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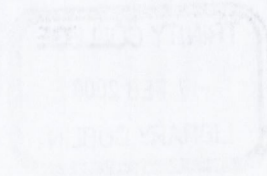
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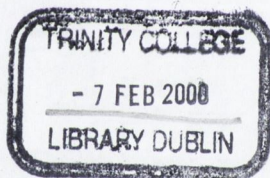
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**On the Development of Animal Models for
Degenerative Retinal disease.**

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Ph.D. Thesis
Submitted 12th October, 1999**





Thesis
5333

Declaration.

I hereby certify that this thesis submitted for examination for the degree of Doctor of Philosophy to the University of Dublin, has not been previously submitted for a higher degree in this or in any other university. The work presented here is entirely my own, except where clearly stated within individual chapters.

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Summary.

Retinitis pigmentosa (RP) is one of the leading causes of inherited blindness within the working population of many developed countries. There is presently no cure for this disorder. The projects outlined in this thesis were undertaken in an attempt to generate and analyze mouse models with RP. Such models are centrally important to the development of methods for the prevention of the disease, as they provide a means to investigate the pathophysiology of the disorder and systems in which to begin assessing retinal therapies.

A major objective of this thesis was the development, on site, of procedures for the maintenance and growth of embryonic stem (ES) cells, their genetic manipulation, and the generation by gene targeting of a mouse model for autosomal dominant retinal degeneration. Specifically a 1bp deletion (CTG to C-G) at codon 307 of the mouse peripherin gene was successfully carried out in R1 embryonic stem cells. These cells were used to generate 21 chimeric mice carrying the targeted deletion. To date, two of these mice have passed the 3codon 307 mutation on to the next generation, demonstrating germline transmission of the cell line. In man, the codon 307 mutation within the RDS-peripherin gene results in a slowly progressive form of adRP. A similar phenotype may be produced in mice. If so, these animals would provide a valuable model for dominant disease. The relatively slow retinopathy may possibly provide a broader window of time to realise the potential of certain gene therapy vectors (i.e AAV).

Animals carrying a targeted disruption of the rhodopsin gene, ($\rho^{-/-}$), are currently used in studies to evaluate various potential gene therapies as a means to treat human retinal disease. Histopathological, immunocytochemical and electrophysiological data are presented demonstrating substantial structural and functional rescue of rod cells in $\rho^{-/-}$ mice retinas. This rescue was brought about by crossing these animals onto a transgenic strain of mouse expressing human rhodopsin. Such information is required for optimal use of $\rho^{-/-}$ mice in exploring methods for suppressing human degenerative retinal conditions whose molecular pathologies are based either upon loss of rhodopsin gene function or on the manifestation of dominant mutations.

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Abbreviations.

AAV	Adeno-associated virus
CGMP	cyclic 3', 5' -guanosine monophosphate
ERG	electroretinogram
ES cells	Embryonic stem cells.
G418	Gancycloivr
GFP	green fluorescent protein
INL	inner nuclear layer
IS	inner segment
LIF	leukemia inhibitory factor
Neo	neomycin
ONL	outer nuclear layer
OS	outer segments
PCR	polymerase chain reaction
PEFs	primary embryonic feeders.
RDS	retinal degeneration slow.
RK	rhodopsin kinase
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
rho-/-	wild type mouse
rho+/-	mouse heterozygous for a targeted disruption in the rhodopsin gene.
rho-/-	mouse homozygous for a targeted disruption in the rhodopsin gene.

Publications from work in this thesis:

Retinopathy induced in mice by targeted disruption of the rhodopsin gene.

Humphries, M. M., Rancourt, D., Farrar, G. J., Kenna, P., Hazel, M., Bush, R. A., Sieving, P. A., Sheils, D. M., Mc Nally, N., Creighton, P., Erven, A., Boras, A., Guyly, K., Cappecchi, M. R. and Humphries, P.
Nature Genetics (1997), 15: 2, 216 - 219.

Structural and functional rescue of murine rod photoreceptors by human rhodopsin transgene.

Mc Nally, N., Kenna, P., Humphries, M. M., Hobson, A.H., Khan, N.W., Bush, R.A., Sieving, P.A., Humphries, P., and Farrar, G.J.
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Chapter 1.
General Introduction.

Introduction.

Retinitis pigmentosa (RP) is one of the leading causes of inherited blindness within the working populations of many developed countries. Investigations of how retinal degeneration occurs have been hampered by a lack of autopsy material from genotypically characterised patients with early disease. Animal models can help to overcome the problem, and also provide vehicles through which experimental gene therapies can be evaluated. Up to 30 genes involved in the etiology of RP have now been identified, many encoding proteins of the visual transduction cascade (RetNet, 1999). While mutations may act in a recessive or dominant manner, observations in a number of animal models, and in one case of autosomal dominant RP in man (adRP caused by a threonine -to-methionine substitution at codon 17 of the rhodopsin gene) indicate that a general mechanism of photoreceptor cell death is apoptosis (Li Z Y *et al.* 1994, Weiss *et al.* 1995, Portera-Caillau *et al.* 1994). Given that efficient methods of gene delivery are likely to be developed (studies at this laboratory and many others, have resulted in long term expression of reporter genes carried by AAV vectors and we have achieved expression of CAT and luciferase reporter genes following systemic gene delivery) the concepts underlying approaches to gene therapy appear to be quite simple; either replace a defective gene, suppress a dominant mutation or suppress apoptotic process, or use a combination of said approaches (Millington - Ward *et al.* 1997, Ali *et al.* 1998). In practice, however, major problems are likely to be encountered. For example, over-expression of a normal gene product may induce degenerative changes (Olsson *et al.* 1992), hence, extreme care must be undertaken such that accurate regulation and expression of the therapeutic gene is achieved. Already, reports have appeared in which a ribozyme directed towards a mutant rod opsin gene, has rescued photoreceptors in a transgenic rat (Lewin *et al.* 1998). However, the rhodopsin gene carries over one hundred mutations in cases of RP. Owing to constraints on ribozyme design, only a minority of these mutants could ever be targeted by ribozymes. Suppressors of apoptosis are numerous, including absence of c-fos in light-induced apoptotic models, up-regulation of the Bcl2 gene product, expression of the P35 survival factor in some systems, and so on (Hafezi *et al.* 1997., Joseph *et al.* 1996, Ali *et al.* 1998, Davidson *et al.* 1998.) However, the long-term effects on retinal tissues of suppression of apoptotic photoreceptor cell death remain to be investigated. To address these, and many

other issues, transgenic animals, and mice carrying targeted modifications of retinopathy genes are required. In the work presented in this thesis, I have established mouse embryonic stem cell technology on site, to the extent of having obtained chimeric mice from morula fusions, and having obtained (in collaboration with Dr. Derrick Rancourt) 21 chimeric mouse founders carrying a targeted dominant mutation of the peripherin/rds gene ($\Delta 307$). In addition, effective photoreceptor cell rescue has been demonstrated in rho-/- mice by a normal human rod-opsin transgene, a first major step towards generating a mouse model for adRP expressing both normal and mutant rod opsins on the rho-/- background. Much of the etiology of RP centers on defects in genes encoding proteins involved in visual transduction. Hence, a brief review of this process will be provided together with an introduction into clinical and genetic aspects of RP and the current state of art in respect to availability of animal models and progress in gene therapy using viral systems.

Visual transduction in rod cells.

The retina represents an important model for studies of neurodegeneration not only in view of the fact that it represents “an accessible piece of the brain”, but also, in a very practical sense, because degenerative diseases of photoreceptor neurons represent the most prevalent cause of registered blindness in many developed countries of the world. An haematoxylin and eosin (H+E) stained section of a mouse retina, viewed by light microscopy, is illustrated in figure 1. The outer and inner nuclear layers define the nuclei of the primary (rod/cone photoreceptors) and secondary retinal neurons respectively. Mouse and human retinas are anatomically similar, although the human retina is very much more cone-rich. A diagrammatic representation of a rod photoreceptor cell is illustrated in figure 1.2. The human retina transmits light impulses to the brain by absorption and transduction of light energy by the rod and cone photoreceptors (see also Chapter 5). Visual transduction takes place within the outer segments of the photoreceptor cells. The outer segments of rod cells are comprised of stacked disc-like structures, of which the protein rhodopsin is the major transmembrane component. The stacked discs of the rod outer segments are clearly visible in the electronphotomicrograph of a mouse retinal section shown in figure 1.3. Rhodopsin

is a photoreactive protein consisting of an opsin molecule bound to the chromophore 11-*cis* retinal (Yau, 1994). Absorption of light results in the conversion of 11-*cis* retinal to the all-*trans* form, enabling the rhodopsin molecule to initiate an enzyme cascade resulting in the visual transduction signal (Djamgoz *et al.* 1995, Molday, 1998- see fig. 1.4). A series of conformational changes in the rhodopsin protein result in the formation of metarhodopsin, which has the ability to bind transducin (Wright, 1992). Transducin is a member of the heterotrimeric G-protein family and consists of α , β and γ subunits and non-covalently bound GDP. Photo-isomerisation of rhodopsin catalyzes the exchange of guanine diphosphate (GDP) to GTP. Once bound to GTP the α subunit of transducin dissociates from its inhibitory β and γ subunits (Hargrave and Mc Dowell, 1992). This dissociation is catalyzed by a retinal RGS, regulator of G-protein signaling (Palczewski and Saari 1999). Phosphodiesterase interacts with the β and γ subunits of transducin preventing reassociation of the subunits until depolarisation occurs. The activated α subunit of transducin in turn activates the heterotetrameric enzyme, cyclic GMP phosphodiesterase (PDE). PDE is comprised of one α , one β , and two γ subunits. A σ PDE subunit has also been identified. PDE activation occurs following displacement of two inhibitory λ subunits. PDE hydrolyzes cGMP bound to a cGMP-gated channel transmembrane protein. Such hydrolysis causes the closure of cGMP-gated ion channels and hyperpolarization of the plasma membrane, generating the neural current.

The cGMP-gated channel controls calcium and sodium ion influx into the cell. In the depolarised state ($\sim 40\text{mV}$) Na^+ and Ca^{2+} ions flow into the rod outer segments through the channel (Koch, 1992). A dark current loop is maintained by the flow of K^+ ions out of the inner segment through voltage-gated K^+ channels. Na, K ATPase maintains the Na^+ and K^+ gradients in the inner segment while the Ca^{2+} concentration in the outer segment is controlled by the Na/Ca-K exchanger in the ROS plasma membrane. There is a continuous release of glutamate transmitter from the synaptic region of the photoreceptor cell during the depolarised state (Kawamura, 1995). The closure of the cGMP-gated channel prevents the entry of the Na^+ and Ca^{2+} ions while the Na/Ca-K exchanger continues to extrude Ca^{2+}

from the ROS, and K^+ is still able to pass out of the cell through the plasma membrane. When the rod cells become hyperpolarised the glutamate release at the synaptic region of the rod cell is inhibited and a build up of positive charge occurs outside the cell informing the neighbouring cells of the light stimulus.

The shut down of the visual cascade and the increase in cGMP levels allows the photoreceptor cells to return to a depolarised state (Yau, 1994). The pumping of calcium ions across the membrane provides the necessary feedback signal. Recoverin activates guanylate cyclase (Djamgoz *et al.* 1995). Recoverin is phosphorylated in conditions of low Ca^{2+} concentration. Guanylate cyclase restores the cGMP quantity in the cell, allowing the reopening of the cGMP gated channels. Rhodopsin kinase and arrestin co operatively deactivate rhodopsin by phosphorylation of the carboxy-terminus of the protein. Conformational changes, which take place in rhodopsin (probably metarhodopsin III), allow it to interact with rhodopsin kinase (RK) (Hargrave and McDowell, 1992) . The latter enzyme phosphorylates threonine and serine residues in the -COOH - terminal region of rhodopsin. Once phosphorylated, rhodopsin binds to arrestin. Final quenching of the rhodopsin protein occurs when all-trans retinal is hydrolysed to all-trans retinol by all-trans retinol dehydrogenase. Dissociation of all-trans retinal from rhodopsin also results in the release of arrestin. Phosphatase 2A then de-phosphorylates rhodopsin. Regeneration of 11-cis retinal from retinol occurs in the retinal pigment epithelial layer. Diffusion is probably mediated by the interphotoreceptor retinoid binding protein, IRBP (Eriksson 1997). Reassociation of 11-cis retinal returns rhodopsin to the photosensitive state, leading to the completion of the transduction cycle (Hargrave and McDowell 1992). As will be outlined below, mutations within a variety of genes encoding enzymatic components of the visual transduction and retinoid cycles have now been encountered in human degenerative retinopathies.

Retinitis pigmentosa.

Retinitis pigmentosa (RP), a disease involving progressive degeneration of the photoreceptor cells of the retina, is the most prevalent of the Mendelian retinopathies. The initial clinical symptom of the disease is usually night blindness (nyctalopia) which is brought about by the early death of the rod photoreceptors cells (Dryja and Li 1995, Wright 1992, Humphries *et al.* 1990). Additional symptoms of the disease include a "waxy pallor" of the optic disc, attenuation of the retinal vasculature, accumulation of pigmentary deposits on the retinal surface and a flat electroretinogram (see figures 1.5 and 1.6 - fundus photographs and a picture of an ERG). While up to fifty percent of cases of the disease are sporadic, the remainder generally segregate in an autosomal dominant, recessive or X-linked manner (Humphries *et al.* 1992, Kaplan *et al.* 1990). However, more recently two additional patterns of inheritance have been documented. A few patients have been identified with a "digenic" form of RP, involving co-inheritance of mutations within both the peripherin/rds and rom-1 genes (Dryja *et al.* 1997). In addition, mitochondrial inheritance of RP, as part of a syndrome incorporating sensorineural deafness and mild muscle myopathy, has also, recently been described (Mansergh *et al.* 1999). Globally, approximately 115 genetic loci involving some form of retinopathy have been identified (see RetNet, 1999). Physical mapping and candidate gene studies have now resulted in the identification of approximately 35 genes involved in the etiology of RP (RetNet), the majority encoding proteins that are expressed either within the photoreceptor cells or, in some cases, the pigmented epithelium. The first RP gene to be localised was for an X-linked form of the disease (Wright *et al.* 1987). In that study the probe used to detect linkage to the disease locus mapped on the short arm of the X chromosome at Xp11. The first linkage of a genetic marker to a gene for autosomal dominant RP(adRP) was obtained as a result of studies on an Irish family (TCD M). Linkage of the disease locus in this family to the DNA marker D3S47 on 3q was reported in 1989 (Mc William *et al.* 1989). The marker co-segregated with the disease locus at zero percent recombination and a lod score of 14.7. Notably, the gene encoding rhodopsin also mapped on 3q (Nathans *et al.* 1986) although the precise location of the gene was unknown. This observation stimulated the search for mutations within the rhodopsin gene of patients with autosomal dominant RP. The first adRP mutation to be reported was a single amino acid substitution at codon 23 in the first exon of the

rhodopsin gene (Dryja *et al.* 1990). The C-A transversion resulted in a proline-histidine substitution toward the amino terminus of the protein. These findings stimulated an extensive search for rhodopsin mutations in banks of DNA from RP patients. At present, over 100 rhodopsin mutations have been linked to autosomal dominant forms of RP (HGMD, 1999). Additionally, a few rhodopsin mutations have been identified in autosomal recessive forms of RP and in autosomal dominantly inherited congenital night blindness (RetNet, 1999).

Rhodopsin is the major transmembrane protein of the rod photoreceptor outer segments and therefore may play a role in the physical stabilisation of the outer segment disk membranes as well as its role in visual transduction. The protein is a member of the G-coupled receptor family that typically consists of proteins with 7 membrane spanning domains (Shastri 1994, Bird 1995). The acetylated amino end of the protein is located on the intradiscal side and the carboxy terminus on the cytoplasmic side of the membrane. (see Fig. 1.7) The rhodopsin molecule contains 348 amino acids with two asparagine -linked oligosaccharide side chains (Bird 1995). Peptide folding results in seven predominantly α -helices being embedded in the disk membrane bilayer, with interconnecting hydrophilic segments protruding from the membranous surface. Two highly conserved cysteines on the intradiscal surface are important for structural stability. The lysine at codon 296 is the 11-cis retinal binding site. 11-cis retinal also interacts with other helices to form a cage around the chromophore (Bird 1995). Photon absorption brings about rearrangement of the helices and exposure of amino acids encoded by codons 231 to 252 to the cytoplasmic surface of the discs, thus allowing the binding of the G protein, transducin (Franke *et al.* 1992). The carboxy terminus of rhodopsin contains serines and threonines, which are phosphorylated by rhodopsin kinase, thus enabling the binding of arrestin (Bird 1995). Mutations within each of the regions of the protein outlined above can result in RP. Mutations in the hydrophobic region or near to the oligosaccharide attachment sites may interfere with rhodopsin folding and glycosylation, preventing the correct incorporation of the protein into the photoreceptor membrane (Bird 1995). A diagram of the rhodopsin protein indicating the position of many of the mutations identified to date is illustrated in figure 1.7.

Sung *et al* (1991), reviewed rhodopsin mutations responsible for autosomal dominant RP and studied the functional characteristics of many of them. Specific mutations were introduced into human rhodopsin cDNA by site-directed mutagenesis and the functions of the encoded proteins examined by transfection of a human embryonic kidney cell line and COS cells with mutant constructs. On the basis of these studies in cultured embryonic kidney cell lines, dominant rhodopsin mutations were divided into class I and class II. In Class I mutations, rhodopsin is expressed normally on the plasma membrane and binds 11-cis retinal, creating a chromophore with an absorption spectrum similar to that of wild type rhodopsin (Sung *et al.* 1991). Class II mutations on the other hand, are defective in thermal stability and folding, malfunction in respect to the binding of the chromophore 11-cis-retinal, and fail to reach the plasma membrane. Deletion mutagenesis experiments have highlighted the importance of the intradiscal domain in maintaining the correctly folded protein structure (Bird 1995). Proper folding of the protein also requires the presence of a disulphide bridge between cysteines at 110 and 187. Mutant rhodopsin tends to accumulate in the rough endoplasmic reticulum, thus indicating a transport problem (Dryja and Li 1995). Improperly transported rhodopsin in adRP may bring about photoreceptor degeneration owing to an insufficiency of rhodopsin in the outer segments (haploinsufficiency). It is also possible that the mutant protein may interfere with cellular function by accumulating near the endoplasmic reticulum (Bird 1995). Mutations, which effect rhodopsin transport, have been studied in some detail through the use of animal models. (This will be considered later in the section of the introduction dealing with animal models). Rhodopsin mutations within the cytoplasmic domain and at the carboxy terminus of the protein may affect protein structure and folding or may disrupt phosphorylation or protein-protein interactions of the visual transduction cycle. The interference with protein-protein interactions necessary for the visual transduction cycle may create cytotoxic problems for the photoreceptor cells, hence causing degeneration (Bird 1995, Shastry 1994).

While, the mechanisms of retinal degeneration caused by rhodopsin mutations are not fully understood, it does seem likely that for some mutants aberrant intracellular processing of rhodopsin or disc instability brought about by the mutated protein are the likely causes of cell mortality. Irrespective of the nature of the mutation (this probably applies also to mutations within other retinopathy

genes), the “final common pathway” of photoreceptor cell death appears to be apoptosis. Much of the evidence in support of the apoptotic death process comes from the study of animal models (see following section), although apoptotic demise of photoreceptor cells has been observed in at least one human post-mortem retinal specimen (Portera- Cailliau *et al.* 1994, Chang *et al.* 1993, Li Z Y *et al.* 1994).

The gene encoding Peripherin/RDS was the second to be implicated in the etiology of adRP. The “retinal degeneration slow” (rds) mouse was identified in 1989 (Travis *et al.*). In the naturally occurring mouse model, (retinal degeneration slow), the gene encoding peripherin, a structural component of the rod and cone outer segment disc membranes, is transcriptionally inert. The human homologue of murine peripherin, the so-called peripherin/RDS gene, has been physically mapped on 6p. Farrar *et al.* (1991) reported the cosegregation of a late-onset adRP gene with genetic markers from 6p, and the subsequent identification of a mutation within the peripherin/RDS gene in a patient with adRP. The peripherin protein is comprised of 346 amino acids with four predicted hydrophobic transmembrane domains and two putative N-linked glycosylation sites (Arikawa *et al.* 1992). Peripherin/RDS is present in the rim of the outer segments of the rod photoreceptor cells (see fig. 1.8). The first RP causing mutation within the peripherin/RDS gene (Farrar *et al.* 1991) was a three base-pair deletion removing one of a pair of cysteine residues at codons 118/119. Kajiwara *et al.* (1991) subsequently reported three mutations at the peripherin/RDS locus in autosomal dominant RP patients. These were a trinucleotide deletion at codon 219 resulting in the removal of a proline residue and single base codon changes at 216 and 185 which brought about proline - leucine, and leucine - proline substitutions respectively. Subsequent screening of banks of DNA from patients with various retinopathies revealed that mutations within the peripherin/RDS gene can give rise to a variety of ocular symptoms including autosomal dominant RP, retinitis *punctata albescens*, patterned pigment dystrophy of the fovea and macular dystrophy (Felbor *et al.* 1997, Kajiwara *et al.* 1993, Nichols *et al.* 1993, Weleber *et al.* 1993, Wells *et al.* 1993). To date over 60 mutations have been found within the peripherin/RDS gene in patients with retinopathies (HGMD).

In 1996 Keen and Inglehearn re-evaluated the 43 mutations that had been described in the human peripherin/RDS gene at that time. Thirty of these were missense mutations, 2 single base substitutions resulting in termination

codons, 7 small in-frame deletions and 4 insertion/deletion mutations that break the reading frame. Of these mutations, 39 were associated with retinal phenotypes that could be grouped into 4 broad categories, dominant RP, progressive macular degeneration, digenic RP or pattern dystrophies. The mutations underlying dominant RP and severe macular degeneration were largely missense or small in-frame deletions in the large predicted intradiscal loop between the third and fourth transmembrane domains of the molecule. In contrast, mutations causing other phenotypes were largely non-sense mutations and were scattered throughout the gene (Keen and Inglehearn 1996).

Digenic RP is the term given to the condition where two genetic components are required to give rise to the disease pathology (Kajiwara *et al.* 1994). Rom-1 was identified in 1992 as a protein structurally similar at the amino acid level to peripherin/RDS (Bascom *et al.* 1992). This protein has been located in the rim of rod photoreceptor discs (Moritz and Molday 1996). Peripherin/RDS can bind non-covalently to rom-1, forming a tetrameric complex consisting of two peripherin/RDS and two rom-1 proteins linked by disulphide bonds (Goldberg *et al.* 1995). It has been proposed that the formation and stability of the bend in the disc membrane in rod photoreceptors depends upon the association between rom-1 and rds proteins. In the absence of rom-1 the tetrameric structure can be formed by two homodimers of peripherin and vice versa. Kajiwara *et al.* (1994) demonstrated that RP could be caused by double heterozygosity of the leu85-pro mutation in combination with a null mutation of the rom-1 gene. Interestingly no case of RP has been identified which is due to a rom-1 mutation alone. The precise role of this protein, or indeed peripherin/RDS, while speculated as structural, is unknown. Simultaneously, mutations in both proteins may de-stabilise the rod outer segment disc structure. In summary, mutations in the peripherin/RDS gene can cause a range of clinically variable inherited retinal degenerations.

Three mutations within the PDEA gene, encoding the α subunit of rod-specific phosphodiesterase (PDE) have been identified in patients with RP (Huang *et al.* 1995). Two nonsense mutations, Tyr 583 ter and Trp 561 ter, bring about early termination of translation of mRNA. The Ser 344 Arg mutation occurs at a highly conserved site in the second non-catalytic cGMP-binding domain (Huang *et al.* 1995).

The β -subunit of rod PDE is also a likely candidate for mutations in RP owing to the fact that mutations within this gene are known to bring about naturally occurring retinopathies in the dog and mouse. The human PDEB gene is located on 4p16. To date 1 splice site mutation, one 1bp deletion, 3 nonsense mutations and 5 missense mutations in the PDEB gene have been associated with arRP (Mc Laughlin *et al.* 1993, HGMD). No mutations in the PDEG gene, encoding the PDE γ subunit, have yet been identified. Exactly how mutations in the PDE β and PDE α genes bring about RP is not understood, however it has been suggested that raised cGMP levels brought about by these mutations may be harmful to the photoreceptors. High cGMP levels may force the cGMP gated channel open and thereby cause a strain on the entire phototransduction system (Tsang *et al.* 1996). The CNCG protein (the cGMP-gated cation channel), is comprised of an α and a β subunit. The α subunit gene (CNCG1) has been mapped to 4p14-q13 and consists of 10 exons. Five mutations within the α subunit gene have been associated with arRP (Dryja *et al.* 1995). Two of these are nonsense mutations, one a frameshift mutation resulting in a truncated protein, another a deletion which removes a large portion of the gene, and one mutation is a Ser 316 Phe missense mutation. The mis-sense mutation and the c-terminal truncation result in mutated protein which, when expressed *in vivo* is not transported to the plasma membrane.(Dryja et al 1995).

Not all genes involved in the etiology of RP are confined in their expression to the photoreceptor cells. Three notable cases are genes involved in the X-linked forms of RP (RP2/ RP3) and the gene encoding protein Rpe65, resulting in an autosomal dominant form of adRP (Battacharya *et al.* 1986, Meindl et al. 1996, Musarella et al. 1990). The first of these genes to be characterised (RP3) was at Xp21.1 (Wright *et al.* 1987). The gene has now been identified by positional cloning, and shows homology to the regulator of chromosome condensation gene (RCC1) (Meindl *et al.* 1996). RCC1 belongs to a family of guanine nucleotide exchange factors (GEFs) of Ran, a small Ras-like GTPase (Egan and McInnes 1996). The gene is called RPGR, retinitis pigmentosa GTPase regulator. RPGR may play a role in regulation of Ran or a similar protein. The role of Ran in the cell appears to be the activation of RNA transport out of the nucleus

through the nuclear pore complex. The RPGR protein contains sites, which suggest that it is membrane-bound, unlike the RCC1 protein, indicating a rather more specific role for RPGR. However it is of note that expression of RPGR is not limited to the retina, and as yet the mechanism by which the protein causes disease is unknown. A second XIRP gene maps to Xp11.3 (RP2). Mapping studies based on three deletion patients pinpointed this location for RP2 on Xp (Bhattacharya *et al.* 1984). Schwahn *et al.* (1998) isolated the gene encoding RP2 by positional cloning. It is of interest that the amino acid sequence of the RP2 gene has significant homology to human cofactor-C. Cofactor-C is involved in the final stage of β -tubulin folding. Therefore, a possible role for the RP2 protein in the correct folding of photoreceptors or neuron-specific tubulin isoforms has been proposed by Schwahn *et al.*

The RPE 65 protein is a microsomal protein specific to the RPE (Hamel *et al.* 1993). The gene has been mapped to the human chromosome 1p31 (Hamel *et al.* 1994, Nicolletti *et al.* 1995). A role for the protein in the metabolism of vitamin A was initially proposed due to its biochemical association with the retinal binding protein RBP and 11-cis-retinol dehydrogenase (Bavik *et al.* 1992). The generation of an RPE 65 knockout mouse demonstrated the necessity of the RPE65 protein in the production of 11-cis-retinal, (Redmond *et al.* 1998). Over accumulation of all trans-retinal esters in this mouse, indicates a role for the RPE65 protein in the isomerization of all trans-retinal to the all 11-cis form. Research is ongoing into the precise role played by RPE65. This study highlights the value of animal models in retinal research.

Animal models of retinitis pigmentosa.

Animal models of Retinitis pigmentosa are centrally important to the development of methods for the prevention of the disease. Naturally occurring retinal degenerations have been identified in mice, rats, cats, and dogs (Carter-Dawson *et al.* 1978, Mullen and LaVail 1976, Suber *et al.* 1993, Narfstrom and Nilsson 1987). Such naturally occurring models provide a means to investigate the pathophysiology of these disorders, and systems in which to begin assessing retinal therapies. A range of animal models mimicking human disease is required to undertake such studies. Mouse strains from repositories such as the Jackson

laboratory have been analysed to identify new animal models simulating human disorders (Chang *et al.* 1993). The recent development of transgenic technologies and the ability to manipulate the mouse genome through the use of embryonic stem cell technology has greatly increased the range of animal models available for study.

The mouse continues to be the most widely used animal for research into retinitis pigmentosa. The advantages of the mouse as a model include the following: the mouse breeds easily, has a short gestation period, and requires relatively little living space when compared to other animals. In addition, the genetics of the mouse is well documented, and the genome size and number of genes are similar to human, as are patterns of development (Hogan *et al.* 1986). Transgenic technology with mouse embryos is highly developed and until quite recently the mouse was the only animal for which embryonic stem cells could be maintained in culture. The mouse is presently the only mammalian species that gene targeting and gene knockouts have been successfully carried out in (see for example Humphries *et al.* 1997). The disadvantages of the mouse as an animal model for ocular disease investigations include the small size of the mouse eye and the nocturnal behaviour of the animal. The small size of the eye complicates administration of therapies via, for example, ocular injection and in addition makes accurate electroretinography more difficult. The nocturnal behaviour of the mouse is a reflection of a higher rod: cone photoreceptor ratio in mouse as compared to humans. There are other disadvantages to the use of the mouse as a model for human disease. For example, cases have arisen where the knockout of a gene involved in human disease has little or no phenotypic effect in the mouse. For example, mutations in the *Hexa* and *Hexb* genes in man cause Tay-Sachs and Sandoff diseases (Phaneuf *et al.* 1996). Knockout of the *Hexa* gene in the mouse however, does not result in severe neurological problems. Removal of the mouse *hexb* gene on the other hand does induce a phenotype similar to that observed in man. Investigation of ganglioside metabolism in the mouse uncovered differences in the ganglioside degradation pathway between mouse and humans, explaining the discrepancy observed with the targeted *Hexa* mice. Additionally, problems can arise owing to the nature of the transgenes used to create the animal model, resulting in a less than perfect model for the human disorder. For example, regulatory elements may be missing from the construct, or the site of integration of

the transgene may affect subsequent expression, preventing accurate simulation of human disease in resulting mouse lines (Melet *et al.* 1996). Although the genetics of mouse and man are similar, genes are nevertheless present in humans that are not present in the mouse, and in such cases the mouse will most probably not be a suitable animal model for human disease. Also, the short lifespan of the mouse can be problematic in cases where a degenerative disease which progress over a period of years in humans may not have sufficient time to develop in the mouse. The rd mouse was identified in 1978 by Carter-Dawson *et al.* and exhibits an autosomal recessive retinopathy similar to that observed in human retinitis pigmentosa. Retinal degeneration in the rd mouse is a rapid process; the majority of photoreceptors have degenerated by postnatal day 47. The outer segments of the rod and cone cells develop normally until postnatal day 7-8 at which time degeneration of the outer nuclear layer begins. Degeneration is characterised by changes in retinal ultrastructure such as mitochondrial swelling and disorganisation of the outer segment disc membranes. The rate of degeneration of the rod cells differs from that of the cones. Rod cells degenerate essentially completely by 7 weeks. Cone cells, however, survive for up to 18 months. The photoreceptor degeneration has a central-peripheral gradient for both rod and cone cells. A cone electroretinogram can be detected from the periphery of the retina up to postnatal day 132. In contrast, the central retina loses its cone ERG response at day 24. Farber and Lolley (1974, 1976) showed that retinal degeneration in the rd mouse is preceded by a build up of cyclic GMP in the retina, which correlates with abnormal activity of rod photoreceptor cGMP-phosphodiesterase. The causative gene in rd mice was identified as an *ochre* mutation (C-A transversion at codon 347) within the β -subunit of the cGMP-phosphodiesterase gene which is located on mouse chromosome 5 (Bowes *et al.* 1990). The photoreceptor cells in rd $\pm$ mice are normal in morphology but show a reduction in levels of retinal cGMP, altered phosphodiesterase kinetics, abnormalities of cGMP binding and somewhat abnormal electroretinograms. The rd mouse has been widely used to assess various gene delivery systems for retinal tissues and the viability of gene therapies. For example, Bennett *et al.* (1996) delivered the wild type PDE β gene in an AV vector to rd mice via subretinal injection and showed some rescue of the disease pathology.

The *rds* mouse expresses a semi-dominant mutation, which results in a biphasic retinal degeneration, that is, the rate of degeneration is most rapid over the first three months, after which it becomes slower. In *rds*^{-/-} mice rod and cone photoreceptor do not develop outer segments (van Nie 1978). Rhodopsin-containing vesicles are present in the space between the photoreceptor cell bodies and the pigmented epithelial cells. The presence of rhodopsin suggests the existence of an outer segment layer, which is unable to fold into discs in the absence of peripherin. Retinal degeneration becomes apparent 2 weeks postnatally in this animal model. The rate of rod degeneration differs between the peripheral and the central part of the retina. Peripheral degeneration progresses more rapidly, resulting in a loss of photoreceptors by nine months, while photoreceptor cells are present for up to 12 months after birth in the central regions of the retina. The ERG response from the *rds* mouse shows a slight reduction in amplitude of both the a- and b- waves from the start of the degeneration. However the response time-to-peak is similar to that recorded in wild type mice. Readings in older mice show a decrease in amplitude of both a and b waves and increase in time-to-peak. Mice of 6-7 months of age still generate the b-wave at high light intensities, however the a-wave is no longer detectable (Reuter and Sanyal 1984). In contrast to homozygous *rds* mice, heterozygous *rds* mice form photoreceptor outer segments. However, morphology is somewhat abnormal, with reduced length and a disorganised structure. The process of disc shedding and their uptake by phagosomes also shows differences between *rds* and wild type mice. The phagosomes in the retinal pigment epithelium are much bigger in size in *rds* mice and appear to be present in larger numbers than in normal mice. The *rds* gene has been identified and is transcriptionally inert owing to insertion of a 9.2kb repetitive genomic element similar to the t haplotype-specific element within exon II of the H2 complex (Connel *et al.* 1991). *Rds* mice, like *rd* mice, should prove valuable in the exploration of therapeutic approaches for retinopathies.

In 1993 Chang *et al* identified a naturally occurring mouse model with a retinal degeneration termed *rd*-3. In these mice the retina develops normally for the first two weeks, and rapid photoreceptor degeneration beginning in the third week postnatally. The majority of the rod photoreceptors are lost by 5 weeks of age. However, cone photoreceptors survive until 8 weeks postnatally. The electroretinogram never shows a normal response but a maximum peak is recorded

at 4 weeks after birth. Rd-3 mice show no ERG response by 7 weeks postnatally. The rd-3 locus has been localised to mouse chromosome 1, distal to Akp-1, but has not yet been identified. This region is syntenic with human chromosome 1q, close to the region where the gene for Usher syndrome type II is located (Kimberling *et al.* 1990). While this model may assist research into Usher syndrome type II, it may also be a good model for retinitis pigmentosa owing to the initial normality of the photoreceptor cells over the first weeks, thus resembling the human disorder more closely in terms of morphology than other naturally occurring animal models. A dominant retinal degeneration (rd4) associated with a chromosomal inversion has been identified in the mouse (Roderick *et al.* 1997). Rd4 is homozygous lethal. In heterozygotes photoreceptor cell death begins 10 days after birth and is complete after 6 weeks. Electroretinograms are difficult to detect at 6 weeks. The mutation was found in a stock of mice carrying the inversion In(4)56Rk (chromosome 4), and a human homologue is therefore expected on the corresponding human chromosomes 1p or 8q. This example highlights the benefits of screening available mouse stocks for retinal disease. The characterisation of this mouse may assist in the identification of a new human RP locus.

The rd-5 mouse was also found as part of an active program to screen mouse strains and stocks for ocular disorders. The homozygous rd5 mouse represents a model for Usher syndrome type 1 (Heckenlively *et al.* 1995). The ERG is never normal, with reduced amplitudes that are no longer detectable by 6 months. Shortening of the photoreceptors, and disorganisation of the inner and outer segments are observed by 3 weeks. Retinal abnormalities such as arteriolar attenuation, venous dilation and a granular appearance are visible by 6 weeks and by 8 months no photoreceptors are present. Again the discovery of this mouse is an example of the benefits of searching stocks for suitable animal models. Larger mammals will undoubtedly prove to be of value as intermediary models in the development of human therapies. The RCS rat displays abnormal choroidal angiogenesis and a retinal pigment epithelium (RPE) which is unable to phagocytose shedding rod outer segment membranes (Mullen and LaVail 1976). This animal has been widely used in studies of transplantation of RPE, a strategy that has been shown to have a rescuing effect on the photoreceptor cells. The RCS gene remains to be identified, although it has been resolved to an interval of approximately 400kb (Dr. Marc Abitbol, personal communication). Retinal disorders

leading to blindness are also common in dogs, the Irish Red Setter being an example. Suber *et al* 1993, identified a nonsense amber mutation at codon 807 within exon 21 of the gene encoding the β -subunit of rod phosphodiesterase in the animals. Progressive rod-cone dystrophy in dogs has also been mapped to chromosome 9, a region syntenic with human chromosome 17q22, where an RP gene has been localised in a large South African pedigree. However, neither the human nor the dog gene has yet been characterised. Other animals such as the Abyssinian cat show a progressive form of rod/cone degeneration inherited in a recessive fashion, the gene causing this degeneration having not yet been found (Narfstrom and Nilsson 1987).

Transgenic mice.

In 1993 Olsson *et al.*, succeeded in generating transgenic mouse lines expressing mutant and wild-type alleles of the rhodopsin gene (the mutant allele was P23H). These mice displayed variable expression and copy number of the transgenes. Three lines of mice carrying the mutant allele developed a photoreceptor degeneration similar to that observed in human RP patients. The least severe case of retinal degeneration occurred in the mouse with the lowest level of transgene expression. Thus it was possible to formally validate the assumptions made by linkage studies, that is, that mutations within the rhodopsin gene cause retinal degeneration.

This animal model also permitted analysis of the mutated protein in an *in vivo* environment. As mentioned previously, *in vitro* studies have been carried out involving the expression of rhodopsin mutants in embryonic kidney cell lines. Rhodopsin mutants were sub-divided into three classes based on such studies. The conclusion from these initial cell culture investigations was that rhodopsin containing the P23H mutation binds 11-cis-retinal poorly and is not transported from the endoplasmic reticulum to the plasma membrane (Sung *et al.* 1991). This observation led to the hypothesis that the degeneration caused by P23H was due to the failure of newly synthesised rod opsin to leave the Golgi apparatus and travel to the rod inner segments (Olsson *et al.* 1992).

The information obtained as a result of the *in vivo* transgenic studies contradicted these original assumptions (Roof *et al.* 1994). Analysis of

degenerating retinas showed mutated rhodopsin to accumulate in the outer nuclear layer and within the photoreceptor synaptic terminals of the P23H mouse. This new observation demonstrated that transport of P23H rhodopsin did occur out of the golgi apparatus and rhodopsin moved both to and away from the rod outer segments. Roof *et al.* have suggested the unusual location of the mutated rhodopsin to be due to intracellular routing of the mutated protein along a pathway not used by normal rhodopsin, a loss of polarity. This finding highlights the importance of *in vivo* mouse studies and the presence of the correct environment for analysis of the properties of mutated proteins. In the case of the P23H rhodopsin mutation, the *in vivo* analysis highlighted the putative role of the carboxy terminus in intercellular transport.

In 1994 a transgenic Glutamine-344-ter mouse was produced which demonstrated a requirement for the carboxy terminus of the protein for efficient rhodopsin transportation to the rod outer segments (Sung *et al.* 1994, Liu *et al.* 1997). Immunocytochemical studies of the retinas of these mice showed abnormal accumulation of the Q-344-ter protein in the plasma membrane of the photoreceptor cell bodies and the inner segments. It was proposed that loss of the 5 amino acids at the carboxy end of the protein impaired the binding of one or more components that act to transport rhodopsin. In the Q-344-ter mouse in addition to a number of animal models, photoreceptor cell death appears to be brought about by apoptosis, as measured by inter-nucleosomal DNA fragmentation and TUNNEL labeling (Portera-Cailliau 1994). Intracellular congestion may be the cause of retinal degeneration in these animals.

The proline at position 347 in rhodopsin is conserved in all known visual pigments and mutations at this position have been shown to cause RP in humans (Berson *et al.* 1991). In P347S transgenic mice there is no accumulation of mutated protein in the inner segments but rhodopsin-containing vesicles were observed in the extracellular space, again suggesting inefficient transport of the protein from inner to outer segments (Li *et al.* 1996). It has been proposed that photoreceptor degeneration occur due to a failure to renew outer segments at the normal rate. The photoreceptors of transgenic mice expressing pro347Ser degenerate slowly. This mouse model was used to study the mechanisms of retinal degeneration by aggregation of embryos of normal and transgenic mice to form chimeras (Huang *et al.* 1993). Chimerism /mosaicism was observed in coat colour, RPE and retina. In

the retina, the genotype of adjacent patches of normal and transgenic photoreceptors was determined by in situ hybridization with a transgene-specific RNA probe. Photoreceptors in chimeric retinas degenerated uniformly, independently of the genotype and similar to the photoreceptors of transgenic mice. Chimeric retinas with nearly equal proportions of normal and transgenic photoreceptors displayed uniform but slower degeneration. These observations imply that cellular interactions between photoreceptors probably play a role in degeneration (Huang *et al* 1993).

In vitro biochemical studies have indicated that phosphorylation of the carboxy terminus of rhodopsin is crucial in the quenching of rhodopsin activity during the phototransduction cycle. The binding of arrestin to the protein follows phosphorylation of rhodopsin. Transgenic mice have also been employed to investigate the importance of the carboxy terminus of the rhodopsin protein *In vivo* (Chen *et al.* 1995)

Transgenic mice expressing a truncated form of rhodopsin, Ser 334 ter, and therefore lacking the phosphorylation sites at the carboxy terminus of the protein were generated and by Chen *et al.* Comparison of the wt mouse ERG and that of the transgenic rhodopsin Ser 334 ter mouse proved interesting. Both normal single photon responses after photoisomerisation and prolonged responses were observed in the transgenic mouse. The prolonged responses, which became evident about 0.1 sec after the stimulatory flash of light, were brought about by the presence of the truncated protein. The prolonged response of the truncated protein indicates a role for the phosphorylation sites *in vivo*. A sudden shut off of the photoexcited ser 334 ter rhodopsin was observed after the prolonged responses. This shut off of the rhodopsin photoresponse in the absence of phosphorylation sites highlights the importance of other factors in this process (Chen *et al.* 1995). Lysine 296 is the rhodopsin chromophore attachment site. A Lys-296-Glu rhodopsin mutation *in vitro* causes light-independent activation of rhodopsin and in turn, the constitutive activation of transducin (Robinson *et al.* 1994). Introduction of this mutation into transgenic mice brought about a retinal degeneration as expected (Li *et al.* 1995). The mutation did not, however, constitutively activate rhodopsin according to ERG studies. The mutant protein was found to be phosphorylated and stably bound to arrestin, indicating efficient rhodopsin deactivation. Rhodopsin deactivation in the presence of this mutation was not observed *in vitro*. The

interaction of other proteins in the retina may be necessary for efficient deactivation of rhodopsin. It is also possible that deactivation of rhodopsin may have occurred *in vitro* at a rate too slow to observe (Li *et al.* 1995). The mechanism of retinal degeneration caused by this mutation remains to be established.

Transgenic mice have also been produced expressing other mutated retinopathy genes. For example, mice carrying a P216L substitution within the peripherin/RDS gene have recently been generated (Kedzierski *et al.* 1997). Proline 216 is present within the highly conserved D2 loop of peripherin. This transgene was analysed both on a wild type, *rds*^{-/-} and *rds*^{-/+} genetic backgrounds. The transgene on the wild type background resulted in a retinal degeneration with a rate roughly proportional to the level of transgene expression. The photoreceptor cells were shortened and a large number of lacunae were present in the outer segments. The mutation in the D2 Loop may disrupt the secondary structure of the D2 domain, inhibiting interaction with other proteins and causing the lacunae. The phenotype observed in the *rds*^{-/+} mouse expressing the transgene was slightly more severe than that of the *rds*^{+/-} heterozygote. Increased outer segment disorganisation was apparent, as was an acceleration of photoreceptor degeneration. Severely dysplastic outer segments were detected in P216L transgenics on a *rds*^{-/-} null background (Kedzierski *et al.* 1997).

Transgenic analysis of peripherin/*rds* N- glycosylation has also been carried out by Kedzierski *et al.* (1999). A number of secreted and transmembrane proteins are modified by N-linked glycosylation. Such modification plays an important role in intracellular trafficking, stability, and secretion. N-glycosylation may also be important for dimerization of proteins. A highly conserved N-glycosylation site is present at the asparagine 229 site within the intradiscal D2 loop of peripherin. To examine the effect of this site on peripherin/*rds*-rom-1, and peripherin/*rds*/peripherin/*rds* dimerization, an S-231-A substituted peripherin/*rds* transgenic mouse was generated. No abnormal phenotype was observed in this mouse, indicating a non-essential role of this posttranslational modification in the normal function of peripherin (Kedzierski *et al.* 1997).

Both transgenic rats and pigs expressing retinal genes are now available (Li *et al.* 1998, Petters *et al.* 1997, Liu *et al.* 1999). As mentioned previously such larger mammals may play an important role in the investigation of human therapies owing to their size and in the case of the pig, their increased numbers of cones.

Mice generated by gene targeting.

Gene targeting is the name given to the technique which permits a genetically modified gene to be introduced, by homologous recombination, into mouse ES cells, and thence into living mice. A number of modified ocular genes have been introduced into mice in this way (Humphries *et al.* 1997, Xu *et al.* 1997, Tsang *et al.* 1996). Inactivation of a gene (knockout) is technically the easiest defect to achieve. However an increasing number of animals with more subtle targeted mutations have been made although none to date in genes causing retinitis pigmentosa (Gu *et al.* 1994). The alteration of the mouse genome by gene targeting confines the level of gene expression to that of the endogenous gene. The importance of this control is reflected in the effect of increased rhodopsin expression on photoreceptor viability in transgenic mice. Gene targeting allows investigation of mutated or altered proteins alone, without the background effect of the endogenous protein (see chapter 2 or 3)

A mouse strain lacking the λ subunit of rod cGMP phosphodiesterase was generated by introducing a neomycin resistance cassette into the third exon of the gene (Tsang *et al.* 1996). Mutations in the λ subunit of this gene were expected to result in constitutive expression of PDE $\alpha\beta$, decreased cGMP levels and a resultant closure of the cGMP-gated channel. However, this was not found to be the case. An increase in cGMP levels of up to five times that of the wild type was observed. A quantitative change in levels of PDE α and PDE β was shown to be the cause of elevated cGMP, rather than an alteration in cGMP production. Despite the unexpected cGMP levels, the phenotype of homozygous mutated mice resembled human retinitis pigmentosa. The photoreceptor outer segments did not develop normally and a progressive degeneration, more severe in the central retina, was observed. The conclusion from this study was, that an interaction between the λ subunit and PDE $\alpha\beta$ is essential for cGMP hydrolysis, since lack of λ causes a decrease in both α and β subunits. The high cGMP level was proposed to cause degeneration due to an "excessive energy load on the rod photoreceptors" (Tsang *et al.* 1996).

We have also exploited gene targeting technologies, in this case to generate a null mutation within the rhodopsin gene (Humphries *et al.* 1997). A

neomycin resistance cassette was incorporated into exon II of the gene, and the disrupted gene was electroporated into R1 embryonic stem cells after incorporation into the TK-containing targeting phage vector, λ Syrinx 2 TK. The frequency of homologous recombination was high, 8% of selected ES cell clones showing evidence of targeted gene replacement (see Fig. 1.9). In homozygous mutant mice there was a complete absence of rod outer segments. No rhodopsin was detectable with an anti-opsin antibody and there was a lack of rod ERG response in 8 week old pups. These results demonstrated that rhodopsin is necessary for the maintenance of disc morphology and structure. Pathological effects were also observed in heterozygous mice, including disorganisation of the photoreceptor outer segments and a reduction in the thickness of the outer/nuclear layers. This observation suggests a role for rhodopsin in the maintenance of rod outer segment length. The rhodopsin retinopathy, although affecting the same pathway as that of the λ cGMP phosphodiesterase subunit, exhibited a slightly different phenotype, reflecting the wide clinical heterogeneity of retinopathies. Photoreceptor degradation was of a slower nature in the rhodopsin knockout than in the λ cGMP phosphodiesterase knockout. Furthermore the homozygous rhodopsin mouse had no rod outer segments, in contrast to the PDE λ mouse (Humphries *et al.* 1997).

A third mouse model has recently been generated by Xu *et al.* (1997). Knock-out technology was employed to introduce a neomycin cassette in replacement of a 1.2 kb fragment of the arrestin gene which encodes some 5' promoter sequence as well as the first two exons of the gene. Western blot analysis confirmed the successful generation of mice lacking the rod arrestin protein. Retinal degeneration in these mice was present only in homozygous knockouts. By 4 weeks of age the photoreceptor outer segments became shortened and disordered. The phenotype was similar to that observed after chronic strong photic stimulation. Xu *et al.*, proposed that lack of arrestin might prolong the light response owing to defective rhodopsin shut-off. Electroretinographic responses for wild type mice, +/- and -/- arrestin knockouts were compared. The responses of the +/- arrestin mice were indistinguishable from wt controls, indicating that arrestin quantity and binding to rhodopsin is not rate limiting in the recovery of the rod flash response. In arrestin -/- mice there was no effect on the rising phase of the flash response but the recovery phase was severely affected as expected, confirming the role of arrestin in

quenching rhodopsin activity.

Two further knockouts have recently been reported. Gene targeting techniques were used to delete exon 1 of the rom-1 gene in order to examine its role in the formation and maintenance of outer segments. It is of interest that no known mutations in human rom-1 lead to retinal degeneration (Bascom *et al.* 1995). In the case of the mouse knockout however, retinal degeneration was observed. The rom-1 protein was not detectable in retinas of rom-1 $-/-$ mice while levels of peripherin/rds remained normal (McInnes *et al.* 1999). 25% of photoreceptors died during the first 6 months and by 18 months 40% were dead. The photoreceptor outer segment layer was 25% shortened in the knockout animals. A triphasic pattern of disorganisation was observed which indicated the rate of photoreceptor death to be greatest in younger animals. These results indicate that rom-1 is essential for photoreceptor viability. A knockout of the α subunit of rod transducin has also been reported by Lem *et al.* (1999) Photoreceptor cell function was assessed by single cell electrophysiology at 15, 30 and 90 days post birth. The readings from both hemi- and homozygous knockout mice were normal, showing no significant effect of this knockout despite α transducin subunit levels being reduced to between 25-40% in hemizygous mice and a complete absence of the sub-unit in homozygous knockouts.

With the success of the gene targeting approach, it is now possible to produce a wide range of mouse strains reflecting the diverse molecular pathologies of retinitis pigmentosa and other hereditary blinding diseases, an endeavor which is necessary if a full understanding of the molecular pathology of these diseases is to be reached. Employment of techniques such as the cre-lox P system to incorporate more subtle mutations such as point mutations, deletions and insertions should allow the generation of animal models closely resembling the human disease (Orban *et al.* 1992). Such models are necessary if potential gene therapies are to be adequately assessed.

Gene therapy.

Numerous gene transfer strategies are now being explored in an attempt to correct mutations, which result in heritable retinopathies. Adenoviral (AV), adenoassociated-viral (AAV) and lentiviral vectors have all been used to target ocular tissues in mice and rats (Ali *et al.* 1998, Ali *et al.* 1996, Bennet *et al.* 1994, Grant *et al.* 1997, Jomary *et al.* 1994, Li *et al.* 1994, Mansour *et al.* 1994, Miyoshi *et al.* 1998). AV delivery systems have been widely shown to express reporter genes in retinal cells for up to 100 days post injection (Borras *et al.* 1994). Adenoviral vectors expressing ocular proteins have also been shown to result in a retardation of photoreceptor death in some mouse retinopathies (Bennet *et al.* 1996). The naturally occurring mouse model of mucopolysaccharidosis VII (MPS VII) exhibits reduced levels of β -glucuronidase (Watson *et al.* 1998). β -glucuronidase degrades glycosaminoglycans within the retinal pigment epithelium (RPE). Deficiency of this enzyme results in abnormal lysosome vacuole formulation and inhibits the photoreceptor supportive functions of the RPE, causing photoreceptor degeneration. Adenovirus-mediated gene transfer of the human β -glucuronidase gene to the eyes of the MPS VII mice has been undertaken by intraocular injection (Watson *et al.* 1998). A significant correction of the lysosomal storage phenotype was observed after 3 weeks. MPS VII mice display numerous other symptoms including premature death, therefore it was not possible to follow the success or otherwise of the therapy after 3 weeks.

Bennett *et al.* (1996) used the rd mouse, which, as previously described carries a null mutation in PDE β subunit gene. Sub-retinal delivery of an adenovirus carrying the missing cDNA driven by a cytomegalovirus promoter gave promising results. 19 days after injection of homozygous rd mice, 3-4 rows of photoreceptor nuclei were observed, in comparison to a single row in non-injected mice. A concomitant increase in β PDE transcript and enzyme activity was also observed. An overall delay of total photoreceptor degeneration of 6 weeks was achieved. To quote the paper; "This is the first report describing the therapeutic effects of somatic gene delivery in an inherited retinal degeneration caused by a genetic defect expressed specifically in photoreceptors"

Adenoviruses are a common human pathogen and *in vivo* delivery may be compromised by host immune responses. On the other hand adeno-associated

viruses (AAV) are nonpathogenic human parvoviruses, which are dependent on helper AV virus for replication. AAV have been used with some success for gene delivery to ocular tissues. Expression of reporter genes including β -gal and green fluorescent protein, were visible for at least three months after injection (Ali *et al* 1998, 96, Grant *et al* 97, Bennet 97)

Jomary *et al.* (1994) also demonstrated significant rescue of photoreceptor function, again in the rd mouse, by AAV-mediated gene transfer. Injection of a human PDE- β AAV construct with a CMV promoter induced a two fold increase in photoreceptor survival of 17-18 day old rd mice. An increase in expression of immunoreactive PDE protein was also observed, as was two- folds increase in ERG response.

Retroviral vectors have been used more than any other gene transfer vehicle. Until recently retroviruses were not considered suitable for retinal tissue owing to the non-dividing nature of the cells. Recent reports have described the generation of a new series of retroviruses based on the HIV virus, which are capable of infecting and stably integrating into non-replicating neurons. Miyoshi *et al.* injected such an HIV vector containing a gene encoding green fluorescent protein into the subretinal space of rat eyes. In cases where the vector contained GFP under the control of a CMV promoter, expression was observed in the photoreceptor cells and the retinal pigment epithelium. Under the control of the rhodopsin promoter expression was found mainly in the photoreceptor cells, as expected. GFP expression was visible for up to 12 weeks within up to 80% of the area of the whole retina. This efficient delivery may prove important for future therapies. The importance of animal models here is obvious, since HIV-based vectors for use in man will need extremely careful evaluation.

The use of nonviral vectors as a means for gene delivery to the retina is also being investigated. Liposomes, for example, have been shown to protect intraocularly injected DNA, allowing expression of Lac Z by a CMV promoter for 30 days after injection (Hangai *et al.* 1996)

Down regulation of the expression of mutant alleles will be necessary in the case of dominant retinitis pigmentosa. A range of methods is currently available for suppression of gene expression. Suppression can be achieved by use of antisense RNA/DNA, ribozymes, a triple helix approach or by the use of peptide

nucleic acids. Of these methods, some success in retardation of photoreceptor cell death has been reported with the use of ribozymes. Ribozymes are RNA molecules with autocatalytic enzyme activities, which can be designed to cleave target RNAs in a sequence-specific manner. Lewin *et al.* (1998) designed a ribozyme to cleave at a histine-to-proline substitution at codon 23 of the human rhodopsin transgene transcript in their transgenic rat model. The transgenic rat displays a photoreceptor degeneration caused by the presence of mutated rhodopsin. An AAV-vector carrying a ribozyme targeting P23H was injected into the eyes of the transgenic rats. *In vivo* expression of the ribozyme was achieved, as was a reduction in the rate of photoreceptor degeneration for a period of three months.

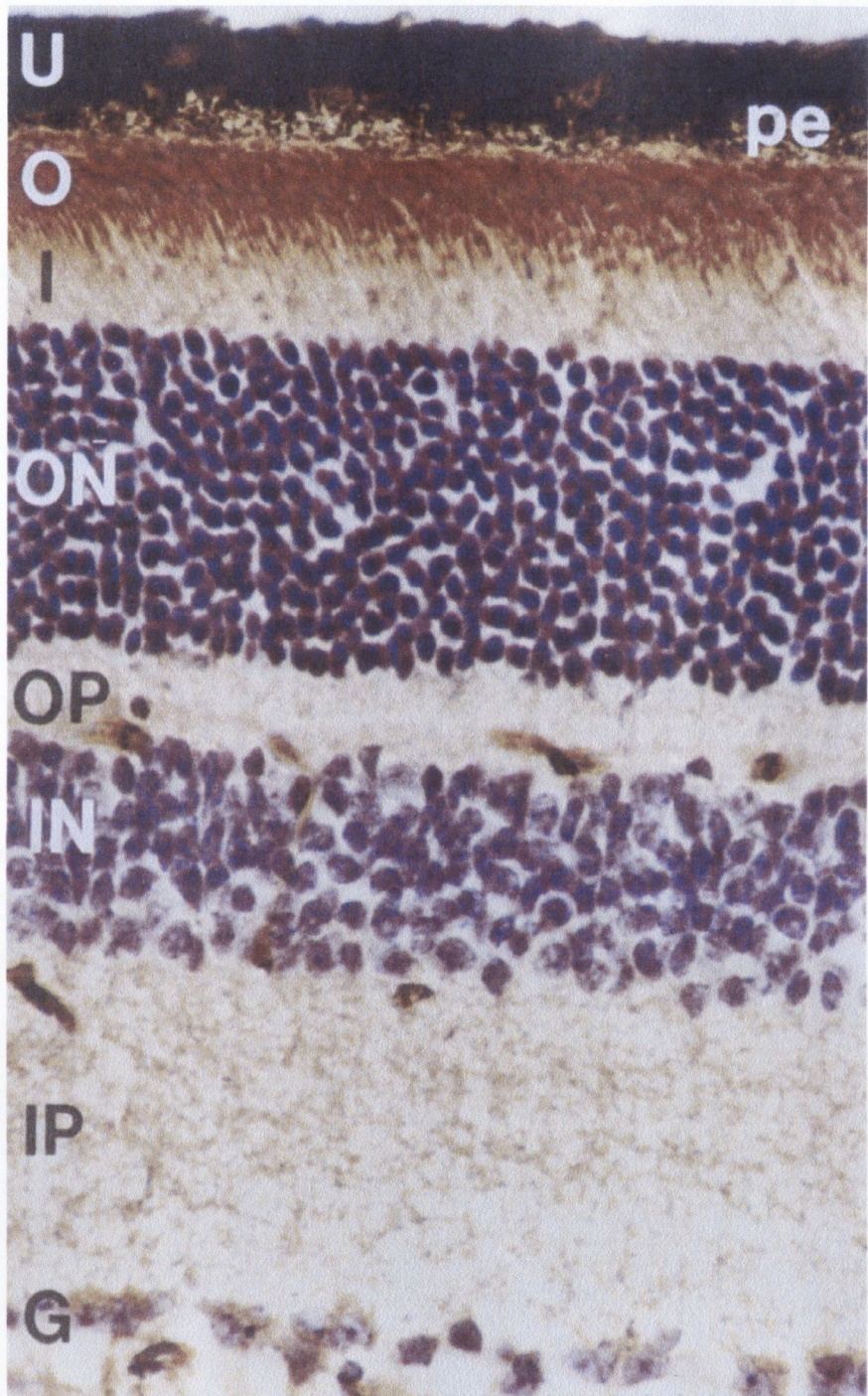
Retinitis pigmentosa is an extremely heterogeneous disorder, with up to 90 known mutations causing the disorder in rhodopsin alone. Dominant gene therapy strategies designed to target specific mutations will not prove cost effective or, indeed, be feasible owing to design constraints on suppressors of mutant transcripts. In an effort to overcome the problem of heterogeneity a number of mutation-independent gene therapy strategies employing ribozyme technology are presently being developed at this laboratory.

For example Millington-ward *et al.* (1997) have reported three strategies for dominant gene therapy in which ribozymes were designed to target transcribed but untranslated regions (UTRs) of peripherin and rhodopsin. A co-introduced therapeutic gene with an altered UTR could, in principle, escape suppression. Similar strategies designed to target degenerate third nucleotides, or common polymorphisms within the coding region, have also been explored. Such strategies present the investigator with a wider choice of target sequence for ribozyme design than those offered by targeting the mutation alone. Hammerhead ribozymes designed to target rhodopsin and peripherin transcripts have been shown to function successfully *in vitro*. The availability of suitable mouse models will allow extrapolation of this research to the *in vivo* environment.

A wider range of animal models than those currently available will be required to investigate the effects of particular mutations and potential therapies. The range of available models is slowly increasing as a result of the ongoing genetic characterisation of existing naturally occurring models and the application of developing transgenic technologies. Although a naturally occurring rds mouse is currently available, a more specific model for dominant disease based on mutations

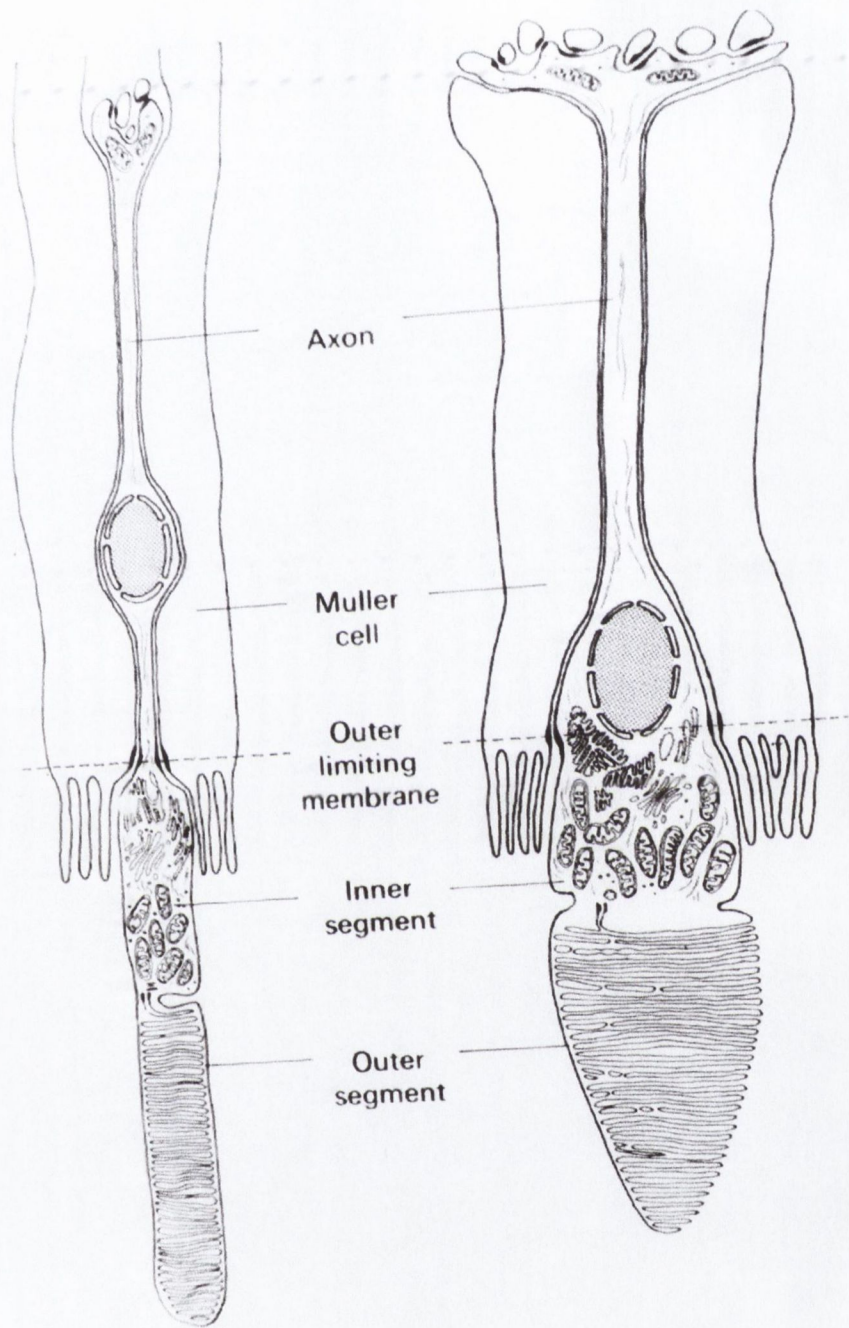
known to cause the disease in man, would be extremely useful. For this reason I have undertaken the challenge of generating a mouse model of autosomal dominant RP caused by a mutation in exon III of the rds-peripherin gene, a mutation which has previously been shown to cause adRP (Aplfelsted-Sylla *et al.* 1995). This endeavor required the development and establishment for the first time in Ireland, of embryonic stem cell and targeting technologies. Chimeric mice have been generated, and germline transmission of the modified peripherin allele has been demonstrated. I have also further exploited the rho-/- mouse by generating a model for dominant RP in which a normal human rod opsin gene is expressed on the rho-/- genetic background.

Fig. 1.



Haemotoxylin and eosin stained cross section of a normal mouse retina. The layers are as follows: retinal pigment epithelium (PE), photoreceptor outer segment layer (O), inner segment layer (I), outer nuclear layer (ON), outer plexiform layer (OP), inner nuclear layer (IN), inner plexiform layer (IP) and the ganglion cell layer (G).

Figure 1.2



Schematic diagram of rod and cone photoreceptors taken from Wheater's Functional Histology, Third Edition, 1993

Figure 1.3

Outer segment layer

Inner segment layer

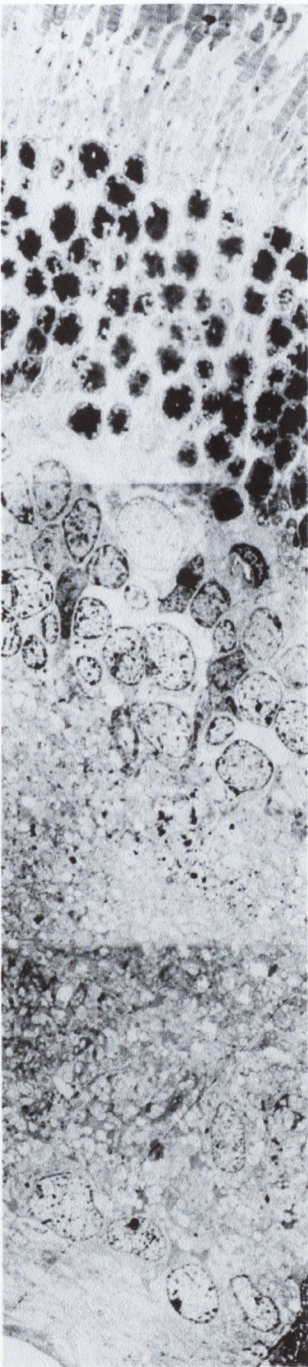
Outer nuclear layer

Outer plexiform layer

Inner nuclear layer

Inner plexiform layer

Ganglion cell layer



Electron micrograph of mouse retinal section kindly provided by Dr. E. Fekete

Figure 1.4

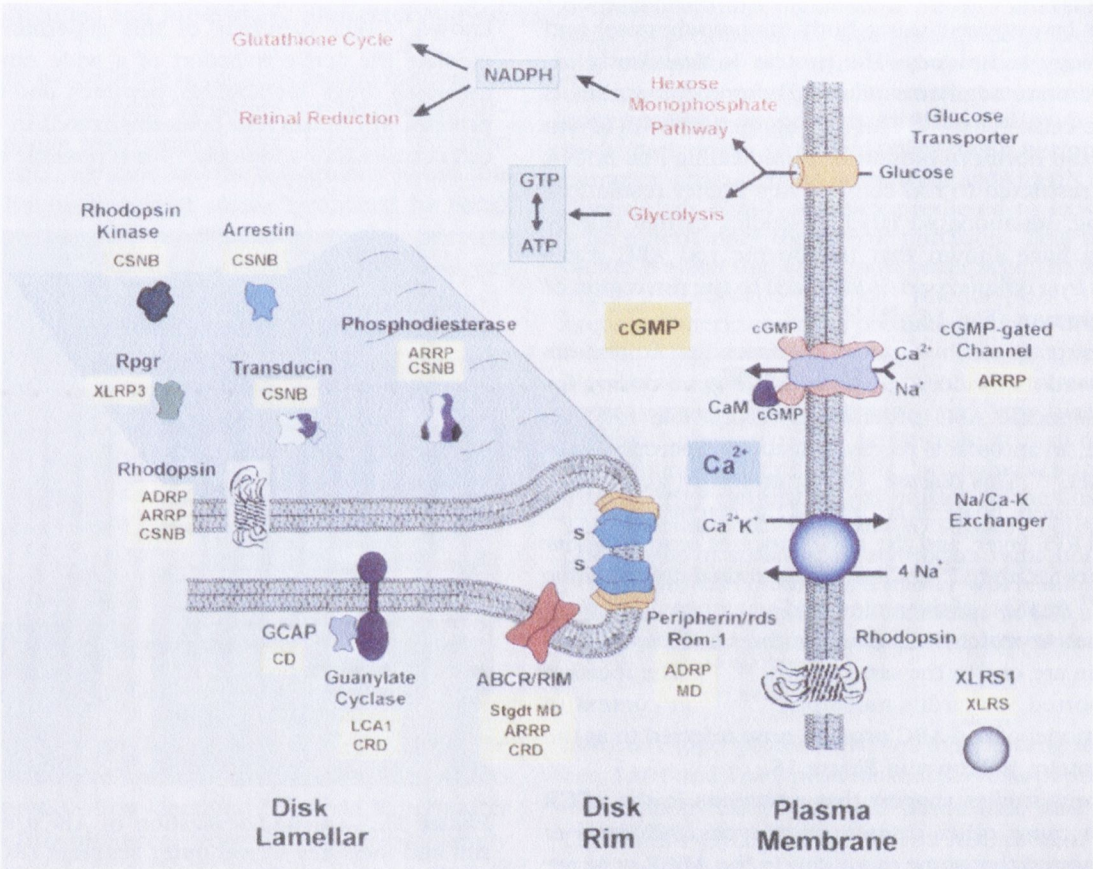
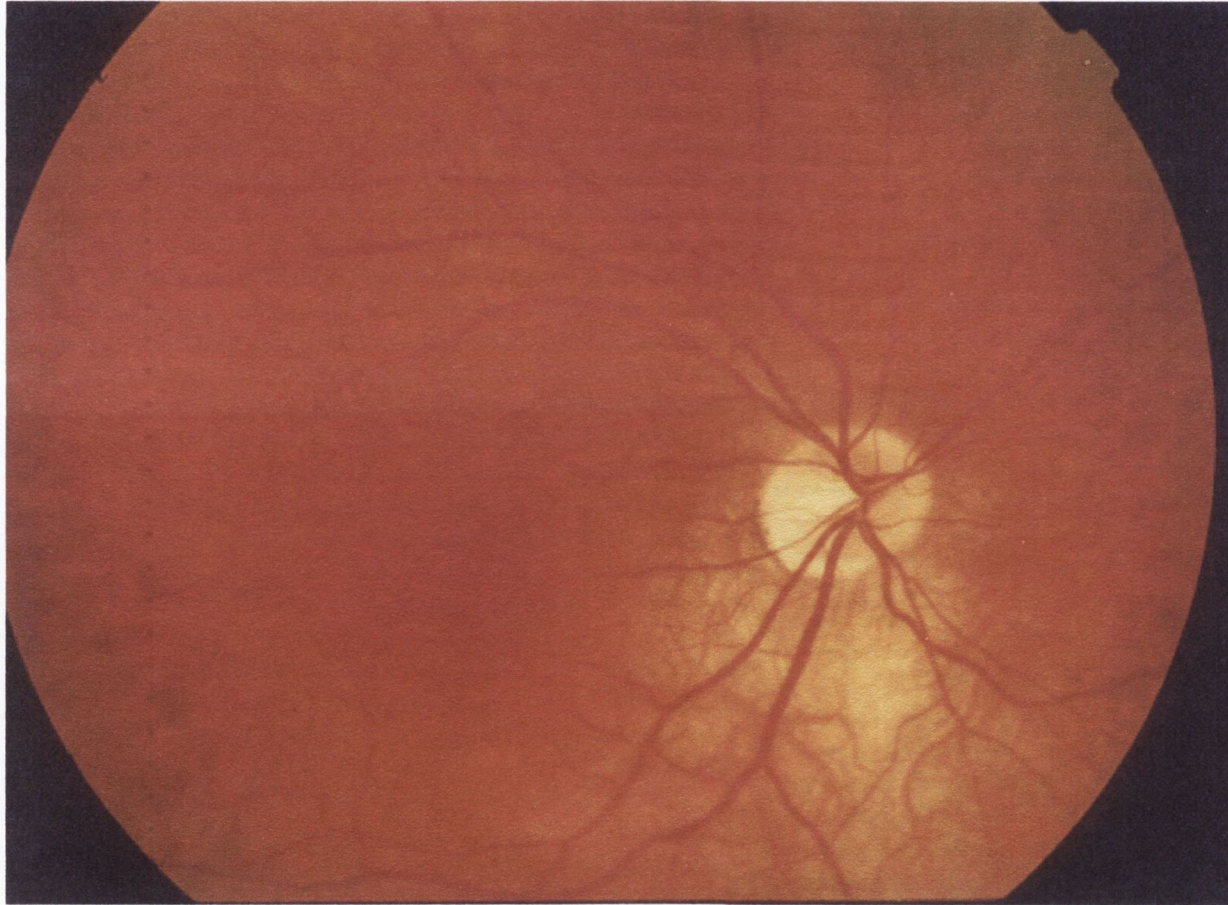


Diagram of visual transduction taken from Molday R, IOVS, Vol 39, (1999), 2493 - 2513

Figure 1.5a



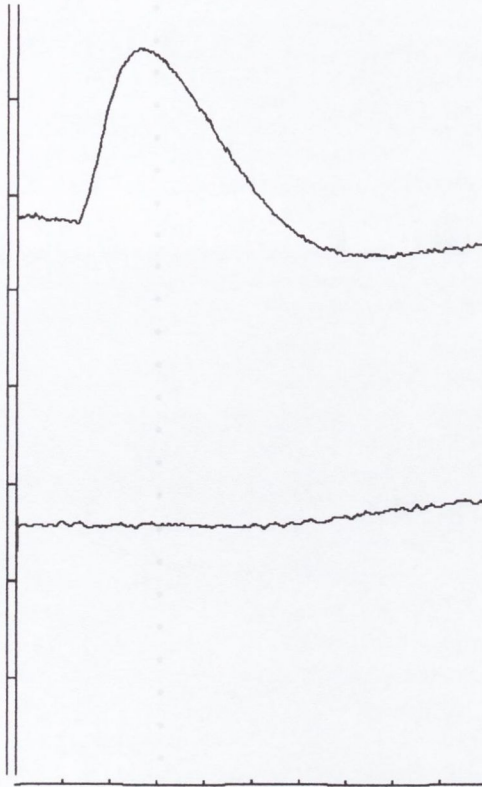
Photograph of the fundus of a normal human eye (kindly provided by Dr. P. Kenna). The central yellowish disc represents the optic nerve head from which the optic nerve fibres and blood vessels emanate and the darker area to the left represents the macula.

Figure 1.5b



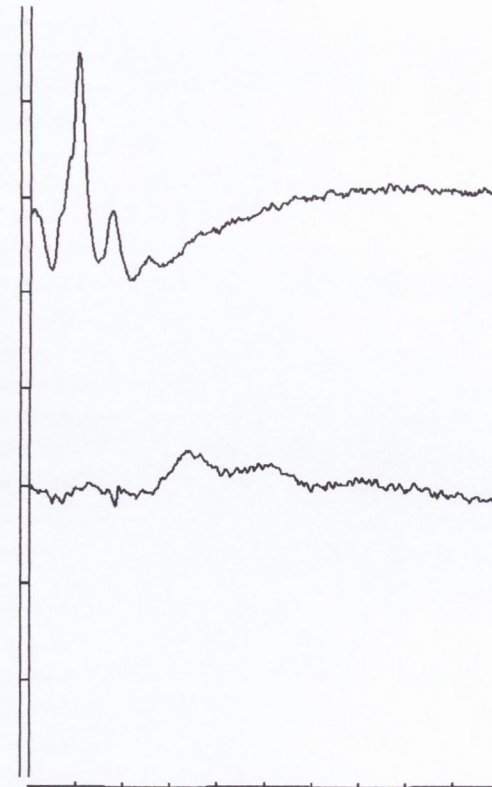
Retinal photograph of an individual with advanced Retinitis Pigmentosa, kindly provided by Dr. Paul Kenna.

Figure 1.6a



Example of dark adapted rod-isolated electroretinogram (ERG) tracings. Top, normal tracing; bottom, non-recordable response from an RP patient.

Figure 1.6b



Example of light adapted cone-isolated electroretinogram (ERG) tracings. Top, normal tracing; bottom, very delayed and grossly reduced amplitude generated by an RP patient.

Fig 1.7.

A diagram of the rhodopsin protein indicating the position of many of the mutations identified to date circled in bold type.

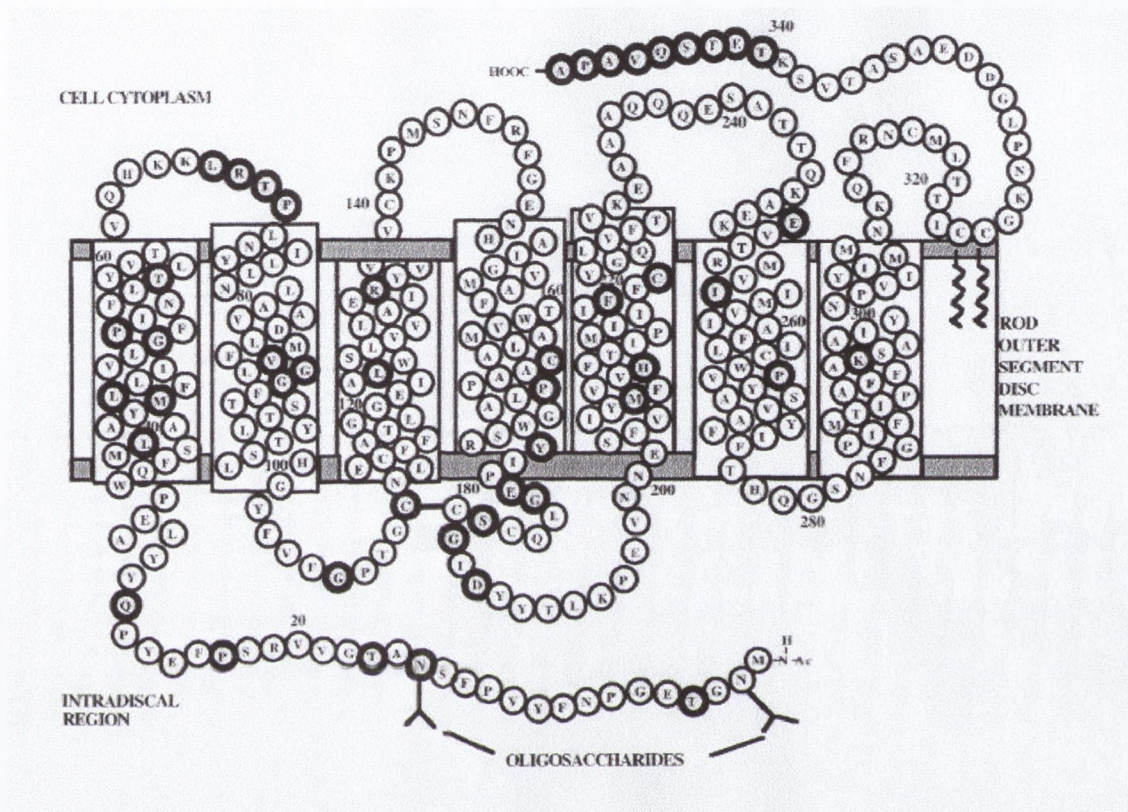


Fig. 1.8.
A diagram of RDS peripherin.

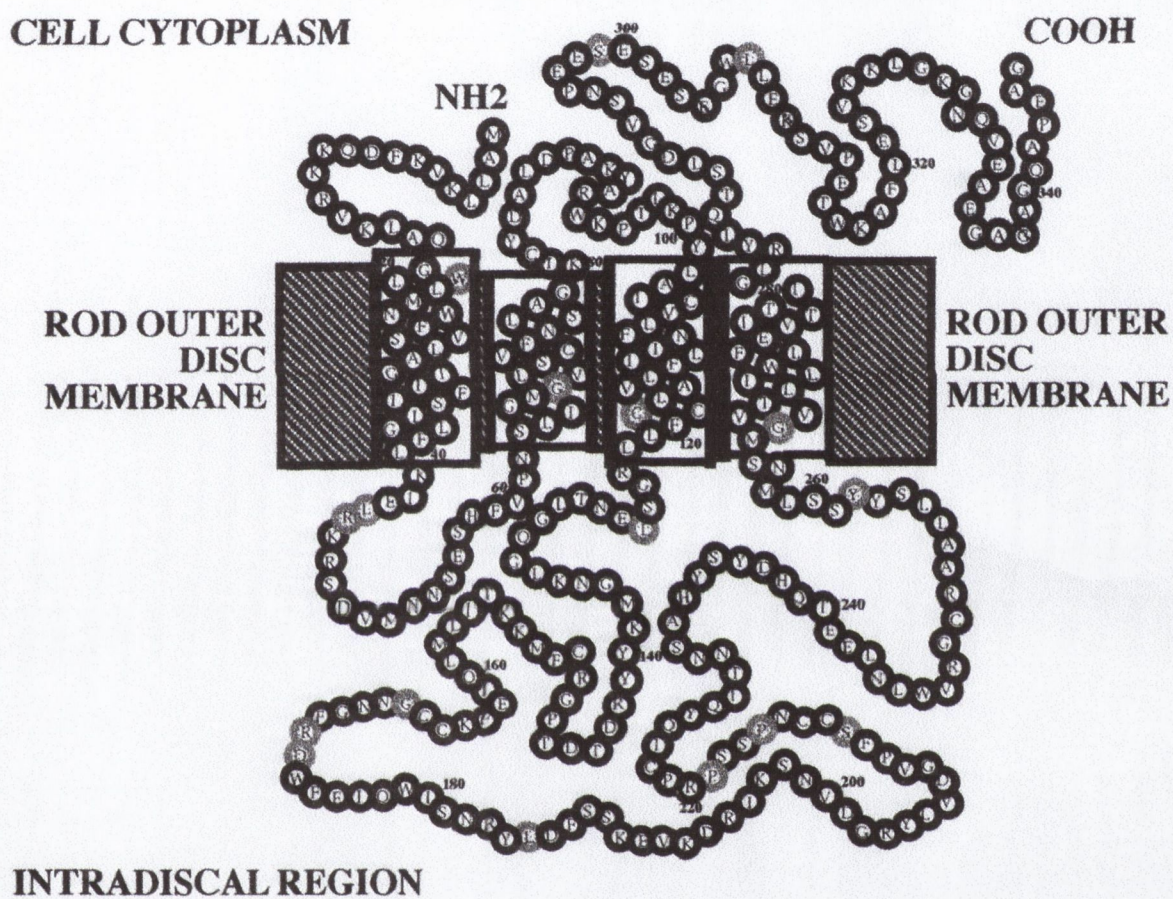
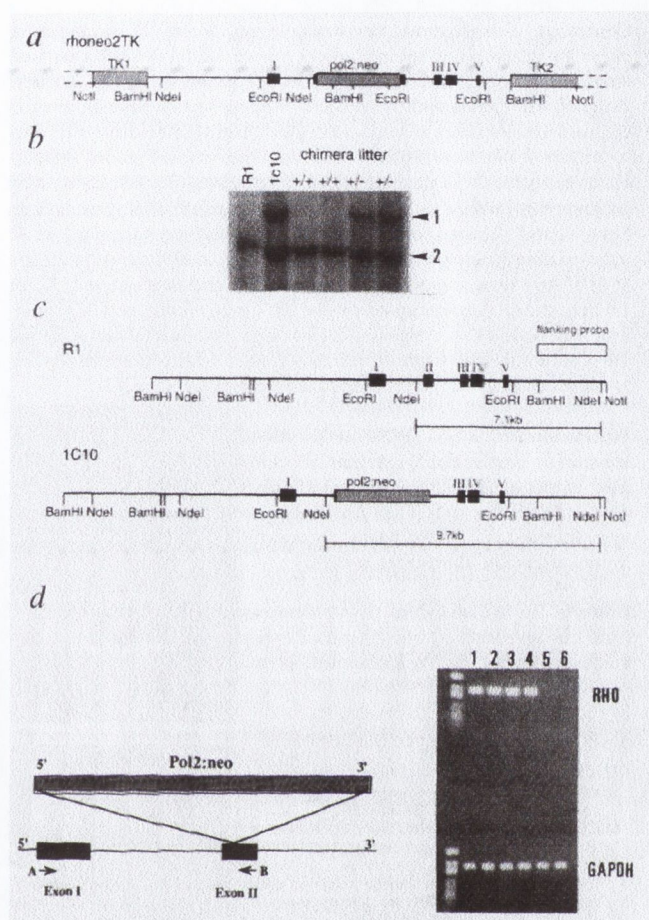


Figure 1.9



Construction of Rhodopsin gene targeting vector. **a**, targeting vector; **b**, Southern analysis of ES clones by probe flanking targeting vector, showing diagnostic shift-up of *NdeI* restriction fragment, from 6.7 kb (1) to 9.8 kb (2); **c**, restriction map illustrating *pol2:neo* insertion into exon 2 of the rhodopsin gene, resulting in a 3.1 kb *NdeI* shift-up; **d**, RT PCR analysis of total RNA extracted from ocular tissues derived from *Rho*^{+/+} mice (lanes 1 and 2), and *Rho*^{+/-} and *Rho*^{-/-} mice (lanes 3 and 4 and 5 and 6 respectively) using primers specific for the rhodopsin gene (above) and constitutively expressed gene GAPDH (below). Left most lane, *Msp1* – cut pGEM plasmid DNA marker. The positions of primers within exons 1 and 2 of the rhodopsin gene are illustrated.

Chapter 2

On-site development of embryonic stem cell technologies.

Introduction.

At the outset of this project, no research employing embryonic stem cell, or gene targeting technologies, was being undertaken in Ireland. In order to extend our retinopathies program into areas in which problems relating to the development of gene therapies could be addressed, we needed such technology. Thus, my initial research objective was to establish, on site, methods for the culture of pluripotent embryonic stem cells and conditions for successful gene targeting.

Mouse embryonic stem (ES) cells were first isolated and conditions for their maintenance in culture defined, in 1981 (Evans and Kaufman, 1981). When grown under rigorously defined conditions such cells maintain their ability to develop into any of the multitude of cell types of a developing embryo even after being subjected to a number of passages and to genetic manipulation. Research on ES cells was largely a continuation of earlier work on embryonic carcinoma stem cells (EC) derived from teratocarcinomas. EC cells proved to be difficult to work with owing to the occurrence of abnormal karyotypes and a tendency for these cells to undergo spontaneous differentiation into epithelial, skeletal muscle and neuronal cells (Robertson, 1986). In an attempt to overcome such problems, two research groups independently derived embryonic stem cell lines directly from the inner cell mass of mouse blastocysts (Evans and Kaufman 1981). EC cells were most commonly found in the testes of 129 mice and therefore the earliest ES cell lines were also produced from the 129 strain (Robertson 1986). A variety of additional ES cell lines are now available from other mouse strains, for example, TT2 and ES632 cell lines both from C57BL/6 mice (Yagi *et al.* 1990; Doetschman *et al.* 1987).

Embryonic stem cells expressing markers such as green fluorescent protein and Lac Z have been produced (Hadjahtonakis *et al.* 1998, Lallemand *et al.* 1990.). Such cell lines allow the identification of tissues into which the ES cells have been incorporated during development, which is especially helpful in cases of embryonic lethality. Interestingly, embryonic stem cells can not be derived from all mouse strains, such strains being termed "non-permissive". A number of research groups are exploring such animals in an attempt to identify factors, which might allow, or prevent, differentiation (Mc Whir *et al.* 1996.). For example, Oct 3/4 is a transcription factor that contains a structural motif present in members of the POU gene family, a gene which is expressed in undifferentiated ES cells. The regulation of OCT 3/4 may be of importance for the development of ES cell lines for larger mammals, as

well as for “non-permissive” mouse strains (Tomilin *et al.* 1998). We chose R1 cells as our stock ES line because of its proven success in contributing to the germ cells of chimeras both by aggregation and micro-injection, (Nagy *et al.* 1993) unlike ES cell lines, such as D3 and AB1 (Doetschman *et al.* 1987; Mc Mahon & Bradley 1990). The R1 ES cell line was derived from 3.5 day male blastocysts from a 129/Sv x 129/Sv-CP cross (Nagy *et al.* 1993). The pluripotency of R1 cells was evaluated by measuring the success of germline transmission of the R1 genotype mice produced by tetraploid embryo and R1 aggregation (Nagy *et al.* 1993). Cells older than passage 14 did not produce live pups by aggregation: when the R1 cell line was subcloned at this late passage number, only about 20% of the subclones had the original R1 developmental potential. Subcloning of healthier colonies and monitoring these by karyotypic analysis resulted in a substantial increase in the developmental potential of these cells. The R1 cell line has been used widely and successfully used in gene targeting and has also proven to be useful in explorations of controlled differentiation into cell types through which cell specific gene regulation can be investigated (Yagi *et al.* 1990).

It is of interest to note that human pluripotent stem cells have recently been generated, and maintained in culture for up to 5 months, retaining developmental potential and forming derivatives of all three embryonic germ layers (Thomson *et al.* 1998, Shamlott *et al.* 1998). Such cell lines may hopefully contribute to future therapies and undoubtedly to the further understanding of human developmental biology.

Chimeric transgenic calves have also been obtained from somatic cell-derived stem- like cells, exemplifying the feasibility of production of transgenic livestock with site-specific, rather than random insertions of DNA (Cibelli *et al.* 1998). Numerous research groups are working on the generation of ES cell lines from other mammals, including rats and pigs (Takahama *et al.* 1998, Piedrahita *et al.* 1997). Conditions for the culture of ES cells are complex, reflecting a requirement for a range of undefined, though essential factors. Cell maintenance requires a high concentration of foetal calf serum and a feeder layer of fibroblasts or other supplements (Scherer *et al.* 1979, Smithies *et al.* 1985). The feeder layer provides anchorage for the cell line and secretes components into the culture medium which assist in preventing differentiation. Early ES cell lines were established on permanently growing STO fibroblast feeder cells, the latter a

thioguanine / ouabain-resistant subline of SIM mouse fibroblasts (Evans & Kaufman 1981). The disadvantage of using these cells is in their inability to maintain pluripotency (Martin 1981). Doetschman *et al.* (1987) discovered that primary mouse embryonic fibroblasts (PMEF) could serve as an alternative to STO cells, PMEFs having a more stable euploid karyotype. ES cells can also be grown in the absence of feeder cells on gelatinized tissue culture dishes if supplemented with Buffalo-Rat liver (BRL) cell-conditioned medium or leukemia inhibitory factor (LIF) (Ware & Axelrad 1972). LIF is one of the differentiation-inhibiting components provided by feeder cells (Wasserman & De Pamphilis 1993). BRL contains ES cell differentiation-inhibitory activity (DIA), which is identical to LIF (Doetschman *et al.* 1987).

While early experiments relied on the use of calcium phosphate / DNA precipitation techniques or retroviral vectors for introducing DNA into ES cells, electroporation is currently the standard method used (Doetschman *et al.* 1987). The electroporation procedure involves opening of pores in the membrane of the cells by applying a brief high voltage shock. Transformation efficiencies for electroporation are usually between 10^{-4} and 10^{-5} (Potter *et al.* 1984). Titration curves to determine the optimal strength and duration of the voltage shock are necessary to establish maximum transformation efficiency with minimal cell death. It is interesting to note that DNA microinjection into ES cells has been described with transformation rates of over 20% (Zimmer and Gruss 1989). However microinjection is technically difficult and high success rates have not been reported. In this chapter I describe the experiments carried out in order to establish a working ES cell laboratory here in Ireland. The maintenance of ES cells in a pluripotent state is of the utmost importance if the cells are to contribute to a germline mouse. It was therefore necessary to identify the criteria for the successful growth of these cells and to optimise electroporation conditions to allow the maximum efficiency of gene targeting.

Materials and Methods.

For solutions & suppliers please refer to Appendix 1.

Cell counting.

Cells were counted using the Neubauer chamber cell counting grid (see Fig.2.1)

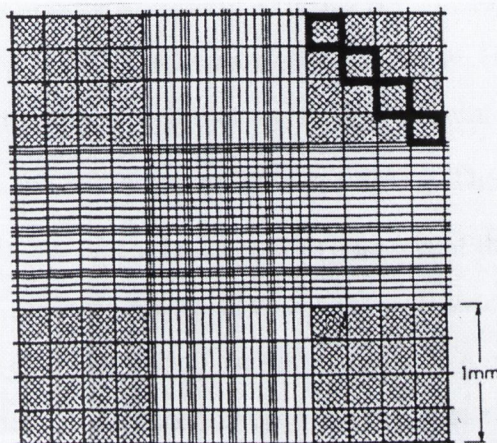


Fig. 2.1

Each corner square consists of sixteen smaller squares which is equivalent to $1\text{mm}^2 \times 0.1\text{mm}$ deep, which equals 0.1mm^3 or 1ml . Therefore each small square contains a volume of $1/160\text{ml}$. Cell numbers were counted in all sixteen squares and multiplied by 10^4 to establish the number of cells per ml.

Plating efficiency.

Plating efficiencies of cells were calculated by counting viable colonies and then dividing this value by the number of cells plated.

Screening batches of serum for optimal ES cell growth.

R1 cells (3×10^4) were plated onto 60mm dishes (Nunc) containing feeder layers in 10%, 15% and 30% foetal calf serum. Colonies were grown for seven days. Plates were rinsed with Phosphate buffered saline and 2mls of methylene blue was added to each plate. After rinsing the plates with water, colony morphology was examined using an inverted microscope.

Preparation of feeder layers from primary embryonic fibroblasts.

Primary embryonic fibroblasts were prepared following the protocol suggested by E.J. Robertson in "Teratocarcinomas and Embryonic Stem Cells, A Practical Approach". Pregnant female transgenic mice expressing Neo (Harlan. U.K.) were sacrificed on the thirteenth or fourteenth day of pregnancy. Foetuses were dissected out of the uterus and placed in a 10cm Petri dish containing PBS. Embryos were dissected away from the uterine wall and transferred to a fresh PBS-containing dish. The head and internal organs were discarded and carcasses washed twice with 50mls of PBS. Carcasses were then placed into a dish containing 2ml of trypsin EDTA solution, minced finely using sharp scissors and incubated at 37°C for 5minutes. The trypsin was neutralized by addition of 5ml DMEM /10% Foetal Calf Serum. The contents of the dishes were then transferred to a 50ml Falcon tube. Large pieces of debris were allowed to settle for 2mins and the supernatant was then transferred to a fresh tube. The suspension was made up to the 10mls per original carcass. 10mls of supernatant was added to each 150mm tissue culture plate. The medium was changed 24hrs later. When plates were confluent the cells were detached by trypsinization. This process involved aspirating the feeder medium and washing the cells twice with PBS to remove all traces of serum. 10mls of trypsin/EDTA was then added to each of the plates, which were then incubated for 5mins. When the cells became detached, 10mls feeder medium was added to neutralise the trypsin /EDTA. The cells were centrifuged (1000rpm, 5mins) and resuspended in feeder medium. An aliquot of cells was counted. Plates (150cm) were seeded with 5×10^6 cells. After three days of culture the EMFIs (primary embryonic fibroblasts) formed a confluent monolayer and were trypsinized. The cells from each plate were frozen in 1ml 1 x freezing medium and stored at -70°C.

Preparation of EMFI feeder layers.

Feeder cell layers (Primary Embryo Fibroblasts) were prepared using a method kindly supplied by Magoshia Kownaka (Mt. Sinai Hospital, Toronto). A vial of primary feeders was thawed, washed with DMEM / 10% FCS and centrifuged gently. Each vial was plated onto four 150mm plates in feeder cell medium. When confluent (3days) the plates were passaged by trypsinization onto four 150mm

plates, grown to confluency and passaged to a further four 150mm plates. Confluent plates were treated with mitomycin C. Cells were removed by trypsinization, counted, diluted to a concentration of 2×10^5 cells/ml and plated. Feeders were allowed to attach to the base of plates overnight before use. The medium was removed from the plates and 10mls DMEM, 10% FCS containing 100ml 2mg/ml mitomycin C solution was added. The plates were then incubated at 37°C for 2hrs. Following incubation, plates were rinsed twice with PBS.

The culture of ES cells.

Dr. Andreas Nagy, Mount Sinai Hospital Toronto, kindly donated R1 stem cells at passage 14. ES Cell expansion was carried out following guidelines established by E.J Robertson (1987). Briefly, a vial of R1 ES cells was thawed rapidly, diluted in ES medium and centrifuged for 5mins at 1000rpm. The medium was aspirated off and the cells were resuspended in 10mls fresh medium. Cells were counted and seeded onto 60mm plates containing confluent mitomycin C-treated feeder cells at a concentration of 1×10^6 . Once confluent (3 - 4 days), cells were trypsinised with 2 mls of trypsin/EDTA and incubated for 5 minutes at 37°C / 5% CO₂. The cells were then pipetted gently to produce a single cell suspension. ES cell medium (5mls) was added to neutralise the trypsin/EDTA. The suspension was centrifuged for 5 mins at 1000rpm and resuspended in 5-7mls of ES medium. Cells were counted before transferring them to a plate containing a feeder layer at a concentration of 2.5×10^5 R1 cells per ml.

Production of leukemia inhibitory factor (LIF).

LIF was generated by transient high level expression of a LIF-containing vector, pXMT2 mLIFD. The method used for transfection of COS cells was electroporation. pXMT2 mLIFD plasmid was a gift from David Staunton CRC, Biochemistry Oxford. Frozen ampules of COS-7 cells were obtained from Dr. A.S.Whitehead, T.C.D. An ampule of COS cells was seeded onto a 150mm plate and grown to confluency with feeder medium. COS cells were trypsinized with 10mls of trypsin/EDTA to obtain a single cell suspension. The cells were then washed twice in PBS, centrifuged at 100rpm for 5mins and resuspended at a concentration of 2×10^7 cells/ml in DMEM.

This suspension (0.75ml) was placed in an electroporation cuvette. Plasmid DNA (50µg) was mixed gently into the suspension by pipetting. The cells were electroporated at 320V, 500mF and the cuvettes left at room temperature for 15 minutes. The suspension was then made up to 20mls with feeder medium and seeded onto two 150mm plates. The plates were incubated at 37°C, 5% CO₂ overnight. The medium was then aspirated and replaced with 30mls of 10% FCS medium. The cells were then incubated for a further three days. The LIF containing medium was filter sterilized using a 0.22µm nitrocellulose filter and stored in 1ml aliquots.

LIF batch testing.

R1 (3×10^4) cells were seeded onto a 60mm gelatinised plate, prepared by covering the surface of a 60mm dish with 0.1% gelatin solution and allowing the dish to stand for 5 mins before aspirating the gelatin solution and leaving it to dry. The ES cell medium on each plate was supplemented with various dilutions of LIF-containing feeder medium. Plate 1 received a 1- 1/100 dilution, plate 2, a 1/40 dilution and plate 4, a 1/10 dilution. The medium was changed daily and the R1 colonies were monitored for any signs of differentiation.

Electroporation procedures.

Antibiotic Resistance Test.; ES Cell medium containing, 0, 150, 200, 250, and 500 µg/ml of G418 (Gibco) and 2µM Gancyclovir (Cytovene, Syntex.) was prepared. R1 cells (3×10^4) were plated onto 60mm plates on feeder layers in ES cell medium containing LIF and each of the concentrations of G418 or the gancyclovir medium. Control plates containing ES cell medium only, were also prepared. The colonies were grown for seven days and Methylene blue (2mls) was added to each plate. The colonies were counted after the plates had been rinsed with water.

Optimisation of electroporation.

Four electroporation cuvettes containing 7×10^6 R1 cells were prepared. An aliquot of these cells (0.8mls) was placed in a Biorad 0.4 width cuvette. Pulses of 0, 50, 240 and 450V, 500µF capacitance were applied. Surviving cells were counted

after staining by addition of 2mls of methylene blue and rinsing with water

Results and Discussion.

A primary concern in any ES culture work is to ensure that cells do not differentiate and that they retain a normal karyotype. Both of these requirements are essential if germ-line transmission of modified ES cells is to be achieved. To prevent ES cell differentiation, cells must be grown on a mitotically inactivated feeder layer or alternatively on gelatinised plates in the presence of leukemia inhibitory factor (LIF). Use of feeders is often the preferred method for long-term culture and maintenance of ES cells. LIF, however is necessary for growth of cells used for DNA isolation and is frequently added as a supplement to feeder layers in everyday culture. The feeder layer is comprised of Sto cells or primary embryonic fibroblasts. I chose the latter to maintain the ES cell lines, following advice from Dr. Andras Nagy (Toronto). I received the R1 cell line from Dr. A. Nagy at passage 14. The same group also kindly provided a small number of ampules containing EMFI and LIF. I found that the R1 cells grew well using standard supplemented DMEM, and incubation conditions of 37° C and 5% CO₂ using the Canadian feeders and LIF (see Fig. 2.2). R1 cells require a pure water source. Therefore, only millipore Q-filtered water was used. Alkaline detergent residues were also found to cause variability in ES cell growth. Hence a mild detergent (Liposol) was chosen and glassware was carefully washed and rinsed by hand. A plating efficiency (PE) of between 35% and 40% was routinely achieved using these conditions (see Fig.2.3)

Owing to the fastidious serum requirements of ES cells, initial investigations were required to test a variety of sample batches of ES cell-certified Foetal Calf Serum (FCS) supplied by Gibco. The consensus appears to be that the only reliable indicator of a "good" batch of serum is that ES cells grow well and have a comparable PE and morphology as that obtained with an established "good" batch of serum over a variety of concentrations. Gibco serum Cat. no. 10502 - 045, Lot. no. 30F70435 fulfilled this criterion well and was used routinely. R1 cells were grown in 10%, 15% and 30% Foetal Calf Serum. All concentrations gave a similar PE, although colony size and morphology were superior in 15% serum. At the higher concentration of 30% any toxic components within the serum may become detectable by their effect on ES cell growth and morphology.

Toxicity of serum is thought to be partially due to high levels of complement. An example of a colony growing with a good morphology is shown in Fig. 2.4.

An important aspect of ES cell propagation is to ensure that the culture does not become overcrowded or too sparse during routine maintenance. If ES cells are allowed to form aggregates in suspension they will often begin to differentiate. It was necessary therefore to establish optimal conditions for detachment and replating of R1 cells. 1×10^6 R1 cells per 60mm plate was found to be an adequate density to create a confluent plate every three or four days. If the cells required a more frequent passage or a higher dilution, the cells were discarded and a new vial seeded. This minimised the likelihood of selecting a mutated subclone (see Chapter 4).

When passing R1 cells embryoid bodies can occasionally form. These may arise when clumps of ES cells are passed onto a new plate (as illustrated in figure 2.5.). This problem can be overcome by ensuring that a single cell suspension is produced before passing the cells. When generating such a suspension a balance is required between the amount of trypsin needed to separate the colonies and the amount of cell death brought about by the chemical. Excessive trypsinization also causes attachment problems for R1 cells. The optimal quantity of trypsin for the passage of a 60mm plate was found to be 2mls, while 3mls was necessary for the 90mm plate of cells.

For the routine propagation of the R1 cell line, it was necessary to establish a constant source of both EMFI and LIF. G418-resistant primary embryonic fibroblast cells were made from transgenic foetuses expressing a neomycin resistance gene. A stock of EMFI cells was prepared and frozen down in 1ml ampules. Each ampule was capable of producing sixteen confluent 150mm plates within three days of growth (see Fig.2.6). Cell densities of an average of 6×10^6 were observed per plate and R1 cells were maintained on these feeders as efficiently as stocks received from Toronto .

LIF concentration testing.

Recombinant LIF is commercially available. However, it is expensive and effectively doubles the price of every bottle of medium. A high level transient LIF-expressing vector was used to generate LIF-conditioned medium from transfected COS cells. LIF conditioned medium was diluted 1/100, 1/40, 1/20 and 1/10 in DMEM-supplemented medium and used for maintenance of R1 cells on gelatinized plates. Control plates containing 1000U/ml LIF from Dr. A.Nagy's laboratory, 1000U/ml Gibco Esagro LIF and no LIF were also grown. Dilutions 1/40, 1/20 and 1/10 were found to maintain the pluripotency of the R1 cells. Although no distinct differentiation was observed in cells grown in a 1/100 dilution, some cells appeared somewhat flattened and the edges hinted at the start of endoderm formation (Fig. 2.7). The success of the majority of the dilutions, was not unexpected since under efficient transfection conditions the concentration of LIF in COS cell medium has been reported to approach 1µg/ml. A wide variety of differentiated cells were observed in control plates containing no LIF (see Fig.2.8)

Electroporation conditions.

Preliminary experiments indicated that electroporation at 240V and a capacitance of 500µF resulted in death of 50% of the R1 cells. Others have found that the percentage of cell death for optimal transfection efficiency is 40 - 60%. The results obtained here are therefore compatible with those obtained from other centres engaged in ES technology (see Table 2.1). Different batches of G418 vary in potency. Each batch needs to be titrated on the target cell type to determine the optimal G418 concentration for selection (see Table.2.2). In this series of experiments 250µg was found to be optimal, since it was the lowest concentration to give total cell death after seven days culture. Batches of gancyclovir that are toxic to R1 cells are quite frequently encountered. I tested 2µM gancyclovir on non-targeted ES cells and no unnatural cell death was observed. A visible difference in cell death between plates of targeted cells containing G418 and plates containing both G418 and Gancyclovir is considered to be a sufficient test for Gancyclovir activity. I therefore concluded from the targeting experimental results that 2µM gancyclovir was a suitable concentration for negative selection.

Conclusion.

Precise procedures for culturing embryonic stem cells vary significantly from laboratory to laboratory. Tissue culture conditions must be adopted that work best in the individual laboratory and with a particular ES cell line. Establishment of such conditions can be a laborious task involving a large amount of trial and error. As a result of the research described above I have established a working embryonic stem cell laboratory at the Ocular Genetics Unit at T.C.D., generating the capacity to exploit genome manipulation in embryonic stem cells and begin to generate animal models suitable for assessment of retinal gene delivery and expression.

Figure. 2.2.
Healthy ES cells growing on a primary embryonic fibroblast
monolayer received from A. Nagys lab. Toronto (Magn. 100X)

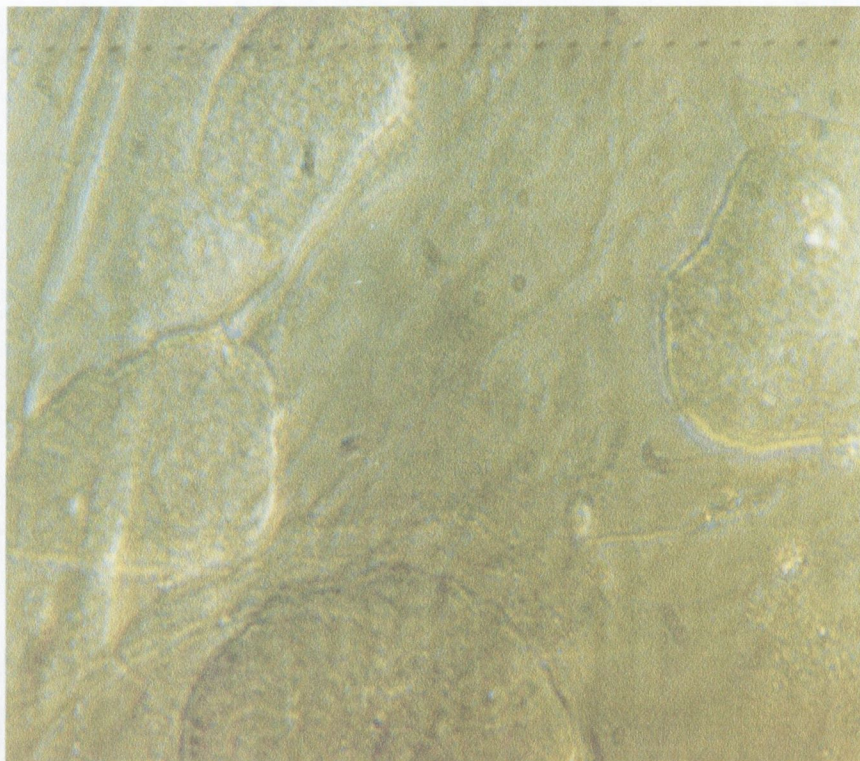


Figure. 2.3.
R1 Cells growing with a plating efficiency of 40%.
(Magn. 40X)



Figure. 2.4.

Typical morphology of R1 cells, with individual cells of the colonies difficult to distinguish and smooth defined edges. (Magn. 400X)

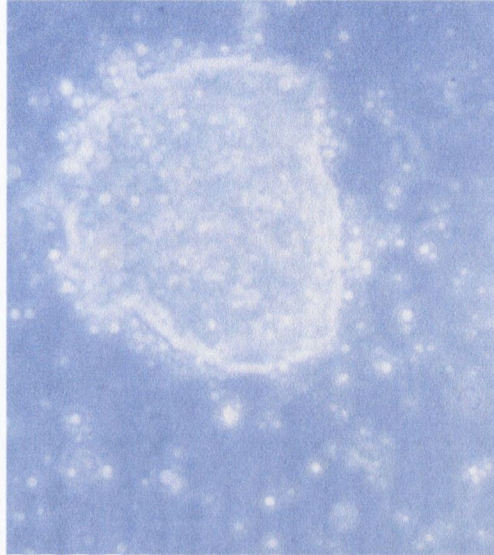


Figure. 2.5.

Illustrated below is an example of an embryoid body beginning to form (—▶) A layer of endoderm has started to form around the colony. (Magn. 400X)

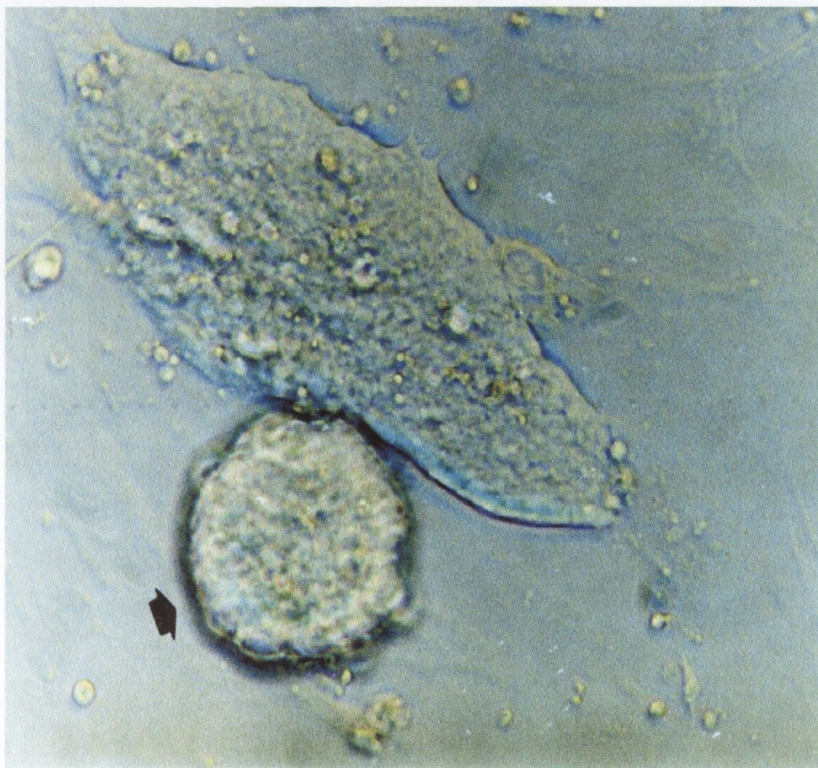


Figure. 2.6.
A monolayer of Primary Embryonic Fibroblasts.
(Magn. 40X)

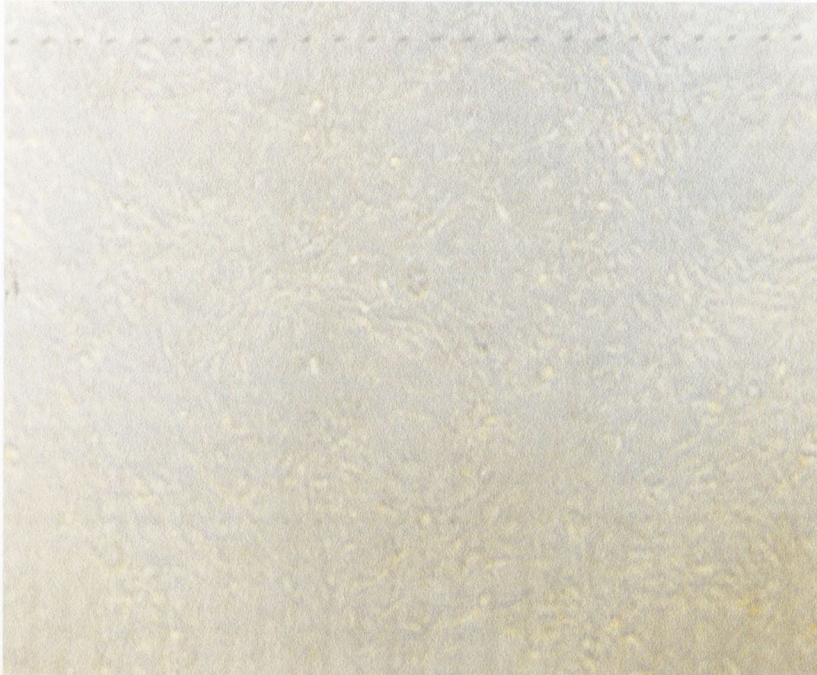


Figure. 2.7.
The colony shown in this figure, has become somewhat flattened in appearance, and has begun to spread out, this is indicative of cell differentiation. Furthermore endoderm appears to be forming in the area depicted by the arrow. This colony was grown in medium containing limited LIF (A 1 in 100 concentration) Magn 400X.

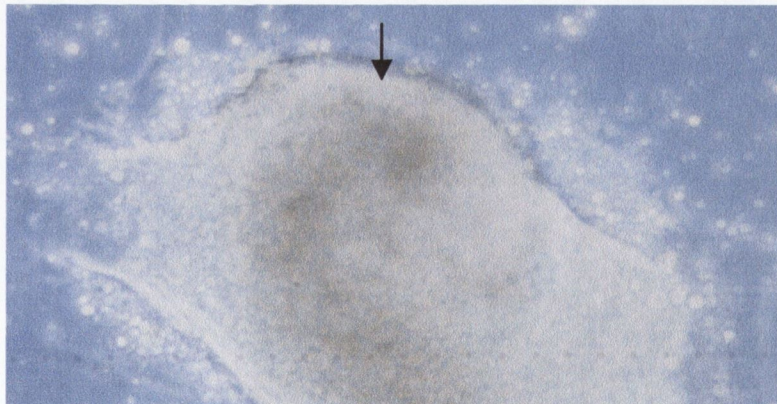


Figure.2.8.1.

Differentiated R1 cells - In the absence of a sufficient quantity of LIF the R1 cells have differentiated into Epithelial like cells (Magn. 400X)

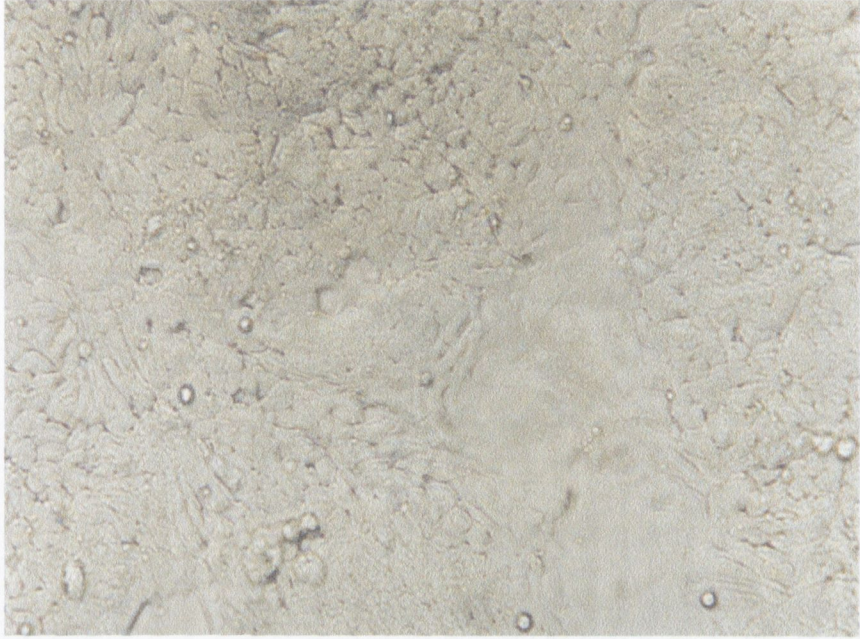


Figure. 2.8.2.

R1 cell differentiated to Fibroblast- like cells.
(Magn. 400X)

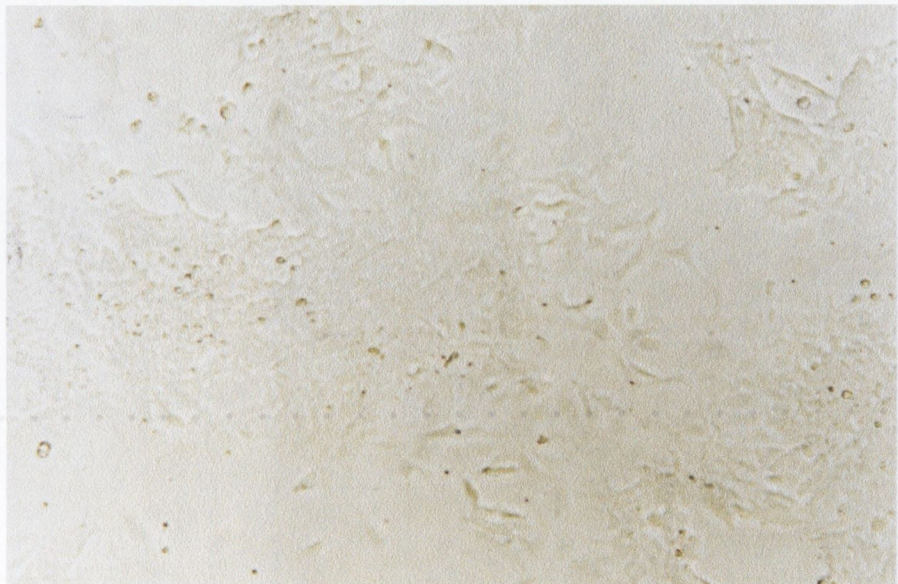


Figure. 2.8.3 –
Differentiated R1 cells - The R1 cells have differentiated
into Trophoblast-like cells

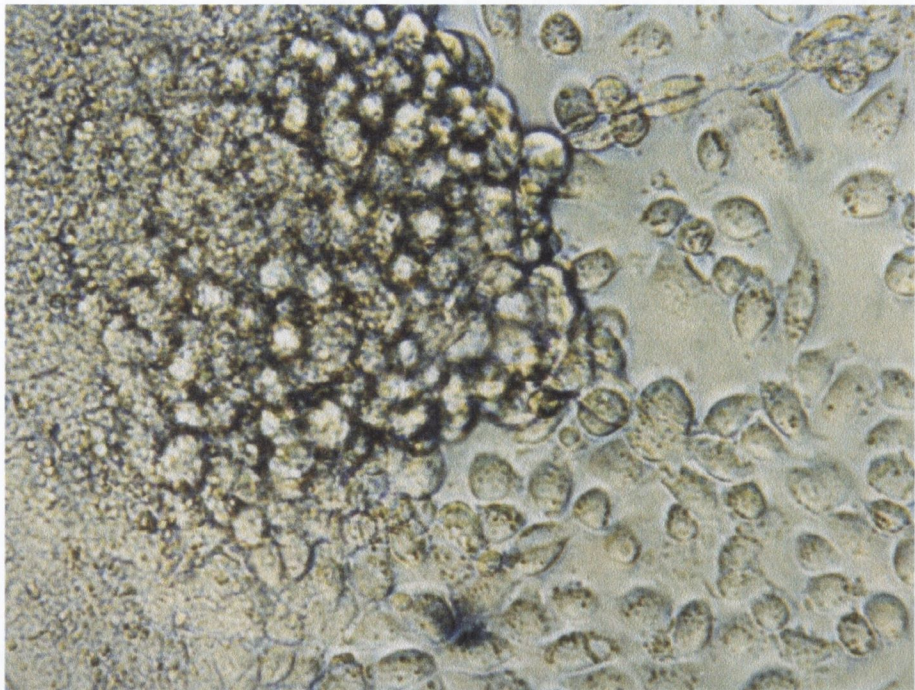


Figure. 2.8.4 -
Differentiated R1-Cells – Endoderm-like cells.

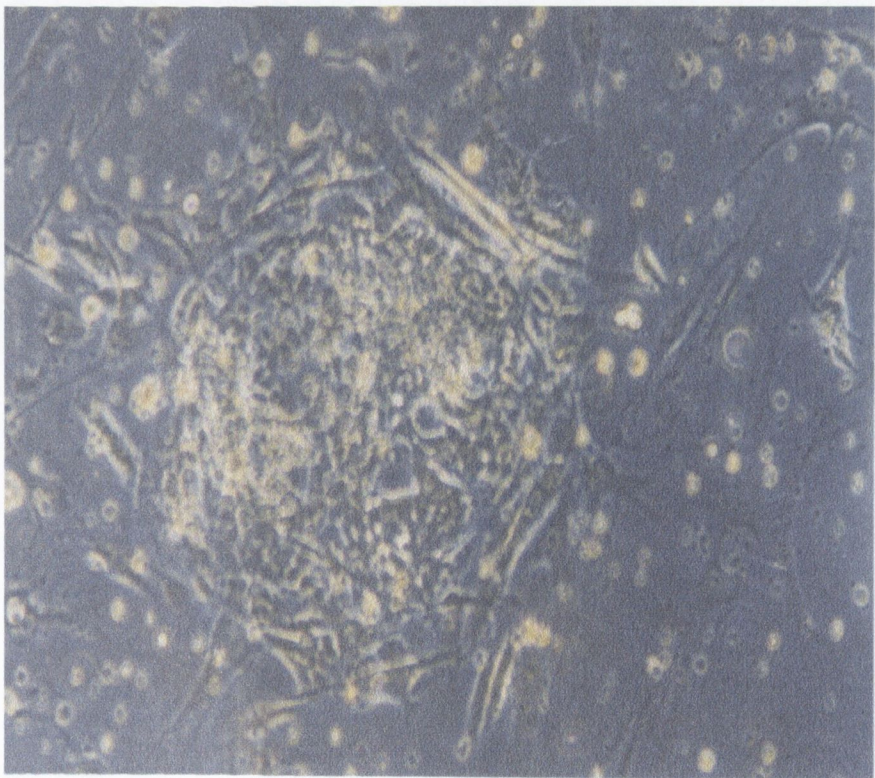


Table 2.1: 7

Four cuvettes containing 3×10^7 R1 cells were electroporated under various conditions. Pulses of 0, 50, 240 and 450 volts, 500 μ F capacitance respectively were delivered. The remaining cells were counted. This experiment demonstrated that electroporation at 240V, 500 μ F gave a 50% cell death. This is the required cell death for an effective EP.

Voltage	Number of cells surviving electroporation.
0 V	3×10^7
50V	3×10^7
240V	1.5×10^7
450V	1.9×10^7

TABLE 2.2.

Batches of G418 vary in potency. Each batch needs to be titrated on the target cell type to determine optimum concentration for selection. ES cell medium containing, 0, 150, 200, 250 and 500 µg/ml of G418 (Gibco) respectively was added to plates of R1 cells. The colonies were grown for seven days. The addition of 250mg/ml of G418 was shown to be optimal, as it was the lowest concentration to result in total cell death.

G418 Conc.	Colonies surviving selection.
500 µg/ml	0
250 µg/ml	0
200 µg/ml	12
150 µg/ml	30

Chapter 3.

The generation of stable embryonic stem cell lines carrying a targeted dominant mutation of the rds-peripherin gene.

Introduction.

A major problem in respect to the development of genetic therapies for RP (and indeed many other hereditary conditions) is the extensive genetic heterogeneity encountered in the condition (RetNet, 1999). For example, one hundred mutations have at this stage been encountered within the rhodopsin gene (largely in adRP). Moreover, between thirty and forty mutations have been found within the peripherin/RDS gene in adRP and also in a number of hereditary molecular dystrophies (RetNet, 1999). Mutation-independent methods for the suppression of dominant mutations have been described (Millington-ward *et al.* 1997). However, animal models are a necessary requirement for the *in vivo* workup of such methodologies. While a number of rodent models carrying dominant mutations within the rhodopsin gene have been described, no mouse models have yet been generated by targeted dominant modification of known retinopathy genes. We selected, for gene targeting, a 1 base-pair deletion (CTG to C-G) at codon 307 of the mouse peripherin/rds gene. Peripherin, and this specific mutation, were selected for the following reasons. Firstly the gene in question is of historic interest in terms of retinopathy research at this unit. Following the localisation of the first adRP gene in 1989, which lead to the implication of mutations within the rhodopsin gene (Mc William *et al.* 1989), the efforts of the team focused on a second large adRP pedigree, and the gene was localised on 6p in 1991 (Farrar *et al.* 1991a). Very shortly after this members of the group identified a 3-base pair deletion at codons 118-119 of the peripherin/RDS gene in an Irish family (Farrar *et al.* 1991b). The mutation resulted in the deletion of a cysteine residue at the boundary of the second proposed transmembrane domain and the larger of the two proposed intradiscal loops of the protein. This was the first mutation to be characterised within the peripherin/RDS gene in a human retinopathy. Secondly, while a number of methods for gene delivery to the retina have, and are being worked up, we anticipated that AAV vectors might prove to be of particular value. (We have now succeeded in obtaining long-term expression of reporter gene constructs following sub-retinal inoculation of AAV viruses in mouse retinas). However, mice can not be injected until they are at least one month of age. Furthermore, maximal expression in our hands, and in those of some other groups, takes up to 8 weeks. We anticipated that the rd, the rho^{-/-} and even the rds^{-/-} retinopathy models might be too rapid to fully exploit the rescuing potential of these viruses. In man, the codon

307 mutation within the peripherin/RDS gene results in a slowly progressive form of adRP (Apfelstedt-Sylla *et al.* 1995). The mutation causes a frame-shift, and as a result creates a stop codon after a further 16 triplets in man. The expected protein is therefore, 26 amino acids shorter than human wild-type peripherin/RDS. The mutation was identified in a German patient with classical RP. Symptoms in this patient began with a decline in visual acuity and loss of peripheral vision in her forties, and onset of night blindness in her fifties. Hence, the disease caused by the mutation is relatively mild. The frameshift brought about in the mouse genome by the 1bp deletion at codon 307 results in a stop codon a further 11 triplets after the normal peripherin/rds exon II stop codon. While it can not be assumed that a similar phenotype to that observed in man will manifest in mice, the probability is, nevertheless, that a relatively slow retinopathy will occur. If so, this would possibly provide a broader window of time in which to realise the maximal potential of the AAV vector systems. In addition, the model would in all likelihood manifest symptoms in heterozygous mice and thus provide a model for dominant disease through which various strategies for the suppression of dominant mutations could be addressed. Finally, by crossing these mice onto the natural rds^{-/-} genetic background, studies of the biochemical properties of the mutated protein, for example whether it migrates from the nuclear compartment to the rod outer segments, could be investigated for the first time. For these reasons, I have undertaken a project to generate a mouse carrying a targeted mutation at codon 307 of the rds-peripherin gene.

Many of the present day gene targeting vectors are based on the results of studies conducted in yeast and other fungi. For example, Hinnen *et al.* (1978) first described a single step, so-called O-type insertional event of foreign homologous DNA into yeast in 1978. O-type insertions result in duplication of the region of homology. A different form of integration, single step replacement or so-called Ω -type insertion, was later described by Rothstein *et al.* (1983). Unlike O-type insertions, the latter targeting procedure results in integration, replacement or deletion, as opposed to duplication of the target sequence, depending on the size and design of the incoming DNA. These single step events form a basis for the most common vector designs in current gene targeting technology. A more complex two step "in - out" targeting process was also observed in yeast in 1979 as

a result of experiments described by Scherer & Davis. The “in” step involved incorporation of foreign DNA into the target locus by an O-type insertion, i.e. duplication. The “out” step involved intra-chromosomal homologous recombination of the duplicated target sequence, which was brought about by single reciprocal recombination or uneven sister chromatid exchange. The method has been used in a number of gene targeting experiments to introduce subtle mutations into the mouse germline.

The first gene to be targeted into mouse ES cells was that encoding HPRT. Doetschman *et al* (1985) used an O -type plasmid to correct a defective HPRT gene in a 6 -TG (thioguanine)-resistant ES cell line. Targeted cells were readily selectable owing to the fact that they survived in HAT (hypoxanthine, aminopterin, and thymidine) - containing medium. The frequency of targeting was found to be 1 per million electroporated cells. Thomas and Capecchi (1987) used two types of targeting construct to inactivate a wild type HPRT gene. Both constructs were found to target with similar efficiencies, that is, of 1 per 2 million ES cells that survived electroporation in these experiments. The plasmid targeting vector, contained a neomycin resistance gene driven by a promoter from the herpes simplex thymidine kinase gene and an enhancer region. Inclusion of such cis-acting sequences were of substantial relevance to the design of future experiments, since their inclusion presented a means of selection of targeted events within transcriptionally inactive genes and in those genes which did not possess a selectable phenotype.

Much work has been carried out on vector design in order to improve both selection techniques and frequencies of homologous recombination. The two most commonly used targeting vectors are replacement and insertional vectors the design of which are based on the original Ω and O type recombination studies carried out in yeast. Both of these types of vector are basically comprised of sequences homologous to the target locus, and contain markers for positive and sometimes negative selection and also bacterial plasmid sequences (see chapter 2). The main difference between the two types of vector is in the position of the site of linearization, which influences the manner in which recombination occurs. In replacement vectors the site of linearization lies outside of the region of the homology between the vector sequence and that of genomic DNA, and targeting

occurs by double reciprocal recombination between the vector and the chromosomal sequences. When insertion vectors are used, a single reciprocal recombination event occurs, which is initiated by a double stranded break in the vector. Although plasmid vectors are by far the most commonly used, a number of vectors based on adenovirus- or bacteriophage- backbones have also been developed (Mitani *et al.* 1995, Tsuzuki *et al.* 1998) The frequency of adenoviral vector integration by homologous recombination in ES cells is usually very high, with 25 - 40% of the cells being targeted, even without the enrichment effect of negative selection. Targeting vectors can be generated more easily and rapidly in bacteriophage by the use of novel procedures involving phage-plasmid recombination that require less genomic sequence or restriction site information than that needed for the production of plasmid vectors.

A number of researchers have explored the relationship between length of homology in targeting vectors and the frequency with which homologous recombination takes place between the vector and its target sequence in embryonic stem cell DNA. Experiments carried out by Thomas *et al.* (1987) demonstrated a 40 fold increase in targeting frequency when the homologous region within the targeting vector was increased from 4kb to 9kb. Hasty *et al.* (1991) found a staggering 190-fold difference in targeting efficiency between vectors of 1.3 and 6.8 kb in length. The results of these, and other studies clearly indicate that there is an exponential relationship between the extent of target homology and targeting frequency. The ideal length of homology is probably between 5 and 10 Kb. While vectors containing as little as 500 base pairs of flanking homology have been successfully targeted, ideally the vector should be designed with at least 2Kb of homology on each arm (Bradley *et al.* 1984, Deng *et al.* 1992).

While the extent of homology is clearly important in respect to gene targeting other factors may also be important. A syngenic background has been emphasised as being important by some groups but not by others. For example, te Riele *et al.* (1992) reported a 20 fold improvement in targeting by the use of isogenic DNA (129 sv- derived) compared to non isogenic DNA (BALB\C derived) when using 129-derived ES cells. However, Thomas *et al.* (1987) found no such variation.

A variety of selectable markers are now available for incorporation into targeting vectors (Yagi *et al.* 1990, Reid *et al.* 1990). Markers are referred to as positive when they allow the identification of transfected cells and negative when

they cause the death of non-specifically targeted cells (Mansour *et al.* 1988).

Opinions vary on which selection processes are optimal. (see chapter 4).

A number of regulatory elements are now available which result in increased expression of selectable markers. For example, targeting frequencies of vectors containing neomycin resistance genes driven by the phosphoglycerate kinase gene promoter, the RNA Pol II promoter or the pyF441 polyoma enhancer/ TK promoter, have been reported by Soriano *et al.* (1990). The use of Neo vectors containing PGK - 1 or pol II promoters resulted in a higher frequency of targeting. The Soriano team also demonstrated an increased frequency of transformation following the addition of extra polyadenylation sequences at the 3' terminus of the neomycin resistance marker.

So-called enhancer and promoter-trap vectors have been designed to decrease the number of random integration events into ES cell DNA and hence decrease the number of clones that need to be screened in order to identify those in which homologous recombination has occurred. Such vectors rely on homologous recombination with the target gene to supply cis- acting regulatory regions. For example, Schwartzberg *et al.* (1991) reported gene targeting with a promoter-less neomycin resistance gene yielding a 100-fold enrichment for targeted clones. This form of enrichment for targeting has been shown to be successful even for genes with low expression levels in targeted cells. Enhancer-trap targeting vectors result in a smaller reduction in nontargeted G418 clones. In such vectors a selectable marker with a weak position-dependent promoter is used, and therefore the system relies largely on endogenous enhancer sequences (Gossler *et al.* 1986, Ng *et al.* 1998).

In the further workup of gene targeting technologies, a negative selection technique has been developed by Mansour *et al.* (1988), which selects against non-homologous incorporation into the genome of Ω -replacement vectors. This selection procedure requires incorporation of a negatively selectable marker into the arms of the Ω vector loop, outside of the region of homology. The negatively selectable marker is lost as a result of homologous crossovers, allowing most targeted cells to survive selection. When non-homologous recombination takes place, i.e. at a random location, the vector still contains the selectable marker and thus the cell is killed. Negatively selectable markers include those encoding herpes

simplex thymidine Kinase, diphtheria toxin or hypoxanthine phosphoribosyl transferase. Cassettes containing such genes are widely available. Enrichment of transfected cells using the negative selection technique is very variable, reports ranging from 2 to 20 fold (Joyner, 1993).

In the generation of animal models of human diseases it is often necessary to introduce small changes such as point mutations, insertions or deletions at a given locus as opposed to a complete functional knockout. Introduction of a marker gene and its transcriptional control elements may influence the expression of both the targeted gene and possibly other closely linked genes. Such disruptions may alter the phenotype of the animal and hence a number of techniques have been developed to overcome such problems. For example, attempts have been made to avoid a requirement for a selectable marker at the target site. As an example, DNA delivered by microinjection was reported by Zimmer *et al.* (1989) to result in the targeting of the Hox 1 gene in 1 of 150 cells. Owing to the efficiency of this method, targets were detectable directly by Southern blot analysis. As mentioned in chapter 2, the latter technique is difficult and is therefore not commonly used in ES cell work. Co-introduction into ES cells of two vectors, one of which contains a selectable marker, can result in the identification, by positive selection, of cells that have taken up DNA. Reid *et al.* (1990) also demonstrated a 100-fold enrichment for targeted events using this technique.

A so-called hit and run two step selection method based on "in out" recombination events that have been observed in yeast can also be used in gene targeting. This process involves two steps. In the first, an insertion O type vector carrying markers for both positive and negative selection that are located outside of the homologous genomic sequence is used, resulting in the duplication of genomic sequences and inclusion of the marker gene into the genome. Intra-chromosomal recombination results in removal of the selectable markers together with plasmid sequences, leaving only one copy of the duplicated homologous region at the targeted locus. In 1990 Valancius *et al.* (1991) successfully introduced a four nucleotide insertion into an hprt locus using this procedure. The method was problematic owing to the low frequency of successful recombination events. The positioning of the mutation of interest relative to the double strand break is of the utmost importance. This is because numerous integration products can be formed owing to gap formation and repair, mismatch heteroduplex repair or the branch

migration of Holliday junctions, which occur during the integration of an insertion vector (Steeg *et al.* 1990).

Systemic expression of mutated genes of unknown function may result in embryonic lethality. However, a technique has recently been developed which allows targeted inactivation in a cell-, or tissue- specific manner. This procedure is based on the cre-loxP recombination system of bacteriophage P1. Cre recombinase catalyzes site specific DNA recombination between 34 base-pair repeats named loxP through a transient DNA-protein covalent linkage. Orban *et al.* (1992) demonstrated the utility of this technique by producing two strains of transgenic mice, one transgenic for a β -galactosidase gene flanked by loxP sequences and a mouse transgenic for the P1 cre recombinase driven by a tissue specific promoter. Doubly transgenic mice were shown to exhibit β -galactosidase expression in a tissue-specific manner owing to cre expression. Gu *et al.* (1994) used a similar approach to create an animal model in which the *Pol b* gene was targeted in a specific tissue. In this study targeted ES cells were produced by homologous recombination, with loxP sites flanking both the gene of interest and the incorporated neomycin resistance and herpes thymidine kinase selectable markers. Cells were selected by their resistance to G418 and following such selection, transient expression of cre recombinase facilitated homologous recombination between the two loxP sites. Deletions were negatively selected for using gancyclovir. Type I deletion resulted in a knockout of the targeted gene. Type II resulted in a loxP -flanked targeted gene (*pol b*^{lox}). Strains of mouse containing both mutations were generated. Mice homozygous for the *pol b* - knockout displayed an embryonic lethal phenotype. Tissue-specific homozygous knockout mice were generated by interbreeding *pol b*^{lox} strains with mice transgenic for the cre recombinase enzyme driven by the *lck* proximal promoter specific for T cells. This method of gene modification is extremely powerful. It should be noted however that this sort of specific gene targeting requires the generation of a large number of mouse strains and considerable knowledge of tissue-specific promoters. The cre recombinase strategy can also be used to remove lox P -flanked selection cassettes, by expression of cre recombinase at the embryonic stem cell stage or by breeding the targeted mouse with a transgenic mouse expressing the cre recombinase in the tissue of interest (Gu *et al.* 1994).

Markers used in selection and unwanted plasmid sequences can also be removed using this strategy. Cassettes are available in which the marker used for positive selection is flanked by *lox P* sites. When targeted ES cells are identified it is possible to transfect such cells with a plasmid containing a cre recombinase gene together with a transient drug resistance marker such as puromycin or hygromycin. Cells are then selected for drug resistance and hence cre recombinase is expressed. However, in this approach, it is still necessary to confirm the presence of the targeted mutation, which can usually be achieved by restriction enzyme analysis or sequencing.

Furthermore, genetic modification of cell lines that have already been subjected to gene targeting are sometimes necessary. Low frequency of targeting can, however, become a significant problem in consecutive rounds of targeting. One method of overcoming this problem is to introduce I-Sce I recognition sequences at the site of interest during the first round of targeting (Cohen-Tannoudji *et al.* 1998). I-Sce I is a yeast meganuclease that induces double-strand breaks in order to facilitate intron homing in yeast mitochondrial DNA. Specific double strand breaks can also be produced in ES cells using this method. Such breaks stimulate DNA repair by recombination with a donor molecule homologous to the regions flanking the break. This strategy has been used by a number of groups and has been shown to produce targeted integration into genomic DNA with frequencies increased by of 100- to 5,000- fold.

Following enrichment for targeted integration of genes into ES cells it is necessary to screen transfectants for actual targeted events. Southern blot analysis and PCR have both been used for this purpose (Nitschke *et al.* 1993 , Humphries *et al.* 1997). Before these techniques can be employed, DNA of sufficiently high quality must be prepared, especially for Southern blotting. Choice of restriction enzyme and probe is extremely important in distinguishing wild type from targeted clones. The ideal probe is a flanking one, which is not present in the targeting vector, since internal probes also detect random integrations. Difficulties often arise in the selection of restriction enzymes. Those which cut within the vector are not ideal since they do not distinguish between integrants of the whole vector (i.e including plasmid or phage component) or concatemers, or the correct single gene replacement. If an ideal probe or enzyme can not be found, extensive probing of both the 5' and 3' regions of the endogenous and targeted sequences can

sometimes be required.

PCR analysis is also useful owing to the rapidity with which it can be used, even with low quality DNA. This procedure requires the design of primers from the targeting vector and the endogenous target sequence, a combination of which should result in a product only after homologous recombination has occurred. If PCR analysis is used, the vector should ideally be designed with an arm of 1Kb or less of homologous sequence. However, long range PCR methods have been reported which have enabled this problem to be overcome (Joyner, 1993) . A major disadvantage of the PCR technique is the occurrence of PCR artifacts or PHLOPs (Polymerase-halt-mediated linkage of primers). PHLOPs are caused by an initial reaction, which extends each primer into a region of DNA , the sequence of which is shared both by the target locus and the targeting vector (Reid *et al.* 1990). The next step can result in the annealing of these two DNA fragments which, following amplification by the PCR process can result in a false positive signal. It is important to devise and test a screening procedure for targeted events before electroporation of ES cells since the development of an assay can be time consuming and tedious.

In this chapter, I describe the successful development of a robust (Southern blot-based) assay for targeted integration of the $\Delta 307$ peripherin/rds clone into the ES cell genome. Following the development of the assay, a series of electroporations of the targeting vector into ES cells was undertaken, and the cells were subjected to either single or double-selection. Ten clones were identified in which the $\Delta 307$ mutation was targeted at the peripherin/rds locus - the first step, toward the generation of a mouse expressing the mutation.

Materials and Methods.

Peripherin/rds $\Delta 307$ VECTOR.

The peripherin/rds $\Delta 307$ vector was constructed as a collaborative effort with supervisor Dr. G.Jane Farrar (see fig.3.1). A 17kb clone was screened out of a λ dash phage library containing 129Sv- derived mouse genomic DNA using PCR primers designed in the 5' region of the mouse peripherin gene. The insert was removed from λ dash and cleaved into two fragments of 5kb and 12kb by digestion with *Sal*I and *Spe*I restriction enzymes. These fragments were subcloned into Bluescript plasmid. The 12kb clone was 5' of the 5kb clone and of importance for the establishment of a Southern blot assay. The 5kb rds clone included exon III of the peripherin gene. A 1bp deletion known to occur in an adRP patient (see Introduction to chapter 3) was introduced into Exon III by PCR. The construct was cloned into pPNT, a vector containing an HSV thymidine kinase gene for negative selection (see fig.3.2). The pPNT vector was digested with the restriction enzymes *Xba*I and *Xho*I and ligated with the mutagenised 5Kb peripherin/rds fragment which had been removed from Bluescript by double digestion with *Spe*I and *Sal*I. The neomycin resistance gene (*plox Neo*) required for positive selection was cloned into an *Hpa*I site approximately 500bp away from the mutation (see fig.3.3). The neomycin gene flanked by *plox neo* was removed from the *ploxPneo-1* plasmid by digestion with *Xho*I and *Sma*I. Care was taken to ensure that the neomycin-resistance gene was cloned into the vector in an antisense orientation. Vector DNA was linearized with the Not I restriction enzyme. Prior to the electroporation the linearised DNA was extracted with phenol/ chloroform, precipitated with ethanol, and dissolved in sterile water at a concentration of 1mg/ml. Sections of the vector were sequenced in order to ensure that both the $\Delta 307$ mutation and the *ploxneo* insert was present.

Electroporation and ES cell selection procedure.

Electroporation (EP) was carried out using a Biorad II Gene Pulser. The cell media was changed one hour prior to electroporation. Fifteen confluent 10cm plates of R1 cells were required for the EP.

Each plate was trypsinized in a volume of 3mls of trypsin to break up cellular aggregates into a single cell suspension, 7mls of ES media was added and the cells were pre-plated for 15 mins at 37°C and 5% CO₂. Media containing the R1 cells was removed from these plates and centrifuged in a 50ml Falcon tube for 5 mins at 1000 rpm. The supernatant was aspirated and the cells resuspended in cold PBS, 1ml per 10cm plate. The R1 cell concentration was then counted and adjusted to 6×10^6 cells per ml. The cell suspension (0.8ml) was mixed with 20µg of linearized vector DNA and transferred into an electroporation cuvette. A control cuvette was also prepared containing R1 suspension only. The cells were electroporated at 240 V and a capacitance of 500µF. After electroporation, cuvettes were placed on ice for 20mins. Cell suspensions were then mixed with 20mls of ES cell medium, 10mls of which were aliquotted onto two 10cm plate coated with a 0.1% gelatin. G418 was added for selection two days later at a concentration of 200µg /ml. After 7 days of selection, drug resistant colonies were picked.

Picking of ES cells into 98 well plates.

Drug resistant colonies were circled using a marker pen. 50µl of 0.1% Gelatin was placed in every well of every other row of a flat-bottomed 96-well plate using a multichannel pipette. The gelatin was aspirated and 200µl of LIF/G418 media added to each gelatinized well. A V- bottomed 96-well plate with alternate rows of wells containing 50 µl of trypsin/EDTA 0.05% was also prepared. Media was aspirated from colony-containing plates and replaced with 10ml PBS. An eppendorf pipettor set to 10µl was used to scrape off drug resistant colonies and to transfer those to one of the wells of trypsin in the V-bottom plate. One 12-well row at a time was picked. Cells were left in trypsin at 37°C for 5-10mins. A multi-pipettor set to 200µl was then used to pick up medium from one row of the gelatinized flat bottom 96-well plates and to transfer it to one row of the cells in the trypsin plates. Cells were resuspended by pipetting before transfer back to the same row in the gelatinized plates.

Growth of ES cells for freezing and for preparation of ES cell DNA.

The G418-resistant colonies on 96-well plates were grown for approximately 4 days or until the majority of the colonies were confluent, they were then passed by trypsinization onto two 96-well plates. Once confluent, one set of colonies was frozen and one used in the preparation of DNA.

Freezing of ES cells on 96-well plates.

Media was changed in all wells two hours before freezing. At the time of freezing the media was removed by aspiration and the colonies washed with 100µl of PBS. Cells were trypsinized by addition of 50µl trypsin/EDTA 0.05% and incubated at 37°C for 5 - 10 mins. While on ice, 50µl LIF MEDIA was added to the wells to neutralize the trypsin, and 100µl of 2 x freezing media was added (1 part DMSO, 1 part FCS, 3 parts complete Media). Each well was covered with 80µl of cold sterile mineral oil (Sigma). Plates were wrapped in foil placed in the prepared box that had previously been cooled to -70°C, and stored in the -70°C freezer.

Purification of ES cell DNA from 96 well plates.

Media was removed by aspiration and colonies washed with 100µl of PBS. Lysis buffer (50µl), was added to each well and the plates incubated in a humid chamber O/N at 37°C. 2 x volumes of 100% ethanol were added to each well and the DNA was allowed to precipitate for 1h at room temperature. The ethanol was removed by blotting the plates on layers of tissue. DNA was washed three times with 70% ethanol and air -dried for 20mins. DNA was resuspended in 20µl of 0.1 x TE or alternatively restriction digest cocktails were added directly.

Restriction enzyme digestion of DNA in 98 well plates. Restriction digests were carried out in individual wells of the 96 well plates in a total volume of 30 or 100µl. A typical mix contained : 1 x restriction buffer for the enzyme to be used (NEB) , 1mM spermidine (Sigma), 100µg / ml BSA (NEB), 50µg/ml RNase (NEB), 15 units of enzyme (NEB).

Electroporation and selection procedure.

Method 2.

Again, the electroporation was carried out using a biorad II gene pulser.

R1 cells were prepared as before. 7×10^6 cells were used for each electroporation.

Two electroporations at 240V and 500 μ F capacitance were carried out on Day 1.

Cells from each electroporated cuvette were resuspended in 32 mls of ES Cell media.

Five 10cm plates were seeded with electroporated ES cells from each electroporation, 10mls of the resuspended cells were added to three 10 cm plates to be used for G418 and Gancyclovir selection, and 1ml of resuspended cells was added to two 10cm plates containing 9ml of ES media for G418 only selection. On day four, three more EPs were carried out. Various concentrations of targeting vector DNA were used in the EPs, specifically, 20 μ g of DNA was used in the first three EPs and 40 μ g and 50 μ g respectively in the third and fourth. Selection drugs were added into the media two days after each EP. G418 was added at a concentration of 300 μ g/ml to all plates and 2mMols of Gancyclovir was added to selected plates. Drug resistant colonies were picked after 12 days and all the plates were released from drug selection. Colonies appearing on the plates over the following four days were also picked.

Picking of ES cells into 12-well plates.

ES cells were picked following the same protocol as that for 96-well plates but without the use of a micropipette, the main difference being that the colonies were picked over a number of days since they appeared more clearly upon release from drug selection.

Freezing of ES cells on 12-well plates.

ES cells were trypsinized and then removed from 12-well plates into labeled cryotubes. An equal volume of 2 x Freezing solution was added to each tube and these were placed in a "Mr Frosty" (A cryotube holder surrounded by isopropanol, which allows uniform freezing of the cells) in the -70°C freezer.

Purification of ES cell DNA from 12 well plates.

Media was removed by aspiration and colonies washed with PBS. 500µl of Lysis buffer (see Appendix 3) was added to each well and plates were left at room temperature for 1hr. Lysis buffer and DNA were removed from wells with a 1ml pipette tip and placed in a 1.5ml eppendorf tube. Proteinase K was added to each tube at a concentration of 200µg/ml. Cells were then incubated O/N at 55°C. DNA was cleaned by 1 x Phenol/ Chloroform extraction followed by Ethanol precipitation. The DNA was collected by spooling with a glass rod and resuspending in 100µl TE at 37°C overnight.

Restriction enzyme digestion.

DNA was incubated at 65°C for 2-3 hrs prior to restriction enzyme digestion. 1/10 Vol. of DNA was used for each digestion with the same restriction digest cocktail as described previously.

Southern blotting.

Electrophoresis.

Loading buffer was added to digested DNA and samples loaded on a 1% agarose gel in TAE (0.04M Tris-acetate, 0.001M EDTA). Electrophoresis was carried out at 40V for 18 - 36h. Gels were subsequently photographed on a U.V. lightbox.

Depurination of DNA in gels was carried out in 0.2M HCL for 15mins. Denaturation was achieved by agitating the gel in an alkaline solution (0.2 M NaOH, 1.5 M NaCl (BDH)) for 1h. Gels were then placed in neutralizing solution (1M Tris pH7.5, 1.5 M NaCl, 0.001M EDTA (BDH)) for 1h. If the gel was to be alkaline blotted only the depurination step was necessary. DNA was transferred to nylon filters overnight at room temperature by capillary action Southern blotting. Eight squares of Whatman 17 Chr 25 cm x 25cm were placed in a plastic tray 30 x 30 cm containing 20 x SSC (3M NaCl, 0.4M Tri-sodium citrate (BDH)). The gel was placed on top of the filter paper. The gel was covered with a Hybond™-N nylon membrane and a sterile 10ml pipette was rolled over the membrane to remove air bubbles. Parafilm was placed around the edges of the gel and the tray to ensure that all the SSC passed through the gel. Two dry pieces of Whatman chromatography paper were used to

cover Hybond™-N nylon membranes. Finally gels and subsequent layers were covered by a glass plate and a 500g weight. Membranes were air dried, DNA side up the following day.

Alkaline transfer.

Alkaline transfer was carried out using 0.4N NaOH in place of the 20 x SSC in the traditional protocol. Denaturation, transfer and fixation was carried out in single step using this method. The Hybond™-N + nylon membrane irreversibly binds nucleic acids in alkaline solution due to its positive charge.

Fixation.

The DNA was fixed to Hybond™ N membranes by one of the following methods, U.V. Crosslinking, (the membrane was U.V. irradiated on a transilluminator, DNA side down for 5 mins), Baking at 80° in an oven for 2hours, or incubation in 0.05 N sodium hydroxide solution for 5min at room temperature followed by a wash in 2 x SSC.

Prehybridization.

Membranes were prehybridized in a plastic heat sealed bag at 65°C overnight. Prehybridization solution (6 x SSC, 5 x Denhardt's solution, 0.5% SDS) and 20µg/ml boiled and sheared salmon sperm DNA, was added at a concentration of 5mls per 100cm² membrane. In order to reduce the amount of background hybridization a number of blocking agents were also added to the prehybridization solutions including 10x Dextran Sulphate, 30%Formamide, 0.5M Na₂HO₄ /7% SDS and 25mg/ml sheared mouse DNA.

PCR product probes.

Oligonucleotide primers.

Name	Primer Sequence
------	-----------------

Xba I External.

Forward Primer.	AGC AAC GTG GAT GGG CGG TA
-----------------	----------------------------

Reverse Primer.	TGA ATT CAA GGG TAG CAT GG
-----------------	----------------------------

A 100µl PCR reaction mix contained 200ng of template DNA, 200mM each dNTP (Boehringer Mannheim), 15mM MgCl₂, 100mM Tris pH 9, 50mM KCl, 1 x Triton (BDH) and 50pM forward and reverse primers as listed above. One unit of Taq polymerase was added to each reaction at a temperature of 80°C following a denaturation at 94°C for 5mins in a DNA thermal cycler (Biometra Triothermoblock). Thirty cycles of amplification were carried out using a step program (denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min). The products of these amplifications were analysed on ethidium bromide-stained 1.5% agarose minigels.

Radiolabelled PCR reactions.

A 50µl PCR reaction mix contained 10ng DNA, 12kb Peripherin-rds vector, 200mM dATP, dGTP, and dTTP, 15mM MgCl₂ PCR Buffer, 50pM forward Ex500 primer, 0.5 units of Taq polymerase and 0.5µCi of [α -³²P]dCTP (Amersham). The amplification was carried out on a Perkin Elmer Cetus thermocycler. DNA amplification was performed using the following parameters; 30 cycles of 94°C for 1 min., 55°C for 1min. and 72°C for 1min. 20 sec.

Removal of unincorporated nucleotides.

Sepharose Spin Columns.

Spin columns consist of 200 µm glassbeads resuspended in TE overlaid with Sepharose CL 6B (Pharmacia) in a 0.5ml eppendorf pierced with a 20 gauge needle. The labeled probe is spun through the column in a microcentrifuge at 2000 RPM for 2mins. The probe was used for hybridization if 30 - 50% incorporation was achieved.

Sephadex G50 columns.

Glass wool was placed in the bottom of a 1ml syringe and overlaid with sephadex G50 resuspended in 3xSSC. Probes were washed through the columns by addition of 100µl aliquots of 3xSSC .

Hybridization.

Probes were boiled for 5mins then snap cooled on ice before adding to the prehybridization solution. Hybridizations were carried out in a water bath or a Hybond Oven at 65°C overnight.

Stringency of washing.

Variations in the concentration of SSC, SDS and washing times were made in an attempt to eliminate backgrounds. The following wash procedure was found to be optimal. Filters were initially rinsed in 2x SSC at room temperature and then washed as follows for 30 mins at 65°C. (a) 2 x SSC/ 0.1% SDS, (b) 1 x SSC/ 0.1% SDS, (c) 0.2 x SSC / 0.5% SDS, then 0.01% Na₂PO₄ was then added to the wash to cut down on background.

Autoradiography.

Filters were sealed in a plastic bag and overlaid with two sheets of autoradiographic film (Agfa). Films were processed usually three days later in an RG II Fugi X-ray film processor and the second sheet was allowed to remain in contact with the X-ray film for up to 3 weeks if more intense autoradiographic signals as required.

Sequencing.

Radioactive 5' end labeling of oligonucleotides.

Oligonucleotides were labeled by mixing 1pmol of primer with 1µl of 10X polynucleotide kinase buffer and 2µl of sterile dH₂O. 5µl of radioactive *Redivue* γ³²P dATP (Amersham) and 1µl of polynucleotide kinase (NEB) were then added. The end-labelling reaction was mixed thoroughly and incubated at 37°C for 1-2

hours. The polynucleotide kinase was heat-deactivated at 95°C for 5 mins.

Sequencing of PCR products.

DNA from each targeted cell line was PCR amplified, using amplification conditions previously described, with a forward primer 5' to peripherin/rds exon III and a reverse primer ~ 100bps into the *ploxneo* sequence which had been inserted into the cells by targeting.

pLox F 5'

Reverse Primer CAC ATA CAC TTC ATT CTC AG

2051

Forward Primer GCA GAG AGT ATC AGA TGT TC

PCR products were Phenol /Chloroform extracted and ethanol precipitated. Each sample was incubated with 5µl of 200µg/ml Proteinase K for one hour at 55°C and run through a Sepharose column. 0.5µl of the purified product was run out on a 1% agarose minigel beside a control sample of known concentration in order to check the quantity of DNA in each sample. 500ng of template DNA was placed in a small eppendorf tube and volumes made up to 8ml with H₂O. Both DNA and end-labelled primer were heated to 94°C and 2µl of end-labeled primer was then added to the DNA. DNA and primer mixtures were kept at 94°C for 5-7 mins before snap chilling on ice for 2 mins. 8µl of 2X sequencing buffer (see Appendix) and 2µl of a 1/8 dilution of *Sequenase* V7.0 (USB) in *Sequenase* dilution buffer (USB) was added. Samples were mixed thoroughly and incubated at 37°C for 5 mins. 4.35µl of this mix was then added to 3µl of ddGTP, ddATP, ddTTP and ddCTP respectively. Samples were incubated at 37°C for 10mins before the addition of 5µl of formamide stop solution. Samples were overlayed with 2 drops of paraffin oil. 3-5µl of each sample was loaded on to a preheated 8% denaturing polyacrylamide gel. Electrophoresis was carried out at 40mA and voltage of 2000 V. The bromophenol blue dye in the stop solution was used as a indicator of how far the sequence had run. Gels were subsequently placed on 3mm filter paper (Whatman). Each gel was covered in cling film and dried on a Savant gel drier at 80°C for

approximately 1 hour. Once dry, gels were placed in cassettes and overlayed with Agfa X ray film.

***Hae* II digestion.**

Digestion was carried out in a 20µl volume containing 1µg of PCR product obtained by PCR amplification of each targeted R1clone as described for sequencing, 1 x *Hae* II restriction enzyme buffer and 5 units of *Hae* II.

Results.

A map of the targeting vector is illustrated in figure 3.1. In order to develop an assay for targeted integration of the manipulated peripherin gene at the peripherin locus in embryonic stem cell DNA, at least one probe in the flanking genomic DNA, capable of detecting either a "shift-up" or a "shift-down" of a specific restriction fragment as a result of the homologous integration event is required. To this end, approximately 2Kb of genomic mouse DNA immediately 5' of the sequence of homology in the targeting vector was sequenced (data compiled in collaboration with Dr. Fiona Mansergh and Dr. Sophie Kiang). The sequence is shown in figure 3.4. As illustrated in figure 3.5, this sequence is contained within a portion of an *Xba* 1 fragment, and immediately flanks the targeting vector at its 5' end. As will be noted from the sequence, a region of approximately 200 base-pairs bearing strong homology to a murine equivalent of the human consensus *Alu* 1 dispersed repetitive element was revealed within this region. The position of the repetitive sequence is illustrated on the map of the targeting vector in figure 3.6. Primers were thus designed (a and b, figure 3.5) such as to enable the amplification of a segment of ES DNA approximately 500bp in length, immediately flanking the targeting vector at the 5' end and avoiding the repetitive region. When used as a probe in Southern blotting of ES DNA cleaved by *Xba* 1 a fragment of 5.01 Kb, representing the segment of genomic DNA containing exon III of the peripherin gene, is predicted to be detected (see fig. 3.5). Since the *plox* neo marker within the targeting vector contains an *Xba* 1 site, a "shift-down" in the exon II-containing *Xba* 1 fragment of 4.18 Kb is predicted to occur. Thus, ES cells in which homologous recombination has taken place between the targeting vector and the endogenous gene, should display the 5.01 Kb allele together with the 4.18Kb allele, diagnostic of the endogenous and targeted alleles respectively. Genomic ES DNA was digested with *Xba* 1, subjected to Southern blotting and probed with the 500 bp probe. A fragment of 5 Kb was obtained, suggesting that the assay should be effective in screening for targeted events (see fig. 3.6).

Five electroporations, each with 20µg of the peripherin/rds Δ307 vector DNA were initially carried out. Only those cells with a healthy appearance were counted when preparing ES cells for electroporation. 6 x10⁶ healthy cells were required to survive the electroporation. Thus, up to four times this number of cells

was used in each EP. Drug selection was carried out for seven days using 200µg/ml G418. On average, 600 colonies survived selection. In this experiment 480 G418-resistant colonies were picked onto 96 well plates. Each 96 well plate was expanded on two further plates once the majority of the wells were confluent. DNA was retrievable from only 140 wells, which may be a reflection of the method of DNA preparation. The method used in this experiment was a rapid one, as described by Ramirez-Solis *et al.* (1992) This procedure relies on adhesion of the DNA to the surface of the wells. In some instances this may not occur adequately and the DNA may have been lost at the inversion stage (see Materials and Methods). Each colony grows at a slightly different rate and it is not beneficial to allow cells to remain in a confluent state while other colonies catch up, since they may lose their pluripotency (see Chapter 1). It was necessary therefore to proceed to the DNA stage once the majority of the wells were confluent, which meant losing a number of slow-growing wells. The major problem in this initial series of experiments, in addition to poor yields of DNA, was incomplete digestion of the DNA by *Xba* 1 (the rapid method used did not include phenol /chloroform extraction). A number of restriction enzymes have been found to cut poorly when crude preparations such as those in the experiment, are used (Dr. Andras Nagy, Personal communication). A list of such enzymes is shown in table 3.1. As will be noted, *Xba* 1 has a bad rating. Largely owing to problems encountered in DNA cleavage, and the amount of time that had elapsed in attempting to develop this targeting assay, the initial series of experiments were discontinued.

Table 3.1

96 Well Plate genomic digests
ES CELLS

Enzyme Performance

Rating:	Rating:	Rating:
GOOD	MAYBE	BAD
Asp718	Eco RI	Bsp DI
BamHI		Cla I
Bgl II		Sac I
Eco RV		Sal I
Hind III		Xho I
Kpn I		Sac II
Pst I		Not I
Pvu II		Sma I
Sca I		Sme I
Stu I		Xba I
Nco I		

Expertise has shown that it can take some time to establish a suitable assay to confirm the genetic nature of a given clone.

In a second attempt to electroporate the vector, both neomycin and gancyclovir were used in the selection process. Double selection has been reported to increase the probability of finding targeted events by 2 - 20 fold. It is worth noting that a number of gene targeting groups do not favour double selection since there are concerns over decreased R1 cell pluripotency. Also in this second series of electroporations the 96 well rapid method was not used because of the problems outlined above.

Five electroporations were carried out on two alternative days. ES cell cycle rates are believed to effect gene targeting frequencies and as a result, the frequency of targeting of the same vector may vary if electroporations are carried out on different days (Bill Colledge, Personal communication). On Day 1, two electroporations were undertaken with 20µg of peripherin/rds Δ307 vector DNA and 7x 10⁶ R1 cells under conditions described in Materials and Methods.

On Day 2, three electroporations were undertaken using the same procedure, with 20µg, 30µg and 50µg of peripherin/rds Δ307 vector DNA respectively. Following both neomycin (300µg/ml) only, and double selection using neomycin and gancyclovir (2µM), 181 neomycin resistant colonies and 114 Neomycin (Neo) Gancyclovir (Ganc.)- resistant colonies were picked. Colonies were expanded in duplicate for freezing and DNA preparation. Following Southern analysis, signals were obtained from 96 and 32 neo^R and neo^R and neo^R / ganc^R colonies respectively (Fig.3.7). Thus, the vector was found to target well, with a frequency of 8 out of 32 Neomycin/Gancyclovir-resistant colonies having undergone the homologous recombination. Two clones out of 96 of the colonies selected with neomycin resistance alone also targeted, giving a frequency of targeting 1 in 48.

DNA from the following clones gave bands of 5kb and 4.2kb in size, indicative of targeted events, when digested with XbaI and Southern blotted.

N2E1	N3E3
N3B8	N4B4
N3C3	N4E11
N3D1	N5E1
N3D3	N3E2

Clones were then sequenced to assay for the presence of both the neomycin resistance gene and the Δ307 mutation. Two clones, N4E11 and N3D3, did not contain the Δ307 mutation but did have the neomycin resistance gene. (see fig. 3.8)

Discussion.

The peripherin/rds $\Delta 307$ vector targeted with high efficiency, with a frequency of 1 in 48 of the Neomycin-selected, and 2 in 7 of the doubly selected colonies. The efficiency of targeting may have been influenced by a number of factors. Our targeting vector was of the replacement Ω -type. Such vectors are superior to insertion-type vectors in their ability to replace existing sequence rather than to duplicate it. The length of homology between targeting vector and target locus certainly influences targeting frequencies (see introduction). The vector used here has approximately 2.5 kb of homologous sequence either side of the non-homologous neomycin resistance marker. The success in targeting of the $\Delta 307$ vector further demonstrates the utility of vectors with relatively short stretches of genomic homology. Some disagreement has arisen over the benefits of double selection using both neomycin and gancyclovir to enrich for targeted events, since the increased strain on the cell line may cause abnormalities in cell karyotypes. In the current experiments double selection increased targeting frequencies approximately-12 fold. The high frequency of targeting observed in these experiments may be partly due to use of the PGK-1 promoter and terminator in the neo^R cassette as described by Soriana *et al.* (1991) The neomycin cassette was inserted in an antisense orientation, since this is believed to enrich for the selection of targeted events (Skryabin, BV and Schmuss C, 1997). Interestingly, two of the clones containing the neomycin cassette did not contain the 1bp deletion. There is a distance of approximately 500bps of DNA homologous to the target site between the mutation and the neomycin cassette. It is therefore probable that homologous recombination occurred in the region between the deletion and the neo^R insertion. The two stable cell lines containing the neomycin cassette only, could be used to generate a germline mouse, which would assist in verifying the cause of phenotype observed in the case of insertion of the entire targeting vector. Although the neo^R cassette was inserted in the 3' untranslated region of the peripherin gene, it is possible that the insertion may effect the phenotype of the mouse. A way to overcome this problem would be to breed any mouse produced from these stable cell lines with a transgenic mouse expressing cre recombinase. Another possibility would be to induce recombination by transient expression of cre recombinase in the targeted embryonic stem cells. Transient gene integration markers can also be

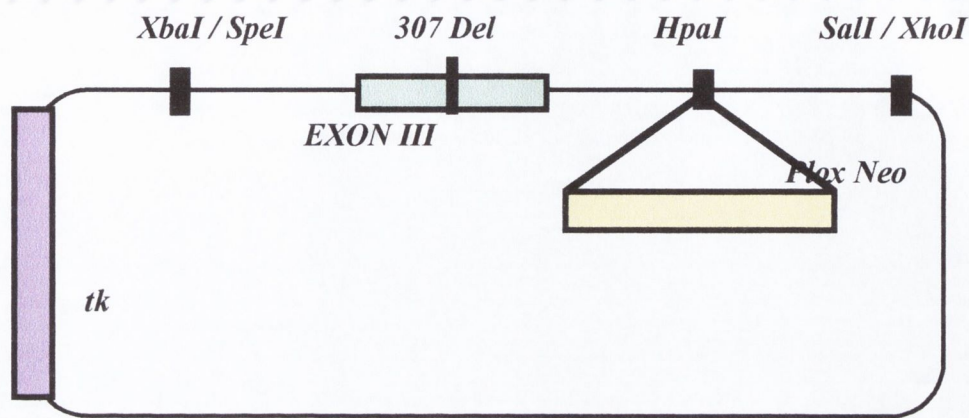
incorporated into the cre recombinase vectors, allowing identification of transfected colonies. Removal of the neomycin gene would require a new assay for mice or cell lines containing the 1 bp deletion. Fortunately the $\Delta 307$ mutation creates a new *Hae* II restriction enzyme digestion site, which would allow this distinction, as shown below.

Codons	305	306	307	308	309	310	311
Wild Type	GGC	TGG	CTG	CTG	GAG	AAG	AGC
	Gly	Trp	Leu	Leu	Glu	Ser	
1bp deletion	GGA	TGG	CGC	<u>TGG</u>	<u>AGA</u>	<u>AGA</u>	GC
	Gly	Trp	Arg	Trp	Arg	Arg	

The stable ES cell lines generated in this series of experiments, were further characterised in terms of karyotypic stability and subsequently used in the generation of chimeric mice (see Chapter 4).

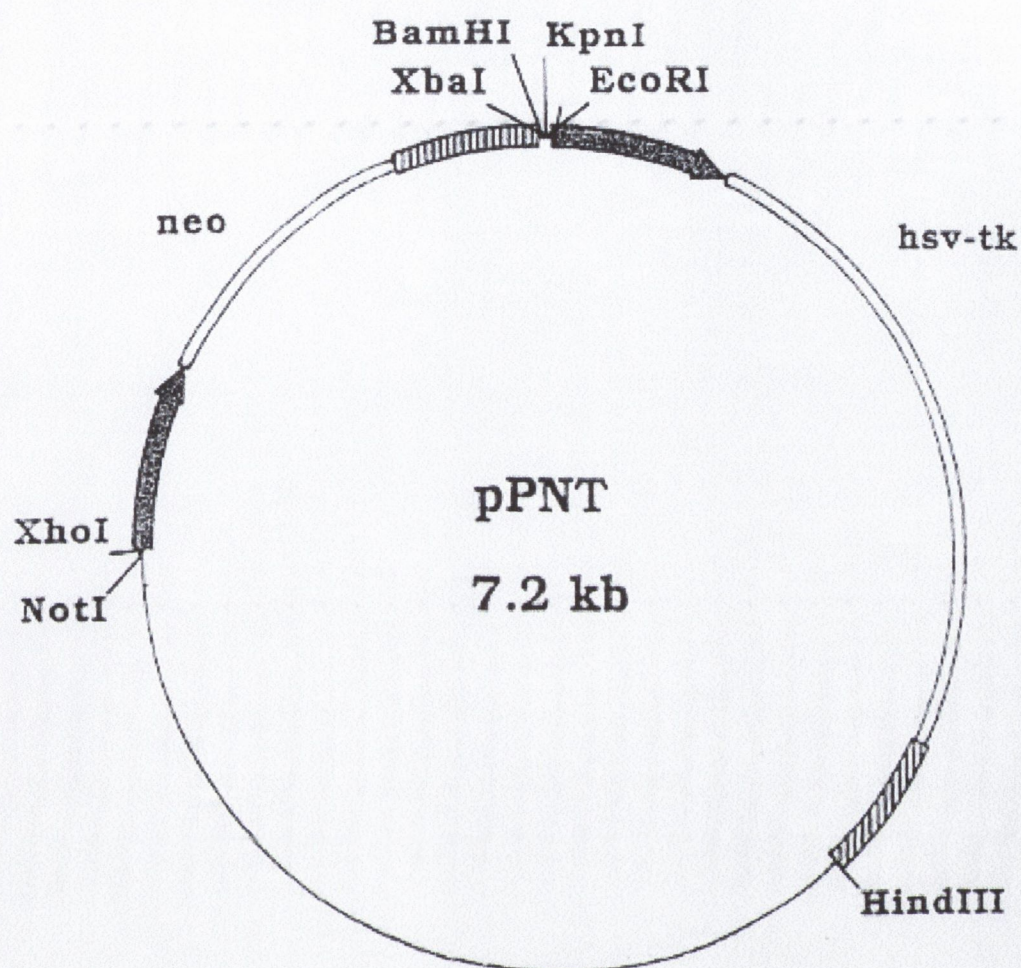
Figure 3.1.

pPNT RDS 307



A mouse genomic RDS clone of 5kb including exon III of the gene has been mutagenised and a 1bp deletion found in an adRP patient placed into exon III. The neomycin gene (plox Neo) enabling positive selection has been cloned into a *HpaI* site approximately 500bp away from the mutation. The construct is in pPNT, a vector containing the HSV thymidine kinase (*tk*) gene for negative selection.

Figure 3.2.



Structure of pPNT

The shaded arrows represent the PGK-1 promoter, the hatched boxes represent the PGK-1 poly (A) addition sequences, the open boxes are the neo and HSV-tk genes as labelled, and the line represents the plasmid backbone. Unique restriction sites are indicated. The precise nature of each of the fragments is described in Experimental Procedures.

Figure 3.3.
The *Plox P neo-1* vector kindly supplied by Dr. Nagy (Toronto).

Plox P neo - 1 (4965 bp)

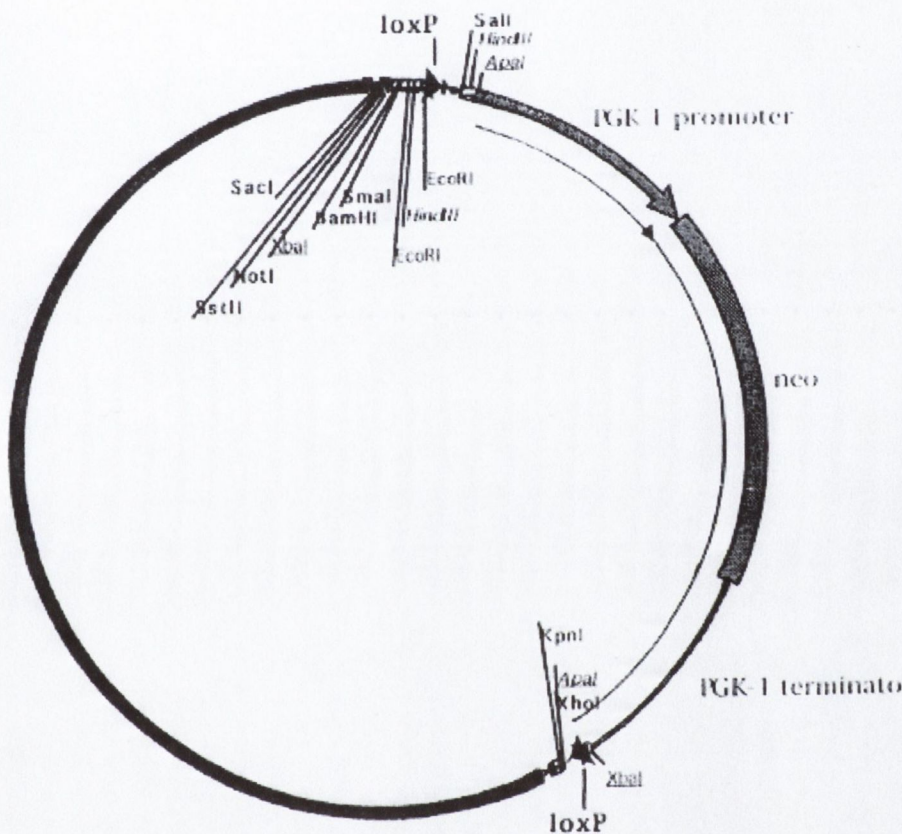


Figure.3.4

1	CCACCTCCTC	CTCTTCCTAC	TCCTCCTCCC	CCCTCCCGCT	CCTTCTCCTC
51	CTCACAAACAG	AGTATCTCTC	ACTGGCCTGG	GAATACACT	AGCTAGCCAG
101	TGAGTTCCAG	GAATCTATCT	GAGTCCCTAA	AGCCCCAGCT	GCCACATCAG
151	CAGATGGAAC	TCAGATCCTT	CTGTCTAGCT	CTTTACCAAG	AGGNCCTGTCT
201	CTTTAACCTT	TATTTATATA	TTTATCTCTG	GAGACAGCCT	TTTGTAAATCT
251	GAGCTGGCCC	AGAAGGCGCT	GTGTAGCTGA	GGGTAACNT	AAACTGATCC
301	TGCTGCCTCC	ACCTCCCGAG	AGTCTCGATT	GCAGGATGCT	CCAGCATGCC
351	CAGCCAAGCA	CACACACATC	TTAAGCGTGC	TATTGGAGGA	ATTATGTGNC
401	AGCCCTGGTA	CAGTGTGTGG	GGGCTGGAAG	GACCTTTNCA	CTGTAAANCT
451	TCCCTCTGGG	ACTGTGGATC	ACTGTGAAGG	CACAGCAGAC	CTTAAAGACA
501	CATGAATTAA	TACAGACATG	TGGGGTGGCT	GGGAGCCTTG	CCTGGTGGCT
551	CAGTGAGCAG	AGGACACAGC	CTTGCCCTTC	AAAGTCAGTA	AGGAGACTCT
601	GCTGTCACTG	TGTTTATAGA	GGTGAGGGCT	CTGCCCTGTCC	CCAGCCCCCA
651	TGCNCTGCCC	ATCCCCAGCC	TCCATNCCCT	GCCTCTGCAC	AGGCCCTGAG
701	CCACAGCAGA	CGTTGGTCAT	GAGTGATCTA	TGCAGGGGAG	AGATGACTGT
751	TGNAAGGGGT	GAGGGANTAA	ATACTGCCTC	CACCTTCTAG	AANANCCACT
801	CCAGGAAATA	GGGTNCTCTG	TCCTATATGT	AGTTCCTAGT	ACAGCGGCTG
851	NCATGTGGTG	GGCTGTATAT	GAATGNAAAT	CTAGGTTCCCT	CGGGGCATGG
901	GAGGATCTCC	TCCTTGGTGA	GATGTCCCGG	GAACCTGAGC	CAGNCTGTTT
951	CTCTTCCAGT	CGCATCAAGA	GCAACGTGGA	TGGGCGGTAC	CTGGTGGACG
1001	GCGTCCCTTT	CAGCTGCTGC	AACCCAGCT	CCCCGGGCTC	CTGTATCCAG
1051	TACCAGCTCA	CCAACAATC	GGCGCACTAC	AGCTATGACC	ATCAGACTGA
1101	GGAGCTCAAC	CTCTGNCTGC	GGGGCTGCAG	GGCCGCTCTG	CTGAATTACT
1151	ACAGCAGCCT	CATGAATTCC	ATGGGCGTCG	TCACACTTCT	CGTCTGGCTC
1201	TTTGAGGTAG	GCCACAGAG	GGCTGGGGAA	GGGAGCCTGC	CAGACTTGGG
1251	TTTAACATGG	TGAAGACAGA	CCTGGTGAGC	AAACCCTACA	GGGTCTCAGG
1301	TAATCTGGGC	TAGCCTTGAA	CTTGATAAGT	AGCCAAGGCT	AGCCAAGGCT
1351	AGCCCAGAAT	TCCTTATCCT	TCCCAGTACC	AGGAAAGAGG	TGTCCACCGC
1401	CATGCAGTTG	AGACATACTC	TCATTATGTT	<u>CACCATGCTA</u>	<u>CCCTTGAATT</u>
1451	CATGATCCTC	TGCCCTCAGT	TCCCCAAAAA	GCTGGGATCA	CAGATGTGCC
1501	ATCGTGTCTG	<u>GTGTCATTAC</u>	<u>TTACAAAAAA</u>	<u>GTATTTAGGT</u>	<u>AAAAATCAAC</u>
1551	AACAACANAA	AAATAGACTT	TNTCTTCCTN	TCCTTNCCTT	TTNTATTTNT
1601	GAAATAGGGT	CTNNCTATAT	AGAGAGCTGG	NTGCTCTGGG	<u>AACTCCCCAA</u>
1651	<u>GTAGGACCAG</u>	<u>GCTGGCCTCA</u>	<u>AACTCACAGA</u>	<u>TATCTGCCTG</u>	<u>CCCCCACCT</u>
1701	CCTAAATGCT	GGGATTAAAG	GCATGTNCAA	AATTAAGCCG	TTTATTATAT
1751	TCACAGTAGC	ACATATCCAC	CACCTCAACC	TAGTTCTAAA	ACATTTTATT
1801	CTCGCCCCC	ACCCCAAGAA	AACTTCACAC	CCACTAAACA	GTAACACTCA
1851	TTTGTAAGTG	TGCCCGCGTG	TGTACCTATT	GTGGACAAC	GGTATGCATG
1901	GACCAATGGG	AAGCTACACA	ACCTCTTGTG	CTTGACTTTT	CATTAGCCA
1951	GACCTTGGGG	TTCCCAGTAC	TTTCCCTGTN	TGTTTCTGAG	GCCTTGGATG
2001	GTTACGTAAA	CTGTTACACG	GGGGATGATG	GCATNACCTC	TGGGGGACTG
2051	CTGGGAGTCC	CTGTGTACGT	ATATGNTGGG	ANCTTCATT	AGGNAAGAGN
2101	GTCTAAATCG	ATTTGTACTT	TTGGAAGTCT	AGTTGGTGTT	TACATGCTAG
2151	TCTAATCTTA	TGTCATCTGT	AAGGCTCAAT	TCCCTTTTCT	GTAGTTTCTG
2201	GGAAGGATAC	GCTAACACAT	GGTGCTAGGA	GCTGTTCTAG	GTGCTGAACA
2251	GGCTACAAAC	<u>TAGT</u>			

2.1Kb of genomic mouse DNA sequence immediately flanking the targeting vector at the 5'end. It was necessary to sequence this region to establish a suitable Southern blot assay. The *Xba* I site starting at base 786 is underlined in blue.

The sequence used to design primers to produce a PCR product probe for Southern blotting are underlined in green.

The *Spe* I site starting at base 2107 is underlined in yellow.

The sequence with homology to a repetitive mouse Alu like sequence is underlined in red.

Figure. 3.5

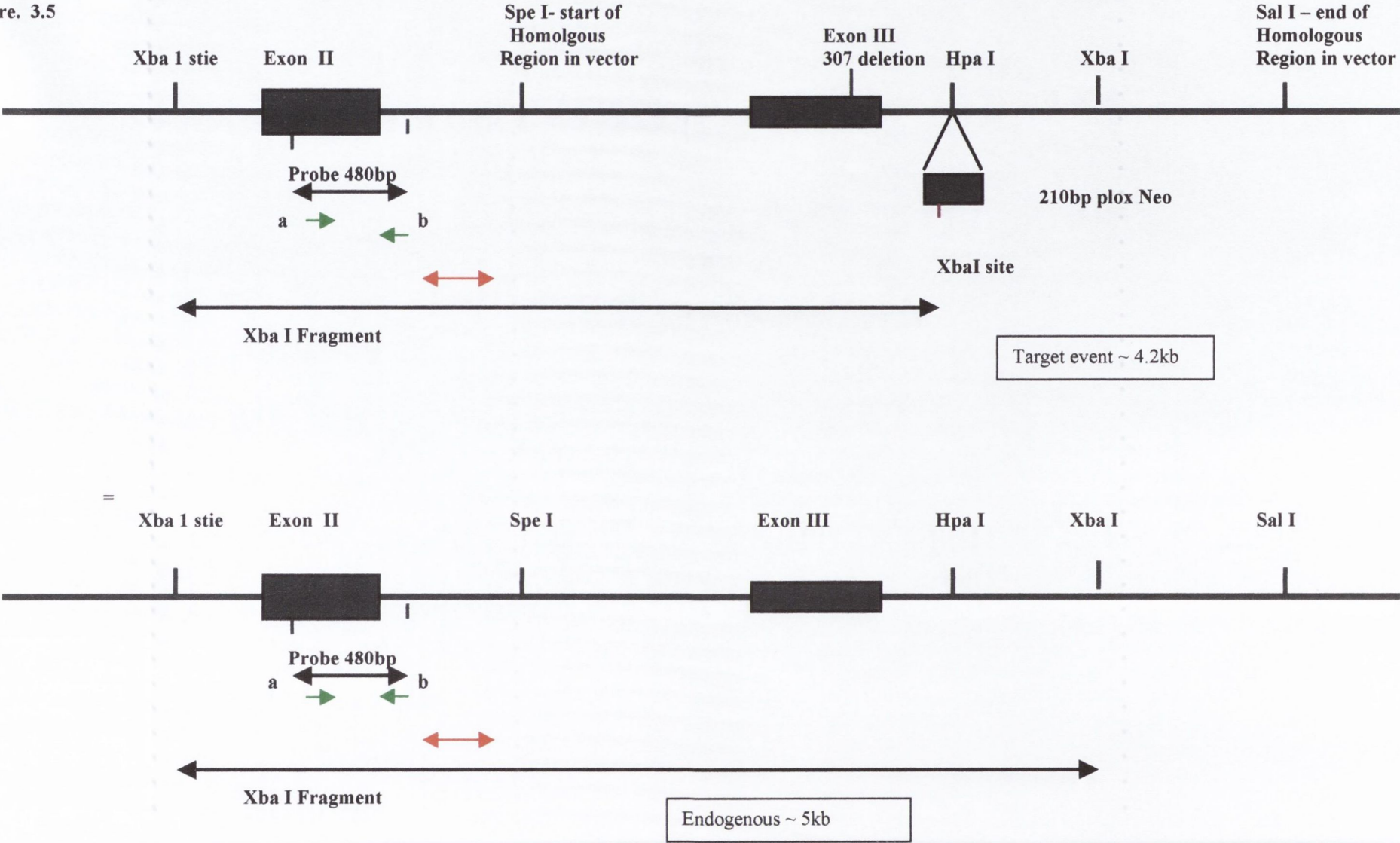


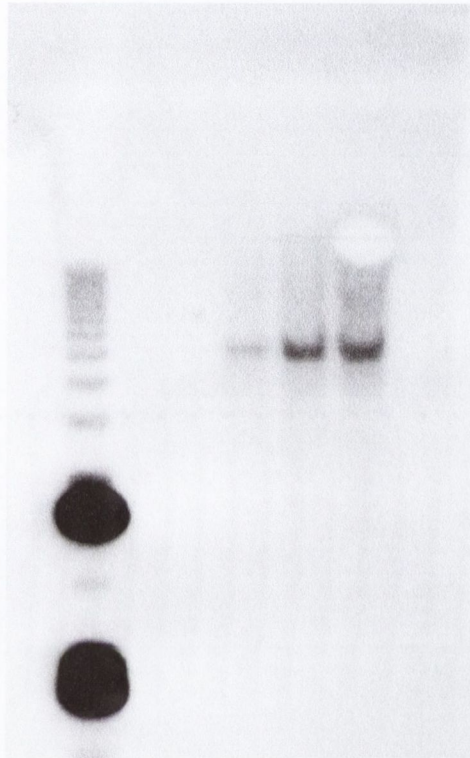
Fig. 3.5.

This figure is a restriction map illustrating a 1.8kb shift down in size of a *Xba* I fragment in the *rds-peripherin* gene as a result of gene targeting. The *plox* neo insertion into a *Hpa*I site and the $\Delta 307$ deletion in exon II in the targeted allele are shown.

The probe (probe 480) used for Southern blotting and the primers used to generate it are highlighted in green. An area of sequence with homology to a mouse *Alu* like repetitive sequence is underlined in red .

Figure. 3.6

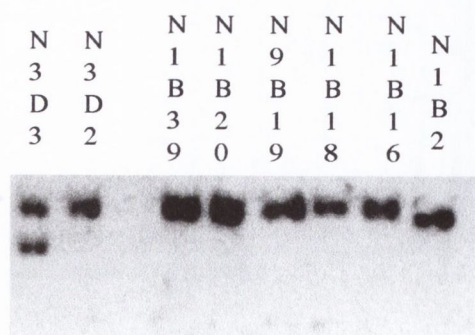
1 2 3 4



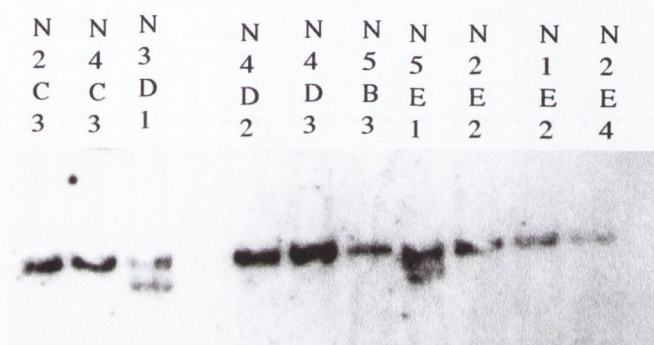
Southern analysis of ES clones by a PCR probe flanking the region to be targeted showing the endogenous band of 5Kb in ES cells. Lane 1 contains a 1Kb DNA ladder. Lanes 2, 3, 4 contain 10μg of *Xba* I digested RI DNA.

Figure 3.7

a



b



c

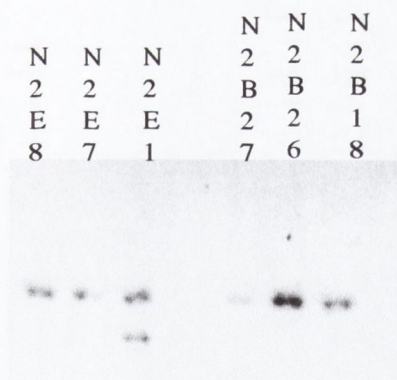


Figure 3.7d

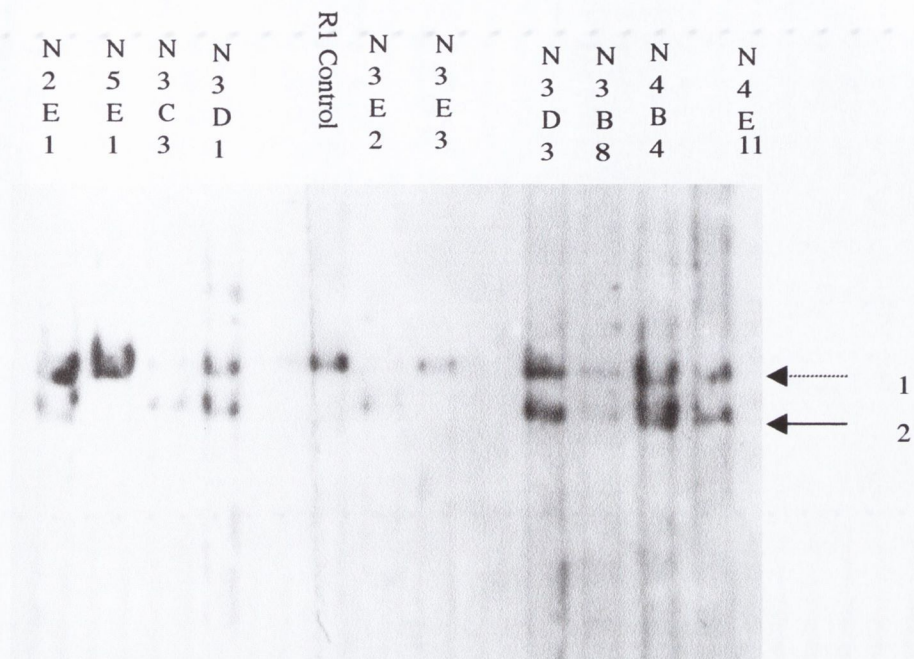


Figure 3.7.

Southern analysis of ES clones by a probe flanking the targeting vector showing diagnostic shift down of a *Xba* I restriction fragment from 5kb (1) to 4.2kb (2).

Each lane contained 10µg of *Xba* I digested R1 DNA from clones which survived drug selection, i.e contain the neomycin resistance gene.

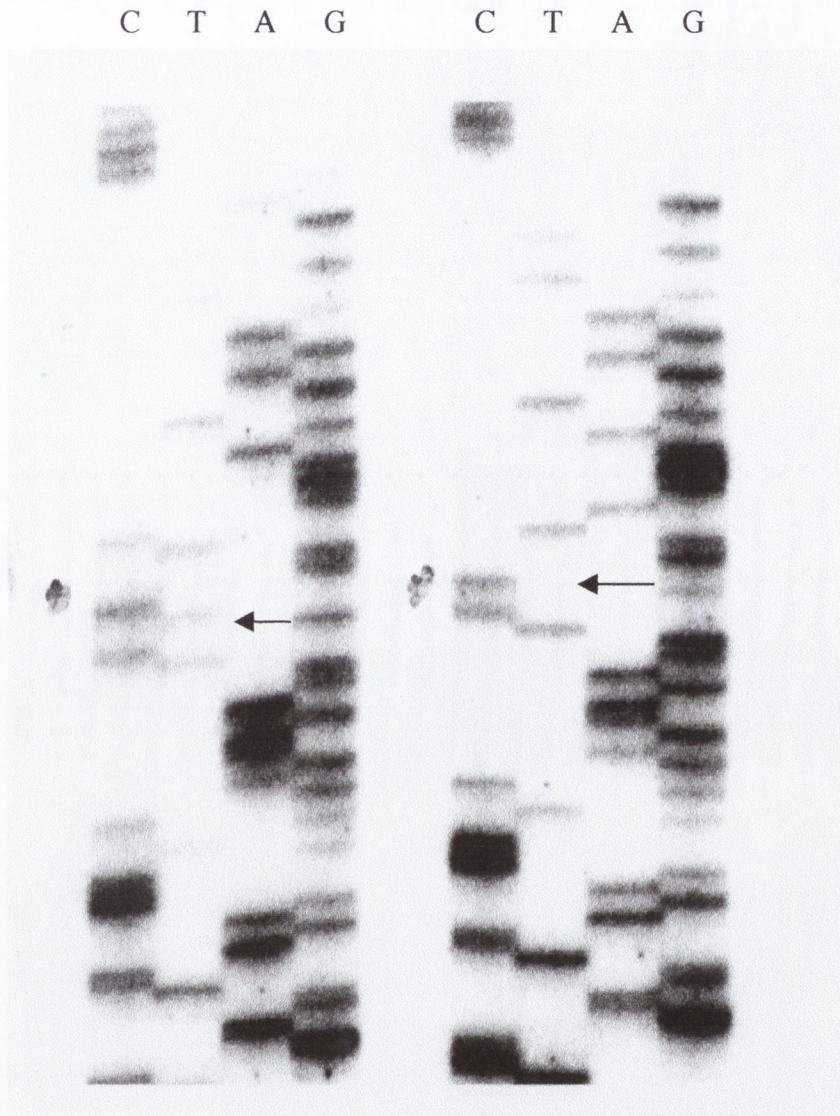
a, b and c represent the initial screening of the ES DNA.

Fig.3.7d is the Southern blot done to confirm the targeted event .

Figure 3.8.

Endogenous ES cell
rds/peripherin sequence

Targeted R1 cell
rds/peripherin sequence



In the targeted ES cells which contain the $\Delta 307$ deletion the sequence surrounding the mutation reads:

GGA TGG CGC TGG AGA AGA.

The deletion is highlighted with an arrow.

The cells without the $\Delta 307$ deletion contains the following sequence:

GGC TGG CTG CTG GAG AAG AGC.

The targeted sequence also contains a polymorphic change in the codon coding for glycine.

Chapter 4.
The generation of chimeric mice.

Introduction.

Three techniques are available for the production of ES Chimeras- blastocyst injection, morula injection, and morula aggregation. Initially we chose morula aggregation as our method of chimera production. This choice was based on literature demonstrating the efficiency of aggregation in the production of chimeras. A common problem in ES cell technology is the lack of capacity of a proportion of ES cells to contribute to the germ line. Low efficiency of germ line contribution by a given targeted cell line may be due to a loss of pluripotency or to karyotypic abnormalities. Therefore, it is advisable to karyotype ES cell lines on a regular basis in order to ensure the presence of the correct chromosome complement. Giemsa staining is usually sufficient to determine chromosome number, and to estimate the range of variability, if any, within the population of cells. A recent study by Longo *et al.* (1997) confirmed the importance of stable ES cell karyotypes in the generation of somatic and germ cell chimerism. A correlation was established between the percentage of chimerism of ES cell-embryo chimeras, the absolute numbers of chimeras and the percentage of euploid metaphases in the ES cell line used to produce chimeras. Euploid ES cells cultured for more than 20 passages tended to become aneuploid, and the ability of these cell lines to contribute to the germ line was lost if the percentage of euploid cells in the population fell below 50% (Longo *et al.* 1997). Passage number appears to be important in respect to karyotypic stability. Fedorov *et al.* (1997) tested CJ7, D3 and R1 ES cell lines of different passage number for the ability of these cells to contribute to the germline. They found the maximum germ line transmission passage number for all three cell lines to be passage 32 for targeted clones. It is interesting to note that ES cells with Trisomy 8 can generate chimeras, but rarely contribute to the germline. This may explain the low incidence of germline transmission obtained from some targeted cell lines (Liu *et al.* 1997.)

The first chimeric mouse generated as a result of mechanical aggregation of two pre-implantation cleaving embryos was produced in 1961 (Tarkowski *et al.* 1988). Genetic markers were used to distinguish the genetic contribution of the two embryos involved. In the early 1970's a number of experiments were undertaken in which attempts were made to produce chimeras containing tumor-derived teratocarcinoma cells (EC) (Papaioannou *et al.* 1975, Evans and Kaufman, 1981). While production of chimeras was achieved, the

frequency was extremely low owing to the unstable and differentiating nature of the cells and the unpredictable contribution of these cells to specific tissues.

In 1984 Bradley and colleagues investigated the potential of ES cells to form chimeric mice. Chimeric pups with a good ES cell contribution were obtained, and a high germline transmission was obtained in subsequent generations. In further experiments, ES cells were infected with retroviruses containing a neomycin resistance gene and cells were selected for G418 resistance (Robertson *et al.* 1986, Gossler *et al.* 1986). Such cells were presumed to have become G418-resistant as a result of integration of the retrovirus into genomic DNA. Resistant cells were fused with developing embryos and animals were obtained that transmitted G418 resistance to their offspring. Gossler *et al.* (1986) carried out similar experiments, this time introducing a neomycin-resistance gene into an ES cell line using the calcium chloride-DNA precipitation technique. Germline-competent chimeras were obtained. Specific mutations were introduced into the mouse germline for the first time in 1987. For example, Hooper *et al.* (1987) isolated spontaneous ES mutants for 6-TG resistance and successfully transferred an ES cell line containing a characterised deletion within the HPRT gene to the germline using the blastula injection technique.

As outlined above blastocyst injection, morula injection or morula aggregation can generate ES Chimeras. Methods for blastocyst and morula injection involve introduction of ES cells into pre-implantation embryos at different stages of development. The morula aggregation method is based on the principle that when ES cells are in contact with an 8 -cell embryo they can adhere to the embryo and eventually become internalised. Two methods of morula aggregation have been developed, so-called, “co-culture”, and “darning needle aggregation” (Wood *et al.* 1993, Nagy *et al.* 1993) Both involve removal of the zona pellucida surrounding the embryo, followed either by aggregation upon a lawn of ES cells (co-culture) or with a clump of ES cells in a depression in the base of a petri dish (darning needle aggregation) and co-incubation until ES cells and morulas fuse and develop to the blastocyst stage. The morula aggregation technique is technically simpler in comparison to injection methods. Micro-injection, while highly effective, requires months of practice in order to ensure efficient introduction of ES cells into the blastocoel. Skill is also required in the production of high quality holding and injection needles. Morula aggregation, does, however present a

number of challenges. Owing to the fact that ES cells are introduced into embryos at an earlier stage of development, chimeras tend to be born either with higher, or lower levels of chimerism than those obtained by blastocyst micro-injection. While high level chimeras increase the probability of colonisation the germline, if the contribution of the ES cells is too high the result can be abnormal development and re-absorption of mid gestation embryos. It is believed that both completely, and largely ES cell-derived embryos may suffer from abnormalities owing to loss of some pluripotency in the cell line and hence a reduction in the ability of the embryo to develop extra-embryonic membranes. Limiting the numbers of ES cells introduced into morulas to 5-10, rather than the 10-15, usually used for blastocyst injection usually address this. The media used in both techniques favours growth of the embryo in order to slow down the growth of ES cells. This prevents overgrowth of the ES cells, and hence problems associated with high levels of ES cell contribution.

Following ES cell incorporation by any of the above methods, chimeric blastocysts are injected into the uterus of a pseudopregnant foster mouse. The production of chimeras by incorporation of ES cells is not a straight forward procedure and varying levels of success have been achieved. For example, Wood *et al.* (1993) reported frequencies for generation of germline-competent chimeras of approximately 5% of blastocysts into which ES cells have been transferred. The majority of groups using blastocyst injection methods have achieved similar frequencies. However germline transmission rates of up to 33% have been reported by some experienced researchers (McMahon *et al.* 1990). Frequencies of blastocyst implantation, and hence birth rates, have been shown to be dependent upon a number of other variables, including ES cell line pluripotency, karyotypic normality and the choice of mouse strain for both donor and foster mothers. Techniques for introduction of ES cells into the germline have become very much more sophisticated owing to a greater understanding of both the properties of ES cells and the mechanisms of embryo development. As mentioned previously, ES cells are capable of contributing to all tissues of a developing embryo. However their contribution to extra embryonic membranes has been shown to be more limited. Nagy *et al.* (1993), have described a way to exploit such properties by using tetraploid embryos to create offspring which are completely ES cell derived. Tetraploid embryos rarely develop to healthy pups, yet are capable of

contributing to the extra-embryonic tissues, thus presenting a perfect partner for ES cell aggregations. Offspring were generated by combining normal embryonic cells with tetraploid morulas. The resultant embryos were completely ES derived, while the embryonic membranes remained tetraploid. Several different methods have been used to produce tetraploid mouse embryos. These techniques involve the use of agents such as colchine or cytochalasin B, which inhibit cytokinesis and induce fusion of normal diploid embryonic cells (Snow, 1973.). Fusion can also be mediated by agents such as Sendai virus and polyethylene glycol or by electrofusion techniques (O'Neill *et al.* 1990, Eglitis, 1980). Germline transmission of genetically manipulated ES cells following incorporation into a normal developing embryo is not easy. The production of completely ES cell derived foetuses according to the above techniques may help to alleviate or overcome this problem.

The choice of embryo donor is of particular importance since both the ES cell line and the donor embryo will compete for contribution to the chimera. A high percentage contribution of ES cells to chimeras is necessary to increase the probability of germline transmission. Schwartzberg *et al.* (1989) have reported substantial variance between mouse strains in their ability to contribute to the germline when combined with ES cells. Such variability can result in a competitive advantage either for ES cells or host blastocysts (morulas) in embryo development. For example, ES cells derived from 129/J or 129/SV strains of mice have been shown to predominate in chimeras where the host embryo was of the C57 BL/6J strain (see Wood *et al.* 1993.)

In order to recognize chimeras by coat colour it is necessary to introduce 129-derived ES cells onto an albino or non-agouti mouse strain (see Beddington *et al.* 1989) C57BL/6J inbred mice are widely used as a source of donor embryos due to the difference between this strain and the 129 in coat colour and other genetic markers. The C57BL/6J inbred mouse line is well proven as a compatible host for 129 ES cells in leading to chimera formation and germline transmission. Random-bred mice do not offer the same number of genetic markers to allow identification of the ES cell contribution to chimeras, and have been shown when used by a number of groups, to result in poor chimeras with minimal contribution to the germline. (Joyner 1993). Inbred strains of mouse, such as CD-1, do however, display good maternal behaviour in comparison to other inbred strains and their good

reproductive performance results in the availability of a larger number of blastocysts suitable for manipulation. Genetically altered lines derived from R1 cells have been reported to give a high efficiency of germline transmission when injected into C57 blastocysts or when aggregated with CD-1 or ICR-outbred, 8 cell stage embryos. However as mentioned previously, high quality ES cells are required for aggregations, hence a low passage number of R1 cells is believed to be optimal (Nagy *et al.* 1993).

In respect to characterisation of chimeras, early experiments relied on assessment of coat colour, based on the assumption that if coat colour was 50 - 60% ES-derived, the contribution to the germline would be of a similar level (Tarkowski *et al.* 1977). While pigmentation methods including eye colour are still used, newer methods have now been developed to detect chimerism. Methods such as isozyme difference between strains allows the determination of ES contribution at any stage of development (Robertson, 1986). A substantial number of knockouts result in embryo lethality. A method based on coat colour would be useless in such circumstances. Glucose phosphate isomerase is most commonly used in such situations owing to the fact that its three structural variants are easily distinguishable by their electrophoretic mobilities (Mann *et al.* 1993). Cell markers have also been devised which involve prior incorporation of genomic DNA into ES cells, such as the integration of a mouse β globin gene at high copy number into either the ES cell line or the donor embryo, or the incorporation of the gene encoding the β galactosidase enzyme (Lo, 1986, Friedrich and Soriano. 1991). The DNA can then be identified by *in-situ* hybridization or appropriate staining procedures.

To assay for germline chimerism, test breeding with a suitable mouse strain is required. A number of genotypes generated by gene targeting have been found to express different phenotypes on different inbred mouse backgrounds (Lyon and Searle, 1990). Breeding schemes to alter genetic background require seven or eight generations of back-crossing to mice of a given inbred strain. Such back crossing results in approximately 99% homozygosity for loci unlinked to the mutant allele. After ten generations, mice are considered co-isogenic (Lyon and Searle, 1990).

The majority of available ES cell lines are of male origin. The use of male-derived ES cells in the production of chimeras results in a sex bias towards male offspring. The conversion of a developing female embryo to a male phenotype by the inclusion of XY ES cells was first reported by Iannaccone *et al* (1985). The conversion of female blastocysts is advantageous since XX germline cells cannot undergo spermatogenesis and only ES cell-derived genes are passed to the next generation. It is of interest however that transmission of the male-derived ES cell genotype has been reported to occur from female chimeras (Kuehn *et al*. 1987). This is believed to be due to the loss of the Y chromosome, resulting in XO cells capable of undergoing oogenesis.

Materials and methods.

Preparation of mice for embryo flushing.

On day one, 15 female mice (CD1, HARLAN UK.), 4-6 weeks old were intraperitoneally injected with 10IU pregnant mare serum gonadotropin (FOLLIGON 1500IU with solvent. INTERVET) in order to begin superovulation.

Two days later, the mice were injected intraperitoneally with 10IU human chorionic gonadotrophin (CHORULON 15000IU with solvent. INTERVET) and subsequently mated with one stud male each. Although a stud male is capable of plugging more than one female a night, a second plugging would contain a limited quantity of sperm resulting in a lower yield of morulas. The following day donor plugs were checked before 10AM, as they fall out in the early afternoon. Mating was indicated by the presence of a white or yellow vaginal plug composed of male secretions. Plugs were either loose and clearly visible or sealed the vagina shut. The plugs were checked both visually and by insertion of a probe (Spatula, Forceps) to check for blockage as plugs are harder than surrounding tissue and can be positioned deep inside the vagina.

Sixteen 10 week old females (CD1) were mated with vasectomized males to act as pseudopregnant recipients. These females were checked for oestrus prior to mating. External vaginal epithelial characteristics were used to select females for mating. A female mouse in oestrus possesses a dry, swollen, wrinkled vagina, which is pink in colour. Two females were placed in each vasectomized male cage. The following day the potential pseudopregnant females were checked for plugs.

Morula flushing and aggregation.

Our Male CD1 mice were vasectomized by Caroline Woods (Bioresources, Trinity College Dublin).

The mated superovulated females were killed by cervical dislocation on day 2.5. The oviducts were dissected out and placed in drops of M2 medium in a petri dish (see fig.4.1 for dissection procedure). Under a light microscope (NIKON SMZ-2B) the fimbrial end of the oviduct was located and a 30-gauge needle attached to a 1ml syringe filled with M2 was inserted. The needle was held in position with a No.5 forceps and a 0.05mls of medium was flushed through. The morulas were collected with a mouth pipette attached to a drawn out Pasteur pipette and

transferred into an organ culture dish containing medium M16 and placed into the incubator at 37°C, 5% CO₂ in air.

Aggregation plate.

Assembly and overnight culture of aggregates took place in an aggregation plate. Four rows of M16 microdrops, three drops in the first and fourth rows and five drops in the second and third rows were made with an M16 filled syringe in a 35mm tissue culture dish. Six small depressions were made in each drop of M16 with a darning needle (DN-09, BLS HUNGARY). The zona pellucida of the embryos was removed by washing through drops of acid tyrodes solution. Dropping two embryos into each well or one to two embryos and 5 -10 R1 cells assembled aggregation sandwiches. To obtain R1 cells in clumps suitable for aggregation the trypsinization process which is normally 5mins was reduced and the cells were only left in trypsin/EDTA until they formed clumps of loosely connected cells. Aggregates formed compact morula or early blastocysts after overnight culture.

Embryo transfer to the uterus.

The pseudopregnant female was weighed and anesthetized with avertin at 0.2ml per 10g body weight. The embryo transfer pipette was connected to a mouth pipette and used to take up a column of air, medium, a small air bubble and the embryos to be transferred. The transfer pipette was made by drawing out a micropipette over a Bunsen flame. The mouse was prepared for transfer by swabbing with alcohol and making a 1cm transverse incision in the skin at the level of the first lumbar vertebra (see fig. 4.2.) Excess hair was rubbed away with an alcohol pad. The incision was moved laterally until the left or right ovarian fat pad and ovary were visible. A small incision was made through the peritoneal wall and the fat pad pulled out using a blunt forceps. The ovary was stabilized outside the peritoneum using a serrefine clip. The uterus was held lightly with a blunt forceps and an opening made using a 21-gauge needle, taking care to avoid blood vessels under an operating microscope (WILD WEERBURG M650). The pipette tip was inserted into the uterus through the hole made by the needle. The embryos were blown into the uterus along with the small air bubble behind them. The transfer pipette was then checked to ensure that no embryos remained in the pipette. The tract was replaced with care

into the peritoneum and suturing closed the skin incision. The weight of the mice was recorded every day following transfer for 10 days.

Analysis of implantation success.

If a gain of weight was followed by a rapid loss in the ten days following uterine transfer the female mice were sacrificed by cervical dislocation. The uterus was dissected out and examined for developing fetuses. If present, the foetus was dissected away from the uterine tissue and analysed under the inverted microscope. The identification of a beating heart was used to distinguish if the foetus was still living.

Recovery of blastocysts.

The mated superovulated females were killed by cervical dislocation on day four. The uterus was dissected out, and placed in a dry sterile Petri dish. The cervix was cut from the uterus to expose the entrances to both uterine horns. 0.5 mls of M2 medium was flushed through each horn by inserting a 30 gauge needle attached to a 1ml syringe into the cervical end of the horn. The blastocysts were collected with a mouth pipette attached to a drawn out Pasteur pipette and transferred into an organ culture dish containing medium M16 and placed into the incubator at 37°C, 5% CO₂ in air.

Preparation of cells for microinjection.

ES cells in exponential growth phase were fed one hour prior to microinjection. The cells were then trypsinized and stored as a single cell suspension in M16 medium.

Blastocyst injection with targeted ES cell lines was carried out by Brenda Lee Carson and Jeannine Turnbull following the Robertson procedure (Robertson , 1987)

Uterus transfer was carried out as described previously.

Karyotype analysis.

Slide preparation.

Slides (Corning microslides , Single frosted 0.96 - 1.06mm, 75 x 25mm) were cleaned by leaving overnight in a mixture of alcohol and concentrated HCL (1:1). The following day the slides were washed in running tap water and rinsed in distilled water, before storing in distilled water.

Preparation of mitotic spreads.

ES cells were passaged and grown in 6cm plates for 2 days. On day 2 Colcemid (Sigma) was added to the medium at a final concentration of 0.05 μ g/ml. The cells were incubated at 37°C for 1hr before trypsinization and resuspension in 10mls of 0.56% (w/v) KCl solution for 6mins. The cells were pelleted by centrifugation (800rpm, 5min) and the KCl solution was aspirated off. Cold freshly prepared Carnoy's fixative (3:1 volumes of absolute methanol to glacial acetic acid) was added to the cells and the cells resuspended. The cells were pelleted by centrifugation and fresh fixative added twice before the final centrifugation and resuspension in 1ml of Carnoy's fixative. A slide was removed from the distilled water with a forceps and placed on tissue paper to absorb excess fluid. A Pasteur pipette was used to deliver three drops of cell suspension onto the slide. The slide was quickly placed upside down on a piece of tissue paper and the back dried with a Kim wipe. The drops were allowed to dry until interference rings were visible at their periphery, and then blown on. The slides were placed on a 37°C hot plate overnight.

Staining.

The slides were stained by placing in Giemsa (Gurr BDH) for 3 minutes, washing in 0.005M Hepes and rinsing in distilled water.

Photography of karyotypes.

The karyotype spreads were photographed using a Leica Laborlux S microscope and Leica (Wild MSP 48.52 Photoautomat) camera. The Kodak slide film ASA 100.

Results.

We initially chose the “darning needle” morula aggregation technique to produce chimeric mice. Preliminary experiments were carried out by fusing black C57 and white LACA mouse embryos at the eight cell morula stage to produce viable chimeric pups (see Fig 4.3). Although our embryo culture and morula aggregation techniques proved successful, our success rate on pregnancies following uterine transfer was low (1/20; see fig. 4.4). To optimize our implantation frequencies we began implantation of unfused or modified CD1/ICR blastocysts into the uterine horn of CD1/ICR pseudopregnant females. CD1 mice were used owing to their suitability for aggregation experiments (see Nagy *et al.* 1993). When weight gain was followed by a rapid loss in weight, animals were sacrificed in order to determine whether perinatal embryonic death had taken place (see figure 4.5) Perinatal embryonic death occurred most frequently in the early stages of these experiments. Our developing experience in surgical techniques resulted in higher frequencies of live births, three in four transfers yielding live pups. By conducting implantation experiments on a regular basis a number of factors which appeared to influence implantation were identified (see Table 4.1.) For example, we found that pseudopregnant females of weights varying between 25 - 31g were the best recipients of blastocysts. Recipient foster mice 2.5 days post coitus were found to be at their most receptive to incoming blastocysts. These observations can probably be explained by the fact that there is a requirement for transferred embryos to re-synchronise with the recovering uterus following microsurgery. Perinatal death was observed in females which were 3.5 days post coitus and no implantations were observed in 1.5 days p.c. females. (Initial experiments were carried out using M2 medium and a drawn out Pasteur pipette to transfer the blastocysts to the uterus. Later, DMEM-supplemented transfer medium was used) As a result of the above, we succeeded in producing an R1 cell derived chimera by morula aggregation. The coat colour of the pup was approximately 90% brown/black, signifying a large ES cell contribution (see fig.4.6). The R1 cell line is male derived and unfortunately the sex of our chimera was female and the ES cells in the mouse did not contribute to the germline. The fact that the R1 cells did contribute to the developing embryo and resulted in a live birth and a healthy mouse indicated that our tissue culture techniques were of high quality. Since these techniques were being worked up for the first time in Ireland, we regarded this

result as a milestone in the overall development of our capacity to generate mice by targeted gene modification.

Reports have been made of female chimeras transmitting the male ES cell genotype to the next generation (Kuehn *et al.*, 1987). As mentioned previously, this is thought to be a result of the loss of part, or all of the Y chromosome, permitting the cells to form ova. This phenomenon would not be expected to occur with high frequency. Furthermore, a female chimera will obviously parent pups with a much lower frequency than is possible for a male, making it difficult to assess the germline contribution of the ES cells in such animals. It is quite likely that our chimera was a somatic, as opposed to a germline chimera, since a high contribution of R1 cells to the chimera should influence the tissue which determines the sex of the pup causing a sex conversion.

As a result of the research reported in the previous chapter, 10 embryonic stem lines showing targeted modification of the peripherin/rds gene locus were identified. However, before proceeding further, it was critically important to ensure that the karyotypes of these targeted cell lines were sufficiently stable to permit their ongoing use in the generation of mouse chimeras. Thus, a systematic analysis of chromosomal structure was undertaken in the cell lines (see Table 4.2, Fig. 4.7).

The normal mouse mitotic karyotype contains 40 acrocentric chromosomes. Upon karyotype analysis of a number of our clones, both aneuploidy and the presence of a metacentric chromosome were observed. The metacentric chromosome may have been generated by the fusion of two chromosomes. Care must obviously be taken to differentiate a metacentric chromosome from two chromosomes that are simply adjacent to one another on the spread. Two of our clones, N3D1 and N2E1 contained a reasonably pure population of cells with 40 acrocentric chromosomes. In a few of the spreads, 38 or 39 acrocentric chromosomes were counted. Such loss may in fact have been due to spreading of the chromosomes when dropped onto the slides. Hence, further karyotype analysis was undertaken on these cell lines in order to verify our results. A second count of the karyotype of 20 random cells from lines N3D1 and N2E1 as shown in table 4.3 was carried out. Again, the bulk of the spreads examined were karyotypically normal. Cell lines, which contained the neomycin insertion only and did not possess the $\Delta 307$ mutation were also karyotyped since it would be useful to

generate a mouse model carrying the neo^R insertion only. If a retinopathy were observed in mice, carrying only the neo^R insert, it would in principle be possible to remove the neomycin resistance gene by breeding these mice onto a transgenic strain expressing cre recombinase, thus hopefully excising the neo^R cassette. Cell line N3D3 contained a high percentage of cells (16/20) with a karyotype of 40 acrocentric chromosomes. Two of the cells in the population appeared to contain 38 acrocentrics and one metacentric chromosome, and two other cells contained 39 acrocentrics. As mentioned previously, stable cell lines with half the total number of cells showing abnormal karyotypes have been shown to go germline. Thus cell line N3D3 and N3D1 appeared sufficiently stable to warrant their use in chimera production. The frequent occurrence of aneuploidy and the appearance of metaphase chromosomes might have been a result of the high passage number of the targeted cell lines. At this stage in the work up the passage number of these was approximately 20. Numerous studies of the effect of passaging on karyotypic abnormalities have been reported (see introduction). For example, Longo *et al.* (1997) reported that cells passaged twenty times or more started to lose their euploid karyotype and needed to be investigated for abnormalities. However, cells of high passage number and cell populations containing a large number of normal karyotypes routinely went germline.

Eighty morula aggregations were carried out with the cell line N3D1 and six blastocysts were transferred to eleven pseudopregnant females the following day. Four pups were born from these transfers, but none of these were chimeras. The success of morula aggregation is highly dependent on the quality of the ES cell lines owing to the high contribution made by the ES cells to the development of the embryo from the early morula stage. In the case of completely ES-derived embryos, i.e. those produced by tetraploid embryo aggregation, no live offspring can be obtained with cells older than passage 14. Such cells can however, go on to create healthy germline chimeras and ultimately produce a successful mouse lines when the blastocyst-injection method is used. For these reasons we decided to attempt to introduce our R1 targeted cell lines into the germline by blastocyst injection.

The N3D1 cell line was microinjected into CD1 blastocysts. These injections resulted in the birth of three live pups. One of these pups was a female chimera with approximately 5 -10% ES cell contribution to coat colour (see fig.4.8). We bred this

female chimera with C57 males. The chances of this female chimera contributing to the germline were however low for the reasons mentioned previously and the low percentage of ES cell contribution to coat colour was not encouraging. No germline offspring resulted from these crosses. There is some dispute about the suitability of outbred mouse strains such as CD1 to act as donor blastocysts for ES cell microinjection. The colouring of our CD1/N3D1 chimera is similar to that observed in a number of aggregation experiments (personal communication with Dr. D. Rancourt). A number of research groups have reported the use of outbred mice blastocysts to result in poor chimeras with minimal contribution to the germline which may explain this observation (Joyner, 1993). With this information in mind inbred C57 mice were used as our donors for the next set of microinjections. In this set of experiments the N2E1 and N3D1 cell line were microinjected into C57 blastocysts. These injections resulted in the birth of 86 live pups, 21 of which were chimeras (see fig.4.9). The ES cell contribution to the coat colour of the chimeras varied from 5-90%. Ten of the chimeras displayed a substantial contribution of ES cells to coat colour (50 – 90%) and were subsequently test bred to ascertain contribution of the ES cell to the germline. To date two out of the 21 chimeras have been shown to contain ES cell derived germ cells, which have successfully contributed to the following generation. At the time of submission of this thesis, 9 offspring from these germline chimeras were shown by PCR to contain the targeting vector. Microinjection of the ES cell line N3D3 into C57 blastocysts has also been carried out. The N3D3 cell line carries the neomycin resistance gene, but not the $\Delta 307$ mutation. Four chimeras have been produced, three male and one female. The ES cell contribution to coat colour is low in all the chimeras (under 50%), injections are therefore ongoing to produce a chimera which will produce pups containing the N3D3 genotype.

Conclusion.

Now that two germline chimeras have been identified, the first priority is to obtain and maintain the targeted allele in a living animal colony. Analysis of the phenotype of the mice will be carried out by electroretinogram, immunocytochemistry and histopathology studies similar to those used on the rhodopsin knockout mouse and the transgenic rescue of this phenotype (see Chapter 1 & 5).

It will be of interest to see if the disease symptoms are similar to those observed in man. The onset of retinal degeneration is in the fourth decade in humans and the phenotype is quite mild in comparison to other cases of retinitis pigmentosa. As yet we do not have a germline chimera which contains the neomycin resistance gene and does not contain the deletion. It is quite likely that the incorporation of the neomycin gene in the 3' untranslated region will play no part in altering the phenotype of the mouse. Various other strategies, such as cross breeding the targeted mouse with a transgenic mouse expressing cre recombinase are also under consideration to verify this assumption (see Chapter 3).

Our next priority will be to put the $\Delta 307$ peripherin/rds allele onto a number of genetic backgrounds. The cystic fibrosis transmembrane conductance regulator deficient mouse shows increased life spans on different inbred strains, suggesting a role for genetic background in the disease severity. To transfer our mutant allele we will backcross the mouse with the strain of interest for 10 - 12 generations and analyze the effect of the deletion on various genetic backgrounds (Grub *et al.* 1999). This analysis may prove interesting as was the case for the rds mouse. The phenotype of the rds^{-/-} mouse on both a pigmented and albino genetic background shows variations. Photoreceptor cell loss in the pigmented rds^{-/-} is slightly slower than in the albino rds^{-/-} mouse. This observation is probably due to the susceptibility of the albino strain to increased light damage (Schalken *et al.* 1990, Sanyal *et al.* 1986). Furthermore, in man a different diagnosis is often assigned to patients with the same rds mutation, this phenomena may also be visible in mice (Dryja and Li 1995).

Peripherin /rds has been shown by immunochemical studies to be localized to the rim region of the photoreceptor outer segment disk membrane and is thought to create the bend at the periphery of disk membranes or to anchor the

disks to the cytoskeleton (Shastry 1994, Bird 1995). The pathogenesis of peripherin mutations may be due to destabilization of the disk membrane, or insufficient transport as in the case of some rhodopsin mutations, the precise mechanism of pathogenesis is unknown. We intend to use our $\Delta 307$ rds mouse model to study the effects of mutant peripherin on transport of the protein from the nucleus to the outer segments.

Cellular expression of the mRNA for peripherin/rds was studied in rat retinas by *in situ* hybridization histochemistry with antisense and sense probes prepared from polymerase chain reaction amplified cDNA of the bovine peripherin/rds. Predominant mRNA signals were detected in the inner segments and distal region of the outer segment disc membranes of photoreceptor cells. (Yanagit 1993). *In situ* hybridization could also be used to locate mutated peripherin transcripts in our mouse model.

Fariss *et al.* (1997) investigated the transport pathways of both rhodopsin and peripherin in normal photoreceptors and degenerating photoreceptors due to retinal detachment. Laser scanning confocal microscopy and immunolocalisation studies were carried out. Both proteins were shown to normally target to the newly forming discs of the outer segment. Photoreceptor degeneration resulted in accumulation of the proteins in different cellular compartments. Peripherin immunolabelling located the protein to the cytoplasmic vesicles while opsin immunolabelled within the photoreceptor cells plasma membrane. These observations are indicative of two different transport pathways for the proteins. Farris *et al.* (1997) proposed protein transport of rhodopsin and peripherin occurs via two different post golgi transport vesicles and are separately inserted into the plasma membrane. It is also possible that the proteins are cotransported but rhodopsin finds its way into the plasma membrane and peripherin does not remaining with in the cytoplasmic vesicles. It is of interest that the peripherin/rds positive vesicles are located beside the striated rootlet, further studies are required to establish the role of this cytoskeletal element in peripherin transport. These studies demonstrate the usefulness of mutated animal models for the peripherin gene for analysis of the protein transport pathway, which is not yet fully understood (Li *et al.* 1996). Immunocytochemical studies have been carried out on the transgenic mouse carrying the dominant rhodopsin mutation P347S and have

demonstrated the defective vectorial transport. Such experiments encourage the analysis of our dominant $\Delta 307$ rds mutation.

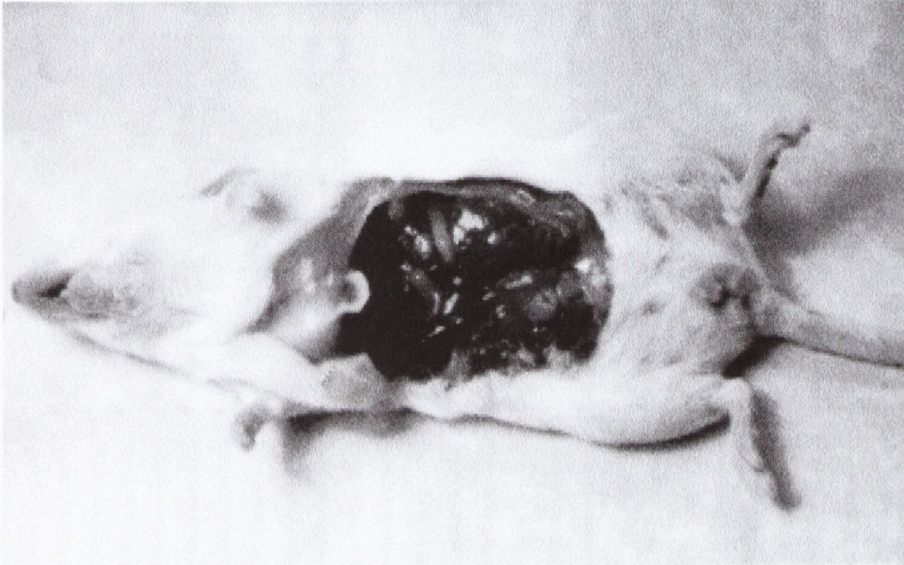
We will also investigate the capability of the mutated protein $\Delta 307$ to partially rescue the $\text{rds}^{-/-}$ phenotype in a hemizyous state when crossed onto the $\text{rds}^{-/-}$ background. We will compare this phenotype to that observed in the heterozygous $\Delta 307^{-/+}$ mouse (C57B/6/129-TgH [rdsT307] mouse). The 307 deletion is disease causing in man even in the presence of a normal allele. We will therefore need to establish if the mutation still has a dominant effect on the mouse genetic background. No outer segments are present in the $\text{rds}^{-/-}$ mouse however, in the heterozygous $\text{rds}^{-/+}$ mice abnormal outer segments of a reduced length are formed (Hawkins et al.1985). Photoreceptor degeneration does occur in the $\text{rds}^{+/+}$ starting later than observed in the $\text{rds}^{-/-}$ mice and progressing slower (Sanyal, 1986). The phagosomes in the RPE appear bigger in size and larger in number than normal mice. Furthermore the process of disk shedding and uptake of phagosomes appears to be different to that observed in a normal mouse. These observations demonstrate the effect of peripherin /rds haploinsufficiency. It would be of interest therefore to examine the phenotype of both $\Delta 307^{+/-}$ and $\Delta 307^{+/+}$. The P216L mutant peripherin/rds transgenic mouse was generated on wild type, heterozygous $\text{rds}^{-/+}$ and homozygous $\text{rds}^{-/\text{rds}^{-}}$ genetic backgrounds. Increased severity of photoreceptor degeneration outer segment dysplasia and shortened b-wave amplitudes were observed in $\text{rds}^{-/-}$ mice expressing the transgene, ERG decreases were observed in the $\text{rds}^{-/+}$ mouse expressing the transgene but not outer segment dysplasia. Kedzierski *et al.* (1997) believe the dominant effect caused by the combination of the two mutations displays a phenotype similar to that observed in patients with the P216L mutation and therefore represents a good animal model for the disease. Alleviations of the effects of dominant retinopathies presents a major challenge to the scientific community. Dominant animal models present a means to study developing antisense, antigene and ribozyme strategies for dominant suppression. We intend to use the $\Delta 307$ rds peripherin mouse to further the laboratories ongoing ribozyme studies. Dr.G.J Farrar, Sophia Millington Ward and Brian O'Neill have designed a number of ribozymes, which cleave sites within the mouse peripherin gene. It would be of great interest to explore the

cleavage ability of these ribozymes *in vivo* and the effect of this cleavage on the mouse phenotype. The introduction of these ribozymes could be accompanied by the delivery of a rds peripherin gene, which does not contain the ribozyme cleavage site, and could therefore in principal rescue the retinopathy. It may also be of interest to design a ribozyme to cleave the mutated targeted allele specifically. This may be possible due to polymorphic differences between the targeted and endogenous mouse sequence as well as the 1bp deletion (see Chapter 3)

Figure. 4.1

This figure depicts the method of dissection used for the recovery of morulas and blastocysts.

4.1(a); The outer skin and peritonium of the pregnant mouse is opened



4.1 (b); The uterus is lifted and each horn is freed by cutting the mesentry and finally cutting at the utero-tubal junction.

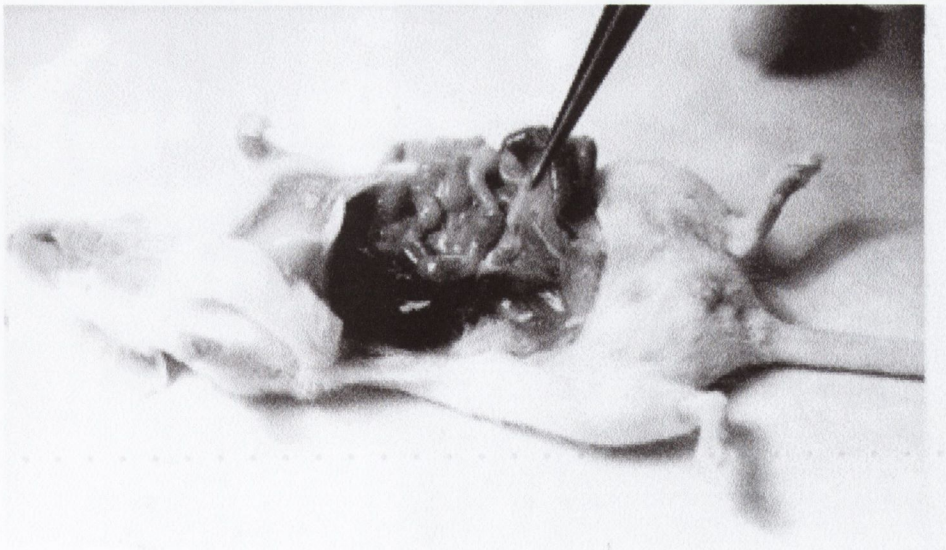
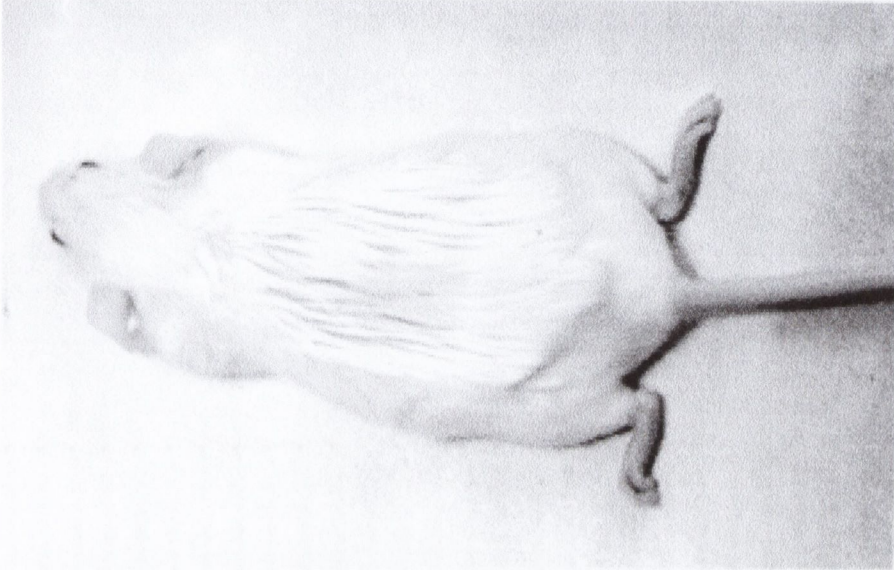


Figure. 4.2

Figure 4.2 depicts the process of embryo transfer to a pseudopregnant mouse.

(a) An anesthetized mouse prepared for transfer.



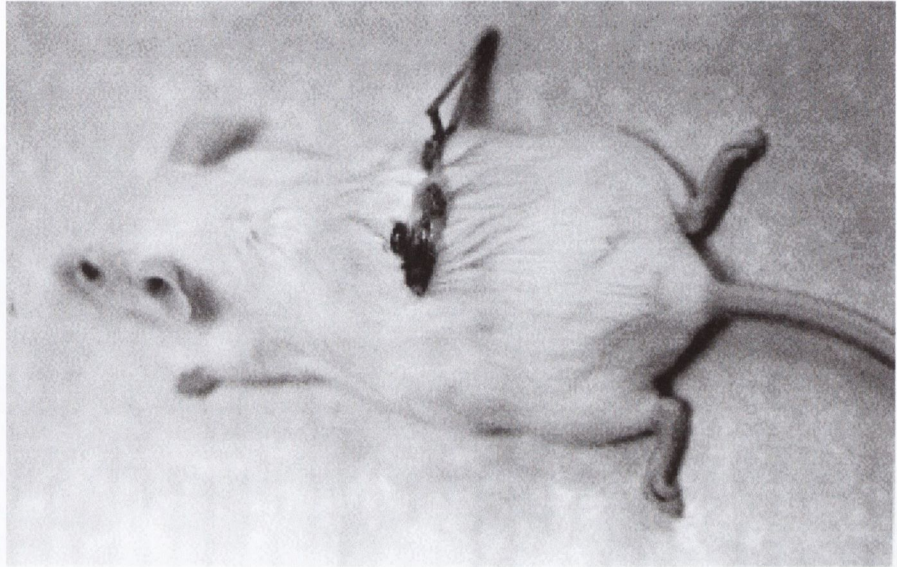
(b) A dorsal incision is made to allow access to both the left and right uterine horn.



Figure. 4. 2

(C)

The left ovary and uterus are secured with a serrefine clip.



(d). A hole is made in the uterus for embryo transfer.



Figure. 4.2 (d).
Transfer of embryos into the uterine horn.

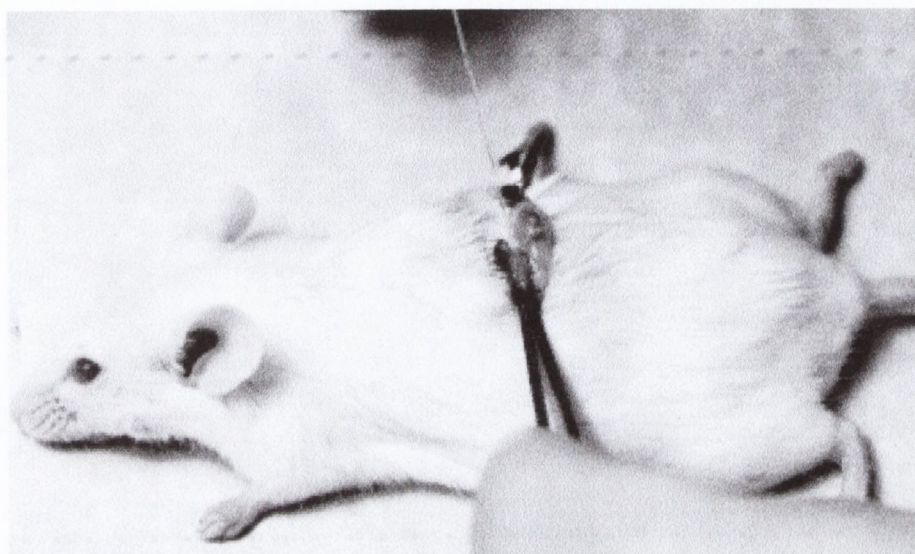


Figure 4.3.
Viable chimeric pups generated by the fusion of a black C57 and white LACA mouse embryos.



Figure 4.4
Morula Fusion

Fig. 4.4a Two morulas and a clump of ES cells in a needle depression.

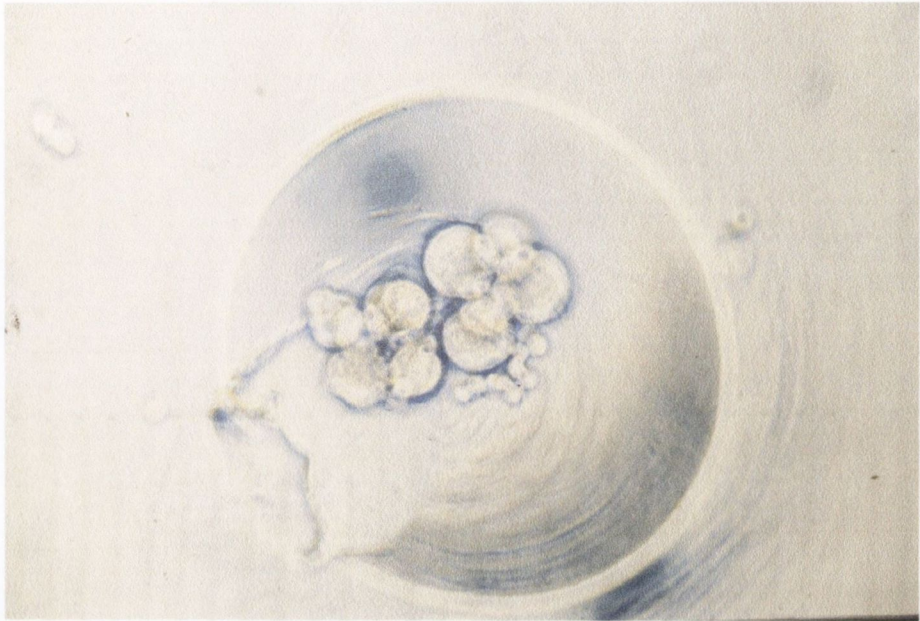


Fig. 4.4(b) A compact morula following fusion of the above.

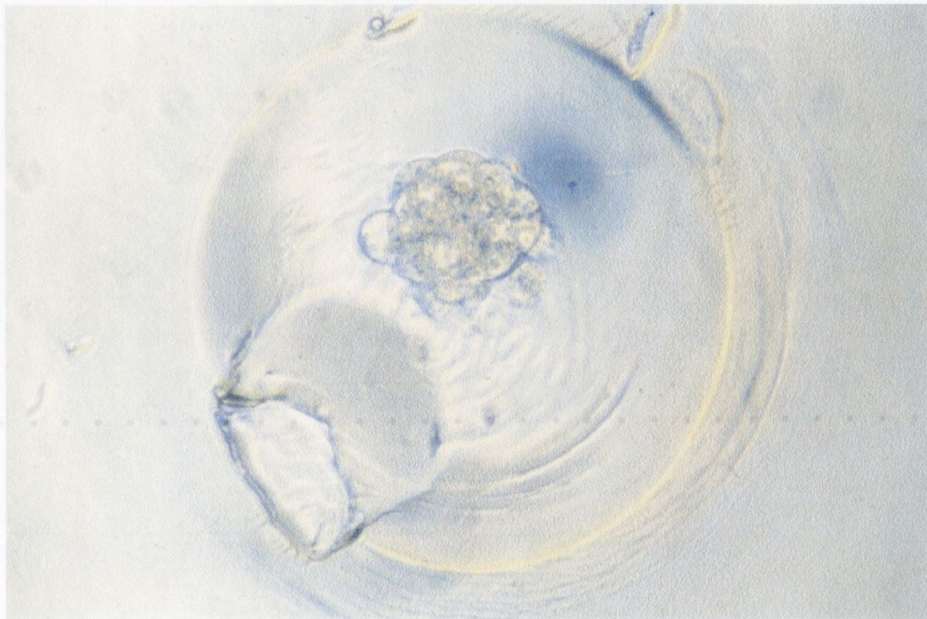


Figure. 4.4 (C);
A healthy developed blastocyst resulting from morula fusion.

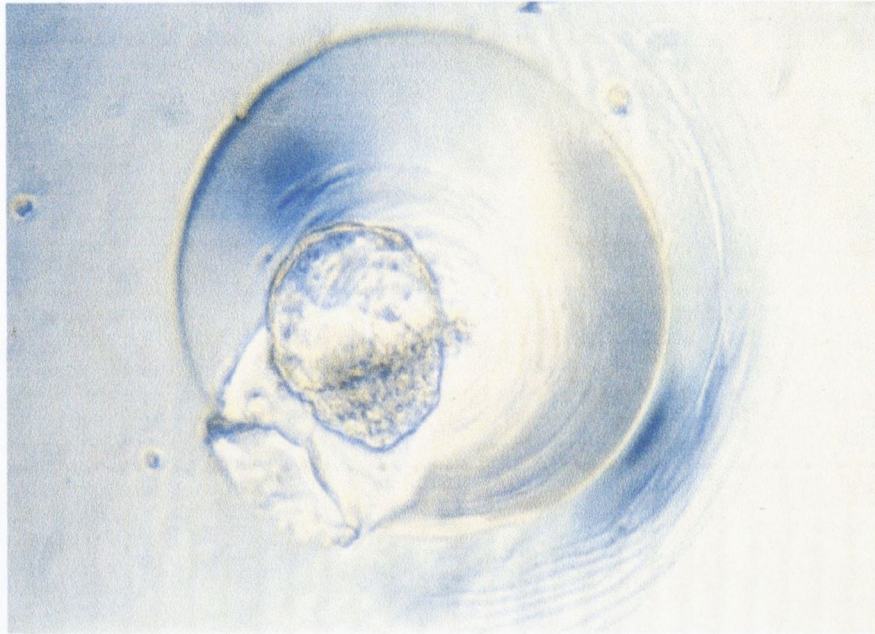


Figure. 4.5

A 10 day old foetus. A beating heart can be observed as a result of careful dissection. It is therefore possible to identify perinatal embryonic death.

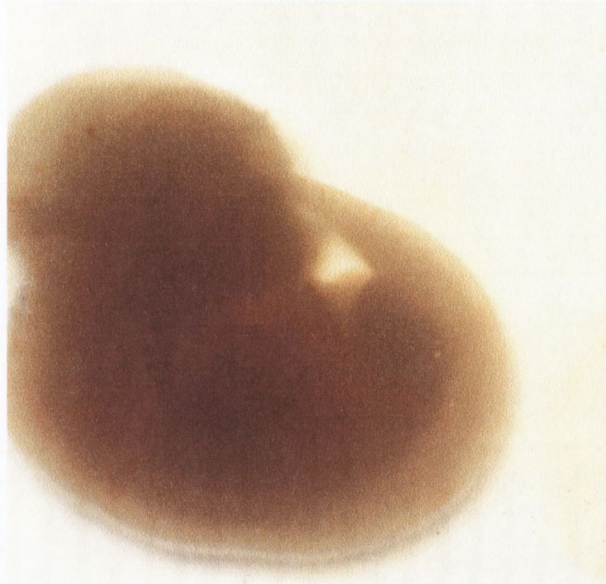


Figure 4.6 :

A chimera generated by aggregation of a white Laca embryo and R1 ES cells.

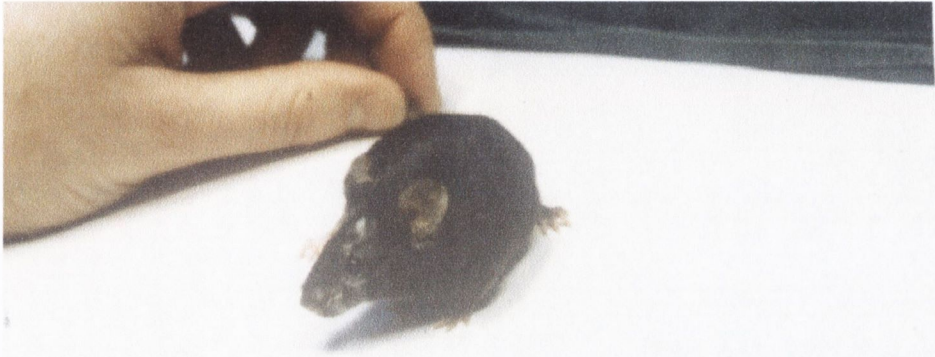
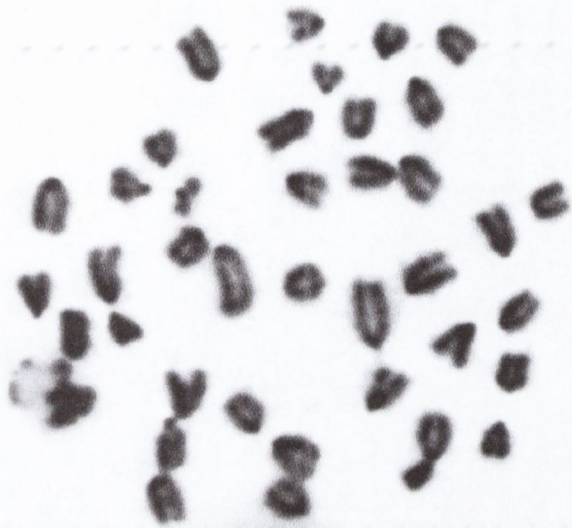


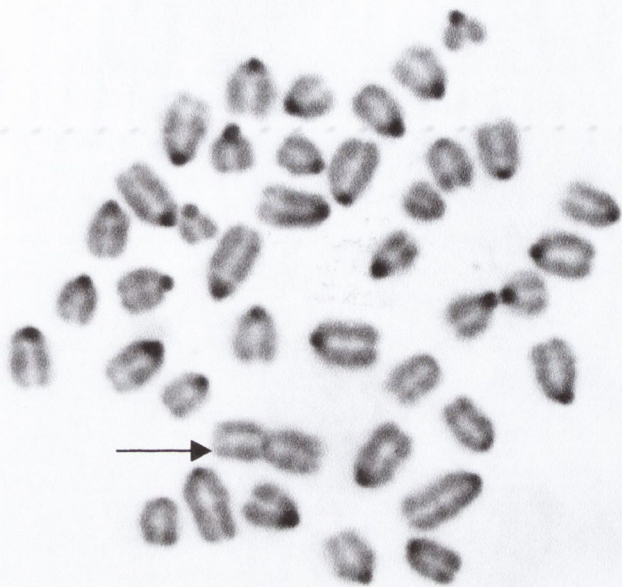
Figure 4.7.
The cell lines containing the targeted alteration were karyotyped in order to ascertain which would be the mouse likely to contibute to a germline chimera.



N3D1 (above) and N2E1(below) contained the largest number of cells with the normal number of mouse chromosmes



Figure 4.7b



A metacentric chromosome was observed in a number of the karyotypes examined as illustrated (—▶) above in cell line N3B8.
The cell line N3D3 (Karyotype below) was also used in attempts to produce germ line chimeras. This cell line contains the neomycin resistance gene only.

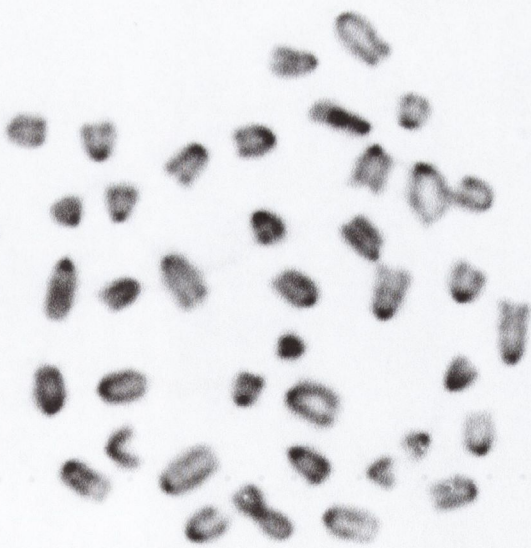


Figure 4.8.
A female chimera with 5-10% Es cell contribution to coat colour was born as a result of microinjection into a CD1 blastocyst.



Figures 4.9.
Chimeric mice generated from blastocyst injection of the N3D1 and N2E1 cell line.

a.

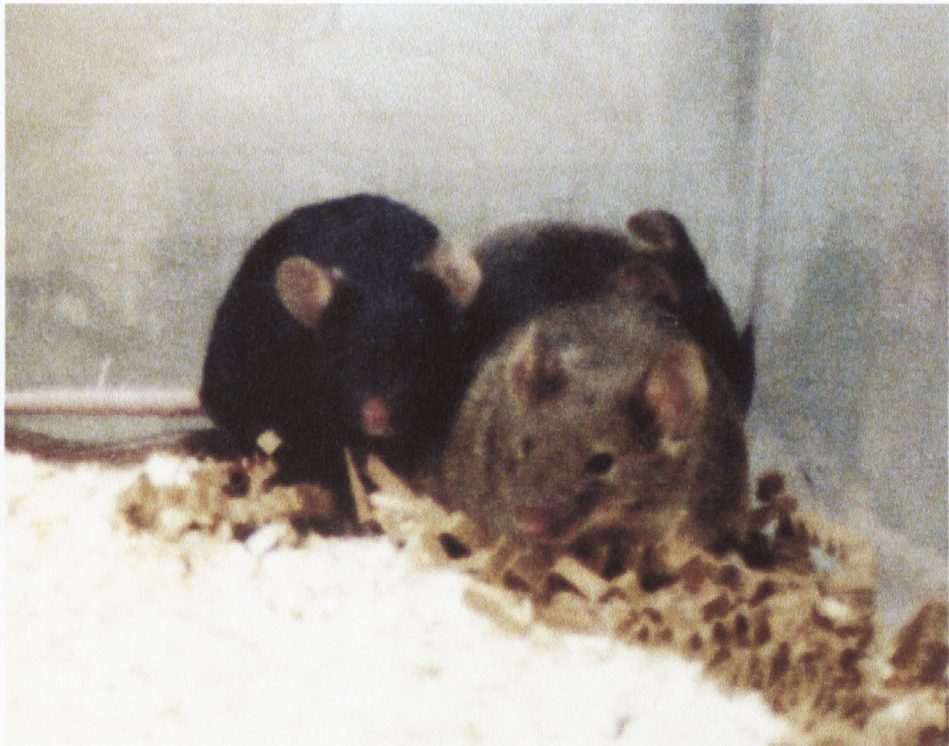


Figure 4.9 b.
The chimeric mouse is agouti in colour.



Figure 4.9 c
A chimeric $\Delta 307$ mouse.

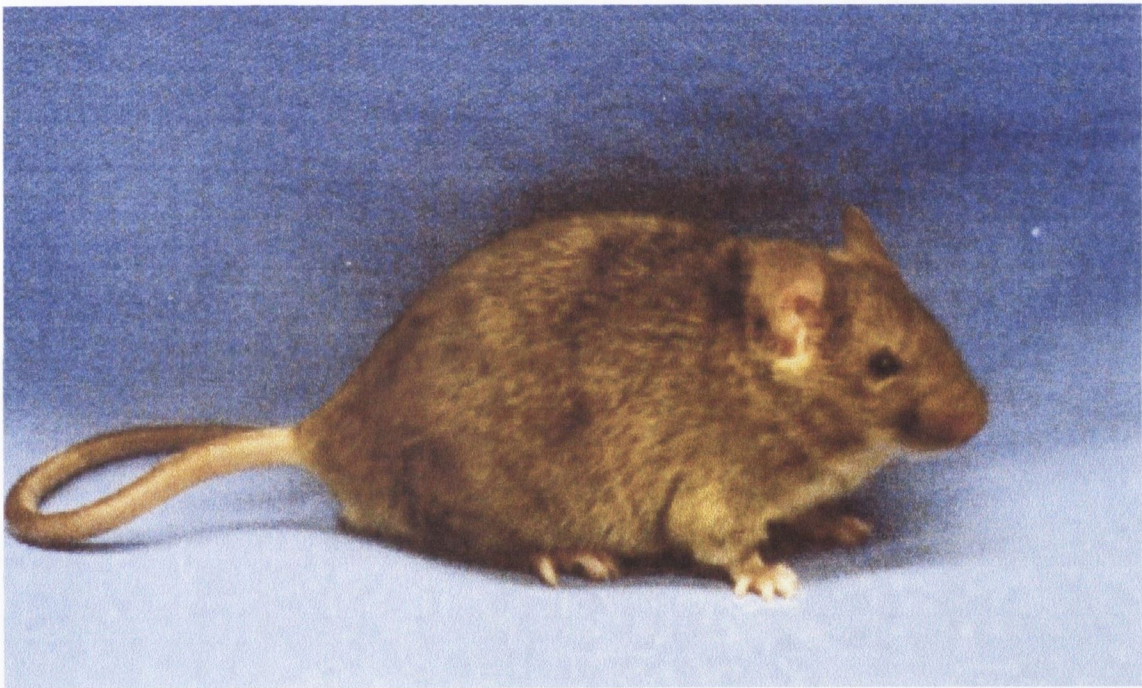


TABLE 4.1:

Blastocyst Development post uterine implantation

Age of Recipient female.	Original Weight.	No. of days Pseudopregnant.	Perinatal Death.	Healthy Foetuses.	Pups Born
10 wks.	30.05g	4	4 (7 days)		
10 wks.	32.4g	4	3		
10 wks.	30.66g	2			2
10 wks.	30.05g	2			
10 wks.	29.75g	2			4
10 wks.	28.84g	2			
10 wks.	27.87g	2			4
10 wks.	27.78g	2			
10 wks.	27.40g	2			4
10 wks.	27.37g	2			6
10 wks.	26.99g	2			4
10 wks.	26.45g	2			6
10 wks.	26.43g	2			4
10 wks.	25.87g	2	2	2	
10 wks.	23.6g	2	1		
10 wks.	22.9g	2			
10 wks.	29.56g	1			
10 wks.	26.54g	1			
12 wks.	30.76g	2			5
12 wks.	30.15g	2			4
12 wks.	29.66g	2			
12 wks.	29.65g	2			4
12 wks.	28.07g	2			
12 wks.	25.28g	2			4
12 wks.	25.14g	2			
13wks.	36.50g	2			
13wks.	35.55g	2			
13wks.	29.00g	2			4

TABLE 4.1 : Implantation experiments were carried out on a regular basis. Age, weight and number of days Pseudopregnant were noted. It was therefore possible to establish which combination would result in the birth of the highest number of healthy pups.

Table 4.2.

The ES cell clones are listed in the left column.
The chromosome content of twenty cells for each clone was counted. A putative metacentric chromosome was observed in a number of chromosomal spreads. The presence of the metachromosome is depicted in the table by an x. c/some = chromosome.

Clone	41 c/somes	40 c/somes	39 c/somes
N3D1		13	6x and 1
N2E1		17	2x and 1
N3B8	8	10	2
N3C3	15	3	2
N5E1	8x	11x	1x
N3D3		16	2x and 2
N4B4	10	8	2
N3E2	2	18	
N4E11		14	3x and 3

Table 4.3.

The karyotype s of ES cell clones N3D1 and N2E1 were counted for a second time.

Clone.	40 c/somes	39 c/somes	38 c/somes
N3D1	17	1 and 1x	1
N2E1	16	4	

Chapter 5.

Structural and Functional Rescue of Murine Rod Photoreceptors by a Human Rhodopsin Transgene.

Introduction.

In 1997 we reported the generation of a rhodopsin gene “knockout” mouse by introduction of a targeted disruption into exon 2 of the gene (Humphries *et al.* 1997). Homozygous mutant mice displayed a complete absence of rod outer segments, no rhodopsin as assayed using an anti-opsin antibody, and an absence of rod ERG responses in eight week old mice. Heterozygous mice also displayed some abnormal pathology including, for example, some degree of disorganisation of the photoreceptor outer segments and a reduction in the thickness of the outer-nuclear layer. A detailed description of the disease pathology for both homozygous and heterozygous *rho*^{-/-} mice is provided in chapter 1. Comparison of the protein sequences of murine and human rhodopsin reveals that there are 18 amino acid differences between proteins (Mc Nally *et al.* 1999). It is significant to note that to date, the effect that these differences have on the capacity of human rhodopsin to substitute structurally and functionally for mouse rod opsin is unknown. This is particularly relevant in view of the fact that such polymorphic variation is found near the chromophore binding site. *Rho*^{-/-} mice are presently being used in studies to evaluate various potential gene therapies as a means to treat human retinal disease using a range of viral and non-viral vectors for gene delivery. *Rho*^{-/-} mice carrying either a normal human rod opsin transgene, or carrying simultaneously both normal and mutant human rod opsins are likely to be of substantial value in the work-up of gene therapies. However, it must first be demonstrated that normal human rhodopsin can substitute, both structurally and functionally, for the normal murine counterpart on a murine rod-opsin-less background. Therefore, we decided to cross *rho*^{-/-} mice with transgenic mice expressing a human wild type(wt) rhodopsin transgene and to evaluate retinal histology and function in these animals. Immunocytochemical techniques were also used to assess the time course of human rhodopsin expression in the retinas of our animals.

Rhodopsin is the major transmembrane component of the outer segments of the rod photoreceptor cell (see introduction). The importance of this protein in photoreceptor function is reflected by the large number of mutations that have been identified within the rhodopsin gene, which can cause various forms of retinal degeneration (see Fig.1.7). At least 90 disease-causing mutations have been identified, the majority of which cause adRP, although a few mutations have been implicated in recessive forms of RP and congenital night blindness (RetNet. July.

1999). The nucleotide- and amino acid sequences of rod opsin have been established in a large number of organisms (Bird 1995, GenBank.). While variations in amino acid sequence are evident, the overall predicted structure of the protein remains similar between species. (see Fig.5.1).

The proposed model for the topography of rhodopsin is based on extensive biochemical, biophysical and mutagenic data. Mutagenesis, involving both double cysteine replacements followed by cross-linking and double histidine replacements followed by metal ion binding have been used to yield information on the topography of the rhodopsin protein in solution (Sheikh *et al.* 1996, Cai *et al.* 1997). A model for packaging of the transmembrane helices has been derived from electron cryo-microscopy (Kim *et al.* 1997, see fig 5.2). Recently, the tertiary structure of rhodopsin has been explored using magnetic resonance imaging studies of individual peptide segments. However, MRI techniques cannot be used on the entire protein owing to its size and insolubility (Yeagle *et al.* 1997). Site directed spin labeling studies of rhodopsin have provided information on both the dark structure and the rigid movements of the helices relative to one another once photoactivated (Cai *et al.* 1997). Details of structural reorganisation during photo-stimulation and consequent alterations in the 3D structure have not been fully elucidated, hence it is not possible to predict accurately any subtle alterations in protein function which may be induced by changes in amino acid sequence.

As mentioned above, it is notable that human and mouse rod opsins differ in their amino acid sequence at 18 residues. It will be evident from the diagram that the locations of these amino acid differences do not coincide with the known positions of mutations that cause retinal degenerations. However, some inter-species variability is seen around the chromophore-binding site at codon 296 (lysine), mutations at this site having been implicated in human disease (Shastri, 1994). The arrangement of the seven helices of rhodopsin is believed to hold retinal in position (the so-called helical bundle model). Rhodopsin binds 11 - *cis* retinal by a Schiff base linkage to lysine 296 within the hydrophobic portion of the lipid bilayer (Nathans, 1990). Opsin mutants, where Lys-296 is replaced either by Glu (K296E) or Gly(K296G) constitutively activate transducin and are no longer substrates for rhodopsin kinase due to loss of the chromophore. (Robinson, 1994)

Visual pigments from different organisms absorb light at different wavelengths. The amino acids in the vicinity of retinal are believed to specify the protein's absorption

spectrum. The counter-ion for the protonated Schiff base, glutamic acid, plays an important role in regulating the wavelength of maximal absorption of rhodopsin. This has been demonstrated by the rhodopsin spectrum shift from 498 to 380 nm upon replacement of the Glu¹¹³ with an uncharged glutamine amino acid. (Sakmar *et al.* 1989, Nathans, 1990). More subtle changes in the amino acid environment have also been shown to affect the wavelength of absorption of visual pigments. For example Neitz *et al.* demonstrated a shift in absorption spectra in monkey red-green sensitive visual pigments as a result of replacement of three non-polar amino acids with structurally similar hydroxy-amino acids. The positions of the amino acids studied in the monkey pigments are comparable to positions 164 in helix IV and 261 and 269 in helix VI in bovine rhodopsin (Rosenfeld *et al.* 1995). A further example of the importance of the amino acid environment of the binding pocket is the observation that if Trp²⁶⁵ in helix VI is replaced by alanine the spectrum of rhodopsin is blue-shifted by 30nm. Additionally, mutation of Trp²⁶⁸ to Phe decreases the ability of rhodopsin to bind transducin and is believed to contribute to conformational changes of rhodopsin linked to retinal isomerization as well as to the formation of the retinal binding pocket. (Rosenfeld *et al.* 1992). Given that the role of each amino acid within rhodopsin has not as yet been clearly defined, it is somewhat difficult to predict how each of the 18 a.a. differences between mouse and human rod opsin would affect the functioning of the protein. However, in the light of the studies outlined above, it is worth noting that in the mouse rod opsin sequence the nonpolar alanine residues present in human rod opsin at positions 298 and 299 are replaced with two polar serine amino acids (McNally *et al.* 1999). In the mouse rod opsin sequence the nonpolar alanine residues at positions 298 and 299 are replaced in human rhodopsin by two polar serine amino acids. Such amino acid substitutions may possibly cause some structural modification to the retinal binding pocket and/or the wavelength of absorption of rhodopsin.

Whether or to what extent the sequence variability observed between mouse and human rhodopsin has any influence on the ability of the human rhodopsin protein to couple with similar efficiency as that of the endogenous mouse protein, components of the mouse visual transduction cascade (for example, transducin) and requires further investigation. Data generated from this study of rescue of the rho^{-/-} phenotype using a human rhodopsin transgene has resolved this issue (McNally *et al.* 1999).

The transgenic mice utilised in the study were generated by Olsson *et al.* (1992). In that study, various transgenic mice carrying either a mutant rhodopsin (P23H) transgene or the wild type allele, were created using genomic DNA from a patient with autosomal dominant RP. Two lines of mice expressing the wild type human rhodopsin allele were produced, one expressing approximately equal amounts of human and endogenous murine rod opsins, the other expressing approximately 5 times more human than murine opsin. The transgenic mouse expressing approximately equal amounts of human to mouse rod opsins retained normal retinal function and structure. In contrast, over expression of human wild-type rhodopsin resulted in transgenic mice with a retinal degeneration. The latter retinopathy is probably brought about by over expression of the wild-type protein. This observation has clear implications in respect to accurate control of therapeutic gene expression in future therapies, which may involve rhodopsin gene replacement. Transgenic mice can be used to provide an indication as to whether the disease physiology observed in a dominant disorder is the result of haploinsufficiency (lack of wild-type protein) or due to a dominant-negative effect of the mutant protein, or indeed to a combination of both mechanisms. In the case of rhodopsin, a number of transgenic mice carrying mutant human rhodopsin known to cause adRP in humans have been generated. For example, expression of the human P23H mutation at levels as low as one-sixth that of endogenous rod opsin mice expressing two copies of endogenous murine rhodopsin, results in a severe retinal degeneration. The data suggest that the human mutation causes the disease pathology owing to a dominant-negative effect as opposed to a simple loss of function. (An additional study that is ongoing at this research unit is to establish whether the P23H mutant protein is partially functional and whether it can rescue to any degree the disease pathology in rho^{-/-} mice, which completely lack endogenous mouse rhodopsin).

Transgenic rescue of the wild type phenotype has been demonstrated in a number of murine retinopathies, including the rd and rds mouse (Travis *et al.* 1992, Lem *et al.* 1992). In the current study transgenic rescue of rho^{-/-} mice with a human rhodopsin transgene has been explored. Demonstration that a human transgene homologous to the gene mutated in a murine retinopathy model can suppress the degeneration of the mouse photoreceptors is important for a number of reasons. For example, such studies provide us with insights into the

conservation of retinal proteins between species at the functional level.

Human transgenes have been shown to rescue a variety of disease phenotypes in mouse models. For example complete genetic rescue of adenosine deaminase - deficient mice has been achieved through expression of the human gene (Migchielsen *et al.* 1996). An important requirement in such experiments is adequate expression level of the transgene may only result in partial rescue of the phenotype as has been observed in one attempt at rds mouse rescue (Travis *et al.* 1992). Incomplete rescue may also be observed as a result of inter-species differences in gene regulation and expression, which appears to have been the case in attempts to suppress the cystic fibrosis phenotype in CFTR- deficient mice by the human cDNA homologue (Rozmahel *et al.* 1997). Failure of the transgene to correct the intestinal pathology in these mice was probably due to inefficient functional expression of the human protein in the mouse.

In the present study suppression of photoreceptor degeneration in the rho-/- mouse by a human rhodopsin transgene was monitored using a number of techniques, including electroretinography, detailed histological analysis and immunocytochemistry. Some of these techniques, together with a description of some retinal pathology are described below as an aid in interpretation of the results section of this study.

ERG.

The electroretinogram (ERG) is used to diagnose retinitis pigmentosa by measuring the electrical activity generated by the retina in response to flashes of light (Karpe, 1945). Rod ERGs are often delayed and reduced in amplitude in RP patients. The Cone ERG can be normal or abnormal depending on the case in question. Patients with advanced RP show loss of all ERG responses even when some central vision remains. The rod ERG is established by dark adapting the subject prior to exposure of a series of light flashes, ranging from dim to very bright, on a dark background (Newell, 1982). The dimmest flashes result in pure rod-mediated responses, the brightest flashes in both rod and cone responses. After light adaptation by exposure to bright light for approximately 10 minutes, bright flashes are administered in the presence of a background light which suppresses the rod cell ERG. This permits analysis of pure cone-mediated responses. The light flashes induce an electrical response from the retina, which passes through the optic nerve to the brain. This current also travels through the ocular fluids of the eye. An

electrode is placed on the cornea to record this response. An ERG records both an a-wave and a b-wave. The a-wave is caused by the re-polarisation of photoreceptor cells and the b-wave is the response of the cells in the bipolar region of the retina (Heckenlively, 1988).

Histology of the eye.

The eye is the organ that converts light energy into nerve action potentials, which are relayed to the brain. It is composed of three layers; the corneo-scleral layer, the uveal layer and the retinal layer (Djamgoz *et al.* 1995, Junquera *et al.* 1995, see fig 5.3). The sclera is a tough fibro-elastic outer layer that contains the extra ocular muscles. The cornea is a transparent extension of the sclera directly in front of the iris and the pupil. The function of the cornea is to assist in focusing incoming images onto the retina. The uveal layer contains the choroid, ciliary body and the iris. The choroid is a highly pigmented vascular layer found between the sclera and the retina that provides nutritional support and absorbs any stray light, which passes through the retina. The ciliary body contains smooth muscle which allows it to control the shape and hence the focus of the lens via the suspensory ligament. The lens is a transparent biconvex structure, which provides a fine focus for the corneal image onto the retina. The iris is positioned in front of the lens and regulates the amount of light reaching the retina through the pupil. The lens, suspensory ligament and ciliary body separate the eye into a posterior compartment and anterior compartment. The iris separates the anterior compartment into anterior and posterior chambers. The anterior compartment contains aqueous humor, which is secreted by the ciliary body. The aqueous humor gives shape to the eye, acts as a source of nutrients for the non vascular lens and cornea and is the optical medium through which the light can pass. The posterior compartment contains the vitreous body, which consists of vitreous humor. The vitreous body supports the eye from within and provides a non refractive optical medium. The main function of the cornea, vitreous humor, lens and aqueous humor is to change the angles of incoming light and focus them onto the retinal layer (Djamgoz *et al.* 1995, Junquera *et al.* 1995).

Retinal layer.

The retinal layer converts incoming light impulses to chemical messages, which can be perceived by the brain. The foveal depression in the retina is the area of greatest visual acuity and is surrounded by the yellow pigmented macula lutea.

The nerve fibres from the retina leave the eye through the sclera at the lamina cribrosa and form the optic disc. There are no photoreceptors at this point and it is therefore referred to as the blind spot.

Histologically the retina can be divided into ten different layers (Djamgoz *et al.* 1995, Junquera *et al.* 1995). (see fig.5.3)

1. The pigment epithelial layer.

This layer rests on Bruchs membrane and separates the retina from the choroid.

2. The rod and cone inner layer.

3. The outer limiting membrane

This structure separates the inner and outer photoreceptor layer.

4. The outer nuclear layer

The outer nuclear layer contains densely packed nuclei, which are the cell bodies of the photoreceptor layer.

5. The outer plexiform layer.

The synaptic connections between the short axons of the photoreceptors and integrating neurons are present in this layer.

6. The inner nuclear layer.

This layer contains the cell bodies of the integrating neurons.

7. The inner plexiform layer.

The integrating neurons make connections with dendrites within this layer. The axons of these dendrites form the optic tract .

8. The ganglion layer.

Cell bodies of dendrites (ganglia) form this layer.

9. The layer of afferent fibres.

Afferent fibres passing toward the optic disc to form the optic nerve.

10. The inner limiting membrane

This final layer separates the retina from the vitreous humor

The retina is made up of three cell types, neurons, pigmented epithelial cells and neuron support cells. The neurons can be further divided into three

groups, the photoreceptor cells, the optic nerve fibres and the integrating neurons. The integrating neurons join the photoreceptor cells and the optic nerve fibres, which transmit the incoming signal to the cerebral cortex. Integrating neurons consist of bipolar cells, horizontal cells and amacrine cells.

Given that the primary pathology in RP is found at the level of the photoreceptor cells, a description of photoreceptor pathology is now provided.

Photoreceptor cells.

The photoreceptors are of two different types rods and cones. Cone cells consist of three types (short, medium and long wavelength) and allow detailed colour vision in bright illumination. Rod cells function in dim illumination and are responsible for peripheral vision (Djamgoz *et al.* 1995, Junquera *et al.* 1995).

Rod photoreceptors are long thin bipolar cells consisting of inner and outer segments connected by strands of cytoplasm comprising nine microtubule doublets (see Fig. 1.2.). Energy generation and protein synthesis occur within the inner segments. The outer segments have a cylindrical shape comprising of stacks of flattened disks which contain, amongst other proteins, the visual pigment rhodopsin. The discs are continuously shed from the ends of the rods and phagocytosed by the retinal pigment epithelium, the discs being replaced from the inner segments. The visual transduction process involves interaction of light with rhodopsin and the subsequent generation of an action potential due to conformational change of the protein. The action potential then passes inwards along the dendrite and axon of the photoreceptor to the layer of integrating neurons.

Cone cells are very similar to rod cells. The cone outer segment is a long conical structure approximately two thirds of the length of the rod outer segments. The disc membranes are continuous with the plasma membrane in cones, unlike the rods. Cones contain pigments similar to rhodopsin which are sensitive to red, blue and green light.

Each individual possesses approximately 126 million photoreceptor cells, which pass their sensory output on to only 1 million ganglion cells (Djamgoz *et al.* 1995, Junquera *et al.* 1995). The information is amalgamated by intervening neurons, that is, bipolar, horizontal and amacrine cells.

Bipolar cells are the most common of the latter three cells types and make direct connections between one or more photoreceptors and the optic tract neurons.

Horizontal cells consist of several short processes and one long process, one of which makes lateral connections between adjacent and distant photoreceptor cells. The amacrine cells consist of one or two short dendritic trunks, which give rise to numerous branches. These branches connect with bipolar cells, optic tract neurons and sometimes with other photoreceptor cells.

Neuron support cells.

Glial cells, Muller cells, astrocytes and microglia cells span the complete thickness of the retina. These cells support the retina and insulate the axons. During and after illumination they clear the potassium ions, which are released, into the intra cellular space. Owing to their presence throughout the retina these cells also aid in establishing retinal orientation for histology purposes. Muller cells provide structural support and may transfer essential metabolites such as glucose to the retinal neurons. They are also believed to play a role in Vitamin A metabolism (Bunt-Milam & Saari 1983; Das *et al.* 1992).

Pigmented epithelial cells.

The retinal pigment epithelium lies immediately beneath the retina. It consists of a monolayer of pigmented cells. The outer segments of the photoreceptor cells are partially embedded in this layer. RPE cells phagocytose the shedded photoreceptor discs, provide a blood-retina barrier, supply nutrients and generate 11-cis-retinaldehyde from vitamin A absorbed from the blood stream. RPE also absorb any light, which may have passed through the retinal layers.

Material and methods.

All chemicals were obtained from BDH unless otherwise stated.

Tail assays.

Approx. 1cm of mouse tail was used in each assay. Lysis buffer (50mM Tris pH 8, 100mM EDTA pH8, 100mM NaCl and 1%SDS) and 30µg of Proteinase K were added to each tail (400µl) and incubated overnight at 55°C. DNA was phenol/chloroform washed and ethanol precipitated the following day. Genotypic analysis of mice.

Oligo.A 5'TTCAAGCCCAAGCTTTCGCG3' is an antisense primer for pol-II-neo, Oligo B and C are sense and antisense primers for exon II of the rhodopsin gene, B 5'AGGTTAGAGCTGGAGGACTG3'; C,5'TAAGACTGATTGGACCATTTC3'.

A 25µl PCR reaction mix contained 100ng DNA, 50pmol sense and antisense primers B and C for exon II, 25pmol of antisense pol-II-neo primer A, 200µM each of dGTP, dATP, dTTP and dCTP, 1.5mM MgCl₂, 100mM Tris pH 9, 50mM KCL, 1 x Triton and 0.75 unit Taq polymerase. PCR conditions were ; 94°C, 1min;55°C 1min;72°C, 1.2min;35cycles. Fragments of 394 and 135 bp are diagnostic of wild-type and mutant opsin alleles respectively.

Primers specific for amplification of exon 5 of the human rhodopsin gene are , sense 5'-TTCCAAGCACACTGTGGGCA-3' and antisense 5'-

TGTGACTTCGTTTCATTCTGC-3'. A 25µl PCR reaction mix contained 100ng DNA, 50pmol sense and antisense primers, 200µM each of dGTP, dATP, dTTP and dCTP, 1.5mM MgCl₂, 100mM Tris pH 9, 50mM KCL, 1 x Triton and 0.75 unit Taq polymerase. PCR conditions were ; 94°C, 1min, 55°C, 1min;72°C ,1.2min;30 cycles.

The amplification product obtained with these primers is 274 base pairs in length.

Dr. T.Dryja kindly supplied transgenic mice expressing levels of human opsin approximating those of the endogenous protein in the wild type mouse. Rhodopsin -/- mice were crossed with mice homozygous for the human rhodopsin transgene. The F1 generation, heterozygous for the mouse rhodopsin gene and containing

one copy of the human transgene, was self crossed. F2 mice of genotype of rho-/- and carrying either one or two copies of the human rhodopsin transgene were identified by PCR amplification for the present study.

Histopathological analyses of mouse retinas.

Mice were humanely sacrificed by CO₂ gas inhalation. The superior portion of the cornea was marked with a hand-held cautery. Corneas of eyes were punctured and eyes were soaked overnight in a mixture of 2% paraformaldehyde /2.5% gluteraldehyde in 0.1M phosphate buffer, pH7.4. The following day Dr. Paul Kenna bisected the eye through the superior/inferior axis. Eyes were washed six times in 0.1M phosphate buffer/ 5% sucrose over a period of 1hr and post fixed in 2% osmium tetroxide/0.1M phosphate buffer/5% sucrose for a further hour. Tissues were dehydrated in alcohol at room temperature; 50% alcohol for 1hr, 75% alcohol for 1hr, 95% alcohol for 1hr, and 100% alcohol for 1hr. Eyes were then placed in propylene oxide for 30mins. Fresh propylene oxide was then added for a further 45mins. Eyes were left overnight in a mixture of 50% Agar resin and 50% propylene oxide. The following day this mixture was replaced with full-strength resin for 4 hours, before the eyes were placed in beem capsules (The Bronx EM Company) with fresh resin and fixed at 60°C overnight.

Semi thin sections cut from resin blocks.

Semi-thin sections 1 micron thick were cut from resin blocks in a Reichert-Jung, ultracut microtome with a glass knife. Sections were heat- fixed onto glass microscope slides. Toluidene blue was used to stain sections. Coverslips were mounted with DEPX.

Sections were photographed using a Zeiss KS 300 camera. Photographs were printed on Lexmark, Ink Jet glossy film paper .

Immunocytochemical assay for rhodopsin.

Mice were sacrificed by CO₂ inhalation or decapitation, depending on age. It is deemed more humane to sacrifice pups under the age of five days by decapitation. Eyes were enucleated and corneas punctured. Eyes were fixed in 4% paraformaldehyde and 0.5% gluteraldehyde for 1hr. The fixative was then changed

to 4% paraformaldehyde overnight while rotating at 4°C. The fixative was washed out serially with 0.1M phosphate buffer /1% sucrose. Eyes were stored in 30% Sucrose/0.1% Na-azide.

Cryostat sectioning

Eyes were placed on blotting paper prior to sectioning to remove excess sucrose. Once blotted they were placed in a mould and covered with OCT solution for 30min. Tissue were snap-frozen by floating the molds in liquid nitrogen and were then placed in the cryostat chamber (Bright OFT model) for 30mins to equilibrate to the cryostat temperature of -24°C. Sections of 10µm were cut with a Bright 5040 microtome and thaw-mounted on poly-L-lysine slides (BDH). All sections were stored at -70°C.

Immunocytochemistry.

Sections were outlined with a Pap pen and 50-100µl of blocking solution was placed over the tissue for 15 mins at room temperature. Fluorescently labeled anti-rhodopsin antibody 4D2 was diluted 1:250 in labeling solution and placed over the sections. Each section was incubated overnight, in the dark, in a humidified container at room temperature. Slides were washed twice in 0.1M PBS and the remainder of the blocking solution to initially wash away the labeling solution, and subsequently slides were washed every 15mins for 1hr. Excess wash solution was absorbed with a tissue and each section was overlaid with 10-20µl of Movial mounting solution (Calbiochem, La Jolla, U.S.A) and a clean coverslip. Slides were stored in the humid chamber o/n and sealed with nail varnish the following day. Immunofluorescent signals were observed under a Zeiss immunofluorescent microscope (Axioplan,2 Attoarc HBO 100w).

Blocking Solution consisted of 10% goat Serum;0.5% triton x in 0.1M phosphate buffer. Labeling Solution contained 3% goat serum, 0.1% triton x in 0.1M phosphate buffer.

Electroretinographic analysis of mouse retinas.

Electroretinograms were recorded on 8 mice from the F2 generation. 4 mice were 20 weeks old and 4 were 16 weeks old. Each age group contained two rho-/- Transgene +, 1 rho-/- Transgene- sibling and 1 wild-type mouse. ERGs were performed according to previously described procedures (see Humphries *et al.* 1997). Mice were dark adapted overnight, exposed to dim red light and again dark-adapted for a further 20min. Anesthetic was administered: 500mg/kg urethane, 11mg/kg xylazie and 14mg/kg ketamine. Pupils were dilated with 0.05% phenylephrine and 0.05% atropine. A bite bar and nose clamp were used to position the mice. ERGs were recorded using a 1mm silver-silver chloride wire loop electrode on the cornea, a stainless steel subcutaneous needle on the forehead as the reference and a ground on the foot. Signals were amplified 10,000 times at 0.1 to 1000 Hz and passed through a 60Hz notch filter. One hundred traces were averaged. Single traces were collected over the brightest 2 log units of intensity for animals with large responses, with 50ms interstimulus intervals. Intensity of 50ms flashes were regulated with Wratten (Kodak) neutral density filters over more than 6 log units with a maximum intensity of 130cd/m^2 . Dark-adapted ERGs were recorded beginning at threshold. Light adapted ERGs were recorded at 0 neutral density flashes on a background of 37cd/m^2 to suppress rod responses and reveal cone responses after 10 min of exposure to the background light. (ERG analysis was carried out by Dr. Paul Sieving and Colleagues).

Results and Discussion.

Suppression of the severe retinopathy observed in the rhodopsin knockout mice has been explored using a human rhodopsin transgene. Rho^{-/-} mice do not elaborate rod outer segments and display a marked decrease in numbers of photoreceptor cell nuclei with age. At 3 months of age the outer nuclear layer of the retina (ONL) is reduced to a single layer of cells, which probably represent surviving cone photoreceptors. Using immunocytochemical assays, rhodopsin is undetectable within the developing photoreceptor layer at any time postnatally. Moreover, rho^{-/-} mice have no electroretinographic (ERG) responses below 0.3cd/m² (Humphries et al.1997).

Sections of retinas of 3 month old rho^{+/+}, rho^{-/-}, and mice expressing the human rhodopsin transgene are shown in figure 5.4. Notably, both wild type retinas and rho^{-/-} retinas expressing the human rhodopsin transgene show an equivalent number of photoreceptor nuclei within the outer nuclear layers. Clearly rho^{-/-} mice expressing the human transgene elaborate normal looking photoreceptor outer segments (fig 5.4 c.). The morphologies of the inner and outer segments of the photoreceptors of both the wild-type and knockout mice expressing the human transgene were indistinguishable, indicating significant recovery of the wild type phenotype in rho^{-/-} mice expressing the human opsin transgene.

Immunocytochemical investigations were carried out on cryo-sectioned retinas with the fluorescent anti-rhodopsin antibody, 4D2, kindly supplied by Drs. Robert and Lauri Molday. This antibody recognises an epitope at the amino terminus of the rhodopsin protein (Molday, 1998). No immunoreactivity was detectable in the retinas of the rho^{-/-} knockout mice at any stage (see fig. 5.5). In contrast, at 3 months of age immunofluorescence was clearly visible in both wild type retinas and in rho^{-/-} knockout retinas expressing the human rhodopsin transgene. Rhodopsin expression was investigated at various times post-natally in rho^{-/-} mice, in wild type mice and in rho^{-/-} mice expressing the human rhodopsin transgene. These studies indicate that expression of the human transgene in rho^{-/-} mice occurs at a similar stage to that of the endogenous murine rod opsin. That is, although immunoreactivity was not detected in the retinas of 1 day old mice, it was clearly visible in wild type mice and in rho^{-/-} mice expressing the human rhodopsin transgene by post natal day three (see fig. 5.5). The human transgene expressed in the rho^{-/-} mouse encodes the entire transcriptional unit of the human rhodopsin

gene together with 4.8kb and 6.2kb of upstream and downstream sequences respectively. Hence, the present study indicates that sufficient genomic DNA is present to direct photoreceptor-specific expression of the human rhodopsin gene in the mouse and to enable the human opsin gene to respond adequately to the mouse expression control factors.

Dr. Paul Sieving's group at the University of Michigan, Ann Arbor, carried out ERG studies. Figure 5.6 shows a typical dark-adapted ERG series recorded over 6.4 log units of flash intensity for wild type mice on the left and for rho-/- mice carrying the human transgene at the base of the figure. The dimmest flashes of light show primarily the b-wave and a negativity that is the scotopic threshold response from the proximal retina. Notably, for both groups ERG thresholds of 10 microvolts were at about -5.14 log units cd-s/m^2 . In contrast, homozygous mice had no detectable ERG response for any stimulus, including the brightest flash for both dark- and light- adapted conditions.

Figure 5.6 shows the intensity-response functions for the normal wild-type and transgenically rescued rho-/- mice. Wild-type mice had somewhat larger b-wave amplitudes above -1 log cd-s/m^2 than transgenically rescued rho-/- mice. However when standard deviations were taken into consideration this difference was not significant. Such sizable standard deviations are common for recordings of the mouse ERG when small sample numbers of animals are used. The maximum b-wave amplitude measured at a flash intensity level of 0.61 log cd-s/m^2 was 792.49 $\pm$ 487.64 μV for wild-type mice and 615.81 $\pm$ 183.79 for transgenically rescued mice ($p=0.52$ calculated by a two tailed t-test). A 50 μV criterion was used to identify b-wave threshold and the corresponding flash intensity level was measured from the intensity-response curve for each animal. The mean for the normal mice was -3.99 $\pm$ 0.75 log cd-s/m^2 and the mean for the transgenically rescued mice was -3.91 $\pm$ 0.04 log cd-s/m^2 . There was no significant difference between the mean b-wave threshold for the two groups ($p=0.93$ using a two tailed test). The maximum amplitude of the photopic ERG for a 0.61 log cd-s/m^2 was 150.099 $\pm$ 70.91 for the wild types and 156.07 $\pm$ 113.03 for the transgenic mice ($p= 0.94$ using the two tailed test. Previously, the dark adapted b-wave threshold of rho-/- mice was found at -1.07 log cd-s/m^2 for animals younger than 6 weeks of age;

since this represents the threshold of cone photoreceptors, all responses at dimmer intensities must be rod driven and are not present in rho-/- mice. By 12 weeks of age, the retinal degeneration in the rho-/- mouse has progressed such that no detectable ERG was found. It is of note that the rho-/- mice carrying the human rhodopsin transgene in the present study had thresholds 3 log units more sensitive than the cone dark-adapted b-wave threshold of rho-/- mice from previous studies; these observations clearly suggest that this response must be rod driven. Furthermore, these recordings were made in 20 week old mice, by which age rho-/- animals have been clearly shown to have no detectable response both in the current study and in the original study of rho-/- mice.

This study demonstrates conservation of the structural and functional properties of human and mouse rhodopsin proteins. In spite of the 18 a.a. differences between the two proteins, interaction with other proteins involved in visual transduction does not appear to be compromised, as evidenced by the ability of human rhodopsin to substitute for the mouse rhodopsin. Furthermore, transcription factors controlling tissue specificity and timing of expression during photoreceptor development apparently interact with the human gene in the murine environment. It is interesting to note the differences between the human and murine rod opsin at positions 298 and 299. Human rhodopsin contains two nonpolar alanine residues at these positions while in the mouse two polar serines are present. This change does not affect the ERG readings and could possibly represent some subtle difference between the mouse and human in the sense that mice are nocturnal.

This study also highlights the fact that 4.8kb upstream and 6.2kb downstream of the transcriptional start site is sufficient to allow developmental and tissue-specific control of human rhodopsin in the mouse retina. This information is extremely important in respect to assessment of the amount of sequence required for rhodopsin gene delivery in attempted therapies.

Conclusion.

The histopathological, immunocytochemical and electrophysiological data presented here demonstrate the structural and functional rescue of the rho-/- mouse retina by expression of a human rhodopsin transgene. The results unequivocally establish that the deficiency of rhodopsin in the rho-/- mouse causes the observed phenotype. This information is important for the development of gene therapies . We have established the rho-/- mouse as a suitable model for the exploration of human gene delivery and expression studies. These data also help to reinforce the argument that mice are suitable models for the initial examination of therapies, which will ultimately be developed for patients with retinal degenerations.

Since it has now been shown that the retinopathy that develops in rho-/- mice can be effectively suppressed by the presence of a human rhodopsin gene, it would be of substantial value to undertake further breeding experiments in which rho-/- mice expressing the normal human rhodopsin transgene are crossed with mice expressing a dominantly mutated human rhodopsin transgene. Such experiments are in fact now in progress at this laboratory. Rho-/- mice simultaneously expressing both normal and mutated (in the case P23H) human rod opsins should provide a useful model for studies of suppression of dominant –acting mutations.

CELL CYTOPLASM

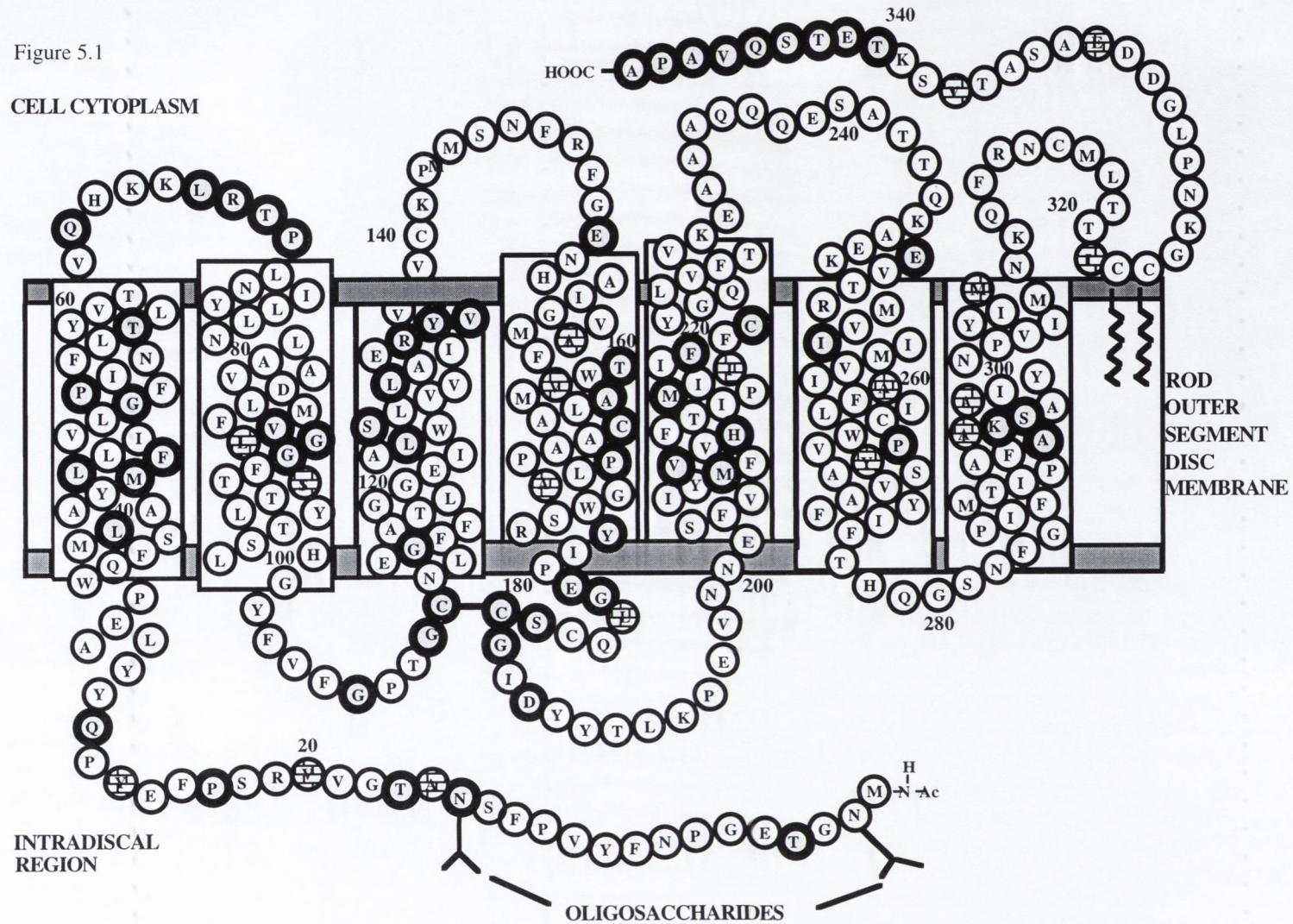


Figure 5.1.
The human rhodopsin protein sequence differs from its murine counterpart at 18 amino acids.
The differing amino acids are filled in the figure, with the following pattern: 

A number of the mutations known to cause RP are also highlighted by a thick black circle.

Figure 5.2.

A structural model of the rhodopsin helix packaging in a projection near the cytoplasmic membrane-aqueous boundary based on cryoelectron microscopy data. (Taken from Kim *et al.* 1997)

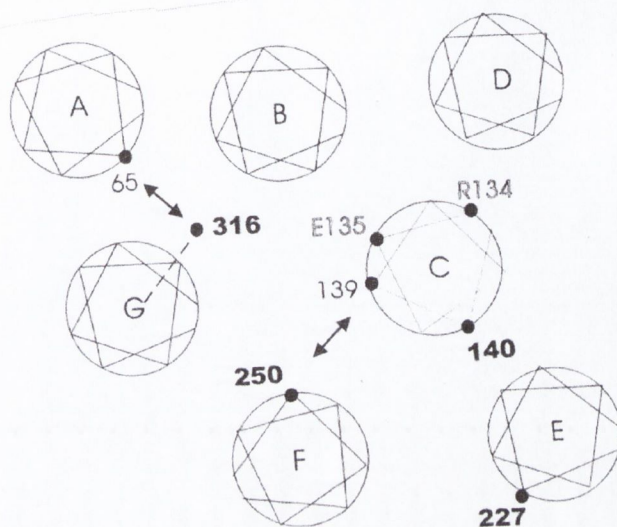


Figure 5.3.
Histologically the retina can be divided into ten different layers as illustrated below
(Taken from Wheaters *Functional Histology*)

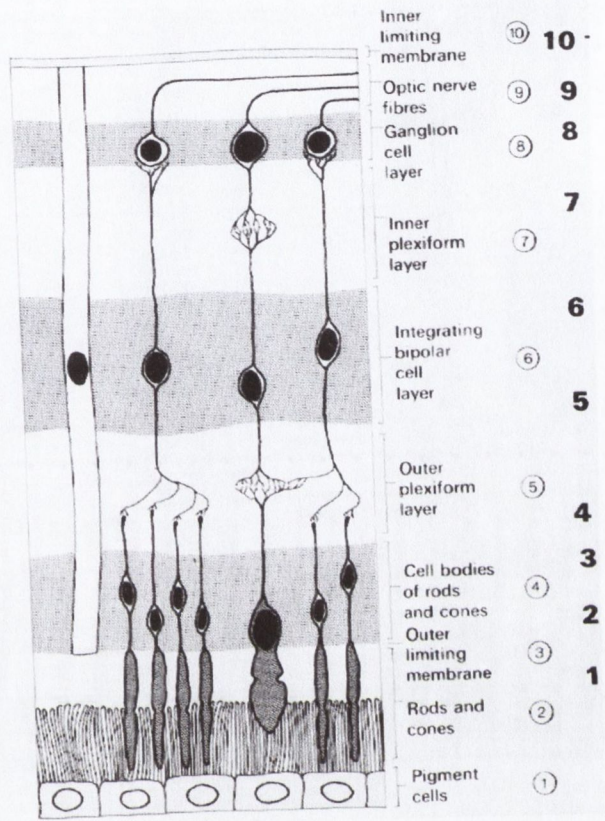


FIGURE 5.4

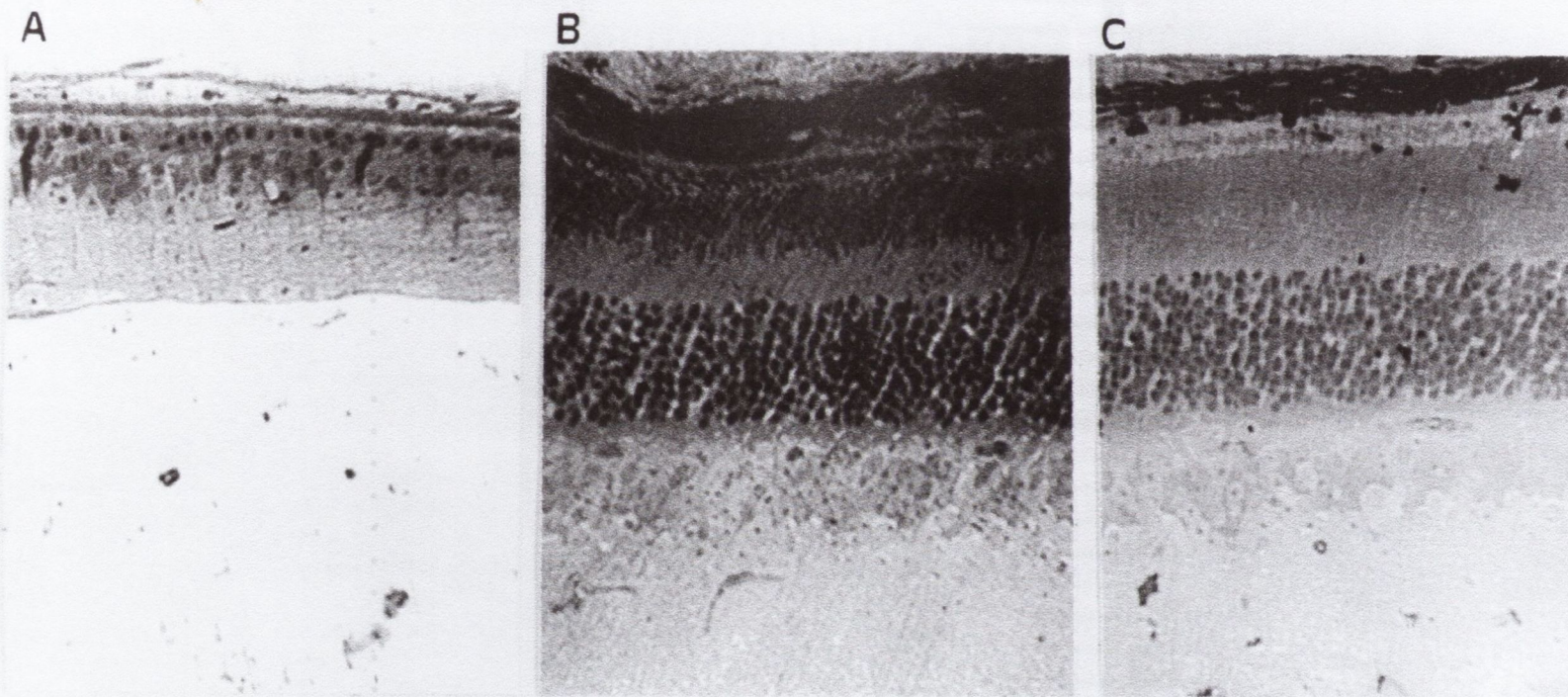


FIGURE 5.5

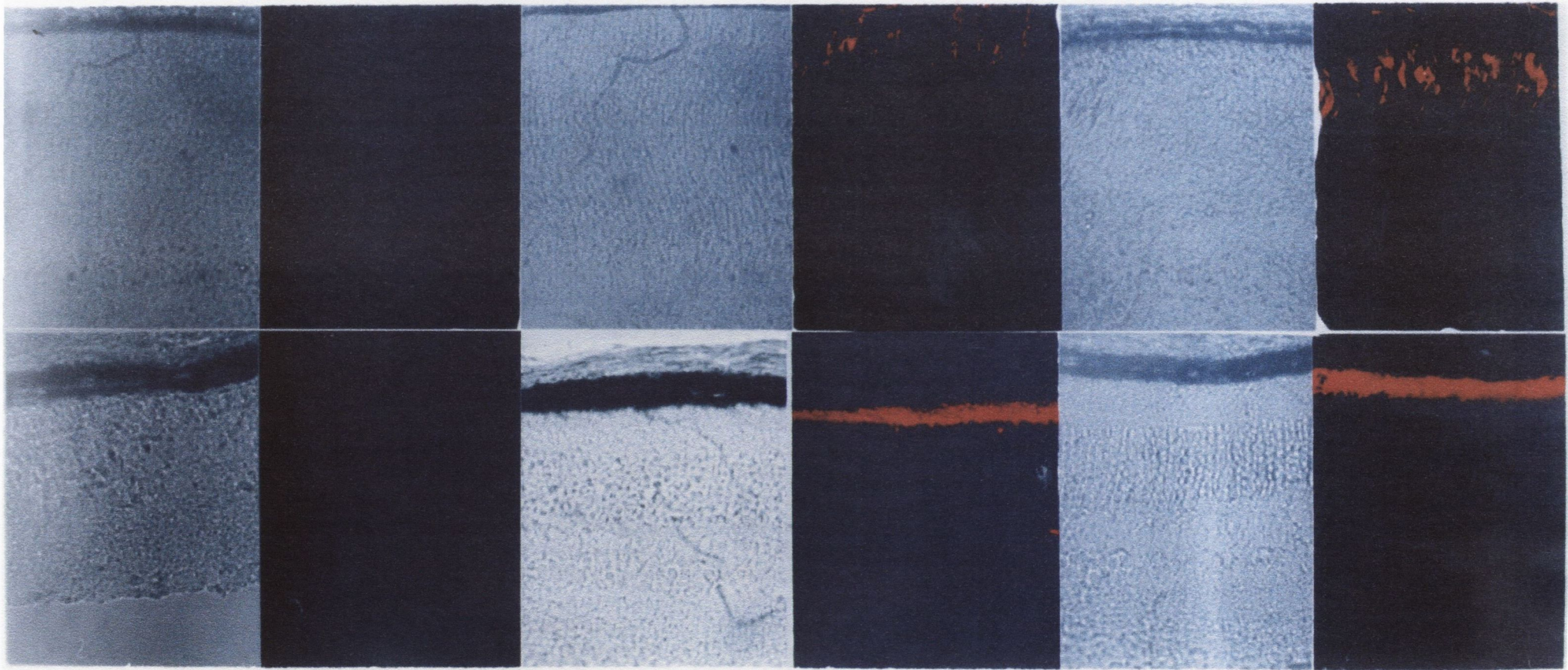
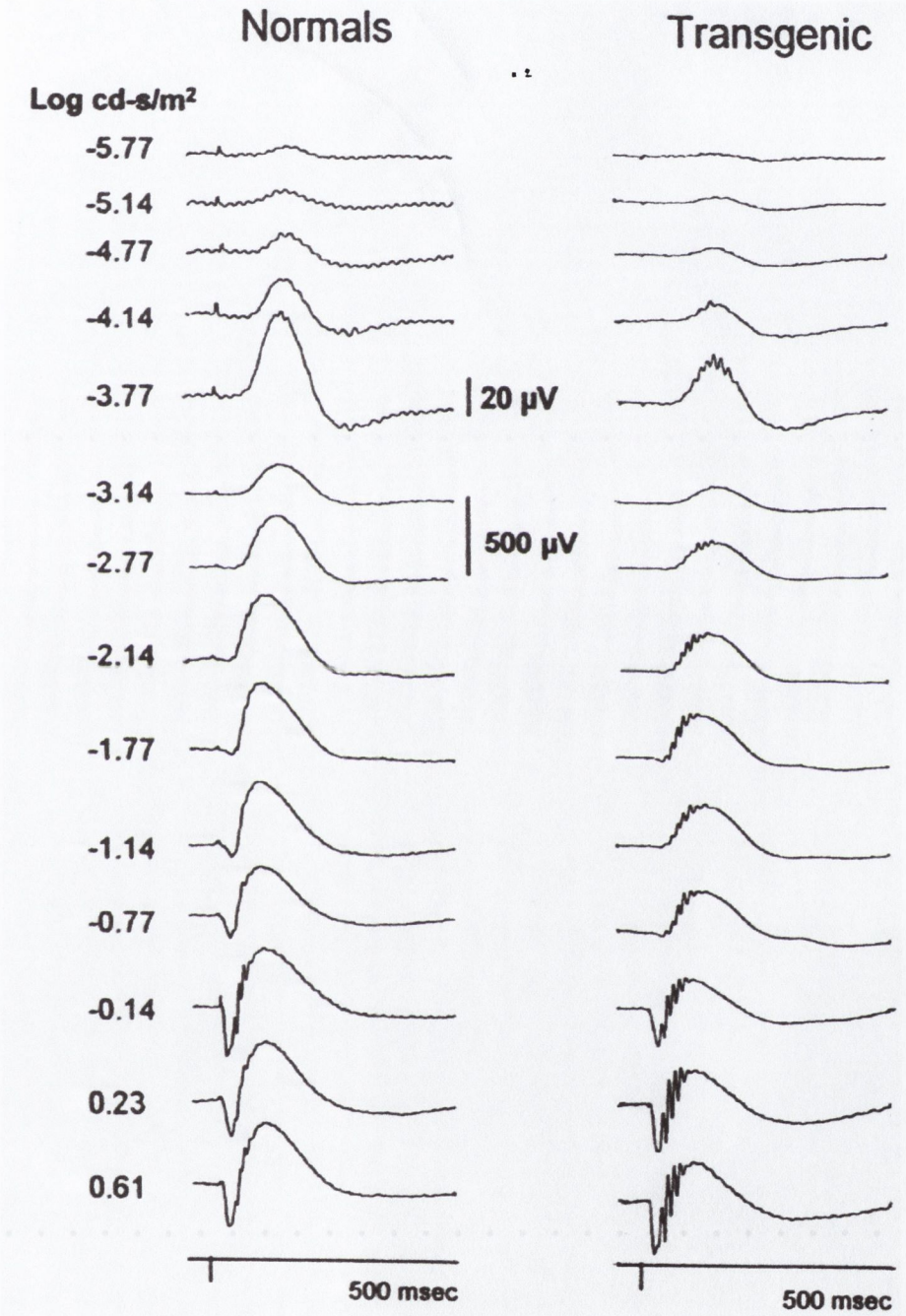


FIGURE 5.6



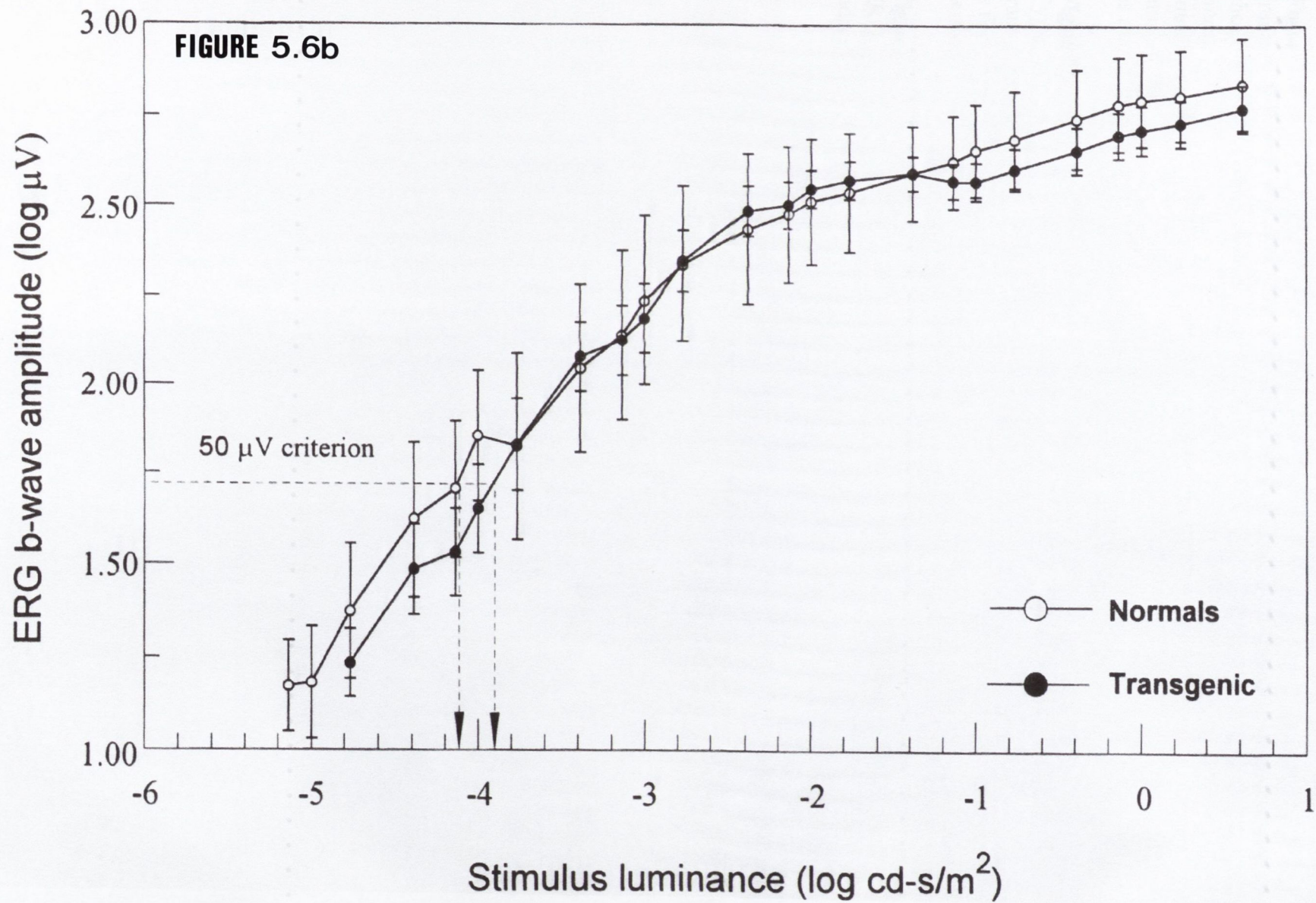


Figure 5.4.

Epon-embedded sections of the retinas of 3 month old Rho^{-/-} (A) wild-type (B), and of retinas from Rho^{-/-} mice rescued by human transgene (C).

Figure 5.5.

Immunocytochemical analysis of cryosectioned mouse retinas using fluorescent anti-rhodopsin antibody 4D2. Upper panel, left to right: DIC image of 3-day Rho^{-/-} mouse retina; immunoreactivity toward same retinal section; DIC image of 3-day wild-type mouse retina; immunoreactivity toward same retinal section; DIC image of 3-day Rho^{-/-} retina rescued by human rhodopsin transgene, and immunoreactivity toward same retinal section. Lower panel; as above sequence but with retinas of 3 month old animals.

Figure 5.6a.

Typical dark adapted ERG intensity series from a normal wild-type mouse (left) and a transgenic mouse (right), closest to the means of the respective groups. Amplitude calibration is for adjacent and lower traces for 500 μ V, and adjacent and upper traces for 20 μ V. The vertical mark on the time scale indicates the stimulus flash.

Figure 5.6b.

ERG intensity-response curve of the mean b-wave amplitude for each group, with bars indicating $\pm$ SE. 50 μ V threshold criterion is shown by dashed line.

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Appendix 1.

ES cell culture media and solutions.

5ml DMEM (Dulbecco's modified Eagles medium).

1 x 5L pk. Dry powder DMEM W/4500mg/L: W/O Na pyruvate, W/O NaHCO_3

7.5% Na bicarbonate. (Sigma)

Penicillin / Streptomycin 50 lu /ml (Gibco)

The pH should be adjusted to 7.2 with HCL and then the solution filtered through a 0.2 μm nitrocellulose filter (Gelman Science) into sterile bottles.

Feeder Medium.

500ml DMEM and 10% Foetal calf serum (Gibco. Serum Cat. 10502-045).

ES Medium.

500ml DMEM

15% Foetal Calf Serum.

60 μl 10^{-6} M β mercaptoethanol

6ml 2mM L-Glutamine (Gibco)

1000 U/ml LIF (Gibco, ESGRO) or Cos/LIF medium at established dilution.

Foetal calf serum was heat inactivated prior to use by heating at 56°C for 30 minutes.

1L Phosphate buffered saline (PBS) made in Mili Q filtrate H_2O .

10g NaCL (BDH LTD.)

0.25g KCL (BDH LTD.)

1.44g Na_2HPO_4 (BDH LTD.)

0.25g KH_2PO_4 (BDH LTD.)

The pH was adjusted to 7.2 with HCL and the solution autoclaved.

Freezing medium.

DMEM

25% Foetal calf serum

10% Dimethyl sulphoxide (cryoprotectant)

Trypsin EDTA.

0.25% (W/V) Trypsin in 0.04% (W/V) EDTA. 1x solution (Sigma)

Methylene blue (Sigma) 3.3g Methylene blue. 1.1g fuchin (Sigma) in 1 L of methanol.

On the Development of Animal models for Degenerative Retinal Diseases.

Niamh Mary Mc Nally

Abstract.

The major objective of this thesis was the development, on site, of procedures for the maintenance and growth of embryonic stem (ES) cells, their genetic manipulation, and the generation by gene targeting of a mouse model for autosomal dominant retinal degeneration. Specifically, a 1bp deletion (CTG to C-G) at codon 307 of the mouse peripherin gene was successfully carried out in R1 embryonic stem cells. These cells were used to generate 21 chimeric mice carrying the targeted deletion. To date, two of these mice have passed the codon 307 mutation on to the next generation, demonstrating germline transmission of the cell line. In man, the codon 307 mutation within the RDS-peripherin gene results in a slowly progressive form of adRP. A similar phenotype may be produced in mice. If so, these animals would provide a valuable model for dominant disease. The relatively slow retinopathy may possibly provide a broader window of time to realise the potential of certain gene therapy vectors (i.e AAV).

Animals carrying a targeted disruption of the rhodopsin gene, (rho-/-) are currently used in studies to evaluate various potential gene therapies as a means to treat human retinal disease. Histopathological, immunocytochemical and electrophysiological data are presented demonstrating substantial structural and functional rescue of rod cells in rho-/- mice retinas. This rescue was brought about by crossing these animals onto a transgenic strain of mouse expressing human rhodopsin. Such information is required for optimal use of Rho-/- mice in exploring methods for suppressing human degenerative retinal conditions whose molecular pathologies are based either upon loss of rhodopsin gene function or on the manifestation of dominant mutations.