytes are given. 4 mice of each type were used for the analysis. The F- test and t-test were applied for the significance of variance and change between the two samples at a level of 90%-95%.

Ubi-loxP-Id4#37/lck-Cre adults			
positive		negative	
CD4	10.5	CD4	13.4

DP	73.2	DP	77.5
DN	6.3	DN	4.5
CD8	10.4±7	CD8	4.55±1
αβCD4	9.78	αβCD4	9.7
αβDP	71.1	αβDP	58.1
αβDN	3	αβDN	3.6
αβCD8	2.7	αβCD8	2.7
γδCD4	7.7	γδCD4	9
γδDP	51.8	γδDP	51
γδDN	0.2	γδDN	0.3
γδCD8	3.2	γδCD8	3.2

Appendix C, Table IV: FACS analysis in thymus of Ubi-loxP-Id4#37/lck-cre embryos. Average percentages of total thymocytes are given. 3 mice of each type were used for the analysis. The F- test and t-test were applied for the significance of variance and change between the two samples at a level of 90%-95%.

Ubi-loxP-Id4#37/ lck-Cre 15.5 dpc embryos			
positive		**control**	
CD4	2.1	CD4	2.5
DP	0.05	DP	0.1
DN	97.55	DN	96.3
CD8	0.33	CD8	0.4
αβCD4	0.2	αβCD4	0.15
αβDP	0.02	αβDP	0.03
αβDN	2.6	αβDN	2.9
αβCD8	0.09	αβCD8	0.08
γδCD4	0.8	γδCD4	0.4
γδDP	0.01	γδDP	0.03
γδDN	13.4	γδDN	7.2
γδCD8	0.03	γδCD8	0.06
NKCD4	0.3	NKCD4	0.23
NKDP	0.01	NKDP	0.01
NKDN	4.1±2	NKDN	3.6±0.1
NKCD8	0.03	NKCD8	0.01

APPENDIX D

STRAINS OF MICE

The mice used for the establishment and the subsequent crosses of the transgenic mice were C57BL/6 X DBA/2 wild type mice. These have been derived from crosses between the C57BL/6 and the DBA/2 strains of mice. All the information provided below has been reprinted from http://www.informatics.jax.org and references provided there.

C57BL/6

C57BL

Black, a. Origin: Little 1921 from the mating of female 57 with male 52 from Miss Abbie Lathrop's stock.. C57BL is probably the most widely used of all inbred strains, (substrain C57BL/6 alone accounts for over 14% of occasions on which an inbred strain is used) though in many ways it seems to be atypical of inbred strains of laboratory mice. In contrast to 36 other standard inbred strains, it carries a Y chromosome of Asian *Mus musculus* origin (c.f. AKR and SWR)), and a LINE-1 element derived from *Mus spretus* the frequency of which suggests that up to 6.5% of the genome may be of *M. spretus* origin (A probe designated B6-38 to the pseudoautosomal region of the X and Y chromosome has a characteristic Pst I pattern of fragment sizes which is present only in the C57BL family of strains.
It usually has a good breeding performance, depending on substrain, and has been used as the genetic background for a large number of congenic strains covering both polymorphic and mutant loci.

SubstrainC57BL/6
Inbr (J) 150. This substrain is now probably the most widely used of all inbred strains. Maintained. by J,N, Ola.

Behaviour
High alcohol (ethanol) preference (1/4) .Achieve blood alcohol levels of 60 mg% when access to alcohol is restricted to 60 mins. per day.Alcohol preference may be associated with strain differences in mesolimbic enkephalin gene expression A quasi-congenic QTL introgression strain carrying a low alcohol consumption gene from BALB/c has lower voluntary alcohol consumption than C57BL/6, with 96% of loci in common Low severity of ethanol withdrawal symptoms compared with DBA/2, possibly associated with differences in neuroactive steriod sensitivity Alcohol preference is due to at least two recessive quantitative trait loci that are sex-restricted in expression.
Low 'emotionality' (12/15), high open-field exploration (2/15). High spontaneous locomotor activity (8/9).Short time of immobility in a forced swimming test (8/9). Low shock-avoidance learning (7/9) Low shuttle-box avoidance (5/5), high wheel activity (1/5) Rapid shock-avoidance learning
(2/7) and slow extinction (6/7) High shock-avoidance learning (1/8) High radial-arm maze learning (1/3)
High locomotor activity (1/5) High locomotor activity when grouped (2/6) and single (1/6) Resistant to audiogenic seizures (11/11) Relatively insensitive to the primary odorant isovaleric acid (contrast seven other strains) and may provide an animal model of specific anosmia . Low balsa-wood gnawing activity (2/16) Fawdington and

Festing (1980). High preference for sweet tasting substances (saccharin, sucrose, dulcin and acesulfame, averaged) (1/26) Rejects saline at moderate concentrations (contrast 129)). Feed restriction for nine days failed to cause stereotypic cage cover climbing (contrast DBA/2).

Life-span and spontaneous disease

Primary lung tumours 1% in males, 3% in breeding females and zero in virgin females. Lymphatic leukaemia less than 2%, mammary adenocarcinomas less than 1% Leukaemia 7%. Rare "lipomatous" hamartomas or choristomas have been noted.
Congenital abnormalities 10%, including eye defects, polydactyly and otocephaly. Microphthalmia and anophthalmia 8-20% and hydrocephalus 1-3% . Occular defects appear to be due to defects in development of the lens.
Develop spontaneous auditory degeneration with onset during young adulthood, with enhanced susceptibility to acoustic injury and delayed effects of toluene (contrast CBA/Ca) Carry a single recessive gene different from that found in BALB/cBy and WB/ReJ, causing age-related hearing loss.Hearing loss is caused by degeneration of the organ of Corti, originating in the basal, high frequency region and then proceeding apically over time. This results in a severe sensorineural hearing loss by 14 months of age.
Life-span above average in both sexes in conventional conditions Gross tumour incidence 70%, maximum life-span about 1200 days in SPF conditions.
Dermatitis with intense pruritis leading to self-mutilation and death, and sometimes associated with the mite *Myobia musculi* appears to be more severe in this strain than others.Impaired axonal regeneration involving multiple genetic loci

Anatomy

Small kidney/body weight ratio (19/21).Large thyroid. High total leukocyte count (2/18), low erythrocyte count (14/18). Small hippocampus (9/9) . Accessory spleens in about 32% of mice (2/9) and low number of Peyer's patches (7/7).Higher bone mass than A/J. Low number of haematopoetic stem cells in bone marrow (contrast DBA/2). Hematopoetic stem-cell pool 11-fold lower than in DBA/2. This is largely due to loci on chromosome 1 (Mullersieburg and Riblet, 1996).

Immunology

High susceptibility to induction of amyloid by casein (1/6) Poor immune response to type III pneumococcal polysaccharide (4/5) Poor immune response to synthetic double- stranded RNA (6/7).Good immune response to cholera A and B antigens (2/8). Resistant to induction of anaphylactic shock by ovalbumin. Rapid rejection of about 76% of male skin isografts by females by 25 days (1/10). Poor immune response to GAT (random terpolymer of Glu^{60}, Ala^{30}, Tyr^{10}) (9/10) (, 1974). Good immune response to *Salmonella senftenberg* (1/5) and *S. anatum* (2/5) lipopolysaccharide. Non-responder to synthetic polypeptide Glu^{57}, Lys^{38}, Ala^{5} (cf. 4/7. High sporadic occurrence of natural haemagglutinins to sheep red blood cells. Precipitating and skin sensitising antibodies have slow electrophoretic mobility (6/6). Resistant to anaphylactic shock. Susceptible (1/5) to induction of autoimmune prostatisis (contrast BALB/c). High expression of neutral glycosphingolipid GgOse(4)Cer in concanavalin A stimulated T lymphoblasts (cf 3/6).
Anti-BPO IgE monoclonal antibody produced potent systemic sensitization sufficient for provocation of lethal shock in most aged (6 to 10 months) mice (c.f. 3/8) Susceptible to immunosuppression of contact hypersensitivity by ultraviolet B light (cf 3/18)).

Reproduction

Good reproductive performance (3/8). Litter size 6.2 _ 0.2, sterility . Large litter size (1/6), mean 6.2 . Has longer and more regular oestrus cycles than DBA/2 and C3H/HeJ .Late opening of vagina and first cornification (3/3), but early onset of cyclicity compared with C3H .

Mice carrying the Y-chromosome from *Mus musculus domesticus* from Tirano (Italy) or *M.m. poschiavinus* from Poschiavo (Switzerland) fail to develop normal testes but instead develop ovaries and ovotestes. Some hermaphroditic males become fertile, but the XY females lack normal gonadal steroids and can not carry pregnancy to term. There is delayed expression of IGF-I which may be responsible for the low setroid expression (Villapandofierro et al, 1996).

DBA

rey: *a,b,d.* Origin: Little 1909 from stock segregating for coat colour. Oldest of all inbred strains of mice. In 1929-30 crosses were made between substrains, and several new substrains established, including the widely used substrains /1 and /2. Differences between the substrains are probably too large to be accounted for by mutation, and are probably due to substantial residual heterozygosity following the crosses between substrains. Thus DBA/1 and DBA/2 differ at least at the following loci: *Car2, Ce2, Hc, H2, Ifl, Lsh, Tla,* and *Qa3.* With such large differences, they should probably be regarded as different strains rather than substrains of the same strain. In this listing the two are listed separately. DBA/LiA differs from /1 and /2 at the *Gpdl* locus, and is similar to DBA/2 at the *Tla* locus. Note that unfostered substrains carry the mammary tumour virus and have a high indicence of mammary tumours.

DBA/2

Inbr (J) 150. Origin: Substrain maintained at the Jackson Laboratory. Maint. by J,N, Ola.

Behaviour

Low alcohol preference (4/4), (18/18), (5/5) (McClearn, 1965). High severity of ethanol withdrawal symptoms compared with C57BL/6, possibly associated with differences in neuroactive steriod sensitivity. High shock-avoidance learning (2/9)), (1/9). Low avoidance conditionability (7/9) (Royce, 1972). Long time of immobility in a forced swimming test (3/9).Low shuttle-box avoidance (4/5), high wheel activity. Good long-term memory compared with C3H/He. Slow extinction of learned conditioned avoidance response (7/7). Susceptible to audiogenic seizures (2/11). Long latency to attack crickets (6/7). High rearing (1/7), low defaecation (6/7) in Y-. Low locomotor activity when grouped (6/6) but not when single (3/6). Low social dominance of males in competition for females (6/6). Low balsa-wood gnawing activity (4/16). Low preference for sweet tasting substances (saccharin, sucrose, dulcin and acesulfame, averaged) (20/26).

DBA/2 mice failed to react to a spatial change of objects in an open field, and therefore resemble rats with dorsal lesions of the hippocampus. They may represent a

model of hippocampal dysfunction. Feed restriction for nine days causes a high incidence of stereotypic cage cover climbing (contrast C57BL/6) .

Life-span and spontaneous disease

Primary lung tumours l% in males, 2% in females. Lymphatic leukaemia zero in males, 2% in females and 3% in virgin females. Mammary adenocarcinomas in unfostered substrains l% in males, 72% in breeding females and 48% in virgin females. A high proportion of mammary tumours are of the acinar type (1/7) . Overall tumour incidence 15% in males, 49% in females, including lymphomas 10% in males and 12% in females; mammary tumours zero in males and 31% in virgin females. Leukaemia 3% .

Long life-span in SPF fostered conditions (12/17 = 629 days in males, 15/17 = 719 days in females) with 6-35% liver and 1-23% lung tumours. Long life-span in conventional conditions (21/22 = 707 days in males, 20/22 = 714 days in females) . Life-span 722_30 days in males and 683_26 days in females (Goodrick, 1975).

High incidence of expression of RNA tumour virus group-specific antigen (2/5). Type B reticulum cell neoplasms 18% at about 20 weeks.

Carry three separate recessive genes similar to those found separately in C57BL/6J, BALB/cBy and WB/ReJ, causing age-related hearing loss.

Anatomy

Large testes weight (2/8). Low brain weight (18/18 in males, 15/18 in females). Low brain weight. High erythrocyte count (3/18), low haematocrit (15/18), low mean corpuscular volume (18/18) and low haemoglobin (16/18 or 15/18, depending on substrain).

Small forebrain (9/9), neocortex (9/9) and hippocampus volume (8/9). Cerebellum has an intraculminate fissure between vermian lobule IV and vermian lobule V (the ventral and dorsal lobules of the culmen) (contrast SJL, C57BL/10 and BALB/c). Large heart/body weight (1/5). High number of haematopoetic stem cells in bone marrow (contrast C57BL/6). High level of spontaneous sister chromatid exchange (3/4).

Hematopoetic stem-cell pool 11-fold higher than in C57BL/6. This is largely due to loci on chromosome 1.

Immunology

Resistant to experimental allergic encephalomyelitis (cf. 7/18). Low lymphocyte phytohaemagglutinin response (43/43). Serum antinuclear factor 26% incidence (3/17). Poor immune response to type III pneumococcal polysaccharide (5/5). Good immune response to synthetic double-stranded RNA (2/7) . Poor immune response to cholera A and B antigens (8/9 B, 6/8A). Poor immune response to both ovomucoid and ovalbumin (cf. 2/12) .Precipitating and skin-sensitising antibodies have fast electrophoretic mobility (2/6).Non-discriminator between `H' and `L' sheep erythrocytes (cf. 6/18).Low anti-DNP antibody concentration (7/7). Develops a lethal form of syngeneic graft-vs-host disease when treated with cyclosporine (unlike 5 other strains). Erythrocytes have a high agglutinability (cf. 14/25). Poor immune response to *Salmonella strasbourg* lipopolysaccharide (5/7 to 7/7, depending on substrain). Low PHA-stimulated lymphocyte blastogenic response (5/6High expression of neutral glycosphingolipid GgOse(4)Cer in concanavalin A stimulated T lymphoblasts (cf 3/6).

Reproduction

Poor breeding performance (18/25). Colony output 0.85 young/female/week. Low litter size at weaning of 4.7 (17/26) . Poor breeding performance (8/8). Litter size 4.2_0.3, sterility 31%. Intermediate breeding performance (13/24). Has shorter and

less regular oestrus cycles than C57BL/6J. Susceptible to foetal resorption resulting from restraint-induced stress when mated to C3H/HeJ males, in contrast with CBA/J and A/J. This was reduced by alloimmunization with C3H cells.

Miscellaneous

Recommended host for the following transplantable tumours: fibrosarcoma SaD2, lymphatic leukaemia P1534 and mammary adenocarcinoma CaD2. Hybrids involving DBA/2 are recommended host for transplantable leukaemia L1210, melanoma S91 and MOPC myeloma used as models in screening potential anticancer drugs. The *Fv2*^{r} allele appears to be lethal on the DBA/2 genetic background. High mortality after neonatal thymectomy (5/6) (Law, 1966a).

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