HPRT DEFICIENCY IN CELLS AND MICE

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DECLARATION

The composition of this thesis and the work presented within it are my own, unless otherwise stated. The experiments presented were devised in collaboration with my supervisor, Dr. David W. Melton.

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ABSTRACT

The hypoxanthine guanine phosphoribosyltransferase (HPRT) gene is a housekeeping gene, located on the X chromosome in both human and mouse. In humans, HPRT deficiency causes Lesch-Nyhan syndrome which is characterised by behavioural alterations, including self-injurious behaviour and mental retardation, while partial deficiency causes gouty arthritis. The use of homologous recombination to delete specific parts of the HPRT gene with the aim of studying the control of gene expression has been investigated. The size of the deletion that could be made using a simple procedure of homologous recombination was limited and there was a preference for an insertion mechanism if the targeting vectors were designed to delete more than 20 kb. However, combined with intrachromosomal recombination, the deletion size could be enlarged to at least 30 kb. Using this deletion targeting strategy, a mouse embryonic stem (ES) cell clone with a targeted deletion of the promoter and exons 1-2 in one allele of the adenine phosphoribosyltransferase (APRT) gene was also constructed. This deletion targeted ES clone provides the opportunity to generate APRT knock-out and HPRT/APRT double knock-out animal models for Lesch-Nyhan syndrome. Until recently no spontaneous behavioural abnormalities had been reported in HPRT-deficient mice generated using the embryonic stem cell system. To resolve the asymptomatic ambiguity of HPRT-deficient mice, a hypothesis that mice were more tolerant of HPRT deficiency because they were more reliant on APRT than HPRT for their purine salvage was proposed. The administration of an APRT inhibitor to HPRT-deficient mice induced persistent self-injurious behaviour. This combined genetic and biochemical model will facilitate the study of Lesch-Nyhan syndrome and the evaluation of novel therapies. A novel therapeutic strategy involving the intracerebral transplantation of ES cells was evaluated. HPRT activity was observed in the brain of HPRT-deficient mice which had received intracerebral ES cell transplantation. Some of the implanted ES cells were committed to differentiate down the neural pathway into either neurons or glial cells.

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ABBREVIATIONS

A	adenosine
amp	ampicillin
APRT	adenine phosphoribosyl transferase
ATP	adenosine-5´-triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
cDNA	complementary deoxyribonucleic acid
Ci	Curie
cm	centimetre
CNS	central nervous system
cpm	counts per minute
dATP	deoxyadenosine-5´-triphosphate
dCTP	deoxycytidine-5´-triphosphate
dGTP	deoxyguanosine-5´-triphosphate
DHFR	dihydrofolate reductase
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-5´-triphosphate
dpm	decay per minute
dTMP	deoxythymidylate
DTT	dithiothreitol
dTTP	deoxythymidine-5´-triphosphate
EDTA	[ethylene diamine] tetraacetic acid
EGTA	[ethylene bis(oxyethlenenitrilo)] tetraacetic acid
g	gram
G	guanosine
GTP	guanosine-5´-triphosphate

HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulphonic acid
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPRT	hypoxanthine phosphoribosyl transferase
hr	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kD	kilodalton
l	litre
min	minute
M	molar
MOPS	3-[N-Morpholino]propane-sulphonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NP-40	nonidet P-40
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
rpm	revolutions per minute
S	Svedberg unit
SDS	sodium dodecyl sulphate
T	thymidine
TCA	trichloroacetic acid
TK	thymidine kinase
Tris	tris(hydroxymethyl)-amino-methane
Triton X-100	octylphenoxypolyethoxyethanol
Tween 20	polyoxyethylenesorbitan monolaurate
U	unit
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indol-β-D-galactopyranoside

CHAPTER 1. INTRODUCTION

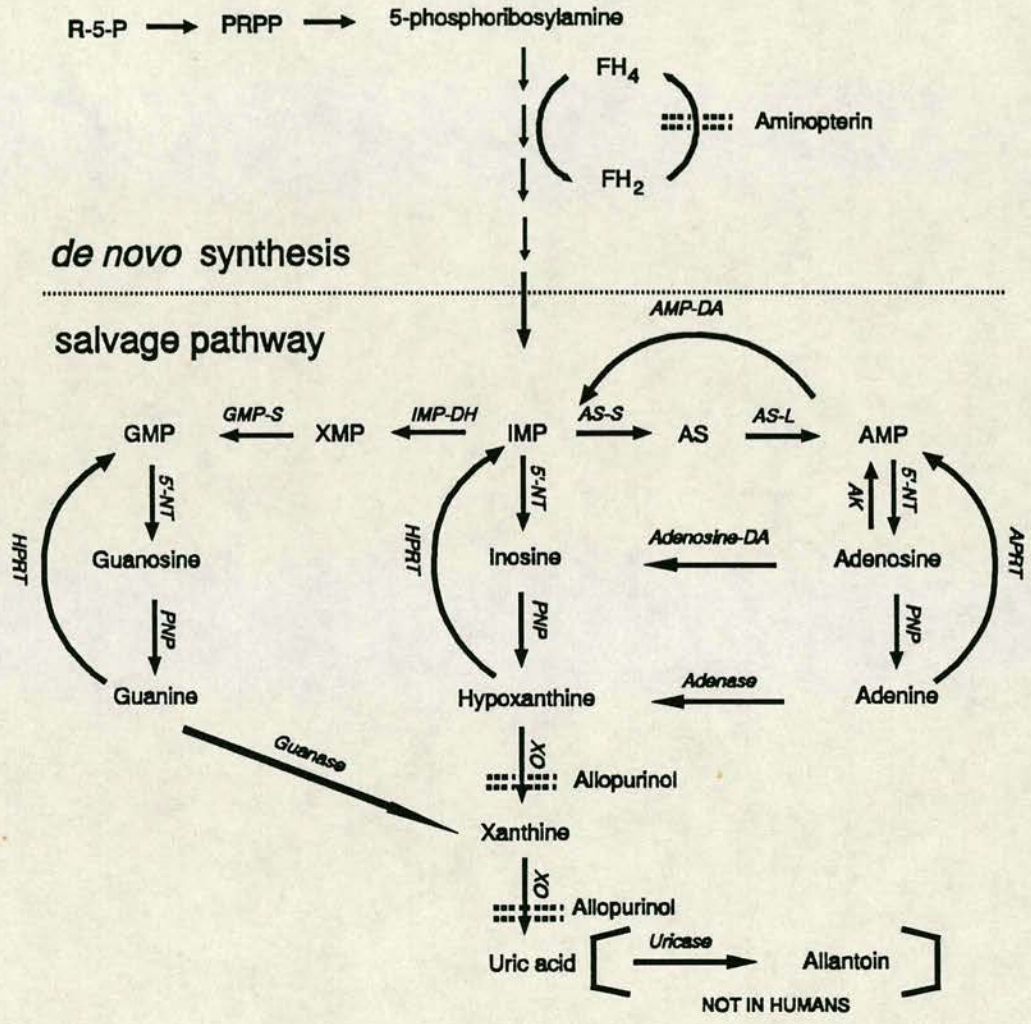
1.1 Foreword

Remarkable discoveries over the last few years in biological sciences have made a major impact on clinical practice. It is now apparent that new methods in recombinant DNA technology and cell biology are going to play an increasingly important role in medicine, and that medical research may well be moving into the most exciting phase of its development. Over the next few years there will be a change of emphasis in clinical research from whole patient physiology and pathology to the definition of disease at the cellular and molecular level. More and more diseases are being identified which are due to either single gene or multiple gene disorders. Animal models for these diseases are being created, which provide either an understanding of the pathogenesis of disease itself or the opportunity to evaluate therapeutic strategies. As befits the position of purines and pyrimidines at the centre of biochemistry, there has been steady scientific investigation suggesting their involvement in many human diseases.

1.2 Purine nucleotide metabolism

Nucleotides play an important role in a variety of metabolic functions in all living organisms, including (1) energy metabolism (2) as monomeric units of nucleic acids (3) as physiological mediators such as cAMP (4) as components of coenzymes and (5) as activated intermediates. Nucleotide metabolism therefore has an influence on most cellular reactions, even in non-dividing cells and in consequence, many clinical symptoms result from disorders of nucleotide metabolism (Wada, 1988). Nucleotides are divided into two classes-purines and pyrimidines. These nucleotides may be synthesized either by *de novo* biosynthesis or by a salvage pathway (Figure 1.1). In humans, all the enzymes of purine metabolism are found in the cytoplasm. The commitment step in purine nucleotide *de novo* biosynthesis is the formation of 5-phosphoribosylamine. The PRPP (phosphoribosyl pyrophosphate) amidotransferase, which catalyses this reaction, appears to be controlled through an allosteric mechanism that depends on the interaction of PRPP concentration. The pyrophosphate group of PRPP is displaced by the amide group of glutamine with an inversion to yield the β-D configuration found in all nucleotides. PRPP also functions in several other

Figure 1.1 The metabolic pathway of purine nucleotides. 5′-NT, 5′-nucleotidase; AK, adenosine kinase; AMP, adenosine 5′-monophosphate; APRT, adenine phosphoribosyltransferase; AS, adenylosuccinate; AS-L, adenylosuccinate lyase; AS-S, adenylosuccinate synthetase; DA, deaminase; DH, dehydrogenase; FH_2, dihydrofolate; FH_4, tetrahydrofolate; GMP, guanosine 5′-monophosphate; HPRT, hypoxanthine phosphoribosyltransferase; IMP, inosine 5′-monophosphate; PNP, purine nucleoside phosphorylase; PRPP, phosphoribosyl pyrophosphate; R-5-P, ribose 5-phosphate; S, synthetase; XMP, xanthosine 5′-monophosphate; XO, xanthine oxidase. Blocking of xanthine oxidase by allopurinol and *de novo* biosynthesis by aminopterin is indicated by the parallel broken lines.


R-5-P
PRPP
5-phosphoribosylamine
FH4
FH2
Aminopterin
de novo synthesis
salvage pathway
AMP-DA
GMP
GMP-S
XMP
IMP-DH
IMP
AS-S
AS
AS-L
AMP
5'-NT
Guanosine
HPRT
PNP
Guanine
5'-NT
Inosine
HPRT
PNP
Hypoxanthine
Adenosine-DA
Adenase
AK
5'-NT
Adenosine
APRT
PNP
Adenine
Guanase
XO
Allopurinol
Xanthine
XO
Allopurinol
Uricase
Uric acid
Allantoin
NOT IN HUMANS

ribosylating reactions, including pyrimidine nucleotide synthesis, NAD^+ synthesis, histidine biosynthesis, and the conversion of purines to purine nucleotides via the purine salvage pathway. The first atoms incorporated into the purine ring are derived from glycine. The enzyme glycinamide ribotide synthetase catalyses the addition of glycine to 5-phosphoribosylamine. ATP provides the driving force, probably by forming a high-energy glycyl phosphate intermediate. The reaction is reversible, however, since the product, glycine amide ribotide, is a high-energy compound. Glycine amide ribotide is formylated at the expense of 5,10-methenyl tetrahydrofolate, which can in turn be formed from formic acid by way of 10-formyl tetrahydrofolate. Another amination reaction with glutamine as the nitrogen donor allows the imidazole portion of the purine ring to be closed by an enzymatic reaction driven by ATP. The subsequent carboxylation reaction occurs on an enzyme that does not appear to contain biotin, even though the precursor, 5-aminoimidazole ribotide, accumulates in biotin-deficient animals. The amination of this carboxyl group occurs in two steps in which aspartate is the nitrogen donor. The aspartate carbon atoms are released as fumarate, and 5-aminoimidazole-4-carboxamide ribotide is formed. Following the formylation of 5-aminoimidazole-4-carboxamide ribotide, the formylated intermediate, 5-formylamidoimidazole-4-carboxamide ribotide, undergoes enzymatic ring closure with the formation of IMP. Between the formation of 5-phosphoribosylamine and IMP, there is no known regulation step. However, regulation is present at the branch point of IMP to GMP and IMP to AMP. The two enzymes which utilise IMP at this branch point, IMP dehydrogenase and adenylosuccinate synthetase, have similar Km's for IMP. AMP is a competitive inhibitor of adenylosuccinate synthetase, while GMP is a competitive inhibitor of IMP dehydrogenase. Two levels of control are therefore in effect at the IMP branch point. GTP serves as an energy source for the adenylosuccinate synthetase reaction, while AMP is a competitive inhibitor of this step; and ATP serves as the energy source in conversion of XMP to GMP, while GMP acts as an inhibitor of XMP formation.

The ribonucleotides and deoxyribonucleotides derived from the hydrolysis of nucleic acids are catabolised to form the corresponding sugar, phosphate, and purine

and pyrimidine bases. In humans and other primates the purine bases are catabolised to uric acid. AMP deaminase catalyses the synthesis of inosine 5′-monophosphate, and 5′-nucleotidase converts IMP and GMP to their respective ribonucleosides or deoxyribonucleosides. These in turn are converted to the free bases by the enzyme purine nucleoside phosphorylase. Mechanistically, this enzyme acts like glycogen phosphorylase, as it removes a sugar 1-phosphate derivative through a phosphorolytic reaction utilising inorganic phosphate. The bases guanine and hypoxanthine have two fates; they may be reconverted to their 5′-ribonucleotides or they may be converted to xanthine. The oxidation of hypoxanthine to xanthine and subsequent oxidation to uric acid are catalysed by xanthine oxidase.

From a quantitative point of view, the most important pathways for the generation of nucleotides are the *de novo* synthesis of purine and pyrimidine rings. Cellular metabolism, under normal conditions, is also influenced by the presence of the so-called "salvage pathways" for bases. Free purines can react directly with PRPP to yield nucleoside 5′-monophosphates resulting in salvage of pre-synthesized purines. There are several well-known enzymes that play this role in salvage pathways, such as adenine phosphoribosyl transferase (APRT), and hypoxanthine-guanine phosphoribosyl transferase (HPRT).

1.3 Hypoxanthine guanine phosphoribosyl transferase

1.3.1 Enzyme and gene

Hypoxanthine guanine phosphoribosyl transferase (HPRT; IMP pyrophosphate phosphoribosyltransferase; EC 2.4.2.8) is the key enzyme in the purine salvage pathway. It is expressed in all mammalian cells as a "housekeeping enzyme" and occurs at an elevated level in the basal ganglia of the brain. The enzyme occurs in low abundance, constituting between 0.005 and 0.04% of total cellular protein in the cytoplasm and is composed of four identical subunits of 217 amino acids with a molecular weight of 24,500 (Wilson *et al.*, 1982). HPRT cDNA sequences were first isolated from a mouse neuroblastoma HPRT revertant cell line, NBR4 that had amplified the HPRT locus (Melton, 1981), and were analysed by Brennand *et al.*

(1982) and Konecki *et al.* (1982). Using mouse HPRT cDNA as probes, the HPRT cDNA sequences of Chinese hamster and human have been isolated and published (Konecki *et al.*, 1982; Brennand *et al.*, 1983). The coding sequences of mouse, human and Chinese hamster have greater than 90% homology at the nucleic acid level. There are only seven positions where the amino acid sequences of mouse and human HPRT differ. A high level of nucleotide sequence conservation between species is also found in the 3′ untranslated regions of the HPRT cDNA, suggesting a role in gene expression for this region. HPRT cDNAs have been used as hybridisation probes to isolate the HPRT genes which consist of nine exons spanning 33 kb in the mouse (Melton *et al.*, 1984), and 42 kb humans (Kim *et al.*, 1986; Patel *et al.*, 1986)(Figure 1.2). Although the human gene is larger than the mouse gene, the exon sizes are the same except for exons 1 and 9 (Melton, 1987). The complete sequence of the 57-kb-human HPRT locus including 1676 bp 5′ to exon 1 and 15,238 bp 3′ to exon 9 has been determined using automated fluorescent DNA sequencing (Edwards *et al.*, 1990).

1.3.2 Promoter and its divergent characteristics

The use of S1 nuclease protection and primer extension analyses have located the promoter of the mouse HPRT gene (Melton *et al.*, 1984). Like many other housekeeping genes, HPRT has its 5′ end located in a GC-rich area of the genome and produces transcripts with heterogeneous initiation sites. The first 100 bp upstream of the major transcription initiation site, designed +1, in the mouse are 80% GC rich. There are no sequence matches for either the TATA box, usually located 20 to 30 bp upstream of the transcription initiation site and thought to be responsible for positioning of transcription initiation (Corden *et al.*, 1980), or the CAAT box, typically located at around -80 and believed to play a role in the frequency of initiation (Benoist *et al.*, 1980). Analysis of the nucleotide sequence of the promoter region revealed a complex pattern of direct repeats. There are two imperfect direct 18 bp repeats and a third incomplete repeat between nucleotide positions -80 and -15. In the region between 30 and 60 bp upstream of the major transcription initiation site, there are two 12 bp perfect repeats in tandem (Melton, 1987) (Figure 1.3). HPRT minigenes have been constructed to study HPRT expression in cultured cells. The

Figure 1.2 Organisation and restriction maps of the human and mouse HPRT genes. The human and mouse genes are drawn to the same scale, with the exons depicted as numbered vertical bars. The size of each intervening sequence (in kb) is also indicated. Selected restriction sites within the two genes are shown and for the mouse, the size of each restriction fragment is given. (Adapted from Melton, 1987)

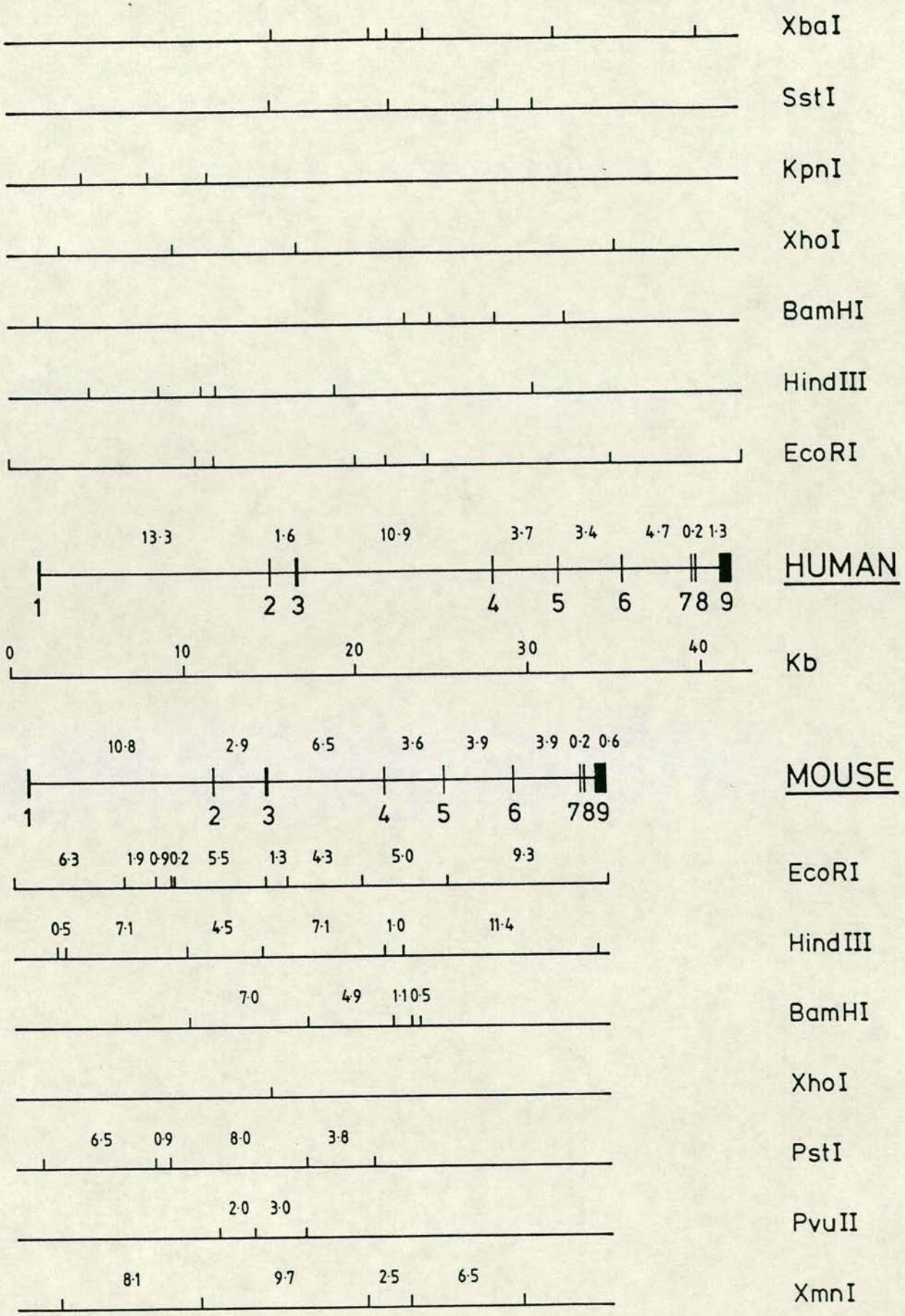
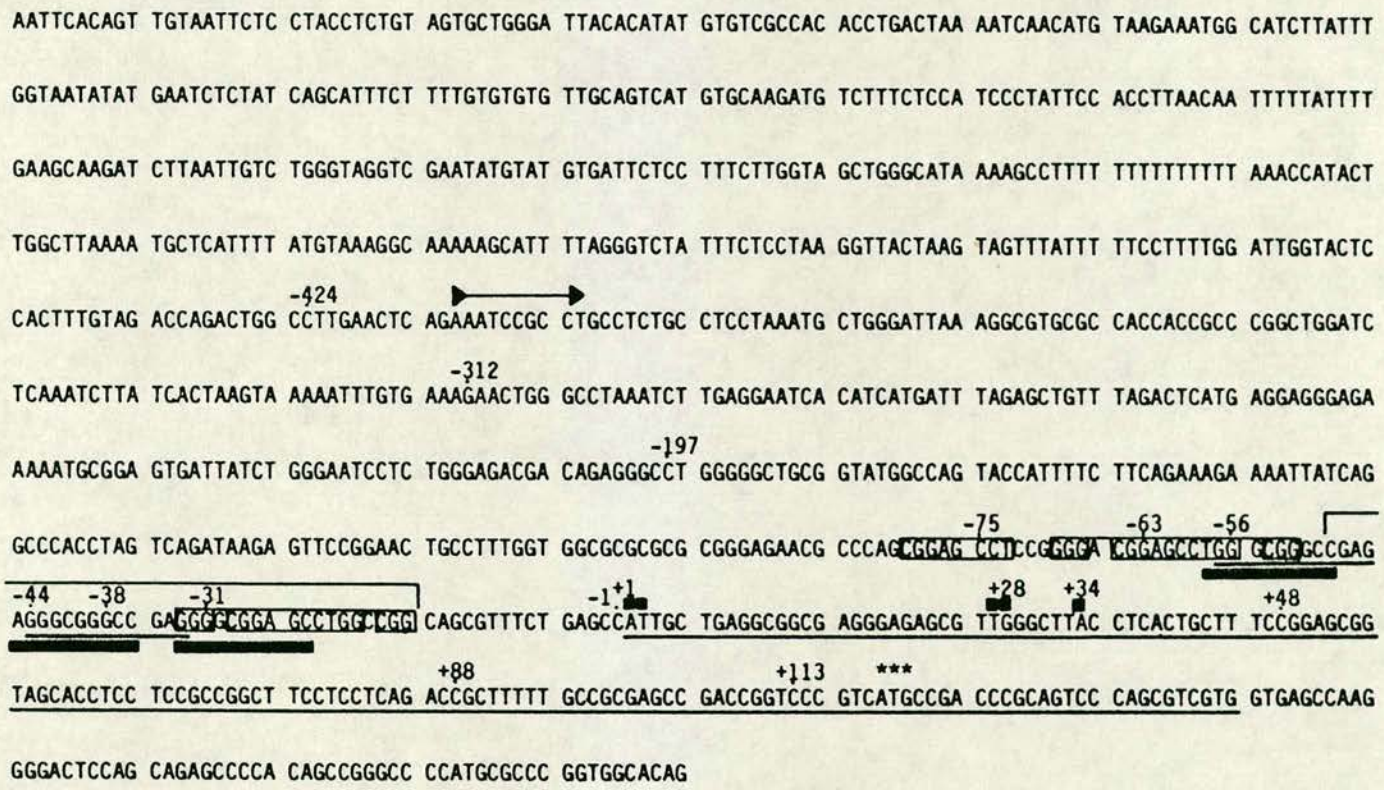

XbaI
SstI
KpnI
XhoI
BamHI
HindIII
EcoRI
13·3
1·6
10·9
3·7
3·4
4·7
0·2
1·3
1
2
3
4
5
6
7
8
9
HUMAN
0
10
20
30
40
Kb
10·8
2·9
6·5
3·6
3·9
3·9
0·2
0·6
1
2
3
4
5
6
789
MOUSE
6·3
1·9
0·9
0·2
5·5
1·3
4·3
5·0
9·3
EcoRI
0·5
7·1
4·5
7·1
1·0
11·4
HindIII
7·0
4·9
1·1
0·5
BamHI
XhoI
6·5
0·9
8·0
3·8
PstI
2·0
3·0
PvuII
8·1
9·7
2·5
6·5
XmnI

Figure 1.3 Structure of the mouse HPRT promoter region. The sequence is numbered from the main transcription initiation site (+1). The 145 bp first exon is underlined and within it the transcription initiation sites at +28 and +34 are indicated. Three asterisks indicate the translation initiating codon. The 18 bp imperfect direct repeats and the third repeat of part of central section are boxed. The 12 bp direct repeats are underlined. Three matches to the consensus Sp1 binding site are heavily underlined. The sequence enclosed by the square bracket has been used as a synthetic promoter. The end points of 5′ deletions used to map functional elements are indicated by a dot over the position of the first base present. The arrow indicates the 5′ boundary of the GC-rich region. (Adapted from Melton, 1987)


AATTCACAGT TGTAATTCTC CTACCTCTGT AGTGCTGGGA TTACACATAT GTGTCGCCAC ACCTGACTAA AATCAACATG TAAGAAATGG CATCTTATTT
GGTAATATAT GAATCTCTAT CAGCATTTCT TTTGTGTGTG TTGCAGTCAT GTGCAAGATG TCTTTCTCCA TCCCTATTCC ACCTTAACAA TTTTTATTTT
GAAGCAAGAT CTTAATTGTC TGGGTAGGTC GAATATGTAT GTGATTCTCC TTTCTTGGTA GCTGGGCATA AAAGCCTTTT TTTTTTTTTT AAACCATACT
TGGCTTAAAA TGCTCATTTT ATGTAAAGGC AAAAAGCATT TTAGGGTCTA TTTCTCCTAA GGTTACTAAG TAGTTTATTT TTCCTTTTGG ATTGGTACTC
-424
CACTTTGTAG ACCAGACTGG CCTTGAACTC AGAAATCCGC CTGCCTCTGC CTCCTAAATG CTGGGATTAA AGGCGTGCGC CACCACCGCC CGGCTGGATC
-312
TCAAATCTTA TCACTAAGTA AAAATTTGTG AAAGAACTGG GCCTAAATCT TGAGGAATCA CATCATGATT TAGAGCTGTT TAGACTCATG AGGAGGGAGA
-197
AAAATGCGGA GTGATTATCT GGGAATCCTC TGGGAGACGA CAGAGGGCCT GGGGGCTGCG GTATGGCCAG TACCATTTTC TTCAGAAAGA AAATTATCAG
-75 -63 -56
GCCCACCTAG TCAGATAAGA GTTCCGGAAC TGCCTTTGGT GGCGCGCGCG CGGGAGAACG CCCAGCGGAG CCTCCGGGGA CGGAGCCTGG GCGGGCCGAG
-44 -38 -31 -1 +1 +28 +34 +48
AGGGCGGGCC GAGGGCGGA GCCTGGCCGG CAGCGTTTCT GAGCCATTGC TGAGGCGGCG AGGGAGAGCG TTGGGCTTAC CTCACTGCTT TCCGGAGCGG
+88 +113 ***
TAGCACCTCC TCCGCCGGCT TCCTCCTCAG ACCGCTTTTT GCCGCGAGCC GACCGGTCCC GTCATGCCGA CCCGCAGTCC CAGCGTCGTG GTGAGCCAAG
GGGACTCCAG CAGAGCCCCA CAGCCGGGCC CCATGCGCCC GGTGGCACAG

first HPRT minigene consisted of 845 bp of 5′ flanking sequence fused onto the wild-type human HPRT cDNA sequence and Chinese hamster 3′ untranslated region (Melton *et al.*, 1984). Other constructs derived from this were published later (Melton *et al.*, 1986). The essential elements of the HPRT promoter have been characterised either by 5′ deletional analysis of the minigene (Melton *et al.*, 1986) or 3′ deletional analysis of fusions with the neomycin phosphotransferase (*neo*) gene (Melton, 1987). It was shown from the 5′ deletional analysis that it is possible to delete as far as nucleotide -39 and still retain greater than 30% activity assayed as [^{3}H]-hypoxanthine incorporation and expressed relative to the undeleted minigene. Incorporation however decreased to 2.5% of the original value for the construct that was deleted to -27, suggesting that the 5′ boundary of the mouse HPRT promoter is located between -39 and -27. Deletions from the 3′ end of the promoter region fused to a *neo* gene lacking its own promoter were assayed for the ability to transform cells to G418 resistance. Deletion to -38 gave 40% survival in a colony forming assay compared to that of a construction containing HPRT sequences from -638 to +113 fused to the *neo* gene. The survival rate was reduced to 5% if the construct was deleted to -44 from the 3′ end of the HPRT promoter region. Thus the 5′ boundary of the mouse HPRT promoter is located between -39 and -27 and the 3′ boundary of the promoter is located between -38 and -44. That is, a region defined as essential by deletional analysis in one direction can be lost, without detrimental effect, in deletions proceeding from the opposite direction. Consequently, it is believed that the mouse HPRT promoter is functionally duplicated (Melton, 1987). For this reason, the 34-bp "core" promoter sequence between position -49 to -16, was synthesized (termed the synthetic promoter) and used for further studies.

RNA polymerase II is responsible for mRNA synthesis. Transcription factor Sp1 is a protein that binds to specific sequences and activates RNA synthesis. It enhances transcription by RNA polymerase II 10- to 50-fold from a select group of promoters that contain at least one properly positioned Sp1 motif, GGGCGG (Kadonaga *et al.*, 1986). Several cellular Sp1-responsive promoters, such as those of the mouse dihydrofolate reductase (DFHR) gene and human metallothionein gene,

have been studied by assaying transcription *in vitro* and by DNase foot-printing experiments. There are three good matches to the Sp1 consensus-binding site in the region 100 bp immediately upstream of the main initiation site of the mouse HPRT gene (Melton *et al.*, 1986). One of these three matches is located between -36 and -44, a region demonstrated to be part of the promoter by 3′ deletion analysis. Another Sp1 site, located in the region of -24 to -33 may also play a significant role as indicated in the 5′ deletion studies. A similar situation was also found in the human HPRT promoter region which contains six Sp1 recognition sites (Patel *et al.*, 1986; Kim *et al.*, 1986). Housekeeping genes are usually defined as genes that are expressed in all cell types. In conclusion, the HPRT gene displays many typical characteristics of housekeeping genes such as an upstream GC-rich region and lacking TATA and CATT boxes.

Bidirectional transcription is a property that has been demonstrated for some housekeeping promoters. That is, a single promoter directing the transcription of two RNA's in opposite direction to each other and on opposite DNA strands. Two groups independently demonstrated that sequences upstream of the DFHR promoter were transcribed. The DFHR gene has two classes of divergent transcripts, one is a polyadenylated mRNA and the other is a non-polyadenylated small nuclear RNA (Crouse *et al.*, 1985; Farnham *et al.*, 1985). More recently, Linton *et al.* (1989) have cloned cDNAs corresponding to the divergent transcripts of the DHFR gene, which they noted have sequence similarity with bacterial genes involved in mismatch repair. Furthermore, studies of CpG rich islands surrounding the 5′ ends of housekeeping genes and of several tissue specific genes, have identified HpaII tiny fragments (HTF) that mediate divergent transcription (Lavia *et al.*, 1987).

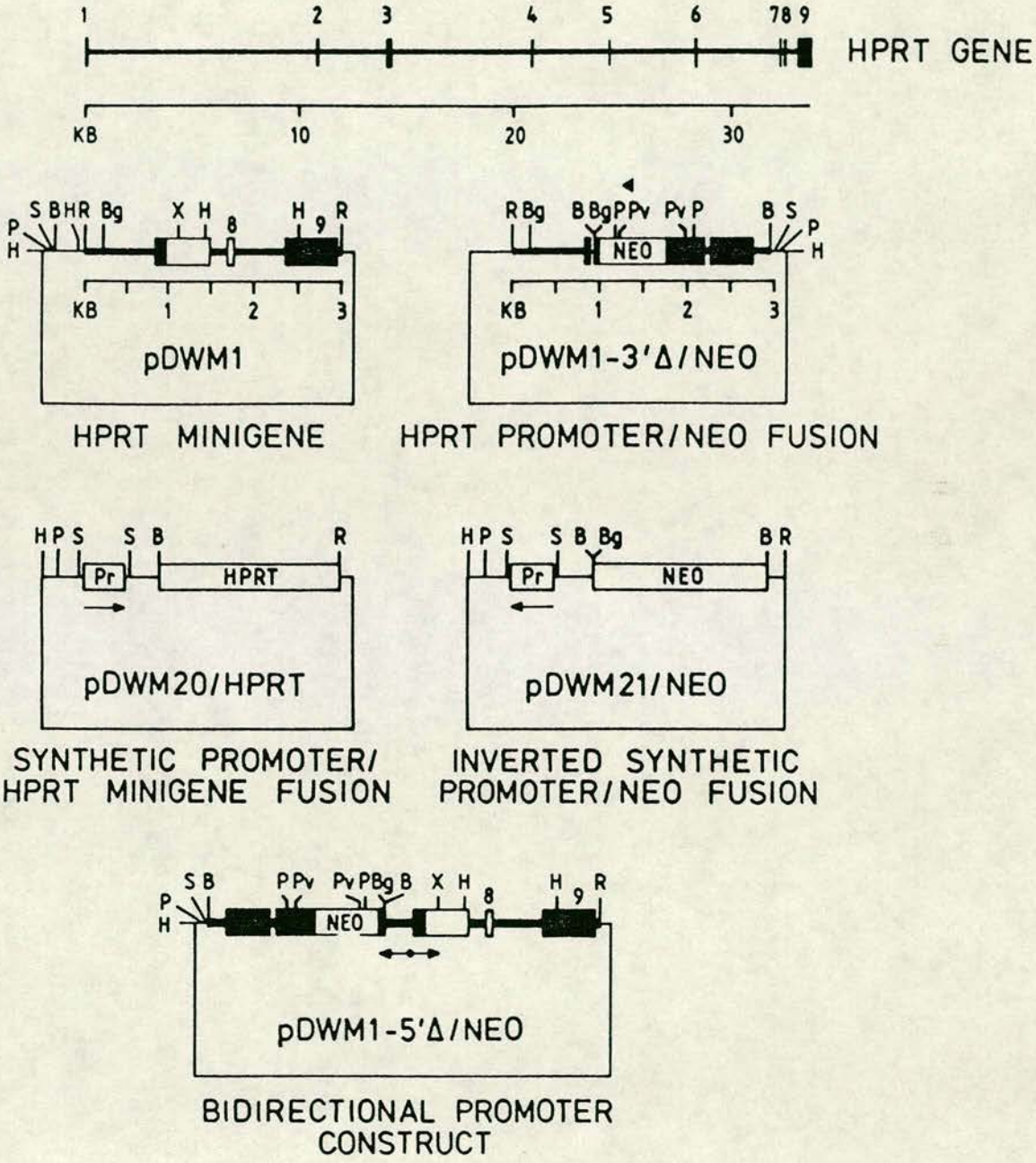
The similarities between the HPRT gene promoter and the DHFR gene promoter suggest that the promoter of the HPRT gene may also mediate bidirectional transcription. Functional duplication within the HPRT promoter has been demonstrated by deletional and linker-substitution analysis (McKnight and Kingsbury, 1982; Melton *et al.*, 1986). Melton (1987) showed that a 34-bp fragment, consisting

of the sequence between -49 and -16 of the mouse HPRT promoter, can act bidirectionally and proposed a model by which housekeeping promoters may initiate transcription in both directions. When the 34-bp synthetic promoter element was linked to a promoterless HPRT minigene or *neo* gene, it directed transcription which initiated from the normal sites and was unaffected by a subtle change in spacing between the promoter and initiation sites. The same results were obtained irrespective of the orientation of the synthetic promoter element to the adjacent gene. Further evidence is derived from the effective transfection of a construct consisting of the 34-bp synthetic promoter sandwiched between a promoterless HPRT minigene and a promoterless *neo* gene, into a HPRT-deficient cell line (Figure 1.4). These cells were effectively G418 and HAT resistant, indicating the expression of both *neo* and HPRT genes (Melton *et al.*, unpublished observation). The bidirectional function of the HPRT promoter was also demonstrated by Johnson and Friedmann (1990) by the expression of luciferase reporter genes in cells transfected with constructs controlled by a human 376-bp HPRT promoter fragment in both orientations. Since a naturally occurring divergent transcript from the HPRT promoter has not been detected, the significance of this bidirectional promoter activity remains unclear.

1.3.3 Introns as regulation elements

It has been demonstrated in transgenic mice that intron-containing constructs of several genes result in higher levels of mRNA production compared to corresponding intron-deficient constructions, suggesting that introns play a regulatory role (Brinster *et al.*, 1988). HPRT minigene expression in HPRT deficient cells or antisense sequence inhibition may allow us to identify the role played by introns in the regulation of HPRT expression. Ao *et al.* (1988) demonstrated that negation of HPRT expression from injected HPRT minigene DNA is mediated by simultaneous injection of HPRT antisense DNA. More recently, it has been demonstrated that antisense mouse HPRT RNA leads to an inhibition of HPRT synthesis in human and in mouse cells (Stout and Caskey, 1990). Reid *et al.* (1990) have constructed a series of human HPRT minigenes to address this question. They claimed that there are two elements which influence HPRT minigene expression in mouse HPRT-deficient

Figure 1.4 Constructions used for mouse HPRT gene expression studies. The structure of the mouse HPRT gene, with the nine exons depicted as vertical bars, is shown for comparison with the basic mouse HPRT minigene, pDWM1, which is cloned in plasmid pUC8. Note the different scales used for gene and minigene. Open boxes, HPRT coding sequence; closed boxes, untranslated regions; thick lines, HPRT flanking and intervening sequences; thin lines, vector sequence. Restriction sites: B, BamHI; Bg, BglII; H, HindIII; P, PstI; R, EcoRI; S, SalI; X, XhoI. 5′ deletional derivatives of pDWM1 were assayed directly by measuring HPRT expression following introduction of the constructions into cultured cells. The effect of 3′ deletions was analysed in constructions containing the *neo* transcription unit, under the control of 3′ deleted HPRT promoter segments. The constructions containing the synthetic promoter element (Pr) are not drawn to scale and the restriction sites within minigene and *neo* modules are omitted. In the pDWM20 series the synthetic promoter is in its natural orientation (indicated by the arrow), with respect to the downstream gene; the element is inverted in pDWM21 constructions. The bidirectional promoter construct was derived from the pDWM1 by inserting the BamHI-BamHI fragment of *neo* coding region into its BglII-BamHI site. (Adapted from Melton, 1987 and unpublished data)

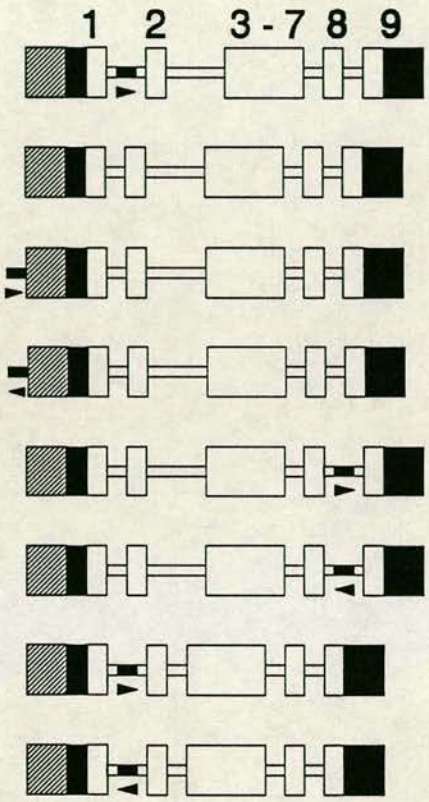

1
2
3
4
5
6
7 8 9
HPRT GENE
KB
10
20
30
S B H R Bg
X H
8
H 9 R
P
H
KB
1
2
3
pDWM1
HPRT MINIGENE
R Bg
B Bg P Pv
Pv P
B S
NEO
pDWM1-3'Δ/NEO
HPRT PROMOTER/NEO FUSION
H P S
S B
R
Pr
HPRT
pDWM20/HPRT
SYNTHETIC PROMOTER/
HPRT MINIGENE FUSION
H P S
S B Bg
B R
Pr
NEO
pDWM21/NEO
INVERTED SYNTHETIC
PROMOTER/NEO FUSION
S B
P Pv Pv P Bg B
X H
8
H 9 R
NEO
pDWM1-5'Δ/NEO
BIDIRECTIONAL PROMOTER
CONSTRUCT

embryonic stem (ES) cells. An element located in intron 1 and another in intron 2 were both indispensable for gene expression. This demonstration might not address the regulation of the human HPRT gene *in vivo* because the authentic HPRT promoter was replaced with the TK promoter and a mutant polyoma enhancer. Stout and Caskey (1990) suggested that a stretch of 373-bp from the junction of exon 1 and intron 1 was important in the antisense RNA inhibition assay. Furthermore, a 420-bp element from the first intron is required for efficient HPRT expression in ES cells (Figure 1.5). It has also been demonstrated that the intron 1 element plays a position- and orientation-dependent role in stimulating HPRT mRNA synthesis in ES cells (Magin *et al.*, 1992a). Based on the necessity of HPRT expression, a vector containing an HPRT minigene with the intron 1 element was constructed and served as a negative selection module to target the murine *ERCC-1* gene in HPRT-deficient ES cells (Selfridge *et al.*, 1992).

1.3.4 X-chromosome inactivation and DNA methylation

HPRT is located on the long arm of the mammalian X chromosome. Due to X-chromosome inactivation which occurs early in embryogenesis, only one copy of the HPRT gene is expressed in female mammalian cells. With the exception of the extraembryonic membranes, the decision of which of the two X-chromosomes to inactivate is made at random in individual cells and, once made, is stably transmitted to clonal descendants. In cells of the germ line, reactivation of the inactive X precedes meiosis. A discrete locus on the X-chromosome is responsible for the initiation of inactivation (Brown *et al.*, 1991). Several reports indicated that DNA from the active X-chromosome was much more efficient in transforming HPRT deficient cells to HPRT expressing cells than that from the inactivated X-chromosome (Liskay and Evans, 1980; Chapman *et al.*, 1982; Lester *et al.*, 1982; Venolia and Gartler, 1983). DNA methylation, which results in modified cytosine residues in CG couplets, is considered to be part of the inactivation process. The region around the 5′ end of both the mouse and human HPRT genes is GC-rich and it was shown that methylation occurred at an AvaI site in this region in female mouse DNA (Melton, 1987). Using methylation sensitive enzymes, such as MspI, it has also been

Figure 1.5 Defining the role of the HPRT intron 1 element in ES cells. The position (closed bar) and orientation (arrowhead) of the 420-bp AvaI-PstI fragment from intron 1 in a series of HPRT minigenes is shown. Numbered boxes, exon elements; open boxes, coding regions; closed boxes, untranslated regions; shaded box, promoter region; open bars, intervening and flanking sequences; closed bar, control element within intron 1. Notes. [a] Plasmid pDWM110 was derived by removing a PstI-PvuII fragment from the intron 1 region and a PvuII-EcoRI fragment from the intron 2 region of plasmid pDWM100 (Thompson *et al.*, 1989). Plasmid pDWM111 was constructed in the same way except that the intron 2 region was further reduced in size. Plasmid pDWM112 was constructed in the same way as pDWM110, except that the exon 1 module only extended to the AvaI site and lacked the 420-bp AvaI-PstI fragment. The 420-bp AvaI-PstI fragment from intron 1 was inserted into the unique BamHI site in the 5′ polylinker sequence of pDWM112 to produce plasmids pDWM117 and pDWM118. The 420-bp AvaI-PstI fragment was cloned into the unique NcoI site within intron 8 of pDWM112 to produce plasmids pDWM121 and pDWM122. Plasmid pDWM120 was constructed by removing the 420-bp AvaI-PstI fragment from pDWM111 leaving a BglII linker in its place and then reinserting the fragment, in the opposite orientation, into the unique BglII site. [b] [^{3}H]-hypoxanthine incorporated/2.5 x 10^6 HPRT-deficient ES cells electroporated/8.5 nM DNA expressed as % of the value with pDWM111 DNA. Values shown are the average from two separate determinations or, where n > 2, the standard deviation, is also shown. (Adapted from Magin *et al.*, 1992)



MINIGENE [a]	EXPRESSION ES(%) [b]
pDWM110	85±8
pDWM112	3±2
pDWM117	4
pDWM118	5
pDWM121	2
pDWM122	3
pDWM111	100
pDWM120	45±10

POSITION AND ORIENTATION DEPENDENCE OF INTRON 1 ELEMENT

demonstrated that several CpGs around the HPRT promoter are methylated on the inactive but not on the active X-chromosome (Yen *et al.*, 1984; Lock *et al.*, 1986; Wolf *et al.*, 1984). A female patient with Lesch-Nyhan syndrome was demonstrated with a wild-type HPRT on the non-random inactive paternal X-chromosome and an HPRT mutation on the active X-chromosome (Ogasawara *et al.*, 1989). Therefore, the HPRT gene provides a suitable system to study X-chromosome inactivation during development. 5-azacytidine, a potent inhibitor of DNA methylation (Mohandas *et al.*, 1981; Lester *et al.*, 1982; Graves, 1982), has been used to induce reactivation of the selectable HPRT gene on the inactive chromosome in a diploid female cell line. Using methylation-sensitive restriction enzymes, Driscol and Migeon (1990) found that the MspI sites in CpG islands of the HPRT gene were unmethylated in germ-cell fractions of fetal ovary and adult testis and suggested that the reversibility of X inactivation occurred in those tissues. In the process of mouse embryonic development, it was found that the inactivation of the HPRT gene on the inactive X-chromosome occurred several days before methylation was detectable in the GC-rich island (Lock *et al.*, 1987). Kratzer *et al.* (1983) claimed that inactivation was not strongly associated with methylation in the early extra-embryonic tissue. Recently, it was suggested that the female germ cells remain unmethylated, but that methylation in male germ cells occurs postnatally, prior to or during the early stages of spermatogenesis (Driscol and Migeon, 1990). Hence HPRT has proved to be useful and will continue to be of use in addressing the complex question of methylation and its role in controlling gene transcription. In addition, the HPRT gene also acts as a model in studies of mutagenesis rate (Grant and Worton, 1989) and DNA synthesis asynchronism (Schmidt and Migeon, 1990) between the active and inactive X-chromosome.

1.3.5. HPRT as a tool for screening mutagens and carcinogens

Purine analogues, such as 8-azaguanine, 8-azahypoxanthine and 6-thioguanine, have been used for isolating HPRT-deficient cells for 3 decades (Szybalski and Smith, 1959; Szybalski, 1992). Analogues of folic acid such as aminopterin and methotrexate are powerful inhibitors of dihydrofolate reductase which converts folic acid to

dihydrofolate and subsequently to tetrahydrofolate. The inhibition of dihydrofolate reductase would be expected to affect every reaction requiring folate coenzyme. Purine nucleotide biosynthesis and thymidylate synthetase requiring 5,10-methylene tetrahydrofolate as a coenzyme are inhibited by aminopterin. The hypoxanthine-aminopterin-thymidine (HAT) selective medium was designed to select for HPRT dependence by blocking the purine *de novo* synthesis and for thymidine kinase dependence by blocking thymidylate synthetase (Szybalska and Szybalski, 1962). HAT medium and 6-thioguanine respectively allow selection both for and against HPRT. X-linkage means that in male (XY) and X-inactivated female (XX) cells, selection depends on a single gene copy. Thus, HPRT provides the most convenient system for somatic cell genetics.

To avoid exposure to carcinogens, it is necessary to examine the carcinogenic properties of compounds or drugs. The identification of carcinogens is time-consuming, costly and usually requires animal experiments. The close correlation between carcinogen and mutagen is well known (Temin, 1988). It is therefore sensible to screen first by using mutagen tests and confirm by a carcinogen test if required. A mutagen test system has been developed by using *Salmonella typhimurium* as the test organism (Ames *et al.*, 1973; Maron and Ames, 1983). Although the incubation of rat liver extracts was included in this test system to simulate the condition of mammalian metabolism, incorrect classification is possible due to the different metabolism of prokaryotes and eucaryotes. For example, a potential antidepressant drug, U-48753, was subjected to genetic toxicity assays and found negative in the Ames test but positive in the CHO/HPRT assay (Aaron *et al.*, 1989a). Therefore, another system using eukaryotes as test organisms would be a better alternative. Taking advantage of bidirectional selection and X-linkage, a quantitative assay of mutation induction at the HPRT locus in Chinese hamster ovary (CHO) cells has been introduced and routinely used in some pharmaceutical companies for the screening of drugs before marketing (O'Neill *et al.*, 1977; Aaron *et al.*, 1989b).

1.4 Inborn errors of purine metabolism

1.4.1 Inborn errors of metabolism

Elucidation of an inherited defect in an enzyme which is part of a well-understood metabolic pathway, often allows a clear description of how the genetic defect produces its manifold clinical effects. At its simplest, the clinical manifestations of an enzyme defect can be seen as a combination of deficiency of substances beyond the block in the metabolic pathway and accumulation of substances prior to the block. Sometimes alternative metabolic pathways will allow the block to be bypassed, however, this compensatory mechanism may fail under conditions of stress. Inborn errors of purine metabolism can be divided into 3 types according to their pathways, (1) the defects in biosynthesis, such as PRPP synthesis abnormality and adenylosuccinase deficiency, (2) the defects in catabolic pathway including AMP deaminase deficiency, adenosine deaminase (ADA) deficiency, purine nucleoside phosphorylase (PNP) deficiency, and xanthine oxidase deficiency and, (3) defects in the salvage pathway, such as HPRT deficiency and APRT deficiency (van der Berghe, 1990). (see Table 1.1.)

PRPP synthetase converts α-ribose-5-phosphate to 5-phospho-α-ribosyl-1-pyrophosphate, which is the start of purine *de novo* synthesis. A defect in regulation of PRPP synthesis leads to the superactivity of PRPP synthetase and an increase in purine *de novo* synthesis, and uric acid precipitation leading to gouty arthritis is observed subsequently. Adenylosuccinase cleaves the C-N bond of adenylosuccinate, yielding AMP and fumarate. Adenylosuccinase deficiency results in the accumulation in cerebrospinal fluid, plasma and urine of two normally undetectable compounds, succinylaminoimidazole carboxamide riboside and succinyladenosine. These succinylpurines are the products of the dephosphorylation, by cytosolic 5′-nucleotidase of the two substrates of the enzyme. Patients with adenylosuccinase deficiency suffer from psychomotor retardation, in severe cases displaying autistic features such as failure to make eye-to-eye contact, repetitive movements and manipulations of objects, and occurrence of temper tantrums upon interference with repetitive behaviour.

Table 1.1 Clinical disorders of purine metabolism.

Biochemical disorder	Primary symptom	Reference
PRPP synthetase superactivity	Gouty arthritis	Becker *et al.* 1986
Adenylosuccinase deficiency	Mental retardation	Jaeken & Berghe 1984
AMP deaminase deficiency	Muscular weakness	Fishbein *et al.* 1978
Adenosine deaminase deficiency	SCID[a]	Giblett *et al.* 1972
PNP[b] deficiency	Immunodeficiency	Giblett *et al.* 1975
Xanthine oxidase deficiency	Mental retardation	Dent & Philpot 1954
HPRT partial deficiency	Gouty arthritis	Kelley *et al.* 1969
HPRT deficiency	Self mutilation	Lesch & Nyhan 1964
APRT deficiency	Kidney stone	Van Acker *et al.* 1977

a. Severe combined immunodeficiency disease

b. Purine nucleoside phosphorylase

AMP deaminase hydrolyses AMP into IMP and NH_3. By removing AMP,which was formed during exercise, the reconversion of ADP into ATP is stimulated and glycolysis is promoted in the presence of IMP and NH_3. The symptoms of AMP deaminase deficiency are muscular weakness, fatigue, cramps, or myalgias following moderate to vigorous exercise. Adenosine deaminase catalyses the conversion of adenosine to inosine and deoxyadenosine to deoxyinosine. Purine nucleoside phosphorylase catalyses a phosphorylytic cleavage of inosine and deoxyinosine to yield hypoxanthine and either ribose 1-phosphate or deoxyribose 1-phosphate. Defects in these enzymes should result in the accumulation of adenosine, deoxyadenosine, and, in the case of purine nucleoside phosphorylase, inosine. Patients with ADA or PNP deficiency suffer from a profound impairment of both humoral and cellular immunity, known as severe combined immunodeficiency disease. Xanthine oxidase converts hypoxanthine to xanthine and subsequently to uric acid. Xanthine oxidase deficiency can be completely asymptomatic and is discovered fortuitously by routine measurement of plasma uric acid. However, in about one-third of the cases, xanthine stones are formed. Abnormalities of the purine salvage pathway including Lesch-Nyhan syndrome and gouty arthritis are discussed below.

1.4.2 Gouty arthritis

Gout is associated with either increased formation of uric acid or its decreased renal excretion. Its incidence is relatively high, occurring in about 0.3 % of the population. Gout is classified into two broad types: primary and secondary. Primary gout, of which there are several subtypes, is inherited. The familial incidence of all cases of gout may be as high as 75 % to 80 %. Secondary gout is brought on by a variety of disorders such as leukaemia and polycythemia (increase in RBC mass) or by antimetabolites used in the treatment of cancer. Primary gout is most often found in men over 30 years of age. When women are affected, the onset is usually postmenopausal. Secondary gout occurs in both sexes and at younger ages. It is believed that most cases of primary gout are caused by excessive purine synthesis rather than increased purine nucleotide breakdown. Consistent with this view is the observation that some patients with gout have PRPP amidotransferase that is resistant

to feedback inhibition by purine nucleotides. The regulatory sites on this enzyme are similar to those of other allosteric enzymes in that they are separate from the catalytic sites. Consequently, a defect in a regulatory site as a result of a mutation could lead to the overproduction of purines as seen in gout. Snyder *et al.* (1989) examined 3 brothers who developed acute gouty arthritis at ages 16,20, and 26 years and found increased levels of plasma urate. Erythrocyte HPRT activity was less than 1% of normal and adenine phosphoribosyltransferase activity increased 2-3 fold. Lymphoblasts from these patients had 0.9-1.6% of control HPRT activity which was 8-fold more labile than control activity at 75 °C but had normal amounts of the expected 1.6-kb mRNA by Northern blot analysis. A better genetic basis of HPRT deficiency correlating to gout has been identified by nucleotide sequence analysis of HPRT cDNAs cloned from patients with gout (Davidson *et al.*, 1989a).

1.4.3 Lesch-Nyhan syndrome

Lesch-Nyhan syndrome, first described by Lesch and Nyhan in 1964, is a rare sex-linked recessive disease with an incidence rate of about 1 in 100,000. The syndrome is usually manifest in young boys displaying delayed mental retardation at three to four months of age. The specific character of Lesch-Nyhan syndrome is compulsive self-injurious behaviour, and onset of this can be as early as 1 year or as late as 16 years of age. It has been demonstrated that all patients lack HPRT activity in their cells by both biochemical and molecular biological analyses (Davidson *et al.*, 1988a,b,c; 1989b; Fujimori *et al.*, 1989; Gibbs *et al.*, 1989; Keough *et al.*, 1988; Sinnett *et al.*, 1988).

Although there is no clear direct evidence to explain the connection between HPRT deficiency and the behavioural abnormality of Lesch-Nyhan syndrome patients, some experiments indirectly demonstrated that this correlation is the basis for the syndrome. Rats pretreated with low doses of clonidine, an antihypertensive drug has been reported to possess marked sedative effects through stimulating central noradrenergic receptors, and administered with caffeine produced a high frequency of self-biting behaviour. Clonidine was less effective in potentiating amphetamine

induced self-biting (Mueller and Nyhan, 1983). The chemical structure of caffeine is very similar to purines and it may play an antagonistic role in purine metabolism, when administered at high doses, to produce the observed abnormal behaviour. The dopamine metabolism of HPRT-deficient variants of PC12 pheochromacytoma cells, a dopaminergic cell line, is not affected after regaining HPRT activity by virtue of transformation with a recombinant retrovirus containing the human cDNA for HPRT (Bitler and Howard, 1986). This *in vitro* study does not correlate with data from patients where levels of the neurotransmitter dopamine in the basal ganglia of Lesch-Nyhan patients are consistently lower than 30% of normal levels (Rosenblom *et al.*, 1967). Biochemical studies found that synthesis of dopamine requires biopterin , which is a purine derivative. This implies that dopamine synthesis may directly or indirectly be linked to purine metabolism. In addition, microinjection of rats with a dopamine agonist into the intrastriatal area elicits self-biting behaviour (Goldstein, 1989). These experiments suggest the imbalance of purine metabolism induced by HPRT deficiency would consequently influence the production of the neurotransmitter dopamine and hence cause abnormal self-mutilation behaviour.

Hypoxanthine and xanthine concentrations in plasma, cerebrospinal fluid and urine of Lesch-Nyhan syndrome patients with and without allopurinol (a xanthine oxidase inhibitor which prevents the accumulation of uric acid) treatment have been examined by using high performance liquid chromatography. Accumulation of hypoxanthine was more marked in urine and in cerebrospinal fluid than in plasma, this was consistent with the most metabolically active tissue for HPRT, brain, showing the most marked functional changes in its absence. The function of HPRT was suggested to be the recycling of hypoxanthine which is released from tissues in increasing quantities as energy use and ATP 'turnover' in the tissue increases (Harkness *et al.*, 1988). Some laboratories began to use microencapsulated xanthine oxidase as an experimental therapy in Lesch-Nyhan disease (Palmour *et al.*, 1989). This attempt to decrease the metabolic accumulation of hypoxanthine may have failed due to the normal absence of xanthine oxidase in the central nervous system (Lopez *et al.*, 1989).

1.4.4 Transgenic animal model for Lesch-Nyhan syndrome

In an attempt to produce a genetic model for Lesch-Nyhan syndrome, two groups independently used the embryonic stem (ES) cell system and HPRT-deficient ES cells selected in culture to produce HPRT-deficient mice (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). These were the first demonstrations that ES cells could be used to generate animals with the same genetic alteration associated with a human inherited disease. Surprisingly, the HPRT-deficient mice showed no evidence of spontaneous self-mutilation, no detectable motor impairments in tests to monitor dysfunction of the basal ganglia, and a normal response to apomorphine which is a dopamine receptor agonist that could induce stereotyped behaviour (Dunnett *et al.*, 1989; Finger *et al.*, 1988). Administration of high doses of amphetamine, which stimulates release and inhibits uptake of monoamine neurotransmitters, did cause significantly increased stereotypic and locomotor behaviour in HPRT-deficient mice compared to control animals (Jinnah *et al.*, 1991). In addition, spontaneous behaviour alteration was observed in old (22-24 months) HPRT-deficient mice which developed trauma to ears and flanks caused by overgrooming (Williamson *et al.*, 1992a). These results suggested that HPRT-deficient mice do have the potential to serve as a good animal model for Lesch-Nyhan syndrome.

Differences in purine metabolism between rodents and man could be responsible for the different consequences of HPRT deficiency (Figure 1.1). It has been suggested that uricase, which is present in rodents, but not in primates, and which catabolises uric acid to allantoin, may prevent the accumulation of neurotoxic levels of uric acid in HPRT-deficient mice. This explanation for the failure to observe self injury behaviour in HPRT-deficient mice is not compatible with the inability of allopurinol (a xanthine oxidase inhibitor which prevents the accumulation of uric acid) to improve the condition of Lesch-Nyhan syndrome patients. In addition, the hypothesis that accumulation of neurotoxic uric acid during brain development does not happen in rodents was weakened by the observation of low xanthine oxidase activity in brain. A second possibility is that the regulation of the nucleotide pool differs between rodents and humans. Compared to man, very little circulating

hypoxanthine is salvaged by HPRT in wild-type mice (Moyer and Henderson, 1983), suggesting that the mouse is less dependent on HPRT than man to maintain purine levels and as a result should be less affected by its loss. The reported ratio of the activities of the two key enzymes in the purine salvage pathway, HPRT/APRT, are quite different between rodents and humans; 1.86 in whole brain extract from 14-day-old neonatal mice and 11.66-51.38 in brain tissue of human autopsy (Brosh *et al.*, 1990; Rosenbloom *et al.*, 1967).

1.5 APRT and clinical symptoms of its deficiency

The mammalian adenine phosphoribosyltransferase (APRT; AMP pyrophosphate phosphoribosyltransferase; EC 2.4.2.7), which like HPRT is a member of a family of phosphoribosyltransferases, is a component of the purine salvage pathway that catalyses the magnesium-dependent transfer of the ribose-5-phosphate moiety of 5-phosphoribosyl-1-pyrophosphate to the 9 position of purine base adenine to form AMP. This occurs in all tissues examined with highest specific activity in nucleated cells. Mammalian APRT is found exclusively in the cytoplasm, while in bacteria APRT activity appears to be loosely associated with the cell membrane in the periplasmic space. The native human APRT enzyme has a molecular weight of 38,200 and is composed of 3 subunits of 143 aa with molecular weight 18,000 which appear to be associated by noncovalent forces (Thomas *et al.*, 1973; Holden *et al.*, 1979). Cells deficient in APRT can be selected by incubation with 2,6-diaminopurine (DAP) that is toxic to cells in the presence of APRT. The antibiotic alanosine has been shown to act as an aspartate analog and prevent the formation of AMP from IMP, probably by inhibiting adenylosuccinate synthetase. Alanosine interferes to a lesser extent with formation of UMP by inhibiting aspartate transcarbamylase. The action of alanosine appears specific for reactions involving the condensation of aspartate with a carbon atom adjacent to a nitrogen. In the presence of alanosine, cells should be dependent upon AMP synthesized either from exogenous adenine via APRT, or from exogenous adenosine via adenosine kinase (Kusano *et al.*, 1971). Murine Ltk$^-$aprt$^-$ cells were selected and maintained in medium supplemented with diaminopurine at 50 μg/ml (Wigler *et al.*, 1979b), which provided the demonstration

of DNA mediated transfer of the gene coding for APRT against the selection of alanosine and adenine (Wigler *et al.*, 1979a). Therefore, by exploiting the capacity of a functional APRT gene to transform APRT-deficient recipient cells to an $APRT^+$ phenotype, the Chinese hamster APRT gene in λ Charon 4A was cloned and subsequently subcloned in pBR322 (Lowy *et al.*, 1980). The isolation of human and mouse DNA coding for APRT was done by using a fragment of hamster APRT gene to screen a genomic library (Sikela *et al.*, 1983; Dush *et al.*, 1985). The functional human adenine phosphoribosyl transferase gene is < 2.6 kb in length and contains five exons. The amino acid sequence of APRT has been highly conserved throughout evolution. The human enzyme is 82% and 90% identical to the mouse and hamster respectively (Broderick *et al.*, 1987). By means of somatic genetic methods and restriction fragment length polymorphism, the APRT gene was located on chromosome 16 in man (Murray *et al.*, 1984) and chromosome 8 in the mouse (Nesterova *et al.*, 1987).

In APRT deficiency, adenine is oxidised by xanthine oxidase to the highly insoluble and nephrotoxic derivative, 2,8-dihydroxyadenine. The accumulation of this compound in the kidney can lead to stone formation and eventual renal failure. There are two different types of inherited APRT deficiency which have been described. In type I APRT deficiency, enzyme activity is practically undetectable either *in vivo* or *in vitro*. Type I deficiency is seen predominantly in Caucasians but also in Japan. Type I patients are homozygotes or compound heterozygotes for a variety of null alleles collectively designated APRT*QO. Type II deficiency, in which there is complete enzyme deficiency *in vivo* but only partial deficiency in cell extracts, has been found only in Japan. Type II patients are homozygotes or compound heterozygotes for the APRT*J missense mutation in exon 5 (Sahota *et al.*, 1991a,b).

1.6 Transgenic mice as animal models

A transgenic animal is characterised by the presence of foreign DNA sequences integrated in the genome by laboratory techniques. The first transgenic mice were produced by microinjecting SV40 viral DNA into the blastocoel cavity of early

embryos (Jaenisch and Mintz, 1974). Subsequently, transgenic mice were produced by exposing early embryos to infectious retroviruses (Jaenisch, 1976). The majority of transgenic animals have since been generated by pronuclear injection with linear DNA (Gordon *et al.*, 1980). Another approach involves transfection of DNA into totipotent teratocarcinoma cells, followed by injection of selected cells into the blastocyst; alternatively, nuclei from such cells can be introduced into fertilised eggs from which the pronuclei have been removed (McGrath and Solter, 1983). Foreign DNA introduced by one of the above routes integrates into the chromosomal DNA of mice and is carried in germ cells as well as somatic cells. The efficiency of producing transgenic mice averages about 25% of live births when linear molecules are microinjected (Gordon *et al.*, 1980). A comparable efficiency is possible with viral vectors when they are used to inject preimplantation embryos. However, although the conventional approach of generating transgenic animals has led to important conclusions, it suffers from restrictions inherently associated with the methodology. Firstly, the copy number of the injected gene and, consequently that of its product cannot be controlled which may lead to alterations never observed under the appropriate gene dosage. Secondly, the injected gene copies integrate randomly into the host genome. This may either cause unexpected mutations in the host or modulate the expression of the introduced gene by involving dominant control elements of the host chromatin found close to the integration site. The way to improve the control of transgene expression is to introduce an opportunity for early selection of transgenic phenotypes by manipulating the gene in cultured cells. The main advantages of cell lines over embryos as targets for gene transfer are that cells whose genomes have been identically altered could be generated by screening and cloning and that the full range of somatic cell genetic techniques can be employed.

Embryologists have been fascinated for a long time with the complex mechanism underlying the developmental program of multicellular organisms. In mouse embryology, *in vitro* models have been a prerequisite for understanding the molecular processes during development. One major outcome was the establishment of embryonic stem (ES) cell lines that retain their embryonic properties when cultured

under appropriate conditions (Martin, 1981; Evans and Kaufman, 1981). Embryonic stem cells are permanent cell lines established directly from the inner cell mass of the preimplantation embryo. These cells represent an ideal link between the situation *in vitro* and that *in vivo*, since they can develop into a variety of cell types upon *in vitro* differentiation and they participate in the formation of all tissues, including the germ line, when introduced into a blastocyst. Martin and Evans (1975) showed that pluripotent embryonic stem cells can be maintained in the undifferentiated state only by culture on feeder layers of growth-arrested embryonic fibroblasts. However, more recent data indicate that totipotency and inhibition of differentiation can be maintained in cell culture using medium supplemented with either medium conditioned by incubation with Buffalo Rat liver cells (Smith and Hooper, 1987) or leukaemia inhibition factor (LIF) (Pease and Williams, 1990). Leukaemia inhibition factor is a polypeptide growth factor with a seemingly remarkable range of biological actions in different tissue systems and its importance for embryo development has been confirmed by gene knock out. Males homozygous for LIF deficiency were fertile and able to sire offspring from both wild-type and heterozygous females, while none of the females homozygous for LIF deficiency became pregnant after repeated matings (Stewart *et al.*, 1992). Hence, the isolation of ES cells from preimplantation embryos can also be completed without the cooperation of other cells (Pease *et al.*, 1990; Nichols *et al.*, 1990).

ES cells provide a new alternative method to generate transgenic mice. DNA can be introduced into ES cells in culture and the desired transformants can be selected *in vitro*. By implanting the genetically altered pluripotent embryonic stem cells into mouse blastocysts where they can colonise all tissues, a chimeric animal is obtained. Transgenic animals that carry the transgene in all cells can be produced by breeding chimaeric mice which have viable germ cells derived from the injected ES cells (Bradley *et al.*, 1984). To distinguish among the offspring derived from ES cell and embryo, the genes encoding for different coat colours or isoenzymes (e.g. glucose phosphate isomerase) can be used (Bradley, 1987). For example, mouse strain 129/Ola is homozygous for the white bellied agouti (A^w) allele, at the agouti locus and

homozygous for the chinchilla (c^{ch}) allele at the albino locus. The chinchilla allele confers a modification of the agouti phenotype to give a light brown (chinchilla) coat colour. It also carries a homozygous recessive allele known as pink-eyed dilute at a third locus (*P*), resulting in pink eyes. When 129/Ola derived ES cells are injected into albino Balb/c embryos, this particular genetic constitution allows for easy visual identification of chimaeras and of the transmission of ES-derived germ cells from test matings of chimaeras. Mouse strain Balb/c is homozygous for the wild-type alleles at the A and P loci and for the recessive (c) allele at the albino locus resulting in white coat and pink eyes (Jackson, 1991). Chimaeras with chinchilla coat patches are easily distinguished from the albino host. If chimaeras are test mated to Balb/c, the offspring carrying ES-derived chromosomes (genetic constitution c^{ch}/c, p/P, A^w/A) will have grey coat and black eyes. Host embryo-derived pups are distinguished by their white coat and pink eyes (Selfridge *et al.*, 1992). Hooper *et al.* (1987) selected spontaneous mutations at the HPRT locus by culturing male ES cells in medium containing 6-thioguanine, while Kuehn *et al.* (1987) obtained an HPRT deficient ES cell mutant by retroviral insertion. Both of them successfully constructed transgenic mouse strains lacking HPRT activity by blastocyst injection. In combination with the recent advances in gene targeting, ES cell techniques open up the possibility of making pre-determined alterations in the mouse genome in order to study gene function and regulation *in vivo*.

1.7 Homologous recombination

There are various methods, such as viral infection, lipofection, microinjection, calcium phosphate precipitation and electroporation, available for introducing exogenous DNA into mammalian cells which is then stably incorporated in the genome. When DNA molecules are introduced into the nuclei of mammalian cells by microinjection, about 20% of these cells stably integrate the exogenous DNA into the genome (Capecchi, 1980) and only 0.1% by electroporation under optimal conditions (Mansour *et al.*, 1988). The integration sites are apparently distributed randomly over the whole genome and this type of DNA integration is called random integration (Murnane *et al.*, 1990). The site of incorporation however could not be controlled

until Smithies *et al.* (1985) suggested the utilisation of homologous recombination. Uptake of introduced DNA by homologous recombination into the cell genome was first demonstrated by transformation of *Saccharomyces cerevisiae* (Hinnen *et al.*, 1978). Most transformants (revertants of the *Leu2*$^-$ mutation) were generated as a result of integration of the selectable *Leu2* gene-plasmid construct at the *Leu2* site due to homology. Twenty per cent of the revertants were generated as a result of a double cross-over event at the site of homology. Microinjection of the herpes simplex virus thymidine kinase gene into nuclei of cultured Ltk$^-$ cells led to the formation of concatomers via homologous recombination between injected DNA sequences, that raised the possibility of recombination between chromosome and incoming DNA (Folger *et al.*, 1982). Mouse L cells with truncated *H-2* genes produced full H-2 antigens following transfection with complementing sequences. This demonstrated recombination between incoming DNA and chromosomal sequences (Goodenow *et al.*, 1983). Mammalian cells in which a plasmid carrying globin sequences had integrated into the human β-globin locus were subsequently isolated (Smithies *et al.*, 1985). The great advantage of this approach is that it eliminates position and copy number effects which complicate analysis in more conventional studies (Capecchi, 1989).

As discussed above, the HPRT gene has provided an ideal model system for positive and negative selection which makes it ideally suited to gene targeting. A series of experiments using the mouse HPRT gene as target locus in ES cells to investigate the mechanism of homologous recombination have been described. Capecchi and colleagues introduced by electroporation a functional *neo* gene flanked by some parts of the HPRT gene into ES cells to inactivate HPRT (Thomas and Capecchi, 1987). In this experiment, G418 resistant clones were obtained at a frequency of 10^{-3} and the majority of the resistant clones exhibited random integration (non-homologous recombination). Only 1 in a 1000 of the G418^r colonies were targeted as demonstrated by resistance to 6-thioguanine. Further strategies for either inactivating the HPRT gene in normal ES cell lines or correcting the HPRT gene in HPRT-deficient ES cell lines by gene targeting have been developed (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987; Capecchi, 1989). Transgenic mice have been

successfully produced after correcting a HPRT gene deletion in ES cells by homologous recombination, creating a chimaeric mouse that had a contribution to its germ line from ES cells, and breeding from this (Thompson *et al.*, 1989; Koller *et al.*, 1989).

A variety of strategies, which have been generated for targeting other genes, either in ES cells (Mansour *et al.*, 1988; Joyner *et al.*, 1989; Zimmer and Gruss, 1989; DeChiara *et al.*, 1990; Thomas and Capecchi, 1990) or somatic cells (Sedivy and Sharp, 1989; Adair *et al.*, 1990; Zheng and Wilson, 1990) are listed in Table 1.2. Steeg *et al.* (1990) have introduced a specific point mutation which results in resistance to α-amanitin into the endogenous murine gene that encodes the largest subunit of RNA polymerase II. The use of the *Escherichia coli lacZ* gene as a reporter gene provides an excellent system to study embryonic development. Several promoters have been studied *in vivo* using this strategy after gene targeting (Greenberg *et al.*, 1990; Le Mouellic *et al.*, 1990; Mansour *et al.*, 1990). Direct functional selection schemes as for HPRT are not available for most genes. In order to detect targeting of such genes several procedures have been developed. Apart from the positive-negative selection which is widely used in homologous recombination and is discussed in detail in Chapter 9, the uses of a promoterless positive selection module and screening by the polymerase chain reaction (PCR) method are generally applied.

Fusion protein studies showed that proteins carboxyl-terminal fused with *neo* protein still conferred kanamycin resistance in bacteria. Several plasmids, in which the coding sequence for the amino-terminal region of the *neo* protein lacking the ATG translation initiation codon was fused to foreign DNA sequences coding for 3-300 amino acids were constructed and examined for the expression of the fusion proteins in bacteria. Although the level of kanamycin resistance is varied, all the fusion proteins provided some resistance (Reiss *et al.*, 1984). The idea of a functional *neo* fusion protein was applied to enrich for targeted events in the homologous recombination procedures. The promoterless *neo* gene was introduced into the targeting construct such that *neo* could be activated by the target gene promoter or in

rare cases, a random promoter. Because activation by cellular promoters occurs in only about 1 in 100 random integration events, selection for G418 resistance should enrich for homologous recombination events approximately 100 fold (Jasin and Berg, 1988; Doetschman *et al.*, 1988; Sedivy and Sharp, 1989; Charron *et al.*, 1990; Schwartzberg *et al.*, 1990; Jeannotte *et al.*, 1991). PCR provides a quick detection method from a very small sample in a very short time (Saiki *et al.*, 1988). Using one primer specific for the incoming DNA and another specific to the target locus, the targeted events could be diagnosed after PCR amplification (Zimmer and Gruss, 1989; Kim *et al.*, 1991; Selfridge *et al.*, 1992; Saga *et al.*, 1992). The major advantage of the PCR technique is its sensitivity, allowing the detection of events from less than 100 cells (McMahon and Bradley, 1990). As a result, targeted ES cells can be injected into blastocysts after only a short period of cell culture to take the advantage of avoiding loss of pluripotency by careless culture or generation of unwanted spontaneous mutations during extended culture.

In the last 2 years mice heterozygous and homozygous for targeted mutations have been generated from ES cells. Phenotypes have been studied in the hope of gaining some insight into the function of mutated genes. Heterozygous mice carrying a paternally transmitted null mutation in the insulin-like growth factor II gene were found to be 60% normal size (DeChiara *et al*, 1990). Homozygotes were also 60% normal size, while heterozygotes born to female carriers were normal size (DeChiara *et al.*, 1991). This study demonstrates that insulin-like growth factor II is indispensable for normal embryonic growth and is subject to parental imprinting. Mutation in the immunoglobulin μ gene leads to abnormal B-cell development (Kitamura *et al.*, 1991) and mutation in the β2-m gene leads to absence of certain T-cell populations due to lack of MHC-1 presentation (Zijlstra *et al.*, 1990). The importance of the transcription factor GATA-1 in erythrocyte development was demonstrated in ES cell-derived chimaeric mice. Erythrocytes failed to develop from the ES cells, in which the X-linked GATA-1 had been mutated by homologous recombination (Pevny *et al.*, 1991). Homozygotes for a null mutation in *c-abl* (Tybulewicz *et al.*, 1991) and a deletion of the C-terminus of *c-abl* (Schwartzberg *et*

al., 1991) result in similar phenotypes, demonstrating that the C-terminus is very important for *c-abl* function. Homozygotes die 2-3 weeks after birth. The immune system, thymus and B-cell population are severely affected.

Homozygotes for a disrupted *Hox 1.5* gene die neonatally due to pulmonary defects (Chisaka and Capecchi, 1991). Absence of thymus and parathyroid, coronary defects and throat region abnormalities may be due to the lack of *Hox 1.5* in branchial arches and pharyngeal pouches prior to migration or mixing of cell derivatives. Homozygotes for *Wnt-1* (*int-1*) mutations have been generated independently by Thomas and Capecchi (1990) and McMahon and Bradley (1990). The majority of homozygotes die neonatally, though one mouse surviving to adulthood suffered severe ataxia (Thomas and Capecchi, 1990). Degrees of penetrance may vary due to mouse strain background. The dorsal and ventral caudal mid brain were found to be absent at 9.5 days *p.c.* (McMahon and Bradley 1990). This correlates with a broad band of *Wnt-1* expression observed on *in situ* hybridisation. CNS development, especially of the cerebellum, is defective. The *Hox 1.5* and *Wnt-1* mutants show severe phenotypes in regions that would be predicted to be affected because of the previously observed pattern of gene expression. However, the genes are also expressed in regions which appear unaffected by the mutation. The gene product may have no role in these areas despite its expression. A more likely explanation is that of redundancy of function - the gene function may be replaced by similar or related proteins. Functional redundancy between 2 highly conserved homeobox-containing genes *En-1* and *En-2* in shared regions of expression is the probable explanation for the normal development of *En-2* null mutation homozygotes (Joyner, 1991). A subtle phenotype is observed in the cerebellum, the only region in the developing mouse embryo where *En-2* is expressed and *En-1* is not. *En-2* may have developed a special function in this region. If redundancy is operating, it may be predicted that upon breeding of *En-1* mutant with *En-2* mutant mice to generate mice homozygous for both mutations, a more severe phenotype than either mutation alone would be observed. Unfortunately, the *En-1* gene has not been targeted to generate transgenic mice. Overlap in function between other protein kinases may also explain why a null mutation in *c-src* does not

lead to a more extensive phenotype (Soriano *et al.*, 1991). The *c-src* gene is expressed widely in the mouse and yet the mutant phenotype is limited to bone. Other widely expressed protein kinases may compensate for the shortage of the *c-src*-encoded protein kinase in other tissues.

1.8 Gene therapy

Sooner or later, gene replacement therapy should be feasible, at least for a number of single gene disorders. So far, the major difficulty has been in transfecting 'foreign' genes into cells and, once inserted, in persuading them to come under the control of the regulatory machinery of the cells. Recently, it has been possible to overcome some of the transfection difficulties by attaching genes to viral vectors or by utilising physical methods. Gene therapy is a medical intervention based on modification of the genetic program of living cells. There are two basic strategies for gene therapy. First, cells are modified *ex vivo* for subsequent administration to humans. This may be carried out by using either autologous cells derived from the recipient of therapy or cells from other individuals or species. Second, the cells are altered *in vivo* by gene therapy administered directly to the individual. These genetic manipulations may be intended to have a therapeutic effect, or they may be used for marking cells to gain an understanding for future therapeutic interventions. Several proposals for human gene therapy in the United States have been approved by the Food and Drug Administration (Anderson, 1992; Miller, 1992). However, there is still a long way to go.

1.8.1 Gene therapy using recombinant virus

In the past few years, the use of recombinant virus for introducing foreign genes into cells, tissues, and whole animals has increasingly been employed. Although there are several viral vectors available to transfer genes into mammalian cells, the majority of attempts have used retroviruses due to their high efficiency of infection and integration (Dick *et al.*, 1985; Bender *et al.*, 1989; Correll *et al.*, 1989; Lim *et al.*, 1989; Moore *et al.*, 1989; Roux *et al.*, 1989; Drumm *et al.*, 1990; Li *et al.*,

1990; Novak *et al.*, 1990). It has been demonstrated that the human factor IX cDNA can be expressed in rabbit hepatocytes after infecting with recombinant virus containing human factor IX DNA sequences. This *in vitro* experiment provided the possibility of gene therapy for haemophilia B. Current treatment for haemophilia B involves administration of either plasma or crude preparations of plasma fractions enriched in prothrombin complex proteins to patients but this has the risk of exposing them to viral contamination such as human immunodeficiency virus and hepatitis virus (Armentano *et al.*, 1990). Murine systemic lupus erythematosus (MRL/lpr mouse), as an animal model for human systemic lupus erythematosus, a severe abnormal immune disease in Caucasians, has been investigated for gene therapy. Gutierrez-Ramos *et al.* (1990) demonstrated that the survival rate of MRL/lpr mice was increased after infection with an interleukin-2/vaccinia recombinant virus.

Lesch-Nyhan syndrome is considered as a neurological disease and has been suggested as a candidate for initial attempts in somatic cell gene therapy. Retrovirus-derived vectors capable of expressing the human HPRT cDNA in cultured fibroblasts and lymphoblasts have been derived (Miller *et al.*, 1984). Palella *et al.* (1988) claimed that the use of retrovirus-derived vectors to correct the enzyme deficiency in lymphoblasts may not result in restoration of neurological function. Hence these workers constructed a recombinant herpes simplex virus type 1 vector containing human HPRT cDNA sequences and successfully expressed the HPRT mRNA both in the cultured HPRT deficient rat neuronal cells (Palella *et al.*, 1988) and in the brains of mice infected with the recombinant virus (Palella *et al.*, 1989).

1.8.2 Correction by gene targeting

An HPRT-deficient male ES cell line, E14TG2a, was produced by Hooper *et al.* (1987) and the structure of the HPRT gene in this mutant was analysed by Southern hybridisations (Thompson, 1989). By using the HPRT cDNA as a probe and 2 genomic DNA fragments, Southern hybridisation revealed that E14TG2a carried a deletion of the first two exons of the HPRT gene and at least 12 kb of 5′ flanking sequence compared to its wild-type cell line, E14 (Handyside *et al.*, 1989).

Consequently, E14TG2a was used to generate a HPRT-deficient mouse strain, $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$. The ability to correct a dysfunctional gene by gene targeting and then to introduce this to the germ line and produce healthy offspring was first demonstrated by Thompson *et al.* (1989). Two similar vectors were utilised to correct the HPRT mutation in E14TG2a. Thirty corrected clones were recovered from 3 different experiments at a frequency of 4×10^{-8} to 2.2×10^{-7} by using HAT selection. Northern hybridisation analysis revealed expression of HPRT mRNA in the corrected clones, whereas no expression was observed in the deficient cell line, E14TG2a. Cells from one corrected clone have been introduced into mouse blastocysts, and germ line transmission of the ES cell-derived corrected gene has been achieved. The corrected gene has the same pattern of expression as the wild-type gene, with the characteristic elevated level of expression in brain tissue. The feasibility of introducing targeted modifications into the mouse germ line by homologous recombination in ES cells has been demonstrated.

Sickle cell anaemia, caused by homozygosity for a point mutation in the β-globin gene, is the most common genetic disorder in persons of African descent. Homozygotes for the β^S allele usually have serious clinical problems and a shortened lifespan, while the β^S allele is asymptomatic when heterozygous with a normal β^A allele. A mouse-human hybrid cell line BSM which was derived from a mouse erythroleukaemia cell line and carried a single human chromosome 11 with the β^S-globin allele was tested for the possibility of correction by homologous recombination. A β^A-globin targeting construct containing a unique oligomer and a neomycin-resistance gene was electroporated into the BSM cells, which were then placed under G418 selection. After PCR screening and Southern hybridisation analysis, clones where the β^S allele was corrected to β^A were detected (Shesely *et al.*, 1991).

1.8.3 Cell transplantation after gene correction or gene therapy

Organ transplantation has been widely used as a therapeutic approach for many diseases such as kidney transplantation for uraemia. Genetic diseases can be classified into those arising from disordered cellular function or from the absence of specific

proteins. Organ transplantation can treat disorders of cellular dysfunction by replacing the abnormal cells with transplanted normal cells. The abnormal cells may be red cell (sickle-cell thalassaemia), liver (tyrosinaemia), or heart (muscular dystrophy). On the other hand, abnormalities of specific proteins, specifically enzymes, may be successfully treated if cells capable of producing the normal protein are transplanted. Transplantation of the normal cells does not require transplantation of the organ in which the clinical symptomatology occurs, since the normal proteins derived from the transplanted cell may be transported to the non-transplanted organ and result in clinical improvement. After birth, blood formation is normally restricted to haematopoietic cells of the bone marrow. Displacement bone marrow transplantation (DBMT) is a technique that aims to obtain 100% donor-type marrow so that the future immune processes of the recipient will be those of the donor. The method has reached a stage of practicality that can be applied to many genetic diseases of marrow-derived cells and not solely to leukaemia or marrow aplasia. Work from many laboratories using recombinant retroviruses to infect haematopoietic stem cells suggests that (i) it is possible to infect efficiently mouse bone marrow cells and (ii) upon reconstitution of the infected bone marrow cells, the expression of foreign genes carried by means of the retroviral vectors is generally very poor. Recently, Wong *et al.* (1989) generated a recombinant retroviral vector containing the murine interleukin-3 (IL-3) gene as a means to create an animal model for myeloproliferative disorder. After introducing the IL-3 gene into mouse haematopoietic cells, integration and expression of the gene were observed in spleen foci from which could be derived IL-3 factor-independent, continuously proliferating cell lines. Irradiated or genetically anaemic recipient mice transplanted with these haematopoietic cells developed a myeloproliferative syndrome characterised by a marked elevation in leucocyte count, bone marrow hyperplasia, and enlargement of the liver and spleen. Animal models have also been developed for the therapy of disease by bone marrow transplantation. The human glucocerebrosidase (GLC) gene has been transferred efficiently into spleen colony-forming unit (CFU-S) multipotential haematopoietic progenitor cells by recombinant retroviral vectors, and production of human GLC RNA and protein has been achieved in transduced CFU-S colonies. Transfected bone marrow cells were

transplanted into recipient mice to generate long-term reconstituted mice. Human GLC was detected in transduced bone marrow and spleen cells 5 months after transplantation (Correll *et al.*, 1989). One 22-year old Lesch-Nyhan syndrome patient has had a DBMT from his matched sibling, which completely corrected the metabolic derangements, but has appeared to have little effect on his long-standing psychosis. Because purine metabolism can be corrected, it would be important to evaluate if correction by DBMT at a younger age could avert the psychosis (Hobbs, 1988). This clinical trial showed it was possible to correct a defect by bone marrow transplantation. If a good animal model of Lesch-Nyhan syndrome was available, then in conjunction with DBMT it could be used to assess potential therapies. Bone marrow haematopoietic stem cells could be isolated from the limbs of transgenic mice after treatment with 5-fluorouracil to remove suppressor cells. The defective HPRT gene of the isolated deficient bone marrow cells could then be corrected by gene targeting *in vitro*. Subsequently, the corrected bone marrow stem cells could be used as donor cells for DBMT into HPRT deficient mice both with and without prior irradiation. The effect of therapy could then be monitored either by enzyme analysis or by Northern hybridisation of RNA from peripheral blood lymphocytes.

1.9 Aims of this study

When this research project was initiated, homologous recombination was just emerging as a powerful tool for the study of gene function in cells and animals. There were then few published results and very little was known about the mechanism of homologous recombination in mammalian cells (Smithies *et al.*, 1985; Thomas and Capecchi, 1987; Doetschman *et al.*, 1988; Mansour *et al.*, 1988; Zimmer and Gruss, 1989; Joyner *et al.*, 1989; Thompson *et al.*, 1989; Sedivy and Sharp, 1989; Ellis and Bernstein, 1989). Homologous recombination technology allows alteration to a gene without removing it from its position and sequence context. Instead of the conventional replacement and insertion strategies, the first part of these studies is an attempt to establish and study targeting deletions using an HPRT gene in ES cells as a model. This approach should allow a better understanding of the mechanism of homologous recombination and provide a strategy for the inactivation of the APRT

gene in the later parts of the studies.

The unexpected lack of phenotype in both strains of HPRT-deficient mice which were the first genetically altered animal models for a human inherited disease, was puzzling (Finger *et al.*, 1988; Dunnett *et al.*, 1989). To resolve this enigma, the hypothesis that rodents are less dependent on HPRT in their purine salvage pathway than humans is proposed. There are two possible ways to increase the dependence on HPRT in mice. One is biochemical inhibition of APRT by administration of an APRT competitive inhibitor and the other is by using a genetic approach to generate APRT-deficient mouse. Caffeine, which has been used for generating an animal model for Lesch-Nyhan syndrome (Peters, 1967) and a novel more specific inhibitor of APRT, are used to test this hypothesis both in cell cultures and in animals. For genetic alteration of APRT gene in ES cells, the targeting deletion strategy is applied to delete the promoter and exons 1-2.

HPRT-deficient mice provide not only a system to study the regulation of the HPRT gene and Lesch-Nyhan syndrome but also a system to evaluate the strategies of gene therapy. Concentrating on establishing the possible therapeutic strategies which could be used in Lesch-Nyhan patients, these studies are restricted to the neuronal lineage. The use of the techniques of homologous recombination allowed me to learn how to produce animal models of human disease. The main objective of this project has been to develop an animal model for Lesch-Nyhan syndrome by a combined genetic and biochemical approach.

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells.

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
β-globin	insertion	4.6 kb	electroporation	fibroblast	+ 13.1 kb	Smithies *et al.* 1985
HPRT	replacement	4.0 kb	electroporation	ES	4.0 kb ==> 5.0 kb	Thomas & Capecchi 1987
	insertion	9.3 kb			+ 9.3 kb	
HPRT	replacement	1.3 kb	electroporation	ES	+ 2.0 kb	Doetschman *et al.* 1988
HPRT	replacement	9.1 kb	electroporation	ES	4.0 kb ==> 5.0 kb	Mansour *et al.* 1988
int-2	replacement	10.0 kb	electroporation	ES	6.2 kb ==> 7.4 kb	Mansour *et al.* 1988
Hox 1.1	replacement	3.6 kb	microinjection	3T3, P19, ES	+ 20 bp	Zimmer & Gruss 1989
En-2	replacement	4.0 kb	electroporation	ES	1.1 kb ==> 1.5 kb	Joyner *et al.* 1989

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
HPRT	insertion	9.0 kb	electroporation	ES, mice	correction + 11.7 kb	Thompson *et al.* 1989
polyoma integration	replacement	4.8 kb	transfection	MT 1.4		Sedivy & Sharp 1989
retrovirus integration	insertion	0.9 kb	transfection	DL 22		Ellis & Bernstein 1989
Class II MHC E_α		2.6 kb	Microinjection	fertilised egg	correction	Brinster *et al.* 1989
c-*abl*	replacement	7.5 kb	electroporation viral capsid	ES, mice	6.5 kb ==> 7.1 kb	Schwartzberg *et al.* 1989

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
β_2-microglobulin	replacement	10.0 kb	electroporation	ES, mice	4.0 kb ==> 5.1 kb	Zijlstra *et al.* 1989
HPRT	insertion	9.7 kb	electroporation	ES, mice	correction + 12.4 kb	Koller *et al.* 1989
DHFR	replacement	4.6 kb	electroporation	CHO	4.2 kb ==> 5.1 kb	Zheng & Wilson 1990
APRT	insertion & ICR[a]	2.6 kb	$CaPO_4$ precipitation	CHO		Adair *et al.* 1990
HPRT	replacement	165 bp	electroporation of viral capsid		correction by cDNA	Hunger-Bertling *et al.* 1990

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
N-*myc*	replacement	5.0 kb	electroporation	ES, Pre-B	3.9 kb ==> 4.6 kb	Charron *et al.* 1990
c-*abl*	replacement	7.5 kb	electroporation	ES	6.5 kb ==> 7.1 kb	Schwartzberg *et al.* 1990
IGF-II	replacement	9.7 kb	electroporation	ES, mice	8.0 kb ==> 8.9 kb	DeChiara *et al.* 1990
RNA pol. II	replacement	5.8 kb	electroporation	ES	4 bp change	Steeg *et al.* 1990
Hox-3.1	replacement	8.3 kb	electroporation	ES	40 bp ==> 7.2 kb	Le Mouellic *et al.* 1990
int-1	replacement	13.5 kb	electroporation	ES, mice	3.0 kb ==> 4.0 kb	Thomas & Capecchi 1990
int-2	replacement	10.0 kb	electroporation	ES	6.2 kb ==> 11.6 kb	Mansour *et al.* 1990

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
c-*fyn*	replacement	6.3 kb	electroporation	ES	3.7 kb ==> 4.5 kb	Yagi *et al.* 1990
pim-1	replacement	5.8 kb	electroporation	ES	3.6 kb ==> 4.1 kb	te Riele *et al.* 1990
CD4	insertion	3.5 kb	electroporation	T-cell line	+	Jasin *et al.* 1990
GATA-1	replacement	4.7 kb	electroporation	ES, mice	4.5 kb ==> 5.6 kb	Pevny *et al.* 1991
Hox-2.6	insertion & ICR	3.1 kb	electroporation	ES	+ 14 bp	Hasty *et al.* 1991a
HPRT	insertion & ICR	5.0 kb	electroporation	ES	+ 4 bp	Valancius & Smithies 1991
ig-μ	replacement	9.0 kb	electroporation	ES, mice	5.2 kb ==> 5.4 kb	Kitamura *et al.* 1991

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
Hox-1.5	replacement	11.5 kb	electroporation	ES, mice	14.8 kb ==>16.8 kb	Chisaka & Capecchi 1991
T-cell receptor	deletion	8.5 kb	electroporation	ES	- 15 kb	Mombaerts *et al.* 1991
β^S-globin	replacement	4.7 kb	electroporation	BSM	correction	Shesely *et al.* 1991
IL-2	replacement	6.0 kb	electroporation	ES, mice	6.0 kb ==> 7.1 kb	Schorle *et al.* 1991
G-protein	replacement	5.0 kb	electroporation	ES	6.5 kb ==> 8.1 kb	Mortensen *et al.* 1991
CD4	replacement	2.8 kb	electroporation	ES, mice	8.8 kb ==> 10 kb	Rahemtulla *et al.* 1991
Hox-1.3	replacement	7.4 kb	electroporation	ES	3.7 kb ==> 11.3 kb	Jeannotte *et al.* 1991

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
Cftr	replacement	7.8 kb	electroporation	ES	3.0 kb ==> 5.7 kb	Koller *et al.* 1991
C-μ	insertion replacement	4.3 kb	electroporation	igm482	correction + 10.0 kb correction	Kang & Shulman 1991
ig-*μ*	replacement	3.9 kb	electroporation	myeloma	2.7 kb ==> 4.1 kb	Smith & Kalogerakis 1991
Hox-1.6	replacement	11.8 kb	electroporation	ES, mice	6.8 kb ==> 8.0 kb	Chisaka *et al.* 1992
p53	replacement	3.7 kb	electroporation	ES, mice	5.0 kb ==> 6.5 kb	Donehower *et al.* 1992
Prn-p	replacement	8.9 kb	electroporation	ES, mice	3.5 kb ==> 4.0 kb	Büeler *et al.* 1992
glucocerebro-sidase	replacement	6.1 kb	electroporation	ES, mice	6.1 kb ==> 7.5 kb	Tybulewicz *et al.* 1992

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
Ren-1D	replacement	5.5 kb	electroporation	ES	6.7 kb ==> 8.7 kb	Miller *et al.* 1992
apolipoprotein A-1	replacement	9.0 kb	electroporation	ES, mice	12.0 kb ==> 13.1 kb	Williamson *et al.* 1992b
LIF	replacement	5.6 kb	electroporation	ES, mice	3.0 kb ==> 3.8 kb	Stewart *et al.* 1992
Cftr	insertion	3.5 kb	electroporation	ES, mice	+ 7.3 kb	Dorin *et al.* 1992
Rb	replacement	8.0kb	electroporation	ES, mice	10.0 kb ==> 11.5 kb	Lee *et al.* 1992
Rb	replacement	9.6 kb	electroporation	ES, mice	10.0 kb ==> 11.2 kb	Jacks *et al.* 1992
Rb	replacement	15.0 kb	electroporation	ES, mice	4.9 kb ==> 7.3 kb	Clarke *et al.* 1992

Table 1.2 Compilation of published gene targeting experiments in ES and somatic cells (continued).

Gene	Mechanism	Homology	Introduction	Cell, Organism	Remark	Reference
TGF β-1	replacement	4.0 kb	electroporation	ES	3.9 kb ==> 4.8 kb	Shull *et al.* 1992
Tenascin	replacement	5.5 kb	electroporation	ES, mice	5.0 kb ==> 7.0 kb	Saga *et al.* 1992
ERCC-1	replacement	2.4 kb	electroporation	ES, mice	2.6 kb ==> 5.0 kb	Selfridge *et al.* 1992

+, inserted with; –, deleted with; ==>, replaced by

a, ICR; intrachromosomal recombination

CHAPTER 2. MATERIALS AND METHODS

2A MATERIALS

2A.1 Suppliers of laboratory reagents

Restriction endonucleases:

Boehringer Mannheim plc: Mannheim, Germany

GIBCO BRL Life Technologies: Paisley, U.K.

New England Biolabs Inc.: Beverly, Massachusetts, U.S.A.

Pharmacia LKB Biotechnology: Milton Keynes, U.K.

E. coli DNA polymerase I (Klenow large fragment), T4 DNA ligase:

GIBCO BRL Life Technologies

Thermus aquaticus (*Taq*) DNA polymerase:

Boehringer Mannheim plc

Standard laboratory reagents:

BDH Chemicals Ltd: Poole, U.K.

Calbiochem-Novabiochem Co.: La Jolla, U.S.A.

Fisons Chemicals: Loughborough, U.K.

GIBCO BRL Life Technologies

ICN Flow Limited: Rickmansworth, U.K.

Sigma Chemical Co.: Poole, U.K.

Bacterial media reagents:

Becton-Dickinson U.K. Limited: Oxford, U.K.

Difco Laboratories: East Moseley, U.K.

Reagents for mammalian cell culture:

GIBCO BRL Life Technologies

ICN Flow Limited

Sera-lab: Sussex, U.K.

Sigma Chemical Co.

Radioactive reagents:

Amersham International plc.: Aylesbury, U.K.

[8-^{3}H]-Adenine	Specific activity	23 Curies/mmole
[2-^{3}H]-Adenine		22 Curies/mmole
[U-^{14}C]-Adenine		270 mCuries/mmole
α-^{32}P-dCTP		~ 3,000 Curies/mmole
[G-^{3}H]-Hypoxanthine		7.9 Curies/mmole
[methyl-^{14}C]-L-Methionine		57 mCuries/mmole
[methyl-^{3}H]-Thymidine		5 Curies/mmole
[methyl-^{14}C]-Thymidine		58 mCuries/mmole

Antibiotics:

aminopterin - Sigma Chemical Co.

ampicillin - Beecham Research Laboratories: Brentford, U.K.

G418 - GIBCO BRL Life Technologies Ltd.

gancylovir - Syntex Laboratories Inc.: Palo Alto, U.S.A.

gentamicin - David Bull Laboratories Pty. Ltd.: Mulgrave, Australia

penicillin G - Sigma Chemical Co.

streptomycin - Sigma Chemical Co.

tetracyclin - Sigma Chemical Co.

Antisera:

Amersham International plc.

Boehringer Mannheim Laboratories

Scottish Antibody Production Unit (SAPU): Lanarkshire, U.K.

2A.2 Media

2A.2.1 Bacterial media

Luria Broth:

Difco Bacto-tryptone, 10 g

Difco bacto-yeast extract, 5 g

NaCl, 5 g

per litre adjusted to pH 7.2.

Luria Agar:

As Luria broth with 15 g per litre Difco agar.

Terrific Broth:

Difco bacto-tryptone, 12 g

Difco bacto-yeast extract, 24 g

glycerol, 4 ml

per 900 mls mixed with 100 mls sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 after autoclaving

M9 Minimal Agar:

Difco Bacto Agar, 15 g

10X M9 salts, 100 ml

20% glucose, 20 ml

1 mg/ml Vitamin B_1, 10 ml

0.1 M $MgSO_4$, 10 ml

0.1 M $CaCl_2$, 10 ml

per litre.

10X M9 Salts:

Na_2HPO_4, 60 g (0.423 M)

KH_2PO_4, 30 g (0.220 M)

NaCl, 5 g (0.086 M)

NH_4Cl, 10 g (0.187 M)

per litre.

Antibiotics:

Ampicillin to a final concentration of 100 μg/ml or tetracyclin to 10 μg/ml was added to media immediately prior to use when required.

2A.2.2 Mammalian tissue culture media

Glasgow Modified Eagles (BHK21) Medium (McPherson and Stoker, 1962; with modifications by W. House, Medical Research Council, Institute of Virology, University of Glasgow, Scotland, 1964) was supplied by GIBCO BRL Life Technologies as 10X concentrate medium. The working medium for general culture was supplemented with 1X non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 5% foetal calf serum (Sera-lab; GIBCO BRL) and newborn calf serum (ICN FLOW; GIBCO BRL).

Medium RPMI-1640 which was used in splenocyte culture, was supplied by GIBCO BRL Life Technologies as 10X concentrate medium and buffered with HEPES at the final concentration of 20 mM.

2A.3 Solutions

20X SSC: 3 M NaCl; 0.3 M tri-sodium citrate pH 7.0

10X TBE: 0.9 M Tris-HCl; 0.9 M boric acid; 20 mM EDTA (pH 8.0)

50X TAE: 2 M Tris; 1 M glacial acetic acid; 50 mM EDTA (pH 8.0)

10X MOPS: 200 mM 3-(*N*-morpholino) propane-sulphonic acid (MOPS); 50 mM sodium acetate; 10 mM EDTA; to pH 7.0 with NaOH

TE: 10 mM Tris-HCl pH 8.0; 1 mM EDTA

STE: 10 mM Tris-HCl pH 8.0; 1 mM EDTA; 150 mM NaCl

PBS: 8 mM K_2HPO_4; 1.5 mM KH_2PO_4; 150 mM NaCl

PCA: 25 parts redistilled phenol: 24 parts chloroform: 1 part isoamylalcohol

CA: 24 parts chloroform: 1 part isoamylalcohol

Mowiol mounting solution: Added 2.4 g Mowiol 4-88 (Calbiochem) to 6 g glycerol and stirred for 1 hr, then added 6 ml distilled water and stirred for another 2 hr. Twelve ml of 0.2M Tris-HCl (pH8.5) was added and incubated at 50 °C in a waterbath with occasional stirring. After clarification of the mixture by centrifugation at 5,000 x g for 15 min, the solution was aliquoted and stored at -20 °C. (The solution is stable for at least 2 weeks at room temperature after thawing).

2A.4 Bacterial strains

NAME	GENOTYPE	REFERENCE
JM83	*ara, Δ(lac-pro), strA, thi, lacZΔM15*	Vieira & Messing 1982
DH 5α	*supE44, ΔlacU169(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1*	Hanahan 1983
NM522	*hsdΔ5, Δ(lac, pro), supE, thi, F´[proAB$^+$, lacZΔM15, lacI^q]*	Gough & Murray 1983
AG1	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1*	Bullock *et al.* 1987
XL1-Blue^r	*supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1 lac, F´[proAB$^+$, lacI^q, lacZΔM15, Tn10(tet^r)]*	Bullock *et al.* 1987
TG1	*supE, hsdΔ5, thi, Δ(lac-proAB), F´[traD36, proAB$^+$, lacI^q, lacZΔM15]*	Gibson 1984

2A.5 Plasmids

NAME	DESCRIPTION	REFERENCE
pUC18, pUC8	General cloning vector.	Norrander *et al.* 1983
pBR322	General cloning vector.	Bolivar *et al.* 1977
pBluescriptII	General cloning vector.	Thummel *et al.* 1988
pPolyIII	General cloning vector.	Lathe *et al.* 1987
pJ3	Eucaryotic expression vector, utilising the SV40 early promoter and SV40 T polyA signal.	Morgenstern & Land 1990
pMT142	Eucaryotic expression vector, utilising the metallothionein promoter and the human growth hormone polyA signal.	R. Palmiter (personal communication)
pHPT4	Construct of full-length mouse HPRT cDNA without polyA sequences.	Konecki *et al.* 1982
pHPT5	Construct of full-length mouse HPRT cDNA with polyA sequences.	Konecki *et al.* 1982
pDWM100	Mouse HPRT-minigene functioning in ES cells.	Thompson *et al.* 1989
pDWM101	HPRT targeting vector for correcting E14TG2a.	Thompson *et al.* 1989

NAME	DESCRIPTION	REFERENCE
pλ1RI, pλ13RI, pλ17RI, pλ2RI, pλ20RI pλ23RI, pλ32RI, pλ4RI	Mouse HPRT genomic DNA subclones, isolated from Balb/c mouse.	Melton *et al.* 1984
pHygro	Hygromycin phosphotransferase gene driven by rat β-actin promoter with SV40 T antigen polyA signal.	T. Magin (personal communication)
pSPTK	Herpes simplex viral thymidine kinase gene driven by its own promoter.	Colbere-Garapin *et al.* 1979
pGKTK	Herpes simplex viral thymidine kinase gene driven by PGK promoter and polyA signal.	T. Magin (personal communication)

2A.6 Cell lines

NAME	COMMENTS	REFERENCE
BRL	Cell line isolated from Buffalo rat liver and used to produce conditioned medium for culturing ES cells.	Smith & Hooper 1987
E14	Wild-type male ES cell line isolated from 129/Ola blastocysts.	Handyside *et al.* 1989
E14TG2a	An HPRT-deficient mutant ES cell line derived from E14 by selection in 6-thioguanine.	Hooper *et al.* 1987
HeLa	A human cervical carcinoma cell line.	Puck & Marcus 1955
HeLa-TG^r	An HPRT-deficient mutant HeLa cell line derived from HeLa cells by selection in 6-thioguanine.	R.T. Johnson (personal communication)
HM-1	An HPRT-deficient mutant ES cell line isolated from blastocysts of 129/Ola HPRT-deficient mice.	Magin *et al.* 1992b
LMTK$^-$	Thymidine kinase-deficient cell line derived from L cells.	Kit *et al.* 1963
COS-7	Fibroblasts of African green monkey cells transformed with an ori$^-$ SV40.	Gluzman 1981

NAME	COMMENTS	REFERENCE
RJK0	An HPRT wild-type subclone of Chinese hamster cell line V79.	Gillin *et al.* 1972
RJK88	An HPRT-deficient Chinese hamster lung fibroblast cell line derived from RJK0.	Fuscoe *et al.* 1983

2A.7 Oligonucleotides

NAME	SEQUENCE 5′-3′	COMMENTS
PE10	GGG ACT GGC CGT CGT TTT AC	PCR oligonucleotide from base 740 to 721 on pUC8 sequence.
PE11	CAG AAG TGG TCC TGC AAC TT	PCR oligonucleotide from base 2151 to 2170 on pUC8 sequence.

2A.8 Antisera

Antiserum	Description	Source
Anti-neurofilament 68 kD	Rabbit polyclonal antibody to neurofilament 68 kD	J. Polak
Anti-neurofilament 160 kD	Monoclonal antibody to neurofilament 160 kD from mouse-mouse hybrid cells; diluted ascites	Amersham cat. RPN 1104
Anti-neurofilament 160 kD	Rabbit polyclonal antibody to neurofilament 160 kD	J. Polak
Anti-glial fibrillary acid protein	Monoclonal antibody to glial fibrillary acidic protein from mouse-mouse hybrid cells; mouse IgG1	Boehringer Mannheim cat. 814369
Anti-vimentin 1118	Monoclonal antibody to vimentin from mouse-mouse hybrid cells	T. Magin
FITC anti-rabbit IgG	The IgG fraction of donkey anti-rabbit IgG conjugated to FITC	SAPU cat. S076-201
Texas Red anti-mouse IgG	The whole antibody of sheep anti-mouse IgG conjugated to Texas Red	Amersham cat. N 2031

2B METHODS

2B.1 Bacterial culture

2B.1.1 Growth of *E. coli* bacterial cultures

Liquid culture of *E. coli* was either in Luria broth or Terrific broth by inoculating from a single colony. Cultures with volumes greater than 10 ml were grown in conical flasks with a total capacity of 5-10 fold to that of the culture volume to ensure good aeration. Cultures were shaken at 37 °C for an appropriate length of time.

2B.1.2 Storage of *E. coli* bacterial cultures

For long term storage, 900 μl of fresh overnight or 6 hr culture of bacteria grown in Luria broth supplemented with antibiotic if necessary, was mixed with 100 μl of glycerol (autoclaved) or dimethyl sulphoxide (DMSO), and stored in a sterile vial at -70 °C. Upon recovery, the surface of frozen culture was scraped with a flame sterile inoculating loop and directly streaked out onto a Luria broth agar plate, with antibiotic if required. Bacteria strain TG1 was streaked onto minimal agar to maintain the F´ plasmid. After overnight incubation at 37 °C, a single colony was picked to propagate a fresh bacterial culture.

For short-term storage (4-6 weeks), bacteria were restreaked onto agar plates and stored at 4 °C after overnight incubation.

2B.1.3 Transformation of *E. coli* with DNA

2B.1.3.1 Calcium chloride method

This uses the method of Mandel and Higa (1970) with the modifications of Dagert and Ehrlich (1979). To 50 ml of LB (supplemented with 1 ml of 1 M $MgCl_2$) was added 0.5-1 ml of an overnight culture of the *E.coli* strain to be transformed. Growth was allowed at 37 °C with vigorous shaking to 0.2 OD_{600nm}, the cells chilled on ice for 5 min, and pelleted by centrifugation at 3,000 rpm (1,250 x g; Denley BR401; Sussex, U.K.) at 4 °C for 15 min. The cell pellet was resuspended in 20 ml ice-cold transformation buffer (50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5), and exposed to this buffer for 30 min on ice. The cells were repelleted by centrifugation for 15

min at 3,000 rpm at 4 °C, and resuspended in 2ml ice-cold transformation buffer. Typically the cells were left overnight (12-16 hr) on ice (or at minimum for 2 hr) before use. Cells were transformed by adding 10 ng of DNA (typically a ligation mix), in a volume of 5-10 μl, to 100 μl of competent cells, mixing and holding on ice for 30 min. Cells were heat shocked for 5 min at 37 °C, 2ml warm LB (at 37 °C) added, and incubated for 1 hr to allow for expression of genes coded by the transformed plasmid DNA (i.e. antibiotic resistance). Cells were pelleted by centrifugation (3,000 rpm) at 4 °C and resuspended in 250 μl of LB. Fifty microlitre aliquots were then plated to dryness on LB agar, with appropriate antibiotic selection and X-gal/IPTG if needed. The X-gal/IPTG allowed recombinants to be identified; recombinant colonies appearing white while non-recombinant colonies appeared blue. This selection is only suitable for some vectors *i.e.* pUC8. Cells were incubated overnight at 37 °C for growth.

2B.1.3.2 DMSO method

This method for transformation of *E. coli* was initially described by Chung and Miller (1988). Cells were grown to the same OD_{600nm} as for the $CaCl_2$ method, kept for 10 min on ice, and sedimented from 10 ml of culture by centrifugation (3,000 rpm) for 5 min at 4 °C. The cell pellet was resuspended in 1 ml of transformation buffer, and incubated for 30 min on ice. DNA was added to 100 μl of cell suspension, and the mixture was left for 30 min on ice. Nine hundred μl of 20 mM glucose in transformation buffer was added to the transformed cells, cells were shaken for 1 hr at the appropriate temperature to allow for expression of antibiotic resistant gene and plated onto selective medium immediately after incubation.

Transformation buffer:

10% (w/v) polyethylene glycol, molecular weight 3000

5% (v/v) dimethylsulphoxide

10 mM $MgCl_2$

10 mM $MgSO_4$

in L-broth

2B.2 Nucleic acid isolation

2B.2.1 Small scale preparation of plasmid DNA

The method used for small scale preparation of plasmid DNA was a modification of that described by Ish-Horowicz and Burke (1981). Five ml of Luria broth or Terrific broth, supplemented with ampicillin to a final concentration of 100 μg/ml, was inoculated with bacteria from a single colony and incubated at 37 °C overnight with aeration. One and a half ml of the culture was pelleted by centrifugation in an Eppendorf tube and resuspended in 300 μl of Solution P1. Cells were lysed by adding 300 μl of lysis solution P2 and incubated at room temperature for 5 min. Following the lysis 300 μl of solution P3 was added and mixed by gentle inversion. Precipitated complex of chromosomal DNA, SDS and protein was sedimented by centrifugation (17,000 x g) in a microcentrifuge for 12 min. Contaminating proteins in the clarified supernatant were extracted with phenol. The upper, aqueous phase was recovered and nucleic acids were precipitated with 600 μl of isopropanol. The DNA was washed twice with 70% ethanol, dried under vacuum, and resuspended in 50 μl sterile distilled water. Plasmid "miniprep" DNA was then stored at -20 °C.

Solution P1: (kept at 4 °C)

50 mM Tris-HCl pH 8.0

10 mM EDTA

100 μg/ml RNase A

Solution P2: (Keep in air-tight bottle)

20 mM NaOH

1% (w/v) SDS

Solution P3:

2.55 M potassium acetate pH 4.8

2B.2.2 Large scale plasmid preparation

2B.2.2.1 CsCl method

A 100 ml culture of bacteria carrying the desired plasmid was incubated overnight at 37 °C with vigorous shaking in Terrific broth supplemented with 100

μg/ml of ampicillin. The cells were pelleted by centrifugation at 5,000 rpm (4080 x g; GSA rotor, Sorvall RC5B; Wilmington, U.S.A.) and resuspended in 10 ml of solution 1 . Cells were lysed on ice by adding 10 ml of lysis buffer and left on ice for 20 min. Addition of 10 ml of 5 M potassium acetate pH 5.0 precipitated complex of chromosomal DNA, SDS and proteins which was spun down at 15,000 rpm (27,000 x g; SS34 rotor, Sorvall) for 30 min at 4 °C. To the supernatant 11 ml of isopropanol were added and left at room temperature for 30 min to precipitate plasmid DNA. The DNA was pelleted by centrifugation at 10,000 rpm (12,000 x g; SS34 rotor) for 20 min at 4 °C. The pellet was washed with 70% ethanol, dried under vacuum, resuspended in 10 ml of TE buffer plus 10 μg/ml RNase and incubated at 37 °C for 30 min. 9.4 ml of plasmid DNA solution was transferred to a fresh tube to which 100 μl of 10 mg/ml ethidium bromide and 9.02 g of CsCl were added, giving a density of 1.55 g/ml. The DNA was banded by centrifugation at 38,000 rpm (95,000 x g; 50Ti rotor, Sorvall OTD50B) for 40-48 hr at 20 °C. DNA was visualised by side illumination with UV light. The lower band containing supercoiled plasmid DNA was removed by puncturing the tube with a 21-gauge needle and syringe. A second 21-gauge needle was inserted at the top of the tube to allow pressure release. The ethidium bromide was removed by extraction several times with butanol, and the CsCl was removed by dialysis against 2 litres of TE buffer for 8 to 15 hr at 4 °C. The TE buffer was changed 3 to 4 times during dialysis. The plasmid solution was then phenol extracted, and residual phenol removed by extraction with an equal volume of chloroform-isoamyl alcohol solution. Plasmid DNA was precipitated, washed and resuspended in sterile distilled water and stored at -20 °C.

Solution 1:

25 mM Tris-HCl pH 8.0

10 mM EDTA

50 mM glucose

Lysis buffer:

0.2 M NaOH

1% SDS

2B.2.2.2 Column method

The large scale preparation of plasmid by this method followed the culture conditions of the CsCl method and the plasmid isolation procedures of the small scale plasmid preparation except using 10 ml of each solution instead of 300 μl. The chromosomal DNA, SDS and protein complex was sedimented by centrifugation at 15,000 rpm (27,000 x g, SS34 rotor) for 30 min at 25 °C. The supernatant was applied onto the Qiagen-tip 500 column (Diagen; Dusseldorf, Germany) which had been equilibrated by 10 ml buffer QBT. After washing three times with 10 ml buffer QC, the DNA was eluted with 10 ml buffer QF. DNA was then precipitated with 0.5 volume of isopropanol and washed twice with 70% ethanol. Dried DNA was resuspended for PCA and CA extraction, followed by ethanol precipitation and washing. DNA samples were redissolved in sterile distilled water and stored at -20 °C.

Buffer QBT:

750 mM NaCl pH 7.0
50 mM MOPS
15% ethanol
0.15% Triton X-100

Buffer QC:

1.0 M NaCl pH 7.0
50 mM MOPS
15% ethanol

Buffer QF:

1.25 M NaCl pH 8.2
50 mM MOPS
15% ethanol

2B.2.3 Preparation of genomic DNA from mammalian cultured cells

The method of DNA extraction is basically that of Pellicer *et al.* (1978). Tissue culture cells were harvested by scraping with a plastic policeman (Costar; Cambridge,

U.S.A.), and collected in 10 ml PBS. Typically four 64 cm^2 dishes were used, yielding some 10^7-10^8 cells, depending on the cell line. The cells were pelleted by centrifugation at 1,300 rpm (304 x g; MSE, Mistral-1000; Crawley, U.K.) and the supernatant discarded. The cells were rinsed three times in PBS by pelleting and resuspended in 10 ml PBS. After the washes the cells were gently pelleted in 10 ml hypotonic solution (10 mM Tris-HCl pH 8.0, 10 mM NaCl and 3 mM $MgCl_2$). The expanded cells were pelleted by centrifugation at 1,300 rpm and resuspended in 10 ml hypotonic solution with Triton-X100 (0.2% (v/v)). This caused cell lysis, and the intact nuclei were pelleted by centrifugation at 1,300 rpm. The nuclei were resuspended in 7.6 ml of 10 mM Tris-HCl pH 8.0, 400 mM NaCl, 10 mM EDTA. Additionally, 0.4 ml 10% SDS was added to the lysate with 100 μl proteinase K (30 mg/ml). This mixture was incubated at 37°C overnight, and RNA degraded by addition of 200 μl of RNase (10 mg/ml) with further incubation at 37 °C for 1 hr. Residual proteins and RNase were then hydrolysed by addition of 100 μl Proteinase K (30 mg/ml) with further incubation at 37 °C for 1 hr. The solution was then phenol extracted twice by addition of an equal volume of PCA and mixed by gentle inversion for 15 min. The organic and aqueous phases were separated by centrifugation at 3,600 rpm (2,300 x g). The aqueous layer was then extracted with an equal volume of CA. DNA was precipitated by addition of 0.25 volumes of 5 M NaCl and 2 volumes of cold 100% ethanol and was seen to precipitate out of solution as a white globular mass. This was recovered by spooling onto a sealed pasteur pipette and rinsed by immersing into cold 70% ethanol. The DNA was air dried for 10-20 min, dissolved into 1 ml of sterile distilled water, and stored at 4 °C. This method typically yielded 1-2 mg of high molecular size DNA.

2B.2.4 Preparation of RNA from mammalian cells

RNA was prepared using modifications of methods described by Strohman *et al.* (1977) and MacDonald *et al.* (1987). Tissue culture cells were harvested by scraping with a plastic policeman, and collected into 10 ml PBS. Typically four 64 cm^2 dishes were used, yielding some 10^7-10^8 cells, depending on the cell line. The cells were pelleted by centrifugation at 1,300 rpm (304 x g), and the supernatant

discarded. The cells were washed three times in PBS by pelleting and resuspension in 10 ml PBS. After the final wash the cells were resuspended in 8 ml of a guanidine hydrochloride solution (6 M guanidine hydrochloride, 10 mM DTT, 25 mM EDTA, pH 7.0). The cell suspension was transferred to a glass homogenizer and the cells macerated with 30 strokes of the homogenizer, and the homogenate transferred to a centrifuge tube. The homogenizer was rinsed out, and residual homogenate recovered with 2 ml of the guanidine hydrochloride solution. One thirtieth volume of 3 M potassium acetate (pH 5.0) and 1/2 the volume of 100% ethanol were added to the homogenate, mixed and the solution left at -20°C for a minimum of 4 hr, to precipitate RNA. The RNA was pelleted by centrifugation at 12,000 rpm (17,300 x g; SS34 rotor) for 20 min at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml of the guanidine hydrochloride solution. The RNA was re-precipitated by addition of 1/30 volume of 3 M potassium acetate and 1/2 volumes of 100% ethanol. The mixture was incubated at -20°C for a minimum of 4 hr, and the RNA repelleted by centrifugation at 12,000rpm for 20 min at 4°C. The pellet was resuspended and repelleted again as above. The resulting compact, white pellet was suspended in 3 ml of a 0.1 M Tris-HCl (pH 8.9), 0.1 M NaCl, 1 mM EDTA and 1% SDS solution. To this was added an equal volume of PCA and the sample phenol extracted by vigorous shaking for 10 min and centrifugation at 3,600 rpm (2,300 x g) for 10 min. The aqueous layer was removed and RNA precipitated from it by addition of 1/5 volume of 3M sodium acetate (pH 5.0) and 2 volumes of 100% ethanol. This mixture was left at -20°C for 4 hr. The RNA was pelleted by centrifugation at 15,000 rpm (27,000 x g; SS34 rotor) for 25 min, and washed twice with 70% ethanol. The pellet was suspended in 0.5 ml sterile distilled water, and precipitated by addition of 1/10 volume 3M sodium acetate (pH 5.0), 2 volumes 100% ethanol, and incubation at -70°C for 15 min. RNA was pelleted by centrifugation in a microfuge, washed twice with 70% ethanol, dried and resuspended in 50-100 μl of sterile distilled water. This method yielded 50-200 μg of RNA, depending on the cell line used.

2B.2.5 Preparation of high molecular weight DNA from mouse tails

Tail DNA was prepared by a method modified from that of Laird *et al.* (1991). One to 2 cm of mouse tail was placed in an Eppendorf tube containing 1.5 ml of tail buffer (0.3 M sodium acetate, 10 mM Tris-HCl, 1 mM EDTA, 1% SDS, pH 8.0) supplemented with 15 μl of proteinase K solution (final concentration 0.2 mg/ml) and incubated overnight at 37 °C with gentle shaking by drum roller. Subsequently, each sample was split into two 0.75 ml aliquots. One aliquot was stored at -20 °C, the other was used to prepare DNA. The suspension was mixed thoroughly but gently with an equal volume of PCA. Following a 5-min spin in a microcentrifuge the aqueous (top) layer was transferred to a fresh tube and the PCA extraction was repeated. The aqueous layer was then extracted with an equal volume of CA. One-thirtieth volume of 3 M sodium acetate pH 5.0 and 1 volume of isopropanol were added to the aqueous layer to precipitate DNA, which was then pelleted by spinning in a microcentrifuge for 30 seconds. The pellet was washed twice with 70% ethanol, dried, resuspended in 0.2 ml distilled water and stored at -20 °C.

2B.2.6 Quantification of nucleic acids

2B.2.6.1 Estimation of DNA concentrations

The DNA sample was diluted in 1ml of distilled water and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometre (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometre). An OD_{260nm} value of 1.0 represents a concentration of 50 μg/ml for DNA. The ratio OD_{260nm}/OD_{280nm} provides an estimate for the purity of the nucleic acid. A value around 1.8 indicates pure preparations of DNA.

2B.2.6.2 Estimation of RNA concentrations

The quantity of RNA was measured spectrophotometrically at wavelengths of 260nm and 280nm as for the estimation of DNA concentration. An OD_{260nm} value is equivalent to about 40 μg/ml RNA with the value of 2.0 for OD_{260nm}/OD_{280nm} indicating pure preparations of RNA.

2B.3 DNA manipulation

2B.3.1 Digestion of DNA with restriction endonucleases

Most of DNA restrictions were performed using BRL enzymes and buffers. DNA was digested with approximately 5 U of restriction endonuclease per μg of DNA using buffer and temperature conditions recommended by the manufacturer. For double digests involving enzymes with different recommended buffers, the buffers were checked individually in double digests to determine which gave most efficient digestion. If the optimal digestion conditions varied for buffers, the DNA was digested with enzyme at lower salt concentration, the enzyme reaction stopped by heat denaturation or phenol extraction, and the buffer concentration altered by addition of sufficient salt solution so that the final concentration was appropriate for digestion by the next enzyme. Digestion reactions were terminated by heating for 10 min at 65 °C, by phenol extraction, or by addition of DNA sample buffer for agarose gel electrophoresis.

2B.3.2 Dephosphorylation

Bacterial alkaline phosphatase is a phosphomonoesterase that hydrolyses 3′ and 5′ phosphates from DNA and RNA. It is suitable for removing 5′ phosphates prior to end labelling and for dephosphorylating vectors prior to insert ligation. The enzyme is active at 65 °C for at least 1 hr and can be inactivated by phenol extraction. Bacterial alkaline phosphatase is sensitive to inhibition by micromolar amounts of inorganic phosphate. Calf intestinal alkaline phosphatase is a phosphomonoesterase that hydrolyses 3′and 5′ phosphates from DNA and RNA. It can be used to remove 5′phosphates before end-labelling and to dephosphorylate vectors before insert ligation. This enzyme can be inactivated by heating for 15 min at 75 °C in the presence of EGTA and 10 mM sodium salt. Phenol extraction is not required.

The terminal 5′ phosphates were removed from DNA (< 5 pmole) by treatment with 0.1 U (1 μl) of calf intestinal alkaline phosphatase (CAP) (Boehringer Mannheim) in a reaction mixture containing 5 μl 10X CAP buffer (10X: 0.5 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$; 10 mM spermidine) and distilled water in

a total volume of 49 μl. After successive incubation at 37 °C for 30 min, the reaction was terminated by inactivating the enzyme at 75 °C for 15 min in a solution containing 10 μl of 10X STE (10X: 100 mM Tris-HCl pH 8.0; 1 M NaCl; 10 mM EDTA) and 2.5μl of 20% SDS and distilled water in a total volume of 100 μl. The dephosphorylated DNA was gene-cleaned (see Section 2B.4.3) and extracted into 10 μl of water.

2B.3.3 Filling-in 3´ recessed termini of DNA

Overhanging ends, generated by restriction endonuclease were converted to blunt ends by 'filling-in' with the Klenow fragment of *E.coli* DNA polymerase I. Typically a reaction consisted of 39 μl of DNA (~ 1 μg), 5 μl of 10X Nick translation buffer (500 mM Tris-HCl pH 7.5, 100 mM MgCl,, 10 mM DTT, 500 μg/ml BSA), 5 μl 5 mM dNTP's and 1 μl Klenow (6 U/μl). The reaction mix was incubated for 1 hr at room temperature and the reaction stopped by phenol extraction.

2B.3.4 Ligation

The vector and insert DNA were cut to completion with appropriate restriction endonucleases. After restriction and removing the restriction endonucleases, DNA was ethanol precipitated. Typically, between 100-200 ng insert DNA were ligated in a reaction with vector to insert DNA concentration at a 1:3 molar ratio. Ligations were carried out in 10 μl reaction volume containing 50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 5 % polyethylene glycol molecular weight 8000 and incubated overnight at 15 °C (for cohesive ends), or room temperature (for blunt ends). One unit of T4 DNA ligase was used for blunt end ligations, and 0.1 units for cohesive end ligations. Ligation products were then transformed into *E. coli.*

2B.4 Electrophoresis of nucleic acids

2B.4.1 Electrophoresis of DNA in agarose gels

DNA was separated in 0.7-1.2% (w/v) BRL electrophoresis grade agarose with

0.5 μg/ml ethidium bromide in 1X TBE buffer (for Southern blotting and routine diagnosis gel) or 1X TAE buffer (for band-recovery gels). Prior to loading, DNA samples were mixed with 1/10 volume of sample buffer (20% glycerol, 100 mM EDTA, 0.1% bromophenol blue). Electrophoresis was carried out horizontally across a potential difference of 1-10 v/cm. Bacteriophage λDNA cut with HindIII and ϕχ174 RF DNA cut with HaeIII were used as size markers. DNA was visualised by UV illumination and photographed.

2B.4.2 Electrophoresis of RNA in agarose gels

Denaturing agarose gels were used for electrophoresis of RNA samples. Gels were made up with 1.4% (w/v) agarose in 1X MOPS buffer and 0.66 M formaldehyde with 0.5 μg/ml ethidium bromide. To 20 μl of total RNA (1 μg/μl) was added an equal volume of formamide sample buffer (47 mM MOPS, 12 mM sodium acetate pH 7.0, 2.3X MOPS, 50% deionized formamide, 11% formaldehyde), and 1/4 volume of loading buffer (0.2 M EDTA pH 7.0, 30% (w/v) Ficoll type 400, 0.1% (w/v) bromophenol blue). Samples were heated for 5 min at 60 °C and snap chilled on ice for a few min. Samples then were loaded onto the gel and run in 1X MOPS buffer at 100 V for 3 to 4 hr.

2B.4.3 Recovery of DNA from agarose gels

DNA was electrophoresed through 0.8% regular melting point agarose in 1X TAE, 0.5 μg/ml ethidium bromide. The desired fragment was visualised by UV illumination, cut out, and extracted from the agarose using Geneclean (Bio101; La Jolla, U.S.A.). The agarose was weighed and 2-3X volumes of 6 M NaI added. The agarose was dissolved by heating to 55 °C for 5 min with occasional mixing, and then cooled on ice for 5 min. Five μl of "glassmilk" (a silica matrix suspended in water) was added, and left on ice for 5 min with occasional mixing to allow DNA to bind to the silica matrix. The "glassmilk" was pelleted by centrifugation in a microfuge, the supernatant discarded and the pellet washed three times with 500 μl of NEW wash (NaCl/ethanol/water mix). After a final spin all the NEW wash was discarded completely and the DNA eluted from the "glassmilk" in 5-10 μl of TE buffer at 55 °C.

The mixture was spun in a microfuge, and the supernatant, containing the DNA, transferred to a fresh Eppendorf tube and stored at -20 °C. Fragments less than 300 bp long were electrophoresed through 2% ultrapure agarose (Mermaid Biogel; Bio101) in 1X TAE, 0.5 μg/ml ethidium bromide. Agarose containing the DNA fragment was cut out, weighed, and mixed with 3X volumes of high salt binding solution (concentrated sodium perchlorate) in an Eppendorf tube. Eight μl of 'glassfog' (a silica based matrix in water) was added, the agarose melted and DNA bound to the 'glassfog' by incubation at 55 °C for 5 min. Adsorption was allowed to continue at room temperature for 5 min, with occasional mixing to keep the 'glassfog' in suspension. The 'glassfog' was centrifuged, the supernatant discarded, and the pellet washed three times with 300 μl of ethanol wash. After the final wash the tube was spun again to ensure removal of all residual ethanol. The pellet of 'glassfog' was resuspended in 10 μl of distilled water, and the DNA eluted by incubation at room temperature for 5 min. The 'glassfog' was centrifuged and the supernatant containing the DNA transferred to a fresh Eppendorf tube. Recovery of DNA using Geneclean and Mermaid kits was usually around 80%.

2B.5 Nucleic acid hybridisation

2B.5.1 Transfer of DNA from agarose gels to membranes

The transfer of DNA from agarose gel onto filters for the detection of specific sequences among DNA fragments was initially developed by Southern (1975) and modified by Smith and Summers (1980). Genomic DNA was digested with the appropriate restriction enzyme in a total volume of 50 μl for more than 6 hr at 37 °C and separated according to size on a 0.8% agarose gel (w/v) in 1X TBE and 0.5 μg/ml ethidium bromide. Routinely genomic DNA was electrophoresed for 12-15 hr at 1 v/cm then photographed. The gel was then soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) with gentle agitation for 30 min. The DNA was transferred to Genescreen Plus nylon membrane (Du Pont; Stevenage, U.K.) by capillary action using denaturation buffer as the transfer medium (Reed and Mann, 1985). Transfer was allowed to continue for 12-48 hr after which the membrane was neutralised for 30 min in 3 M NaCl, 0.5 M Tris-HCl pH 7.0 and air dried before use.

Prehybridisation of the membrane was done in 30-50 ml of 6X SSC, 1% SDS, 10% dextran sulphate and 100 μg/ml of denatured herring sperm DNA for 2 hr at 65 °C.

2B.5.2 Transfer of RNA from agarose gels to membranes

After electrophoresis, the gel was photographed (with short wave UV illumination to visualise the EtBr chelated RNA), and then soaked for two 20-min periods in 10X SSC, at room temperature with shaking. The RNA was transferred to a nylon membrane (GeneScreen Plus, Dupont), using capillary action, a wick made of wet blotting papers was placed on a platform with both ends of the blotting paper immersed in 10X SSC in a reservoir underneath the platform. The gel was laid on the top of the wick, and a sheet of membrane cut to the same size as the gel was placed on the top of the gel. The edges were sealed with plastic films. Three sheets of blotting paper soaked in 10X SSC were laid on the top of the membrane, and another two sheets of dry blotting paper were placed on the top of the wet blotting papers followed by a stack of paper towels. A sheet of glass plate was placed on the top of the paper towels, and a weight was laid on the top. When transfer was complete, the filter was marked with a pencil for the position of the sample wells. The membrane was retrieved and rinsed with 2X SSC, and the formaldehyde reaction reversed by baking at 80 °C for 2hr.

The prehybridisation and hybridisation were as for Southern blot analysis, except at 60 °C. Similarly the wash procedure was duplicated, except the temperature was lowered to 60 °C. The filter was sealed wet and autoradiographed.

2B.5.3 Transfer of DNA from *E. coli* colonies to membranes

This procedure used the denaturing/fixing procedure of Buluwela *et al.* (1989) and the hybridisation conditions of Church and Gilbert (1984). Colonies harbouring putative plasmids of interest were replica plated by spotting onto two LB plates (which had appropriate selection added), and the colonies propagated overnight at 37 °C. Colonies were lifted, from one plate onto Hybond-N (Amersham International) nylon filters by placing dry filters onto plates to contact colonies, and peeling off the

filter. The filters were laid, colonies face-up, onto Whatman (Maidstone, U.K.) No. 1 paper soaked in 2X SSC with 5% SDS (w/v) for 5 min. The filter was then transferred to a microwave oven and irradiated for 2.5 min at full power setting (650 watts), lysing the cells, and immobilising DNA to the filters. The filter was prehybridised in 7% SDS (w/v) in 0.5 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, at 65 °C for 1 hr and the radioactive probe added to this prehybridisation mix. The probe was left to hybridise at 65 °C for 3 hr, the filter recovered and washed three times in 0.1X SSC at 65 °C for 10 min. The filters were sealed wet in plastic and autoradiographed.

2B.5.4 Labelling DNA by random priming with hexadeoxyribonucleotide primers

DNA labelled to high activities was obtained using the randomly primed DNA labelling method (Feinberg & Vogelstein, 1983). This method enables the labelling of DNA available only in minimal amounts. It is based on the hybridisation of a mixture of hexanucleotides to the DNA to be labelled. Many sequence combinations are represented in the hexanucleotide primer mixture, which leads to binding of primer to template in a statistical manner. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme during which radiolabelled dNTP is incorporated into the newly synthesized DNA strand.

A reaction buffer, OLB, containing nucleotides and random primers is required for this method. It is made by mixing 50 μl Solution A (1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$, 25 mM β-mercaptoethanol, 0.5 mM each of dGTP, dTTP and dATP), 125 μl Solution B (2M HEPES buffer adjusted to pH 6.6 with NaOH) and 75 μl Solution C (random hexanucleotides OD_{260nm} = 90 in TE).

The DNA (100 ng and water to 32 μl) was denatured by boiling for 5 min, spun and chilled on ice briefly. After that, 12 μl of OLB buffer, 2 μl of 10 mg/ml BSA, 5 μl of [α-^{32}P] dCTP and 2 units of Klenow large fragment DNA polymerase were added to the denatured DNA at room temperature. The incubation was continued at room temperature overnight. Unincorporated nucleotides were separated from labelled DNA by chromatography on a Sephadex G-50 column.

2B.5.5 Separation of unincorporated nucleotides from labelled DNA

A Sephadex G-50 (Pharmacia) column was used to remove unincorporated [α-^{32}P] dNTP from labelled DNA fragment (Sambrook *et al.*, 1989). The column was prepared by packing a 1ml blue-tip with Sephadex G-50 in sterile distilled water. The Sephadex was washed with 10 μl of the sonicated herring sperm DNA (10 mg/ml) in water with centrifugation (800 rpm/4min). The labelling mix was applied to the column and eluted into an eppendorf tube by centrifugation. The efficiencies of incorporation and recovery were monitored by TCA precipitation assay. The percentage incorporation of radiolabelled nucleotides into nucleic acids was estimated by comparing acid-precipitable Cerenkov counts to total Cerenkov counts in a given reaction. A small aliquot of the reaction was diluted in distilled water, and 1 μl of the dilution was spotted onto a glass fibre disc (GF/C; Whatman). To 49 μl of 2.5 mg/ml tRNA was added 1 μl of the same dilution, and then 12.5 μl 50% (w/v) TCA was added. The mixture was incubated on ice for 15 min. Precipitated nucleic acid was collected by vacuum filtration onto a glass fibre disc, and washed with 10 ml ice-cold 10% (w/v) TCA and 10 ml ice-cold ethanol. The radioactivity of the two glass fibre discs was measured in a scintillation counter, and the two values were compared to obtain the percentage incorporation. To the eluted probe was added 450 μl of sonicated herring sperm DNA and it was boiled for 5 min to denature the DNA before use in hybridisation.

2B.5.6 Hybridisation

By adjusting the stringency of hybridisation, it is possible to distinguish between closely- and distantly-related members of a sequence family. In practise, to distinguish between the distantly-related members of a family of sequences, hybridisation should take place under permissive conditions followed by washing under progressively more stringent conditions. To identify closely-related members, a stringent hybridisation followed by a stringent wash is better. The stringency of hybridisation depends on a number of factors, the most significant ones are listed below.

Definitions:

*T*m (melting temperature): the temperature at which the strands of a DNA duplex or an RNA/DNA hybrid are half dissociated or denatured. *T*m is dependent on ionic strength, base composition and denaturing agents.

$$T\text{m} = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.72(\%\ \text{formamide})$$

where M is the molarity of the monovalent cation and (%G+C) is the percentage of guanine and cytosine residues in the DNA.

Temperature: The temperature of reaction affects the rate of hybridisation which increases to reach a maximum at 20-25 °C below *T*m. Self-hybridisation is favoured at higher temperature. At low temperature, a high rate of cross-hybridisation is attained. So, ideally, hybridisation should be carried out at a temperature that is 20-25 °C below *T*m. In practice, for well-matched hybrids, the hybridisation reaction is usually carried out at 68 °C in aqueous solution and at 42 °C for solutions containing 50% formamide. For poorly-matched hybrids, incubation is generally at 35-42 °C in formamide containing solutions.

Formamide: Formamide can be used to alter the stringency of the reaction conditions. Formamide destabilises double-stranded nucleic acid. Thus, the temperature can be decreased whilst maintaining the stringency of the nucleic acid interaction. By including 30-50% formamide in the hybridisation solution, the temperature can be reduced to 30-42 °C.

Ionic strength: High salt concentrations stabilise mismatched duplexes, so to detect cross-hybridising species, the salt concentration of hybridisation and washing solutions must be kept fairly high.

Dextran sulphate: Addition of an inert polymer such as dextran sulphate increases the rate of hybridisation. The effect is attributed to the exclusion of the DNA from the volume occupied by the polymer, effectively increasing the concentration of the DNA. This favours the formation of concatenates, *i.e.* extensive networks of reassociated probe which by virtue of single-stranded regions, hybridise to filter-bound nucleic acid and so lead to an increase in hybridisation signal.

Hybridisation with Homologous DNA Probes: Hybridisation with homologous DNA fragments was performed under stringent conditions in 6X SSC, 1% SDS, 10% dextran and 100 μg/ml sonicated herring sperm DNA at 65 °C overnight with a 2 hr pre-hybridisation under the same conditions. Non-specifically bound nucleotides were removed by washing the membrane stringently, twice for 5 min in 2X SSC at room temperature followed by twice for 30 min in 2X SSC, 1% SDS at 65 °C and twice for 30 min in 0.1% SSC at room temperature. Filters were then exposed for autoradiography at -70 °C.

2B.5.7 Autoradiography

Autoradiography was used to visualise and quantitate, on film, radioactive molecules hybridised to membrane. After washing the membrane was sealed in a plastic bag to avoid drying. Films were hypersensitised by exposure to a flash of light provided by a photographic flash unit as recommended by Laskey and Mills (1975). Autoradiography was performed using X-OMAT AR X-ray film (Kodak; Rochester, U.S.A.) in a cassette with intensifying screens (Cronex Lightning Plus; Du Pont). The cassettes were stored at -70 °C during exposure to slow the reversal of activated bromide crystals to their stable form as this results in a sharper signal.

2B.5.8 Removal of probes and re-use of blots

The method used to remove radio-labelled DNA probe hybridised to single stranded nucleic acid immobilised on a nylon membrane was that described in the protocols supplied with Gene Screen *Plus* Membranes. For Southern blot, this was achieved by boiling in 0.1X SSC with 1% SDS for 10 min with gentle shaking. The solution was decanted from the membrane, and the boiling procedure was repeated 3 times. The blots were autoradiographed to confirm that dehybridisation was complete. For Northern blot, 0.1X SSC with 0.01% SDS was used as boiling solution. The membrane was then incubated in pre-hybridisation solution and hybridised as usual.

2B.6 Amplification of DNA using the polymerase chain reaction

PCR analysis to identify DNA injected into tissues was modified from McMahon and Bradley (1990). All reactions were carried out using a Dri-Block cycler (Techne PHC-2; Cambridge, U.K.). Tissues were lysed at 55 °C in 100 μl of lysis buffer for 12-16 hr. Oligonucleotide primers used to identify the injected mini-gene were designed such that they share 20 nucleotides homology to pUC8 sequence. To lysed tissue extracts were added 250 ng of each primer, 2 μl of 5 mM dNTP and 2.5 units of Taq polymerase (Boehringer Mannheim) and the volume was made up to 50 μl with water followed by submersion under 1 drop of mineral oil (Sigma). Conditions for PCR were those recommended by the suppliers (Boehringer Mannheim) and cycle conditions were 1 min at 93 °C, 1 min at 56 °C and 2 min at 72 °C for 35 cycles.

Lysis buffer:

50 mM KCl

1.5 mM $MgCl_2$

1 mM Tris pH 8.5

0.01% gelatin

0.45% NP40

0.45% Tween 20

100 μg/ml proteinase K

2B.7 Cell culture

2B.7.1 Culture cells

Mammalian cells were cultured in the Glasgow's modified Eagles' minimum essential medium supplemented with 5% faetal calf serum and 5% newborn calf serum. Embryonic stem cells were cultured in the presence of DIA/LIF (Smith *et al.*, 1988; Williams *et al.*, 1988) or 60% BRL conditioned medium to prevent their differentiation. BRL conditioned medium was prepared as follows: BRL cells were grown to confluence in 75 cm^2 tissue culture flasks. The medium was discarded and 15 ml of fresh medium was added. After 2 days this was collected and replaced with a further 15 ml of fresh medium. In total 45 ml of conditioned medium was collected

from each flask. The conditioned medium was filtered through a 0.22 μm membrane (Millipore) and stored at -20 °C. To Glasgow's medium supplemented with non-essential amino acids, sodium pyruvate, glutamine, 5% of newborn calf serum and 5% fetal calf serum, was added 60% BRL-conditioned medium and 0.1 mM β-mercaptoethanol to serve as the culture medium for ES cell culture (Smith and Hooper, 1987). Recombinant LIF was prepared as follows: Cos-7 cells were cultured to near confluence and transfected with pC106-R by electroporation. The conditioned media were collected 3 days post-transfection and replaced with fresh medium. After a further 2 day-incubation, media were collected and pooled with the previous collection, aliquoted, and store at -20 °C (Smith, 1991). After titration, the recombinant LIF was added into complete Glasgow's medium supplemented with 0.1 mM β-mercaptoethanol to culture ES cells. The recombinant LIF was kindly provided by Angela Pow.

2B.7.2 Splenocyte isolation

Mice were sacrificed by cervical dislocation and their spleens were removed aseptically and placed in a 100-mm petri dish containing 10 ml PBS. After trimming off any contaminating tissue, spleen was transferred to another petri dish containing 10 ml of culture medium and macerated with the bottom of syringe plunger. To ensure a single cell suspension formed, the cell suspension was passed through a 23-gauge needle twice. Cells were then washed three times and adjusted to a density to 10^6 cells/ml and plated into 24-well microtitre plates (1 ml/well). After 3 days incubation with or without 4 μg/ml concanavalin A (ConA) stimulation, splenocytes were pulsed with radioactive hypoxanthine and thymidine in an incorporation assay.

2B.7.3 Brain cell isolation

Mouse brains were aseptically removed and transferred immediately to a petri dish containing 10 ml cold PBS after sacrifice by cervical dislocation. After trimming off any contaminating tissue and blood, the brain was transferred to a new petri dish containing 10 ml cold PBS to wash off the residual blood cells and contaminants. Brain was then transferred to another new petri dish with 10 ml of cold medium and

mechanically disrupted by chopping. Brain tissue suspension was obtained by passing through a 19-gauge needle several times and washed with medium by centrifugation at 200 x g 3 min for 4 times. The cells were then seeded on either 13-mm glass coverslips or 100-mm dishes that had been coated with 100 μg/ml poly-L-lysine. Cultures were maintained in Glasgow's modified minimum essential medium containing 10% faetal calf serum.

2B.7.4 Incorporation assays

2B.7.4.1 Radioactive purine and pyrimidine incorporations in ES cells

Assays were performed in gelatin-coated 24-well microtitre plates. To assess the short term effect of purine analogues, 5 x 10^4 cells were plated/well and 24 hr later the purine analogue, at a final concentration of 10^{-3} M, was added together with 1 μCi of [^{3}H]-adenine or -thymidine. Cells were harvested after 5 hr-incubation and the radioactivity incorporated into cellular nucleic acid (trichloroacetic acid - precipitable material) was determined by scintillation counting. To assess the cytostatic effect of 9-ethyladenine, 2 x 10^4 cells were plated/well and 10^{-3} M 9-ethyladenine was added 24 hr later. Cells were incubated for 96 hr in the presence of the analogue and then pulsed for 5 hr with [^{3}H]-thymidine as described above to give a measurement of cell growth.

2B.7.4.2 [^{3}H]-Adenine incorporation in mouse brain tissue cultures after administration of 9-ethyladenine

Short-term mouse brain tissue cultures were established by sacrificing animals, removing the brain and mechanically disrupting by chopping and passing through a 19-gauge needle. The resulting brain tissue suspension was then distributed into the wells of a 24-well microtitre plate with culture medium. For determination of hypoxanthine and adenine incorporation, 1 μCi of [^{3}H]-hypoxanthine and [^{14}C]-adenine was added to each well for 20 hr, the incorporation of radioactivity into cellular nucleic acid was determined by scintillation counting and the ratio dpm [^{3}H]-hypoxanthine to dpm [^{14}C]-adenine was calculated. To determine the effect of 9-ethyladenine on APRT activity in mouse brain, age-matched (6-8 weeks old) wild-type

and HPRT-deficient strain 129 male mice were used. Three wild-type and 3 HPRT-deficient mice were injected intraperitoneally on alternate days with 9-ethyladenine. Two wild-type and 3 HPRT-deficient animals were injected in parallel with saline. After the fourth injection brain tissue cultures were established from each animal. One μCi of [^{3}H]-adenine and [^{14}C]-methionine was added immediately to each well and left for 20 hr. The incorporation of radioactivity into cellular nucleic acid and protein was determined and the ratio of dpm [^{3}H]-adenine to dpm [^{14}C]-methionine was calculated.

2B.7.4.3 Scintillation counting

Liquid scintillation counting was used to measure the incorporation activity throughout the study. Quenching results in a decreased number of registered light pulses or in a decrease in their intensities due to the presence of certain substances in the scintillator or in the sample. The quenching leads to inefficiency of both transformations of collisions of β-particles with solvent molecules into light pulses and registration of the latter. Thus, the intensity of all the light pulses and the total number detected decrease, the latter being due to pulses which were weak even without quenching. The amplitude of the energy spectrum of the light pulses decreases and spectrum shift to the lower intensities. Quenching affects the counting efficiency, especially when dual isotope counting is required. Generally, the counts of the lower energy-isotope is artificially higher while that of the higher energy-isotope is lower. This artefact gives a false ratio of the 2 radioisotopes. To correct this, a quench curve was made by simulation of the experimental condition. Two sets of 6 glass fibre discs with different dilutions of tissue-TCA precipitate were added with 0.1 μCi [^{3}H]-hypoxanthine or 0.05 μCi [^{14}C]-adenine. These 2 sets of discs were transferred into counting vials after they were dry and organic scintillation solution was added. The vials were used as standards to correct the counting efficiency of the liquid scintillation counter (Tri-Carb 2000CA; Packard; U.S.A.).

2B.7.5 Calcium phosphate/DNA precipitation

Cells were trypsinised, washed once, counted and plated out on 70-mm diameter dishes at a density of 1.2 X 10^5 cells/ 2ml / dish on the day before transfection. Medium was changed the next morning prior to transfection. To 10-20 μg of DNA (CsCl prepared) was added 1 ml HBS (10X HBS: 8% (w/v) NaCl, 0.37% (w/v) KCl, 0.126% (w/v) Na_2HPO_4·dihydrate, 1% (w/v) D-glucose, 5% (w/v) HEPES, pH to 7.2 with NaOH), and 62 μl 2 M $CaCl_2$. The $CaCl_2$ was added slowly, dropwise, and the solution left to precipitate for 45 min at room temperature. The medium was aspirated off the cells, the DNA precipitate added to the cells, and left at room temperature for 20 min, with frequent agitation of the mix. Ten ml of cell culture medium was added to the dishes, and the cells incubated overnight at 37 °C, 5% CO_2 in a humidified incubator, after which time the medium was aspirated and replaced with medium containing appropriate selection. For selecting HSV-TK transformed LMtk$^-$ cells this was typically HAT selection.

2B.7.6 Electroporation

Construct DNA was linearised by the appropriate restriction enzyme, prior to electroporation. The DNA was extracted by phenol twice and precipitated by ethanol, finally resuspended in water for electroporation. ES cells were electroporated using Gene Pulse (BioRad; Richmond, U.S.A.). Cells were suspended in HEPES buffered PBS with linearised DNA. For targeting experiments, 0.8 ml cells were pulsed with capacitance of 3 μF and voltage of 800 V at path length 0.4 cm (Thompson *et al.*, 1989). Cells were incubated at room temperature 10 min before plating onto 100-mm culture dishes.

2B.8 Immunohistochemistry

Cells grown on coverslips were fixed in methanol for 5 min followed by acetone for 30 seconds at -20 °C. The air-dried coverslips could be stored at -20 °C or -70 °C until staining. Appropriately diluted antibodies in PBS were applied onto the prewetted samples and incubated in a humidity chamber overnight. The cells were washed with PBS three times and then stained for 30 min with fluorescien-labelled

anti-rabbit or Texas-Red-labelled anti-mouse IgG which were both diluted 50-fold with PBS. Coverslips were washed several times with PBS prior to dipping in water and ethanol and then left for air dring. Air-dried coverslips were mounted on glass slides using Citifluor embedding medium (Citifluor Ltd.; London, U.K.) or Mowiol mounting solution. Fluorescence was viewed on a Zeiss microscope (Axioskop, Germany) equipped with phase-contrast and epifluorescence optics. Ilford 400 Delta (400 ASA) film (Mobberley, U.K.) was used for all photomicrography.

2B.9 Animal experiments

2B.9.1 Behaviour observation

Strain 129 inbred male mice, 6-8 weeks old, were caged individually and maintained on a 12 hr light/dark cycle. Mice received 2.5 x 10^{-6} moles of the purine analogues (0.25 ml of a 10^{-2}M solution dissolved in sterile normal saline) by intraperitoneal injection 3 times a week. To make video recordings mice were transferred to a clear cage with bedding but lacking food and water for 10 min before treatment. Recording was initiated 10 min after the animals had been returned to the cage following injection and the frequency of self injury behaviour was determined over a 20 min period. Recordings were made on two mice in each treatment group and the behaviour of each animal was determined on three separate occasions. All animals were monitored weekly for the appearance of physical injury caused by overgrooming.

2B.9.2 Intracerebral transplantation

The microsurgical procedure used to transplant cells into a host was similar to that described by Gage *et al.* (1990). Cells from a near confluent culture were trypsinised and washed twice with PBS before being resuspended in 25 mM glucose-PBS to a desired density. Adult HPRT-deficient mice were anaesthetised with an intraperitoneal injection of Hypnorm/Hypnovel solution (10 μl/g body weight). ES cells were injected into the cerebral cortex using a Hamilton syringe with a bevelled 25-gauge needle. Injection (10 μl) was done over a one min interval, and the needle was kept in place for another 30 sec prior to twisted removal. Surgery was tolerated well by most animals, none of the mice died during anaesthesia.

Hypnorm/Hypnovel solution:

Hypnorm (Janssen; Oxford, U.K.)

fentanyl citrate (0.315 mg/ml)

fluanisone (10 mg/ml)

diluted with 3-fold normal saline before use

Hypnovel (Roche)

Solution was made by mixing equal volume of diluted Hypnorm and Hypnovel. Therefore, the final dose in animals was 0.5 μg of fentamyl citrate, 17 μg of fluanisone and 8 μg of Hypnovel per gram body weight.

2B.10 Statistical analysis

Differences in behaviour alteration were analysed according to the Mann-Whitney *U*-test (rank sum test) by using BMDP statistic package (BMDP Statistical Software Inc.; Los Angeles, U.S.A.; Dixon, 1985). Differences in incorporation assay were compared by Student's *t*-test.

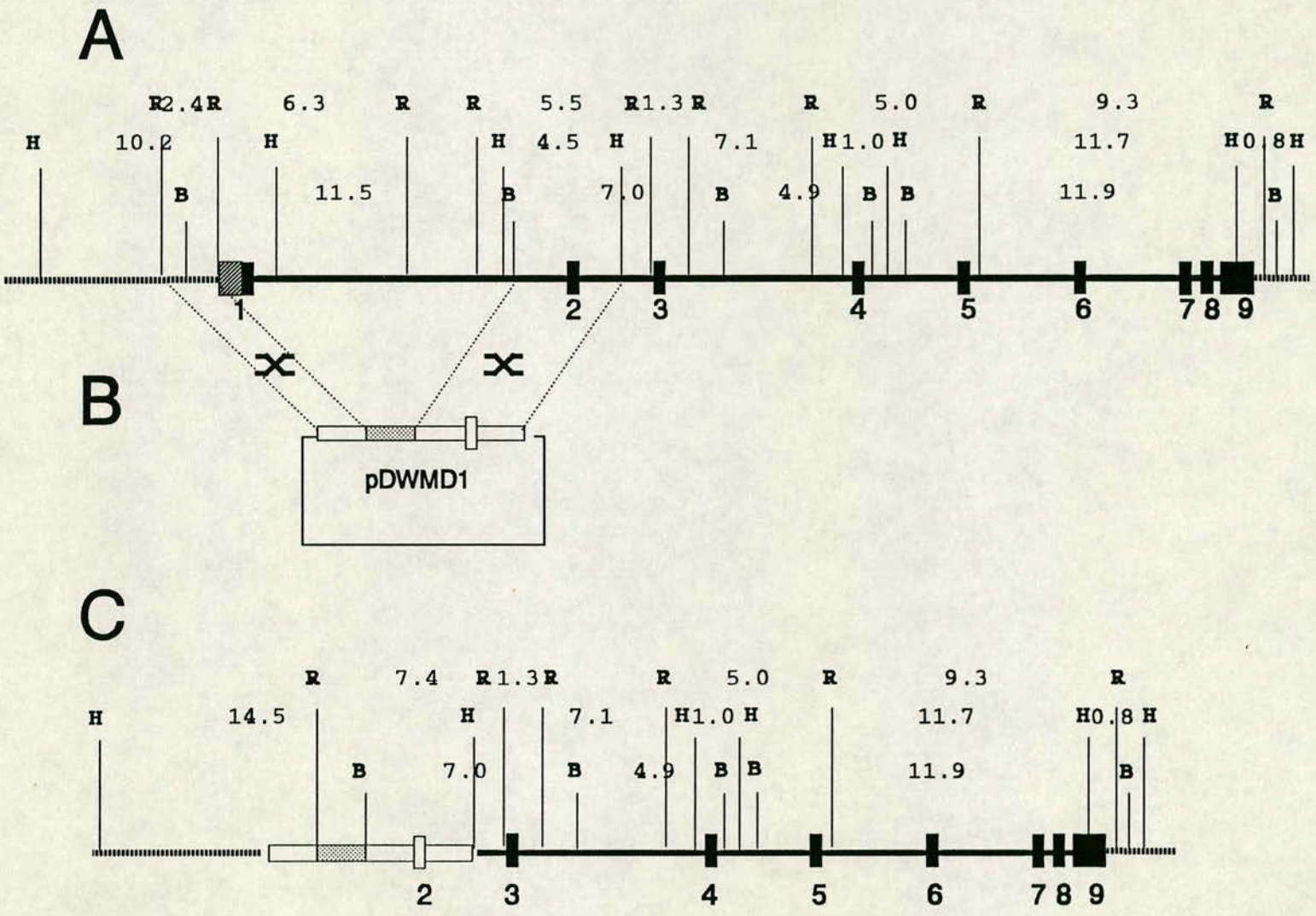
CHAPTER 3. GENERATING DELETIONS USING GENE TARGETING

Deletion mutations play important roles in heritable human diseases, in tumourigenesis, tumour progression, and in genome evolution. Although there is no large deletion of the hypoxanthine phosphoribosyl transferase gene in clinical cases reported so far, small deletions are common in both gouty arthritis and Lesch-Nyhan syndrome patients. Four of 16 patients with Lesch-Nyhan syndrome or severe gouty arthritis carried deletions in the HPRT locus as determined by PCR (Davidson *et al.*, 1989b; Tarle *et al.*, 1991). A Japanese patient with a deletion of 51 nucleotides between nucleotide 747 and 797 of the HPRT gene was also identified (Igarashi *et al.*, 1989). Molecular analysis of the only female Lesch-Nyhan patient has shown a deletion occurred in the maternal HPRT locus while the paternal X chromosome was inactivated (Ogasawara *et al.*, 1989). Characterisation of gene mutations occurring during fetal development was reported by analysing mutant 6-thioguanine resistant T-lymphocytes isolated from placental cord blood samples of 13 normal male newborns. Most of the mutants with structural alterations, 85% of all resistant mutants, consisted of a deletion of exons 2 and 3 (McGinniss *et al.*, 1989). Furthermore, tumour cells and irradiated cells are more susceptible to spontaneous deletion of HPRT (Kaden *et al.*, 1989). Immortalized fibroblasts from a male patient with xeroderma pigmentosum from complementation group D were sensitive to deletion at the HPRT locus induced by treatment with ethyl methane sulphonate or bleomycin (Wood and Moses, 1989). The creation of deletions by gene targeting therefore not only served to establish the methodology for construction of animal models for diseases with gene deletion but also to produce animal models for studying the additional effects of deletion in the HPRT locus apart from Lesch-Nyhan syndrome.

3.1 Vector construction for gene targeting

Gene targeting relies on the recombination between exogenous and endogenous DNA. Homologous recombination can result in a replacement of the endogenous sequence by introduced DNA or in insertion of exogenous DNA into the homologous chromosomal site leading to duplication. The strategy for deletion, diagrammed in Figure. 3.1, was to provide a selectable gene sandwiched by DNA sequences from

Figure 3.1 The strategy for gene deletion in the mouse HPRT locus by homologous recombination. The structures of (A) the wild-type HPRT gene, (B) the deleting vector (pDWMD1), and (C) predicted targeting deletion mutants are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII; B, BamHI. The sizes (in kb) of all EcoRI, HindIII and BamHI restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched box, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette.


A
R 2.4 R 6.3 R R 5.5 R 1.3 R R 5.0 R 9.3 R
H 10.2 H H 4.5 H 7.1 H 1.0 H 11.7 H 0.8 H
B 11.5 B 7.0 B 4.9 B B 11.9 B
1 2 3 4 5 6 7 8 9
B
pDWMD1
C
R 7.4 R 1.3 R R 5.0 R 9.3 R
H 14.5 H 7.1 H 1.0 H 11.7 H 0.8 H
B 7.0 B 4.9 B B 11.9 B
2 3 4 5 6 7 8 9

the flanking regions of the gene to be deleted. As with replacement vectors, double homologous recombination in the flanking sequences would result in the replacement and the deletion of the gene by the selectable marker. The selectable gene used in vectors to delete the HPRT gene was the murine metallothionein promoter driven bacterial neomycin phosphorylase gene (*neo*) with a human growth factor polyadenylation sequence. Three vectors were made to delete different regions of the mouse HPRT gene: pDWMD1 for deleting 10.1 kb, pDWMD5 for 27.2 kb, and pDWMD4 for the entire HPRT 38.6 kb coding region. A 4.3-kb BamHI/HindIII fragment of pDWM101 containing exon 2 (Thompson *et al.*, 1989), a 3.9-kb BglII fragment of pλ17RI9.0 containing exon 5, and a 4.4-kb BamHI fragment of pλ2RI9.5 containing the 3′ flanking region of the HPRT gene (Melton *et al.*, 1984), were each cloned into the 3′ end of a 2.4-kb mouse metallothionein-I gene promoter driven *neo* cassette (Selfridge *et al.*, 1992). The same 2.4-kb fragment of pλ23RI2.4 containing the 5′ flanking region of the HPRT gene (Melton *et al.*, 1984) was inserted into the EcoRI site at the 5′ terminus of the *neo* cassette (Figure 3.1, B; Figure 3.4, B). Hence, the three deletion targeting vectors all contain the same 2.4 kb fragment of 5′ homologous region and a similar length of 3′ homologous region. The design of the deletion vectors with similar length of homology was made to avoid the possibility that the length of homology may effect targeting frequency (Hasty *et al.*, 1991b).

3.2 Production of HPRT deficient clones

Deletion vectors were linearised at a unique HindIII site in the pUC8 polylinker before being introduced into wild-type ES cells which had been incubated in HAT medium. This pre-selection avoids spontaneous mutants being picked up in the 6-thioguanine selection for targeting induced deletions. In the first experiment, 4 X 10^7 E14 ES cells were electroporated in the presence of 200 μg HindIII-digested pDWMD1 DNA. After 24 hr, the treated cells were selected with G418 for 7 days to generate clonal colonies and thus avert toxicity to the targeted cells by intercellular cooperation from surrounding wild-type cells when selection against HPRT was eventually applied. This period also allowed for degradation of active HPRT protein in targeted clones. About 300 G418-resistant colonies were obtained from each dish

plated with 5 X 10^6 cells. On day 7 after electroporation, cells were treated with G418 and 6-thioguanine to select HPRT deficient clones, resulting in eighteen resistant clones from 6 dishes. The frequency of HPRT inactivated clones per cell electroporated was 6 X 10^{-7} while the targeting frequency was 0.92% of G418 resistant clones (Table 3.1). One of these clones, DWMD1-16, was assayed for [^{3}H]-hypoxanthine incorporation. As indicated in Table 3.2, the incorporation of [^{3}H]-hypoxanthine into nucleic acid in DWMD1-16 during a 5-hr incubation was 0.2 % of that of its parental cell line, E14. The incorporation ratio to wild-type cells was similar to that of the spontaneous HPRT-deficient cell line E14-TG2a which lacks both the HPRT promoter and exons 1 and 2 (Thompson, 1989). The results of the incorporation assay suggest that the DWMD1-16 clone, like E14-TG2a is functionally deficient in HPRT.

Electroporations have been done in an attempt to delete different sized fragments from the HPRT locus and the results are summarised in Table 3.1. These results show the generation of HPRT targeted clones was highest with the deletion vector pDWMD1. The frequency of doubly resistant clones against both G418 and 6-thioguanine is lowest using pDWMD4 as the targeting deletion vector. In one case there were no resistant clones following G418 and 6-thioguanine selection from an electroporation that generated a typical colony number under single selection for G418 only. The number of colonies resistant to both G418 and 6-thioguanine generated with pDWMD5 in E14 cells was about 1 colony on average, *i.e.* 17 colonies on 16 dishes, each from 5 X 10^6 electroporated cells. The targeting index is highest using pDWMD1 as the targeting vector and lowest from pDWMD4.

3.3 Southern hybridisation analysis of HPRT deficient clones

3.3.1 Demonstration of targeting deletion using vector pDWMD1

The predicted outcome of deletion from homologous recombination between the vector pDWMD1 and the wild-type E14 target locus, as well as the restriction enzyme sites of the wild-type HPRT gene are shown in Figure 3.1 . The deleted gene should lose a 10.1-kb fragment which includes the promoter region and exon 1, and

Table 3.1 Gene deletion at the HPRT locus by homologous recombination.

Vector	Expected deletion length (kb)	Average resistant colony numbers[a]		Targeting index
		G418	G418 + 6-TG	
pDWMD1	10.1	325	3.00	0.92%[b]
		141	3.13	2.22%
pDWMD5	27.2	282	1.04	0.36%
pDWMD4	38.6	157	0.00	0
		157	0.07	0.04%

a. Average resistant colonies from $5x10^6$ cells/plate.

b. Targeting index: ratio of the targeted colony number against the colony number of effective transfection, *i.e.* the number of G418 and 6-thioguanine (6-TG) resistant colonies/ the number of G418 resistant colonies only.

Table 3.2 Incorporation activities of [^{3}H]-hypoxanthine and [^{3}H]-adenine in wild-type and mutant cells.

Cells	Incorporation (cpm)[a]	
	[^{3}H]-hypoxanthine	[^{3}H]-adenine
E14	273,679 ± 12,185 (100%)[b]	147,786 ± 501
E14TG2a	366 ± 47 (0.1%)	96,254 ± 4,590
DWMD1-16	526 ± 115 (0.2%)	113,464 ± 4,521

a. Incorporation (cpm) of [^{3}H]-hypoxanthine or -adenine into cellular nucleic acid during a 5-hour incubation.

b. Percentage incorporation relative to the incorporation of parental cell line E14.

the deleted allele has a different restriction pattern from that of the wild-type gene. Hence, deletion mutants can be distinguished from the wild type by Southern analysis. In EcoRI restricted DNA from HPRT wild-type cells, 5 different fragments, 6.3, 5.5, 1.3, 5.0 and 9.3 kb, containing exon 1, exon 2, exon 3, exons 4-5, and exons 6-9 respectively hybridise with the pHPT5 cDNA probe in Southern blots. In a deletion mutant generated with pDWMD1, the 6.3-kb fragment is lost and a novel 7.4-kb fragment containing exon 2 replaces the 5.5-kb fragment. The targeted deletion can also be demonstrated using BamHI or HindIII restriction mapping. In the BamHI restriction map, the 11.5-kb fragment containing exon 1 is missing in the targeted deletion allele, whereas the 7.0-kb, 4.9-kb and 11.9-kb fragments remain unchanged. The 10.2-kb fragment which includes exon 1 is lost and the 4.5-kb fragment containing exon 2 is extended to 14.5 kb in the HindIII restriction when probed with mouse HPRT cDNA. Two gene targeting experiments have been performed using pDWMD1. Eighteen colonies were resistant against the selection by both G418 and 6-thioguanine in the first experiment while 20 colonies were generated in the second experiment. Southern analysis suggested that only one from 6 analysed HPRT-deficient clones had a different structure at the HPRT locus to that predicted. Two novel bands, 7.4-kb and 8.2-kb, are visible while the 5.5-kb band is missing in the EcoRI restricted Southern blots from this particular clone. With HindIII restriction, a 12.1-kb band is noticed without any alteration of the wild-type bands (Figure 3.2). These two restriction enzyme patterns suggest an insertion at the 3′ end of the region of homology. The 5.5-kb EcoRI fragment containing exon 2 has been extended to 8.2-kb by inserting pUC8 sequences and a novel 7.4-kb fragment by inserting both the *neo* cassette and exon 2 is generated in this insertion event. The insertion mutation was confirmed by Southern analysis of HindIII restricted DNA, which generated a novel 12.1-kb fragment from the insertion of the pDWMD1 sequence directly from the cutting site of vector into the 3′ end of homology with the target locus (Figure 3.3).

Figure 3.2 Southern hybridisation analysis of clones resistant to G418 and 6-thioguanine generated by electroporation with pDWMD1 and pDWMD5. Genomic DNA samples from wild-type parental E14 cells (lanes 1,5), targeting deletion by pDWMD1 (lanes 2,6) and insertion mutants generated with pDWMD5 (lanes 3,7) or with pDWMD1 (lanes 4,8) were restricted with EcoRI (panel A) or HindIII (panel B), electrophoresed, transferred and hybridised with a full-length HPRT cDNA probe. The sizes (in kb) of hybridising bands from the wild-type gene are shown adjacent to each panel. The fragments containing the HPRT pseudogene sequences are indicated by PG. The exon elements present in each band can be determined by consulting Figures 3.1, 3.3, and 3.5.

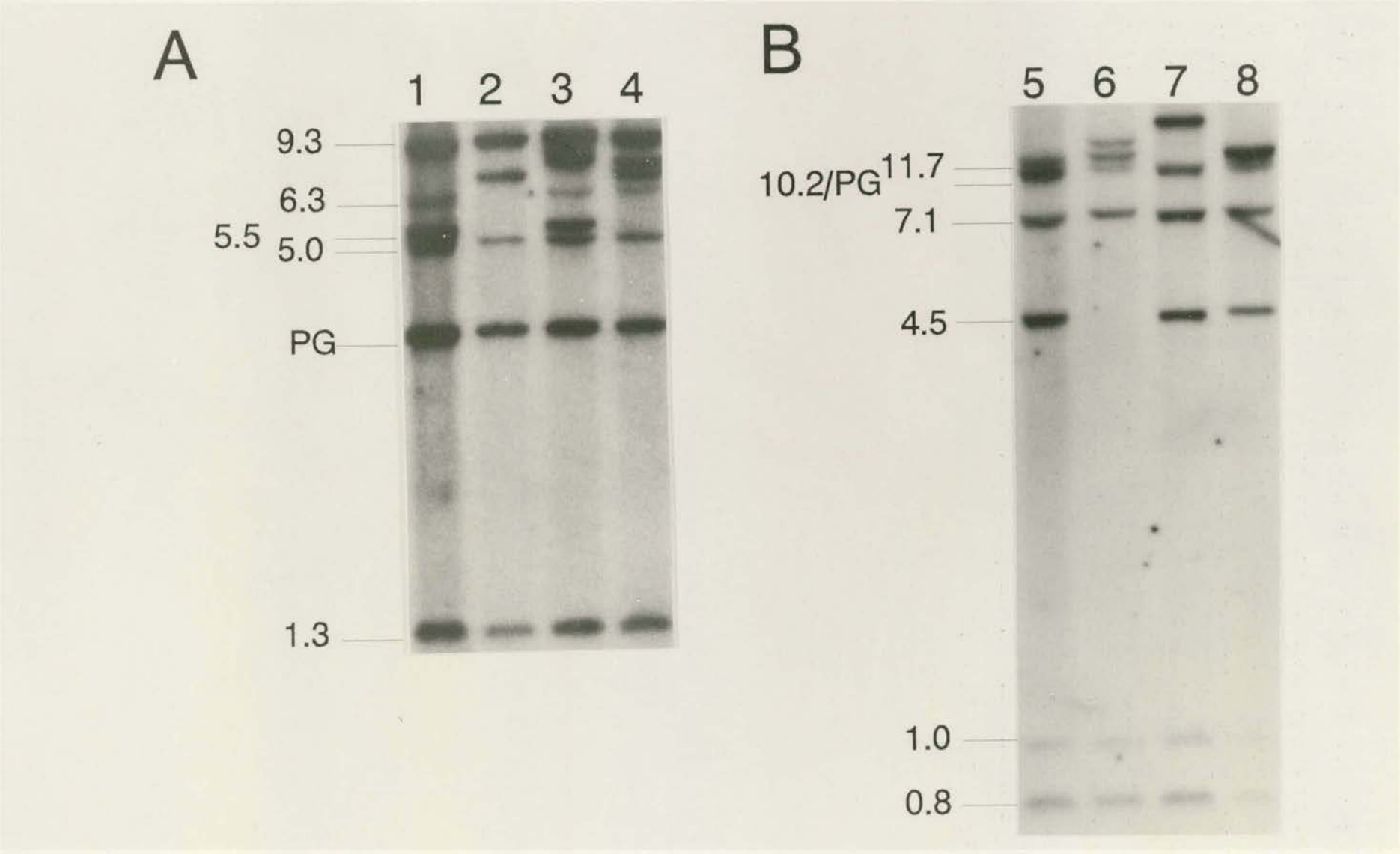
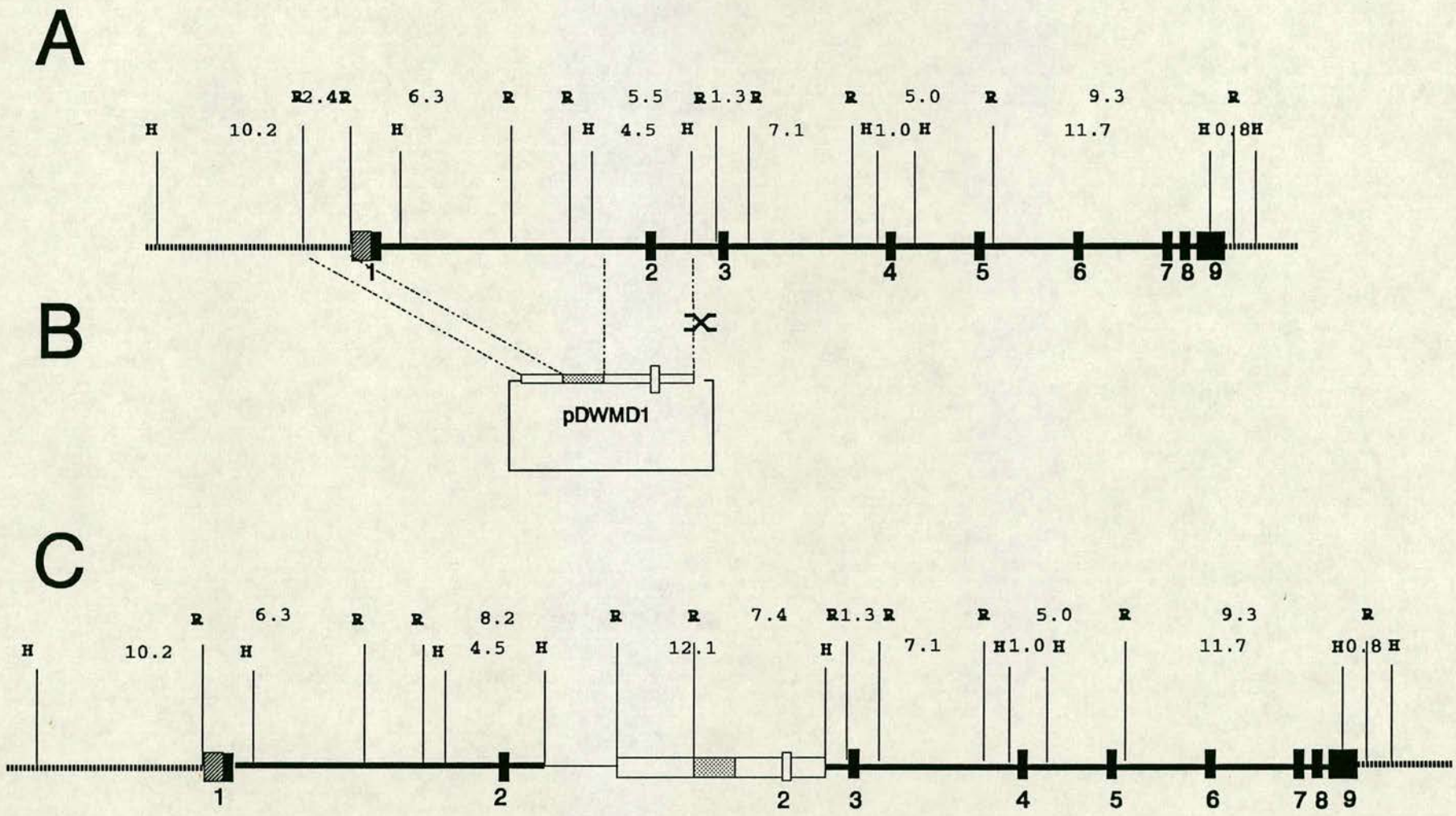

A
1
2
3
4
9.3
6.3
5.5
5.0
PG
1.3
B
5
6
7
8
10.2/PG
11.7
7.1
4.5
1.0
0.8

Figure 3.3 The insertion mutant generated by electroporating pDWMD1 into E14 cells. The structures of (A) the wild-type HPRT gene, (B) the vector (pDWMD1), and (C) the insertion mutant generated by electroporating pDWMD1 are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII; B, BamHI. The sizes (in kb) of all EcoRI, HindIII and BamHI restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched box, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette.


A
B
C
pDWMD1
H
R
10.2
2.4
6.3
4.5
5.5
1.3
7.1
1.0
5.0
11.7
0.8
9.3
8.2
12.1
7.4
1
2
3
4
5
6
7
8
9

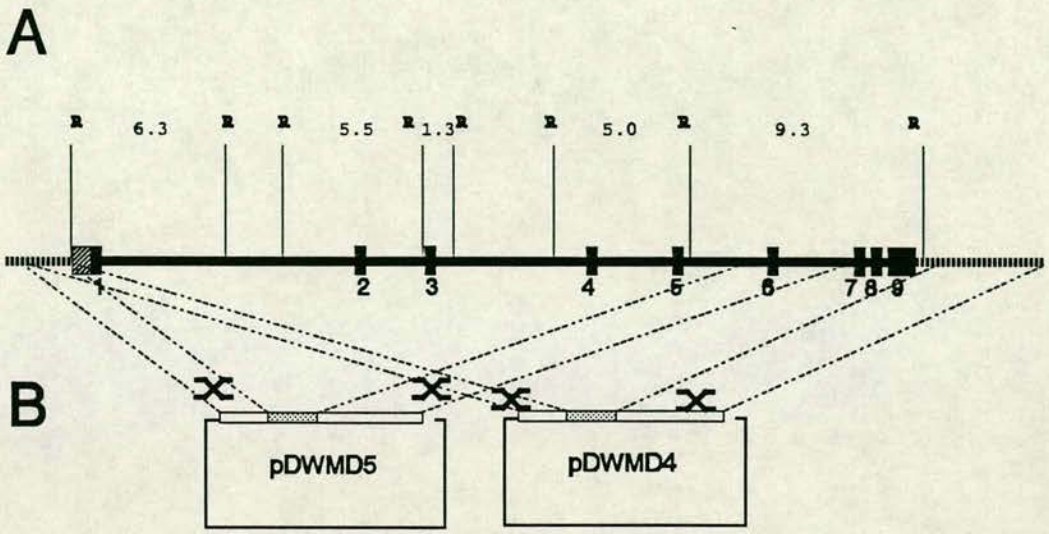
3.3.2 Gene targeting using pDWMD4 and pDWMD5

The vectors pDWMD5 (designed for 27.2-kb deletion) and pDWMD4 (38.6-kb deletion; see Figure 3.4), were electroporated into E14 cells. Clones resistant to G418 and 6-thioguanine were picked and analysed as described. The Southern patterns obtained from all 3 resistant colonies generated in 2 different experiments with pDWMD4 were unexpected and disparate from each other (data not shown). The confusing pattern and rare incidence of doubly resistant colonies suggest that the attempt to delete a large (38.6 kb) fragment by targeting , is much more difficult. Although the targeting index from the electroporation of pDWMD5 into E14 cells, measured by the frequency of HPRT inactivation, is much less than that from pDWMD1, homologous recombination did occur (Table 3.1). All HPRT inactivated clones generated from the electroporation of pDWMD5 showed an identical pattern in the Southern analysis. In the blot with EcoRI restriction, 2 novel bands, 9.9 kb and 8.5 kb, are visible with the 9.3-kb band missing from the wild-type pattern. This result suggests that the inactivated clones generated from the electroporation of pDWMD5 are insertion mutants rather than deletion mutants. The 8.5-kb EcoRI fragment containing exon 6 was generated by insertion of the pUC8 sequence in the intron 6 region. The 9.9-kb fragment which contains *neo* sequences, is an extension of the 9.3-kb EcoRI fragment containing exons 5-9 (Figure 3.2 and 3.5). These insertion phenomena were also confirmed by Southern analysis of HindIII (Figure 3.2) and BamHI (data not shown) digests. The 23.4-kb HindIII fragment containing exons 6-9 and pDWMD5 sequence replaces the 11.7-kb fragment in insertion inactivation mutants.

3.4 Positive-negative selection for HPRT deletion

The use of a well-established cell selection system makes the study of the HPRT gene very convenient. For most genes of interest a direct selection system as described for HPRT does not exist and hence it is impossible to isolate directly a targeted clone with the low frequency of gene targeting currently achieved. Mansour *et al.* (1988) described a general method, termed positive-negative selection, for isolation of cells containing targeted mutations in any gene, regardless of its function.

Figure 3.4 Schematic diagram of strategies for deletion in the mouse HPRT locus using the vectors pDWMD5 and pDWMD4. The structures of (A) the wild-type HPRT gene, (B) the deletion vectors (pDWMD5 and pDWMD4), and (C,D) predicted targeting deletion mutants are shown schematically. The number of each exon is shown directly below it. Restriction sites for EcoRI in the HPRT locus are shown as "R" and the sizes (in kb) of EcoRI restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched box, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette.


A
R
6.3
R
R
5.5
R
1.3
R
R
5.0
R
9.3
R
1
2
3
4
5
6
7
8
9
B
pDWMD5
pDWMD4

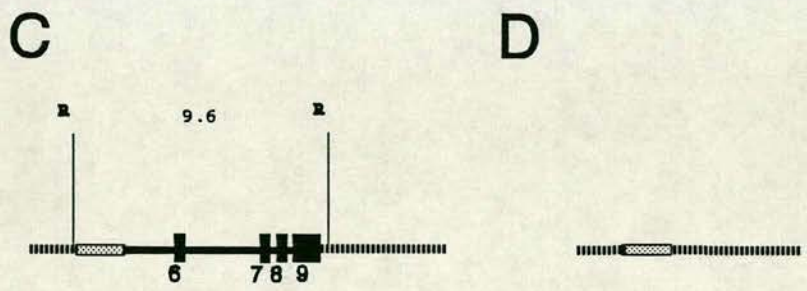
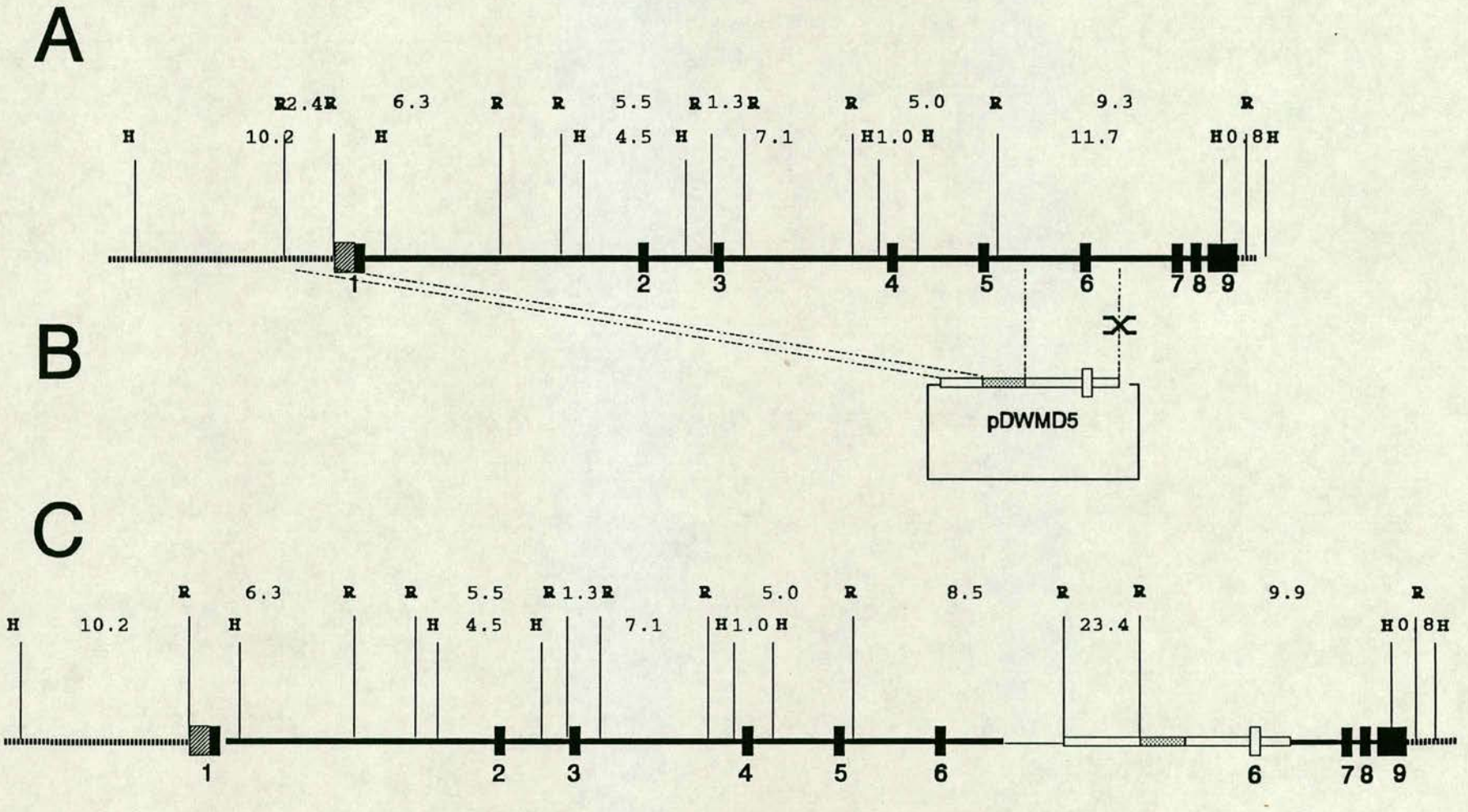

C
D
R
9.6
R
6
7
8
9

Figure 3.5 The insertion which occurred after electroporation with the vector pDWMD5. The structures of (A) the wild-type HPRT gene, (B) the vector-pDWMD5, and (C) insertion mutant generated by electroporating pDWMD5 are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII. The sizes (in kb) of all EcoRI and HindIII restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched box, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette.


A
B
C
pDWMD5
H
R
10.2
2.4
6.3
5.5
4.5
1.3
7.1
5.0
1.0
9.3
11.7
0.8
23.4
8.5
1
2
3
4
5
6
7
8
9

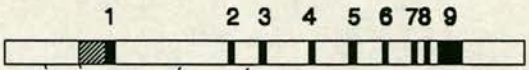
A marker gene (*e.g. neo* or the hygromycin resistance gene) containing all the necessary control signals for efficient expression acts as positive selection and is used to disrupt the target locus. Random integrations of the targeting vector are eliminated by negative selection since these cells retain a second, negatively selectable, marker gene, included in the vector, after the recombination process. The thymidine kinase gene from herpes simplex virus (HSV-TK) is used as the second marker gene, and its presence can be selected against due to its higher enzymatic activity for thymidine analogues such as gancyclovir compared to its mammalian counterpart (Figure 3.6). This method has been successfully used in many different homologous recombination experiments and indicated that up to 2000-fold enrichment can be achieved for those cells that contain a targeted mutation (Mansour *et al.*, 1988; DeChiara *et al.*, 1990).

To determine the possibility of using positive-negative selection to facilitate targeting deletion experiments, the deletion vector pDWMD3 was constructed and introduced into E14 cells. The structure of pDWMD3 is identical to pDWMD1 except for the addition of a gene cassette containing the herpes simplex virus thymidine kinase gene driven by its own promoter to provide a negative selection marker for homologous recombination. HindIII linearised pDWMD3 DNA was electroporated into E14 cells. The electroporated cells were divided into 4 groups. Cells treated with G418 only were analysed to examine the efficiency of electroporation. The second group was incubated with G418 and gancyclovir for testing the possibility of positive-negative selection. The groups with 6-thioguanine treatment were to select HPRT-deficient clones directly after electroporation. Numbers of resistant colonies selected with G418 and gancyclovir however, were not greatly different from those selected in G418 alone, suggesting that in this case positive-negative selection was not working efficiently to reduce the number of surviving random integrants (Table 3.3).

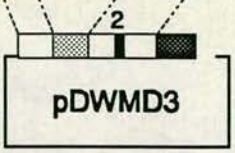
Using homologous recombination techniques, a novel strategy to introduce predesigned deletion mutations into the HPRT locus in ES cells was proposed. A 10-kb deletion including promoter and exon 1 region was constructed, although the positive-negative selection scheme did not function efficiently. This approach

Figure 3.6 The strategy for positive-negative selection in deletion targeting the HPRT gene (A). The deletion targeting vector (B) carries a positively selectable *neo* gene (dotted box), flanked by 5′ and 3′ regions of homology. The HSV-TK gene (crosshatched box) lies 3′ to the 3′ region of homology. Homologous recombination between the vector pDWMD3 and HPRT locus results in replacement of target gene sequences by incoming vector and loss of the flanking TK sequences. Targeting HPRT deletion cells (C) are neo^+ and HSV-TK^-. (D) as a result of random integration the entire targeting vector integrates into the genome. Such cells are not only neo^+ but also HSV-TK^+ which render them sensitive to gancyclovir.

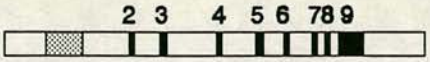
A



B

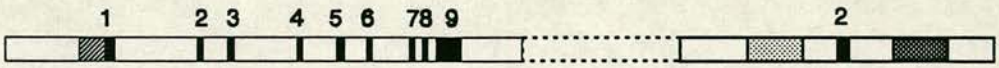


C



neo^{+}, *hprt*$^{-}$, *HSV-tk*$^{-}$

D



neo^{+}, *hprt*$^{+}$, *HSV-tk*$^{+}$

Table 3.3 The effect of positive-negative selection on targeting events with the deletion vector, pDWMD3.

Selection	Colony number[a]			
	Experiment 1		Experiment 2	
G418	1095	(100%)[b]	378	(100%)
G418+GANC[c]	585	(53.42%)	143	(37.83%)
G418+6-TG[d]	1.25	(0.114%)	3.00	(0.797%)
G418+GANC+6-TG	2.00	(0.183%)	1.25	(0.307%)

a. Average resistant colonies from $5x10^6$ cells/plate.

b. Percentage of surviving colony number against the colony number of effective transformation.

c. GANC, gancyclovir.

d. 6-TG, 6-thioguanine.

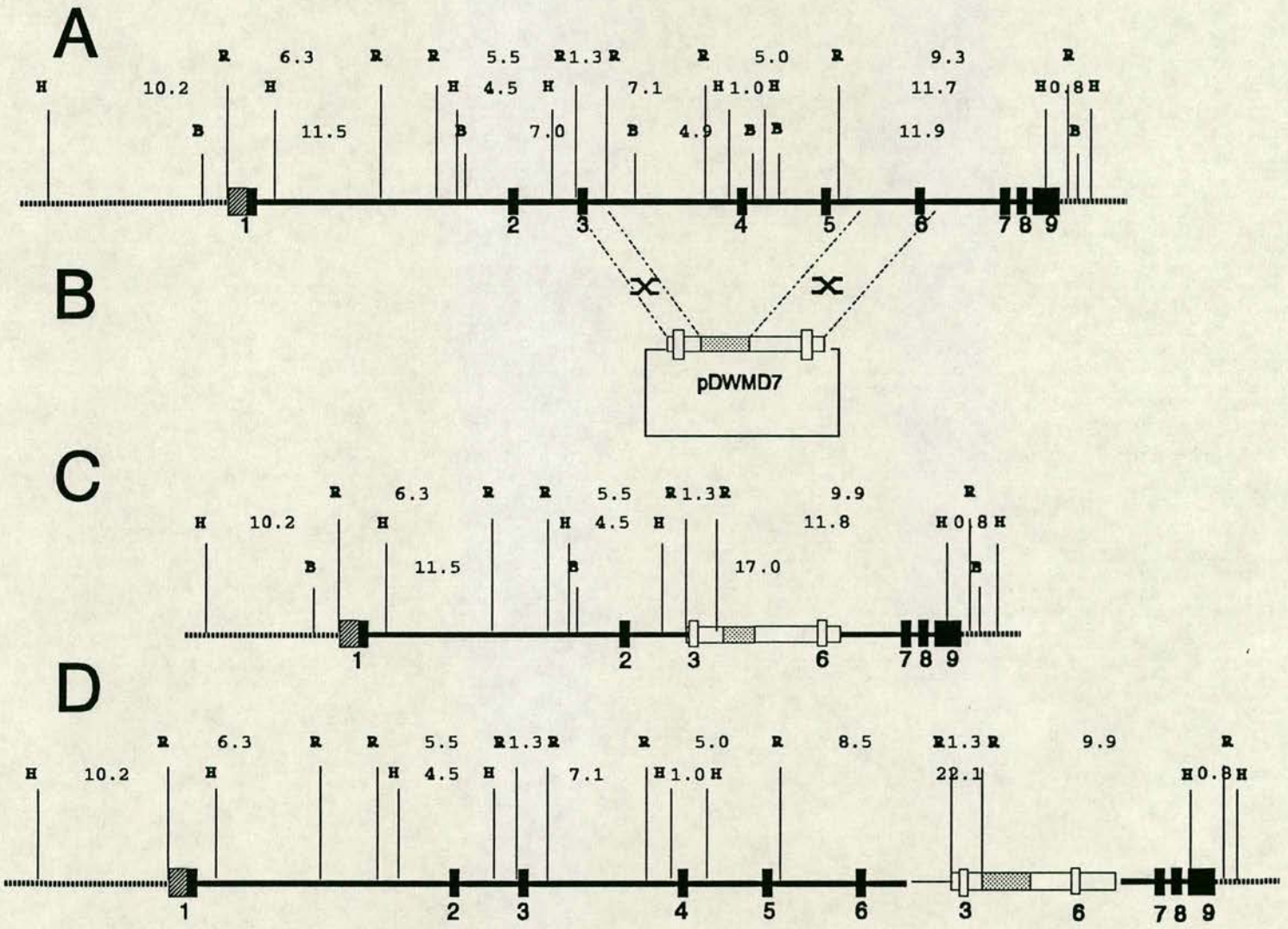
CHAPTER 4. DELETION MECHANISMS CREATED BY GENE TARGETING

4.1 Sequence effect on deletion by gene targeting

Results described in Chapter 3 showed that it was not difficult to perform targeting deletions of the 10.1-kb containing promoter region and exon 1 of the HPRT locus. Nevertheless, an attempt to delete a 27.2-kb sequence containing promoter region and exons 1 to 5 failed. Because both vectors, pDWMD1 and pDWMD5, designed to make deletions of 10.1-kb and 27.2-kb respectively, shared identical 5′ homology and had a similar length of 3′ homology, failure to make the longer deletion might be due to either 3′ sequence differences or the deletion size. To investigate whether the sequence-specificity is important for the targeting deletion, a targeting deletion vector, pDWMD7, was constructed to delete a similar length to that deleted by pDWMD1, but from a different region of the HPRT locus. The 2.4-kb EcoRI fragment holding the upstream flanking region in the targeting deleting vector, pDWMD5, was replaced by the 1.3-kb EcoRI fragment containing exon 3 to build a vector for the deletion of an 11.4-kb sequence encompassing exons 4-5 (Figure 4.1). Targeting vectors pDWMD5 and pDWMD7 share the same 3′ homology but differ by 15.8 kb in the length of DNA to be deleted.

Southern hybridisations were carried out to analyse the structure of the HPRT gene in clones surviving both G418 and 6-thioguanine selection after electroporating with linearised pDWMD7 DNA. Two clones with different patterns were obtained from 1 electroporation of 4 x 10^7 cells. One shows the predicted deletion targeting pattern. The deletion mutant was designed to be diagnosed by loss of a 5.0-kb EcoRI fragment and alteration of the 9.3 kb-fragment to 9.9 kb. The deletion created by pDWMD7 should result in the loss of 11.4-kb containing exons 4 and 5. This leads to loss of a 1.0-kb HindIII fragment and modifications of the 7.1-kb HindIII fragment containing exon 3 and the 11.7-kb HindIII fragment. The 7.1-kb fragment containing exon 3 would lose the 3′ HindIII site and generate a new 11.8-kb fragment containing exons 3 and 6-9 extending to the 3′ HindIII site of the wild type 11.7-kb fragment. The Southern blot of mutant DNA restricted with HindIII and probed with pHPT5 shows 4 bands of 11.8, 10.2, 4.5 and 0.8 kb. Compared to the pattern of wild-type DNA which generates 6 bands with 11.7, 10.2, 7.1, 4.5, 1.0 and 0.8 kb in length, this

Figure 4.1 Strategy for deletion targeting in the mouse HPRT locus using pDWMD7. The structures of (A) the wild-type HPRT gene, (B) the deleting vector (pDWMD7), and targeting mutants are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII; B, BamHI. The sizes (in kb) of all EcoRI, HindIII and BamHI restriction fragments containing exon elements are shown between the restriction sites. The pattern of targeting mutants are shown as (C) predicted deletion mutant, and (D) 3′ insertion mutant. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched boxes, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; dotted boxes, *neo* cassette.


A
B
C
D
pDWMD7

result fits the predicted pattern of deletion targeting (Figure 4.2).

The other pattern shows that clones resistant to G418 and 6-thioguanine are insertion mutants containing an insertion at the 3′ end of the homologous region. The 3′ insertion mutant should have 2 duplicate regions, one containing exon 3 and the other containing exon 6. Therefore, the restriction mapping by EcoRI digestion should identify a novel 8.5-kb fragment holding exon 6 and a repeat 1.3-kb fragment containing exon 3 as well as a modified the 9.3-kb fragment containing exons 6-9 now 9.9 kb in size (Figure 4.1). An EcoRI digest probed with HPRT cDNA shows 7 bands of 9.9, 8.5, 6.3, 5.5, 5.0, 3.5 and 1.3 kb in length in the mutant. There are three differences between the wild-type and mutant restriction patterns. Due to the insertion of an extra 1.3-kb fragment containing exon 3, a 1.3-kb fragment with double density relative to its wild-type counterpart is noticed. Insertion of pUC8 sequences, *neo* module and exon 6 region leads to novel 8.5-kb band and the extension of the 9.3-kb fragment to 9.9 kb. This clone fits the pattern predicted for insertion into the HPRT locus within the region of 3′ homology (Figure 4.3). The insertion event was also confirmed by restriction with HindIII which showed modification of the wild-type 11.7-kb fragment holding exons 5-9 to 22.1 kb due to insertion of vector sequences.

4.2 Effect of deletion size on efficiency of deletion targeting

Although the attempt to delete more than 20 kb from the HPRT locus by gene targeting using pDWMD5 did not succeed, this could have been due to sequence-specific difficulty. To find out whether sequence-specificity or length-limitation influences the feasibility of deletion targeting more than 20 kb in the HPRT locus, a vector, pDWMD8, was constructed. The vector pDWMD8 was designed to delete 21 kb including the 10.1-kb region which was successfully deleted by gene targeting with pDWMD1. Successful targeting deletion by pDWMD8 would give the same EcoRI restriction pattern as the targeting deletion established by pDWMD1 when probed with HPRT cDNA. The construction of the deletion vector, pDWMD8, was performed by inserting a 3.0-kb EcoRI fragment of pλ23RI3.0 into the unique EcoRI site of the intermediate plasmid used in construction of pDWMD8. Plasmid pλ23RI3.0 is a

Figure 4.2 Southern analysis of clones resistant to G418 and 6-thioguanine generated by electroporation with pDWMD1 or pDWMD7 into E14 embryonic stem cells. Genomic DNA samples from wild-type E14 cells (lane 1, 4, 7) and targeting deletion mutants by pDWMD1 (lane 2, 5, 8) or by pDWMD7 (lane 3, 6, 9) were restricted with EcoRI (panel A), HindIII (panel B), or BamHI (panel C), electrophoresed, transferred and hybridised with full-length HPRT cDNA as probe. The sizes (in kb) of hybridising bands from the wild-type gene are shown adjacent to each panel. The fragments containing the HPRT pseudogene sequences are indicated by PG. The exon elements present in each band can be determined by consulting Figures 3.1 and 4.1.

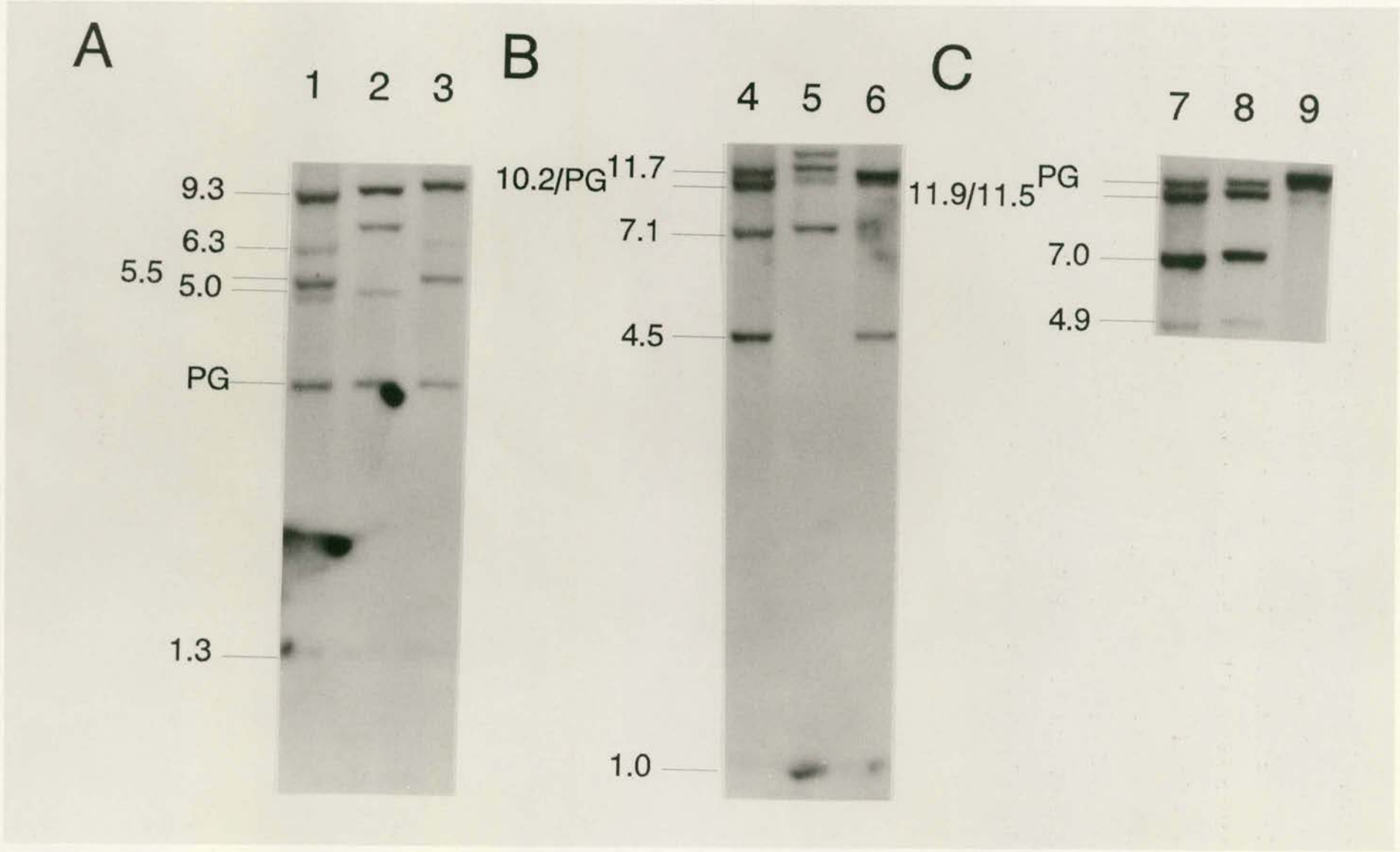
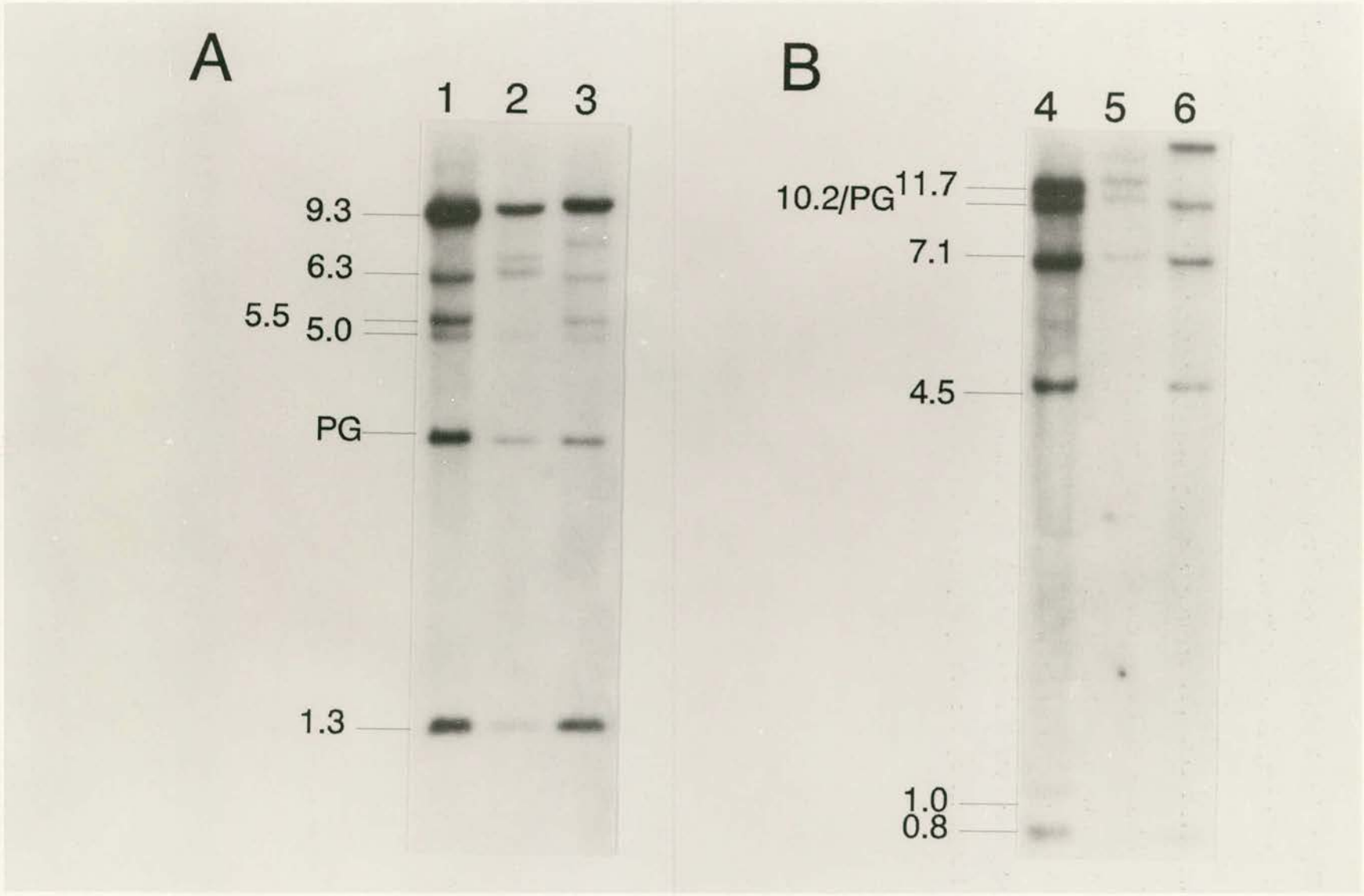

A
1 2 3
9.3
6.3
5.5
5.0
PG
1.3
B
4 5 6
10.2/PG
11.7
7.1
4.5
1.0
C
7 8 9
11.9/11.5
PG
7.0
4.9

Figure 4.3 Southern analysis of insertional inactivation clones resistant to G418 and 6-thioguanine generated by electroporation with pDWMD7 or pDWMD8 into E14 embryonic stem cells. Genomic DNA samples from E14 cells (lane 1) and 3′ insertion mutants produced by pDWMD8 (lane 2) and pDWMD7 (lane 3) were restricted with EcoRI or HindIII, electrophoresed, transferred and hybridised with full length HPRT cDNA as probe. The sizes (in kb) of hybridising bands from the wild-type gene are shown adjacent to each panel. The fragments containing the HPRT pseudogene sequences are indicated by PG. The exon elements present in each band can be determined by consulting Figures 4.1 and 4.5.


A
1
2
3
9.3
6.3
5.5
5.0
PG
1.3
B
4
5
6
10.2/PG
11.7
7.1
4.5
1.0
0.8

subclone of mouse HPRT genomic sequences, located about 11 kb upstream of the HPRT promoter region (Melton *et al.*, 1984).

In a successfully targeted event the 6.3-kb and 5.5-kb EcoRI fragments should be replaced by a novel 7.4-kb fragment due to loss of exon 1 in the targeted deletion mutant (Figure 4.4). Both pDWMD1 and pDWMD8 share the same 3′ homologous region. Two electroporations were carried out to introduce the HindIII linearised pDWMD8 DNA into E14 murine embryonic stem cells. Five clones in total were obtained after selection with G418 and 6-thioguanine in 2 different electroporations. None of the 5 resistant clones fits the expected deletion pattern involving loss of the 6.3-kb EcoRI fragment, suggesting that the attempt to delete 20 kb from the HPRT locus had failed again. Similar to the results with pDWMD5, only insertion events have occurred. In addition to the missing 5.5-kb fragment, the predicted insertion should generate two fragments of 8.2 kb and 7.2 kb in mutant DNA which are not present in wild-type DNA (Figure 4.5). This alteration is caused by the insertion of vector sequences into the 3′ end of the homology. The predicted insertion event should also modify the 4.5-kb HindIII fragment to 14.4 kb by inserting vector sequences. The HindIII digest shown in Figure 4.3 fits the prediction. In the EcoRI digest the 5.5-kb fragment is missing, the 6.3-kb fragment is barely detectable due to underloading, but two novel bands are present. Although their size does not fit the prediction (8.2 and 7.2 kb) particularly well, these results are more compatible with insertion rather than deletion again having occurred in gene targeted mutants.

4.3 Achieving large deletion by intrachromosomal recombination

Until recently, the largest genomic deletion performed by gene targeting techniques in ES cells is about 15 kb (Mombaerts *et al.*, 1991). Some spontaneous mutants as well as human genetic disorders were reported to contain large deletions (Yunis and Ramsay, 1978). It would be useful to develop a method that could create large deletions by gene targeting, so that animal models with such large deletions could be constructed and studied. The capability to generate deletions is limited in size as suggested by results with both pDWMD5 and pDWMD8. There is no simple

Figure 4.4 Strategy for generating deletions in the mouse HPRT locus by homologous recombination. The structures of (A) the wild-type HPRT gene, (B) the deletion vector (pDWMD8), and (C) predicted targeting deletion mutant are shown schematically. The number of each exon is shown directly below it and EcoRI restriction sites are presented as "R". The sizes (in kb) of EcoRI restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched boxes, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; dotted boxes, *neo* cassette.

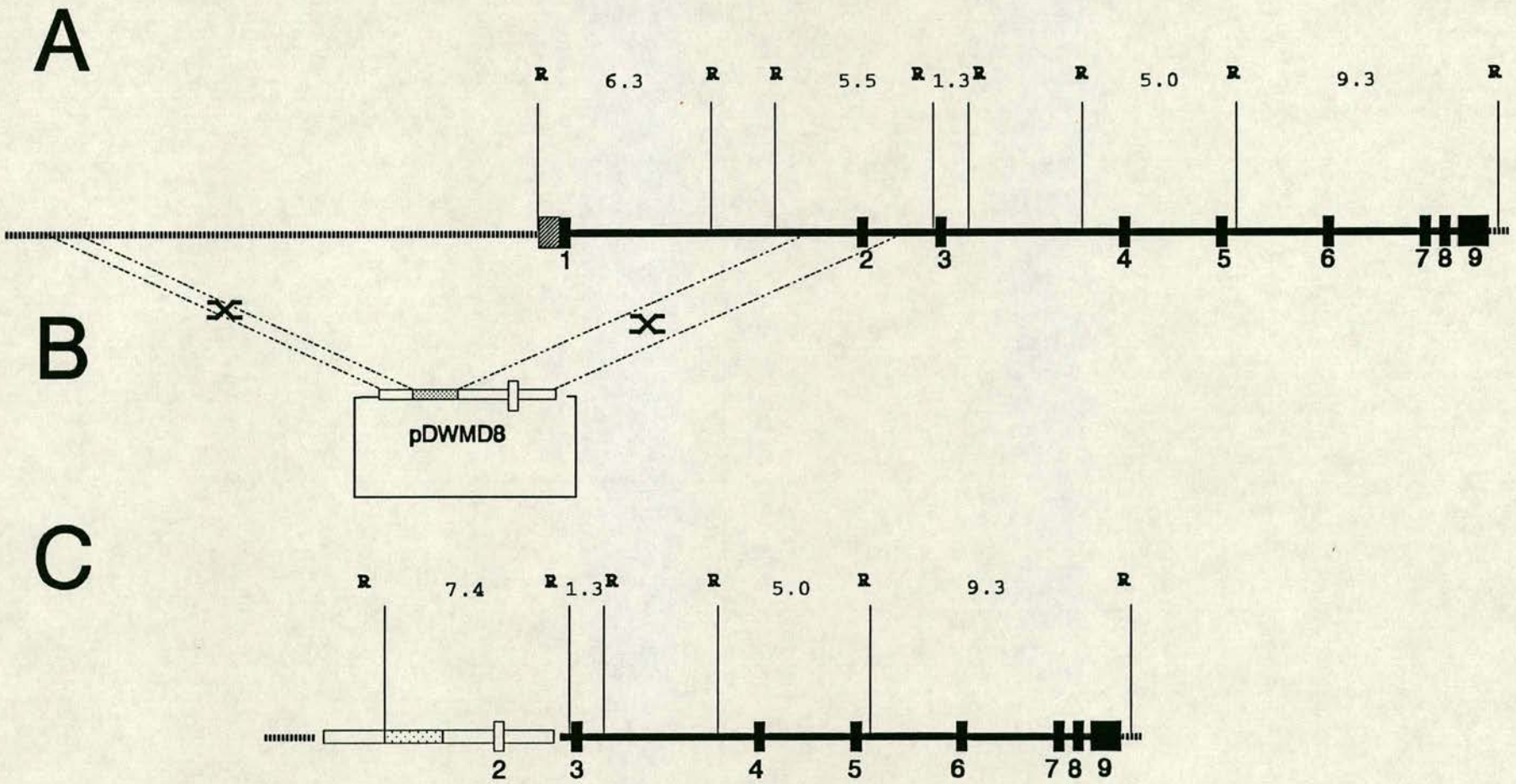
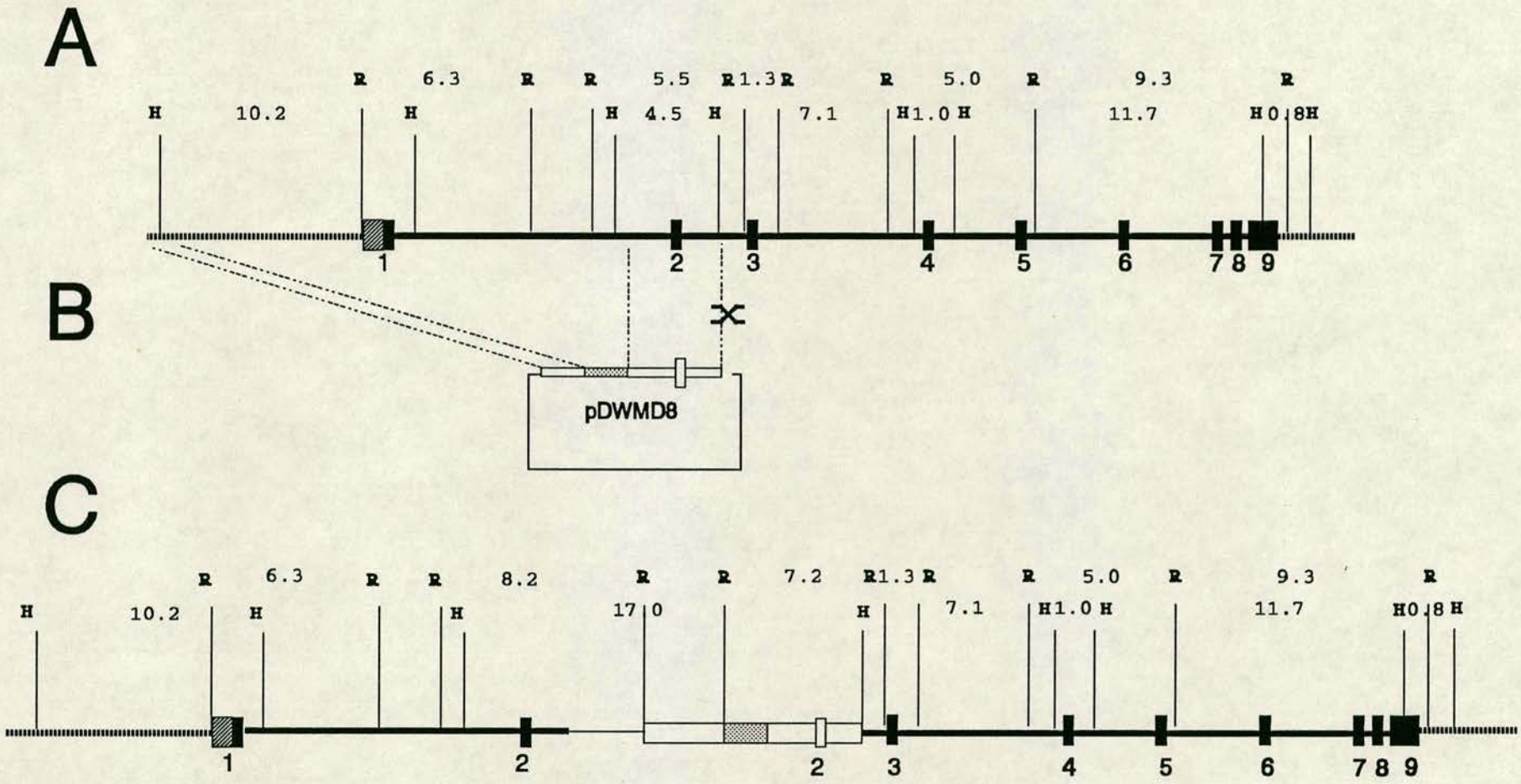

A
R
6.3
R
R
5.5
R
1.3
R
R
5.0
R
9.3
R
1
2
3
4
5
6
7 8 9
B
pDWMD8
C
R
7.4
R
1.3
R
R
5.0
R
9.3
R
2
3
4
5
6
7 8 9

Figure 4.5 The mechanism of generation of insertion mutants by electroporation with pDWMD8 DNA. The structures of (A) the wild-type HPRT gene, (B) the pDWMD8 vector, and (C) insertion mutant generated by electroporating pDWMD8 are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII. The sizes (in kb) of all EcoRI and HindIII restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched boxes, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; dotted boxes, *neo* cassette.


A
R
6.3
R
R
5.5
R
1.3
R
R
5.0
R
9.3
R
H
10.2
H
H
4.5
H
7.1
H
1.0
H
11.7
H
0
8
H
1
2
3
4
5
6
7
8
9
B
pDWMD8
C
R
6.3
R
R
8.2
R
R
7.2
R
1.3
R
R
5.0
R
9.3
R
H
10.2
H
H
17
0
H
7.1
H
1.0
H
11.7
H
0
8
H
1
2
2
3
4
5
6
7
8
9

procedure available to delete a larger region of the HPRT locus by homologous recombination. A two-step recombination procedure used to delete a large fragment is described in Figure 4.6. By the insertion of vector sequences into the 3′ end of homology in the first step, intermediate clones could be selected with G418 and 6-thioguanine for the genotype, *neo*$^+$, *hprt*$^-$, *HSV-tk*$^+$. The second step requires intrachromosomal recombination in the HPRT locus. Clones with the intrachromosomal recombination event can be distinguished from those without recombination by selection against HSV-TK. All cells which lose HSV-TK by intrachromosomal recombination will survive gancyclovir selection. Two different gene structures will be generated from the intermediate clones, if the intrachromosomal recombination occurred. One type, in which the recombination occurred in the 3′ homology will generate a wild-type HPRT with loss of the *neo* module. Those cells in which the recombination occurred in the 5′ homology will produce a mutant with the desired deletion and maintenance of the *neo* module.

An insertion event usually occurs instead of deletion when large deletions are attempted by the gene replacement protocol. The vector pDWMD5 is the best basic design to test the two-step hit and run procedure, as the targeted clones generated from electroporation with pDWMD5 are inserted in the 3′ end sequences of the vector. A negatively selectable gene cassette (PGK promoter driving HSV-TK gene) was cloned into the HPRT 3′ homology of pDWMD5 to construct the 2-step deleting vector-pDWMD5-TK. Intermediate clones with insertion of HSV thymidine kinase, 2.4 kb 5′ flanking sequence, *neo* and a 3.9-kb fragment containing exon 6 would be generated. The intrachromosomal recombination which generates the desired deletion takes place in the two 2.4-kb 5′ flanking regions of the HPRT gene and will delete both promoter region and exons 1-5 (Figure 4.7). This deletion mutant is the only one which can survive under G418, 6-thioguanine and gancyclovir triple selection. The first experiment was performed by electroporating E14 cells with HindIII linearised pDWMD5-TK DNA. Only one colony survived after the selection of G418, 6-thioguanine and gancyclovir. Southern analysis shows that 4 EcoRI bands containing exon 1 to exon 5 were missing and the 9.3-kb fragment holding exon 6 to exon 9 was

Figure 4.6 Strategy for targeting deletion by intrachromosomal recombination. (A) A vector containing the homologous region, positive selectable gene cassette, and negative selectable gene cassette inserted into the 3′ end of homology region. Thereby, an intermediate genotype (B) with *neo*$^+$ *hprt*$^-$ *HSV-tk*$^+$ was generated. After intrachromosomal recombination (C), two genotypes (D) will be created. One is the final deletion mutant and the other is the wild type. Both of them can be distinguished from the intermediate insertion mutants by using gancyclovir selection. Closed boxes, HPRT exons; hatched boxes, promoter regions; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette; cross-hatched boxes, HSV-TK cassette.

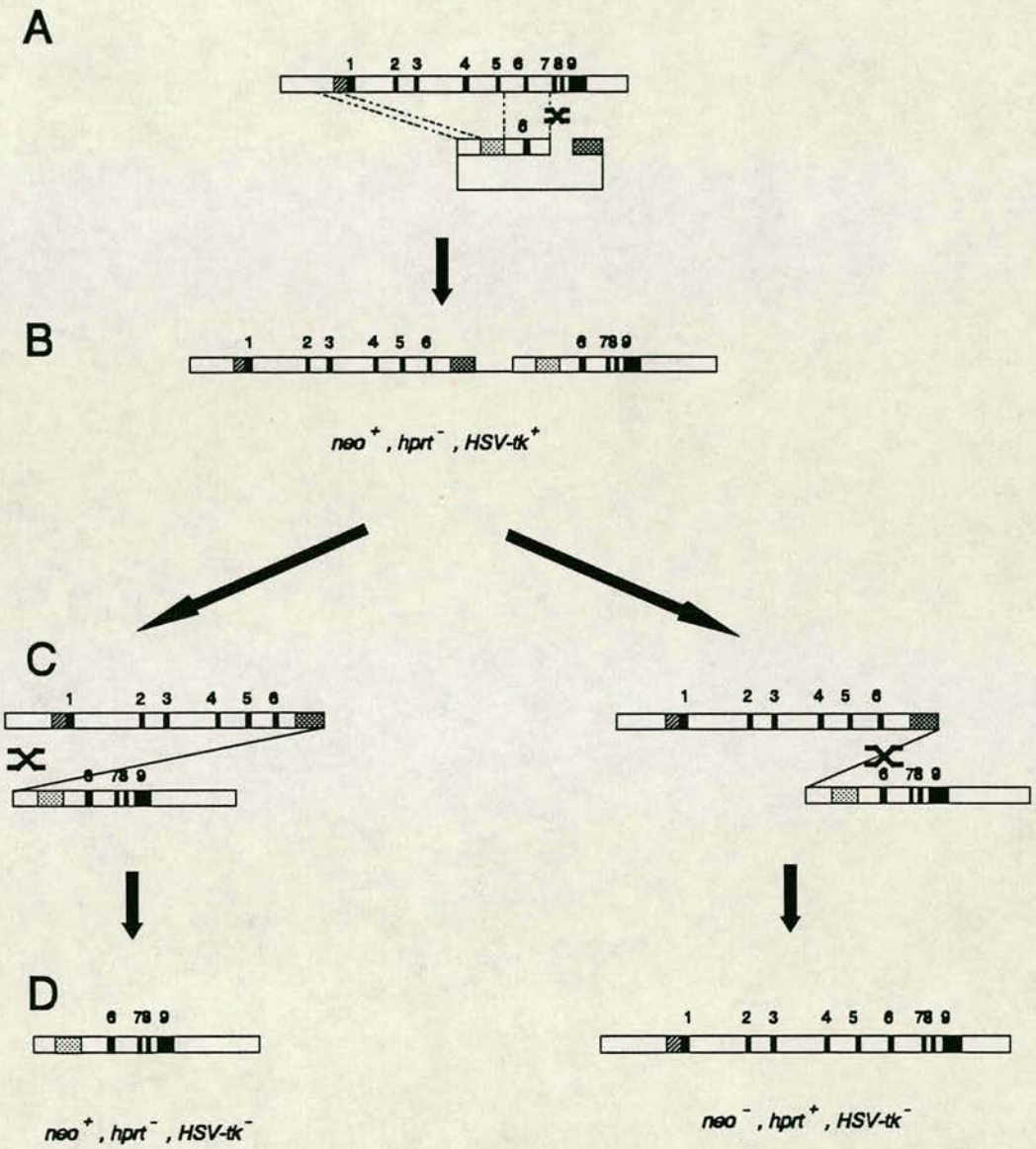
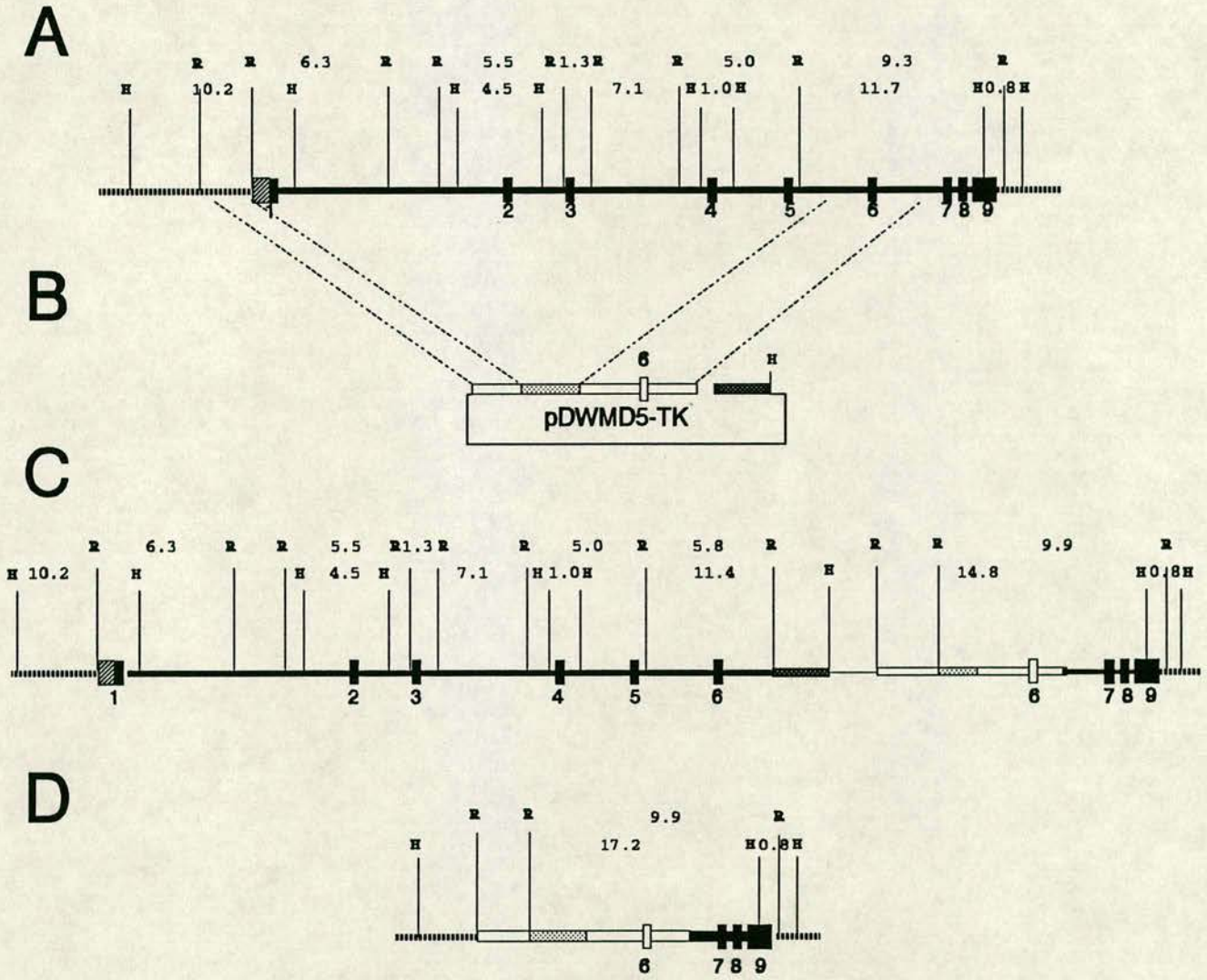

A
1 2 3 4 5 6 7 8 9
6
B
1 2 3 4 5 6
6 7 8 9
neo+, hprt-, HSV-tk+
C
1 2 3 4 5 6
6 7 8 9
1 2 3 4 5 6
6 7 8 9
D
6 7 8 9
1 2 3 4 5 6 7 8 9
neo+, hprt-, HSV-tk-
neo-, hprt+, HSV-tk-

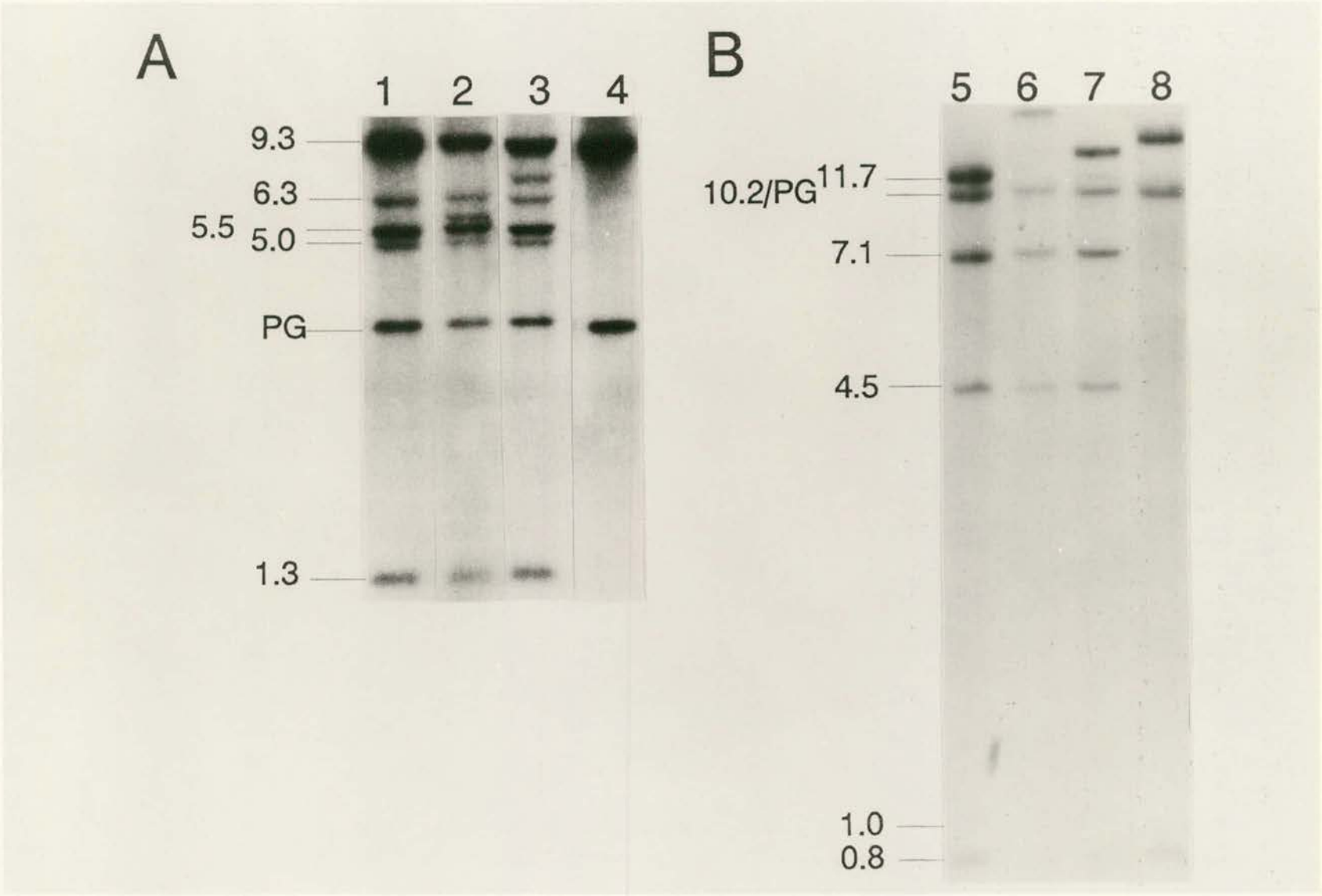
Figure 4.7 Restriction pattern of gene targeting deletion in HPRT locus by pDWMD5-TK. The structures of (A) the wild-type HPRT gene, (B) the deleting vector (pDWMD5-TK), (C) the intermediate insertion mutant and (D) the targeting deletion mutant are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: R, EcoRI; H, HindIII. The sizes (in kb) of all EcoRI and HindIII restriction fragments containing exon elements are shown between the restriction sites. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched boxes, promoter regions; open boxes, vector-derived HPRT sequence; vertically striped line, HPRT-flanking region; thin line, plasmid pUC8 sequence; stippled boxes, *neo* cassette; cross-hatched boxes, HSV-TK cassette.


A
B
C
D
pDWMD5-TK

modified to 9.6 kb in length. These results suggest that a deletion mutant was created by the two-step intrachromosomal recombination. A second experiment was carried out to select the intermediate clones to demonstrate the mechanism of deletion by the selection with G418 and 6-thioguanine only. Five clones resistant to G418 and 6-thioguanine were selected and restriction patterns suggest that 4 clones are intermediate products which have the predicted 5.8-kb novel band as well as the extension of 9.3 kb to 9.9 kb in the EcoRI restriction digest. The fifth clone from the second experiment is identified as a deletion mutant generated by spontaneous intrachromosomal recombination. The deletion mutant with 27.2 kb of the HPRT locus deleted by pDWMD5-TK and its intermediate insertion clones were also confirmed by Southern analysis after HindIII digestion (Figure 4.8). The extra 14.8-kb and 11.4-kb bands with the missing 11.7-kb fragment is shown in the pattern of the intermediate clones. An extension of the 11.7-kb fragment to 17.2 kb and the disappearance of the 10.2-kb, 4.5-kb, 7.1-kb, and 1.0-kb fragments which contain exons 1, 2, 3, and 4 respectively, are also indicated in the Southern blot of deletion mutant DNA restricted with HindIII.

Thus, a 2-step deletion strategy involving targeting insertion and intrachromosomal recombination could provide a solution for the size-limited simple targeting deletion strategy for inactivating genes by deletion in ES cells.

Figure 4.8 Southern analysis of the deletion mutant and intermediate insertion stage generated by gene insertion and intrachromosomal recombination with pDWMD5-TK. Genomic DNA samples from wild type (lane 1, 5), insertion mutants by pDWMD5 (lane 2, 6) or by pDWMD5-TK (lane 3, 7), and deletion mutant (lane 4, 8) were restricted with EcoRI (panel A) or HindIII (panel B), electrophoresed, transferred and hybridised with full-length HPRT cDNA as probe. The sizes (in kb) of hybridising bands from the wild-type gene are shown adjacent to each panel. The fragments containing the HPRT pseudogene sequences are indicated by PG. The exon elements present in each band can be determined by consulting Figures 4.7 and 3.5.


A
1
2
3
4
9.3
6.3
5.5
5.0
PG
1.3
B
5
6
7
8
10.2/PG
11.7
7.1
4.5
1.0
0.8

CHAPTER 5. ADENINE DEPENDENCE OF HPRT-DEFICIENT ES CELLS

5.1 The rescue of HPRT-deficient cells by adenine in the presence of HAT selection

HPRT-deficient cells are unable to grow in HAT medium, which is used widely to select for $HPRT^+$ cells (Szybalska and Szybalski, 1962). However, HPRT-deficient mouse ES cells (E14TG2a) could be rescued efficiently and in a dose dependent manner if HAT medium was supplemented with adenine. The dose-dependent rescue by supplementation of adenine in HAT-treated HPRT deficient cells is not only observed in colony forming assays but also in general growth assays. Growth is totally recovered when the concentration of adenine supplement reaches 10^{-4} M, which is the same as the concentration of hypoxanthine used in HAT medium (Table 5.1). Dose-dependent rescue by adenine did not only occur in HPRT-deficient mouse ES cells but also in HPRT-deficient Chinese hamster (CHO) and human (HeLa) cells (data not shown). In addition, the rescue of HPRT deficient cells by supplementation with adenine also occurred in medium containing aminopterin and thymidine only (data not shown). The supply of thymidine in the medium is to provide TMP via thymidine kinase in cells because aminopterin also inhibits thymidylate synthetase activity. These experiments showed that in HPRT-deficient cells where *de novo* nucleotide synthesis is blocked by aminopterin, adenine can provide the purine nucleotide pool, presumably via the action of APRT and AMP deaminase. Because APRT-deficient mice were not available to investigate the role of this enzyme in preventing self injury behaviour in HPRT-deficient mice, purine analogues were used to inhibit APRT activity and the results show that they did induce profound self injury behaviour in young HPRT-deficient mice.

5.2 Inhibition of APRT activity *in vitro* by the purine analogue, 9-ethyladenine

Most of the purine analogues that have been used to study purine metabolism were inappropriate for this study because they are irreversibly toxic to cells at low concentrations. Instead, two analogues, 9-ethyladenine and caffeine which were expected from their chemical structures (Figure 5.1) to be less toxic but to still act as competitive inhibitors of the purine salvage pathway were used throughout the experiments. The recovery effect by supplementation with adenine on HPRT-deficient

Table 5.1 Growth of the HPRT-deficient ES cell line, E14TG2a, in HAT selective medium supplemented with adenine.

Cell	Treatment	Growth[a]	Colonies number[b]
E14TG2a	Control	0.504	67, 61
	HAT	0.005	0, 0
	HAT + 1.25 x 10^{-5} M adenine	0.077	30, 28
	HAT + 2.5 x 10^{-5} M adenine	0.100	45, 40
	HAT + 5.0 x 10^{-5} M adenine	0.238	N.D.[c]
	HAT + 1.0 x 10^{-4} M adenine	0.496	54, 57

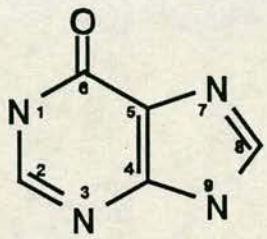
a. Growth was the average of two samples measured with OD_{575nm}. Cells were plated into 24-well microtitre plates and cultured in normal medium for 48 hr. Media were changed to the treatment media on day 2 and the microtitre plates were fixed and stained with crystal violet on day 6. The retained crystal violet in cells was extracted with 70% ethanol and measured by spectrophotometry at 575 nm wavelength.

b. Colony numbers were counted after treatment of 300 cells for 5 days in 24-well microtitre plate.

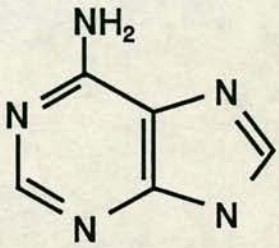
c. N.D., Not determined.

Figure 5.1 The chemical structure of purines and their analogues. A, hypoxanthine; B, adenine; C, 9-ethyladenine; D, caffeine (1,3,7-trimethylxanthine).

A.

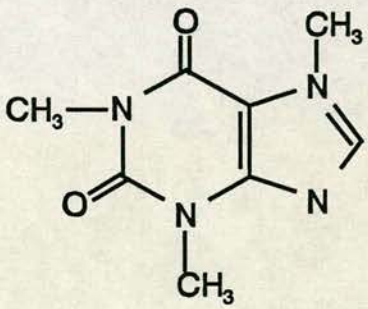


B.



C.

D.



cells under HAT selection was significantly blocked by treatment with 9-ethyladenine. The inhibition of growth by 9-ethyladenine which might be due to interference with APRT activity, is dose dependent (Table 5.2). In a short term (5 hr) *in vitro* assay, the incorporation of [^{3}H]-adenine into nucleic acids in both wild-type and HPRT-deficient mouse ES cells was reduced in the presence of 9-ethyladenine to 35% and 25% of the control value for wild-type and mutant cells respectively. However, the analogue had no effect on [^{3}H]-thymidine incorporation (Table 5.3), suggesting that it was indeed acting as an APRT inhibitor, rather than having a more general cytotoxic effect (Table 5.4). The inhibitor, 9-ethyladenine is shown to have a reversible cytostatic effect on wild-type ES cells by plating 50,000 cells/well to a microtitre plate in the presence of the analogue. Cells were left for 96 hours, with 9-ethyladenine being removed from wells at various times and replaced with ordinary medium, before the amount of growth in each well was monitored by [^{3}H]-thymidine incorporation into nucleic acids (Figure 5.2).

The cytostatic effect of 9-ethyladenine in the 96-hour assay was more pronounced on HPRT-deficient than wild-type ES cells (Table 5.5). [^{3}H]-thymidine incorporation in wild-type (E14) cells was reduced to 36% of the control value by incubation with 9-ethyladenine, while the reductions for two different HPRT-deficient ES cell lines, E14TG2a (18%) and DWMD1-16 (10%) were highly significantly lower. The data suggest that HPRT-deficient cells are more susceptible to 9-ethyladenine than the wild-type cells.

These results indicated that 9-ethyladenine was suitable for administration to mice to block APRT activity without exerting more general toxic effects. Its greater effect on HPRT-deficient cells was predicted from the purine metabolic pathway where the salvage of adenine by APRT and its subsequent conversion to IMP should compensate for the inability to produce IMP from hypoxanthine in HPRT-deficient cells.

Table 5.2 Inhibition of adenine induced rescue of HPRT-deficient ES cells in HAT selection medium by 9-ethyladenine.

Treatment	Growth[a]
Control	0.158
HAT	0.002
HAT + 10^{-4} M adenine	0.120
HAT + 10^{-4} M adenine + 10^{-4} M 9-ethyladenine	0.031
HAT + 10^{-4} M adenine + 10^{-5} M 9-ethyladenine	0.059
HAT + 10^{-4} M adenine + 10^{-6} M 9-ethyladenine	0.074
10^{-4} M 9-ethyladenine	0.123
10^{-5} M 9-ethyladenine	0.150
10^{-6} M 9-ethyladenine	0.162

a. Growth was the average of two samples measured at OD_{575nm} for the dye uptake by surviving cells. Two thousand cells per ml per well were plated into a 24-well microtitre plate and cultured in normal media for 48 hr before treatment. After 5 days of treatment, the cells were fixed and stained with crystal violet. The 70% ethanol extractions of retained dye were measured by spectrophotometry at 575 nm wavelength.

Table 5.3 The competitive effect of 9-ethyladenine on [^{3}H]-adenine incorporation in ES cells.

Cell line	9-ethyladenine	Incorporation (cpm)[a]	
		[^{3}H]-adenine	[^{3}H]-thymidine
E14	–	29,460 ± 4,936 (100%)	33,204 ± 6,859
E14	10^{-3} M	10,291 ± 1,688 (35%)[b]	33,130 ± 232
E14TG2a	–	43,038 ± 6,552 (100%)	20,106 ± 220
E14TG2a	10^{-3} M	11,071 ± 1,674 (25%)[b]	27,225 ± 287
DWMD1-16	–	N.D.[c]	37,790 ± 886
DWMD1-16	10^{-3} M	N.D.	38,557 ± 7,270

a. Incorporation (cpm) of [^{3}H]-adenine or -thymidine into cellular nucleic acid during a 5-hour incubation in the presence or absence of 10^{-3}M 9-ethyladenine.

b. Percentage incorporation in the presence of 9-ethyladenine relative to untreated control cells.

c. N.D., Not determined.

Table 5.4 The competitive effect of 9-ethyladenine on [^{3}H]-hypoxanthine incorporation in ES cells.

Cell line	9-ethyladenine	[^{3}H]-hypoxanthine uptake(cpm)
E14	-	122,042 ± 1,904 (100%)
	1 x 10^{-3} M	66,135 ± 4,184 (54%)
	2 x 10^{-3} M	41,369 ± 1,199 (34%)

a. Incorporation (cpm) of [^{3}H]-hypoxanthine into cellular nucleic acid during a 5-hour incubation in the presence or absence of 9-ethyladenine.

b. Percentage incorporation in the presence of 9-ethyladenine relative to untreated control cells.

Figure 5.2 Reversible cytostatic effect of 9-ethyladenine on ES cells. E14 cells in microtitre plates were incubated in 10^{-3}M 9-ethyladenine for the times indicated and then the medium was replaced with control medium. After 96-hr total incubation, cells were pulsed for 5 hr with [^{3}H]-thymidine and incorporation into cellular nucleic acids was determined. The bars indicate the standard deviation of measurement for each time point.

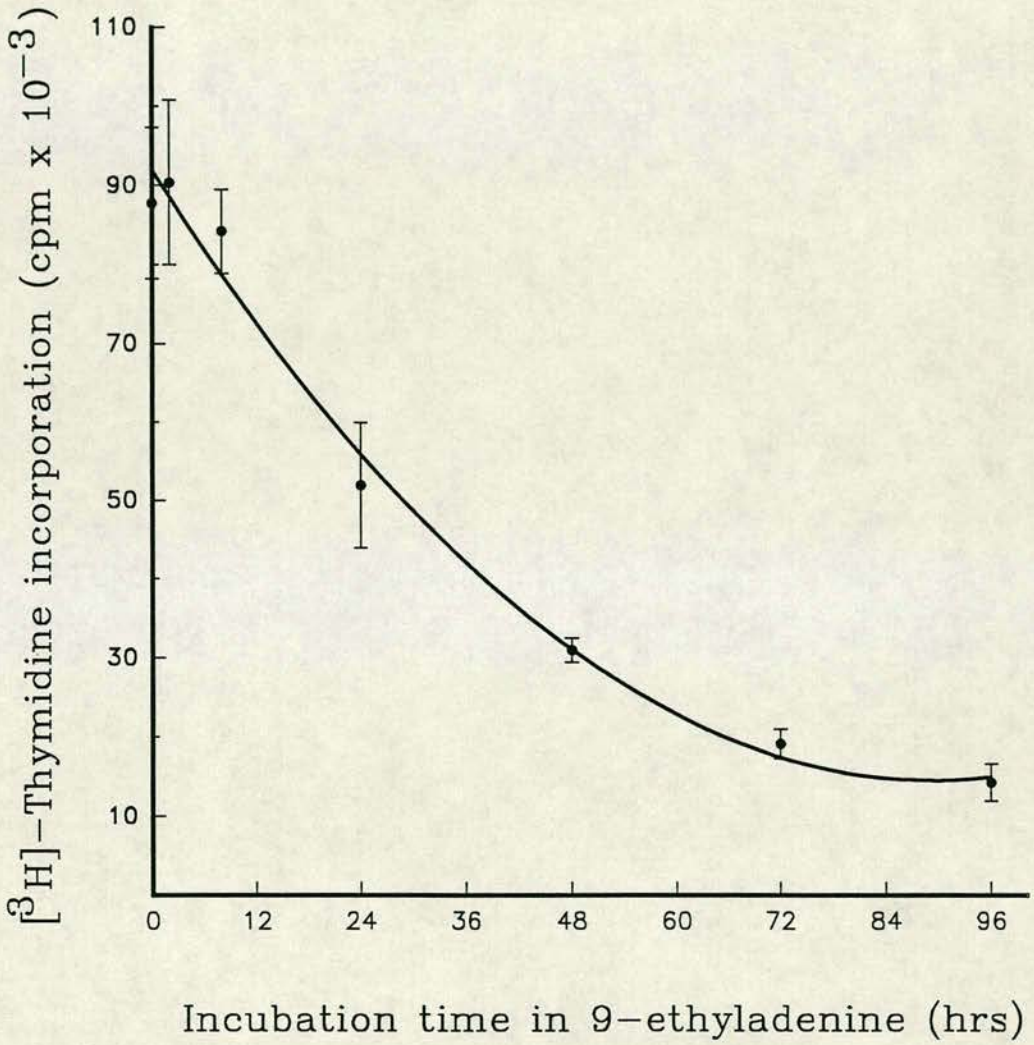

[3H]-Thymidine incorporation (cpm x 10-3)
110
90
70
50
30
10
0
12
24
36
48
60
72
84
96
Incubation time in 9-ethyladenine (hrs)

Table 5.5 The difference in susceptibility to the cytostatic effect of 9-ethyladenine in HPRT-deficient and wild-type ES cells.

Cell line	HPRT	Treatment	[^{3}H]-thymidine incorporation[a]
E14	+	medium	75,046 ± 20,988 (100%)
E14	+	9-ethyladenine	27,029 ± 4,651 (36%)
E14TG2a	–	medium	151,304 ± 8,995 (100%)
E14TG2a	–	9-ethyladenine	27,152 ± 9,478 (18%)**
DWMD1-16	–	medium	219,582 ± 12,798 (100%)
DWMD1-16	–	9-ethyladenine	22,993 ± 9,985 (10%)***

a. Incorporation of [^{3}H]-thymidine (cpm) into cellular nucleic acids in a 5-hr pulse following a 96-hr incubation in control medium or medium containing 10^{-3}M 9-ethyladenine. For each cell line, the percentage incorporation relative to the control culture is also shown.

** $p < 0.01$; ***, $p < 0.001$ compared with E14 cells treated with 9-ethyladenine by Student's *t*-test.

5.3 The effect of caffeine on purine salvage

High doses of caffeine were used to create the first animal model for Lesch-Nyhan syndrome. Self injury behaviour was observed in rats following oral administration of caffeine in a daily dose of 185 mg/kg body weight (Peters, 1967). Self injury behaviour was also noticed in rats intraperitoneally injected with another methylxanthine, theophylline, at 150 mg/kg body weight daily (Sakata and Fuchimoto, 1973). In addition, the methylxanthine effect on self injury behaviour could be enhanced by combined administration with clonidine (Razzak *et al.*, 1977). Although HPRT activity decreased in those rats which received drinking fluid containing 8 g/l caffeine, there was no clear explanation of the correlation between caffeine administration and self injury behaviour (Ferrer *et al.*, 1982). Caffeine was used as a comparative chemical to 9-ethyladenine for the purine incorporation assay. Caffeine caused a smaller reduction in both [^{3}H]-adenine and [^{3}H]-hypoxanthine incorporation, 67% of control levels for adenine compared to 56% for hypoxanthine (Table 5.6). In contrast, 9-ethyladenine inhibited [^{3}H]-adenine incorporation more specifically (35% of control levels) than [^{3}H]-hypoxanthine (54% of control levels) (Tables 5.3 and 5.4). Both HPRT and APRT activities were suppressed in those cells incubated with caffeine, but the inhibitory effect was stronger on HPRT than APRT. The high dose injection of caffeine into animals might cause the depletion of HPRT as well as a decrease of APRT activity. It has been shown that rodents have higher activity of APRT than that of HPRT while man has a higher HPRT activity (Leese *et al.*, 1991; Moore and Whittingham, 1992). Thus, the effect of reducing the APRT compensation in the purine nucleotide pool in rodent by the administration of caffeine was similar to that occurring in patients with Lesch-Nyhan syndrome. In humans, APRT does not play an important role in the purine nucleotide salvage pathway. When HPRT activity was nearly completely inhibited without adenylate pool compensation in animals by treatment with caffeine, then automutilation might be expected. The inhibitory effects of caffeine on HPRT and APRT suggest a possible biochemical mechanism for the animal model that produced self injury behaviour by administering high doses of caffeine to HPRT wild-type animals. In addition, caffeine prompted a smaller reduction in [^{3}H]-adenine incorporation than 9-ethyladenine, 67% of control compared

to 35%. The smaller inhibition by caffeine was correlated to the smaller effect on behaviour alteration in treated animals (see Chapter 6).

Table 5.6 The competitive effects of caffeine on the incorporation of [^{3}H]-hypoxanthine and [^{3}H]-adenine in E14 ES cells.

Caffeine	Incorporation (cpm)[a]		
	[^{3}H]-hypoxanthine	[^{3}H]-adenine	[^{3}H]-thymidine
–	143,374 ± 25,314 (100%)[b]	57,893 ± 415 (100%)	123,111 ± 8,461
10^{-3} M	80,371 ± 9,848 (56%)	38,529 ± 5,336 (67%)	141,020 ± 4,431

a. Incorporation (cpm) of [^{3}H]-hypoxanthine, -adenine or -thymidine into cellular nucleic acid during a 5-hour incubation in the presence or absence of 10^{-3}M caffeine.

b. Percentage incorporation in the presence of caffeine relative to untreated control cells.

CHAPTER 6. BEHAVIOURAL ALTERATION IN HPRT-DEFICIENT MICE

6.1 Decreased APRT activity in brain after treatment with 9-ethyladenine

To evaluate the effects of 9-ethyladenine on APRT activity in mouse brain, 6 HPRT-deficient male mice and 5 age-matched (6-8 week old) male wild-type strain 129 mice were injected with 9-ethyladenine or saline intraperitoneally. Three mice from each genotype were injected with 9-ethyladenine while three mice with HPRT-deficiency and 2 wild-type mice were treated with saline, intraperitoneally 4 times on alternate days. Mice were sacrificed after 7 days to analyse the [^{3}H]-adenine incorporation in the brain cells. After cervical dislocation, mouse brain cells were isolated immediately for analysis of [^{3}H]-adenine incorporation *in vitro*. To correct for the distribution of cell numbers in each sample, the relative activities were calculated as the ratio of the incorporation of [^{3}H]-adenine to the uptake of [^{14}C]-methionine. The incorporation of adenine in brain cells of HPRT-deficient mice was slightly higher than that of wild-type mice although the difference was not statistically significant. The results suggested that APRT activity could be slightly higher in HPRT-deficient mice to compensate for the effect of loss of HPRT activity on the nucleotide pool before drug treatment. This type of compensation has been observed in the erythrocytes of patients with Lesch-Nyhan syndrome (Seegmiller *et al.*, 1967). However, murine APRT activity in the brain of HPRT-deficient mice as measured by Allsop and Watts (1990) was normal. Thus, normal APRT levels may be sufficient to compensate for HPRT deficiency without increased activity in HPRT-deficient mice. The APRT activities were significantly decreased in both HPRT-deficient and wild-type mice after treatment with 9-ethyladenine (Table 6.1). The results suggest that the competitive inhibitor, 9-ethyladenine, of APRT could be used *in vivo* as well as *in vitro* for the blockage of the adenine salvage pathway.

6.2 Pilot study of 9-ethyladenine effect on behaviour alteration in outbred HPRT-deficient mice

To reduce endogenous APRT activity, 9-ethyladenine was administered initially to 5 (2 male and 3 female) outbred HPRT-deficient mice aged 9-12 months. Mice received 2.5 x 10^{-6} moles of 9-ethyladenine (0.25 ml of a 10^{-2} M solution dissolved in sterile normal saline). The analogue was given three times a week intraperitoneally

Table 6.1 The [^{3}H]-adenine incorporation activities in brains of wild-type and HPRT-deficient mice after treatment with 9-ethyladenine.

Strain	HPRT	Treatment	Relative activity[a]
129/Hprt^b/Y	+	saline	1.5840 ± 0.2647
129/Hprt^b/Y	+	9-ethyladenine	1.3055 ± 0.1148*
129 hprt$^{b\text{-}m3}$/Y	–	saline	1.6460 ± 0.2384
129 hprt$^{b\text{-}m3}$/Y	–	9-ethyladenine	1.4016 ± 0.1421*

a Brains were mechanically chopped into small pieces and cultured in medium with 1 μCi of [^{3}H]-adenine and [^{14}C]-methionine for 20 hr. The relative APRT activity was the ratio of the [^{3}H]-adenine incorporation to the [^{14}C]-methionine incorporation by TCA precipitation method.

* $p < 0.05$, compared with the group treated with saline by Student's *t*-test.

between 5 and 20 times in total. All 5 animals developed trauma caused by self mutilation from 48 to 130 days after the injections were started. Injury, which was principally to the ears, neck, back and flanks (Figure 6.1) was caused by overgrooming. These encouraging results provided the confidence to set up more control animal experiments to investigate behavioural alteration in HPRT-deficient mice.

6.3 Administration of purine analogues to mice and measurement of self injury behaviour

Strain 129 inbred wild-type and HPRT-deficient male mice, 6-8 weeks old, were caged individually and maintained on a 12 hr light/dark cycle. The same injection regime described above was used with animals being given saline, 9-ethyladenine or caffeine. To make video recordings, mice were transferred to a clear cage with bedding but lacking food and water for 10 mins before treatment. Recording was initiated 10 mins after the animals had been returned to the cage following injection and continued for 1 hr period. There were two mice in each treatment group and the behaviour of each animal was determined on three separate occasions. All animals were monitored weekly for the appearance of physical injury caused by overgrooming. The frequency of self injury behaviour was determined from video recordings. The definition of self injury behaviour included grooming carried out with fore or hind legs, nibbling and biting (principally of the tail and thorax). One piece of self injury behaviour could include all three activities and would be counted once only unless the period of self injury behaviour was interrupted by another activity such as movement around the cage. Sophisticated observational methods to examine the effects of different agents on discrete forms of stereotypic behaviour in rodents have been developed (Breese *et al.*, 1984; Fray *et al.*, 1980; Lewis *et al.*, 1985). The measurements of stereotypic behaviour here were less extensive and were restricted to identifying changes in the frequency of the three forms of behaviour, grooming, biting and nibbling that were responsible for the self-inflicted injuries observed. Of these, grooming of the head and flanks with the hind legs was the major cause of injury. It is tempting to equate the compulsive overgrooming observed in

Figure 6.1 Injuries to ears and neck caused by self-mutilation behaviour in outbred HPRT-deficient mice. Left, animal treated with 9-ethyladenine; right, untreated control.

these animals with the self-mutilation that is a characteristic of Lesch-Nyhan syndrome: all mice groom, just as many humans nibble finger nails and lick their lips. In HPRT-deficient mice treated with 9-ethyladenine and Lesch-Nyhan syndrome patients, the stereotypic behaviour becomes excessive resulting in physical injury.

The frequency of self injury behaviour in the pilot group of treated outbred animals was up to 4 per minute as averaged from 3 separate observations, with individual bouts of self injury behaviour lasting up to 2 minutes. Behavioural alteration has also been recorded in the second experiment using younger (6-8 week old) wild-type and HPRT-deficient male inbred mice on the strain 129 background (Table 6.2). There was no difference in the frequency of self injury behaviour between wild-type and HPRT-deficient mice injected with saline. There is no difference in the occurrence of stereotypic behaviour after saline injection both in wild-type and HPRT-deficient mice (data not shown). Treatment of wild-type mice with caffeine did not cause a significant increase in the frequency of self injury behaviour, but 9-ethyladenine did cause a significant increase (0.11 to 0.32/min, $p<0.05$). The observation that behaviour alteration did not occur in those mice administered with low doses of caffeine, is in agreement with the threshold setting of caffeine, 140 mg/kg, for the induction of self injury behaviour by clonidine in rats (Mueller and Nyhan, 1983). Both purine analogues had a more pronounced effect on self injury behaviour in the HPRT-deficient mice: caffeine caused an increase in frequency from 0.13 to 0.34/min ($p<0.05$) and 9-ethyladenine had the greatest effect (1.21/min, $p<0.01$). These increases in the frequency of self injury behaviour were observed after the first treatment with the purine analogue and the increased frequency was maintained over the treatment period (3 times/week for 5 weeks). Following treatment, 4 out of 5 HPRT-deficient animals treated with 9-ethyladenine displayed physical signs of overgrooming damage to the ears and neck. None of the other groups of animals showed signs of physical injury (Table 6.3). The injury in these young HPRT-deficient mice treated with 9-ethyladenine, which was observed from 61-105 days after treatment was started, was less severe than in the older group of treated HPRT-deficient animals.

Table 6.2 The occurrence of self-injurious behaviour in wild-type and HPRT-deficient mice after injection with 9-ethyladenine or caffeine.

Strain	HPRT	Treatment	SIB/min***
129/Hprt^{b}/Y	+	saline	0.11 ± 0.14
129/Hprt^{b}/Y	+	caffeine	0.15 ± 0.06
129/Hprt^{b}/Y	+	9-ethyladenine	0.32 ± 0.18*
129 hprt$^{b\text{-}m3}$/Y	–	saline	0.13 ± 0.06
129 hprt$^{b\text{-}m3}$/Y	–	caffeine	0.34 ± 0.11**
129 hprt$^{b\text{-}m3}$/Y	–	9-ethyladenine	1.21 ± 0.42**

* $p < 0.05$; **, $p < 0.01$ compared with the group treated with saline by Mann-Whitney *U*-test.

*** $p < 0.001$ by Kruskal-Wallis *H*-test.

Table 6.3 Observed injury of mice due to self injury behaviour after injection with 9-ethyladenine.

Strain	HPRT	Treatment	Incidence*
129/Hprt^{b}/Y	+	saline	0 / 3
129/Hprt^{b}/Y	+	9-ethyladenine	0 / 5
129 hprt$^{b\text{-}m3}$/Y	–	saline	0 / 3
129 hprt$^{b\text{-}m3}$/Y	–	9-ethyladenine	4 / 5

* $p < 0.05$ by Kruskal-Wallis *H*-test.

Administration of an APRT inhibitor to HPRT-deficient mice induced persistent self-injurious behaviour, providing an animal model for Lesch-Nyhan syndrome. This animal model will facilitate better understanding of the pathogenesis of Lesch-Nyhan syndrome and provide an evaluation system for its therapy.

CHAPTER 7. GENERATION OF APRT-INACTIVATED ES CELLS

7.1 Spontaneous APRT mutation in ES cells

The final test of the hypothesis that the effect of HPRT-deficiency on the nucleotide pool is compensated by APRT, leading to the unaltered behaviour in HPRT-deficient mice, is to create an APRT-deficient animal model. The techniques by which embryonic stem cells are used to construct animal models have been well established. Like the selective effect of 6-thioguanine for HPRT deficiency, 1,6-diaminopurine kills those cells with APRT activity by conversion of 1,6-diaminopurine to a toxic product. The usage of 1,6-diaminopurine as a selective agent to isolate APRT-deficient cells has been widely employed (Turker, 1990). Cloned genomic DNA for gene targeting at the APRT locus was not available when this project was initiated. The only way to create APRT-deficient mice, therefore, was to select spontaneous mutations leading to APRT-deficient ES cells from which chimaeric animals could be generated following blastocyst injection. Selection for spontaneous mutations was used to avoid mutations in other loci that might be induced by mutagens. After gradually increasing the concentration of 1,6-diaminopurine to 50 μg/ml, 2 resistant clones were selected. These two clones were examined for their APRT activity using a [^{3}H]-adenine incorporation assay (Table 7.1). Both clones showed only trace incorporation of [^{3}H]-adenine compared to their parental cell line. The trace amount of adenine incorporation might be due to the incorporation of [^{3}H]-hypoxanthine which is generated from [^{3}H]-adenine by adenase. Therefore, these two clones were identified as APRT-deficient clones. One of the APRT-deficient ES cell clones, DAP1-50, was used in an attempt to produce APRT-deficient mice by making chimaeric animals through blastocyst injection and standard breeding procedures. Chromosomes of the mutant DAP1-50 cells were counted before undertaking blastocyst injection. As the majority of solid stained chromosome preparations contained 40 chromosomes, there was no obvious cytogenetic defect (data not shown). Southern analysis showed that there was no difference between wild-type and DAP1-50 mutant DNA in the restriction patterns of HindIII, PstI, or EcoRV digests probed with a BglII/SphI fragment which contains the whole murine APRT genomic sequence and 5′ region (data not shown). Unfortunately, no chimaeric mice were generated from the injection of 63 blastocysts which was kindly carried out by Jim McWhir,

Table 7.1 Hypoxanthine and adenine incorporation activities in wild-type and APRT mutant cells.

Cell line	Incorporation (cpm)		Corrected adenine uptake
	[^{3}H]-Hypoxanthine	[^{3}H]-Adenine	%
E14	149,327 (100%)[a]	68,974 (100%)	100[b]
DAP1-50	120,943 (81%)	1,494 (2.1%)	2.6
DAP1-40	84,512 (57%)	5,720 (8.3%)	14.6

a. % of the incorporation of mutant cells to that of E14 cells. Cells were plated at 20,000 cells/ml/well and pulsed with [^{3}H]-hypoxanthine or -adenine on day 2 for 5 hr.

b. Incorporation of [^{3}H]-adenine expressed as a percentage of that of [^{3}H]-hypoxanthine to correct for differences in cell number.

suggesting that this clone had lost the potential to contribute to normal development.

7.2 Disruption of APRT in ES cells by gene targeting

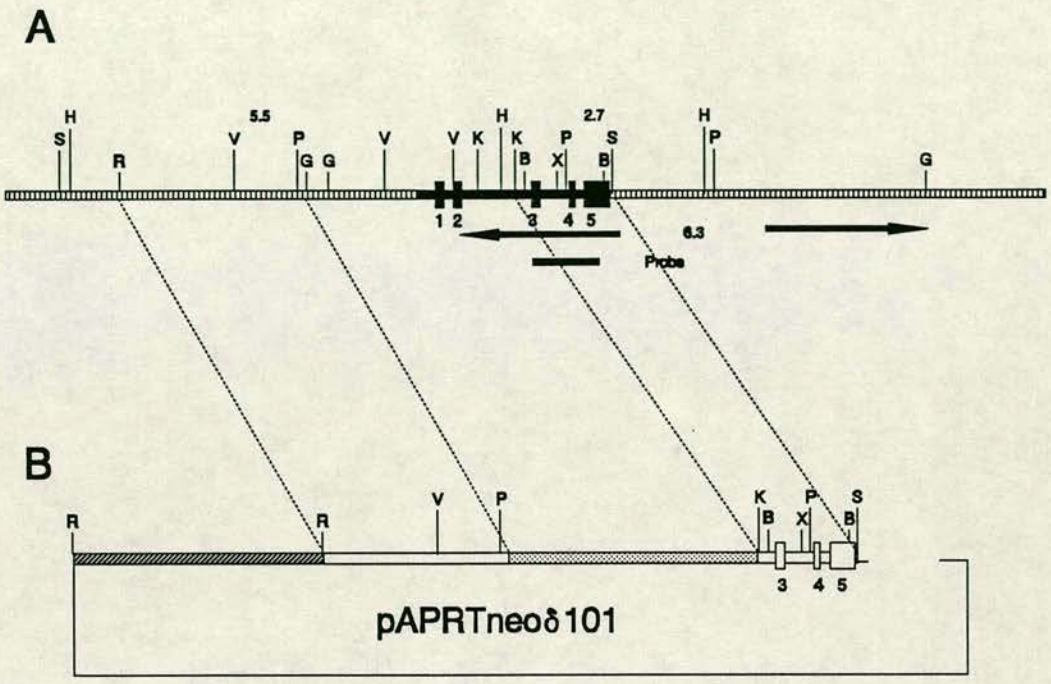
7.2.1 Construction of the targeting vector

A 6.3-kb EcoRI/SalI restriction fragment containing the whole 3.2-kb murine APRT gene and 5′ flanking region from plasmid pSAM6.3, kindly provided by Professor Stambrook (University of Cincinnati, U.S.A.), was subcloned into plasmid pUC8 to generate the plasmid pUCSAM6.3. A 2.4-kb *neo* cassette driven by the mouse metallothionein-I promoter was excised as a KpnI/BamHI fragment to replace the region between the KpnI and BglII sites in pUCSAM6.3 indicated in Figure 7.1 where it served as a positive selection module. An HPRT mini-gene driven by the PGK promoter which had been shown to give good enrichment as a negative marker in positive-negative selection (Selfridge *et al.*, 1992), was inserted into the unique EcoRI site to form the targeting vector. The final structure of the targeting vector, pAPRTneoδ101, includes pUC8, HPRT mini-gene cassette, 2.4-kb 5′ homology, *neo* cassette, and 1.3-kb 3′ homology in order from 5′ to 3′. Vector DNA was linearised with the restriction enzyme SalI before electroporating into the HPRT-deficient ES line, HM-1 (Magin *et al.*, 1992b).

7.2.2 Gene targeting to knock out APRT

Two gene targeting experiments using pAPRTneoδ101 to inactivate the APRT gene in murine ES cells were performed. In the first experiment, 200 μg of pAPRTneoδ101 DNA, linearised with SalI were electroporated into HM-1. To avoid interference by residual APRT, electroporated cells were selected with G418 and 6-thioguanine for 10 days before the addition of 1,6-diaminopurine. No resistant clone was obtained following selection with 1,6-diaminopurine. This result supports the view that it is difficult to generate null mutations at both autosomal loci by homologous recombination in one DNA introduction (Cruz *et al.*, 1991). The second experiment sought to inactivate only one APRT allele using the HPRT mini-gene for negative selection. After introduction of DNA, cells were plated onto dishes and dishes were allocated to 3 groups. One group served as the electroporation control

Figure 7.1 Strategy for APRT inactivation by homologous recombination. The structures of (A) the wild-type APRT gene and flanking region, (B) the APRT inactivation vector with positive-negative selection modules, and (C) predicted targeting mutant are shown schematically. The number of each exon is shown directly below it. Selected restriction sites are shown: B, BamHI; G, BglII; H, HindIII; K, KpnI; P, PstI; R, EcoRI; S, SphI; V, EcoRV; X, XhoI. Closed boxes, endogenous exons; thick closed lines, endogenous introns; hatched boxes, HPRT-minigene cassette; open boxes, vector-derived APRT and its flanking sequence; vertically striped line, APRT-flanking region; thin line, plasmid pUC8 sequence; dotted boxes, *neo* cassette; bar, BamHI fragment of APRT gene used as a probe for Southern and Northern blots; arrow, shows the length of EcoRV/BglII fragment.


A
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S
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5.5
V
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G G
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V K
K
B
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1 2
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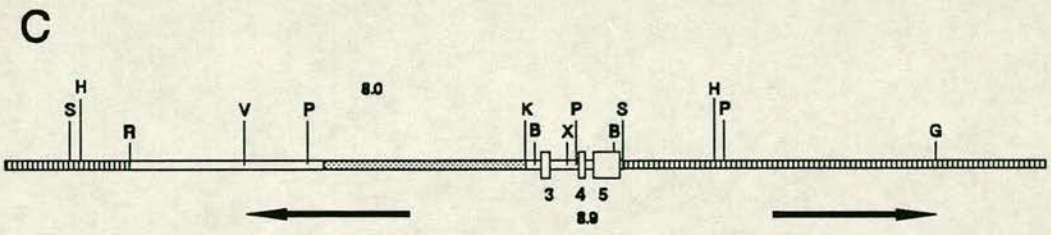
3 4 5
pAPRTneoδ101


C
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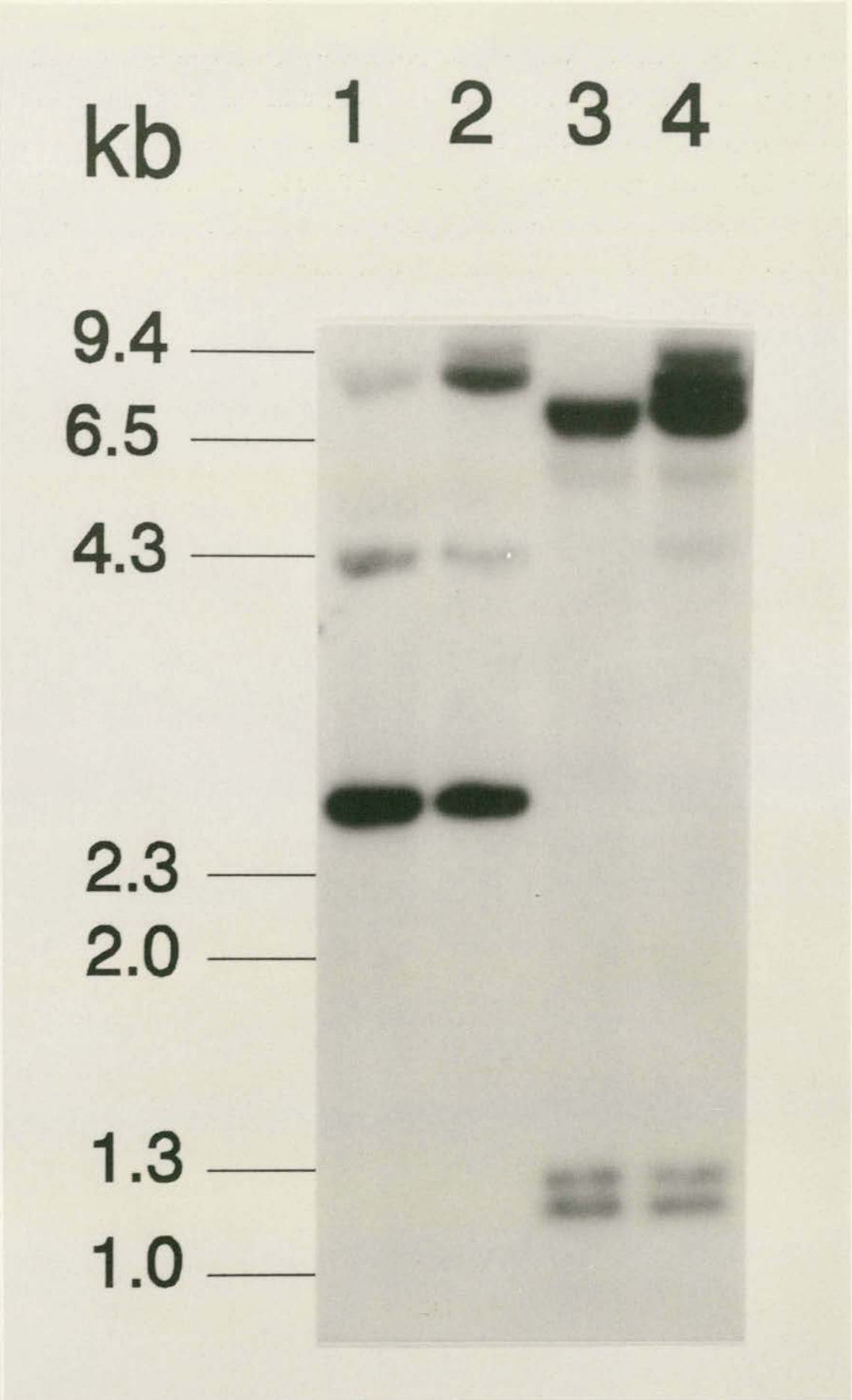
8.9

under G418 selection alone. The other two groups were also counter-selected with 6-thioguanine either concomitantly with G418 selection (group 2) or following a 2-day time lag (group 3). On average there were 904 G418 resistant colonies obtained from 5 X 10^6 cells electroporated with linearised pAPRTneoδ101 DNA. Counter selection with 6-thioguanine enriches for the percentage of surviving colonies that have lost the HPRT mini-gene through homologous recombination at the target locus. An average of 77 resistant colonies were obtained from 5 X 10^6 electroporated cells after positive and negative selection in group 2. The yield of resistant colonies was slightly higher in group 3, with 116 surviving clones. The 6-thioguanine selection schemes indicated a potential enrichment of between 7.8 and 11.7 fold for homologous recombinants. Initially, clones resistant to both G418 and 6-thioguanine were screened for adenine/thymidine incorporation ratio by [^{14}C]-adenine/[^{3}H]-thymidine dual incorporation assay. None of the 124 clones analysed showed significant decrease of adenine incorporation. Southern analysis was then used to screen for targeted clones. After screening 42 clones by Southern hybridisation analysis, one clone, APRT-26 was identified as a targeted mutant.

7.2.3 Characterisation of the targeted ES clone APRT-26

The predicted outcome of homologous recombination between the vector pAPRTneoδ101 and wild-type HM-1 DNA is shown in Figure 7.1. In the targeted allele, the 5.5-kb HindIII fragment containing promoter region and exons 1-2 is extended to 8.0 kb by insertion of the 2.5-kb *neo* module and deletion of an HindIII site. The restriction pattern of APRT-26 DNA which was probed with a 1.1-kb BamHI fragment containing exons 3-5, fits this prediction, giving a novel 8.0-kb band which has the same intensity as the residual 2.7-kb band presumably derived from the non-targeted allele. Further confirmation was done by restriction with EcoRV and BglII, extending a 6.3-kb fragment to 8.9 kb by loss of 2 EcoRV and 2 BglII sites in the 2.7-kb deletion region (Figure 7.2). These digests, in addition to others, with PstI and SphI (data not shown) were all compatible with APRT-26 being a targeted mutant. To further characterise this mutant, Northern hybridisation analysis and incorporation assays were undertaken. The targeting vector had been constructed with the

Figure 7.2 Southern analysis of APRT targeted mutant and wild-type cells. Genomic DNA samples from APRT wild-type parental HM-1 cells (lane 1,3), and targeted mutant by pAPRTneoδ101 (lane 2,4) were restricted with HindIII (lane 1,2) or EcoRV/BglII (lane 3,4), electrophoresed, transferred and hybridised with the BamHI genomic fragment containing exons 3-5 as probe. Molecular size markers are indicated in kb. Some weak additional bands including pseudogene and unknown fragments are also observed.


kb
1
2
3
4
9.4
6.5
4.3
2.3
2.0
1.3
1.0

assumption that deletion of promoter and exons 1 and 2 of the APRT gene would prevent APRT expression. A Northern blot of wild-type and targeted cell RNA was analysed using the same probe used in Southern analysis. RNA samples for the Northern hybridisation were kindly prepared by Jim Selfridge. RNA samples were standardised by spectrophotometry and confirmed by the presence of equal amounts of 18 S and 28 S ribosomal RNA when electrophoresed. As expected, the targeted clone shows a decrease in intensity of APRT mRNA compared to HM-1 cells (Figure 7.3). Surprisingly on reprobing the blot, the targeted mutant APRT-26 also displays a reduction of actin transcripts. To make sure that the APRT targeted ES cells had lower APRT activity than wild-type cells, the adenine incorporation of these cells was analysed. To avoid experimental error due to differences in cell numbers, a dual incorporation assay for APRT was used to examine the difference in activity between targeted mutant and wild-type cells. Adenine incorporation activity in HPRT-deficient cells, HM-1, is not statistically higher than that in wild-type cells, E14. However, the adenine uptake in APRT targeted cells (APRT-26) is significantly lower than that in HM-1 or E14 cells (Table 7.2). Unfortunately no chimaeric mice were produced following repeated blastocyst injection of the APRT-targeted cells, so it was not possible to confirm the essential role of APRT in prevailing the appearance of symptoms of Lesch-Nyhan syndrome in mice.

Figure 7.3 Northern hybridisation analysis comparing APRT mRNA levels in APRT targeted (lane 1,3) and wild-type (lane 2,4) ES cells. Total RNA (30 μg) was prepared from ES cells, electrophoresed on formaldehyde-agarose gels, transferred, and hybridised with the BamHI genomic fragment containing exons 3-5 of the APRT gene, and subsequently with the actin cDNA probe.


1 2 3 4
APRT
actin

Table 7.2 Dual incorporation to identify gene inactivation.

Cell line	Incorporation (dpm)		Relative adenine incorporation[a]
	[^{3}H]-Thymidine	[^{14}C]-Adenine	
E14	162,252 ± 6,454	74,895 ± 2,457	0.4618 ± 0.0056
HM-1	245,818 ± 17,531	113,392 ± 5,115	0.4622 ± 0.0139
APRT26	82,847 ± 6,167	36,108 ± 2,308	0.4362 ± 0.0078*

a Incorporation of [^{14}C]-adenine expressed as a proportion of that of [^{3}H]-thymidine to correct for differences in cell number.

* $p < 0.05$, comparing the relative adenine incorporation of APRT26 with that of its parental cells, HM-1, by Student's *t* -test.

CHAPTER 8. GENE THERAPY IN HPRT-DEFICIENT MICE

8.1 Establishment and evaluation of assay methodology

Advances in understanding the molecular basis of inherited disease have made it theoretically feasible to develop therapies based on gene transfer. Because HPRT is expressed in the brain, HPRT-deficient mice provide a good general animal model for human neurological inherited disease. In order to estimate the HPRT activity in tissues following gene transfer, a more accurate method providing an internal standard was developed to compensate for errors in sampling. The biosynthesis of purine nucleotides is important in cells whether they are proliferative or not, a dual incorporation assay based on the balance of the requirement for AMP and GMP was established. A mouse strain containing a non-X chromosome linked HPRT transgene on the HPRT-deficient background was used to test the assay. The transgene is expressed at around 5% of the level of the endogenous HPRT gene in most tissues examined (Thompson, 1989). Mice heterozygous for the transgene were crossed with HPRT-deficient mice and F1 progeny were examined. Half were expected to have inherited the transgene. One male and 3 female F1 mice were sacrificed to examine the accuracy of the hypoxanthine/adenine dual incorporation assay to identify HPRT expression before animals were genotyped by Southern hybridisation from tail biopsy. Two of the four mice showed positive HPRT activities in both brain samples and in splenocytes stimulated with the lymphocyte mitogen - concanavalin A, whereas the other two mice lacked HPRT activities in both tissues (Table 8.1). These results suggest that two of the four transgenic mice were HPRT positive due to inheritance of the HPRT transgene while the others were HPRT-deficient. Southern analysis confirmed this prediction (Figure 8.1). Therefore, these results suggest that the hypoxanthine/adenine dual incorporation assay is a suitable functional assay for HPRT activity, even for those highly differentiated tissues, such as brain.

8.2 Gene therapy by direct gene injection

Gene therapy is a form of medical intervention based on modification of the genetic program of living cells. There are two basic strategies for gene therapy. Cells may either be altered *in vivo* by gene therapy administered directly to the individual, or alternatively modified *ex vivo* for subsequent transplantation to humans. The

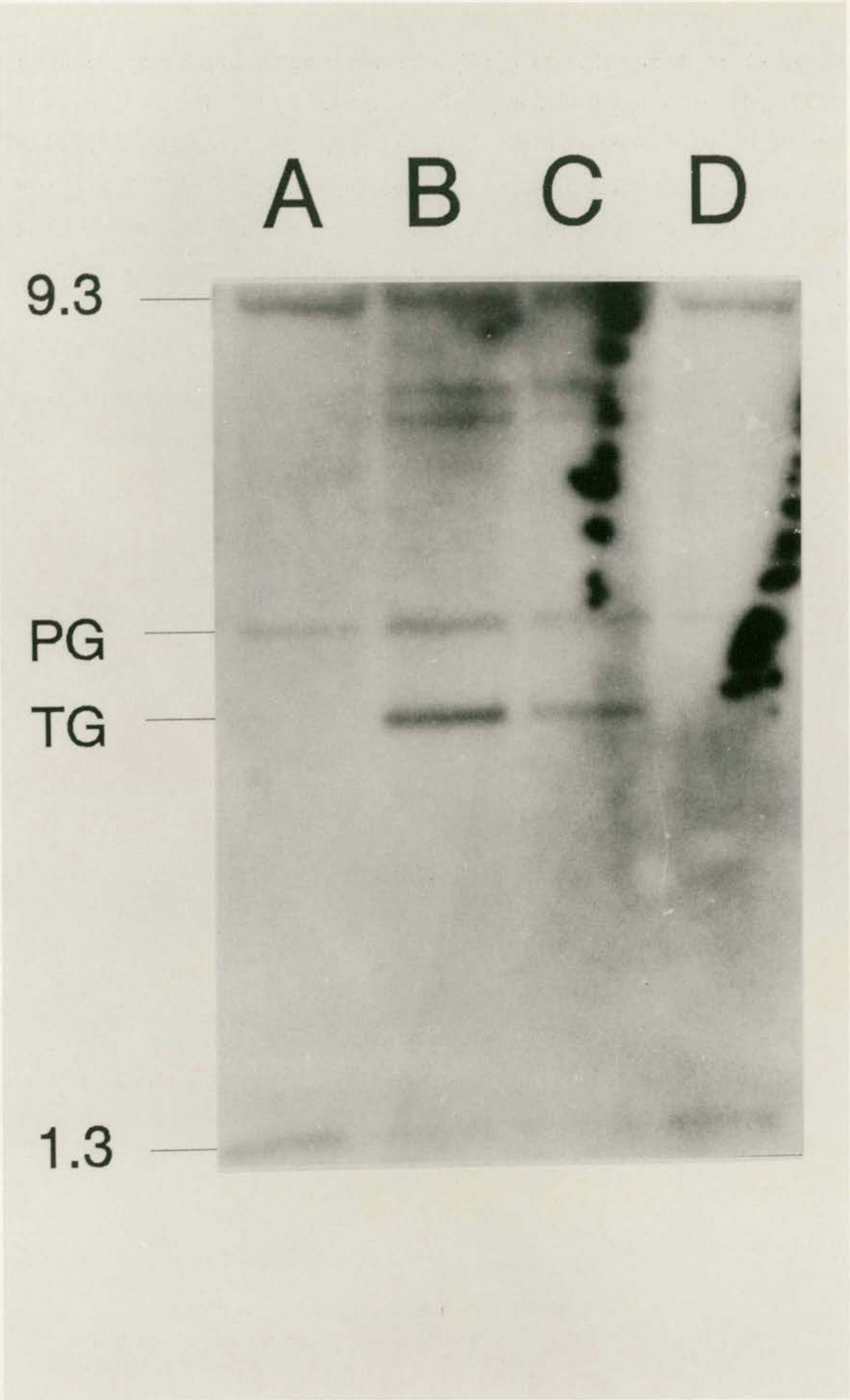
Table 8.1 The incorporation of [^{3}H]-hypoxanthine and [^{14}C]-adenine in the brains and spleens of HPRT deficient mice before genotyping for the presence of the HPRT transgene.

Mouse	Incorporation ratio of Hypoxanthine to Adenine*	
	Brain	Spleen**
A	0.05 ± 0.00	0.05 ± 0.02
B	0.42 ± 0.19	0.47 ± 0.01
C	0.42 ± 0.20	1.49 ± 0.17
D	0.09 ± 0.02	0.02 ± 0.00

* Cells in microtitre plates were pulsed with 2 μCi [^{3}H]-hypoxanthine and 0.5 μCi [^{14}C]-adenine per ml for 30 hrs.

** Splenocytes were stimulated with 4 μg/ml concanavalin A *in vitro*.

Figure 8.1 Southern analysis to identify mice containing the HPRT transgene on the HPRT-deficient background. Genomic DNA obtained by tail biopsy, was digested with EcoRI , electrophoresed, transferred and hybridised to a full-length HPRT cDNA probe, pHPT5. The sizes (in kb) of endogenous gene fragments and the fragment containing the HPRT pseudogene (PG) are indicated to the left of the panel. A fragment containing HPRT transgene sequences is indicated (TG). Mouse A, B, C, D, progeny from cross, see Table 8.1.


A B C D
9.3
PG
TG
1.3

simplest strategy would be to introduce desired DNA into the individual directly. Several virus vectors have been developed to do this and have been optimised for both efficiency and target specificity. Mice were infected with recombinant herpes simplex virus type 1 carrying human HPRT cDNA by intracranial inoculation to evaluate the expression of human HPRT mRNA. Although HPRT mRNA was detected in infected animals, the mortality of the infected mice was very high (Palella *et al.*, 1989). It could be less complicated and less time-consuming to introduce DNA directly by needle injection (Acsadi *et al.*, 1991), particle bombardment (Yang *et al.*, 1990), or jet injection (Furth *et al.*, 1992), compared to the modification of a recombinant virus vector to reduce its pathogenicity. Acsadi *et al.* (1991) successfully detected human dystrophin expression in *mdx* mice after direct intramuscular injection of DNA. Like skeletal muscle, brain is highly organised and stable. The nuclei of mature brain cells do not undergo division. Three HPRT-deficient mice were therefore injected intracerebrally (one side only) with 100 μg of HPRT mini-gene, pDWM100 DNA, after anaesthetisation. Seven and ten days later, the brains were isolated for the dual incorporation assay and a PCR assay to monitor the fate of the injected DNA. Injected brains contained pUC8 sequences as demonstrated by the PCR method (data not shown). Unfortunately, the results from the dual incorporation did not show any significant increase of [^{3}H]-hypoxanthine incorporation following the injection with the HPRT mini-gene (Table 8.2).

8.3 ES cells as a delivery system for gene therapy

Many reports have demonstrated the potential of cell transplantation involving manipulation of a gene *ex vivo* followed by reimplantation of the cells back to the individual to achieve gene therapy. One of the main targets for gene therapy is the haematopoietic system because of well developed procedures for bone marrow transplantation, the many types and wide distribution of haematopoietic cells, and the existence of many diseases that affect haematopoietic cells. The target for gene transfer is haematopoietic stem cells, or long-term repopulating cells, that are present at low frequency in bone marrow and give rise to all myeloid and lymphoid cells over prolonged periods. The first human gene therapy trial involved treatment of adenosine

Table 8.2 [^{3}H]-hypoxanthine and [^{14}C]-adenine incorporation in the brains and spleens of HPRT-deficient mice intracerebrally injected with HPRT minigene DNA.

Mice	Treatments	Incorporation ratio*	
		Brain	Spleen**
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Control	0.05 ± 0.01 (whole brain)	0.02 ± 0.00
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected	0.06 ± 0.01 (whole brain)	0.03 ± 0.02
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected	0.04 ± 0.02 (injection half) 0.04 ± 0.00 (non-injection half)	0.02 ± 0.00
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected	0.06 ± 0.01 (injection half) 0.06 ± 0.01 (non-injection half)	0.02 ± 0.00

* Cells were pulsed with 2 μCi [^{3}H]-hypoxanthine and 0.5 μCi [^{14}C]-adenine per ml for 30 hr. Incorporation ratio: uptake of hypoxanthine/ uptake of adenine.

** Splenocytes were stimulated with 4 μg/ml concanavalin A *in vitro*.

deaminase (ADA) deficiency by transfer of the ADA gene into patients's T-cells (the cells that are most affected by this disorder). ADA deficiency leads to high levels of 2′-deoxyadenosine in the circulation which is toxic to both T and B cells and results in severe combined immunodeficiency. ADA deficiency is a lethal disorder that can be corrected by bone marrow transplantation, but unfortunately only about one third of such patients have suitably matched donors. The disease can also be partially corrected by weekly injections of bovine ADA that has been conjugated to polyethylene glycol to improve stability resulting in increase of the reduced systemic 2′-deoxyadenosine levels and a marked disease improvement. It is becoming clear, in part from this ongoing gene therapy trial, that it is important to produce ADA within T cells for more complete correction of the disease phenotype. Fibroblasts isolated from skin biopsy of rat were transformed with human growth hormone DNA and reimplanted into the donor. Human growth hormone was detectable in the serum of rats with autologous transformed fibroblast implants (Chang *et al.*, 1990). Furthermore, normal dystrophin transcripts were detected in Duchenne muscular dystrophy patients after myoblast transplantation (Gussoni *et al.*, 1992). Unfortunately, the methodology for isolating neuron stem cells from adult brain has not been available until recently (Richards *et al.*, 1992). Thus, other vehicle cells are needed for gene transfer in neuron-associated diseases.

Embryonic stem cells are pluripotent cell lines that are isolated from blastocysts and maintained in an undifferentiated state. Differentiation of ES cells can be prevented either by culturing the ES cells on feeder cells or in the presence of leukaemia inhibitory factor which can be provided in BRL conditioned media or by supplementation with recombinant protein. The differentiation activity of pluripotent embryonic stem cells can be induced either *in vitro* by some chemicals such as dimethylsulphoxide and retinoic acid or *in vivo* by the contribution to embryogenesis after blastocyst injection. In addition, the methodology for genetic manipulation of ES cells is well established not only for gene targeting (Thompson *et al.*, 1989) but also for transgenesis (Gossler *et al.*, 1986). Provided that differentiation can occur in somatic sites other than the blastocoel, ES cells might be used for somatic cell gene

therapy much as they are used in germ line correction.

To investigate this possibility four HPRT-deficient mice were transplanted with 10^5 E14 embryonic stem cells in 20 μl glucose-PBS by intracerebral injection into the brain under anaesthetia. All treated mice recovered from anaesthesia and were sacrificed after several days in order to test HPRT activity in their brains. HPRT activity was detectable on the 7th day (the first sample point) after ES cell transplantation and increased with time (Table 8.3). The increase of HPRT activity in the brain of HPRT-deficient mice suggests proliferation of transplanted ES cells which express HPRT. However, the mice injected with 10^5 ES cells died as a result of tumourigenesis in their brains after 14 days. Teratoma-like tissue was also found in mice after intraperitoneal injection with 5 X 10^7 ES-D3 embryonic stem cells (Sendtner *et al.*, 1992). When 10^3 ES cells were injected intracerebrally into mice, 2 out of 3 mice remained healthy for over 2 months after injection. Tumour incidence seems to be directly related to injected cell number, suggesting that a proportion of injected cells remain undifferentiated rather than differentiating in response to the environment *in vivo*.

8.4 ES cell derivatives are committed to differentiate into the neural lineage.

The neuronal and glial elements of the central nervous system are generated from precursor cells in the neuroepithelium during early development, see Figure 8.2. The fertilised mouse zygote undergoes three indeterminate "cleavage divisions" after which cells become closely apposed, compact to form tight junctions, and become radially polarised with respect to apical microvilli and ligand binding sites and basal nuclei. Concomitant with cavitation, compacted morulae enter the uterus on day 3 and very quickly undergo their first differentiation into inner cell mass (ICM) and trophectoderm. This yields a vesicular, fluid filled blastocyst. Commitment to the ICM lineage is thought to result from an inner location involving complete cell surface apposition. Delamination of primary endoderm from the blastocoelic surface of the ICM occurs on day 4. The precocious mouse blastocyst forms an egg cylinder by day 4 (composed of primary ectoderm, proximal endoderm and extraembryonic ectoderm).

Table 8.3 [^{3}H]-hypoxanthine and [^{14}C]-adenine incorporation in the brains and spleens of mice after intracerebral transplantation with ES cells.

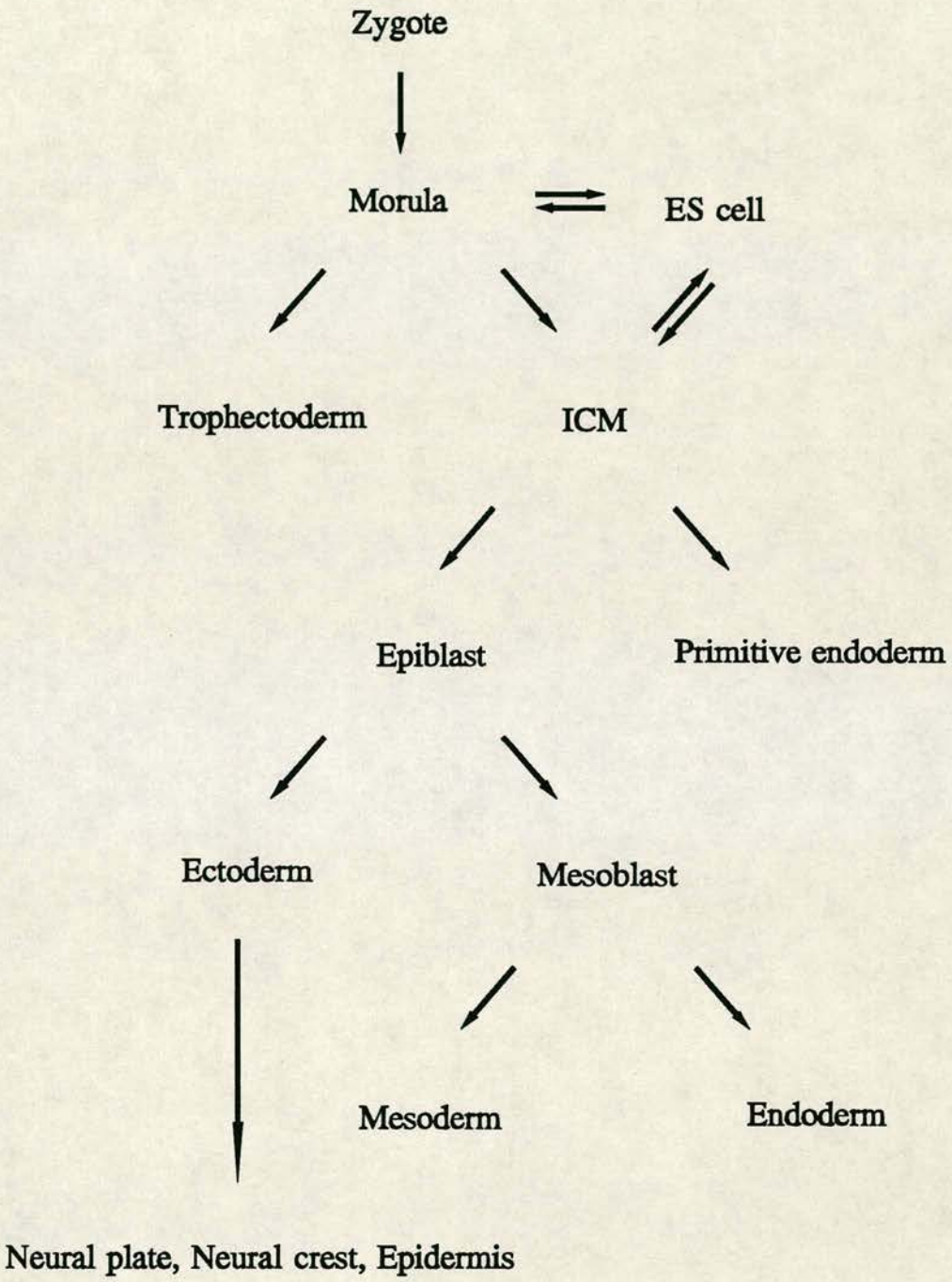
Mice	Treatment	Incorporation ratio*	
		Brain	Spleen**
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	HPRT-deficient uninjected control	0.05 ± 0.01 (whole brain)	0.02 ± 0.01
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected 7 days***	0.12 ± 0.04 (injection half) 0.11 ± 0.01(non-injection half)	N.D.
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected 10 days	0.50 ± 0.16 (injection half) 0.57 ± 0.14 (non-injection half)	N.D.
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected 10 days	0.48 ± 0.08 (injection half) 0.21 ± 0.05 (non-injection half)	N.D.
129 $hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$	Injected 14 days	0.39 ± 0.02 (injection half) 0.47 ± 0.13 (non-injection half)	0.02 ± 0.02
129 $HPRT^{b}/HPRT^{b}$	Wild-type uninjected control	0.56 ± 0.04 (whole brain)	0.47 ± 0.01

* Cells were pulsed with 2 μCi [^{3}H]-hypoxanthine and 0.5 μCi [^{14}C]-adenine per ml for 30 hr. Incorporation ratio: uptake of hypoxanthine/ uptake of adenine.

** Splenocytes were stimulated with 4 μg/ml concanavalin A *in vitro*.

*** Mouse was sacrificed on the 7th day after transplantation approximately 10^{5} ES cells into the brain.

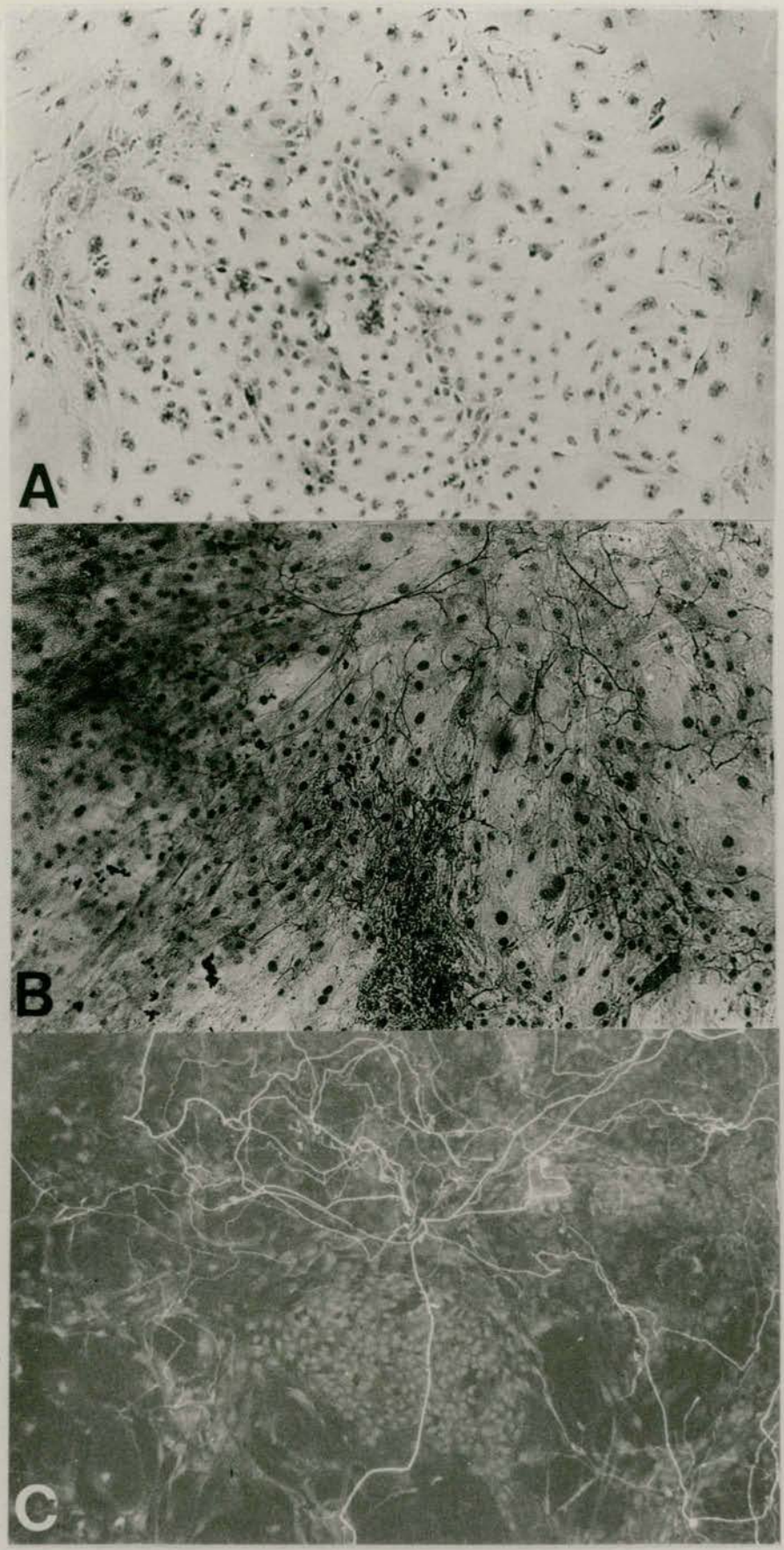
Figure 8.2 Diagram of cell lineages in early mouse embryos.


Zygote
Morula
ES cell
Trophectoderm
ICM
Epiblast
Primitive endoderm
Ectoderm
Mesoblast
Mesoderm
Endoderm
Neural plate, Neural crest, Epidermis

Gastrulation, or first appearance of mesoderm, occurs in mice at about day 8. The majority of neurons are formed by birth in the mouse. In order to understand the development of ES cells after transplantation into the brain, the ES cell derivatives were isolated from the brain of injected mice and selected in HAT media. A control experiment was done to ensure that the HAT resistant cells were derived from E14 ES cells only. The brain cells isolated from HPRT-deficient mice grew in normal media however no cells survived in the presence of HAT medium. Morphological observation showed that HAT-resistant cells, presumably ES cell derivatives of E14 cells intracerebrally injected into the brain of HPRT-deficient mice, lost the special morphological characteristics of embryonic stem cells, even in medium containing LIF which prevents differentiation *in vitro*. Some of the HAT-resistant cells presented a neuron-like axonal morphology with long processes (Figure 8.3).

Immunohistochemical staining with antibodies against different cell markers provides a useful tool to identify the developmental origin and to study the differentiation of the cell. Intermediate filaments are morphologically similar in most eukaryotic cell types but they show a wide heterogeneity in their polypeptide subunits and these have been divided into six classes: types I and II (keratins in epithelial cells), type III (vimentin, desmin, glial fibrillary acid protein (GFAP), peripherin), type IV (the neurofilaments NF-L, NF-M, and NF-H, and α-internexin), type V (nuclear lamins), and type VI (nestin). Since intermediate filament proteins are often specifically expressed in particular cell types, they are useful tools in the study of cell differentiation as well as in tumour identification (Lendahl *et al.*, 1990; Osborn and Weber, 1983). Neuroepithelial stem cells initially coexpress nestin and vimentin, but nestin is subsequently down-regulated and the type IV intermediate filament protein α-internexin appears. α-Internexin is in turn replaced by the type IV proteins NF-L, NF-M, and NF-H that are characteristics of mature neurons. The phenomenon of sequential intermediate filament protein expression during terminal differentiation is also evident in glia. Mature oligodendrocytes lack intermediate filament proteins but their progenitors possess vimentin. Similarly, vimentin is the intermediate filament

Figure 8.3 Morphological difference between E14 cells differentiated *in vitro* and in mouse brain. (a) E14 cells differentiated *in vitro*. E14 cells were cultured in the absence of LIF and any differentiation stimulating agents. (b,c) E14 cells differentiated *in vivo* and cultured in the presence of LIF. E14 cells were transplanted into the brain of an HPRT-deficient mouse for 10 days and reisolated to culture under HAT selection for 7 days. (b) staining with crystal violet. (c) immunostaining with polyclonal rabbit anti-NF-L antibody.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protein of immature Schwann cells although, unlike oligodendrocytes, vimentin is retained in differentiated cells. A second intermediate filament protein, GFAP, appears in Schwann cells at embryonic day 18 (E18) but the expression of GFAP is suppressed upon terminal differentiation of myelin-forming Schwann cells, while it is retained in non-myelin-forming Schwann cells. The sequential appearance and disappearance of different subtypes of neuronal intermediate filament proteins during development which are briefly demonstrated in Figure 8.4, can be partly understood by considering the changing demands for plasticity and stability of cell shape as neurons migrate, elaborate neurites and establish a permanent fibre trajectory.

The HAT-resistant brain cells isolated from HPRT-deficient mice intracerebrally injected with HPRT wild-type ES cells were characterised with different intermediate filament antibodies. Due to the availability of intermediate filament antibodies, four intermediate filament markers, vimentin, NF-L, NF-M and GFAP, were examined. The intermediate filament profile of ES cell derivatives isolated from the brain and resistant to HAT selection shows cells positive for NF-L, NF-M and GFAP but not for vimentin (Figures 8-8.8). Vimentin filaments, which are present in many mesenchymal cell types, are also abundant in most central nervous system neuronal precursor cells before they differentiate. These earliest stages of development are associated with the events of cell division, which involve nuclear rearrangements and changes in cell shape. Vimentin is gradually replaced by neurofilament proteins shortly before neuronal precursor cells stop dividing. The combinations of markers expressed are listed in Table 8.4. ES cell derivatives are a mixture of glia cells, neurons and uncharacterised cells. These immunohistochemical findings, as well as the morphology of the HAT resistant neuron-like cells, suggest that at least some ES cells are committed to the neuronal lineage after transplantation into the brain.

Primary culture cells isolated from mouse brain examined in parallel were positive for the intermediate filament markers, vimentin, NF-L, NF-M and GFAP (Figures 8.9-14). Vimentin filaments are abundant in the earliest stage of development

Figure 8.4 Generalised developmental profile of intermediate filament species of neurons. (Adapted from Nixon and Shea, 1992).

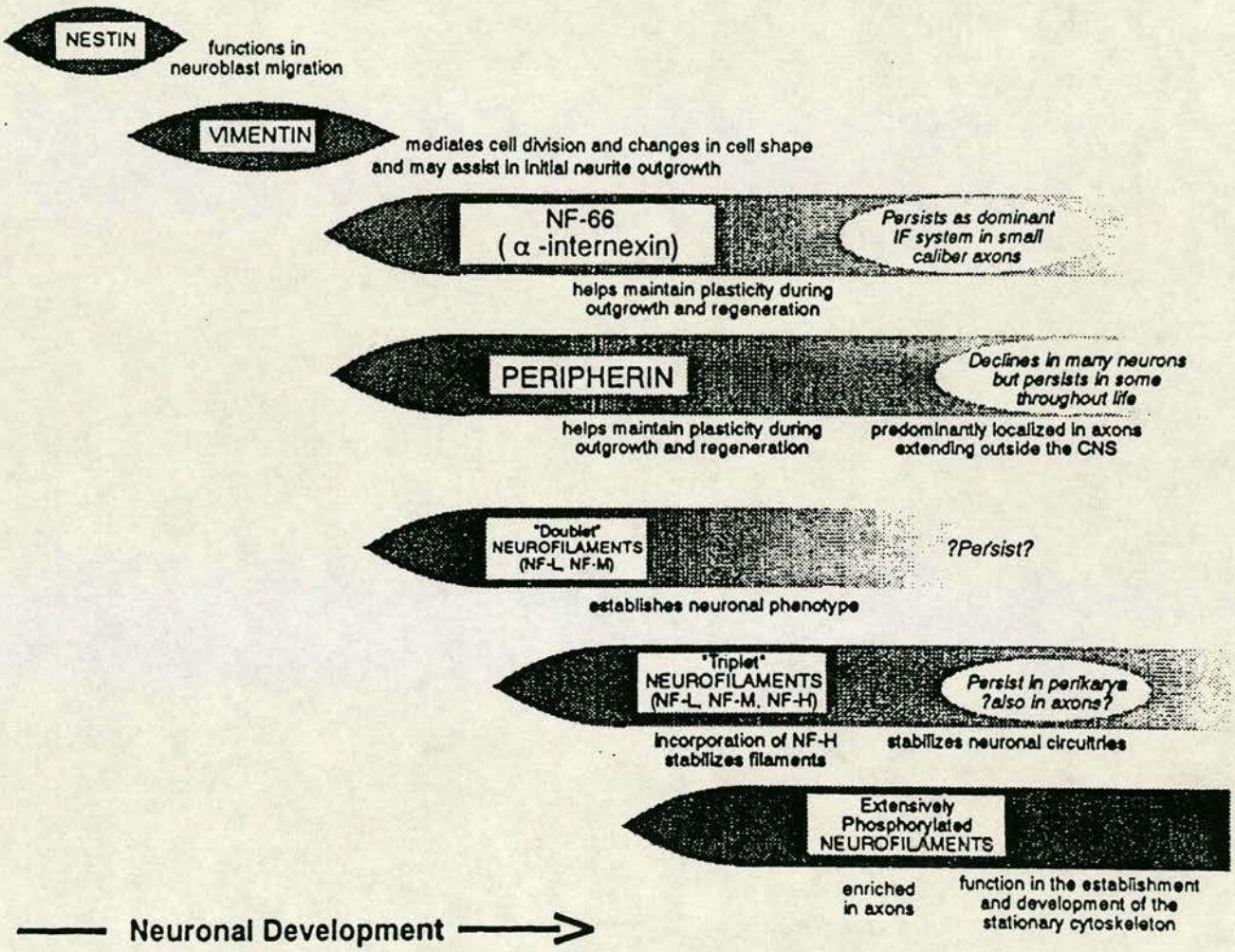
NESTIN
functions in neuroblast migration
VIMENTIN
mediates cell division and changes in cell shape and may assist in initial neurite outgrowth
NF-66 (α -internexin)
Persists as dominant IF system in small caliber axons
helps maintain plasticity during outgrowth and regeneration
PERIPHERIN
Declines in many neurons but persists in some throughout life
helps maintain plasticity during outgrowth and regeneration
predominantly localized in axons extending outside the CNS
"Doublet" NEUROFILAMENTS (NF-L, NF-M)
?Persist?
establishes neuronal phenotype
"Triplet" NEUROFILAMENTS (NF-L, NF-M, NF-H)
Persist in perikarya ?also in axons?
incorporation of NF-H stabilizes filaments
stabilizes neuronal circuitries
Extensively Phosphorylated NEUROFILAMENTS
enriched in axons
function in the establishment and development of the stationary cytoskeleton
Neuronal Development

Figure 8.5 Immunofluorescence microscopy of ES cell-derived brain cells. Double-label immunofluorescence microscopy of HAT resistant cells isolated from HPRT-deficient mice transplanted with wild-type ES cells using a polyclonal rabbit antibody against NF-L (a), and a monoclonal murine antibody against NF-M (b). The neuronal cell bodies and associated axonal networks express both markers. However, some cells show positive for NF-L but not for NF-M (e.g. see bottom right corner of field).

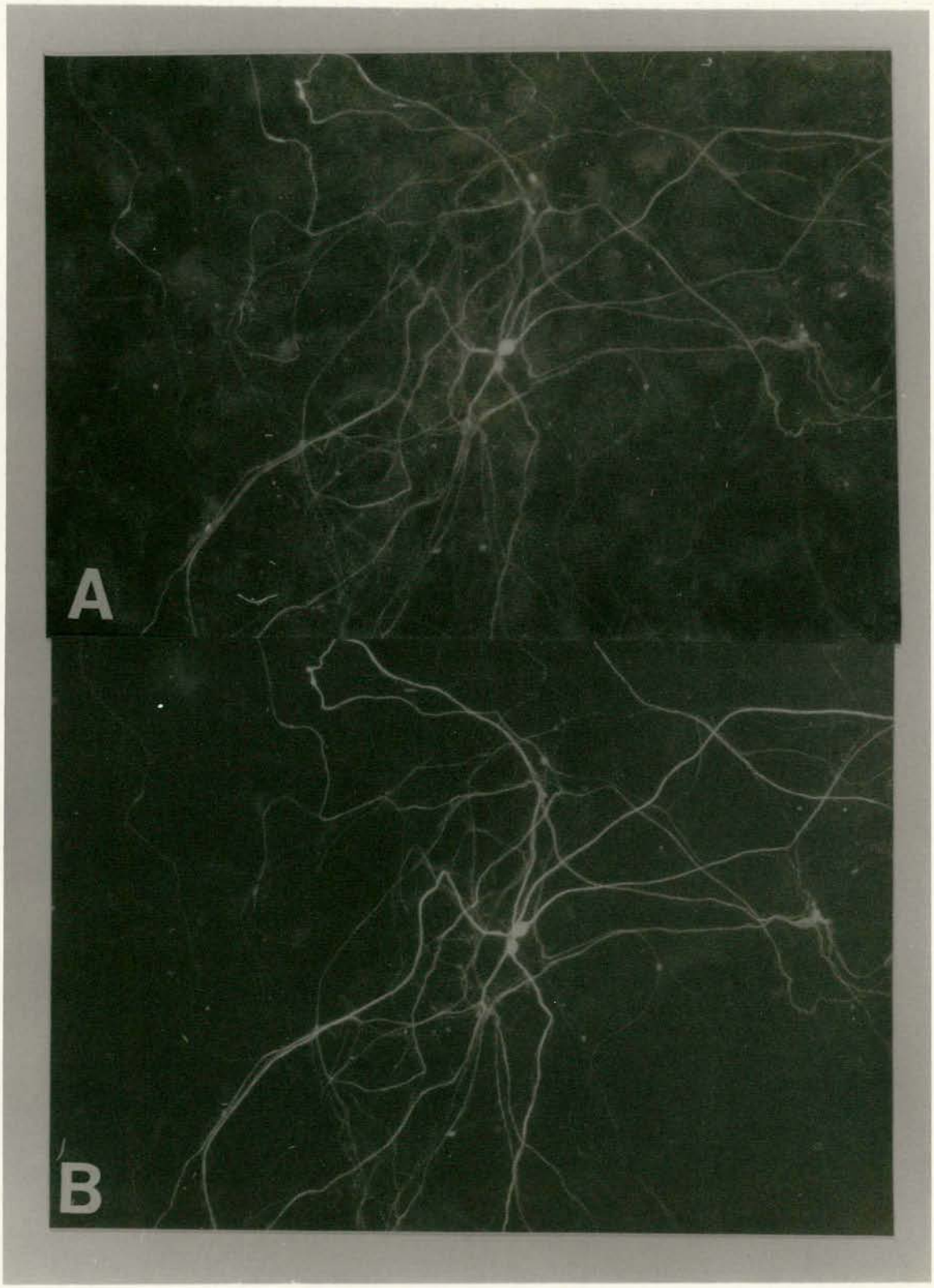
A
B

Figure 8.6 Immunofluorescence microscopy of HAT resistant cells isolated from the brain of HPRT-deficient mice intracerebrally transplanted with wild-type ES cells. Double-label immnofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L (b) monoclonal murine antibody against GFAP (c) phase contrast only of the same field as (a) and (b). Neuronal cell bodies and processes express NF-L but not GFAP.

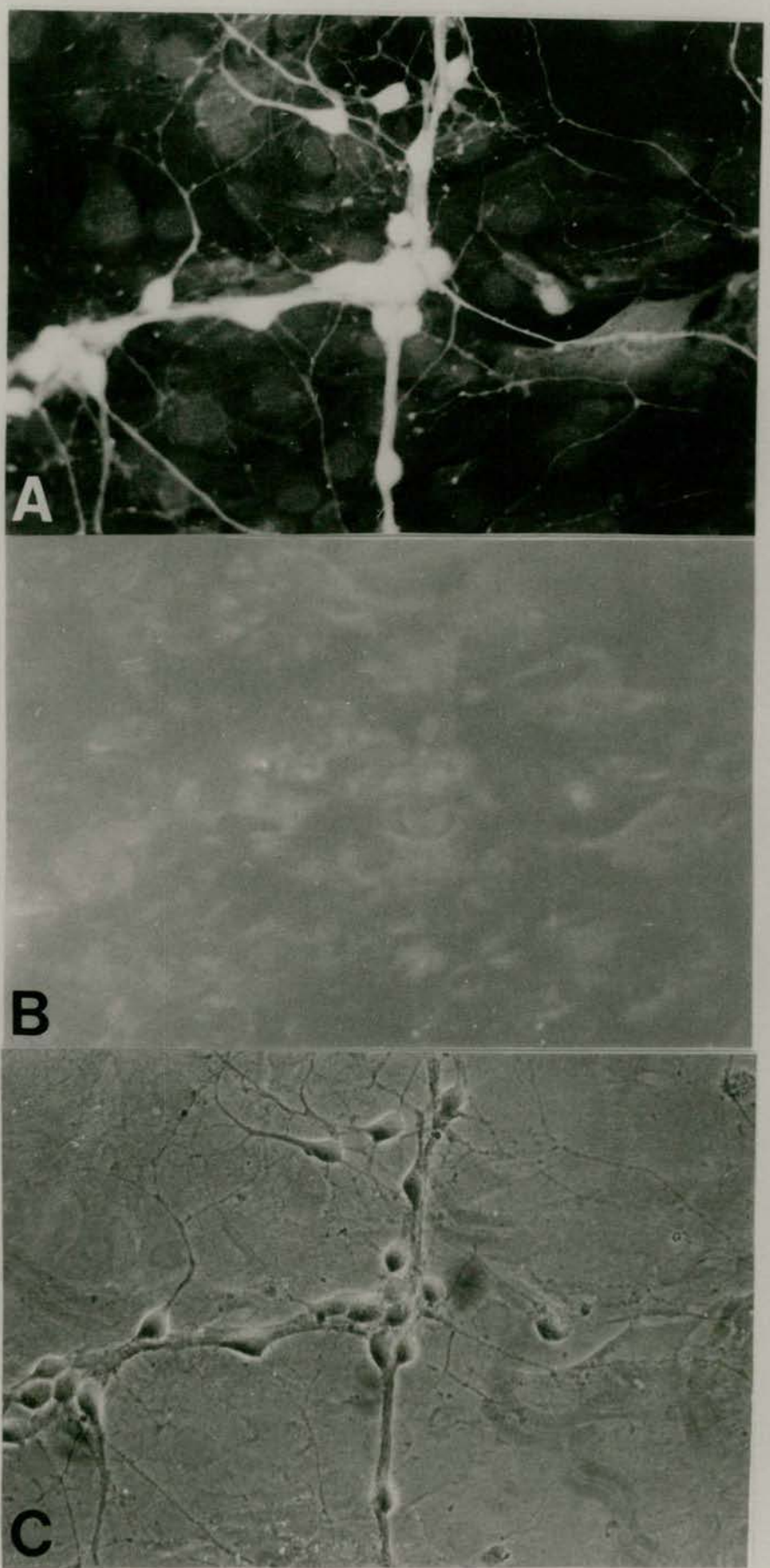
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Figure 8.7 Immunofluorescence microscopy of ES cell-derived brain cells. Double-label immunofluorescence microscopy of HAT resistant cells isolated from HPRT-deficient mice transplanted with wild-type ES cells using a polyclonal rabbit antibody against NF-L (a), and a monoclonal murine antibody against GFAP (b). (c) phase contrast only of the same field as (a) and (b). Neuronal cell bodies and axons expressing NF-L, but not GFAP and glial cells expressing GFAP but not NF-L, are shown.

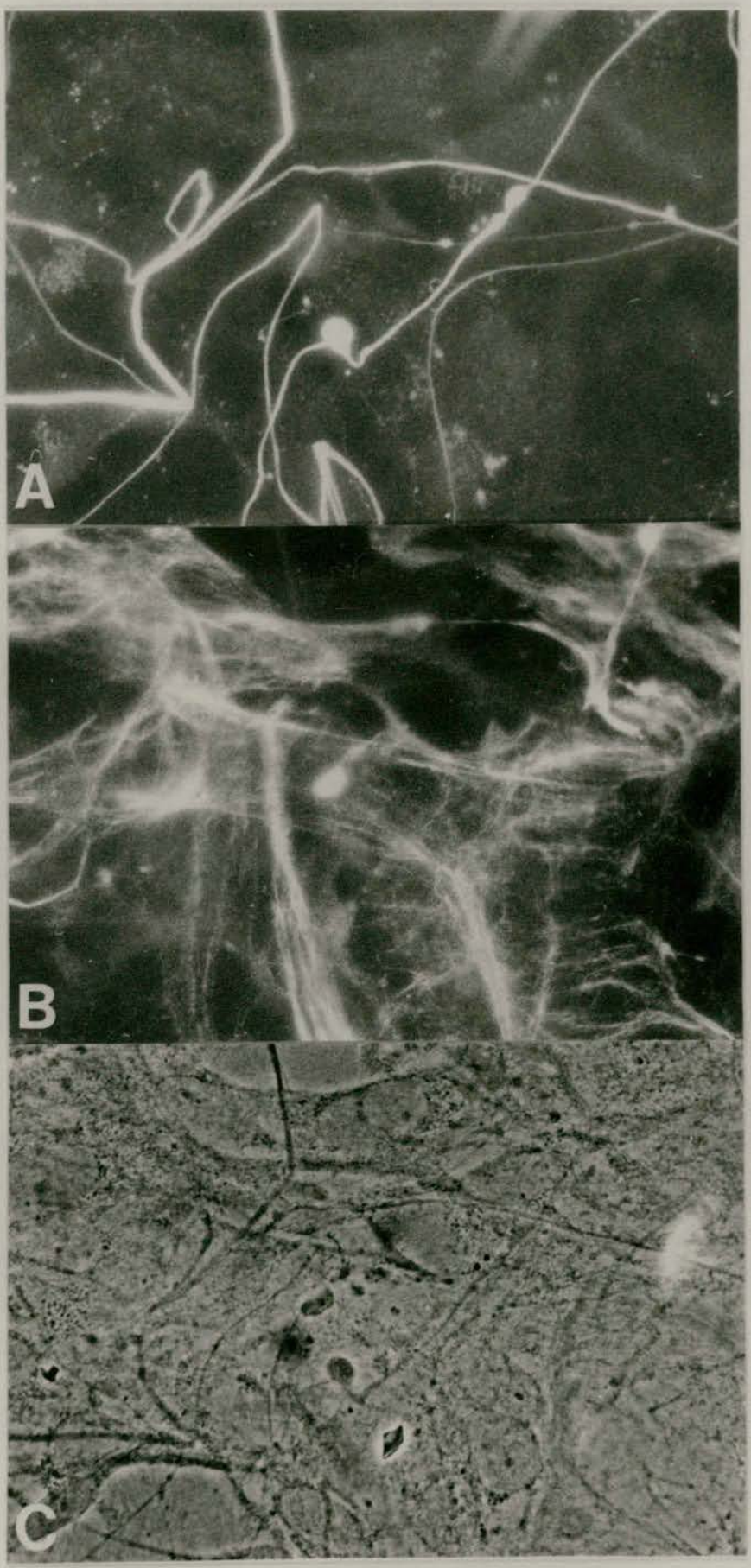
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Figure 8.8 Immunofluorescence microscopy of HAT resistant cells isolated from the brain of HPRT-deficient mice intracerebrally transplanted with wild-type ES cells. Double-label immnofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L (b) monoclonal murine antibody against vimentin (c) phase contrast only of the same field as (a) and (b). The neuronal cell bodies and associated filaments shown express NF-L but not vimentin.

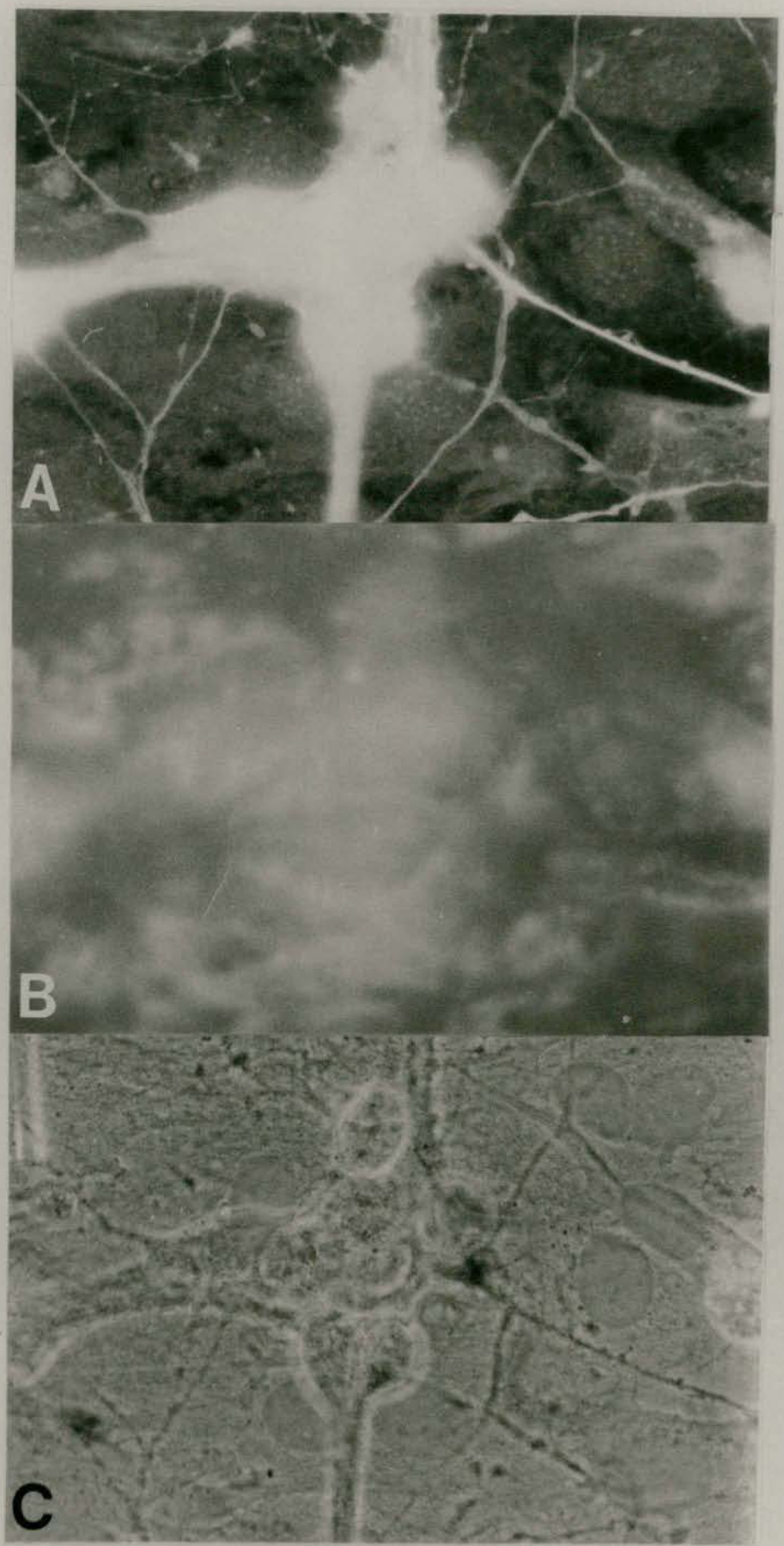
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Table 8.4 The distribution of cell markers in the embryonic stem cell-derived (ES-D) brain cells and cells from primary culture of brain.

Marker	Occurrence		Cell type
	ES-D	Brain	
NF-L$^+$	yes	yes	neuron
NF-M$^+$	yes	yes	neuron
GFAP$^+$	yes	yes	glia
vimentin$^+$	no	yes	
NF-L$^+$NF-M$^+$	yes	yes	neuron
NF-L$^+$NF-M$^-$	yes	no	neuron
NF-L$^-$NF-M$^+$	no	no	
NF-L$^+$vimentin$^+$	no	yes	neuronal precursor ?
NF-L$^+$vimentin$^-$	yes	no	neuron
NF-L$^-$vimentin$^+$	no	no	
NF-L$^+$GFAP$^+$	no	yes	neuronal stem cell ?
NF-L$^+$GFAP$^-$	yes	yes	neuron
NF-L$^-$GFAP$^+$	yes	yes	glia

Figure 8.9 Immunofluorescence microscopy of brain cells in primary culture. Brain cells were isolated from HPRT-deficient mice in the absence of HAT selection. Immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against GFAP. Neuronal cell bodies and axons express NF-L(a) and glial cells express GFAP (b).

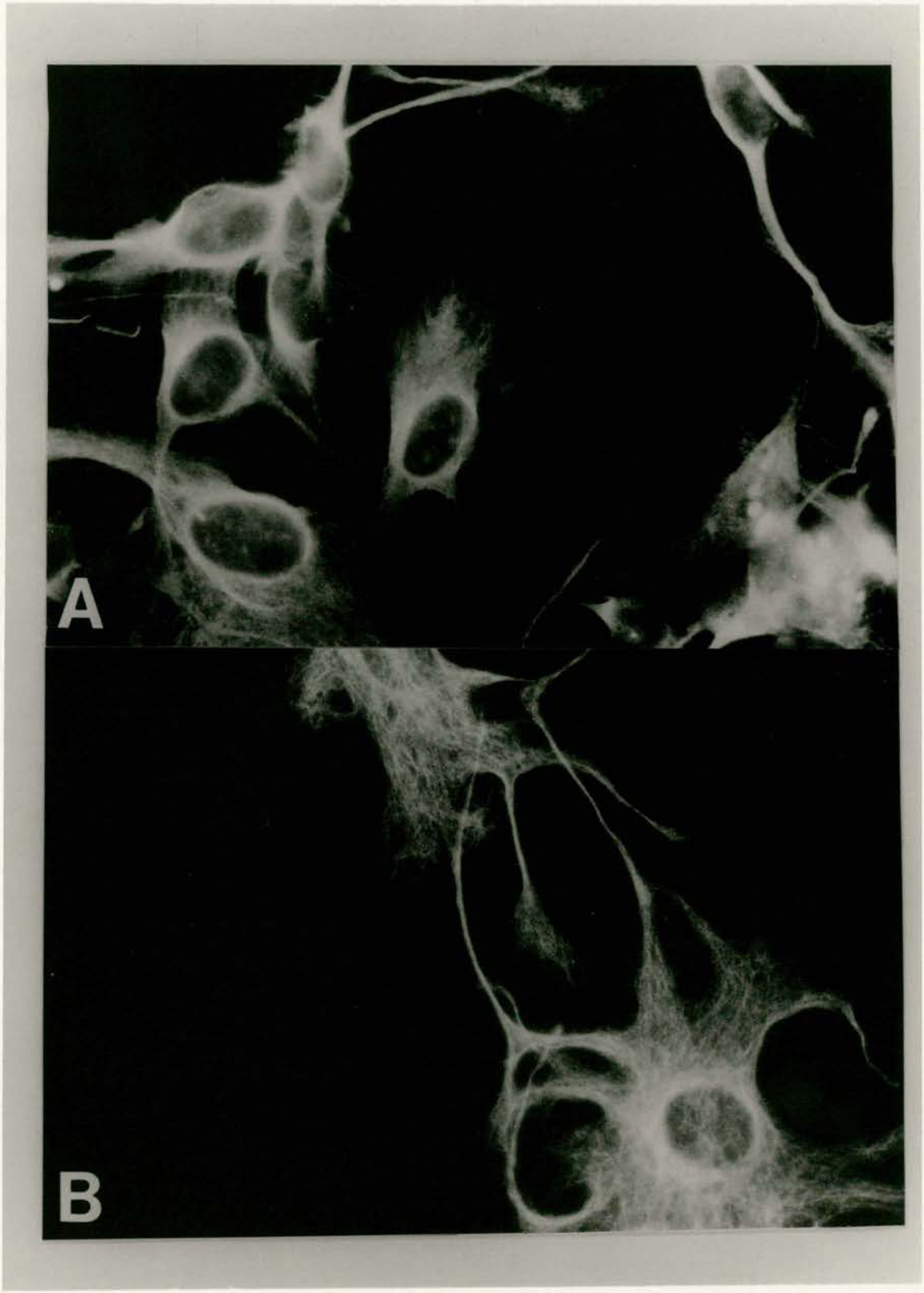
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Figure 8.10 Immunofluorescence microscopy of primary culture of brain cells. Double staining immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against NF-M. (c) phase contrast only of the same field as (a) and (b). Neuronal cell bodies and processing express both NF-L and NF-M are shown.

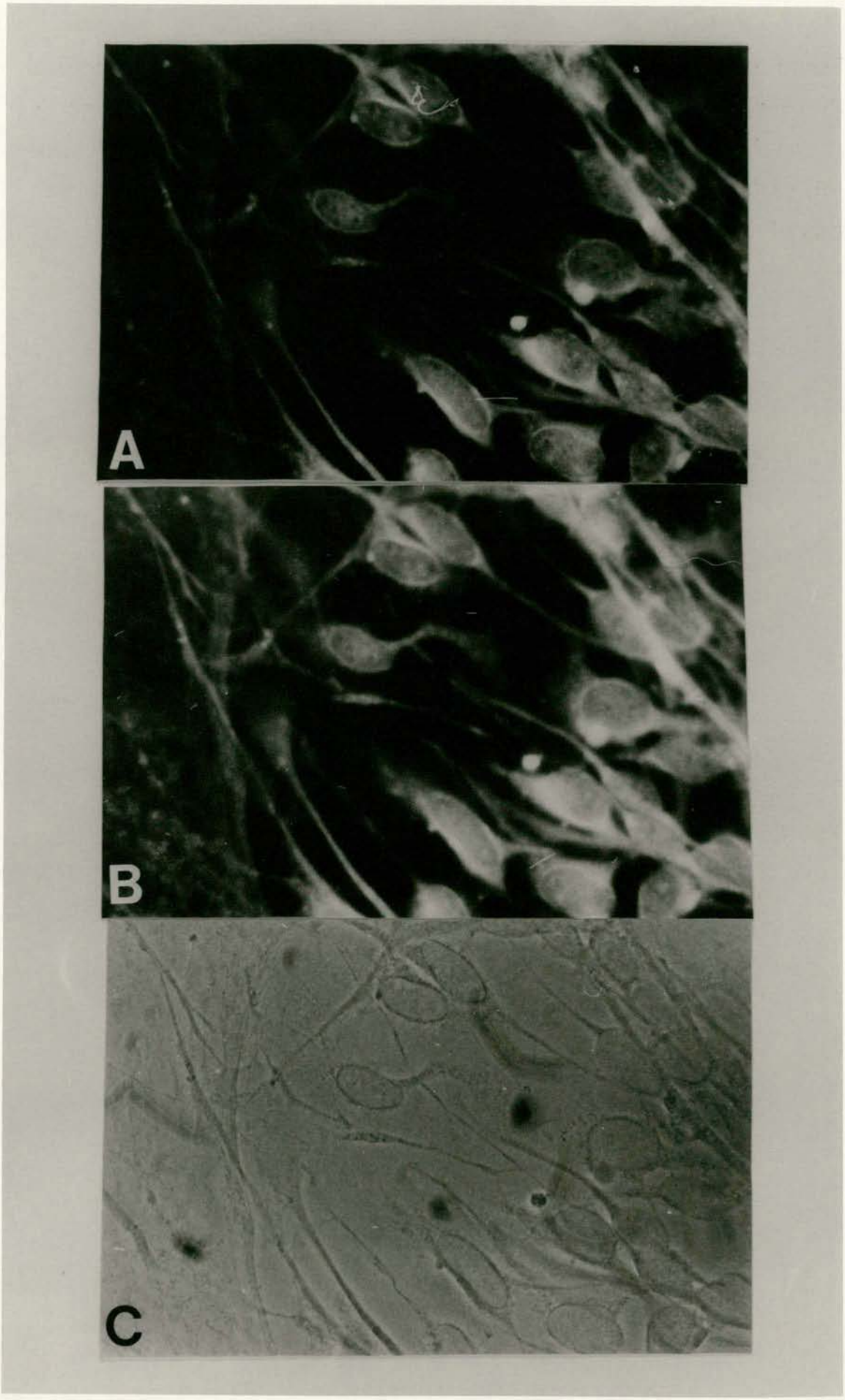
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Figure 8.11 Immunofluorescence microscopy of primary culture of brain cells. Double staining immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against vimentin. (c) phase contrast only of the same field as (a) and (b). Neuronal cells expressing both NF-L and vimentin are shown.

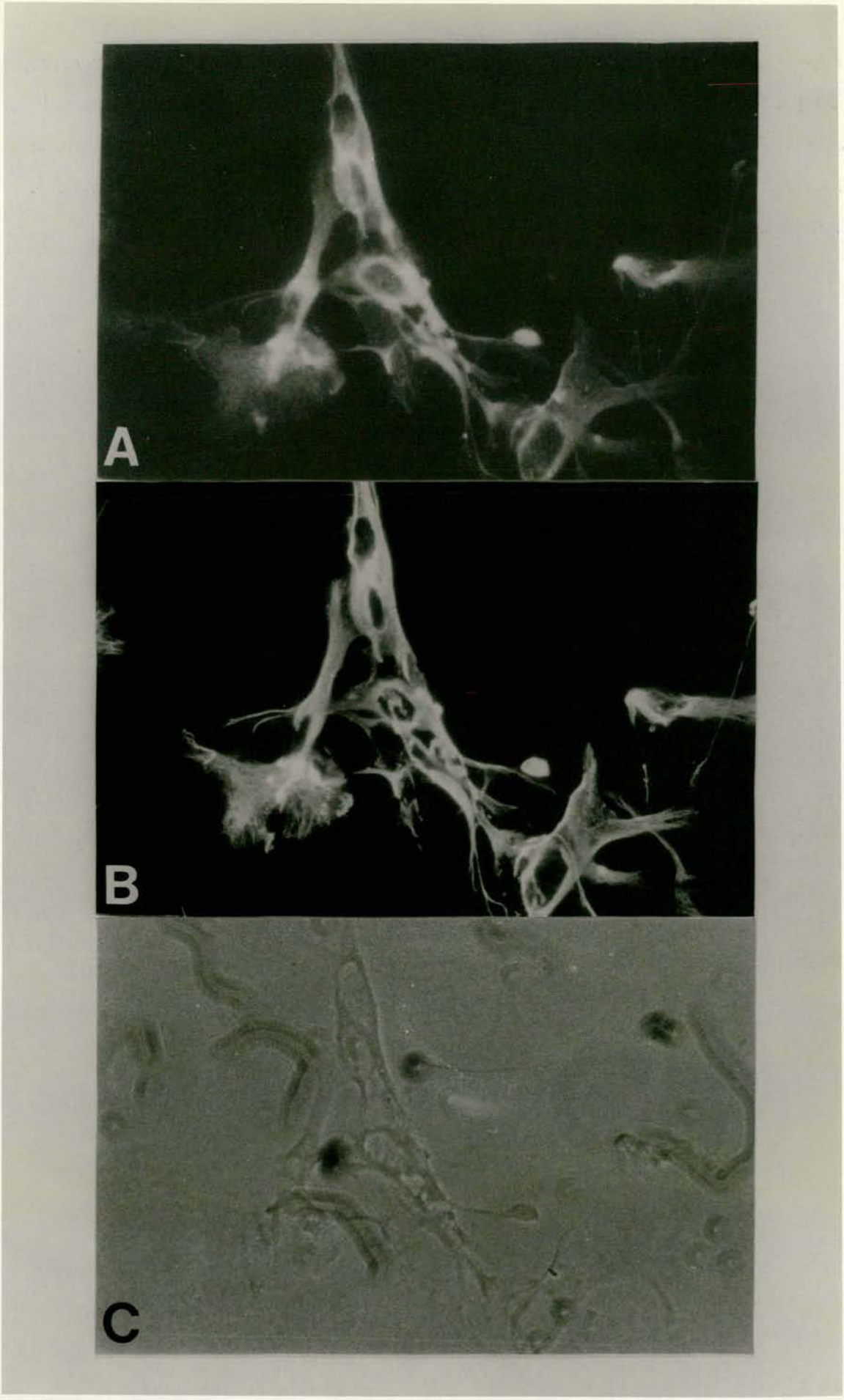
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Figure 8.12 Immunofluorescence microscopy of brain cells in primary culture. Brain cells were isolated from HPRT-deficient mice in the absence of HAT selection. Double staining immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against GFAP. (c) phase contrast only of the same field as (a) and (b). Neuronal cells shown express NF-L but not GFAP.

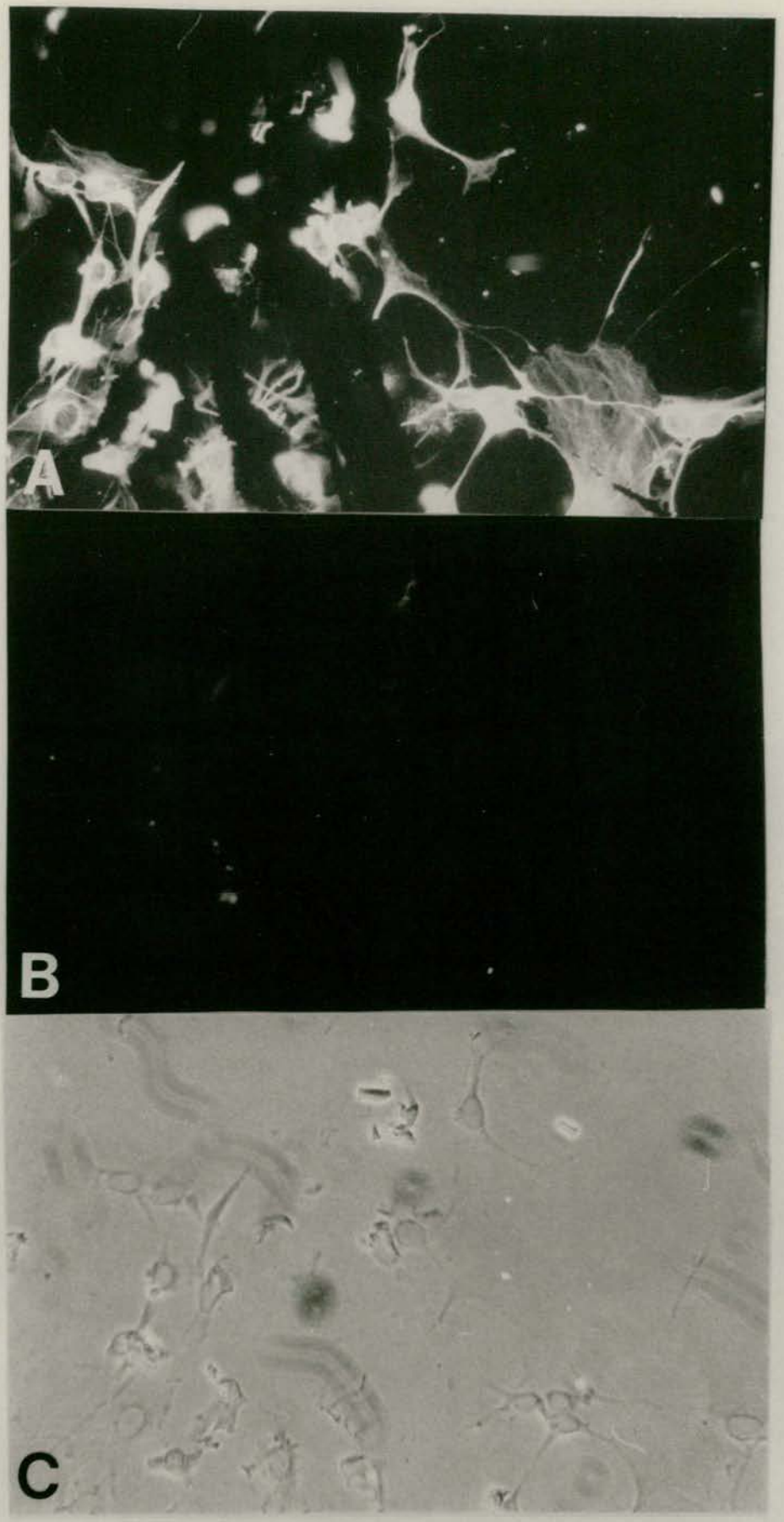
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Figure 8.13 Immunofluorescence microscopy of brain cells in primary culture. Brain cells were isolated from HPRT-deficient mice in the absence of HAT selection. Double staining immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against GFAP. (c) phase contrast only of the same field as (a) and (b). Neuronal cells expressing both NF-L and GFAP are shown.

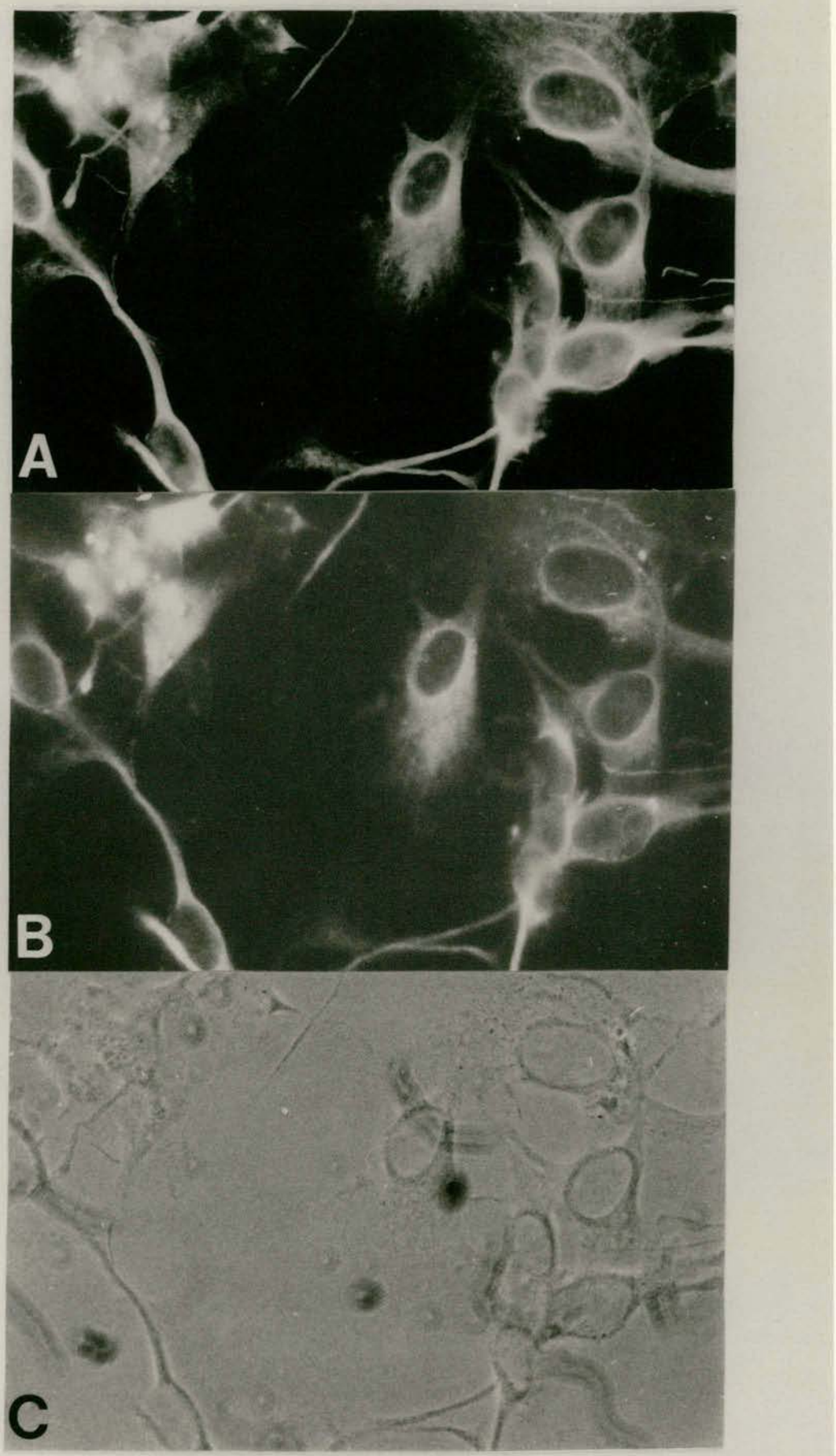
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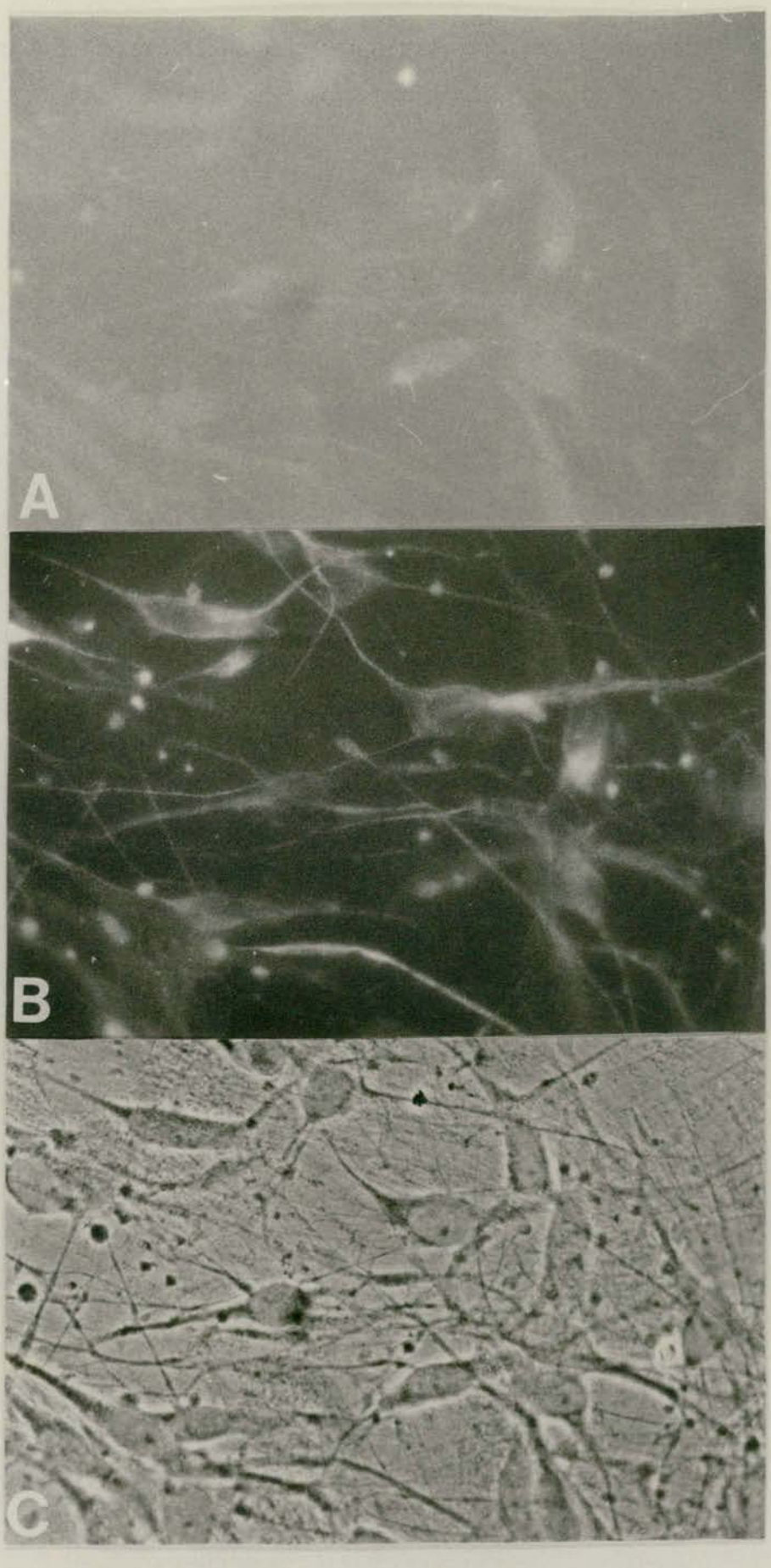
Figure 8.14 Immunofluorescence microscopy of primary culture of brain cells. Double staining immunofluorescence microscopy using (a) polyclonal rabbit antibody against NF-L and (b) monoclonal murine antibody against GFAP. (c) phase contrast only of the same field as (a) and (b). Cells expressing GFAP but not NF-L are shown.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which is associated with the events of cell division in the central nervous system before differentiation. Before vimentin is totally replaced by neurofilament proteins, for a time, both filament protein types may coexist (Nixon and Shea, 1992). The coexistence of NF-L and vimentin in primary culture brain cells (Figure 8.11) suggests the possibility of isolating neuronal precursor cells. In addition, some cultured brain cells express both GFAP and NF-L (Figure 8.13) which coexist only in the early stage of neuronal development. By using retrovirus-mediated gene transfer for marking cell lineages *in vivo*, the persistence of a common progenitor for neurons and glia has been suggested (Turner and Cepko, 1987). Stemple and Anderson (1992) isolated a stem cell for neurons and glia from the mouse neural crest suggesting neuronal and glial cells share the same progenitor. With the treatment of basic fibroblast growth factor and conditioned medium from an astrocyte-like cell line, cells isolated from adult mouse brain express both GFAP and NF-M suggesting that neuronal precursors exist in the adult mammalian brain (Richards *et al.*, 1992).

In summary, the increase of HPRT activity in the brain of HPRT-deficient mice after transplantation with HPRT wild-type ES cells and the commitment to differentiate into the neuronal and glial lineage as well as the use of ES cells for homologous recombination suggest the possibility of a gene therapeutic strategy for inherited neural diseases using ES cells as vehicles, combined with gene targeting techniques.

CHAPTER 9. DISCUSSION

The preceding results are discussed below in 4 sections reflecting, as much as possible, the individual objectives and their interdependence. Gene targeting to generate a deletion at the HPRT locus in murine ES cells is considered in section 9.1, the evaluation and establishment of behaviourally altered mice as an animal model for Lesch-Nyhan syndrome, in section 9.2 and the gene targeting knock out of APRT is covered in section 9.3. Gene therapy by transplantation of ES cells to HPRT-deficient mice is discussed in the final section.

9.1 Gene targeting deletion at the HPRT locus

Some specific deletions cause distinct diseases, such as retinoblastoma (*RB*). Cytogenetic studies of lymphocytes or fibroblasts from retinoblastoma patients showed a small group of patients were found to share a deletion of chromosomal region 13q14 (Yunis and Ramsay, 1978). The *RB* gene has been cloned using esterase D cDNA, which is linked to the retinoblastoma susceptibility locus in band 13q14.11 within an estimated 1,500 kb range, as a probe for chromosome walking. The *RB* gene encodes a nuclear protein that is a substrate for *cdc2* kinase and was found to be composed of 23 exons scattered over more than 100 kb of DNA (Lee *et al.*, 1987). The cloning of the *RB* gene exemplifies a system for cloning a gene defective in a particular disease starting from an observation of cytogenetic deletion. Subsequently, a transgenic animal model was constructed using gene targeting in ES cells, although the animals did not serve as a tumour susceptibility model (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clarke *et al.*, 1992). Homozygous sequence deletions have been reported from several different lung cancer cell lines, suggesting the deleted sequences are derived from independent genetic loci encoding potential tumour suppressor genes other than *RB* and p53 (Wieland *et al.*, 1992). Homozygous deletions with human chromosome band 9p21 were also noticed that have early involvement in development of melanoma (Fountain *et al.*, 1992). Furthermore, other clinical cases with genetic deletion have been reported recently. A patient with typical clinical features of the fragile X syndrome but without cytogenetic expression of the fragile X has been characterised with a submicroscopic deletion of the entire FMR1 gene and about 2.5 megabases of flanking sequences (Gedeon *et al.*, 1992). Seven patients with Kearn-

Sayre syndrome have been identified where the muscle pathology is associated with mitochondrial DNA deletions, 2.3 to 7.1 kb in size (Moraes *et al.*, 1992). In addition, the best way to understand the specific function of one component of a multicomponent system is to take it away and to observe any difference. Many genomic DNAs of unknown function will be cloned and sequenced in the course of efforts to map the human genome. The best way to identify the function of a particular sequence is to make a cell line or a knock-out animal in which the sequence is deleted or inactivated and to observe the physiological and biochemical alteration.

Gene targeting in embryonic stem cells is a valuable system for creating transgenic animals that provide a powerful system for studying specific gene function. The structure of the targeting DNA plays an important role in the efficiency of targeting events. Double-stranded circular DNA molecules were unable to recombine efficiently with each other in either a prokaryotic system (Chua and Oliver, 1992) or a eukaryotic system (Bilang *et al.*, 1992). Targeting efficiency was higher with a linearised than with a circular construct in the targeting insertion of the neomycin phosphotransferase gene into the tubulin gene cluster (ten Asbroek *et al.*, 1990). Purified supercoiled and nicked circular plasmids as well as restriction enzyme linearised DNA were used to determine targeting efficiency in ES cells. It was shown that DNA with a double-strand break in the region of homology targeted at a 10-fold-higher frequency than did nicked circular DNA and a 34-fold-higher frequency than did supercoiled DNA (Hasty *et al.*, 1992). To optimise the frequency of targeting deletion event, all the deletion vectors built for this project were linearised before electroporation.

There are metabolic cooperation phenomena which occur between mammalian cells in culture (Hooper, 1982). This sometimes results in artifacts in the measurement of mutation rates of a large cell population. An artificially low isolation ratio of HPRT-deficient mutants from high inoculation size had been observed, the number of 8-azaguanine resistant clones from ethyl methanesulphonate mutagenised V-79 Chinese hamster cells decreased by increasing the cell amounts plated on the dishes (Chu and

Malling, 1968). Therefore, it is a technical problem to isolate a mutated clone from a large cell population after homologous recombination, especially since this occurs at a low frequency. Hence it is convenient to have some positive selection in the screening procedure even if the gene is directly selectable. Accordingly, more than 90% of the experiments involving homologous recombination use marker genes such as *neo* in their targeting constructs.

When this research project was initiated, only a few reports had been published regarding gene targeting either by insertion or replacement. There was no evidence to suggest the possibility of deletion of particular sequences from the genome by the technique of homologous recombination. The first set of experiments was to test this possibility using 3 different vectors sharing the same 5′ homology to make different size deletions as defined by the 3′ homology. The deletion of 10.1 kb containing the promoter region and exon 1 of the HPRT locus was easily achieved with both pDWMD1 and pDWMD3 at a frequency of about 0.5% G418-resistant clones. The overall targeting deletion efficiency is intermediate to levels reported for 15-kb and 4-kb deletions in the T-cell receptor α subunit gene, 0.4% and 3.3% respectively (Mombaerts *et al.*, 1991). A fragment of similar size (12 kb) to the region deleted by pDWMD1 and pDWMD3 has been inserted into the HPRT gene using both a replacement vector (Mansour *et al.*, 1990) and an insertion vector (Thompson *et al.*, 1989). The attempt to delete the whole HPRT genome from ES cells with deletion vector, pDWMD4, did not succeed. Only one clone resistant to both G418 and 6-thioguanine was isolated from 2 electroporation experiments, however, no deletion was observed from the Southern analysis. Although the attempt to delete 20 kb containing the promoter region and exons 1-5 of HPRT by deletion vector, pDWMD5 failed, the isolation frequency of double resistant clones was much higher than that with pDWMD4. However, the Southern hybridisation patterns show that the mechanism of homologous recombination was by insertion rather than deletion.

The topology of the incoming DNA may play a role, as very different targeting frequencies between an insertion and a replacement vector containing identical

homologous sequences have recently been observed. Using non-isogenic DNA, insertion vectors target at a 5- to 10-fold-higher frequency than replacement vectors do with the same homologous sequences (Hasty *et al.*, 1991b). It is easy to understand that the achievement of replacement, requiring double reciprocal recombination is more difficult than that of insertion which requires only one cross over. It has been shown that the single reciprocal recombination of an insertion vector occurs 92-fold more frequently than double reciprocal recombination of a replacement vector with crossover junctions on both homologous arms. The provision of longer uninterrupted homology in insertion vectors than in replacement vectors plays some role in the higher homologous recombination frequency (Hasty *et al.*, 1991c). However, using isogenic DNA (see later for discussion of isogenic versus non-isogenic DNA and targeting frequency) Deng and Capecchi (1992) showed that sequence replacement and sequence insertion vectors behaved equivalently with respect to the targeting efficiency. The vector used here were derived from non-isogenic DNA. The insertion results from the targeting deletion by pDWMD5 and pDWMD8 which were designed to integrate via a replacement mechanism support the contention that topology of incoming DNA plays a role in homologous recombination frequency.

To ensure that targeting deletion could be accomplished in another region and to clarify whether there were sequence-specific factors leading to the failure of the deletion by pDWMD5, the targeting deletion vector, pDWMD7, was constructed. The successful deletion by pDWMD7 suggests that there was no particular sequence-specific difficulty for deletion in the region that was difficult to delete with pDWMD5. The only difference between pDWMD5 and pDWMD7 is the deletion size desired. Comparing the outcomes of introducing pDWMD1, pDWMD5, or pDWMD7 into ES cells, suggests that there was no difficulty in deleting sequences of 10 kb in size from the HPRT locus and that there were not sequence-specific effects. The non-existence of sequence-specific difficulty was also proved by the achievement of targeting deletion with pDWMD5-TK through the two-step recombination procedure. The failure of deletion by pDWMD5 might be due to a size limitation on the amount of material that could be deleted in a single recombination event. Another targeting

deletion vector, pDWMD8, was constructed to confirm that size limitation is the major reason for failure of larger deletions by a simple homologous recombination protocol. This deletion vector was constructed to delete a 20-kb sequence including the 10-kb region which can be deleted with pDWMD1 without any difficulty. As expected, the 20-kb deletion could not be obtained by the introduction of pDWMD8 into ES cells. The Southern hybridisation patterns of the clones resistant to both G418 and 6-thioguanine isolated from ES cells electroporated with pDWMD8 showed that all the clones contained insertions rather than deletions. Both of two different deletion vectors devised to delete 20-kb sequences from HPRT locus led to insertion events instead of deletions after homologous recombination. This result suggests that there is a size limitation for deleting more than 20-kb sequences by simple homologous recombination. Vector constructs for deletion are very similar to that of conventional replacement vectors rather than that of insertion vectors. The deletion results at both the T-cell receptor β-subunit (Mombaerts *et al.*, 1991) and HPRT locus showed that deletion of less than 15 kb can be achieved without difficulty. However, when larger deletion is desired, the vector recombined by an insertion mechanism instead of replacement mechanism as demonstrated in both pDWMD5 and pDWMD8.

Several studies have illustrated that the targeting frequency in mammalian systems is dependent on the length of homology and is unaffected by the length of nonhomologous DNA. The targeting frequency at the HPRT locus as a function of the extent of homology between the targeting vector and the endogenous target has been reported both in insertion and replacement vectors (Capecchi, 1989). A parallel phenomenon occurs with extrachromosomal intramolecular recombination where a linear increase in the recombination frequency with increased length of homology from 0.25 to 5 kb and a steep reduction in the frequency below 0.25 kb, in mammalian cells (Rubnitz and Subramani, 1984). More detailed analysis of replacement vectors showed that homology of less than 1.7 kb was insufficient to generate targeted events. Homology increasing from 1.9 to 4.2 kb and from 4.2 kb to 6.0 kb resulted in 16- and 3-fold increases respectively in the targeting frequency (Hasty *et al.*, 1991b). The strong dependency of targeting frequency on the length of

homology between the targeting vector and the target locus has been also demonstrated with insertion vectors (Deng and Capecchi, 1992). The lower targeting frequency with pDWMD7 relative to pDWMD1 might be due to the shorter length of homology provided by pDWMD7, 5.2 versus 6.9 kb respectively in the total of 5′ and 3′ homology.

The targeting frequencies of the insertion vector with different linearisation sites indicated that the adjacent homologous ends increased the targeting frequency by 5- and 12-fold over ends at the homologous-heterologous junction and in the heterologous sequences. To determine whether the ends have a rate-limiting role for homologous recombination between introduced and chromosomal DNAs, targeting frequencies with insertion vectors were compared with a double-stranded break either inside the region of homology, at the edge or outside the target homology. The highest frequency was found in the vector with a break inside the region of homology. In addition, size-dependent reduction of targeting efficiency by attaching heterologous sequence to the homologous end was also demonstrated (Hasty *et al.*, 1992). The heterologous end in pDWMD3 might contribute to the lower targeting frequency compared to pDWMD1. The linearised DNA of pDWMD1 provides an homologous end while pDWMD3 has a heterologous end from the HSV-TK module.

The attempts to delete 20-kb sequences from the HPRT locus by simple homologous recombination were disappointing using either pDWMD5 or pDWMD8 as the deletion vector. It is obvious that there is a size limitation for targeting deletions and, as a result, the insertion event takes place. It would be useful to establish a methodology for deleting larger fragments. A modified targeting vector, pDWMD5-TK was therefore constructed to obtain the 20-kb deletion by 2-step homologous recombination at the HPRT locus. This was achieved by a combination of homologous insertion and intrachromosomal recombination. The vector was initially inserted into the 3′ end of the homology. The size of insertion by homologous recombination can be as large as 13 kb if the insertion mechanism is employed (Smithies *et al.*, 1985). A similar two-step recombination procedure

involving intrachromosomal recombination has been used to introduce a subtle mutation in the *Hox-2.6* locus in embryonic stem cells. The initial event involved single reciprocal recombination between the target gene and the insertion vector containing a small mutation in the homologous DNA with *neo* and HSV-TK modules located outside the region of homology. The entire vector integrated at the target gene and created a duplication of genomic sequences conferring a G418-resistant and HSV-TK positive phenotype. The intrachromosomal recombination subsequently occurred within the duplication to create the final mutant construct with the phenotype of HSV-TK negative and G418-sensitive phenotype (Hasty *et al.*, 1991a). A single reciprocal recombination occurred in the 3′ end of homology of pDWMD5-TK to create insertion mutants which were G418- and 6-thioguanine-resistant but gancyclovir sensitive. Mutants with a 20-kb deletion in the HPRT locus were then generated by intrachromosomal recombination in the duplicated region, which could be selected by gancylovir and G418.

In summary, although the generation of deletion by gene targeting is size limited, deletions up to 15 kb could be achieved by a simple protocol. Larger deletions could be accomplished by more complex procedures involving intrachromosomal recombination or by gradual deletion (Table 9.1). The efficiency of targeting deletion, like that of replacement and insertion mechanisms in general homologous recombination, also depends on length of homology, isogenicity, and homology at double-strand breaks.

9.2 Animal model for Lesch-Nyhan syndrome

Patients with Lesch-Nyhan syndrome are usually mentally retarded, but the most unusual feature of the syndrome is the occurrence of compulsive self-injurious behaviour. First attempts to produce an animal model for Lesch-Nyhan syndrome involved chronic administration of high doses of the methylxanthines, caffeine and theophylline, to induce self-mutilation in mice, rats and rabbits (Mueller *et al.*, 1982; Lloyd and Stone, 1981; Peters, 1967). This approach did not produce a useful model due to significant mortality and an induced increase in brain HPRT activity (Minana

Table 9.1 Summary of targeting deletion by homologous recombination at the HPRT locus.

Vector	Expected		Experimental results	
	Remaining exons	Deletion size (kb)	Deletion	Insertion
pDWMD1	2-9	10.1	+[a]	+
pDWMD4	–	38.6		
pDWMD5	6-9	27.2		+
pDWMD7	1-3,6-9	11.4	+	+
pDWMD8	2-9	21.1		+
pDWMD5-TK[b]	6-9	27.2	+	
pDWMD5-TK[c]	6-9	27.2	+	+

a. the occurrence of generating targeted clones resistant to G418 and 6-thioguanine.

b. selected with G418, 6-thioguanine and gancylovir.

c. selected with G418 and 6-thioguanine.

et al., 1984). Caffeine has been used as a comparative agent for studying the behavioural alteration and purine salvage pathway. Although the inhibition of APRT by caffeine is less than that by 9-ethyladenine, caffeine was found to inhibit both HPRT and APRT and it will be discussed later. High doses of clonidine, an agonist for the α-noradrenergic receptor, were able to induce self injurious behaviour in mice housed individually in the absence of objects to bite and the self injurious behaviour was potentiated by pretreatment with caffeine (Razzak *et al.*, 1977; Ushijima *et al.*, 1984). In the most direct Lesch-Nyhan syndrome model to demonstrate the involvement of central dopaminergic neurons, dopamine agonists were used to create lesions in the brain catecholamine pathway of neonatal rats by intracisternal administration. Self injurious behaviour was observed when the neonatally-lesioned rats were challenged as adults with L-dopa, and could be prevented by administration of a dopamine antagonist (Breese *et al.*, 1990; Goldsten, 1989). Other pharmacological models for Lesch-Nyhan syndrome have also been established, but have shed little information on the link between HPRT deficiency and abnormalities in purine and dopamine systems in the brain (Jinnah *et al.*, 1990).

Transgenic animal models make a major contribution to understanding the pathogenesis of human disease and are likely to become increasingly important in the evaluation of novel therapies. It was a particular disappointment, therefore, that the first genetic model for a human inherited disease (Lesch-Nyhan syndrome) to be produced using the embryonic stem cell system did not develop symptoms of the disease spontaneously. Failure to demonstrate the symptoms of Lesch-Nyhan syndrome in HPRT-deficient mice may be understood by comparison of the difference between mouse and human purine metabolism. The extent of dopamine depletion was not as high in the brains of HPRT-deficient mice as in Lesch-Nyhan syndrome patients: up to 50% reduction in mice compared to 70-90% in man (Finger *et al.*, 1988; Williamson *et al.*, 1991). The reduction of dopamine in HPRT-deficient mice could be enhanced by administration of amphetamine. HPRT-deficient mice of either the 129/J or C57Bl/6J strain were more sensitive than their wild-type littermates to the ability of amphetamine to stimulate locomotor or stereotypic behaviours (Jinnah *et al.*,

1991). Amphetamine has been proven to lead to dopamine release in the rat striatum (Butcher *et al.*, 1988). Although these experiments implied that dopamine might influence the incidence of behavioural alteration, the relationship between dopamine levels and HPRT deficiency still remains a puzzle.

Hypoxanthine is an inefficient precursor of purine nucleotides in mouse tissue. *In vitro*, mouse erythrocytes salvage less than 10% of hypoxanthine added to whole blood in 30 min of incubation at 37 °C. *In vivo*, circulating hypoxanthine is rapidly degraded (> 90% in 10 min) to allantoin and uric acid. It is estimated that < 2% of circulating hypoxanthine is salvaged in the mouse, the remainder is catabolised (Moyer and Henderson, 1983). In addition, there is no correlation between HPRT activity and endogenous dopamine levels, dopamine uptake, dopamine release, or monoamine oxidase activity in comparisons of HPRT-wild-type cells with the deficient cell line, PC12, which is a clonal pheochromacytoma line from rat adrenal medulla (Bitler and Howard, 1986).

Following the report that HPRT-deficient mice were more susceptible to amphetamines and observations on the spontaneous occurrence of severe self injurious behaviour in aged HPRT-deficient mice (Williamson *et al.*, 1992a), the hypothesis was proposed that mice were more reliant on APRT than HPRT for their purine salvage. It may, therefore, also be necessary to block APRT activity in HPRT-deficient mice to produce a model for Lesch-Nyhan syndrome. The purine analogue 9-ethyladenine reversibly blocked APRT activity both *in vitro* and *in vivo*, as shown by adenine incorporation assay. 9-Ethyladenine also inhibited HPRT activity. Presumably, 9-ethyladenine was converted by adenase to 9-ethylhypoxanthine that might be an inhibitor of HPRT. However, 9-ethyladenine has a more inhibitory effect on APRT than on HPRT. In contrast to HPRT-deficient mice which did not develop self injurious behaviour spontaneously, the inhibitory effects on both HPRT and APRT led to self mutilation in wild-type mice after administration of 9-ethyladenine. This phenomenon indicates that decrease in APRT activity is more important than that of HPRT in the incidence of self injurious behaviour in mice.

Caffeine also served as an inhibitor of the purine salvage pathway. It inhibited hypoxanthine incorporation more strongly than adenine incorporation. When compared on the basis of their inhibitory effects on adenine incorporation, caffeine is weaker than 9-ethyladenine. Caffeine did not induce self injurious behaviour in wild-type mice when administered at 0.1 mmole/kg body weight. Reduction of purine incorporation in cultured ES cells following caffeine administration at 10^{-3} M was 44% and 33% of control for hypoxanthine and adenine respectively. This level of caffeine administration is about 10-fold less than the dose required for induction of self injurious behaviour in rodents of 185 mg/kg body weight, *i.e.* 0.95 mmole/kg (Peters, 1967). It is therefore easy to understand that the dose required to induce self injurious behaviour would also result in high mortality. By its effect on APRT, caffeine did induce self injurious behaviour after administration to HPRT-deficient mice, although the induction was less severe than that with 9-ethyladenine. The different susceptibility to behaviour alteration with 9-ethyladenine and caffeine did correlate to the effects on adenine incorporation in ES cell culture. The greater severity of self injurious behaviour induced by 9-ethyladenine than by caffeine in HPRT-deficient mice is in agreement with the stronger inhibitory effect on APRT activity by 9-ethyladenine than by caffeine.

The administration of a competitive inhibitor of APRT, 9-ethyladenine, to 9-12 month old HPRT-deficient mice resulted in a high frequency of self injurious behaviour and physical injury. It is tempting to equate the compulsive overgrooming observed in these animals with the self-mutilation that is characteristic of Lesch-Nyhan syndrome. 9-Ethyladenine treatment of 6-8 week old HPRT-deficient animals resulted in less severe physical injury, but a significant increase in the frequency of self injurious behaviour compared to saline-treated control animals. Confirmation of the critical role played by APRT in preventing the appearance of symptoms characteristic of Lesch-Nyhan syndrome in the HPRT-deficient mouse awaits the production of APRT-deficient animals, by gene targeting in embryonic stem cells. The work with 9-ethyladenine and earlier observations on 2-year old HPRT-deficient mice (Williamson *et al.*, 1992a) indicate that it is more difficult to produce the symptoms

of Lesch-Nyhan syndrome in young mice. However, the ability to induce self injurious behaviour in 6-8 week old animals, resulting in a high incidence (80%) of self-mutilation before the mice are 6 months old, indicates that this combined genetic and biochemical model will be valuable for the study of Lesch-Nyhan syndrome. In particular, it will permit the evaluation of novel therapies involving the introduction of functional HPRT genes into the brains of affected animals.

Animal models for human disease, either transgenic or non-transgenic, have provided the opportunity to investigate gene function *in vivo*. As predicted, a null mutation at the immunoglobulin μ locus leads to abnormal B-cell development (Kitamura *et al.*, 1991) and at the β2-m locus leads to absence of certain T-cell populations due to lack of MHC-1 presentation (Zijlstra *et al.*, 1990). A mouse model for Gaucher's disease has a more extreme phenotype than the human condition, with mice dying within 24 hours of birth (Tybulewicz *et al.*, 1992). However, some gene disruptions appear to have no effect on phenotype. The *mdx* mouse, just as the patient suffering from Duchenne muscular dystrophy, is characterised by a complete absence of dystrophin. However, it does not show detectable weakness, nor the progressive degeneration of the limb muscles in adulthood (Bulfield *et al.*, 1984). Stedman *et al.* (1991) have recently shown that the *mdx* mouse diaphragm is more comparable to limb muscle in Duchenne muscular dystrophy, thus extending its applicability as an animal model. More interestingly, surprising differences have been observed in the phenotype of two independent knock-out models for cystic fibrosis. The phenotype of one was severe with most mice dying of intestinal obstruction by 40 days (Snouwaert *et al.*, 1992), while milder symptoms are reported in the other model, including changes in the lung characteristic of the human disease (Dorin *et al.*, 1992). Mice homozygous for a null mutation at the retinoblastoma locus die in utero. Heterozygotes do not develop eye tumours and are thus not good models for familial retinoblastoma (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clarke *et al.*, 1992). On the other hand, mice with a homozygous deficiency for another tumour suppressor gene, p53, develop normally, but succumb to neoplasia with 6 months of birth (Donehower *et al.*, 1992).

These results show that it is important to take differences between man and mouse into account when designing disease models and that the precise nature of the genetic alteration introduced by targeting can have a profound effect on the phenotype. The work with HPRT-deficient mice serves to emphasize the former point. In man HPRT deficiency results in a devastating neurological disorder, while HPRT-deficient mice do not exhibit symptoms of Lesch-Nyhan syndrome spontaneously. In man, APRT deficiency causes difficulties with adenine excretion, rather than behavioural alteration (Simmonds, 1986). The induction of self-injurious behaviour in HPRT-deficient mice treated with an adenine analogue indicates that the relative importance of these two purine salvage pathway enzymes is reversed in the different species.

The animal models for Lesch-Nyhan syndrome are summarised in Table 9.2. Self injurious behaviour was observed in all animal models except untreated HPRT-deficient mice. HPRT-deficient mice carry the identical genetic defect to patients with Lesch-Nyhan syndrome but have shown an incomplete decrease in dopamine levels. The animal model in which neonatal lesions of dopamine receptor are generated pharmacologically showed a correlation between dopamine levels and behavioural alteration. Induction of self injurious behaviour in the 9-ethyladenine-treated HPRT-deficient mouse model will contribute to a better understanding of the pathogenesis of Lesch-Nyhan syndrome.

9.3 APRT inactivation by gene targeting

Although APRT is important in rodents, complete deficiency does not cause any severe symptoms in man (Simmonds, 1986). APRT-deficiency may have contributed to the failure to generate chimaeric mice after injecting APRT null mutant ES cells into blastocysts. HPRT activity is much higher than APRT activity in the human embryo -12.1 to 17.3 pmol/embryo/h between day 2 and 4 after fertilisation, versus 1.3 to 2.0 pmol/embryo/h on day 2-5 for APRT (Leese *et al.*, 1991). In contrast, APRT activity is more important than HPRT activity in mice. HPRT activity in wild-type mice was measured as 0.35 to 0.55 pmol/embryo/h while APRT activity was 0.62 to 1.06 pmol/embryo/h (Moore and Whittingham, 1992). Failure to generate

Table 9.2 Comparison of animal models for Lesch-Nyhan syndrome.

Animal model	Mechanism	Disadvantage	Reference
Administration of caffeine	Inhibition of HPRT and APRT Unknown, probably through adenosine receptor	Self injurious behaviour only occurred at high doses with high mortality	Peters, 1967 this thesis
Administration of clonidine	Blockade of adenosine receptor	No correlation between HPRT-deficiency and adenosine receptor	Razzak *et al.*, 1975
Administration of amphetamine	Stimulation of releasing dopamine	No correlation between HPRT-deficiency and dopamine levels	Brien *et al.*, 1977
Neonatal lesion of dopamine receptor	Dopamine receptor alteration	No correlation between HPRT deficiency and dopamine receptor	Breese *et al.*, 1984
HPRT-deficient mice	Genetic deficiency	No self injurious behaviour occurred except after administration of amphetamine	Finger *et al.*, 1988
HPRT-deficient mice with 9-ethyladenine treatment	Genetic deficiency and biochemical inhibition of adenine salvage pathway	Dopamine level needs to be determined	this thesis

chimaeras with the spontaneous APRT deficient ES cell line, DAP1-50, may be due to loss of pluripotency under 1,6-diaminopurine selection. Alternatively, this failure might have been due to the inability of APRT-deficient ES cells to compete with cells of the host blastocyst.

Although selection for complete deficiency of APRT is possible, it is difficult to isolate heterozygous APRT mutants by diaminopurine selection. Thus, gene targeting was used to isolate ES cells heterozygous for APRT deficiency. To enrich for targeted over random integration events, the positive-negative selection strategy was employed. The thymidine kinase of herpes simplex virus has a much lower Km for thymidine than the thymidine kinase of mammalian cells, 0.6 μM for HSV type 1 kos strain to 2.6 μM for Human AML cells. In addition, the V_{max} value of thymidine kinase in HSV-1 infected BHK cells increased to 26.6 ρmole/sec/mg protein from 8.0 ρmole/sec/mg protein in non-infected cells. The lower Km of herpes thymidine kinase for thymidine and the lower Km of the viral DNA polymerase for deoxynucleoside-5´-triphosphate compared to the mammalian enzymes can be exploited as the therapeutic strategy for herpes simplex virus infections. Acyclovir, 9-(2-hydroxyethoxymethyl)guanine, was the first clinical trial drug for the HSV infection. The positive-negative selection strategy for gene targeting was first introduced by Mansour *et al.* (1988) in their targeting of *int-2* in ES cells. The vector for positive-negative selection was designed using a replacement mechanism when the homologous recombination occurred. It contained homology to the target gene, within which was inserted a neomycin resistance gene as a positive selectable marker allowing selection with G418. An HSV-TK gene was inserted adjacent to either one or both ends of the homology, and was used as a negative selectable marker. When replacement of the endogenous sequences by the vector DNA occurred via homologous recombination, the HSV-TK gene would not be transferred into the target locus while in random integrations the HSV-TK would be retained. Exclusion of the HSV-TK gene during homologous recombination occurred because the HSV-TK gene represented a discontinuity in the homology between incoming vector and the endogenous target sequence. Cells in which the targeting event occurred would

therefore be neo^r and HSV-TK$^-$. On the other hand, random integration of the vector into the recipient cell genome should take place in most cases, resulting in cells that are neo^r and HSV-TK$^+$. Those cells with HSV-TK$^+$ phenotype could be killed by selection with gancyclovir or its analogues (Mansour *et al.*, 1988; McMahon and Bradley, 1990). Mammalian cells, unlike certain bacteria and fungi, do not contain cytosine deaminase and do not ordinarily metabolise cytosine to uracil. Nor do they metabolise the innocuous compound 5-fluorocytosine to the highly toxic compound 5-fluorouracil. As an alternative to HSV-TK, the bacterial cytosine deaminase gene has been used as a negative selection module by supplementation with 5-fluorocytosine in the culture medium (Mullen *et al.*, 1992). The diphtheria toxin A module has been also used as a negative marker to inactivate the *c-fyn* locus of mouse ES cells (Yagi *et al.*, 1990). Those eukaryotic cells containing functional diphtheria toxin A module would commit suicide by inhibiting ADP-ribosylation of elongation factor 2 upon protein synthesis without the requirement of chemical selection.

Owing to the lack of a proper direct selection scheme, the targeting strategy for inactivation of APRT followed the most popular positive-negative selection. Instead of HSV-TK, the HPRT mini-gene was used for counter selection in electroporated HPRT-deficient ES cells. A HPRT mini-gene functional in ES cells, was modified from the original mini-gene which functioned in fibroblasts but not in ES cells (Melton *et al.*, 1986) by the addition of an element from the 5′ end of intron 1 (Selfridge *et al.*, 1992). This modified HPRT mini-gene was as effective as the conventional herpes simplex virus TK gene in targeting vectors designed to inactivate the *ERCC-1* gene using the positive-negative selection procedure. The use of the neo/HPRT mini-gene for positive-negative selection gave 8- to 12- fold enhancement in APRT targeting. Although the targeting efficiency is higher in using insertion vectors than replacement vectors, most targeting experiments use replacement vectors because of the opportunity for positive-negative selection (Table 9.3). Factors which might influence enrichment in positive-negative selection procedures are, marker gene, distance between positive and negative markers, length of homology, end protection of negative marker, and target locus. No effect on enrichment following positive-

negative selection occurs if different positive or negative selection modules are used. No difference was noticed between the *neo* or hygromycin resistance gene modules for positive selection when HSV-TK was used as a negative selection module (Mortensen *et al.*, 1991). Similarly, the enrichment was not affected by using HSV-TK or HPRT mini-gene as a negative selection marker (Selfridge *et al.*, 1992). The length of homology and specific locus effects always play important roles in homologous recombination and they should also contribute to the success of positive-negative selection. Keeping the integrity of the negative selection module could also optimise the enrichment by positive-negative selection. The abundance of endogenous exonuclease in cells will digest some of the incoming DNA to protect the host. This mechanism would decrease the efficiency of negative selection by causing loss of functional negative selection modules before integration. It has also been suggested that the distance between positive and negative gene could be another factor interfering with enrichment. Selfridge *et al.* (1992) showed slight improvement of enrichment on *ERCC-1* gene targeting by reducing the distance between positive and negative markers to reduce the possible loss of negative module spontaneously.

It has been proven that the homologous recombination machinery is very sensitive to base pair mismatches between incoming and target DNAs in prokaryotes as well as in mammalian cells. Although there is only a 2% difference in the sequences of creatine kinase M between strain 129 and Balb/c, this greatly affected the targeting efficiency in 129-derived cells using Balb/c derived vector DNA. Two linearised replacement vectors with identical structures but one derived from strain 129 and the other from Balb/c, were introduced into the mouse strain 129-derived AB-1 cells to evaluate the targeting efficiency. Although the enrichments with positive-negative selection were similar, the targeting frequency with isogenic DNA was 12% of the double resistant clones while no homologous recombinants were observed with non-isogenic DNA (van Deursen and Wieringa, 1992). Different efficiencies of gene targeting between isogenic and non-isogenic DNA were also found for the HPRT (Deng and Capecchi, 1992) and *RB* loci (te Reile *et al.*, 1992). Two similar constructs, with the *neo* gene embedded in 10.5 kb of *RB* sequences around the 19th

and 20th exon, from 129 and Balb/c genomic libraries respectively, were electroporated into the 129-derived ES cell line, E14. Targeting was 50-fold more efficient with the 129-construct than with the Balb/c-construct. The isogenic targeting construct allowed the easy recovery of homologous recombinants without the use of any enrichment protocol. In addition, a *RB* targeting construct containing the HPRT mini-gene was introduced into E14TG2a, an HPRT-deficient mutant derived from E14, to generate HPRT$^+$ *RB* inactivated clones that correctly integrated into the 19th exon of one of the *RB* alleles through homologous recombination. One of these clones was used as the recipient for a second targeting experiment using constructs derived from 129 or Balb/c genomic libraries. Double crossing-over at the previously targeted *RB* allele would substitute HPRT for *neo* and give colonies resistant to G418 and 6-thioguanine. Random integration would gain the resistance to G418 but retain sensitivity to 6-thioguanine. G418 and 6-thioguanine doubly resistant colonies represented 1 in 200 of the singly resistant colonies against G418 when the targeting construct was derived from Balb/c genomic DNA and 1/10 for the isogenic targeting construct. Targeting at the *RB* locus with isogenic DNA was 20-fold more efficient than with non-isogenic DNA (te Reile *et al.*, 1992). The murine APRT gene in the vector pAPRTneoδ101 is derived from the genomic clone pSAM 6.3 which was cloned from Balb/c sperm DNA. Although both 129 and Balb/c strains are *Musculus domesticus* strains, the APRT gene sequence could differ between Balb/c and strain 129. Differences in genomic sequence between strain 129 and Balb/c have been reported in the ERCC-1 gene (Selfridge *et al.*, 1992). Two distinct APRT alleles have been reported in the P19 mouse teratocarcinoma cell line which was established from an embryo of *M. domesticus* strain C_3H mouse (Turker *et al.*, 1989). The low targeting efficiency on APRT by pAPRTneoδ101 might therefore, be due to the non-isogenic origin of the vector.

Reduced transcript levels in cells with heterozygous targeted genes have been demonstrated by Northern analysis, such as G protein αi2 subunit (Mortensen *et al.*, 1991), glucocerebrosidase (Tybulewicz *et al.*, 1992) and Prp protein (Büeler *et al.*, 1992). Primary mouse embryo fibroblasts established from heterozygous mice

generated by gene targeting the LIF gene showed a reduction of the LIF RNA transcript by Northern hybridisation. Heterozygous lines of embryo fibroblast also supported ES cell colony formation to a lesser extent, with about one-third the number of undifferentiated clones obtained compared to the wild-type lines (Stewart *et al.*, 1992). The growth rate decreased in those heterozygous mice carrying an insulin-like growth factor II gene disrupted by gene targeting (DeChiara *et al.*, 1990). Heterozygous APRT targeted ES cells show the expected reduction in intensity of the APRT mRNA compared to the parental HM-1 cells. [^{3}H]-adenine incorporation was also found to be significantly lower in the APRT targeted cells than in parental HPRT-deficient ES cells and in HPRT wild-type ES cells. Decrease in the levels of β-actin transcript in heterozygous APRT targeted cells relative to wild-type cells was also noticed in the Northern blot with well matched 18 S and 28 S ribosomal RNA signals. The reduction of β-actin transcript might be the result of the decreasing *de novo* RNA synthesis in APRT targeted cells. It has been reported that actin mRNA becomes an increasing percentage of total RNA during lymphocyte activation by concanavalin A. As early as 3 hr after mitogen addition, the actin mRNA content per equivalent of total RNA increased substantially (Degen *et al.*, 1983). Opposite to the mitogen activation, the actin transcripts might decrease in the heterozygous APRT mutant which lacks complete purine salvage activity both in HPRT and APRT.

9.4 Gene therapy in HPRT-deficient mice

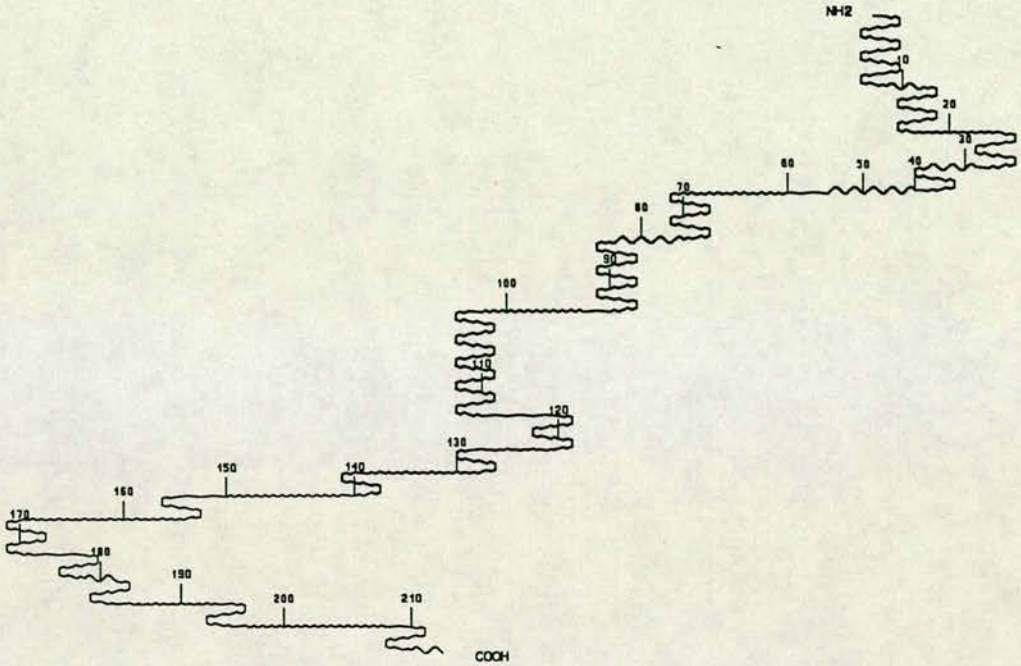
Male mice heterozygous for an HPRT transgene in the HPRT-deficient background (*tg*/+; $hprt^{b\text{-}m3}$/Y) were mated to females homozygous for the null HPRT allele ($hprt^{b\text{-}m3}/hprt^{b\text{-}m3}$). The offspring could be either heterozygous for the transgene or not contain it, but all would be deficient for the endogenous HPRT gene, and could be used to evaluate the usefulness of the dual incorporation assay to detect low amounts of HPRT activity. High hypoxanthine incorporation activities in the brains and concanavalin A-stimulated splenocytes of transgenic mice does not support the finding of low transcript levels in HPRT-transgenic mice (Thompson, 1989). The homozygous transgenic mice contained 9 copies of the transgene but only 12% of normal transcript levels was found. The ratio of hypoxanthine to adenine

incorporation in two transgenic mice was as high as that in HPRT wild-type mice. To understand how the normal incorporation ratio is produced from a low level of RNA transcript, the protein structures of endogenous and transgenic HPRT were compared. Protein derived from the transgene has a different amino acid sequence at the amino-terminus. The mouse endogenous gene codes for a protein which has the amino-terminal amino acid sequence: methionine, proline, threonine, arginine, serine, proline, serine. The initiating methionine is post-translationally cleaved from the HPRT protein (Wilson *et al.*, 1982). The transgene codes for a protein which has the amino-terminal sequence: methionine, proline, threonine, proline, aspartic acid, proline, alanine, serine. The three amino acids, arginine, serine and proline in the endogenous protein which are basic, polar and non-polar respectively, are substituted with proline, aspartic acid, proline and alanine in the transgene which are non-polar, acidic, non-polar and non-polar respectively. Consequently, the predicted secondary structures vary in the amino-terminal region, with one α-helix and β-sheet possibly missing in the mutant protein (Figure 9.1) (Chou and Fasman, 1978). The different activities observed may, therefore, be attributed to their different secondary structures.

The reliable dual incorporation assay combined with HPRT-deficient mice make a good system to evaluate strategies for gene therapy *in vivo* as well as *in vitro*. The idea that human genetic disease and even some degenerative and infectious disease will become amenable to correction at the genetic level has cleared its initial conceptual and technical obstacles and has now become widely accepted in most molecular genetic, medical and public circles (Friedmann, 1992). In general, there are two types of gene therapy depending on the manipulations involved. DNA or a virus vector may be injected directly into the target tissue of the recipient, allowing cells to be altered *in vivo*. Alternatively, vehicle cells either autologous or from an other individual or species may be manipulated *ex vivo* by gene transfer and then transferred into the target tissue by cell transplantation. The most direct and simple delivery system for gene therapy is injection of bare DNA directly into the target tissue. The efficiency of direct injection could vary according to injection methods, simple needle, jet injections (Furth *et al.*, 1992) or particle bombardment (Friedmann *et al.*, 1989).

Figure 9.1 Secondary structure predictions for HPRT wild-type and transgene-encoded proteins. The secondary structure prediction is based on Chou and Fasman (1978) and was computed using an algorithm developed by Jameson and Wolf (1988). Both predictions were plotted using the "Peptidestructure" and "Plotstructure" programs developed by the University of Wisconsin Genetics Computer Group (UWGCG) (Devereux *et al.*, 1984). A: wild-type HPRT protein; B: transgene-encoded HPRT protein; Arrow: the site of different predicted secondary structure of transgene-encoded HPRT protein from that of wild-type HPRT protein.

A


NH2
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40
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60
70
80
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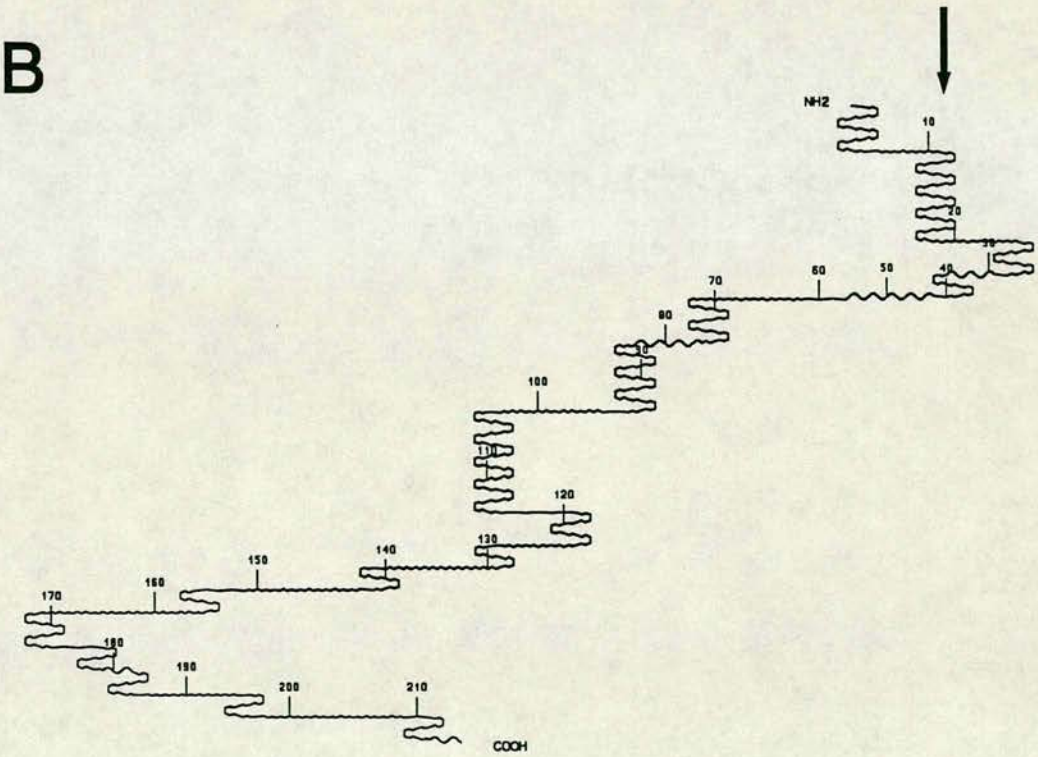
100
120
130
140
150
160
170
180
190
200
210
COOH

B


NH2
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Direct DNA injection was first demonstrated by intramuscular injection of the human dystrophin gene into mice. A human 12-kb full-length cDNA or a 6.3-kb cDNA encoding a functional version of the dystrophin sequence was inserted into an expression vector containing the Rous sarcoma virus promoter. Seven days after intramuscular injection of 400 μg of plasmid DNA containing the dystrophin gene into dystrophin deficient mdx mice, human dystrophin immunoreactivity was detected in some cells located about 5 mm proximal to the injection site (Acsadi *et al.*, 1991). Although expression remained stable one year after injection, the efficiency of this procedure is very poor. Injection of 400 μg of dystrophin cDNA, equivalent to the total number of dystrophin gene copies from several mice, leads to the synthesis of detectable dystrophin in only some 50 fibre profiles out of 5,000 in a single muscle. Failure to detect functional HPRT activity in mice injected with the HPRT mini-gene intracerebrally may result from the same difficulty that arose with intramuscular injection. To improve the efficiency of transfection, a soluble, targetable DNA carrier system consisting of an asialoglycoprotein covalently coupled to a polycation has been developed. A soluble DNA complex was formed by mixing asialoglycoprotein-polycation conjugate with plasmid DNA containing the structural gene for human serum albumin driven by mouse albumin enhancer-rat albumin promoter elements. Nagase analbuminemic rats possess a defect in the splicing of serum albumin mRNA which results in virtually undetectable levels of circulating serum albumin. The DNA/carrier complex was successfully targeted to unique receptors on hepatocytes that internalise galactose-terminal glycoproteins. Partial hepatectomy shortly after infusion of the DNA complex led to hepatocyte replication and prolonged expression of the introduced DNA. Circulating human albumin became measurable at a level of 0.05 μg/ml within 24 to 48 hr after injection and increased in concentration to a maximum of 34 μg/ml by 2 weeks post-injection. Although the level of expression remained stable through 4 weeks after injection and partial hepatectomy, the circulating serum albumin was 1,000 times lower than that of normal mice (Wu *et al.*, 1991). In general, a therapeutic effect would require the increase of activity to more than 10% of the control and there has been no functional recovery following direct gene injection. The efficiency of expression by introducing DNA by direct injection still

requires improvement.

Gene transfer mediated by viral vectors may provide higher target specificity and efficiency. Two genera of virus have been modified as vectors for direct gene transfer *in vivo*. Recombinant adenovirus has been developed as a delivery system for gene transfer to lung tissue *in vivo*. The major advantages of using adenovirus as the vector are that host cell proliferation is not required for expression of adenoviral proteins, and adenoviruses are normally trophic for the respiratory epithelium. Unlike other viruses, adenovirus preferentially integrates into one region in chromosome 19, thus decreasing the risk of unpredictable insertional mutagenesis (Kotin *et al.*, 1990; Samulski *et al.*, 1991). Other advantages of adenoviruses as potential vectors for gene therapy are as follows: (i) genetic variation is rare in contrast to all RNA viruses for the lack of proof-reading repair enzyme activity with reverse transcriptase (Steinhauer and Holland, 1987); (ii) there are no known associations of human malignancies with adenoviral infections despite common human infection with adenoviruses; (iii) the adenovirus genome can be manipulated to accommodate foreign genes of up to 7.0 to 7.5 kb in length; and (iv) live adenovirus has been safely used as a human vaccine. The major disadvantages are the inclusion of many adenovirus genes in current vectors that may stimulate immunity or have other adverse effects, and potential instability of gene expression because the vector does not integrate into chromosomal DNA. α_1-Antitrypsin (α_1-AT) deficiency and cystic fibrosis are the most common hereditary disorders of the lung in human (Crystal, 1992). A replication-deficient adenoviral vector containing an adenovirus major late promoter and a recombinant human α_1AT gene was used to infect epithelial cells of the cotton rat respiratory tract *in vitro* and *in vivo*. Freshly isolated tracheobronchial epithelial cells infected with recombinant virus contained human α_1AT mRNA transcripts and synthesized and secreted human α_1AT. After *in vivo* intratracheal administration of recombinant adenovirus to these rats, human α_1AT was synthesized and secreted by lung tissue, and human α1AT was detected in the epithelial lining fluid for at least 1 week (Rosenfeld *et al.*, 1991). Myoblasts have been used to test the possibility of direct efficient gene expression via adenovirus as a vector. A recombinant adenovirus containing β-galactosidase as a

reporter gene under the control of muscle-specific regulatory sequence was able to direct expression of the β-galactosidase in myotubules derived from rodent myogenic cell lines as well as in mouse muscle *in vivo* up to 75 days after injection (Quantin *et al.*, 1992).

Herpes simplex virus type 1 is a neurotropic virus that establishes latent infection in neuronal cells and, therefore, can establish a nonlytic relationship with the host cells. A recombinant HSV-1 vector containing human HPRT cDNA under the regulatory control of the viral thymidine kinase gene promoter has been demonstrated to direct HPRT expression both in neuronal cells (Palella *et al.*, 1988) and in the brains of mice infected *in vivo* with this vector by direct intracranial inoculation (Palella *et al.*, 1989). The neuropathogenesis of HSV-1 remained the major disadvantage of this gene transfer delivery system, since most of the mice infected with either recombinant or wild-type virus died of neuroparalysis. The latency of HSV-1 infection was then used to develop as a mild transfection route to avoid the cytopathogenesis. No herpes simplex viral proteins are detectable in latently infected cells because none of the genes characteristic of the acute infectious cycle are expressed during latency. Transcription did occur during latency from one region of the viral genome, the latency-associated-transcript sequence. An HSV-1 recombinant containing the β-glucuronidase gene downstream of the latency-associated-transcript sequence of herpes simplex virus type 1 was used to infect MPS VII mice (an animal model for human Sly disease in which deficiency of β-glucuronidase causes the lysosomal storage disease). Cells expressing β-glucuronidase activity were present in the trigeminal ganglia and brainstems up to 4 months after infection by corneal scarification and adsorption (Wolfe *et al.*, 1992). If this procedure were used on patients, recurrence of infection could still occur with the risk of pathogenesis when the patients were under stress or immunosuppressed.

Cell-mediated gene transfer followed by transplantation of modified cells into target tissue, can be carried out in many types of vehicle cells. One of the most promising types of delivery cells for gene therapy is haematopoietic cells because of

well developed procedures for bone marrow transplantation, and the wide distribution of haematopoietic cell types implicated in many human diseases. The target for gene transfer is the haematopoietic stem cell, or long-term repopulating cell, that is present at low frequency in bone marrow and gives rise to all myeloid and lymphoid cells over prolonged periods.

Most gene transfer *ex vivo* trials in humans rely for gene transduction on retroviruses from which all virus genes have been removed or altered so that no viral proteins are made in infected cells. Viral replication functions are provided by the use of retrovirus 'packaging' cells that produce all of the viral proteins but do not produce infectious viruses until the modified retroviral vector is introduced. Introduction of the DNA form of a retroviral vector into packaging cells results in production of virions that carry vector RNA and can infect target cells, but no further virus spread occurs after infection. To distinguish this process from a normal viral infection where the virus continues to replicate and spread, the term transduction rather than infection is often used.

The major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction. The ability to transfer genes efficiently and stably to target cells, especially primary somatic cells, is not shared by other gene transfer techniques and is the major attraction of retroviral vectors for use in gene therapy. Major disadvantages include the apparent inability of retroviral vectors to infect nondividing cells, and an inherent inability to characterise completely the retroviral vector preparations used for gene transduction because retroviral vectors cannot be made synthetically but must be produced by cultured cells. Unlike proteins or other simple compounds, retrovirus vectors are complex mixtures of proteins and nucleic acids and cannot be purified to homogeneity after production. This disadvantage means that vector-producing cell lines must undergo extensive testing for possible adventitious microorganisms, including replication-competent retroviruses. Other contaminants

such as cellular RNAs that are packaged into retroviral vectors cannot be removed. Some of these RNAs can be reverse transcribed and integrated in cells transduced with retroviral vectors, and only experience in animal models and in humans will determine their possible effects.

Two other potential problems with retroviral vectors which warrant discussion, are insertional mutagenesis and potential replication competent virus production. Problems with insertional mutagenesis, such as activation of cellular oncongenes, are shared with any gene transfer technique that results in integration of new sequences into the cellular genome. Although there are many examples of retroviral activation of cellular oncogenes in mice, these events occur in the context of a spreading infection by replication-competent viruses. Whether such events can occur at appreciable rates after infection by replication-defective retroviral vectors remains to be seen. The potential for production of replication-competent virus during the production of retroviral vectors remains a concern, although for practical purposes this problem has been solved. In the human trials so far, none of the production batches of retroviral vectors and none of the human patients have tested positive for replication-competent virus. The potential for replication-competent virus production depends on viral sequences both in the retroviral vector and in the packaging cells used for vector production. The packaging cells contain all sequences necessary for viral protein synthesis and the retroviral vector contains the sequences necessary in *cis* for virus transmission, thus recombination between these sequences has the potential to generate replication-competent virus. To prevent this problem it is particularly important to avoid homologous overlap between helper virus and viral vector (Miller, 1992).

Deficiency of the enzyme adenosine deaminase results in a variant of severe combined immunodeficiency, a lethal disorder usually treated by allogenic bone marrow transplantation. Retroviral vectors can efficiently transduce the human adenosine deaminase gene into established lymphoid cell lines that had been derived from patients and into haematopoietic progenitors (Bordignon *et al.*, 1989; Kantoff,

1986). Retroviral transduced adenosine deaminase restored human peripheral blood lymphocytes which were then injected into BNX immunodeficient mice. Restoration of immune function, including expression of human immunoglobulin and antigen-specific T cells was observed (Ferrari *et al.*, 1991). In skeletal muscle injection of myoblasts obtained from normal mice into the muscles of mdx mice was far more effective than direct transfection at producing dystrophin-positive muscle fibres, injection of 100,000 cells gives up to 30-40 per cent dystrophin-positive fibres, spreading over large areas (Partridge *et al.*, 1989). Myoblast injection also has the advantage of providing new sources of cells for formation of new normal muscle fibres and repair of dystrophic muscle fibre within muscles where the endogenous repair processes are failing (Morgan *et al.*, 1990; Webster *et al.*, 1986). Unfortunately, the myoblast cell line used in these experiments was an immortal mouse myoblast line that can form tumours in recipient animals.

The major difficulty of gene therapy for neurological diseases is obtaining enough cells of the appropriate type for gene manipulation. Neural cells cannot be isolated from adult animals except by supplementation with growth factors. Reynolds and Weiss isolated neurons and astrocytes from the brain striata of 3- to 18-month-old mice by inducing cell proliferation following supplementation with epidermal growth factor. Only about 1% of enzymatically dissociated brain cells survived and underwent cell division (Reynolds and Weiss, 1992). In parallel, neurons and astrocytes could be isolated from the brain of adult mice by supplementation with basic fibroblast growth factor. The efficiency of isolation still remained very low. Fewer than 200 cells survived from 6 X 10^4 cells placed in culture (Richards *et al.*, 1992). Because the efficiency of cell isolation is so low, it is impossible to establish autologous neuron lineage cells for gene manipulation from human biopsy specimens. The best alternative cell to be used in gene transfer for neurological disease in a mouse model might be ES cells. Embryonic stem cells are permanent cell lines established directly from the inner cell mass of the preimplantation embryo. They retain the property of totipotency which implies the capacity to differentiate into all tissues of zygotic origin. Martin and Evans (1975) indicated that pluripotent

embryonic stem cells can be maintained in an undifferentiated state only by culture on feeder layers of growth-arrested embryonic fibroblasts. However, more recent data indicate that totipotency and inhibition of differentiation can be maintained in cell culture supplemented with either conditioned medium (Smith and Hooper, 1987) or leukaemia inhibition factor (Pease and Williams, 1990). Hence, the isolation of ES cells from preimplantation embryos can also be completed without the cooperation of other cells (Pease *et al.*, 1990; Nichols *et al.*, 1990).

Several years ago it was noted that if ES cells were cultured without LIF they would begin to differentiate spontaneously. This differentiation was associated with aggregation of the cells and the production of fluid filled cystic structures composed of many different cell and tissue types. One of the striking findings was that under appropriate conditions the differentiating ES cells would produce structures similar to yolk sac blood islands that contained immature erythrocytes, suggesting that this was an *in vitro* system in which one could investigate the earliest stages of haematopoietic development (Chen, 1992). There is a method for *in vitro* differentiation of ES cells apart from liquid culture system. When ES cells were cultured in methylcellulose, there was a strong transcriptional activation of many haematopoietically relevant genes (Snodgrass *et al.*, 1992). Similarly, when cultured in the presence of 0.3 μM retinoic acid while attached to plastic surfaces, ES cells differentiate into cells resembling fibroblasts which do not express any of the markers characteristic of derivatives of the extra-embryonic endoderm. When aggregated and exposed to 0.3 μM retinoic acid, the ES cells differentiate and develop large numbers of neurons and astrocytes in addition to relatively small numbers of fibroblast-like cells (Jones-Villenuve *et al.*, 1983). Embryonic stem cells are truly pluripotent since they participate in the normal development of all tissues of an animal if implanted back in a developing blastocyst (Martin, 1981; Evans and Kaufman, 1981). The use of ES cells as a gene transfer delivery system has been reported. An expression vector with *neo* selection marker and mouse ciliary neurotrophic factor (CNTF) driven by the CMV promoter was constructed for neurotrophic factor expression , and electroporated into D3 embryonic stem cells. 5×10^7 CNTF-secreting D3 cells were intraperitoneally injected into

pmn/pmn mice, which is an animal model for human spinal motor neuron disease, resulting in prolonged life time. Behaviour and histological studies also showed the progress of the treatment, although intraperitoneal growth of teratoma-like tissue was also observed (Sendtner *et al.*, 1992). In the present study, intracerebral transplantation with 10^5 HPRT wild-type ES cells into HPRT-deficient mice did restore the HPRT activity in the brain effectively. It was also found that transplantation with a smaller number of ES cells could avoid the risk of tumourigenesis. ES-derived brain cells, which are easily distinguished from endogenous brain cells by HAT selection, were committed to differentiate into the neuronal lineage *in vivo*. This phenomenon was proved by both morphological observation and by an immunohistochemical assay. These results suggest that pluripotent ES cells can be educated by the environment and undergo appropriate differentiation *in vivo*.

Correction of defective genes by homologous recombination , which can avoid unexpected mutations occurring during the gene transfer procedure, is well established. In addition to their differentiation ability both *in vitro* and *in vivo*, ES cells could serve as vehicle cells for somatic gene therapy by gene targeting. The isolation of isogenic ES cells for gene manipulation in human is not yet feasible, although there is no difficulty if the mouse is used as a model for gene therapy. Alternatively, mouse oocytes can be induced to initiate development in the absence of fertilisation. Activation of oocytes results in the production of various classes of parthenogenones; either haploid or diploid in genetic constitution. The majority of parthenogenetically-derived embryos will develop normally through the pre-implantation stages of development (Robertson, 1987). If parthenogenetically-derived ES cells could be established from human, it would be possible to do gene therapy for neurological diseases by the manipulation of autologous ES cells *ex vivo* and transplanting the modified ES cells back to the patient, without danger of tissue rejection.

9.5 Conclusion

The major objective of this project was to use HPRT as a model to investigate

mechanisms of homologous recombination for gene deletion and to study the pathogenesis and therapy of HPRT deficiency. The establishment of a method to delete genes by homologous recombination was achieved at both the HPRT and the APRT loci. The success of targeting deletion in murine embryonic stem cells at the APRT locus should lead to the generation of APRT-deficient mice. Subsequently, the APRT-deficient and APRT-HPRT-double deficient mice could be used to confirm the hypothesis that the lack of behaviour abnormalities in HPRT-deficient mice might be due to their greater dependence on APRT than HPRT. Further studies on the regulation of the purine salvage pathway and *de novo* synthesis could be investigated by the provision of APRT-, HPRT-, and double knock-out animal models.

HPRT-deficient mice in conjunction with 9-ethyladenine treatment provides the animal model to study the pathogenesis of Lesch-Nyhan syndrome and to evaluate therapeutic strategies, especially for gene therapy. In this study, the failure to restore the HPRT activity in the brain of HPRT-deficient mice by direct DNA injection has been discussed. Instead, the restoration of HPRT activity in brain has been achieved by transplantation with HPRT wild-type embryonic stem cells. It has also been proven that the ES cells were educated to differentiate along the neural lineage after implantation to the brain. Combined with successful correction by homologous recombination in ES cells, a novel strategy for gene therapy is proposed. Furthermore, wild-type ES cells transplanted into HPRT-deficient mice provides a novel system to investigate tissue development *in vitro*, by making it possible to isolate ES-derived neural cells *in vitro* by HAT selection of explanted brain tissue.

Table 9.3 Comparison of enhancement by positive-negative selection on homologous recombination.

Gene	+[a]	−[b]	Homology(kb)	Distance(kb)[c]	Enhancement	Reference
HPRT	*neo*	tk	9.1	7.8(+[d])	3125	Mansour *et al.* 1988
int-2	*neo*	tk	10	6.8(−[e])	1975	Mansour *et al.* 1988
int-1	*neo*	tk	13.5	10.3(−), 3.2(−)	10000	Thomas & Capecchi 1990
int-2	*neo*	tk	10	9.5(−)	1130	Mansour *et al.* 1990
c-fyn	*neo*	DT-A	6.3	5.3(−)	9	Yagi *et al.* 1990
DFHR	*neo*	tk	4.6	3.5(−)	55-333	Zheng *et al.* 1990
IGF-II	*neo*	tk	9.7	8.7(+)	13	DeChiara *et al.* 1990
GATA-1	*neo*	tk	4.7	1.1(−)	23	Pevny *et al.* 1991
Hox 1.3	*neo*	tk	7.4	5.5(+)	13	Jeannotte *et al.* 1991

Table 9.3 Comparison of enhancement by positive-negative selection on homologous recombination (continued).

Gene	+	−	Homology(kb)	Distance(kb)	Enhancement	Reference
Hox 1.5	*neo*	tk	11.5	4(−), 7.3(−)		Chisaka & Capecchi 1991
G-protein	*neo*, *hyg*	tk	5	2(−) 2(−)	4 3	Mortensen *et al.* 1991
CFTR	*neo*	tk	7.8	0.7(−),7.1(−)	3-8	Koller *et al.* 1991
ig-u	*neo*	tk	9	8.4(+)	8	Kitamura *et al.* 1991
T-cell receptor	*neo*	tk	8.5	6(−), 2.5(−)	2	Mombaerts *et al.* 1991
Ren-1D	*neo*	tk	5.6	1.3(+)	2.7	Miller *et al.* 1992
Hox1.6	*neo*	tk	11.8	7.4(+), 4(−)		Chisaka *et al.* 1992
p53	*neo*	tk	3.7	1.5(−)	3	Donehower *et al.* 1992
apolipoprotein A1	*neo*	tk	9	6(+)	2-4	Williamson *et al.* 1992b

Table 9.3 Comparison of enhancement by positive-negative selection on homologous recombination (continued).

Gene	+	−	Homology(kb)	Distance(kb)	Enhancement	Reference
LIF	*neo*	tk	5.6	4.8(−)		Stewart *et al.* 1992
RB	*neo*	tk	8	1.4(−), 6.2(+)	17	Lee *et al.* 1992
RB	*neo*	tk	9.6	1.1(?)	10	Jacks *et al.* 1992
RB	*neo*	tk	15	12.8(+)	15	Clarke *et al.* 1992
glucocerebrosidase	*neo*	tk	6.1	3.2(+)	8.5	Tybulewicz *et al.* 1992
ERCC-1	*neo*	tk	2.4	1.8(−)	1.5	Selfridge *et al.* 1992
		HPRT	8.0	0.6(+)	15	
				1.3(+)	6.2	
				5.0(+)	4.5	
				5.0(−)	3.8	

Table 9.3 Comparison of enhancement by positive-negative selection on homologous recombination (continued).

Gene	+	–	Homology(kb)	Distance(kb)	Enhancement	Reference
creatine kinase M	*hyg*	tk	8.3	4.8(+)	2.4	van Deursen *et al.* 1992

a Positive selection module

b Negative selection module

c Distance between positive and negative selection module

d with end protection by other sequences

e without end protection by other sequences

Table 9.4 Delivery systems for gene therapy.

Gene delivery	Advantage/Disadvantage	Reference
Direct DNA transfer		
(1) DNA injection	Simple, low expression	Acsadi *et al.* 1991
(2) liposome-DNA	Simple, low expression	Nabel *et al.* 1990
(3) glycoprotein	Simple, low expression	Wu *et al.* 1991
(4) virus-mediated	high expression, risk of viral toxicity	Rosenfeld *et al.* 1992
Cell-mediated gene transfer	Complex, high expression	
(1) haematopoietic		Ferrari *et al.* 1991
(2) myoblasts		Yao & Kurachi 1992
(3) ES cells		

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