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Novel therapeutic strategies for autosomal dominant retinitis pigmentosa

Brian G.D. O’Neill

Thesis submitted to Trinity College Dublin for the degree of Doctor of Philosophy

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DECLARATION

I hereby certify that this thesis, submitted to Trinity College Dublin for the degree of Doctor of Philosophy, has not been submitted as an exercise for a degree at this or any other university. I also certify that the work described here is entirely my own, except where otherwise acknowledged.

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Brian G.D. O'Neill

This thesis is dedicated to my mother and father, Tom and Phyllis O’Neill.

Mam and Dad, without your continued support, encouragement and belief in me, this would not have been possible. Little did we think on the day I went to college for the first time that eight years later I’d be sitting here, writing these lines in a Ph.D. thesis.
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ABSTRACT

A major difficulty associated with the design of gene therapies for autosomal dominant diseases is the immense intragenic heterogeneity often encountered in such conditions. Two strategies which circumvent the difficulties associated with developing multiple mutation-specific therapies for dominant disorders and with discriminating between disease and normal alleles have been explored in this thesis. In the first, normal and mutant alleles are suppressed by targeting sequences in transcribed but untranslated regions (UTRs) enabling introduction of a replacement gene with the correct coding sequence but altered UTRs to prevent suppression. The second approach involves suppression in the coding sequence of a gene and concurrent introduction of a replacement gene by exploiting the degeneracy of the genetic code. Both approaches provide a wider choice of target sequence than those directed to single disease mutations and are appropriate for many mutations within a given gene. The aim of this project was to examine the feasibility of both therapeutic approaches using a group of genetically heterogeneous retinopathies termed retinitis pigmentosa; for example, over 150 mutations have been identified in the genes encoding the photoreceptor-specific proteins, rhodopsin and peripherin in patients with retinal degenerations. Notably, the vast majority of these mutations are known to give rise to autosomal dominant retinitis pigmentosa. In the context of general methods for gene suppression it is of note that such methods may be directed towards the primary defect, in the case of RP for example the rhodopsin gene, or a secondary effect associated with the disease process such as apoptosis. Two general methods targeting the primary defect which overcome the problems of allelic heterogeneity are explored in this thesis using hammerhead ribozymes directed to transcripts from the rhodopsin and peripherin genes. The ribozyme technologies explored in the study could be used to develop therapies for retinopathies which are directed to therapeutic targets other than rhodopsin or peripherin.

Hammerhead ribozymes are catalytic RNA molecules which bind target RNA via two complementary antisense domains and elicit site-specific cleavage of the phosphodiester backbone at an accessible NUX triplet motif. Thus, in principle, it is possible to irreversibly inactivate expression of any target RNA containing a suitable NUX ribozyme cleavage site. A series of hammerhead ribozymes were designed to cleave rhodopsin and peripherin transcripts in vitro in both a mutation-specific and mutation-independent manner (Chapter 3). Replacement genes were modified such that ribozyme binding sites or NUX cleavage sites were removed thereby protecting transcripts from ribozyme-mediated suppression. The mutation-specific ribozyme investigated cleaved mutant transcripts specifically while leaving wild-type transcripts intact. However, notably both mutation-independent approaches were demonstrated in vitro using ribozymes targeting degenerate sites or UTRs in retinal transcripts. All 5' and 3' cleavage products produced were of predicted sizes, moreover, transcripts from modified replacement genes remained intact - they were entirely protected from ribozymes in vitro.
Two classes of multi-target ribozymes exist - the shotgun and connected type. To enhance the probability of attaining efficient target mRNA suppression, a connected-type multimeric ribozyme construct consisting of four monomeric hammerhead ribozymes linked in tandem was designed. This class of ribozyme functions by simultaneously cleaving multiple sites in the target RNA and thus may be in some instances equivalent to administering multiple monomeric ribozymes, each directed towards separate target regions on the RNA.

As kinetic profiles of ribozymes \textit{in vitro} can offer insights into their potential activities \textit{in vivo}, a detailed kinetic evaluation of the mutation-independent ribozymes that proved most efficient in \textit{in vitro} analyses was performed. Kinetic parameters such as $V_{\text{max}}$, $K_m$, $k_2$ and $k_{-1}$ were established for each retinal specific ribozyme. Comparative analyses of ribozyme activity revealed substantial differences in their kinetic performances and clearly identified a number of highly efficient ribozymes. For example, Rz40 was shown to cleave a highly structured RNA efficiently and when compared to many ribozymes reported in the literature was shown to be equally or in some instances more efficient (see Chapter 4 for details).

Results obtained from the \textit{in vitro} studies were used as the criteria to determine which ribozymes were further analysed. Stable rhodopsin and peripherin COS-7 cell lines were generated to facilitate cell culture analyses. In addition, to protect ribozymes from degradation in cell culture systems and thus potentially improve their overall catalytic efficiencies, chemical modification techniques were investigated. A number of cell lines were characterised for expression of each retinal transcript by RT-PCR and/or Northern blotting techniques. Suitable lines were chosen, transiently transfected with the most efficient ribozymes and the levels of retinal transcript monitored for a down-regulation in expression. Non-specific ribozymes were used as controls. Preliminary results have demonstrated up to 30\% reduction in human rhodopsin transcript was observed following transfection with ribozymes when compared to a control ribozyme targeting human peripherin/RDS RNA. Given that optimal transfection of COS-7 cell lines results in only approximately 50-55\% of cells being transfected, this inhibition observed was very encouraging.

In summation, a number of hammerhead ribozymes targeting rhodopsin and peripherin transcripts have been screened both \textit{in vitro} and \textit{in vivo} and a number of extremely efficient ribozymes identified. Data suggests that these ribozymes may provide mutation-independent methods of treating adRP. This work is currently progressing towards the evaluation of these ribozymes in clinical models. Therefore the end of chapter 5 describes the generation of a construct with the most efficient ribozyme targeting human rhodopsin and driven by the mouse opsin promoter for the future generation of a transgenic mouse carrying this ribozyme.
The following abbreviations were used:
RP: retinitis pigmentosa
adRP: autosomal dominant retinitis pigmentosa
CMV: cytomegalovirus
bp: base pairs
dNTPs: dideoxy nucleoside-triphosphates
Rz: ribozyme
S: substrate
Rz-S: ribozyme-substrate complex
PCR: polymerase chain reaction
RT-PCR: reverse transcriptase polymerase chain reaction
TEMED: N, N, N, N-tetramethylethlenediamine
MgCl₂: magnesium chloride
k₂: chemical cleavage rate of substrate RNA
k₄: substrate dissociation rate
t₁/₂: half-life of substrate RNA
V₂₅₀: maximum velocity of ribozyme-substrate reaction
Kₐ: affinity of ribozyme for substrate RNA
MOPS: 3-[N-morpholino]propane-sulphonic acid
APS: ammonium persulphate
EDTA: ethylenediaminetetracetic acid
NaCl: sodium chloride
DMEM: Dulbecco’s modified eagles medium
FCS: foetal calf serum
kDa: kilo daltons
Mₘ: molecular weight
SDS: sodium dodecyl sulphate
SNP: single nucleotide polymorphism
ODN: oligodeoxynucleotide
NCp7: nucleocapsid protein of HIV-1
A1: heterogeneous nuclear ribonucleoprotein A1
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
TNF: tumour necrosis factor
SV40: simian virus 40
BGH: bovine growth hormone
MMLV: Moloney murine leukemia virus
AMV: avian myeloblastosis virus
HIV: human immunodeficiency virus


CHAPTER 1

Introduction
In the last few decades, considerable advances have been made in elucidating the genetic aetiologies of inherited disease. This, together with the generation of efficient gene delivery vehicles, suitable animal models and for example novel therapeutic agents, has enabled researchers to focus on the development of gene therapy approaches for both inherited and indeed infectious disorders. Many investigations performed to date have concentrated on recessively inherited disorders as the delivery of wild-type protein should, in many instances, be adequate to ameliorate disease pathology. However, the development of strategies for dominantly inherited disease is encumbered by the need to suppress the mutant allele and the immense genetic heterogeneity commonly associated with dominant disorders. Two ribozyme-based approaches which are independent of the disease mutation and thereby circumvent complications of intra-allelic heterogeneity are examined in this thesis using a group of hereditary degenerative retinal diseases, termed retinitis pigmentosa.

1.1 RETINITIS PIGMENTOSA

1.1.1 Clinical symptoms associated with retinitis pigmentosa

The term retinitis pigmentosa (RP) was first coined by Donders in 1855 and is used to describe a group of hereditary degenerative diseases of the retina, primarily involving the death of rod photoreceptor cells. It is the most prevalent retinopathy showing clear Mendelian inheritance and currently afflicts approximately 1.5 million people world-wide. RP is both clinically and genetically heterogeneous. Clinical symptoms may vary between patients and even among family members afflicted with the disease. Typically, as rod photoreceptor cells die there is a progressive development of night blindness or nyctalopia. In some instances, progression of the disease is rapid, with nyctalopia developing within the first decade of life whereas in others, night vision problems may not manifest until the fifth decade or beyond (Heckenlively, 1988). Extensive pathological changes in the retina occur following rod cell death. The cone cells begin to degenerate and there is a gradual constriction of the patients visual fields. There is a visible ‘thinning’ of the retina and an attenuation of blood vessels supplying retinal tissue. Retinal pigmentary changes also occur, typically being perivascular and having a characteristic ‘bone-spicule’ appearance [Figure 1.1]. Pigmentary deposition on the retina occurs due to degradation of the retinal pigment epithelial and photoreceptor cell layers (Heckenlively, 1988). As the degeneration
progresses, the pigmentary changes extend both posteriorly and anteriorly culminating in the formation of a ring-like scotoma in the visual field. Further contraction of the visual field leaves a tiny island of central vision remaining, commonly referred to as ‘tunnel vision’ (Kanski, 1984). In many cases, the extensive loss of both rod and cone cells together with substantial tissue degeneration leads to complete blindness (Humphries et al., 1990, 1992). Fundoscopic data characteristically reveals a ‘waxy’ pallor of the optic disc due to gliosis of the nerve head. Electroretinogram (ERG) studies, which measure the electrical response of retinal cells following exposure to flashes of light, show a reduced or extinguished signal depending on the extent of retinal damage (Heckenlively, 1988). ERG analysis normally provides a reliable presymptomatic diagnosis of RP as ERG abnormalities generally precede the onset of symptoms (Humphries et al., 1992). ERG analysis on a patient following light-adaptation allows the cone response to be measured whereas rod responses are measured subsequent to dark adaption. Atypical forms of RP can also occur such as RP sine pigmento, sectoral RP and retinitis punctata albescens. RP sine pigmento patients manifest all the classical symptomatic and electrophysiological features but without any visible pigmentary disturbances. In sectoral RP, the disease pathology seems confined to a localised region of the retina whereas retinitis punctata albescens is characterised by a scattering of white dots in the mid periphery region (Humphries et al., 1990). RP can be inherited in an autosomal dominant (adRP), autosomal recessive (arRP), X-linked (xlRP), digenic and mitochondrial fashion. Applying genetic linkage analysis, a number of retinopathy causing genes have been localised to specific regions of human chromosomes and in some cases the disease mutations identified (for details see http://www.sph.uth.tmc.edu/Retnet/disease.htm).

1.1.2 Rhodopsin and RP

Rhodopsin is a member of the G-coupled receptor family found in the outer segment discs of rod photoreceptor cells. It is a transmembrane photoactive protein composed of an opsin molecule covalently bound to a chromophore 11-cis retinal (an aldehyde of vitamin A). The 38kDa protein extends through the entire thickness of the disc membrane and accounts for approximately 85% of the total protein content of disc structures (Dowling, 1987). The protein structure can be subdivided into three structural regions, the intradiscal, disc membrane and cytoplasmic domains. It consists of seven α-helical transmembrane domains aligned perpendicularly to the rod outer segment disc surface, with the amino terminus of the protein residing in the lumen of the disc and the carboxy terminus lying in
the cytoplasm [Bird, 1995; Figure 1.2a]. This structure resembles a 'cage' which surrounds and facilitates binding at codon 296 of 11-cis retinal. While the carboxyl terminus of the protein is believed to be the site of phosphorylation by rhodopsin kinase, the cytoplasmic side of the molecule is involved in binding of transducin as part of visual transduction [Franke et al., 1992; section 1.1.5]. However, the molecule is not rigidly anchored within the lipid bilayer and has the ability to both rotate and move laterally. The main role of rhodopsin is in the initiation of the visual cascade. However, a study by Humphries et al. (1997) demonstrated that homozygous recessive mice with a targeted disruption of the murine rhodopsin gene did not elaborate rod outer segments (ROS). Heterozygotic animals, while retaining the majority of their photoreceptors, displayed some structural disorganisation of both inner and outer segments. This suggests that rhodopsin may also have a structural role in the proper development and maintenance of ROS.

The first gene implicated in causing RP was localised to chromosome 3q21-q24 using a large adRP family of Irish origin (McWilliam et al., 1989). The rhodopsin gene had previously been localised to this region (Nathans et al., 1986; Sparkes et al., 1986) and because of its role in visual transduction [section 1.1.5] represented a likely candidate for RP. This was confirmed by identification of the first adRP mutation in the rhodopsin gene, a proline-histidine substitution at codon 23 (Dryja et al., 1990). It has been estimated that 12% of American patients suffering from adRP carry this transversion in their rhodopsin gene (Rosenfeld and Dryja, 1995). However, it has never been identified in a European population suggesting that its presence in the American population is due to a founder effect (Farrar et al., 1990b). Further analysis of the Irish pedigree in which adRP had been mapped close to the rhodopsin gene showed that a methionine to arginine substitution in codon 207 was responsible for the disease (Farrar et al., 1992). These studies were but some of the many that highlighted the extensive allelic heterogeneity that exists in rhodopsin related adRP. In fact, over 100 rhodopsin mutations have now been identified and the vast majority of these are known to cause dominant RP (see OMIM database - http://www.bjmu.edu.cn/bi/ncbitest/0000590j.htm).

Many transgenic animal models for the study of rhodopsin-based retinopathies have been generated, some of which should prove valuable for assessing, for example, the potential therapeutic approaches explored in this thesis. Olsson et al. (1992) generated a number of lines of transgenic mice carrying either a Pro23His mutant or wild-type rhodopsin gene.
from a patient with adRP. All three mutant rhodopsin lines manifested a photoreceptor degeneration similar to human adRP; notably, the line least affected expressed the lowest level of the P23H transgene (one-sixth the level of endogenous mouse rhodopsin). Moreover, analysis of two lineages of wild-type rhodopsin mice, one expressing approximately equal levels of transgene and endogenous murine rhodopsin and another expressing approximately five times more transgene, demonstrated a strong dosage effect; while mice expressing equal amounts of both genes maintained normal retinal function and structure, mice over-expressing the transgene manifested a retinal degeneration similar to that observed in mutant rhodopsin lines. This study has serious implications for gene therapy strategies involving delivery of wild-type rhodopsin to mice so as to prevent adRP disease pathology arising (such as those proposed in chapter 3) as it highlights the importance of controlling accurately the levels of expression of the delivered transgene.

Similarly, a transgenic rat model for rhodopsin-linked adRP carrying a human P23H mutant rhodopsin transgene has been generated and used to analyse a mutation-specific ribozyme-based therapeutic approach for combating adRP (Lewin et al., 1998). Moreover, transgenic mice carrying dominant murine rhodopsin mutations such as Val20Gly, Pro23His, Pro27Leu (Naash et al., 1993), Gln344ter (Sung et al., 1994), Pro296Glu (Li et al., 1995), Pro347Ser (Li et al., 1996) have been developed. In one such study, the biochemical defect(s) associated with a naturally occurring stop codon mutation, Gln344ter, was investigated; this mutation results in the removal of the five terminal amino acids of rhodopsin. While mice expressing the Gln344ter transgene showed relatively normal light-stimulated rod responses, antibody studies indicated that the Gln344ter rhodopsin protein (but not endogenous rhodopsin) was abnormally accumulated in the plasma membrane of the photoreceptor cell body. This suggests that the carboxyl terminus of rhodopsin is required for correct transportation to ROS disks (Sung et al., 1994).

Another larger animal model for RP is the Pro347Leu transgenic pig (Petters et al., 1997). This mutation is known to cause severe early onset RP in humans. Morphological and electrophysiological data demonstrated that the pig retinal degeneration closely resembles that observed for human RP; early and severe rod photoreceptor cell loss together with a slow degeneration of cone cells. These animal models may be extremely useful as in vivo systems to analyse rhodopsin-based mutation-independent gene therapy strategies for combating adRP addressed in chapters 3, 4 and 5 of this thesis.
1.1.3 Peripherin/RDS and RP

Many studies performed on adRP families showed no linkage to 3q which suggested that a gene(s) other than rhodopsin was responsible for the disease. One such investigation using another large Irish family revealed a linkage on chromosome 6p, which was in the same region as a suitable candidate gene, peripherin/RDS (Farrar et al., 1991a). For some time, it had been known that the naturally occurring retinopathy of mice called retinal degeneration slow (rds) involved a defective form of peripherin/RDS (Connell et al., 1991). Mice heterozygous for the rds mutation produce distorted and vacuolated discs whereas homozygotic animals do not elaborate ROS (Sanyal and Jansen, 1981; Jansen and Sanyal, 1984; Connell et al., 1991). As expected, analysis of the human homologue in adRP patients showing linkage to 6p revealed the presence of mutations within the peripherin/RDS gene (Farrar et al., 1991b; Kajiwara et al., 1991). To date, over 40 different mutations have been identified in peripherin/RDS that give rise to adRP and various retinal degenerative diseases (Farrar et al., 1991; Kajiwara et al., 1991; Nichols et al., 1993; Wells et al., 1993; Gruning et al., 1994; Molday, 1998). Moreover, up to 30 different genes have now been implicated in the aetiologies of RP and related disorders thus emphasizing the extensive genetic heterogeneity inherent in them (http://www.sph.uth.tmc.edu/Retnet/disease.htm). For example, mutations in the retinal specific CRX and Nrl genes (encoding retinal specific transcription factors) and the βPDE gene (encoding a protein fundamental to visual transduction - section 1.1.5) have been implicated in adRP and arRP respectively (McLaughlin et al., 1995; Sohocki et al., 1998; Bessant et al., 1999). For a complete list of RP related genes see Table 1.1.

The peripherin/RDS gene is composed of three exons coding for 193, 83 and 70 amino acids respectively and is highly conserved in many species including mice, rats, cattle and humans (Connell and Molday, 1990). It encodes a 39kDa glycoprotein located in the rim region of the outer segment discs of both rod and cone photoreceptor cells (Connell and Molday, 1990; Figure 1.2b). Unlike rhodopsin, peripherin/RDS is not involved in visual transduction and is believed to play an essential role in ROS and cone outer segment morphogenesis and stability. The introduction of wild-type peripherin/RDS into the homozygous rds mouse (discussed above) has been shown to completely rescue the photoreceptor degeneration (Travis et al., 1992). The protein is believed to consist of four membrane spanning α-helical domains with both the amino and carboxyl termini facing the cytoplasmic side of the disc membrane (Connell and Molday, 1990). Current models
suggest that it contains two hydrophilic intradiscal loops, one being much larger than the other containing seven highly conserved cysteine residues (Molday, 1998). The precise function of these highly conserved domains is unclear but it has been speculated that the larger segment may be involved in protein-protein interactions which aid in maintenance of the integrity of the disc membrane. Indeed, peripherin/RDS and another retinal protein, rom-1, have been shown to contain homologous subunits and form an oligomeric membrane protein complex found in discs. These proteins form a tetrameric complex which link, through intermolecular disulphide bonding mediated by one of the highly conserved cysteine residues (Cys 150) within the large intradiscal loop of peripherin/RDS, to form higher order oligomers which may uphold disc structure (Molday, 1998). Recently, studies have shown that mutations in peripherin/RDS in conjunction with mutations in rom-1 can cause digenic RP (Kajiwara et al., 1994; Dryja et al., 1997). However, analysis of a rom-1 knockout mouse demonstrated that peripherin/RDS was capable of self-assembly into a homotetramer and of supporting rod and cone outer segment formation (Molday, 1998). This would suggest that peripherin/RDS may be the dominant subunit of the complex required for disk morphogenesis and that rom-1 may serve an auxiliary function such as enhancing stability or fine tuning disk structure (Molday, 1998). A transgenic mouse model of peripherin related adRP which is ideal for the analysis of peripherin-specific therapeutic strategies was generated by Kedzierski et al. (1997). This animal was designed to express a Pro216Leu mutant allele which was known to cause RP in humans. Disease pathology was caused by both the dominant-negative effect of the mutant protein and haploinsufficiency.

1.1.4 Photoreceptor cells

Two populations of photoreceptor cells are found in the retina, namely rods and cones. Rod cells are situated from the far periphery to the centre of the retina, excluding the cone rich macula densa, and operate under dim lighting conditions. The remainder of cone cells are interspersed between rod cells throughout the retinal layers and function in bright light and in colour vision. Cone cells can be sub-classified into red, green and blue categories as determined by the frequencies of light they absorb. It has been estimated that humans possess approximately 150 million rods and 7 million cones (Dowling, 1987). Both photoreceptors are elongated cells composed of several functionally and morphologically distinct regions [Figure 1.3]. The photoreceptor outer segment is a highly specialized compartment in which visual transduction takes place. Electron microscopic analysis has
shown that it contains a column of flattened disk-like structures which arise from invaginations in the plasma membrane (Djamgoz et al., 1995). However, unlike ROS discs which are closed structures, cone discs remain open and part of the plasma membrane (Dowling, 1987). These outer segment disc membranes contain the visual pigments necessary for visual transduction [section 1.1.5]. Photoreceptor outer segments discs are continually renewed with new discs being added at the base of the outer segment and old discs being shed and phagocytosed by the retinal pigment epithelium (Djamgoz et al., 1995; Molday, 1998). The outer segment is linked to the inner segment by a thin non-motile cilium; this compartment contains high concentrations of mitochondria, golgi apparatus, ribosomes and other subcellular organelles and is where the energy required for visual transduction is produced (Djamgoz et al., 1995; Molday, 1998). Adjoining the inner segment is the nuclear cell body which further extends to the synaptic terminus of the photoreceptor cell; this is where the electrical signal produced by the cell is transmitted to other retinal neurons.

1.1.5 Visual transduction cascade

Visual transduction is the mechanism by which photons of incoming light are processed into neural signals which in turn are transmitted to the brain for conversion into visual images. This complex process is a cascade involving the activation of a number of key proteins that in total takes approximately 1-2 msec (Stryer, 1986). Similar transduction mechanisms are found in both rod and cone photoreceptor cells; visual transduction in rod cells is presented below. A diagram depicting the fundamental reactions in phototransduction can be seen in figure 1.4.

The first protein to be activated in the visual cycle is the photoactive pigment - rhodopsin. Absorption of a quantum of light by the visual pigment results in isomerisation of the chromophore from an 11-cis form to an all-trans conformation. This initiates a series of conformational changes in the opsin molecule which ultimately leads to the excitation of the rod photoreceptor cell and separation of the chromophore from opsin. Released retinal may be isomerised back to the 11-cis conformation and therefore spontaneously re-join with opsin to form rhodopsin or alternatively be enzymatically converted to vitamin A for storage. In conditions of continuous bright light, the majority of released chromophore is reduced to vitamin A and stored in the pigment epithelium until required for regeneration of rhodopsin during dark adaptation (Dowling, 1987). Photoactivated rhodopsin
(metarhodopsin II) has the ability to interact with the G-protein ‘transducin’ (Palczewski and Saari, 1997). In an inactive form, this protein consists of three subunits, α, β and γ non-covalently bound to guanosine diphosphate (GDP). Binding of metarhodopsin II catalyses the exchange of a molecule of GDP for a molecule of guanosine triphosphate (GTP) on the G-proteins surface (Molday, 1998). During this process, the GTP charged α subunit of transducin (Tα-GTP) dissociates from its inhibitory β and γ units. While the β and γ subunits interact with another protein, ‘phosducin’ (thereby preventing re-binding of the α subunit and continued reactivation of transducin), the liberated and active Tα-GTP molecule is able to activate the enzyme cyclic GMP phosphodiesterase (PDE). Free metarhodopsin II is then capable of activating additional transducin molecules (Djamgoz et al., 1995). In this primary step of the visual transduction cycle, the visual signal is considerably amplified as one activated rhodopsin molecule can activate up to approximately 500 transducin molecules (Dowling, 1987). Activation of PDE is achieved by the displacement of the enzymes two inhibitory γ subunits from its α and β subunits by Tα-GTP. The Tα-GTP-PDEγ complex rapidly dissociates allowing free Tα-GTP to activate new PDE molecules (Djamgoz et al., 1995). Activated PDEαβ quickly hydrolyses cyclic 3',5'-guanosine monophosphate (cGMP), which binds to the cyclic GMP-gated channel protein. This interaction results in degradation of cGMP to an inactive product, 5'-GMP and further amplification of the visual signal; one PDE molecule can degrade approximately 2000 cyclic GMP molecules per second. It is estimated that between photon absorption and cyclic cGMP inactivation, the visual signal is amplified by approximately $10^6$ (Dowling, 1987). The cyclic GMP-gated channel protein allows the movement of monovalent and divalent cations (Ca$^{2+}$ and Na$^+$) across the rod cell membrane (Djamgoz et al., 1995). While cGMP is bound to this protein, the membrane channel of the rod cell remains open allowing Ca$^{2+}$ and Na$^+$ ions to flow into the rod outer segments (Molday, 1998). As K$^+$ ions flow out of the cell through voltage-gated K$^+$ channels in the rod inner segments, a dark adapted current loop is created; Na$^+$ and K$^+$ gradients are regulated by a Na.K ATPase located in the plasma membrane of inner segments while Ca$^{2+}$ concentrations in the outer segments are maintained by a balanced outflow of Ca$^{2+}$ ions through a Na/Ca-K exchanger in the plasma membrane (Molday, 1998). In the dark state, there is a constant release of glutamate (neurotransmitter) from the synaptic termini of photoreceptor cells so as to second other neurons. However, cGMP hydrolysis by activated PDE results in lowering of intracellular cGMP concentrations, closure of the channel and an inhibition of Ca$^{2+}$ and Na$^+$ ion entry into the cell. However, as Na$^+$, Ca$^{2+}$ and K$^+$ ions continue to extrude
from the cell, there is a substantial build-up of an extracellular positive charge. The difference between intracellular and extracellular charge causes hyperpolarisation of the rod cell, an inhibition of glutamate release from the synaptic region and the initiation of a signal which is transmitted along the axon of ganglion cells to the brain (Djamgoz et al., 1995; Molday, 1998).

After photoexcitation the rod cell is rapidly depolarised such that further visual impulses can be processed. This occurs by complete shutdown of the visual cascade and a calcium mediated feedback mechanism. Closure of the cGMP-gated channel coupled with continued Ca\(^{2+}\) extrusion from the rod cell causes a significant reduction in intracellular Ca\(^{2+}\) concentrations. This results in activation of the calcium binding protein 'recoverin' which in turn stimulates guanine cyclase (GC; inhibited by high Ca\(^{2+}\) concentrations).

Guanine cyclase is responsible for converting GMP into cGMP and is regulated by calcium binding proteins known as guanine cyclase activating proteins or GCAPs (Palczewski and Saari, 1997; Molday, 1998). This leads to an increase in cellular cGMP levels and reopening of the cGMP-gated channels. In the meantime, the action of an enzyme 'rhodopsin kinase' causes deactivation of rhodopsin and restoration to the resting state. In an active form, rhodopsin can bind both transducin and arrestin. However, rhodopsin kinase prevents binding of transducin by phosphorylating serine and threonine residues in the carboxyl terminus of the rhodopsin molecule. Arrestin then complexes with phosphorylated rhodopsin and causes the opsin segment of the molecule to dissociate from the all-trans-retinal unit (Djamgoz et al., 1995). The phosphorylated opsin molecule is subsequently dephosphorylated by protein phosphatase 2A (PrP-2A) and can rebind 11-cis retinal. All-trans retinal is then rapidly reduced to all-trans retinol by retinol dehydrogenase (RDH; Palczewski and Saari, 1997). However, the conversion of all-trans retinol to 11-cis retinal is a complex process involving the retinal pigment epithelium (RPE) cells. All-trans retinol diffuses from the photoreceptor cells into the extracellular space where it complexes with the interphotoreceptor retinoid binding protein (IRBP) and is transported to the RPE cells (Ericksson, 1997). In the RPE, all-trans retinol is esterified to all-trans palmitate by lecithin retinol acetyltransferase (LRAT) and subsequently isomerised and hydrolysed by isomerohydrolase (IMH) to 11-cis retinol. 11-cis retinol is then bound by the cellular retinaldehyde binding protein (CRALBP) and oxidised by 11-cis retinol dehydrogenase (11-cis RDH) to 11-cis retinal (Ericksson, 1997). 11-cis retinal is then transported via IRBP back to the photoreceptor cells for use in the visual transduction cycle (Palczewski
and Saari, 1997). As is evident from the above, the rhodopsin protein is of fundamental importance to the success of visual transduction and it is therefore not surprising that one of the main clinical symptoms in patients afflicted by rhodopsin-linked RP is night blindness. Indeed, many of the proteins involved in visual transduction have now been implicated in causing human retinopathies (http://www.sph.uth.tmc.edu/Retnet/disease.htm).

1.1.6 Gene therapy approaches for the correction of genetic disease

Over the last several decades, the genetic aetiologies of many inherited disorders including RP have been elucidated. With a greater understanding of the molecular components involved, it is now timely to explore methods of therapeutic intervention; this concept has been aptly named ‘gene therapy’. For dominantly inherited diseases this typically encompasses an attack on the mutant gene and the replacement of defective genetic material with functional wild-type material. The development of therapeutic strategies for recessive disorders has been the focus of many investigations as effective therapies should only require the replacement or addition of a wild-type allele. However, therapeutic approaches for dominant disease are more complex. In these disorders, the presence of a normal allele does not suppress the negative effects of the mutant allele. Thus, therapeutic approaches based on the introduction of an additional normal allele would not be sufficient to ameliorate disease pathology. Antisense technologies represent a means of inhibiting the dominant negative effect by sequence specific suppression of the mutant allele thereby allowing expression of protein exclusively from the wild-type allele. A replacement gene would only be required if haploinsufficiency contributed to the disease pathology. Methods of gene suppression include antisense DNA and RNA, peptide nucleic acids, triple helix DNA and ribozymes [section 1.2]. However, the extensive genetic heterogeneity associated with dominant disorders including RP further complicates efforts to develop broad-range therapies. Two ribozyme-based mutation-independent strategies which circumvent the difficulties of genetic heterogeneity associated with rhodopsin and peripherin related dominant RP are explored in detail in chapters 3, 4 and 5 of this thesis.

General approaches for the correction of genetic disease could be targeted towards the primary defect, as discussed above, or to a secondary effect, such as apoptosis. It is now generally accepted that the primary cellular pathogenic event associated with photoreceptor cell death in RP is apoptosis (Chang et al., 1993; Protera-Cailliau et al., 1994; Nickells and
Zack, 1996). Cell death is known to occur via two distinct mechanisms; apoptosis and necrosis. Necrotic death is characterised by random DNA fragmentation events, cell swelling, cell lysis and is always associated with an inflammatory response (Kerr et al., 1972). In contrast, apoptosis is a genetically programmed cell death mechanism that is essential for normal development and health. This form of cell death occurs in two phases: a commitment to cell death followed by the execution phase. The latter phase is accompanied by stereotypical morphological changes in cell structure which would suggest the use of common execution machinery in different cell types (Cohen, 1997). Apoptosis is characterised by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, shrinkage of the cells and finally break-up of dying cells into apoptotic bodies which are phagocytosed by neighbouring cells (Cohen, 1997). Thus, in contrast to necrosis, there is no release of intracellular contents which prevents an inflammatory response. Moreover, the nuclear alterations are often associated with specific DNA fragmentation events in which an endonuclease cleaves cellular DNA internucleosomally. This can be recognised as a ‘DNA ladder’ on standard agarose gels and is frequently used as a biochemical hallmark to identify apoptotic cells (Cohen, 1997).

Inappropriate or deregulated apoptosis has been implicated in many human degenerative diseases such as Huntingtons disease, Alzheimers disease, autoimmune disorders and many forms of cancer (Thompson, 1995; Nicholson, 1996; Wyllie, 1997). This has urged intense investigations into the identification of the underlying genetic components involved in the execution of the cell death programme. The activation of a family of structurally related cysteine proteases known as caspases has proven a common feature in apoptotic cells and represents a major control point of apoptosis (Cohen, 1997). These proteases are synthesised as inactive proenzymes which are activated following cleavage at specific aspartate residues. For example, a recent study performed by Liu et al. (1999) demonstrated the activation of caspase-3 in the retina of transgenic rats with the S334ter rhodopsin mutation during light-induced photoreceptor degeneration. The baculoviral cell survival factor p35 has been shown to inhibit a broad spectrum of caspases and has successfully blocked apoptosis in insects (Clem and Miller, 1994; Hay et al., 1994; White et al., 1996), nematodes (Xue and Horvitz, 1995) and mammalian cell systems (Rabizadeh et al., 1993; Beidler et al., 1995). However, until recently it was unclear whether cells that had already committed to apoptosis could still function despite the physiological stresses that induced cell death. Davidson and Steller (1998) investigated the mechanism of cell death in two Drosophila mutant rhodopsin strains, rdgC306 and ninaE1H27, with age-related
retinal degenerations. The ninAE\textsuperscript{RH27} strain carried a dominant rhodopsin mutation (C200Y) whose equivalent in humans causes a severe form of RP. These strains were crossed with flies carrying a transgene which conferred eye-specific expression of p35. Results demonstrated that the retinal degeneration in both strains proceeded by apoptosis and that the presence of the transgene inhibited programmed cell death. This indicated that expression of mutant rhodopsin ultimately lead to caspase activation which in itself was required for the degeneration. Moreover, ERG and walking optomotor analysis illustrated that visual function was preserved in structurally rescued rdgC\textsuperscript{306} and ninAE\textsuperscript{RH27} cells examined. It is believed that caspase inhibition is a result of the formation of a p35-caspase complex following cleavage of p35 substrate by the caspase which would prevent it from initiating the apoptotic cascade (Cohen, 1997). In another study, light-induced photoreceptor apoptosis could largely be eliminated in wild-type mice when mice were bred onto a \textit{c-fos}\textsuperscript{−/−} background (Hafezi et al., 1997). \textit{c-fos} has previously been shown to promote apoptosis, moreover, \textit{c-fos} has also been shown to be elevated in apoptotic photoreceptors in some retinopathies. Down-regulating \textit{c-fos} expression in photoreceptors may provide a general approach for therapies. Many potential targets for the prevention of apoptosis exist (Chen \textit{et al.}, 1996; Nicholson, 1996), some of which may prove to be appropriate routes for gene therapy. With the elucidation of the hierarchy of the caspase cascade (Cohen, 1997), these executioners of apoptosis may prove particularly suitable. For this reason, ribozyme mediated suppression of caspase expression is currently being evaluated by a member of the Ocular Genetics Unit, TCD.

An alternative approach to therapy for retinal degenerations is the use of growth factors to prolong the life of photoreceptor cells. For example, Cayouette \textit{et al.} (1998) demonstrated that intraocular adenovirus-mediated gene transfer of a ciliary neurotrophic factor (CNTF) to the \textit{rds} mouse (see section 1.1.3) reduced photoreceptor loss and caused a significant increase in the length of photoreceptor segments. Moreover, results demonstrated a redistribution and increase in the retinal content of rhodopsin together with markedly improved scotopic ERG responses. This study suggests that continuous administration of CNTF may potentially be useful for the treatment of some retinal degenerations. Similarly, Chong \textit{et al.} (1999) investigated whether repeated intravitreal injection of a human CNTF analogue (Axokine) or brain-derived neurotrophic factor (BDNF) leads to long-term photoreceptor survival in an autosomal dominant feline model of rod-cone dystrophy. Axokine treatment significantly prolonged photoreceptor survival and reduced the presence
of apoptotic cells whereas BDNF and sham-injected eyes were not significantly different from untreated controls. Moreover, repeated injections of axokine over the course of the experiment (17.5 weeks) was shown to maintain the protective effect. Therefore, it was concluded that axokine but not BDNF delays photoreceptor loss in this retinal degeneration. In another very recent study, the neuroprotective potential of glial cell line-derived neurotrophic factor (GDNF) was examined using the retinal degeneration (rd) mouse model of human retinitis pigmentosa (Frasson et al., 1999). In this study, 13 and 17 day old rd mice received subretinal injections of GDNF whereas control animals received saline injections or underwent no procedure. ERG analysis at 22 days showed that 4 of 10 GDNF-treated mice had detectable a- and b-wave responses whereas no ERGs could be recorded from control animals. Moreover, histological examination of these mice demonstrated a significant increase in rod numbers following GDNF treatment compared to controls. Thus it was concluded that GDNF exerts both histologic and functional neuroprotective effects on rod photoreceptors in the rd mouse model of human RP. The studies described above represent but some of the investigations reported in the literature demonstrating that neurotrophic growth factors may aid in the future development of treatments for some forms of hereditary human blindness.

However, targeting secondary effects associated with a disease pathology may not alleviate or may only partially alleviate disease symptoms or indeed may in some instances have deleterious effects. In such situations, targeting the primary defect may be necessary to ameliorate disease pathology. Alternatively, a combination of therapies directed to both primary defects and secondary effects may be required to treat some diseases. For example, modulating photoreceptor apoptosis may in some cases provide an appropriate time-frame in which to undertake correction of the primary defect. It is unclear at this point in time which therapeutic strategies will prove to be successful for human retinopathies, however, it is highly likely that one or more of the approaches being explored will produce viable future therapies for these debilitating disorders.
1.2 GENE SUPPRESSION

1.2.1 Antisense technology

Transcription from two complementary DNA strands in the same region of a gene was first observed in phage λ by Bovre and Szybalski (1969), although its role in gene regulation was only speculative at the time. One of the first reports of an RNA transcript functioning as a naturally occurring repressor of its own gene was the miRNA found in *Escherichia coli* (Mizuno et al., 1984). Since then, many classes of natural occurring antisense RNAs have been identified (for review see Inouye, 1988). These initial findings prompted investigations into the use of artificially engineered antisense oligonucleotides as a means of selectively inhibiting gene expression. In particular, antisense-based therapeutic strategies for cancer and AIDS have been studied extensively (for reviews see Helene, 1991a, 1994; Agrawal and Tang, 1992; Stein and Cheng, 1993; Wagner, 1994). Antisense oligonucleotides comprised of either RNA or DNA specifically bind target RNA via Watson and Crick base-pairing and exert their effect by (a) inducing cleavage of the oligonucleotide-target mRNA duplex by RNase H (b) blocking the correct assembly of the translation machinery (c) enhancing RNA degradation by altering target RNA secondary structure or (d) inhibiting splicing of the pre-mRNA (Helene, 1994). Antisense RNA strategies have the distinct advantage that plasmids encoding the RNAs can be delivered to cell systems and transcribed using the host machinery to produce continuous supplies of antisense RNA. In contrast, antisense DNA approaches require repeated administration as transient expression can only be achieved. To increase the stability, affinity and overall efficacy of antisense oligonucleotides *in vivo*, chemical modifications such as 2'-O-propyl, 2'-O-methyl, 2'-fluoro and phosphorothioate linkages have been incorporated into their backbone structures (Agrawal and Tang, 1992; Stein and Cheng, 1993; Beltinger et al., 1995; McKay et al., 1996). As 2'-O-alkyl modifications do not support RNase H activity, phosphorothioate alterations are preferred (Stein and Cheng, 1993). However, non-specific binding of oligonucleotides to imperfectly matched target sites causing suppression of unintended targets has been reported (Wagner, 1994). Moreover, the degradation products of chemically protected oligonucleotides, such as mononucleotide phosphorothioates, could potentially be re-incorporated and mutagenise cellular DNA (Stein and Cheng, 1993). Nevertheless, the ability of antisense oligonucleotides to produce sequence-specific
gene inhibition has in many cases been established and with further development may provide a realistic method of therapy for human diseases.

An alternative approach for the selective inhibition of gene expression is the use of oligonucleotides which bind to complementary sequence in the major groove of the double helix thereby forming a triple helix. Target sequence for the most part is limited to homopurine-homopyrimidine stretches to which homopyrimidine oligonucleotides bind (Helene, 1994). This triple helix complex can serve as a steric blockade that inhibits the progress of the transcriptional machinery and therefore suppress target gene expression (Helene, 1991b; Postel et al., 1991; Duval-Valentin et al., 1992; Chan and Glazer, 1997). For example, Porumb et al. (1996) demonstrated that a 28-base phosphodiester triple helix-forming oligonucleotide directed to the promoter region of the human oncogene HER2 reduced both mRNA and protein levels by approximately 42% when analysed in MCF7 breast cancer cells. Oligonucleotides can also be specifically designed to form a 'clamp' triple helix structure on single-stranded nucleic acid to inhibit gene expression (Giovannangeli et al., 1993; Helene, 1994). This mechanism consists of an oligonucleotide which binds complementary sequence through Watson-Crick base pairing and a second oligonucleotide that forms Hoogsteen hydrogen bonds with the preformed double helix. These structures have a higher specificity than conventional antisense oligomers as mismatches can be recognised from both the Watson-Crick and Hoogsteen sides (Helene, 1994). However, the potential application of these 'clamp' structures in gene inhibition has not been explored in the same depth as for regular antisense constructs.

Another method for the suppression of gene expression being explored is the use of peptide nucleic acids (PNAs). These molecules are DNA mimics in which the sugar-phosphate backbone has been replaced with a pseudopeptide backbone consisting of N-(2-aminoethyl)-glycine units (Knudsen and Nielsen, 1996). PNAs bind DNA and RNA with high affinity through complementary base-pairing and have been shown to bind double-stranded DNA by a unique strand displacement mechanism to form triple helix structures (Mollegaard et al., 1994). Similarly, PNAs composed of a mixed purine/pyrimidine sequence form PNA-RNA duplexes whereas homopyrimidine PNAs can form (PNA)/RNA triplex structures and have the ability to arrest the translation machinery at target sites (Knudsen and Nielsen, 1996). However, unlike antisense oligonucleotides, PNAs structures are not substrates for RNase H. Nevertheless, they are substantially more...
resistant to intracellular degradation than their equivalent oligodeoxynucleotides (Taylor et al., 1997) and therefore represent another class of specific gene deactivating reagents. For example, PNAs have been proposed as a means of combating heteroplasmic mitochondrial DNA (mtDNA) disorders (Taylor et al., 1997). Intracellular heteroplasmy is a phenomenon associated with the vast majority of patients suffering from mtDNA defects and is characterised by the presence of both wild-type and mutant molecules within the same cell. As disease pathology depends on the level of expression of mutant mtDNA, the possibility exists to selectively inhibit replication of the mutant molecule while allowing propagation of the wild-type molecule. To examine this strategy, PNAs complementary to human mtDNA templates containing a deletion or a single nucleotide mutation associated with human disease were designed and shown to selectively inhibit replication of mutant mtDNA templates but not wild-type templates in vitro. Interestingly, sequence-specific oligonucleotides had previously been tested but were unable to provide selective inhibition. Antisense oligonucleotides and PNAs will not be discussed any further as an alternative approach for the selective inhibition of gene expression is employed throughout this Ph.D. The molecules used are ribozymes (see below) and have the ability to elicit an irreversible sequence-specific cleavage of target RNA in a catalytic manner and thus may in principle be more favourable than any of the gene suppressors discussed above.

1.2.2 RNA enzymes

Until fairly recently it was assumed that all enzymes were proteins and that proteins were the sole catalytic agents of the cell, thereby reducing nucleic acids to nothing more than repositories of genetic information and structural scaffolding. However, the discovery of RNA molecules with the intrinsic ability to catalyse chemical transformations on themselves and on other RNA molecules, in the absence of protein, revolutionised such thinking and completely reformed the general understanding of chemical reactions in biological systems. These catalytic RNAs were termed ‘ribozymes’. The first ever description of a ribozyme was that of the group 1 self-splicing intron of the ciliate *Tetrahymena thermophila*. In the presence of a guanosine co-factor and divalent cation, a 413bp intron in the nuclear rRNA precursor of the protozoan was precisely removed and the two exons re-ligated (in complete absence of protein); hence the splicing activity was intrinsic to the structure of the RNA. Analysis of the excised intron demonstrated that it was modified during the process with the guanosine covalently coupled to its 5'-terminus (Kruger et al., 1982). These self-splicing introns are examples of ribozymes that act in cis.
Cis-acting ribozymes catalyse only one turnover reaction, are normally modified during the process and when compared to enzymatic reactions are considered quasi-catalytic (Cech and Bass, 1986).

Pioneering research by Cech and colleagues in the late 1970’s generated much interest in the field of RNA catalysis and studies focused on isolating RNA enzymes that could function in trans; the catalytic and target strands being on separate molecules. In 1983, Altman and co-workers, demonstrated that the 377 nucleotide RNA component of E.coli RNase P could itself catalyse the precise cleavage in vitro of pre-tRNA molecules into mature tRNAs, with multiple turnover and without being modified, under elevated Mg\(^{2+}\) conditions. Moreover, the M, 14,000 RNase P protein subunit was shown to lack nuclease activity and to simply provide auxiliary functions such as stabilising the catalytically active RNA structure under intracellular conditions and modifying the substrate specificity of the enzyme (Guerrier-Takada, 1983; Gardiner et al., 1985). This was the first description of a truly catalytic RNA molecule. The self-splicing group I intron and prokaryotic pre-tRNA processing machinery produce cleavage products with 2’-, 3’-hydroxyl groups and usually 5’-phosphorylated hydroxyls (Symons, 1992). However, another class of ribozymes exists in which the RNA molecule, in the presence of Mg\(^{2+}\) or other divalent cations, undergoes self-cleavage to produce fragments with 5’-hydroxyl and 2’, 3’-cyclic phosphate termini. In this instance, the cleavage reaction is a nonhydrolytic transesterification that is theoretically reversible (Symons, 1992). These divalent metal ion dependent, self-catalysing RNA reactions can be sub-divided into four groups; the hammerhead, hairpin, hepatitis delta virus (HDV) and Neurospora VS RNA ribozymes.

1.2.3 Hammerhead ribozymes

Of all the catalytic RNAs, the hammerhead ribozyme is the smallest. Many naturally occurring hammerhead ribozymes have been identified within both plant and animal RNA viruses such as, the avocado sunblotch virus (ASBV), the satellite RNAs of tobacco ringspot virus (sTobRV) and the Lucerne transient streak virus (vLTSV) and are considered to play an essential role in their replicative process (Symons, 1989; 1992). These pathogenic RNAs use the rolling circle mechanism of replication which requires highly specific cleavage within the concatameric RNAs to produce monomeric units (Symons, 1992). Self-catalysed cleavage of these RNAs occurs in cis; a single RNA molecule comprises all the necessary functions required for cleavage. RNAs of both
STobRV and vLTSV provide ideal examples in which a single contiguous stretch of 52 nucleotides is all that is required for self-cleavage to occur (Haseloff and Gerlach, 1988). In contrast, self-cleavage of ASBV requires the participation of two widely separated sequences on the intact RNA (Haseloff and Gerlach, 1988). Comparative analysis of the catalytic domains of naturally occurring RNAs enabled conserved features of the hammerhead structure to be deduced. The structure basically comprises of a single-stranded central core of 13 conserved nucleotides surrounded by three base-paired stems, I, II and III (Symons, 1992; Figure 1.5a). Based on the observation that the interaction of two separate and independent RNA molecules could form ‘active’ hammerhead structures, researchers began to engineer ribozymes to function in trans against other RNA molecules. Uhlenbeck (1987), developed a simple system based on (-) ASBV in which two synthetic DNA templates of 19 and 24 nucleotides were generated and transcribed in vitro. When both RNAs were mixed, they base-paired to form the conserved hammerhead structure and efficient cleavage of the 24 nucleotide RNA occurred. The smaller RNA was capable of promoting multiple-turnover cleavage reactions and was unaltered in the process; thus it possessed all the properties of an RNA enzyme (Uhlenbeck, 1987). However, as the target RNA (24 nucleotide) also contained conserved sequences and secondary structure, this system was unsuitable as a general model for the design of ribonucleolytic RNA enzymes with broad sequence specificity. Extensive investigations into the single self-cleaving domains from (+)STobRV and other naturally occurring self-cleaving RNAs resulted in elucidation of the hammerhead catalytic centre and the design of de novo oligoribonucleotides possessing new and highly specific endoribonuclease activities (Haseloff and Gerlach, 1988). This model ribozyme consists of (a) a region containing the sequence GUC adjacent to the cleavage site, (b) a region of highly conserved sequence and secondary structure and (c) flanking regions of antisense RNA which provide ribozyme specificity [Figure 1.5b].

1.2.4 Hairpin ribozymes

The 359 nucleotide satellite RNA of tobacco ringspot virus (sTobRV) is an intriguing molecule, in that, during rolling circle replication (+) sTobRV self-cleaves via hammerhead catalysis (discussed above) whereas (-) sTobRV was found to undergo site-specific cleavage by an entirely different structure - the hairpin ribozyme (Buzayan et al., 1986; Prody et al., 1986; Haseloff and Gerlach, 1989). Linker insertion mutagenesis and deletion analyses demonstrated that nucleotides 52 to 43 and 222 to 177 of (-) sTobRV
(corresponding to substrate and ribozyme RNA) were required for self-catalysis (Feldstein et al., 1989; Haseloff and Gerlach, 1989). Hampel and Tritz (1989) exploited the separation of both RNA fragments to define the minimum length of ribozyme and substrate necessary for \textit{in trans} reactions; a 50-nucleotide catalytic RNA fragment from (-) sTobRV was shown to be capable of promoting multiple-turnover cleavage of a 14-nucleotide substrate with a \(k_{\text{cat}}\) and \(K_m\) comparable to that of \textit{trans}-acting hammerhead ribozyme reactions (2.1 min\(^{-1}\) and 0.03 \(\mu\)M respectively). Extensive mutagenesis studies of \textit{trans}-cleaving hairpin reactions has resulted in elucidation of the structural framework of the optimised ribozyme-substrate complex. It consists of two short intermolecular helices, H1 & H2, (flanking the N\(^{4}\)GUC cleavage site where N = any nucleotide) and two intramolecular helices, H3 & H4, separated by two internal loops, A & B, which are critical for efficacy (Hampel and Tritz, 1989; Berzal-Herranz et al, 1993; Figure 1.5c). As helices 1 and 2 determine ribozyme specificity, it is possible to target and elicit sequence specific cleavage of any RNA containing an accessible cleavage motif. For efficient catalysis, reactions require divalent cations such as Mg\(^{2+}\), Ca\(^{2+}\) or Sr\(^{2+}\) (Chowrira et al, 1993), as is also the case with hammerhead ribozymes.

1.2.5 Hepatitis Delta Virus ribozymes

Hepatitis Delta Virus (HDV), a satellite virus of hepatitis B, contains a closed single-stranded RNA of approximately 1700 bases. Similar to sTobRV, vLTSV and ASBV viruses, HDV RNA is hypothesised to replicate by the rolling circle mechanism and both genomic (-) and antigenomic (+) RNAs can undergo site-specific self-cleavage (Wu \textit{et al}., 1989; Symons, 1992). Mutational analyses performed to identify the minimum sequence requirements necessary for (-) HDV self-cleavage identified a contiguous stretch of 117 nucleotides with a predicted RNA secondary structure in a cloverleaf arrangement (Wu \textit{et al}., 1989). Using an alkaline degradation protocol, the minimum RNA required was further reduced to 82 nucleotides with evidence suggesting that only one to three nucleotides 5' of the cleavage site was required for self-cleavage (as opposed to three and four for naturally occurring hammerhead and hairpin domains respectively). In contrast to Wu \textit{et al}. (1989), an active secondary structure in the form of a pseudoknot was proposed for the RNA by Perrotta and Been, (1990). However, the observation that both genomic and antigenomic HDV RNAs, which are derived from separate regions of the HDV genome, are approximately 80% homologous provides evidence for the fidelity of a third structural variant - the axehead (Branch and Robertson, 1991). The axehead structure is composed of...
an open region of single stranded RNA surrounded by three base-paired stems [Figure 1.5d,e]. Dissection of the (+) and (-) RNA axehead structures in helix II into a 24 nucleotide ribozyme RNA and a 64 or 65 nucleotide target RNA enabled the development of efficient \textit{in trans} reactions (Branch and Robertson, 1991). Like many other ribozyme motifs, HDV ribozymes require divalent cations for cleavage (Wu et al., 1989) producing products with 2', 3'-cyclic phosphate and 5'-OH termini (Branch and Robertson, 1991). However, the use of \textit{in trans} HDV reactions are not as advanced as, for example, the hammerhead or hairpin ribozymes and therefore have not been as widely employed in gene inactivation studies.

1.2.6 Neurospora mitochondrial VS RNA ribozymes

The Neurospora mitochondrial VS RNA is a single-stranded circular RNA of 881 nucleotides derived from a double-stranded mitochondrial DNA plasmid (VS DNA) which consists of a number of head-to-tail multimeric units (Symons, 1992). Analysis of \textit{in vitro} transcribed full-length and monomeric VS DNA demonstrated that, in the presence of Mg\textsuperscript{2+}, the RNAs self-cleaved producing two products with 5'-hydroxyl and 2', 3'-cyclic phosphate termini (Saville and Collins, 1990). While Guo et al. (1993) has shown that the catalytic core consists of 154 nucleotides, its structure does not appear to resemble any of the other naturally occurring self-cleaving domains (the closest being the group 1 introns) and thus probably represents a previously unidentified and novel self-cleaving molecule (James and Turner, 1997). Further mutational analyses will be required before the VS RNA ribozyme can be used \textit{in trans}. Hammerhead ribozymes were the only ribozymes investigated in this Ph.D and therefore will be the only ribozymes discussed in detail in the following chapters.

1.2.7 Factors influencing hammerhead ribozyme design

The basic principles of hammerhead ribozyme design appear quite simple; identify a suitable NUX target site (N = any nucleotide, X = A, U or C but not G) in a region of an RNA of interest, generate two stretches of antisense oligonucleotides complementary to the target RNA that flank the cleavage motif and place the conserved hammerhead catalytic core sequence in between. However, several factors that can improve the effectiveness of ribozyme functionality should be considered when target sites for ribozyme design are selected.
These include (i) accessibility of the target to the ribozyme, (ii) length and composition of the antisense arms flanking the catalytic core and (iii) the base composition of the cleavage site.

(i) Prediction of mRNA secondary structure:
The catalytic turnover of trans-acting ribozymes both in vitro and in vivo, is dependent on the ability of the ribozyme to bind their target via complementary antisense arms, elicit an intermolecular cleavage reaction at the cleavable motif and dissociate for re-binding of remaining intact RNA. However, the targets for potentially therapeutic ribozymes, large RNAs, readily fold into complex secondary and tertiary structures. Thus, it is to be expected that not all potential target sites in an RNA molecule are equally accessible for ribozyme mediated-cleavage. As target accessibility frequently proves rate-limiting, it is essential for successful ribozyme design, that thermodynamically stable loop regions without base-pairing are identified. Computer algorithms such as MFold or PlotFold (Zucker, 1989) can be employed to assist in the identification of suitable areas in target RNAs. Probable secondary RNA structures are assigned maxima and minima ΔG free energy values that correspond to relatively unstable and stable regions of the RNA respectively. Those with lowest internal energies are most likely to represent the conformational folding pattern of a specific RNA. The use of RNA secondary structure predictions for the selection of suitable ribozyme target sites has previously been reported (L’Huillier et al., 1992; Xing et al., 1992; Hendrix et al., 1996). L’Huillier et al. demonstrated that ribozymes directed towards open-loop regions of the α-lactalbumin mRNA were by far more efficient than those targeting structured areas of the RNA. As most computational algorithms for RNA structure prediction are limited in the length of sequence that can be analysed, distant interactions in a full-length mRNA species cannot be accurately determined. However, it is of note that despite limitations, computer packages like MFold have proven helpful for ribozyme design. More accurate experimental approaches for the identification of cleavable sites within complex RNAs are now available. One such approach utilizes ribozyme expression libraries to identify hammerhead ribozymes targeted to a particular triplet with randomised sequences in the antisense arms (Lieber and Strauss, 1995; Figure 1.6a). This technique enables the screening of whole target RNA molecules without any prior knowledge of the sequence. In principle, substrate RNA can be cleaved at any of the targeted codons, however, more accessible sites are cleaved more efficiently and can be identified from ribozyme cleavage
products. This requires tailing, reverse transcription, PCR and subsequent cloning and sequencing to determine the most efficient cleavage sites (Figure 1.6a). Using this technique and human-growth-hormone RNA as a target, Lieber and Strauss identified seven accessible ribozyme cleavage sites. Ribozymes directed to these sites proved extremely efficient in vitro and successfully inhibited gene expression in cell culture. Moreover, one of these ribozymes was found to inhibit growth-hormone expression in mice (Lieber and Kay, 1996). Another methodology to identify cleavable sites involves the use of a completely randomised oligodeoxynucleotide (ODN) library in conjunction with RNase H to probe for accessible regions within RNA structures (Birikh et al., 1997b; Figure 1.6b). Randomized oligodeoxynucleotides bind accessible areas of transcript that serve as targets for RNase H attack. Analysis of cleavage products allows the identification of open-loop regions within RNA secondary structure for ribozyme design. This technique is based on the assumption that sites accessible to ODN binding will also be accessible to ribozymes. Using a human acetylcholinesterase transcript, Birikh et al. (1997b) generated five ribozymes that proved extremely efficient in vitro. Notably, the most efficient ribozyme designed using this technique was found to be 150-fold more active than the best ribozymes generated using MFold. However, both experimental approaches have their advantages and shortcomings. The method of Lieber and Strauss is difficult and time consuming but enables the identification of precise positions of cleavable triplets whereas the procedure of Birikh et al. although more simple to perform, only offers broad predictions of potential ribozyme annealing sites.

(ii) Length and composition of antisense arms:

The length of the antisense arms flanking the catalytic core of a hammerhead ribozyme is critical to its efficiency. These hybridising antisense arms bind substrate RNA (via complementary base-pairing) and therefore define the specificity of the ribozyme. In general, it is assumed that the longer the flanking sequence, the more specific the ribozyme. However, a balance between providing specificity for the substrate while enabling efficient product dissociation must be achieved. Bertrand et al. (1994) analysed a series of ribozyme/substrate combinations with the same target sequence but different lengths of the ribozyme/substrate duplex. Results indicated that longer flanking regions resulted in reduced ribozyme activity due to an increased binding affinity of the ribozyme for the cleavage products, i.e., a decreased rate of product dissociation. Such a situation results in a stoichiometric cleavage of the target and an inhibition of ribozyme catalysis.
Similarly, a comparison of thermodynamic data obtained for short- and long-armed hammerhead ribozymes indicated that activation entropies became very unfavourable when long-chained ribozyme-substrate complexes were formed (Hammann et al., 1997). Moreover, studies have shown that if the flanking sequence used is too long, the specificity of the cleavage reaction between matched and mismatched substrates can actually be reduced (Werner & Uhlenbeck, 1995). If the cleavage rate of both matched and mismatched substrates is significantly greater than the rate of substrate dissociation, both will be cleaved regardless of the differences in binding affinity. For efficient association in conjunction with fast dissociation (at least in vitro) optimal lengths of antisense flanks on either side of the catalytic core of a hammerhead ribozyme are estimated to be in the range of 6-8 nucleotides (Hertel et al., 1994; Hendry et al., 1997). It has been suggested that asymmetric arms may be more effective than symmetric arms (Hormes et al., 1997; James & Gibson, 1998). Moreover, the chemical composition of the hybridising arms can be varied, for instance by incorporation of DNA. Hammerhead ribozymes with DNA arms are known as DNAzymes and have in some studies been shown to increase the rate of substrate cleavage, stability, and overall rate of turnover when compared to all-RNA ribozymes (Hendry et al., 1992; Taylor et al., 1992; Shimayama et al., 1993; Hendry and McCall, 1995). For example, Goila and Banerjea (1998) compared in vitro the activity of an all-RNA ribozyme and a DNAzyme targeting a region of the CCR5 chemokine receptor transcript (coreceptor essential for HIV-1 entry and fusion). Notably, the DNAzyme was found to be more effective in cleaving the 1376 nucleotide full-length transcript. In addition, cell membrane fusion assays using HeLa-CD4\(^+\) cells demonstrated that cells transfected with the DNAzyme displayed significantly reduced fusion activity by interfering with the functional expression of CCR5 compared to ribozyme controls.

(iii) Base composition of the cleavage site:
In nature, the most commonly found cleavage triplet is GUC, although GUA and AUA sequence permutations have been identified (Keese et al., 1983; Miller et al., 1991). Extensive mutagenesis studies of the GUC motif demonstrated that for a hammerhead ribozyme, a G at the third base inhibited the cleavage reaction, while a U in the central position was necessary for cleavage (Ruffner et al., 1990; Perriman et al., 1992). These findings led to the generally accepted NUX rule (N = any nucleotide, X = A, U or C but not G) which states that any RNA containing an NUX motif can potentially be cleaved by a hammerhead ribozyme. However, marked differences in cleavage efficiencies for each
motif have been reported between research groups. While the naturally occurring GUC motif was always cleaved with a relatively high efficiency (Ruffner et al., 1990; Perriman et al., 1992; Zoumadakis and Tabler, 1995), substrates containing the AUC triplet were cleaved with either a higher efficiency compared to wild-type GUC (Zoumadakis and Tabler, 1995), a reduced efficiency (Ruffner et al., 1990) or cleavage was absent altogether (Perriman et al., 1992). Inconsistencies in data may be explained by differences in structural design and functionality of the hammerhead ribozymes being compared or the reaction conditions used.

An oversight common to all these studies was the reaction scheme employed; to obtain cleavage efficiencies, ribozyme/substrate complexes were allowed to pre-anneal under single-turnover conditions (conditions of ribozyme excess) thereby not allowing for variations in ribozyme hybridisation to substrates containing permutations of the NUX motif which would significantly affect overall efficiency. To address the shortcomings of prior art, Shimayama et al. (1995) performed an in-depth kinetic analysis examining the effect of all possible permutations of NUX on $K_m$ and $k_{cat}$ (the affinity of ribozyme for target RNA and the turnover rate of the reaction respectively). All mutations of the wild-type GUC motif reduced the cleavage efficiency, although some mutations mainly affected $k_{cat}$ whereas others affected $K_m$; for example cleavage triplets with A at the first or third position increased $K_m$ by 35- and 30-fold respectively, while $k_{cat}$ was relatively unaffected.

In fact, while the highest $k_{cat}$ obtained was for the AUC motif (4.4 min$^{-1}$), in agreement with the cleavage efficiency observed by Zoumadakis and Tabler (1995), the $K_m$ value was significantly greater than any other NUX permutation (700nM) indicating a reduction in binding affinity and overall efficacy. $k_{cat}$ and $K_m$ values generated for GUC in the same study were 4.0 min$^{-1}$ and 20nM respectively. While Zoumadakis and Tabler deduced that AUC was a more efficient motif, it is apparent that when kinetic measurements such as $k_{cat}$ and $K_m$ are considered, a more accurate reaction scheme that could not have otherwise been predicted can be obtained. Moreover, the high $K_m$ for AUC together with the high 50°C reaction temperature and longer substrate RNA used in a study by Perriman et al. (1992) may explain the observed abolition of cleavage activity for the AUC motif; the 50°C temperature may have caused the A and U base-paired nucleotides in the cleavage triplet to melt producing an inactive structure (Shimayama et al., 1995). It was concluded that with respect to $k_{cat}$ the optimal cleavage sites are ordered GUC, AUC>GUA, AUA, CUC>>UUC, GUU, UUA, AUU, CUA, UUU, CUU whereas for the overall catalytic
efficiency \( (k_{cat}/K_m) \) the preferred sites are GUC>CUC>>UUC, GUU, AUA, AUC, GUA, UUU, UUA, CUA, AUU, CUU. Eventhough, with respect to \( k_{cat}/K_m \) most mutant substrates were cleaved less efficiently than GUC, the \( k_{cat} \) values generated for AUC, CUC, GUA and AUA motifs in particular were somewhat comparable to the wild-type GUC motif and should operate efficiently under \( k_{cat} \) controlled conditions (saturating conditions of ribozyme or substrate excess).

Furthermore, a study performed by Clouet-d’Orval and Uhlenbeck (1997) demonstrated that two U-A pairs directly 3’ of the cleavage motif can increase chemical cleavage by 10-fold compared to that of conventional hammerhead ribozymes. While both dinucleotide pairs were present in the antisense arm of the ribozyme and not in the catalytic core, it was hypothesised that the fast reaction rate was due to the structure of the two U-A base pairs in the hammerhead, placing the 5’-oxygen leaving group in an optimal position for reactivity. Alternatively, if a structural disruption of one of the base pairs is required for the hammerhead to reach the catalytically active conformation, the U-A pairs may be able to do so more easily than other base pair combinations (Clouet-d’Orval and Uhlenbeck, 1997).

In summation, to optimise the design and efficiency of hammerhead ribozymes, GUC motifs proceeded by two U-A base pairs and present in accessible areas of the target RNA should be chosen. However, if experimental design prevents GUC or U-A dinucleotide usage, AUC, CUC, GUA or AUA motifs may suffice. Moreover, asymmetric antisense arms of 7 and 8 bases respectively which provide adequate specificity but allow efficient product dissociation should flank the catalytic core. The design of hammerhead ribozymes targeting retinal specific transcripts is addressed in detail in chapters 3, 4 and 5 of this thesis.
1.2.8 Role of metal ions in hammerhead ribozyme catalysis

Unlike protein enzymes, ribozymes are always metalloenzymes, demonstrating an absolute requirement of divalent metal ions for efficient catalysis. Dahm and Uhlenbeck (1991) investigated the precise role and effect of using various divalent cations in reactions on the efficacy of the cleavage reaction of a hammerhead ribozyme self-cleaving domain composed of two oligoribonucleotides. The observed rate of cleavage varied depending on the type of metal ion used; cleavage was efficient in the presence of Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\) and Ca\(^{2+}\), slower with Sr\(^{2+}\) or Ba\(^{2+}\) and absent with Cd\(^{2+}\) and Zn\(^{2+}\). However when 0.5mM spermine was included in reactions to stabilise the hammerhead structure, rapid cleavage was observed with Cd\(^{2+}\) and Zn\(^{2+}\) while the rates with Sr\(^{2+}\) and Ba\(^{2+}\) were markedly increased. This would suggest that while certain divalent cations could not promote proper folding of the ribozyme, they were able to stimulate cleavage, hence indicating that ion size is very important for ribozyme functionality. Notably, no cleavage was evident in the absence of divalent cation. Thus, it has been suggested that divalent cations physically aid in structural stabilisation of the hammerhead architecture and are generally required for cleavage chemistry (Dahm and Uhlenbeck, 1991; Pyle, 1993). The hammerhead RNA molecule is an extended polyanion that, for transformation into a catalytically active form, requires a substantial charge neutralisation to occur between its interacting strands. Divalent cations can neutralise this charge and evidence suggests that they can specifically bind sites within complex RNAs (Pyle, 1993). Interaction with functional groups in close proximity, may allow the cations to bridge separate RNA strands and stabilize folded ‘pockets’ thereby representing a form of tertiary interaction reminiscent of the role of Zn\(^{2+}\) ions in the stabilisation of DNA binding domains of Zn transcription factors. In fact, NMR spectroscopic data has demonstrated that Mg\(^{2+}\) ions instigate the structural transformation in the hammerhead ribozyme necessary to generate the catalytically active ribozyme-substrate complex (Orita et al., 1996).

Four mechanisms have been proposed by which a metal ion can potentiate hammerhead ribozyme cleavage of the phosphodiester back-bone of a target RNA molecule (Pyle, 1993; Figure 1.7):

(1) A metal-coordinated hydroxyl group has the ability to serve as a general base, resulting in deprotonation of the 2’-OH group.
(2) The metal ion can directly coordinate with the phosphoryl oxygen. This may leave the phosphorous centre more susceptible to attack or stabilise the oxyanionic negative charge in the trigonal-bipyramidal transition state. Data obtained from a metal specificity switch experiment, in which a single phosphorothioate linkage was inserted at the cleavage site, further implicates direct interaction of the metal group with the phosphate oxygen. The insertion which resulted in substitution of the phosphate oxygen atoms with a sulphur atom caused a strong inhibition of Mg$^{2+}$-dependent ribozyme cleavage; Mg$^{2+}$ cations preferably coordinate oxygen ligands over sulphur ligands (Dahm and Uhlenbeck, 1991). Notably, the cleavage efficiency was completely restored when Mn$^{2+}$ cations, which coordinate both oxygen and sulphur, were utilized. This suggests that the divalent ion directly coordinates with the phosphate at the cleavage site.

(3) Stabilisation of the formation of a negative charge on the leaving group by a directly coordinated metal ion.

(4) A hydrated metal ion can serve as a general acid, donating a proton to the leaving group.

Although it appears that four discrete ions are involved, a single ion has the potential to perform several of the above roles simultaneously (e.g. 1&4 and 1&2).

1.2.9 Protein enhancement of hammerhead ribozyme catalysis

As discussed in section 1.2.7, three fundamental problems with ribozyme functionality which may reduce their effectiveness for use in gene inhibition studies both in vitro and in vivo exist; turnover, specificity and the accessibility of target RNA for efficient ribozyme catalysis. However, proteins such as nucleocapsid protein of HIV-1 (NCp7), heterogeneous nuclear ribonucleoprotein A1 (A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), have been shown to aid ribozymes in overcoming these general limitations (Tsuchishashi et al., 1993; Bertrand et al., 1994; Muller et al., 1994; Sioud and Jespersen, 1996). In one such study, the addition of NCp7 was shown to enhance the cleavage activity of a hammerhead ribozyme (HH16), directed to a short RNA oligonucleotide target (17mer) by 10- to 20-fold. Moreover, the ability of HH16 to discriminate between a fully-matched 17mer target and a smaller mismatched 13mer (with a single base alteration) was increased by ~100-fold in the presence of NCp7 (Tsuchishashi et al., 1993). Similarly,
Bertrand and Rossi (1994) examined the effects of NCp7 and A1, using previously well-defined ribozymes and substrates, on the chemical cleavage reaction, the rate of substrate association and product dissociation. By comparing each reaction rate for transcripts containing the same target sequence but with reduced ribozyme-substrate hybrid lengths, it was possible to elucidate the stage of the reaction and the optimal length of ribozyme flanking sequence at which the effects of A1 and NCp7 were most prevalent. Data indicated while chemical cleavage of ribozyme-substrate duplexes of 13-14bp was unaffected by NCp7 and A1, short unstable (≤12bp) and long (17-20bp) duplex reactions were inhibited by both proteins; it was not determined whether the slight inhibition observed for duplexes ≤12bp was due to an impairment in binding or chemical cleavage. However, it was suggested that as longer helices were not inhibited (13-14bp), it was probably related to the inherent instability of the helix length to support optimal cleavage. In contrast, the 5-fold inhibition observed with ribozyme-substrate helices of 17-20bp was shown to be directly dependent on hybrid length. Moreover, the effect of each protein on the rate of product dissociation was also dependent on the length and stability of ribozyme-substrate duplexes; NCp7 and A1 only enhanced the catalytic turnover of ribozymes with hybridising arms of ≤7bp either side of the catalytic core by increasing rates of product dissociation. Most interestingly, the inhibition of ribozyme binding and catalysis commonly observed with long RNA substrates [section 1.2.7] was significantly alleviated in the presence of A1 and NCp7; ribozyme cleavage of a 950 nucleotide Pit1 mRNA, previously shown to have a reduced catalytic efficiency (10-fold) due to the length of its target (Bertrand et al., 1994), was increased 5-fold by both proteins. This effect was attributed to possible helix-destablising properties of NCp7 and A1 which could result in an unwinding of complex RNA secondary and tertiary structures thereby conferring a structural stability on the target RNA and increasing the probability of successful ribozyme binding and catalysis. It was concluded that optimal protein enhancement of ribozyme binding, product dissociation and overall catalytic efficiency, is attained with ribozymes containing hybridising duplexes of 14 bases (in total). Herschlag et al. (1994) directly demonstrated that NCp7 and A1 enhancement of ribozyme activity was derived from the ability of the proteins to increase the speed of ribozyme-substrate binding and product dissociation from the ribozyme. Moreover, it was demonstrated that NCp7 was capable of resolving a misfolded ribozyme-substrate complex and may possibly serve as an RNA chaperone that prevents misfolding of RNAs or disentangles RNAs already misfolded, thereby ensuring RNA accessibility for its biological functions. In particular, A1, which is
one of the most abundant cellular proteins and as it interacts with almost all mRNAs in vivo, it may play a substantial role in enhancing the intracellular activity of most ribozymes.

Moreover, a study performed by Sioud and Jespersen (1996) with a tumour necrosis factor specific ribozyme (TNF-α-Rz) demonstrated that, like NCp7 and A1, GAPDH possessed unfolding activity and was capable of dramatically enhancing the ribozyme cleavage rate (≤ 25 fold) and overall catalytic turnover. In addition, GAPDH was shown to have the capacity to accelerate ribozyme-substrate binding, particularly when the predicted ribozyme/substrate structure was expected to interfere with the association step. Interestingly, TNF-α-Rz was found to be more stable in vivo than another ribozyme being tested that was specific to interleukin-2 mRNA (IL-2-Rz) and was comparable in length and transfection efficiency. This observation prompted researchers to probe for endogeneous proteins that may be bound to the ribozyme thereby enhancing its stability. The unexpected stability also coincided with the ability of TNF-α-Rz to bind cellular proteins of approximately 38 (± 2) kDa. Purification and analysis of this protein, at the sequence and antigenic level, positively identified it as being GAPDH. This protein has four sub-units, each containing a tightly bound coenzyme molecule (NAD^+). Competition studies demonstrated that NAD^+ and ATP competed with TNF-α-Rz binding. In fact, examination of the ribozymes structure indicated that GAPDH may recognise a putative folding structure that involves the interaction of its 5' end and catalytic core. Moreover, an analysis of various interleukin-2 specific ribozymes showed that the addition of sequence with a high affinity for GAPDH dramatically increased their efficacy in cell culture. Thus, protein enhancement of ribozyme catalysis which was largely assumed to arise from non-specific interactions may in fact be highly-specific. Therefore, it is more than likely that many, as yet unidentified, endogenous proteins analogous to NCp7, A1 and GAPDH exist and may exert similar effects on hammerhead ribozyme cleavage of mRNAs in vivo. One such example, may be a hammerhead ribozyme directed to U3 small nucleolar RNA. Under saturating conditions of ribozyme, only 40% of target RNA was cleaved in vitro. This low activity was presumed to be a function of the folding properties of both ribozyme and substrate RNA. However, when analysed in cell culture, in excess of 90% of target was ablated (Samarsky et al., 1999) which may possibly represent an enhancement of catalysis by intracellular protein interactions. Thus, ribozymes that prove somewhat inefficient in
vitro may through the help of RNA chaperones such as A1 and GAPDH be found to be extremely active in vivo.

1.2.10 Enhancement of ribozyme catalysis by intracellular co-localisation strategies

The cellular compartmentalisation of ribozyme and substrate RNAs has been shown to significantly influence the efficiency of ribozyme reactions in vivo (Sullenger and Cech, 1993; Bertrand et al., 1997; Hormes et al., 1997; Samarsky et al., 1999). While mRNA processing events, such as addition of m7G caps and poly (A) structures and excision of intronic sequence, are known to occur in the nucleus followed by the export of mature RNAs to the cytoplasm for translation into protein, the precise mechanisms of mRNA intracellular trafficking largely remains unclear. However, it is apparent that different mRNA species are sorted to different sub-cellular locations (Kislauskis et al., 1993; Bertrand et al., 1997). Thus, for optimal ribozyme activity both ribozyme and target RNAs should be in the same intracellular compartment, enabling efficient substrate binding and processing.

The importance of co-compartmentalisation on ribozyme functionality was investigated by Sullenger and Cech (1993) using an experimental system that exploited properties of retroviral replication and gene transfer machinery. A murine retroviral construct containing the Moloney murine leukemia virus (MoMLV) packaging signal, \( \psi \), encoding LacZ transcripts with distinct intracellular fates was generated. While some LacZ mRNAs were transported to the cytoplasm for translation into protein, the majority of transcripts served as genomic RNAs for replication of the retroviral vector and localised to sites of viral budding on the surface of packaging cells. In parallel, a \( \psi \)-dependent retroviral construct with a LacZ-specific hammerhead ribozyme sequence embedded in the retroviral genome was created. Thus it was hypothesised that MoMLV encapsidation machinery should co-compartmentalise ribozyme RNA with LacZ containing genomic RNA but not with LacZ mRNA. Therefore, if colocalisation was important for efficient ribozyme catalysis, the reduction in \( \beta \)-galactosidase viral titre should greatly exceed that of \( \beta \)-galactosidase activity in cellular extracts. Notably, the ribozyme was found to reduce infectious LacZ retroviral titres by approximately 90% whereas no reduction in LacZ mRNA was observed.
Similarly, to enhance in vivo ribozyme activity, Samarsky et al. (1999) used U3 small nucleolar RNA as a carrier to localise a hammerhead ribozyme and U3 target RNA to the yeast nucleolus. Using two structural variants of U3 with a unique hybridisation tag, both ribozyme and target RNA were embedded in a common snoRNA structural motif, known as the box C/D motif. The hybrid small nucleolar RNA: ribozyme designated a ‘snorbozyme’ was shown to cleave >90% of target RNA in vivo; in situ hybridisation microscopy demonstrated that both molecules colocalised precisely to the nucleolus as expected. This was reported to be the most efficient in vivo application of any trans-acting hammerhead ribozyme.

To understand the factors involved in RNA localisation and catalytic RNA efficacy in vivo, Bertrand et al. (1997) generated a series of Pol III driven U1 snRNA, U6 snRNA and tRNA<sup>Met</sup> constructs fused to various processing/stabilizing elements and compared their expression levels, sub-cellular localisation and ability to promote efficient RNA catalysis with that of RNA polymerase II (Pol II) driven constructs which included the capping and polyadenylation signals normally required for mRNA production. To facilitate gene transfer in vivo, the cassettes were inserted into Adeno-associated and MoMLV retroviral vectors. When human embryonic kidney cells (293) and African green monkey kidney cells (COS) were transiently and stably transfected with the various expression cassettes, the U1, U6 and tRNA transcripts were shown to express at the highest levels and were nuclear in localisation. In contrast, Pol II promoted constructs were present at lower levels and were localised to the cytoplasm. Interestingly, the absolute levels of expression obtained from all vectors was 20-fold lower in stable lines compared to transiently transfected cells. To assess ribozyme activity in vivo, a ribozyme specific for the TAR region of an SIV-growth hormone reporter gene in a Pol II cassette was stably expressed in 293 cells. Data indicated that while the nuclear localised tRNA, U1 and U6 cassettes did not support ribozyme activity, the expression of Pol II driven RNAs (localised to the cytoplasm) was successfully inhibited. Similar results were obtained in CEM T-cells transduced with a retroviral construct carrying an anti-tat HIV-1 ribozyme when challenged with HIV-1. The combined results demonstrated that the ribozymes tested were only active when present in the same cellular compartment as their respective target mRNAs and that intracellular localisation was dependent on the type of expression cassette employed. Thus, it was likely that the high-degree of co-compartmentalisation observed was induced by a common feature shared by all transcripts and was proposed to be the
poly(A) tail. In accordance with Bertrand et al., sequences in the 3' untranslated regions of, for example, Xenopus, Drosophila and many vertebrate mRNAs have been shown to direct these mRNAs to specific cellular locations (Ding and Lipshitz, 1993). In fact, polyadenylation of mRNAs has been demonstrated to be a necessity for successful export of transcripts from the nucleus to the cytoplasmic compartment (Huang and Carmichael, 1996).

Thus, it is apparent that for efficient ribozyme functionality in vivo, the design of expression cassettes is imperative in that they promote high transcription rates and enable co-compartmentalisation of ribozyme and substrate RNAs. Notably, the same mammalian expression vector (pcDNA3), containing an SV40-derived Pol II promoter and bovine growth hormone polyadenylation signal, [Figures 3.2 and 5.1] was used to generate all ribozyme and target cDNA constructs for use in cell culture studies in this Ph.D; this should promote co-localisation of both RNA species to the cytoplasmic compartment of the cell systems employed and enhance overall ribozyme activity [Chapter 5].

1.3 MULTIMERIC HAMMERHEAD RIBOZYMES

1.3.1 Connected and shotgun multi-target ribozymes

To optimise the efficiency of ribozyme mediated gene suppression, multiple-ribozyme expression vectors containing several ribozymes, each specific for a different target site, have been generated (Chen et al., 1992; Ohkawa et al., 1993; Ramezani et al., 1997; Benedict et al., 1998). Two classes of multi-target vectors exist, the connected type and the shotgun type. The connected vector simply consists of multiple sequence specific ribozymes linked in tandem which are expressed as a single concatameric RNA. In contrast, the shotgun ribozyme combines the self-cleaving capabilities of cis-acting ribozymes (CARs) with trans-acting hammerheads such that several trans-acting ribozymes, directed to the specific RNA of interest, are trimmed at both their 5' and 3' termini by the action of CARs resulting in the liberation of multiple and functionally independent trans-acting ribozymes. As methodologies used to determine RNA secondary structure in vitro can only offer broad predictions of the accessibility of individual ribozyme cleavage sites in vivo, both multi-target constructs, in principle, increase the probability of attaining complete RNA suppression.
For example, the genome or transcripts of the human immunodeficiency virus type 1 (HIV-1) have become prime targets of multimeric ribozyme-based antiviral strategies. While a study using a monomeric ribozyme, directed to the commonly conserved 5' untranslated region of HIV-1, demonstrated a 1000-fold inhibition of HIV-1 replication in primary human lymphocytes for 10-25 days post infection, complete inhibition was not attained (Leavitt et al., 1994). Moreover, as HIV-1 continuously undergoes mutational changes due to the low fidelity of its reverse transcriptase (Preston et al., 1988; Roberts et al., 1988), monomeric ribozyme based targeting strategies may in essence prove unsuitable; if the target RNA or ribozyme cleavage site was to undergo mutation, the monomeric ribozyme would be rendered ineffective. In contrast, if the ribozyme was only a single unit of a multimeric cassette, unaltered sites would still be cleaved by the additional ribozymes present in the construct. Therefore, multimeric ribozyme-based approaches have been proposed as a means of overcoming HIV-1 mutability and addressing overall ribozyme efficacy (Chen et al., 1992; Ohkawa et al. 1993; Ramezani et al., 1997). Chen et al. (1992) generated several mono-, di-, tetra-, penta-, and nonaribozymes directed to relatively conserved sites in HIV-1 envelope (Env) RNA. The connected ribozymes were shown in vitro to retain the specificity of monomeric ribozymes. Furthermore, unlike the monomers, the multimeric ribozymes when embedded and expressed as part of a 3.3Kb transcript were shown to be functionally active. Following co-transfection with the infectious HIV-1 clone pNL4-3, the nonaribozyme (driven by HIV-1 LTR) inhibited virus replication in HeLa T4 cells by 90%. Similarly, Ramezani et al. (1997) engineered a retroviral vector to express a connected multimeric ribozyme targeting nine highly conserved sites in HIV-1 Env RNA. When a human CD4+ T lymphocyte-derived MT4 cell line was stably transduced with the retroviral construct, HIV-1 replication was dramatically inhibited; no viral RNA or protein could be detected for up to 60 days post transfection. In contrast, viral replication was only delayed 9-12 days when the MT4 cell line was stably transduced with a retroviral construct expressing a HIV-1 ENV specific monomeric ribozyme.

In comparison to connected ribozymes, shotgun ribozyme expression systems offer the additional advantage of being able to produce completely trimmed trans-acting ribozymes; extraneous non-specific sequences which flank both ends of ribozyme transcripts can cause deleterious effects on ribozyme efficacy in vivo (Benedict et al., 1998) are completely removed by the specific cleavage action of CARs. Benedict et al. (1998) generated a tetracycline regulated shotgun vector containing two cis-acting ribozymes flanking a trans-
acting hammerhead ribozyme targeted to retinoblastoma (Rb) tumor suppressor RNA. When stably transfected mouse embryo fibroblast cell lines were induced with tetracycline, internal trans-acting ribozymes were efficiently liberated (determined by selective RT-PCR) and substantially reduced Rb mRNA levels by > 70%.

While both shotgun- and connected-type ribozyme expression vectors appear to operate more efficiently than conventional monomeric hammerhead ribozymes, each construct has drawbacks for in vivo utilisation; shotgun ribozymes produce trans-acting ribozymes that lack poly (A) tails thereby reducing their stability and rendering them vulnerable to intracellular degradation. In contrast, whereas connected ribozymes are poly (A) stabilised, the long concatameric RNA transcript may, depending on the RNA in question, adopt an unfavourable (and unpredictable) secondary structure that might inhibit individual ribozyme activity. A connected-type multimeric construct consisting of four trans-acting hammerhead ribozymes directed to human rhodopsin RNA has been generated and analysed both in vitro and in vivo in chapters 4 and 5.

1.3.2 Minizymes and miniribozymes
Mutational analysis of Haseloff and Gerlach’s hammerhead ribozyme model has shown that stem II, consisting of four nucleotides in a loop and eight nucleotides forming a four base paired structure [Figure 1.5], can be completely eliminated or truncated to generate smaller active ribozymes known as minizymes and miniribozymes (McCall et al., 1992; Tuschl and Eckstein, 1993; Hendry et al., 1995). A minizyme is a hammerhead ribozyme in which stem II has been removed and replaced with a short non-base paired linker whereas a miniribozyme is a structure that has just one base pair in stem II. Minizymes with DNA or RNA anisense sequence together with both DNA or RNA linker sequence (typically consisting of 5’- dGTTTT and 5’- rGUUUUC respectively) have been designed (Hendry et al., 1995). Minimised ribozymes have been proposed as a means of overcoming steric hindrance caused by stem II in full length ribozyme-substrate interactions; for example, eventhough cleavage in vitro of an unstructured 13 nucleotide target RNA was slower with a four nucleotide stem II minizyme compared to a full-length ribozyme, the cleavage rate achieved by the minizyme was faster than the full-length ribozyme when a complex 428 nucleotide substrate RNA was used (Hendry et al., 1995). Moreover, minizymes are capable of dimerising and targeting two different cleavage sites simultaneously (Amontov et al., 1996; Kubwabara et al., 1996; Sioud et al., 1997). In one
such study, a number of dimerised minizymes directed to HIV-1 tat mRNA and differing in the number of bases in the short oligonucleotide linker (n = 2-5 bases) were analysed for activity in vitro. All minizymes were shown to elicit two site specific cleavages of HIV-1 RNA. Notably, the dimer with a 5 nucleotide linker proved more active than a full-length hammerhead ribozyme which was only capable of targeting a single site in the HIV-1 RNA (Kuwabara et al., 1996). Like the parental hammerhead structure, single and dimerised minizymes require divalent cations (Mg^{2+}) for cleavage (Amontov et al., 1996). Thus, minizymes represent a third class of multimeric ribozyme.

1.4 RIBOZYME REACTION KINETICS

Many of the experimental approaches used to determine protein enzyme reaction rates also apply to ribozyme analysis. Like enzymes, ribozymes bind their substrate RNA to make bimolecular reactions unimolecular, stabilise the transition state and destabilise the ground state (Cech et al., 1992, 1993; Pan et al., 1993; Hertel et al., 1994; Narlikar et al., 1995). A minimal kinetic description for one turnover of a hammerhead ribozyme reaction involves (a) binding of the ribozyme and substrate to form a ribozyme-substrate complex (b) cleavage of the phosphodiester backbone of substrate RNA to produce ribozyme bound 5' and 3' products (c) two pathways for dissociation of product RNA thereby releasing free ribozyme for re-binding of intact substrate (Hertel et al., 1994). Each step in this pathway, in both forward and reverse directions, is defined by a measurable elemental rate constant. Therefore, by varying the relative molar concentrations of ribozyme and substrate under suitable experimental conditions, the catalytic pathway for the ribozyme can be dissected into individual components and the kinetic constant corresponding to each step determined. For instance, single-turnover experiments in which saturating excesses of ribozyme are used, examines the ability of a ribozyme to promote the conversion of a single substrate to products. Experimentally, by pre-annealing radioactively labeled ribozyme and substrate, all substrate molecules become bound by ribozyme and since substrate association and product dissociation become irrelevant, the rate constant for the cleavage reaction, $k_2$, can be directly measured. Moreover, by comparing the amount of cleaved substrate obtained to that in an experiment in which a large excess of unlabeled substrate is added to the reaction, the rate of substrate dissociation, $k_1$, can be determined. Under these conditions, any labeled substrate that dissociates from ribozyme prior to cleavage is bound by unlabeled RNA and remains uncleaved.
In contrast to single-turnover kinetics, multiple-turnover reactions examine the ribozymes' ability to function as a true re-cycling catalyst. In this case, reactions are performed under conditions of substrate excess and as ribozymes follow Michaelis-Menten kinetic regimes (Fedor and Uhlenbeck, 1992) kinetic parameters such as $V_{\text{max}}$, $K_{m}$, $k_{\text{cat}}$, and $k_{\text{cat}}/K_{m}$ can be determined. Figure 1.8 depicts the classic Michaelis-Menten behaviour of a ribozyme. $k_{\text{cat}}$ is known as the ‘catalytic constant’ or more confusingly as the ‘turnover number’ and defines the capacity of an enzyme-substrate complex, once formed, to generate product. Therefore, the symbol $k_{\text{cat}}$ is often used to represent $k_{2}$ (Cornish-Bowden and Wharton, 1990). For protein enzymes, values of $k_{\text{cat}}$ typically approximate at $10^{3}$ s$^{-1}$, however, for ribozymes the rates are generally several orders of magnitude smaller (Stage-Zimmermann and Uhlenbeck, 1998). However, $V_{\text{max}}$ which defines the ‘limiting rate’ or ‘maximum velocity’ of the ribozyme reaction is probably the most important kinetic parameter as it is a measure of overall turnover. The Michaelis constant, $K_{m}$, is equal to the total of the rates of substrate dissociation, $k_{1}$, and chemical cleavage, $k_{2}$, divided by the rate of substrate association, $k_{1}$ (Cornish-Bowden and Wharton, 1990). $K_{m}$ resembles a dissociation constant in that it has dimensions of concentration and specifies the relative concentrations of free enzyme, free substrate and enzyme-substrate complex under steady-state conditions (Cornish-Bowden and Wharton, 1990). $K_{m}$ values in the nanomolar range are characteristic of most naturally occurring and artificially generated ribozymes (Fedor and Uhlenbeck, 1990). Using a Michaelis-Menten reaction curve, $K_{m}$ can be determined to be equal to the concentration of substrate when the rate of the ribozyme reaction is $0.5 V_{\text{max}}$ [Figure 1.8]. In practical terms, it is a measure of the affinity of ribozyme for substrate RNA. $k_{\text{cat}}/K_{m}$ is a second order-rate constant that combines both ribozyme-substrate binding and cleavage and is frequently used to define and compare the overall efficiency of ribozyme catalysed reactions (Hammann et al., 1997). It has units of concentration and time (M$^{-1}$/min or M$^{-1}$/s) and can be determined from the initial slope of the Michaelis-Menten curve. Michaelis and Menten, the ‘founders’ of enzyme kinetics were first to identify the importance of controlling pH and temperature and using initial rates rather than whole time courses when defining kinetic parameters of enzyme catalysed reactions. Similarly, multiple-turnover experiments to determine ribozyme-substrate parameters should be performed within pre-determined initial rates where no inhibition inherent to the reaction exists. Moreover, as pH, temperature and concentration of divalent metal ion have been shown to effect the rate of ribozyme chemistry, ‘standard’ kinetic reaction conditions of 10mM MgCl$_2$, pH 7.5 and 37°C should be used (Stage-Zimmermann and Uhlenbeck, 1998).
As kinetic profiles of ribozymes in vitro can offer a broad prediction of potential activity in vivo (Birikh et al., 1997a) an in-depth kinetic analysis of multiple hammerhead ribozymes directed to rhodopsin and peripherin transcripts is presented in chapter 4 of this thesis.

1.5 RIBOZYME DELIVERY AND THEIR THERAPEUTIC APPLICATIONS

1.5.1 Targeted trans-splicing

In addition to the application of ribozymes in gene regulation studies, ribozymes can be used as a means of repairing mutant transcripts associated with common genetic disorders (Sullenger et al., 1994; Jones et al., 1996; Jones and Sullenger, 1996; Phylactou et al., 1998a; Lan et al., 1998). This technology utilises the self-splicing ability of Tetrahymena thermophila group 1 introns which consists of a two-step trans-esterification reaction resulting in site-specific excision of the intron and ligation of two flanking exons [section 1.2.2]. The trans-splicing ribozymes are re-engineered such that sections of any RNA (analogous to the 5' exon) can be replaced with 'new' RNA (analogous to the 3' exon) tagged to the 3' terminus of the ribozyme. Typically, the ribozyme is directed to a suitable site upstream of the mutation(s) such that the defective portion can be removed by the cleavage activity of the ribozyme and replaced with an unaffected stretch of RNA to produce a wild-type transcript [Figure 1.9]. As the only sequence required for cleavage is a uridine residue directly preceding the cleavage site and in an accessible area of the target transcript, almost any RNA can be trans-spliced (Phylactou et al., 1998a). For instance, Lan et al., (1998) availed of group 1 trans-splicing machinery to correct mutant β-globin transcripts, associated with sickle cell anemia, in erythrocyte precursors derived from peripheral blood in patients with sickle cell disease. A suitable target site was identified at position 61 of the β-globin transcript (upstream of a point mutation at position 70), cleaved and a 516 nucleotide γ-globin 3’ exon fragment trans-spliced. Thus, the diseased β-globin RNA was converted into an mRNA encoding the anti-sickling γ-globin protein.

Quantitative-competitive RT-PCR demonstrated that approximately 10% of transcripts were corrected. However, trans-splicing ribozymes have been shown to revise up to 50% of substrate RNAs in cell culture conditions (Jones and Sullenger, 1997). Nevertheless, for many genetic disorders the efficiency of RNA repair may not need to be 100% to observe a therapeutic benefit. Sickle cell disease is one such example in that patients expressing γ-globin at 10-20% the level of mutant β-globin in their red blood cells have significantly enhanced clinical prognosis (Lan et al., 1998).
Similarly, Phylactou et al. (1998a) promoted the use of \emph{trans}-splicing technology as a therapeutic strategy for combating diseases associated with trinucleotide repeat expansions (TRE’s). Using myotonic dystrophy as a disease model, they employed an adapted group 1 intron ribozyme (DMPK- Rz1) to modify the number of repeat units at the 3’ terminus of the human myotonic dystrophy protein kinase transcript (DMPK). The ribozyme was shown, both \textit{in vitro} and in a fibroblast cell line, GM04501A, to successfully reduce the 3’ terminus of the artificially generated DMPK transcript from twelve repeat units to five repeats and also to replace the 3’ end of endogenous transcripts containing five CUG repeats with a small fragment containing four units.

However, a major obstacle to overcome with the use of \emph{trans}-splicing ribozymes is their low target specificity (Phylactou et al., 1998). This is particularly relevant when compared to conventional ribozyme technology, in that, non-specific suppression would not merely reduce expression of the non-specific target but would alter it and may lead to the production of novel mutant proteins with deleterious effects. Thus, ribozyme-mediated RNA repair mechanisms still require further optimisation but may in the future provide a therapeutically feasible molecular remedy for the treatment of genetic disease, particularly in situations requiring methods of gene suppression together with gene replacement (such as the proposed therapeutic strategies for combating rhodopsin- and peripherin-linked adRP discussed in chapter 3).

\subsection*{1.5.2 Endogenous and exogenous gene transfer}

The strategies available for delivery of ribozyme/gene suppressors to cell and animal systems can be divided into two broad categories; endogenous and exogenous delivery. Endogenous delivery to cells involves the transfer of a gene encoding a ribozyme/antisense construct present in a suitable mammalian expression cassette such that transcription of the insert can proceed from a promoter of choice; cytomegalovirus (CMV) or SV40 promoters enable constitutive expression of the desired construct whereas tissue-specific promoters such as, for example, rhodopsin and peripherin limit transcription to photoreceptor cells or to cells containing the correct transcriptional machinery to support expression. For instance, Efrat et al. (1994) used an insulin promoter to drive expression of a ribozyme construct in the pancreas of mice to generate an animal model for a type of diabetes named maturity-onset diabetes of the young. Moreover, inducible promoters can be employed to switch on/off gene suppressor expression such that their specific effects can be
experimentally controlled and closely monitored. This system was used to generate transgenic *Drosophila* carrying a heat-inducible driven hammerhead ribozyme specific for the *fushi tarazu* (*ftz*) gene (Zhao and Pick, 1993). Induction of the heat-shock-protein-70 promoter resulted in the reduction of *ftz* protein levels and the generation of an *ftz*-like phenotype in heat-shocked embryos. This experimental system allowed the systematic monitoring of the biological function of the *ftz* gene during various developmental phases and resulted in confirmation of its involvement in neurogenesis during early phase formation of the central nervous system. This study was immensely important for two reasons; it demonstrated the ability of ribozymes to dissect gene function during development *in vivo* and also was the first example of ribozyme activity in a transgenic animal. The main advantage of endogenous delivery is that it allows continued expression of constructs without a need for re-administration of the therapeutic agent. This is particularly important when delivering therapeutic molecules to ocular tissue, as the eye for example, cannot sustain repeated intra-ocular injection (personal communication, Dr. Paul Kenna). In addition, endogenous delivery enables the analysis of suppressor activity in cell-systems by means of transient- or stable-transfection assays [Chapter 5].

Exogenous delivery of ribozymes is an alternative method to introduce pre-synthesised ribozymes into cell systems. In contrast to some of the vectors used for endogenous delivery, this method does not have any effect on the genome and therefore is considered relatively safe. The main advantage of exogenous delivery is that the unstable ribonucleic backbone of ribozymes can be chemically modified to substantially increase their stability in the presence of intracellular nucleases. As RNases require 2'-OH groups for cleavage, ribozymes are normally 2' modified with amino, fluoro, allyl or *O*-methyl derivatives alone or in combination with terminal phosphorothioate linkages or an inverted nucleotide (Pieken *et al.*, 1991; Flory *et al.*, 1996). Some of these modifications have been shown to increase the half-life of unprotected ribozyme RNA in human serum from a matter of minutes to days without any loss of catalytic activity (Taylor *et al.*, 1992; Heidenreich *et al.*, 1994; Burgin *et al.*, 1996; Lierdal *et al.*, 1998). With a view to the development of some of the most efficient ribozymes identified during the course of this study for *in vivo* gene suppression, chemical modifications which aid ribozyme stability have been explored in chapter 5 of this thesis. For example, a 2'-aminouridine modification of a rhodopsin-specific ribozyme is addressed in detail.
However, poor uptake of ribozymes by cells in culture presents a significant challenge for exogenous delivery. Consequently, there are few examples of carrier-free exogenous delivery of ribozymes either to animal or cell culture systems in the literature. One of the few animal studies performed was by Flory et al. (1996); a series of chemically protected ribozymes specific for the metalloproteinase stromelysin (a key mediator in arthritic diseases) were administered by direct intra-articular injection into the knee joint of a rabbit model for interleukin 1-induced arthritis. The ribozymes were shown to be taken up by cells in the synovial lining and were 70-90% intact 3 days post-administration. Significant reductions in the levels of IL-1-induced stromelysin mRNA in the synovium were observed. Similarly, Flores et al. (1997) demonstrated that an exogenously delivered phosphorothioate protected ribozyme, specific for the carbamoyl phosphate synthetase II gene of the human malarial parasite *Plasmodium falciparum*, was capable of reducing malarial viability in infected erythrocytes by up to 55%. This study is the only example to date of successful ribozyme utility in a cell culture system without the use of a carrier to enhance cellular uptake (to the best of the author’s knowledge). Moreover, it is the first reported assessment of the ability of ribozymes to function as anti-malarial therapeutic agents. However, there is some suggestion that this study may possibly be unique in that infected erythrocytes are particularly susceptible to the uptake of extracellular material and that the cells must have possessed low RNase activity as the phosphorothioate modification made to the ribozyme would not routinely provide the level of protection against intracellular degradation required to obtain the presented results (Vaish et al., 1998).

Significant improvement of cellular uptake can be achieved when cationic liposomes are used to aid delivery. These agents have been successfully employed in both cell culture and animal studies (Kisich et al., 1995; Scherr et al., 1997; Gonzalez et al., 1998; Kijima et al., 1998). Scherr et al. (1997) used a cationic lipid (LipofectAMINE) to deliver a ribozyme specific for a point mutation at position 763 of the N-ras gene, to HeLa cells. The experimental design was such that a luciferase gene was placed under the control of the N-ras gene and promoter. Notably, a 54% reduction in luciferase activity was observed when the ribozyme was delivered to HeLa cells. In contrast, no inhibition of expression was detected in a control experiment in which wild-type N-ras was fused to luciferase. However, a significant drawback with liposome-mediated gene transfer is that delivered molecules often become trapped in endosomes and are unable to traverse into the intracellular space (Gregoriadis, 1995; Zelphati and Stoka, 1996). Alternative carriers other than cationic liposomes can be used for ribozyme delivery. For example, hammerhead
ribozymes specific for fibrillin-1 mRNA (FBN-1: mutations in this gene are associated with Marfan syndrome) were administered in the form of ribozyme-transferrin-polylysine conjugates to cultured dermal fibroblasts; FBN-1 mRNA levels were reduced by approximately 50% compared to controls (Phylactou et al., 1998b). There is now a wide array of non-viral systems for gene delivery; these fast-developing tools represent possible alternatives to viral mediated gene delivery systems. The mechanism of cationic lipid transfection of cultured cells, along with their use for delivery of retinal-specific ribozymes to COS-7 cells are addressed in detail in chapter 5 of this thesis.

1.5.3 Viral delivery systems

For successful gene therapy, the choice of vector used to introduce genetic material into cells is of critical importance. Frequently, gene transfer techniques using viral vectors that have the ability to infect and transform a wide array of cell-types are preferred. Four distinct categories of virus are currently being manipulated and tested for high-efficiency gene delivery in both animal and cell-culture systems; Retrovirus, Adenovirus and Adeno-associated virus and herpes virus. Retroviral vectors contain an RNA genome and are probably the most well characterised for in vivo delivery of ribozymes; they can transduce dividing cells in culture with near 100% efficiency and stably integrate into the host genome. However, this mode of delivery has limitations in that retroviruses are difficult to grow in high titres and as their integration is random, they may cause damage to essential genes thus raising concerns regarding their safety of use (Mitani and Caskey, 1993; Vaish et al., 1998). Moloney murine leukemia virus (MoMLV)-based retroviral vectors have been engineered to deliver monomeric and multimeric ribozymes, directed to conserved regions of HIV-1, to cultured T lymphocyte populations and confer partial resistance against HIV-1 infection (Sun et al., 1996; Ramezani et al., 1997; section 1.3). In the context of therapies for retinal degenerations involving post-mitotic photoreceptor cells, lentiviruses have been found to infect non-dividing tissues (Miyoshi et al., 1997). These viruses have been modified to, in principle, enable efficient and safe delivery of therapeutic genes to non-dividing tissues. In the study by Miyoshi et al. a HIV-based lentiviral vector expressing green fluorescent protein (GFP) was injected into the sub-retinal space of rat eyes. CMV driven expression of GFP was shown to be highly efficient in both photoreceptor cells and retinal pigment epithelium. Notably, the use of a tissue-specific rhodopsin promoter limited expression predominantly to photoreceptor cells. Moreover, photoreceptor cells in > 80% of the area of the entire retina showed persistent expression of
GFP for at least 12 weeks post injection of virus. Hence, HIV-based viral delivery systems may offer substantial promise for the future treatment of retinal disease.

Adenoviruses (DNA virus) have also been successfully employed for the delivery of ribozymes to cell culture and animal systems. They can infect post-mitotic cells with high efficiencies and provide high-levels of expression of recombinant genes. However, as these viruses exist extrachromosomally in host cells, they only enable transient expression of delivered genes (Birikh et al., 1997a). Moreover, they often elicit strong immune responses which results in rapid removal of infected cells thus preventing successful repeat administrations (Vaish et al., 1998). Nevertheless, Lieber and Kay (1996) coupled high efficiency adenovirus gene delivery with the ability of ribozymes to down-regulate the expression of specific genes. Ribozyme gene transfer was accomplished in transgenic mice expressing the human growth hormone in both the liver and gastrointestinal tract.

Adenoviruses, carrying a ribozyme directed to human growth hormone (hGH) transcripts, were infused by tail vein injection and resulted in a 96% reduction in hGH mRNA levels for several weeks. Furthermore, the degree of hGH mRNA reduction directly correlated with the concentration of ribozyme RNA delivered. Similarly, Czubayko et al., 1997 used adenoviral vectors as vehicles for the delivery of hammerhead ribozymes targeting tumour promoting factors; the tyrosine kinase receptor HER-2/neu and the growth factor pleiotrophin (PTN), into three human cancer cell lines. Respective mRNA levels were reduced by ≤ 75% with a corresponding inhibition of protein expression. This resulted in near complete abrogation of HER-2/neu or PTN-dependent cancer cell proliferation. With regard to gene therapies for retinal degenerations, Bennett et al. (1996) delivered via sub-retinal injection an adenovirus carrying a murine cDNA encoding wild-type cGMP phosphodiesterase (βPDE) and driven by a CMV promoter to the rd mouse. This mouse model manifests a retinal degeneration similar to human RP caused by a recessive mutation in the βPDE gene - the βPDE protein plays a fundamental role in the visual transduction cascade (for details see section 1.1.5). Intraocular transfer of the virus into neonate rd mice resulted in βPDE transcript production, increased βPDE activity and delayed photoreceptor cell death (by six weeks). This study highlighted the feasibility of using adenoviral delivery systems for treating inherited retinal diseases such as RP. Given the potential utility of adenoviral vectors for gene delivery, second, third and fourth generation viruses which have been modified to reduce potential host immune responses have been generated. Such vectors may enable repeated administration of gene therapies.
Adeno-associated viral (AAV) delivery mechanisms present another alternative system for gene delivery. They are non-pathogenic DNA viruses that allow continued expression of delivered genes; adeno-associated viruses can infect both dividing and non-dividing cells and those AAVs with a rep coding region specifically insert into a region of chromosome 19 (Mitani and Caskey, 1993; Vaish et al., 1998). However, as the virus is replication incompetent, co-infection with a helper adenovirus or herpes virus is required for propagation. For example, an AAV system was used recently as part of a therapeutic strategy for adRP. Both hammerhead and hairpin ribozymes were delivered to the retina of Pro23His transgenic rats by intra-ocular injection. The intracellular production of ribozymes targeting P23H mutant rhodopsin transcripts in photoreceptor cells was accomplished by transduction with recombinant adeno-associated viruses carrying the ribozymes. In vivo expression of either ribozyme was found to significantly retard the rate of photoreceptor degeneration in the transgenic rat model of adRP for at least three months (Lewin et al., 1998). While this study clearly demonstrated the potential use of ribozymes as therapeutic agents for rhodopsin-related adRP, the ribozymes used would not directly be suitable for treatment of human patients as the ribozymes were designed to target artificially engineered ribozyme cleavage sites i.e. the P23H mutation does not create either a hammerhead or hairpin cleavage motif and in fact is completely unsuitable as a therapeutic target for ribozymes.

An additional viral vector for gene delivery has been used in a wide range of studies - Herpes simplex virus type 1 (HSV-1). These viruses, like adenoviruses, remain as episomes in the nucleus and hence have the advantage that insertional mutagenesis of the host genome does not occur. However, as with adenoviruses transferred genes are eventually lost from dividing cells. Notably, HSV-1 has been used to transfect a variety of cell types including post-mitotic neurons (Mitani and Caskey, 1993). The most frequent utility for HSV-1 to date has been for various tumour therapies (particularly brain tumours) as these viruses are more cytotoxic to rapidly dividing cells than non-dividing cells (Kramm et al., 1997). For example, Yoon et al. (1998) investigated the efficacy of a replication competent HSV-1 derived viral vector, hrR3, in destroying colon carcinoma cells in vitro and in vivo. hrR3 also possessed the HSV-thymidine kinase gene which converts ganciclovir into a toxic metabolite. Therefore it was hypothesised that the addition of ganciclovir to hrR3-infected cells may improve the tumouricidal properties of hrR3. Moreover, to increase specificity for tumour cells, hrR3 was defective for ribonucleotide
reductase and therefore replicates selectively in cells containing high levels of endogenous ribonucleotide reductase such as actively dividing tumour cells. Results demonstrated that the levels of expression of ribonucleotide reductase was markedly higher in colon carcinoma cell lines than in primary cultures of human hepatocytes and that hrR3 efficiently destroyed the carcinoma cell lines in vitro. In addition, to assess the efficiency of hrR3 in vivo, the human colon carcinoma cell line, HT29, was injected into the flanks of nude mice followed by intratumoral injection of hrR3. Notably, a single injection of hrR3 significantly retarded tumour growth rate. However, results indicated that the addition of ganciclovir had no beneficial effect.

It is apparent from the discussion provided above that a broad range of viral and non-viral vectors are already available to explore gene delivery to both dividing and non-dividing tissues. Undoubtedly, given the intense activity in the field of vectorology, various modifications to these vectors will be undertaken to optimise their utility for gene delivery. Optimisation of such vectors maybe a prerequisite to the successful development of many ribozyme-based therapies such as those discussed in chapters 3, 4 and 5 of this thesis.

Summary
Since the initial discovery of ribozymes, a vast wealth of information regarding their structure, chemical and physical properties has been accumulated. Hammerhead ribozyme technology has advanced to the stage that it is now realistic to explore their possible therapeutic applications. To date, these molecules have been employed in studies as diverse as dissecting gene function in zebrafish (Xie et al., 1997) to investigating their use as anti-oncogene, anti-HIV-1, anti-arthritis and anti-malarial agents (Kashani-Sabet et al., 1994; Feng et al., 1995; Flory et al., 1996; Czubayko et al., 1997; Flores et al., 1997; Scherr et al., 1997). Moreover, many of the cell culture and animal studies outlined in the introduction and throughout the body of this thesis provide significant optimism for the successful application of these therapeutic tools in vivo. Ribozymes are now being proposed as gene suppressors for the mutation-independent gene-silencing of rhodopsin and peripherin transcripts linked with adRP. Two ribozyme-based strategies which overcome the extensive genetic heterogeneity associated with adRP and offer potential as broad range therapies are explored in this thesis. In chapter 3, the viability of both mutation-independent therapeutic approaches are examined using in vitro transcribed target and ribozyme RNAs. Replacement genes with modified UTR sequence or degenerative
nucleotides were generated such that retinal transcripts were protected from ribozyme-mediated suppression and could in theory provide wild-type protein. Detailed kinetic studies were subsequently performed on the mutation-independent ribozymes that proved most efficient at down-regulating expression of retinal transcripts \textit{in vitro} (Chapter 4). The kinetic profiles generated were used to determine which ribozymes were worth analysing in cell culture systems. Rhodopsin and peripherin expressing COS-7 cell lines were developed to facilitate ribozyme investigations \textit{in vivo}. These cell culture studies are discussed in detail in chapter 5 of this thesis.
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<thead>
<tr>
<th>Gene symbol</th>
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<td>mitochondrion</td>
<td>RP with progressive sensorineural hearing loss; protein: serine tRNA 2 (AGU/C)</td>
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Table 1.1 Loci and genes known to cause RP. This is a modified version of a table by Dr. S. P. Daiger; http://www.sph.uth.tmc.edu/Retnet/disease.htm For references see the original table.
Figure 1.1

(A)-(B) = Comparison of a normal and RP retina respectively. As is evident, the RP retina is visibly thinning, the blood vessels are attenuated and heavy pigmentedy deposits are present. Photographs were kindly provided by Dr. Paul Kenna.
Figure 1.2

(A)-(B) = Presented are diagrams of the rhodopsin and peripherin/RDS proteins respectively. Whereas rhodopsin is only found in rod photoreceptor cells and plays a major role in visual transduction, peripherin/RDS is present in both rod and cone photoreceptors and is believed to be involved in maintaining the structure of outer segment discs. Red circles represent mutations that have been identified in the genes encoding both proteins.
Figure 1.2a
Figure 1.2b
Figure 1.3
Schematic diagram of a rod photoreceptor cell (from Berson et al., 1991). The synaptic terminus, nucleus and inner and outer segments of the rod cell can be seen. The outer segment consists of hundreds of flattened disc-like structures assembled in an ordered axial array that contain the photoreceptor-specific proteins, rhodopsin and peripherin.
Figure 1.3
Figure 1.4

Schematic diagram depicting the principal reactions in visual transduction. Briefly, light initiates the isomerisation of 11-cis retinal to an all-trans form resulting in the activated state of rhodopsin. Activated rhodopsin catalyses the exchange of GDP for GTP on transducin and the dissociation of the $\alpha$-subunit ($T\alpha$) from the $\beta\gamma$-subunits ($T\beta\gamma$). $T\alpha$ subsequently interacts with PDE to release the enzymes inhibitory $\gamma$ subunits from its $\alpha$ and $\beta$ subunits. Activated PDE catalyses the hydrolysis of cGMP to $5'$GMP. The decrease in intracellular cGMP concentration causes the channel to close and the rod photoreceptor cell to be hyperpolarised. Intracellular Ca$^{2+}$ levels decrease as the Na/Ca-K exchanger continues to pump out Ca$^{2+}$ from the outer segment. Photorecovery is initiated by shutdown of the visual cascade and the calcium medium feedback mechanism. The visual cascade is inactivated by (a) phosphorylation of rhodopsin by rhodopsin kinase and subsequent binding of arrestin, (b) hydrolysis of GTP to GDP on $T\alpha$. PDE also returns to its inactive state as a result of this reaction and (c) re-association of $T\alpha$ with its $\beta\gamma$-subunits resulting in deactivation of transducin. Low intracellular Ca$^{2+}$ concentrations lead to (1) activation of guanine cyclase (GC) mediated by GCAP and (2) an increase in sensitivity of the channel to cGMP. As cGMP concentration increases, the channels re-open and the cell is returned to its depolarised state. The increase in Ca$^{2+}$ also converts GC to its inactive state. Solid arrows indicate the photoexcitation process whereas dashed arrows indicate the photorecovery process (taken from Molday, 1998).
Figure 1.4
Figure 1.5

Presented are the hammerhead structures of (A) the naturally occurring cis-acting ribozyme and (B) the Haseloff and Gerlach model of a trans-acting ribozyme. In the naturally occurring self-cleaving domain, two of the three stems have closed loops. In contrast, artificially engineered constructs are designed such that the hammerhead RNA is divided into arbitrary enzyme and substrate strands. Diagrams are from Ohkawa et al. (1995). (C) The structure of the optimised hairpin ribozyme where N = any nucleotide, R = G or A, Y = C or U (from Yu and Burke, 1997). (D)-(E) = Structures of the axehead ribozyme motifs for the genomic and antigenomic hepatitis delta virus RNAs. + and - conserved regions are boxed and the cleavage sites are indicated with arrows (from Symons, 1992).
Figure 1.5a-b
**C**

1. In position 2 and C at +3 are each important for efficient cleavage. They probably interact with A7 and C8 of the enzyme.
2. At position +1, immediately to the 5' site of the scissile bond, is essential for cleavage.

**Helix 1:** The sequence of helix 1 can be changed at the user's discretion. Its length can be four or more base pairs, but six is a good place to start. Cleavage activity will be adversely affected if helix 1 is too short or too long.

**Internal Loops A and B:** The identity of nearly all of the bases in loops A and B of the ribozyme is important for catalytic activity.

**Helix 2:** Choose a substrate with C or A at position +3 or position -2. It is helpful but not essential to have a G or A at position +1.

**Helix 3:** The sequence can vary, so long as five base pairs are maintained. It's probably best to line up with the naturally-occurring sequence.

**Helix 4:** This helix is only 3 bp in the wild type molecule. Extensions work better if helix 4 is saturated by extending it with two or more base pairs, and capping it with a triple-loop structure.

3. The base at position 11 should be G regardless of whether C or U is used at position 3 of the substrate.

4. HeUx 3: Replacing the naturally-occurring G increases the activity of most ribozymes.

**D**

**Genomic (-)**
- R: 24 nt
- S: 65 nt

**Antigenomic (+)**
- R: 24 nt
- S: 66 nt

Figure 1.5c-d
Experimental approaches for the selection of accessible ribozyme binding sites on long RNA substrates. (A) = Ribozyme-library selection which allows the determination of accessible ribozyme cleavage triplets in target RNAs. (B) = Oligodeoxynucleotide (ODN)-library enabling selection of unstructured regions in target RNAs. Ribozymes can subsequently be designed towards accessible areas in the RNA. Diagrams taken from Birikh et al. (1997).
Ribozyme library

Target RNA

Reverse transcription
Tailing of cDNA
PCR
Cloning
Exact position of efficiently cleavable triplets
Design of ribozymes to these triplets

ODN library

RNAse H

Target RNA

RNAse H

Denaturing PAGE

Accessible regions on mRNA
Design of ribozymes cleaving in these regions

Figure 1.6
Role of metal ions in ribozyme cleavage reactions. The four mechanisms by which a metal ion can potentiate a hammerhead ribozyme cleavage reaction are indicated with numbers (taken from Pyle, 1993).
Figure 1.7
Figure 1.8
This curve depicts the rate of a ribozyme catalysed reaction displaying Michaelis-Menten behaviour. Experimentally, the concentration of substrate in the ribozyme reaction is varied, rate constants are obtained for each condition and plotted as a function of substrate concentration. Most ribozyme reactions show a rate dependence on substrate concentration at very low concentrations. However, instead of the rate of the reaction increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no rate dependence on concentration and the reaction becomes saturated with respect to substrate. Kinetic parameters such as $V_{\text{max}}$ and $K_m$ can be obtained from this curve (taken from Cornish-Bowden and Wharton, 1990).
Figure 1.8
Figure 1.9

Reaction scheme for the correction of mutant transcripts by targeted trans-splicing. A group 1 ribozyme can be designed to react with any uridine present in an accessible region of the target RNA by varying the nucleotide composition of the 5’ exon binding-site on the ribozyme. The ribozyme is designed such that it binds upstream of the mutation and mediates transfer of its 3’ exon (wild-type RNA sequence) by cleaving the substrate RNA backbone immediately 3’ of the targeted uridine and ligating its 3’ exon tag onto this new end. This gives rise to a wild-type transcript without the targeted mutation. $X_m =$ point mutation at nucleotide $X$, $X_{wt} =$ wild-type nucleotide (taken from Sullenger and Cech, 1994).
Mutant transcript | Ribozyme with wild-type 3' exon

\[ X_m \]

\[ \text{Ribozyme} \]

3' exon

Ribozyme catalysed cleavage and ligation

\[ X_m \]

Corrected transcript

+ Ribozyme + Mutant 3' exon

Figure 1.9
Chapter 2

Materials and methods
2.1 MATERIALS

2.1.1 Reagents

(I) Chemicals and antibiotics: from BDH Laboratory Supplies (Poole, England), Sigma Chemical Corporation (Steinheim, Germany), Gibco/BRL (Paisley, Scotland) or Prolabo (France).

(II) Enzymes and buffers: Restriction enzymes and buffers from New England Biolabs (Hertfordshire, England), Boehringer Mannheim (East Sussex, England) or Promega (Madison Wisconsin); T4 DNA ligase and RNaseIT (Stratagene); RNasin and calf intestinal phosphatase (CIP) (Boehringer Mannheim).

(III) Size Standards: 1Kb DNA ladder (Gibco BRL) and 0.28–6.58 Kb RNA ladder (Promega).

(IV) Kits: T7 Ribomax large-scale RNA expression and PolyATtract mRNA isolation systems (Promega); Random-priming kit, ‘HighPrime’ dCTP (Boehringer Mannheim); Primer extension system, AMV reverse transcriptase (Promega).

(V) Tissue culture: Dulbecco's Modified Eagle Medium (DMEM), Foetal Bovine Serum (FBS), sodium pyruvate, L-glutamine, penicillin-streptomycin, phosphate buffered saline (PBS), trypsin-EDTA and geneticin (G418): Gibco/BRL.

2.1.2 Solutions

(I) 10XTBE: 55g Boric Acid, 108g TRIZMA base and 40ml 0.5M EDTA (pH8.0). Final volume adjusted to 1000ml.

(II) Acrylamide: 96.5g acrylamide, 3.35g bis-acrylamide, 233.5g urea and 50ml 10XTBE. Final volume made up to 500ml.

(III) 7.75M Urea: 233.5g urea, 50ml 10XTBE and H2O to 500ml.

(IV) Agarose loading dye: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol and 69.5% H2O.

(V) 10X MgCl2 PCR buffers: 500mM KCl, 100mM Tris-HCl (pH9.0), 0.1% gelatin, 1% triton X100 and 10, 12.5, 15, 20 or 25mM MgCl2.

(VI) Miniprep and maxiprep solutions:

Lysis solution 1: 50mM glucose, 25mM Tris-HCl(pH8.0) and 10mM EDTA(pH8.0).

Lysis solution 2: 0.2M NaOH and 1% SDS.

Lysis solution 3: 3M sodium acetate, pH 5.2.

Top-up Solution: 17.6g CsCl, 17.4ml 10mM Tris-HCl (pH 8.0) and 1.4ml EtBr (10mg/ml).
Luria Bertani (LB) broth: 1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 10mM MgSO\textsubscript{4} and 0.2% maltose. For agar plates LB medium contains 1.5% (w/v) agar. LB agar and broth were autoclaved at 15 p.s.i for 20 minutes.

Complete DMEM: DMEM, 10% (v/v) FBS, 1mM sodium pyruvate, 2mM L-glutamine and 0.05mg/ml penicillin-streptomycin.

In situ staining solutions:
0.5M Sodium phosphate buffer (pH 7.3): 36.45% (v/v) 1M di-sodium hydrogen orthophosphate (Na\textsubscript{2}HPO\textsubscript{4}), 13.55% (v/v) 1M sodium di-hydrogen orthophosphate-1 hydrate (NaH\textsubscript{2}PO\textsubscript{4}·H\textsubscript{2}O) and 50% d.H\textsubscript{2}O.

Fix solution: 4ml 25% glutaraldehyde, 100ml 0.5M sodium phosphate buffer, 5ml 0.5M EDTA (pH 8.0), 1.0ml 1.0M MgCl\textsubscript{2} and d.H\textsubscript{2}O to 500ml.

Rinse solution: 100ml 0.5M sodium phosphate buffer, 1ml 1.0M MgCl\textsubscript{2}, 0.05g sodium deoxycholate, 100µl nonidet-P40 and d.H\textsubscript{2}O to 500ml

Stain solution: 0.165g potassium ferricyanide, 0.165g potassium ferrocyanide and final volume adjusted to 10ml with rinse solution. 0.4ml of X-gal (25mg/ml in dimethyl formamide) /10ml was added fresh before use.

2.1.3 Oligonucleotide synthesis and purification

Oligonucleotides were synthesised on an Applied Biosystems 394 DNA/RNA synthesis by Dr. Sophie Kiang. Oligonucleotides were purified by chloroform extraction and dried in a Savant SVC200 vacuum centrifuge. Pellets were resuspended in d.H\textsubscript{2}O to a final concentration of 50pMoles/µl. In addition, presynthesised and purified oligonucleotides were purchased from Gibco/BRL and Genosys Biotechnologies LTD (Cambridge, UK). Concentrations were calculated by: O.D (λ26OnM) X Dilution factor x 33 = ng/µl

2.1.4 Cell lines

COS-7 cells were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, UK. COS-7 cells were derived from CV-1; an African Green monkey kidney cell line, transformed by an origin-defective mutant of SV40. ECACC number: 87021302.
2.2 GENERAL METHODS

2.2.1 Phenol/chloroform extraction of nucleic acids
Samples were mixed with equal volumes of phenol, vortexed for 30 seconds and centrifuged at 13,200 rpm for 5 minutes to separate phases. The upper aqueous phase, containing nucleic acids, was transferred into an equal volume of phenol/ chloroform and again vortexed and centrifuged. Phenol/ chloroform extractions were repeated and added to an equal volume of chloroform. Following centrifugation, samples were ethanol precipitated.

2.2.2 Ethanol precipitation of DNA and RNA
1/10 volume of 3M sodium acetate (pH 5.2) was added to each sample followed by 2 volumes of absolute ethanol (EtOH). 2.5 volumes of EtOH was used for RNA precipitation. Samples were stored at -20°C for 30 minutes and centrifuged at 13,200 rpm. Pelleted nucleic acid was washed with 70% EtOH x 2, dried at 60°C and resuspended in d.H₂O.

2.2.3 Restriction enzyme digests
Restriction digests were typically performed in 100-200 µl volumes depending on the quantity of DNA being cleaved. 1/10 volume of the appropriate restriction buffer was added and enzyme to a concentration of 5U/µg of DNA. The final volume was made up with d.H₂O and the reaction incubated for 2-3 hours.

2.2.4 Polymerase chain reaction (PCR)
Typically, a standard 50µl PCR reaction contained 250ng DNA template 25pmol of forward and reverse primers, 0.2M dATP, dTTP and dGTP and 2.5 units of Taq polymerase. The reaction was performed in a 1X MgCl₂ reaction buffer containing 0.5-2.0mM MgCl₂ and the final volume brought to 50µl with d.H₂O. An overlay of paraffin was added to the final reaction mix to prevent evaporation. PCR cycle: 1 cycle of 94°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1.20 minutes. PCR amplifications were performed using a 'Biometra Trio-Thermo-Block.'
2.2.5 Purification of DNA fragments from agarose gels

Restriction digested or PCR amplified samples were mixed with 1/10 volume of loading dye, loaded on 1-1.5% agarose gels (with 0.45μg/ml EtBr) and electrophoresed at 80mA in 1xTAE. Size standards were also run on the gels. Gels were visualised with U.V. light and fragments of correct size cut out with sterile razor blades. An agarose gel isolation column (Sulpecco, U.K.) was prepared by pre-washing with 100μl TE buffer and centrifugation at 13,200rpm for 30 seconds. The gel slice was placed at the top of the column and the DNA eluted by centrifugation (in a fresh eppendorf) at 13,200rpm for 10 minutes. The purified DNA was re-run on an agarose gel to ensure accuracy of isolation. Gel-purified fragments were used for PCR, cloning or radioactively labeled for generation of probes for Northern blotting.

2.2.6. Generation of molecular weight markers

λDNA and pUC19 were digested with HindIII and MspI respectively. The λ DNA/HindIII molecular weight marker was used for the size estimation of large DNA fragments whereas pUC19/MspI was used for smaller fragments. Fragment sizes are as follows:

- pBR322/HindIII: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bases.
- PUC19/MspI: 497, 489, 404, 331, 242, 190, 147, 110, 102, 67, 34x2, and 26 bases.

2.2.7 Competent cell preparation.

20-30ml of LB broth was inoculated with a single colony of an appropriate E.coli strain (XL-1 blue, DH5αF) and incubated overnight at 37°C. 1ml of the overnight culture was added to 200ml of LB and incubated at 37°C until an optical density reading of 0.2-0.3 at λ600nm was obtained. The flask was subsequently stored on ice for 30 minutes. 2 x 50ml aliquots of culture was centrifuged at 3,000rpm for 5-10 minutes to pellet the cells. Supernatants were removed, pellets resuspended in 50ml of ice-cold 50mM CaCl₂ and left on ice for 30 minutes. Samples were again centrifuged at 3,000rpm for 10 minutes and supernatants removed. Cell pellets were re-dissolved in 5ml of 50mM CaCl₂ and left on ice for 20-30 minutes before use. Alternatively, competent cells were stored at 4°C for a maximum of 24 hours or cryo-preserved at -70°C in 15% glycerol.
2.2.8 Ligations

In 15μl reaction volumes, varying concentrations of insert DNA were added to 100ng of dephosphorylated vector. 1/10 volume of 10X ligase buffer and rATP was subsequently added with 10U ligase enzyme. The final volume was adjusted to 15μl with d.H2O and the mix incubated at 15°C overnight. Phosphorylated and dephosphorylated vector controls were used, both with and without insert DNA. Dephosphorylation of vector DNA by calf intestinal phosphatase (CIP) was performed using standard protocols (Sambrook et al., 1989).

2.2.9 Transformations

5μl of ligation mix (10ng plasmid DNA) was added to 10μl of d.H2O. 200μl of competent cells was subsequently added and samples left on ice for 30-45 minutes. Cells were heat shocked at 42°C for 5 minutes and placed on ice for approximately 1 minute. 1ml of LB was added and samples incubated for 1 hour at 37°C. 200μl of each transformation was plated on LB agar plates containing appropriate concentrations of antibiotic; 50μg/ml ampicillin and 25μg/ml tetracycline.

2.2.10 Plasmid mini-preparations

Overnight cultures (~10ml) of plasmid transformed cells were centrifuged at 4,000rpm for 15 minutes to pellet the cells. Supernatants were removed and pellets resuspended in 200μl of lysis solution 1 and left at room temperature for 5 minutes. 400μl of lysis solution 2 was subsequently added and after 5 minutes on ice, samples were mixed with 300μl of lysis solution 3. Following 5 minutes on ice, 900μl of each sample was transferred to fresh eppendorfs and centrifuged at 13,200rpm for 15 minutes. 600μl of each supernatant was removed and plasmid DNA extracted with phenol x 2, phenol chloroform x 1 and chloroform x 1. Samples were ethanol precipitated and resuspended in 45μl d.H2O. 3 units of RNaseIT was added to samples to degrade any contaminating RNA extracted.
2.2.11 Large-scale plasmid preparations and caesium chloride purification

One litre of LB broth was inoculated with 20ml of an overnight culture of a plasmid transformed *E.coli* colony and incubated overnight at 37°C in an orbital shaker. The culture was sub-divided into 4 x 250ml aliquots and centrifuged at 8,000rpm and 4°C for 10 minutes. Supernatants were removed and 50ml of 0.05M Tris-HCl used to serially resuspend each pellet. Cells were again harvested at 7,000rpm and 4°C for 10 minutes. The resulting pellet was resuspended in 50ml of lysis solution 1 and left on ice for 30 minutes. 80ml of lysis solution 2 was added, the sample mixed well and left on ice for a further 10 minutes. 40ml of cold lysis solution 3 was subsequently added, left on ice for 15 minutes and the total mixture centrifuged at 8,000rpm and 4°C for 15 minutes. The supernatant was decanted into a fresh container through sterile gauze and 0.6 volume of ice-cold isopropanol added. Following centrifugation at 8,000rpm and 0°C for 10 minutes, the supernatant was removed and the pellet air-dried. Exactly 8ml of 10mM Tris-HCl(pH 8.0) was used to resuspend the pellet, therefore raising the final volume to approximately 8.8ml. 8.8g CsCl was accurately weighed, using a fine balance, and 8.7ml of the resuspended pellet added. 700μl of EtBr (10mg/ml) was then added and the mixture transferred to a Sorvall ultracentrifuge tube. The final volume was adjusted with top-up solution and the sample ultracentrifuged at 45,000 rpm for 18 hours. Using a sterile needle and syringe, the ultra-pure plasmid band was removed and washed with equal volumes of H₂O saturated butanol until all EtBr was removed. The sample was dialysed (to get rid of CsCl) and pressure concentrated (to reduce the volume) to approximately 1-2μg/μl. Preparations typically provided 1-2mg of ultra-pure plasmid DNA.

2.2.12 Sequencing reactions

Manual DNA sequencing was performed using the dideoxy chain termination protocol. (A) Alkali denaturation of supercoiled plasmid DNA: 50μl of plasmid DNA (6μg) was mixed with 50μl of a 0.4M NaOH and 0.4mM EDTA solution and incubated for 5 minutes at room temperature. The reaction was neutralised by the addition of 10μl of 2M ammonium acetate (pH 4.6) and subsequently vortexed. 275μl of absolute ethanol was added, placed at room temperature for 10 minutes and centrifuged at 13,200rpm for 15 minutes. The DNA pellet was washed with 70% EtOH and centrifuged at 13,200rpm in duplicate, dried and resuspended in 5μl d.H₂O. (B) Annealing reaction: To 5μl (6μg) denatured plasmid DNA, 2μl of 5X T7 DNA polymerase buffer and 1.5pmol of sequencing primer were added. The volume was adjusted to 10μl with d.H₂O and incubated for 15 minutes at 37°C. (C)
Labeling reaction: To each annealing reaction, 1μl of DTT (1000mM), 2μl of 5X labeling mix, 0.5μl α [32P] dATP (1,000 Ci/mmol) and 3U of T7 DNA polymerase was added and the reaction incubated at room temperature for 5 minutes. (D) Termination reaction: 2.5μl dG, dA, dT and dC were placed in separate eppendorfs with corresponding labels and incubated at 37°C. 3.5μl of the labeling reaction was added to each termination reaction tube and incubated for a further 5 minutes at 37°C. 4μl of stop solution was added to each tube and reactions stored at -70°C. Prior to loading on an 8% polyacrylamide gel, samples were heated to 70°C for 3 minutes. T7 polymerase enzyme and buffer, DTT, 5X labeling mix, dNTP’s and stop solution were provided as components of the USB Sequenase Version 2.0 DNA sequencing kit. Radioactivity was provided by Amersham. Automated sequencing was performed by Dr. Sophie Kiang, Avril Kennan and the author on an ABI automated sequencer using standard protocols. The PCR program used to generate arbitrarily terminated sequence consisted of 21 cycles of: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes.

2.2.13 Polyacrylamide gel electrophoresis
Polyacrylamide gels were made as detailed by Sambrook et al. (1989) and polymerised by the addition of 600μl of 10% ammonium persulphate (APS) and 75μl TEMED. Radioactive DNA and RNA samples were mixed with equal volumes of formamide loading dye, heated to 90°C for 3 minutes, electrophoresed at 2.20kV and 43mA on 4-8% polyacrylamide gels with 6.2M and 4.65M urea respectively and dried under vacuum. Images of radioactive gels were obtained with autoradiographic film (Agfa Curix) and by instant image analysis (Packard instant imager).

2.2.14 Radioactively labeling probes
cDNA and PCR fragments were labeled by random priming using the ‘HighPrime’ dCTP kit. 25ng of DNA (in 11μl d.H2O) was denatured by boiling for 10 minutes and immediately quenched on ice. While on ice, 5μl (50μCi) of [α-32P] dCTP and 4μl of Klenow mix was added, mixed gently and incubated at 37°C for 15 minutes. Reactions were stopped by heating to 65°C for 10 minutes and/ or adding 1μl of 0.5M EDTA.
DNA fragments were Klenow-filled using Klenow enzyme and an appropriate radioactive nucleotide. Typically, reactions contained 0.5-1.0μg DNA, 3μl of a common 10X enzyme buffer (e.g. NE buffer 2, NE Biolabs), 1.5μl of [α-32P] dCTP (10mCi/ml), 0.5 units Klenow and d.H2O to 30μl. Reactions were incubated for 30 minutes at room temperature. Klenow was also used to fill-in restriction endonuclease termini by substituting radioactive nucleotide with 1μl of all four nucleotides - 0.5mM dNTP.

A small hole in the bottom of a PCR eppendorf was made with a 21 guage needle. Using a cut pipette tip, 20μl of 100μm glass beads in TE buffer (Sigma) was added. 1ml of CL6B Sepharose (Pharmacia) was placed on top of the beads ensuring no air-bubbles formed between layers. To solidify the column and remove residual buffer, the eppendorf was centrifuged at 2,000rpm for 2 minutes. The volume of labeling reactions was increased to 100μl with TE buffer, pipetted into the column and centrifuged at 2,000rpm for 2 minutes. Comparing the level of radioactivity of the eluted probe to that remaining on the column enabled determination of labeling efficiency. Typical efficiencies of labeling were ≥ 80%. In addition, PCR products were also put through Sepharose columns to remove residual primer.
2.3 RNA TECHNIQUES

2.3.1 Creating ribonuclease-free conditions
Due to the inherent instability of RNA, it was imperative that all glassware, tips, eppendorfs, solutions and electrophoresis buffers used for RNA work were RNase free. Prior to use, glassware pipettes and benchtops were treated with RNase AWAY (Gibco/BRL). RNase free tips and eppendorfs were provided by Promega and Robbins Scientific (USA) respectively. All solutions except for Tris-HCl (pH 8.0) were treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity. In addition, aseptic techniques were observed and gloves worn at all times.

2.3.2 DEPC - treating solutions
0.2ml of DEPC was added per 100ml of solution and incubated for 2 hours at 37°C in a shaking incubator. Solutions were subsequently autoclaved at 15p.s.i for 30 minutes.

2.3.3 RNA solutions
(I) MOPS: 82.4g MOPS, 21.8g sodium acetate and 7.4g EDTA made up to 800ml in tissue culture H$_2$O. The pH was adjusted to 7.0 with 10M NaOH and final volume raised to 1000ml with H$_2$O. The solution was autoclaved at 5p.s.i for 30 minutes. If the MOPS solutions appeared yellowish in colour, it was disposed of, and fresh stock prepared.

(II) 20X SSC: 88.23g tri-sodium citrate and 175.32g NaCl made up to 800ml with high-grade H$_2$O and the pH checked to be 7.0-8.0. Final volume was adjusted to 1000ml.

(III) RNA denaturing buffer: 10ml deionised formamide (Kodak), 3.5ml formaldehyde (37%), 1.0ml 20X MOPS and DEPC-H$_2$O to 15ml.

(IV) RNA loading dye: 1mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 50% glycerol.

(V) RNA running dye: 1ml denaturing buffer and 200µl loading dye. This running dye was used for Northern gels.

(VI) Formamide loading dye: 1mM EDTA (pH 8.0), 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol and 80% deionised formamide. This loading dye was used for radioactive ribozyme and target RNA reactions on polyacrylamide gels.

(VII) 100X Denhardtts solution: 2g Ficoll400, 2g polyvinylpyrrolidine and 2g BSA adjusted to 100ml with d.H$_2$O and stored in 20ml aliquots at -20°C.
(VIII) Hybridisation solution: 5XSSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 0.5% SDS, 100μg/ml tRNA and 100μg/ml salmon-sperm DNA.

(IX) Post-hybridisation wash solutions: 2XSSC, 0.5% SDS and 0.05% sodium pyrophosphate and 1XSSC, 0.1% SDS and 0.05% sodium pyrophosphate.

(X) Guanidinium isothiocyanate (GTC) solution: 4M guanidinium isothiocyanate, 5mM EDTA (pH 8.0), 25mM citrate (pH 7.0) and 0.5% sarcosyl. 7.1μl/ml β-mercaptoethanol was freshly added before use.

### 2.3.4 RNA extraction from tissue culture cells

Total RNA was extracted from 5X10⁶ COS-7 cells using guanidinium isothiocyanate (GTC). Cells were trypsinised (see section 2.4.1) and centrifuged at 1,000rpm for 5 minutes. 500μl of 4M GTC was added to the cell pellet, vortexed and left overnight at 4°C. An equal volume of phenol chloroform-isoamyl alcohol (50:49:1) was added with 0.2M sodium acetate (pH 4.0), placed on ice for 30 minutes and centrifuged at 13,200rpm for 15 minutes. The supernatant was removed and 1μl of glycogen and 1ml of cold 100% isopropanol added. The sample was stored at -20°C for 2 hours and precipitated. Total RNA extracted was resuspended in DEPC-H₂O, 3U of DNase1 enzyme (Promega) was added and the reaction incubated at 37°C for 20 minutes. Ratios of spectrophotometric readings at λ260nm and λ280nm were used to assess quality of RNA preparations; ratio for high-quality extractions range between 1.5-2.0. Typical preparations provided 150-200μg of total RNA. RNA samples were stored at -70°C.

### 2.3.5 Poly(A) mRNA isolation from total COS-7 Cellular RNA

Poly(A) mRNA was extracted from total COS-7 cellular RNA using the PolyATtract mRNA isolation system. (A) Annealing of probe: 100-200μg of total RNA was made up to a volume of 500μl with DEPC-H₂O and heated to 65°C for 10 minutes. 3μl of biotinylated-oligo(dT) probe and 13μl of 20XSSC was added to the RNA, mixed gently and incubated at room temperature until cool. (B) Washing streptavidin-paramagnetic particles (SA-PMPs): The SA-PMPs were resuspended by gentle vortexing until completely dispersed and collected at the side of the eppendorf using a magnetic stand. The supernatant was removed and SA-PMPs washed 3 times with 300μl volumes of 0.5XSSC (each time capturing particles and removing supernatant). The washed SA-PMPs were resuspended in 0.1ml of 0.5XSSC. (C) Capture and washing annealed oligo(dT)-mRNA complexes: The
contents of the annealing reaction were mixed with washed SA-PMPs and incubated at room temperature for 10 minutes. Following capture of SA-PMPs and removal of the supernatant, particles were washed 4 times with 300μl volumes of 0.1XSSC by gentle vortexing. Without disturbing the SA-PMPs, the supernatant was removed. (D) mRNA elution: The final SA-PMP pellet was resuspended in 0.1ml of DEPC-H₂O by vortexing, captured and the eluted mRNA transferred to a fresh eppendorf. The elution step was repeated with 0.15ml DEPC-H₂O and the pooled 0.25ml centrifuged at 10,000rpm for 10 minutes. The mRNA was removed from residual carried over particles and dried down in a vacuum centrifuge. Poly(A) RNA was resuspended in 15μl DEPC-H₂O and spectrophotometrically analysed. Preparations typically produced 1.0-2.0μg mRNA. Biotinylated-oligo(dT) probe, SA-PMPs and the magnetic stand were provided in the kit.

2.3.6 Analysing RNA by reverse-transcriptase PCR (RT-PCR)

2-5μg of DNase1 treated total RNA was made up to 15μl with DEPC-H₂O and reverse transcribed into cDNA using an M-MLV reverse transcription kit (Gibco/BRL) as follows: A reverse transcription mix was made consisting of 6μl 5X Gibco RT buffer, 10mM DTT, 100μM each dNTP, 1μl Pd(N)₆ random primers, 24U RNasin (Promega) and 400U M-MLV reverse transcriptase. 14.1μl of RT mix was added to DNased samples, incubated at 37°C for 2 hours and terminated by heating to 65°C for 10 minutes (a heated lid was used). RT (-) and housekeeping gene controls were also included. PCRs were set up using 2μl cDNA, 2.5μl of 15mM 10X MgCl₂ buffer, 200μM dNTPs, 25pM forward and reverse primers, 0.1 units Taq polymerase and d.H₂O to 25μl. PCR cycle: 94°C for 5 minutes, 80°C for 7 minutes followed by 26 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. A final 3 minute extension time at 72°C was used.
2.3.7 Analysing RNA by Northern blotting

(A) Electrophoresis of RNA and transfer to nylon membrane:

RNA samples were electrophoresed on formaldehyde/ agarose gels in 1X MOPS running buffer. Gels were composed of 1.2% (w/v) agarose, 1X MOPS, 1.11% (v/v) formaldehyde (37% stock) and DEPC-H₂O. Agarose was boiled in DEPC-H₂O, cooled to 68°C and added to a pre-heated formaldehyde-MOPS mix. The solution was mixed, poured into a NaOH-treated casting apparatus and set for 1 hour. 500ng of Poly(A) RNA was added to 15µl RNase-free running dye [section 2.3.3], denatured at 70°C for 15 minutes and loaded on 1.2% gels which were electrophoresed at 140mA for approximately two and a half hours. Gels were removed and washed for 15 minute intervals in d.H₂O followed by 10XSSC. Meanwhile a sheet of nylon membrane for RNA transfer (Hybond N, Amersham) along with 3 sheets of 3mm Whatmann paper were cut to size (same as gel). 3 larger sheets of Whatmann, placed along the base of the blotting apparatus, (both ends dipped in 10XSCC) served as a wick. Gels were placed on top of the wick and air-bubbles between layers removed using a broken 10ml pipette. To this, nylon membrane presoaked in d.H₂O for 5 minutes and 10XSSC for 3 minutes was carefully added. Following, the further addition of 3 sheets of 3mm Whatmann (pre-soaked in 10XSSC), a stack of absorbent blotting paper was added (Sigma). A glass plate and heavyweight were placed on top of the blotting paper and transfer proceeded overnight. After blotting, RNA was fixed to the membrane by UV cross-linking (120,000J/cm²) and baking at 80°C for 30 minutes.

(B) Prehybridisation, hybridisation and washing membranes:

Membranes were pre-hybridised at 65°C for 2 hours in pre-heated hybridisation solution (0.125ml/cm² filter). This was performed in bottles in a rotating hybridisation oven. Probes were radioactively labeled [section 2.2.14] and unincorporated nucleotide removed, using sepharose columns [section 2.2.16]. Probes and salmon-sperm DNA were denatured by boiling for 10 minutes, immediately quenched on ice and added to fresh pre-heated hybridisation solution. Pre-hybridisation solution was removed and fresh radioactive solution added. Hybridisation proceeded overnight. Blots were washed with post-hybridisation wash solutions (2-5ml/cm²); 2XSSC, 0.5% SDS X 2 at 65°C ad 1XSSC, 0.1% SDS X 2 at room temperature, exposed to X-ray film and quantified by instant image analysis.
2.3.8 Primer-extension assays

An end-labeled oligonucleotide was hybridised to RNA and utilised as a primer by reverse transcriptase, in the presence of deoxynucleotides, to locate the 5' terminus. (A) Primer labeling: 10pmol primer (20mer) was added to 1μl T4 polynucleotide kinase buffer (PNK), 3μl [γ-32P] ATP (3,000 Ci/mmol, 10mCi/ml), 10 units T4 polynucleotide kinase and the final volume adjusted to 10μl with DEPC-H2O. Reactions were incubated at 37°C for 10 minutes, heated to 90°C for 2 minutes to inactivate the T4 PNK and the concentration of primer adjusted to 100fmol/μl by adding 90μl DEPC-H2O. In addition a primer complementary to a region of a 1.2Kb Kanamycin positive control RNA and 250ng of dephosphorylated φX174 HinfI DNA marker was labeled. The volume of labeled marker was made up to 200μl with DEPC-H2O and stored at -20°C. (B) Primer extension of control and sample RNA: 5ng of control RNA and 100ng Poly(A) sample RNA were diluted in 5μl DEPC-H2O and transferred into separate sterile eppendorfs. In addition 5μl of DEPC-H2O was added to a ‘NO RNA’ control tube for each primer. 1μl/100fmol of 32P-labeled primer was added with 5μl of AMV primer extension 2X buffer. RNA and primer were annealed by heating to 58-60°C for 20 minutes and subsequent incubation at room temperature for 10 minutes. 9μl of reverse transcriptase (RT) extension mix (5μl AMV primer extension 2X buffer, 6.2mM sodium pyrophosphate, 1 unit AMV-RT and DEPC-H2O to 9μl) was added to annealed primer and RNA and incubated at 42°C for 30 minutes. Samples were ethanol precipitated, resuspended in 5μl DEPC-H2O and 5μl of formamide loading dye added. 1μl of labeled marker and 5μl of primer extended RNA samples were heated to 90°C for 3 minutes and loaded on 8% polyacrylamide gels. AMV and T4 PNK enzymes and buffers, control RNA and primer and sodium pyrophosphate were supplied as components of a primer extension system-AMV RT (Promega). Details for extension of highly structured RNAs are provided in Chapter 5 of this thesis.
2.4 TISSUE CULTURE PROTOCOLS

2.4.1 Sub-culturing COS-7 cells

Adherent COS-7 cells were grown to confluence in 100mm tissue culture dishes (Nunclon) with complete DMEM at 37°C and 5% CO₂. Medium was removed from the cells and 3ml of warm phosphate buffered saline (PBS) added and rolled over the monolayer to remove traces of DMEM. The PBS was removed and 1ml of 2X trypsin-EDTA subsequently added. Dishes were incubated at 37°C for 5 minutes to dissociate the cell monolayer. 10ml of warm DMEM was added and remaining cells detached from the surface by pipetting medium against the monolayer. Resuspended cells were removed and centrifuged at 1,000rpm for 5 minutes. Pellets were resuspended in 5ml DMEM and 1ml was removed for counting on a haemocytometer. Typically, COS-7 cells were split 1:4 to 1:6 into new dishes containing 15ml DMEM. Medium was renewed every 2-3 days.

2.4.2 Haemocytometer cell counting

Before use the haemocytometer was washed with 70% EtOH and dried. The edges of a coverslip were dampened and placed over the counting area of the haemocytometer chamber. The cell sample was mixed, diluted 1/10 and placed to the edge of the coverslip allowing the suspension to be drawn under the coverslip by capillary action. The haemocytometer was transferred to a microscope and viewed under the 10X objective. The number of cells present in 16 squares (circled below) were noted. This was repeated for the 3 other 16 squared areas and the average number of cells calculated. The number of cells in the sample counted = average cell count X dilution X 10⁹/ml.

Magnified view of one haemocytometer counting chamber
2.4.3 Freezing cells in liquid nitrogen

Cells were grown to 85-90% confluency in 100mm dishes, harvested as in section 2.4.1 and pellets resuspended in 2ml DMEM. 500μl aliquots were removed and mixed with equal volumes of 2X freezing solutions (20% dimethyl sulfoxide [DMSO], 60% FCS and 20% complete DMEM) in colour coded cryo-tubes (Nunclon). Vials were labeled with the name of the cell line, passage number and date. Cryo-tubes were stored at -70°C for up to 3 months thereafter being transferred to liquid nitrogen for long-term storage. Details were recorded in a cell bank record book enabling rapid identification of particular ampoules. The number of vials frozen per tissue culture dish was dependent on the size of the flask; 60cm² dish gives 3-4 vials in a 140cm² dish gives 6-8 vials. For revival of cells, cryo-tubes were removed from liquid nitrogen and thawed for 3-4 minutes in 37°C H₂O. Cells were transferred to 5ml of warm DMEM, centrifuged at 1,000rpm for 5 minutes and plated in 60cm² dishes with 15ml fresh DMEM.

2.4.4 Electroporation of COS-7 cells

COS-7 cells were transfected by electroporation. Cells were grown to confluency in large 140cm² dishes, harvested by trypsinisation and centrifugation and counted by haemocytometry. 5X10⁶ COS-7 cells were resuspended in 0.75ml DMEM (minus serum and antibiotic) or PBS and transferred to electroporation cuvettes (Biorad, 0.4cm gap width). 5-15μg of DNA in PBS (1μg/μl) was mixed with the cell suspension and stored on ice for 30 minutes. Cell controls without DNA were also included. Samples were electroporated at 262V and 500μF (typically for 12.8 seconds) using a Biorad GenePulser and replaced on ice for 20 minutes. Cells were transferred to 15ml of warm complete DMEM in 60cm² dishes and incubated overnight. The following day, medium was removed and replaced with fresh DMEM.

2.4.5 Transfection of COS-7 cells with LipofectAMINE PLUS

The polycationic lipid reagent, LipofectAMINE PLUS (Gibco/BRL), was used to transfect COS-7 cells. A day prior to transfection, cells were trypsinised, counted and plated in 10cm² 6-well dishes (Nunclon) with serum-free and antibiotic-free DMEM so that they would be 50-80% confluent on the day of transfection. 1μg of DNA was diluted in 100μl DMEM minus serum, mixed with 6μl PLUS reagent and pre-complexed at room temperature for 15 minutes. 4μl LipofectAMINE reagent was diluted in 100μl serum-free DMEM, combined with the DNA-PLUS reagent complex and incubated for a further 15
minutes at room temperature. DNA-PLUS-LipofectAMINE complexes (200μl volume) were added to each well containing 0.8ml fresh medium on cells, mixed and incubated at 37°C and 5% CO₂ for 3-5 hours. The volume of medium was increased with complete DMEM to a concentration comparable to that of normal growth medium (10% FCS) and left overnight. The medium containing LipofectAMINE was removed, cells were washed with PBS and fresh DMEM added. 24-28 hours after the start of transfection, cells were assayed by *in situ* staining for reporter gene activity (e.g. β-galactosidase) or harvested for RNA analysis. Modifications to the protocol for optimisation of transfection efficiency are addressed in Chapter 5.

2.4.6 Generation of stable COS-7 cell lines

Stable cell lines, with relevant plasmids integrated into the COS-7 cell genome, were generated by electroporation or by transfection with LipofectAMINE PLUS. 48 hours post-transfection, fresh DMEM supplemented with 600μg/ml G418 was added to the cells. Cells were selected for 3-4 weeks with G418, changing the medium every 2-3 days. When control plates (not transfected with the selected antibiotic-resistance gene) were completely dead healthy G418 resistant colonies were located and marked with a laboratory marker for picking. Medium was removed from the plates and cells were washed with PBS X2. 1.5ml of PBS was added, 35-40 marked colonies carefully removed in 15μl volumes using sterile cut P20 pipette tips and transferred into 15μl of 2X trypsin-EDTA. Following incubation at 37°C and 5% CO₂ for 3 minutes, cells were seeded in 96-well plates. Sterile silicone greased cloning cylinders (autoclaved) were also used for single colony selection. In this case, trypsin-EDTA was directly added to enclosed colonies, inactivated with 7 volumes of complete DMEM and transferred to 96 well plates. After 5 days, amplified colonies were transferred to 24 well plates followed by 12 well, 6 well, 60mm, 100mm and 150mm dishes. Amplification to 150mM dishes (in the presence of antibiotic) took approximately 3-4 weeks. 10-12 stable cell-lines were frozen down and stored in liquid nitrogen for analysis. The same protocol as above but with 400μg/ml Zeocin antibiotic was used to generate stable COS-7 cells expressing genes cloned into the multiple cloning site of plasmids containing a Zeocin resistance gene.
2.4.7 *In situ* staining of transfected cells (6-well plate)

24-48 hours post-transfection of cells with a β-galactosidase reporter plasmid, medium was removed and the cells washed with 2ml PBS X 2. Cells were fixed to the dish by adding 2ml of fix solution (section 2.1.2) and incubating for 10 minutes at room temperature (performed in duplicate). Fix solution was removed and the cells rinsed in duplicate with 2.0ml of rinse solution (section 2.1.2). Cells were stained with 0.42ml of stain solution (section 2.1.2) at 37°C overnight. Following colour development, cells were rinsed with 2ml of rinse solution (2 X 10 minutes) and stored at 4°C. Using microscopy, by counting the number of stained cells compared to clear cells in a number of fields of view, it was possible to quantify the transfection efficiency. Larger numbers of cells can be stained by scaling up reagent volumes in proportion to the increase in plate surface area.
CHAPTER 3

Strategems in vitro for gene therapies directed to dominant mutations
3.1 INTRODUCTION

Many gene therapy studies so far undertaken have primarily focused on recessively inherited disorders, the rationale being that the successful introduction and expression of the wild-type gene may be sufficient to prevent the disease pathology arising, or at least aid in its amelioration. In contrast to recessive disorders, dominant disorders may be caused by a reduction in the level of wild-type protein, the presence of mutant protein (gain of function mutation) or by a combination of both. As for many dominant disorders the presence of the wild-type protein does not prevent the negative effect of the mutant protein, the introduction of an additional copy of the normal allele may in many cases not be sufficient to prevent disease pathology. Gene therapies for dominant diseases, where the pathology is due, at least in part, to a gain of function mutation rather than haplo-insufficiency alone may require suppression of the disease allele while maintaining expression of the wild-type allele. This may, in many instances, be difficult to achieve as frequently disease and normal alleles differ by only a single nucleotide. Furthermore, many dominant disorders are heterogeneous in that a variety of mutations within the same gene may give rise to a similar disease pathology. Designing a therapy directed towards each disease causing mutation may prove difficult, if not impossible, due to the time and cost involved and additionally the inability to discriminate between normal and disease alleles adequately using current technologies.

However, a variety of methods for gene suppression do currently exist. This, in conjunction with an ever-expanding knowledge of the molecular aetiology of disease results in an increasing number of potential disease targets for therapies. This has lead to the exploration of novel experimental approaches to the treatment of inherited disease using for example antisense DNA and RNA, ribozymes, triple helices or peptide nucleic acids. Varying successes have been reported with the aforementioned agents (Postel et al., 1991; Duval-Valentin et al., 1992; Bennett et al., 1994; Efrat et al., 1994; Kashani-Sabet et al., 1994; Khillan et al., 1994; Laitala et al., 1994; Larsson et al., 1994; Valera et al., 1994; Wakita et al., 1994; Resnicoff et al., 1994; Dosaka-Akita et al., 1995; Feng et al., 1995; Quattrone et al., 1995; Jankowsky et al., 1996; Knudsen et al., 1996; Lieber et al., 1996; Ohta et al., 1996; Porumb et al., 1996; Bauer et al., 1997; Lewin et al., 1997; Taylor et al., 1997; Kijima et al., 1998), but in many instances, complete suppression of expression of the target gene has been difficult to achieve. For some dominant disorders, low levels of mutant protein may be tolerated but for others it may be necessary to block expression of
the disease allele completely. However, the therapeutic utility of many gene suppressors, such as antisense oligonucleotides and ribozymes, is potentially limited by their short half-lives in biological fluids. Typically, unprotected RNA would have a half-life of a few seconds to minutes in human serum. To improve stability, oligonucleotide analogues in which the nucleic acid backbone or the nucleotides themselves are chemically modified to confer additional protection from degradation on the oligonucleotide have been designed. Modifications, such as phosphorothioates, have been made to antisense oligonucleotides to increase resistance to nuclease degradation or to alter binding affinity and cellular uptake (Cazenave et al., 1989; Wagner, R.W., 1994; McKay et al., 1996). Ribozymes, RNA enzymes which can elicit sequence specific cleavage of target RNAs (Cech., 1987; Haseloff and Gerlach, 1988; Pley et al., 1994), may also be modified by replacing regions of the catalytic RNA, with DNA or chemically modified nucleotides hence increasing their stability without a loss of catalytic activity (Pieken et al., 1991; Taylor et al., 1992; Heidenreich et al., 1994; Burgin et al., 1996; Lierdal et al., 1998; Sioud et al., 1998). Chemical modification of ribozymes to enhance activity in cell culture is addressed in greater detail in chapter 5 of this thesis. Moreover, hammerhead ribozyme stability and catalysis can be enhanced by proteins such as p7 nucleocapsid protein from HIV-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heterogeneous nuclear ribonucleoprotein A1 (Tsuchishashi et al., 1993; Bertrand et al., 1994; Muller et al., 1994; Sioud and Jespersen, 1996; Chapter 1, section 1.2.9). In addition to their use as tools for gene ablation, ribozymes have been proposed as a means of replacing defective RNAs with correct copies by targeted trans-splicing (Sullenger et al., 1994; Jones et al., 1996; Jones and Sullenger, 1996; Phylactou et al., 1998; Lan et al., 1998; Chapter 1, section 1.5.1).

Despite the availability of methods for efficient gene silencing, the extensive genetic heterogeneity associated with dominant disease makes therapeutic approaches based on targeting specific disease mutations unfavourable. For instance, individual mutations are often rare and can occur in a single family. Given the effort, time and cost involved in developing ‘designer’ therapies for each mutation that gives rise to disease pathology, it is apparent that mutation-independent therapeutic strategies would be required if combating autosomal dominant disease is to become feasible. Towards this end, three strategies for the suppression of dominant disease which are independent of the disease mutation have been developed and explored in vitro using retinal genes implicated in retinitis pigmentosa. Hammerhead ribozymes have been employed in all approaches; however, other
suppression effectors such as antisense RNA or DNA could equally be employed for these approaches. The first strategy involves ribozyme mediated suppression of both the normal and mutant alleles of a gene by targeting sequences in transcribed but untranslated regions (UTRs) and the concurrent introduction of a wild-type therapeutic gene with altered UTR sequence such that the delivered gene is protected from suppression (Farrar et al, PCT/BG96/02357). Targeting UTRs provides flexibility in the choice of sequence for suppression as UTRs are present in all forms of RNA. The approach has the advantage that the same therapeutic agent could be used, in principle, to suppress many different mutations in a given gene. In contrast, strategies directed towards single disease mutations are appropriate for patients only with the specific mutations. Moreover, targeting a specific mutation clearly restricts the sequence that can be chosen to achieve gene silencing whereas the general approaches adopted in this study allow a much wider choice of target sequence.

The second strategy explored in this thesis exploits the degeneracy of the genetic code and involves suppression in the coding region of a gene (Farrar et al, PCT/GB97/00929). Again, both the normal and disease alleles are silenced but in this instance by ribozymes designed to elicit sequence-specific cleavage at third base degenerative sites (wobble positions). Wild-type transcript is provided using a 'masked' replacement gene which has been altered at the targeted wobble position by a nucleotide substitution which does not alter the amino acid. Hence, the modified replacement transcript escapes ribozyme-mediated silencing but still codes for wild-type protein. The approach again provides a wide choice of target sequence which optimises the probability of efficient suppression being achieved. In essence the strategies involve gene suppression and replacement such that the replacement gene is masked from silencing. The same suppression and replacement steps could in principle be used to ameliorate many different mutations in a given gene, circumventing the need for mutation-dependent therapies i.e. the principle of the approaches being that one 'drug' fits all. This is particularly relevant when large numbers of mutations within a single gene gives rise to disease pathology, as is the case with many dominant disorders not least of which is retinitis pigmentosa.
A third strategy to achieve suppression in a mutation-independent manner has also been devised. This approach utilizes intragenic polymorphism (Farrar et al., PCT/GB97/00574). As sequence databases expand, it is becoming apparent that levels of intragenic polymorphism are substantial. Such polymorphism can be used to direct ribozyme cleavage specifically to transcripts from one allele of a polymorphism while allowing expression of the other allele. Again, this mutation-independent approach overcomes the need for developing mutation-specific therapies. The proportion of patients who potentially could be treated would depend on the allelic frequencies of the targeted polymorphism in a population (2pq). Additionally, as the approach enables continued expression of one allele, a replacement gene would only be required for dominant diseases where some disease pathology was due to haplo-insufficiency. The generation of suppressors targeting common intragenic polymorphisms may provide a powerful means of circumventing the extensive genetic heterogeneity encountered in many autosomal dominant disorders. The recent inclusion of the identification of 100,000 SNPs in the human genome as a key aim of the Human Genome Project highlights a growing realisation that such SNPs may be extremely valuable for many purposes - one of which is the utility described above - that is, discriminating between mutant and disease alleles in a mutation-independent manner.

This Ph.D. focuses on the application of the UTR and ‘wobble’ therapeutic strategies outlined above for a group of hereditary degenerative diseases of the retina termed retinitis pigmentosa (RP). Additionally, a number of mutation-specific therapeutic approaches for RP were explored briefly (Millington-Ward et al., 1997; Chapter 3, section 3.3.1). Studies of degenerative eye disorders, including RP and various macular dystrophies, have resulted in a substantial elucidation of the molecular aetiologies of these debilitating human retinopathies (McWilliam et al., 1989; Ott et al., 1990; Farrar et al., 1990, 1991 and 1993; Stone et al., 1992; Jordan et al., 1993; Inglehearn et al., 1993; Weber et al., 1994; Kajiwara et al., 1994; Dryja et al., 1994; Huang et al., 1995; Berson, 1996). RP primarily involves the loss of rod photoreceptor cells followed by cone cell degeneration, whereas macular degeneration largely involves the loss of cone photoreceptors. The availability of large RP pedigrees and the application of genetic linkage analysis has enabled the localisation of X-linked RP genes, autosomal dominant RP (adRP) genes and autosomal recessive RP genes. In some cases, the disease genes have been characterised and specific mutations identified. Mutations in the genes encoding the two main photoreceptor proteins, rhodopsin and peripherin/rds have been implicated in some autosomal dominantly
inherited retinopathies (Dryja et al., 1990; Farrar et al., 1991b; Kajiwara et al., 1991; Nichols et al., 1993; Wells et al., 1993). Together, over 150 rhodopsin and peripherin/rds mutations have been identified in patients suffering from adRP, congenital stationary night blindness and various macular degenerations. Studies using transgenic mice mimicking human rhodopsin- and peripherin-linked adRP [Chapter 1, sections 1.1.2-1.1.3] have indicated that at least some of the disease pathology is due to dominant gain of function mutations (Olsson et al., 1992; Nassh et al., 1993; Kedzierski et al., 1997); however, accurate expression of the wild-type gene may also be required, since over-expression of, for example, wild-type human rhodopsin has been shown to lead to disease pathology in transgenic mice (Olsson et al., 1992). Over the past few years, many other mouse models of human retinopathies have been created (Tsang et al., 1996; Humphries et al., 1997) which, with an ever-increasing knowledge of the molecular aetiologies of inherited disease, should greatly aid in the development of methods of therapeutic intervention for this group of debilitating disorders. In parallel, efficient viral and non-viral vectors for gene delivery will be required [see Chapter 1, section 1.5].

The feasibility of using intragenic polymorphism as a general mutation-independent suppression strategy for dominant disease has been explored by a colleague in the Ocular Genetics Unit, Sophia Millington-Ward. Another disease model was chosen for this study, namely Osteogenesis imperfecta (OI). OI is an autosomal dominantly inherited brittle bone disorder affecting approximately 1 in 12,000 people. Disease pathology in some forms of OI is the result of mutations in the collagen 1A1 and IA2 genes (COL1A1 and COL1A2). Like rhodopsin- and peripherin-linked adRP, OI is genetically heterogeneous; to date over 150 mutations have been identified in the COL1A1 and COL1A2 genes (Prockop et al., 1994 and 1995). Mutations in these genes are also known to cause some forms of Marfans disease and type VII Ehlers-Danlos syndrome (Lehmann et al., 1994). In an effort to circumvent the immense allelic heterogeneity associated with dominantly inherited OI, ribozymes have been generated which target intragenic polymorphisms in COL1A1 and COL1A2. For further details of this mutation-independent strategy see S. Millington-Ward Ph.D thesis and Millington-Ward et al. (1997, 1999).

Notably, the three mutation-independent strategies outlined above could potentially be applied to a wide range of dominantly inherited diseases involving gain of function mutations. Indeed there are over 1,000 dominantly inherited disorders in humans.
Additionally, many of the characterised mutations that give rise to inherited disease are inherited in an autosomal dominant fashion; disorders such as epidermolysis bullosa, hypertrophic cardiomyopathy, Marfans syndrome and Charcot-Mary-tooth disease represent a few of the very large number of examples. Many of these diseases represent potential target disorders for the strategies explored in this thesis.

Therapies for dominant disorders, such as adRP, where extensive genetic heterogeneity has been problematic could be directed to the primary genetic defect by eliminating or reducing the presence of the mutant protein while maintaining expression of the wild-type protein. Alternatively, therapies could be directed to modulating secondary effects that contribute to disease pathology, for example, by targeting mechanisms of photoreceptor apoptosis [see chapter 1] or administering neurotrophic factors which aid in photoreceptor survival (Cayouette et al., 1998; Chong et al., 1999; Lewin et al., 1999). The current study focuses on the former approach - targeting the primary defect. Notably, both mutation-specific and mutation-independent therapeutic strategies for adRP are addressed.

Hammerhead ribozymes have been employed in the study due to minimal cleavage site sequence requirements. Their flexibility in choice of target sequence combined with their high sequence specificity and catalytic activity make ribozymes in many cases favourable as gene suppressing agents when compared to, for example, antisense oligonucleotides and triple helix DNA. A range of hammerhead ribozymes targeting retinal genes have been designed, generated and evaluated both in vitro and in cell culture. The results from this study are presented in this Ph.D thesis.
3.2 MATERIALS AND METHODS

3.2.1 Secondary structure prediction and ribozyme design
Rhodopsin and peripherin/rds (human and mouse) sequences were obtained from the GenBank database. When necessary, genomic sequences were edited by removing intronic regions so as to generate cDNA/mRNA sequence. Sequences were subsequently analysed to identify potential NUX ribozyme cleavage sites. Due to project design, this search was focused on UTRs, motifs where the third base was a suitable degenerative nucleotide and dominant disease mutations. Predicted secondary structures for mRNAs were obtained using the RNAPlotFold program from the GCG package (Genetics computer group, Madison, WI, USA). Hammerhead ribozymes were designed to target suitable NUX cleavage sites present in accessible regions (open-loop structures) of the RNAs. When possible, GUC motifs were chosen, however this was not always possible. The integrity of loops was evaluated by comparative analysis of the fifteen most probable two-dimensional conformations. In addition, predicted structures for truncated RNA products were generated and the integrity of open-loops between full-length and truncated RNAs compared [see Figure 3.1]. All ribozymes were designed with asymmetric antisense flanking sequences of seven and eight bases.

3.2.2 Cloning Vectors
All cDNAs, ribozymes were cloned into the multiple cloning site (MCS) of commercial mammalian expression vectors (pcDNA3, pZeoSV and pZeoSVLacZ), which enable expression in vitro from T7, T3 or Sp6 promoters and in cell culture from CMV or SV40 promoters [Figure 3.2]. Inserts were typically placed at or near the terminal ends of the MCS thereby reducing any possible problems arising with expression efficiency due to the presence of a large proportion of the MCS. Positive clones were identified by restriction enzyme analysis and sequenced using ABI automated sequencing machinery.
Mouse rhodopsin cDNA in pcDNA3

EcoR1

pcDNA3

5'-UTR

Rz3 targets a
GUC motif

1393-1395

Coding sequence

Rz32

UUC

1458-1460

Rz33

UUU

1459-1460

Degenerative nucleotides
at cleavage sites

Mouse peripherin cDNA in pcDNA3

HindIII

5'-UTR

pcDNA3

Rz7

Rz6

Rz17

CTC GTA AUU

153-155 207-209 162-164

Coding sequence

Human rhodopsin cDNA in pcDNA3

HindIII

5'-UTR

pcDNA3

Rz447

GTC

445-447

Rz10, Rz40

GTC

475-477

CUC

544-546

CUC

577-579

GUC

982-984

RzMM

Coding sequence

Mouse peripherin cDNA in pcDNA3

HindIII

5'-UTR

pcDNA3

Rz9

GTT

190-192

Rz8

CTA

234-236

Rz30

CTA

255-257

Rz31

CTA

357-359

Degenerative nucleotides
at cleavage sites
Rz447 and human rhodopsin

(a) Human rhodopsin cDNA

A human rhodopsin cDNA clone was obtained from Dr. W. Baehr. Using primer-driven PCR mutagenesis and a HindIII (in pcDNA3) to BstEII (in exon IV of human rhodopsin) PCR cassette, the full 5’-UTR was inserted into the clone. The human rhodopsin cDNA with full-length 5’-UTR was subsequently cloned into the HindIII and EcoRI restriction sites of pcDNA3 by Dr. Jane Farrar. (Human rhodopsin sequence accession no. KO2281). The clone was digested with Acyl1 prior to expression.

(b) Human rhodopsin hybrid with Gly51Val mutation

A single base change (GlyGGC51ValGTC) which creates a ribozyme cleavage site was introduced into the human rhodopsin cDNA using primer driven mutagenesis and a HindIII to BstEII cassette (by S. Millington-Ward). This clone was digested with BstEII prior to expression.

(c) Ribozyme 447

A hammerhead ribozyme, Rz447, was designed to target an accessible GUC motif at position 445-447 of the human rhodopsin sequence. Subsequent to synthesis and annealing, Rz447 was cloned into the HindIII and XbaI sites of pcDNA3 (by S. Millington-Ward). The ribozyme sequence is given below - antisense flanking sequence is underlined:

```
5'- UGGGGAACUGAUGAGUCCGUGAGGACGAAACCAGCAC -3'
```

7 bases 8 bases
3.2.4 UTR approach: 5'-UTR cleavage and replacement

Ribozyme 3 and mouse rhodopsin; wild-type & mRhoH1 and mRhoH2

(a) Mouse rhodopsin cDNA
The full length mouse rhodopsin cDNA was cloned into the EcoR1 site of the multiple-cloning site of pcDNA3. This cDNA clone was generated using an EcoR1 site at position 1120 5' of the transcriptional start site (accession no. M55171).

(b) Hybrid cDNA with altered 5' non-coding sequence, mRhoH1
A mouse rhodopsin hybrid cDNA with altered 5'-UTR sequence was generated by PCR primer-directed mutagenesis using a HindIII (in pcDNA3) to Eco47III (in exon 2) cassette. The 5'-UTR of mouse rhodopsin cDNA was replaced with human peripherin 5'-UTR sequence. Mouse rhodopsin sequence begins at the mouse rhodopsin ATG start site in the hybrid clone. Eco47III was used to linearise the plasmid prior to RNA production.

(c) Hybrid cDNA with altered 5' non-coding sequence, mRhoH2
A second mouse rhodopsin cDNA hybrid with an altered 5'-UTR sequence was designed to eliminate the GUC ribozyme target motif (Rz3 target site). The GUC→GGC sequence change was generated using a HindIII→Eco47III PCR cassette by primer directed PCR mutagenesis. Fsp1 was used to linearise the hybrid clone for subsequent RNA synthesis.

(d) Ribozyme 3
A hammerhead ribozyme, Rz3, designed to target a predicted open-loop structure in the 5'-UTR of mouse rhodopsin cDNA was synthesised and subsequently cloned into the HindIII and Xho1 sites of pcDNA3. Rz3 is composed of a nucleolytic motif targeting a GUC site at position 1393-1395 of the mouse rhodopsin sequence flanked by two antisense arms of seven and eight bases respectively. Rz3 sequence is given below - flanking antisense arms are underlined:

\[
\text{5'-CUUCGUACUGAUGAGUCCGUGAGGACGAAACAGAGAC-3'}
\]

7 bases 8 bases

The clone was digested with Xho1 prior to RNA synthesis. Mouse rhodopsin hybrids, mRhoH1 and mRhoH2, were generated and subsequently cloned by Dr. Jane Farrar. All
subsequent work such as digestions, purifications and cleavage reactions were performed by the author.

Ribozymes 6, Rz7 and Rz17 and mouse peripherin; wild type and mPerH1

(a) Mouse peripherin cDNA

The mouse peripherin cDNA which was cloned into the HindIII and EcoRV sites of pcDNA3 was obtained from Dr. Jane Farrar. The clone contained the complete 5’-UTR sequence together with twenty-seven bases of additional vector sequence 5’ of the UTR sequence. BglII or NolI were used to linearise the clone for subsequent RNA expression.

(b) Hybrid cDNA with altered 5’-non-coding sequence, mPerH1

A mouse peripherin hybrid cDNA with an altered 5’-non-coding region was generated by primer driven PCR mutagenesis using a HindIII (in pcDNA3) to SacII (in coding sequence of mouse peripherin cDNA) PCR cassette (performed by Dr. Jane Farrar). In this construct, the mouse peripherin 5’-UTR sequence, including the twenty-seven bases of additional sequence, was replaced with mouse rhodopsin 5’-UTR sequence. BglII was used to linearise the vector for RNA synthesis. (Mouse peripherin cDNA sequence accession no. X14770).

(c) Ribozyme Constructs

Three hammerhead ribozymes, Rz6, Rz7 and Rz17 were designed to target predicted open-loop structures in the 5’-UTR of the mouse peripherin gene sequence. Rz6 and Rz7 were directed to GTA and CTC target motifs at positions 207-209 and 153-155 respectively. Rz17 targeted an AUU triplet motif at position 162-164 of the sequence. Whereas, Rz6 and Rz7 were cloned into the HindIII and Xho1 sites of pcDNA3, Rz17 was placed into the HindIII and Xba1 sites. All ribozymes were grown-up in bulk and digested with Xho1 (Rz6 & Rz7) and Xba1 (Rz17) for subsequent RNA production. Rz6, Rz7 and Rz17 sequence is given below - antisense arms are underlined:

Rz6: 5’-GCCAUCGUCAUGAGUGCCUGUGAGGACGAAACCGACU-3’
8 bases 7 bases

Rz7: 5’-UCCGAGUCAUGAGUGCCUGUGAGGACGAAACCGACU-3’
7 bases 8 bases
Ribozymes 8 and 9 and human peripherin; wild-type and hPerH1

(a) Human peripherin cDNA

A full length human peripherin cDNA, with the 5'-UTR, was kindly provided by Dr. Gabriel Travis. The gene was cloned into the EcoRI site of the multiple cloning site in the pBluescript vector. Prior to RNA synthesis, the clone was digested with BglII.

(b) Hybrid cDNA with altered 5'-non-coding sequence; hPerH1

A hybrid human peripherin cDNA clone with an altered 5'-UTR sequence was generated using primer direct PCR mutagenesis of a BamH1 (in 5'-UTR sequence) to BglIII (in coding sequence of human peripherin cDNA) DNA fragment (performed by Jane). This clone contains human peripherin 5'-UTR sequence to the BamH1 site at position 76 and mouse peripherin 5'-UTR sequence to the ATG start site (position 84-250 of mouse cDNA sequence), the remaining sequence is human peripherin sequence. The clone was digested with AarII for subsequent RNA expression. (Human peripherin cDNA sequence accession no. M62958).

(c) Ribozyme constructs.

Two hammerhead ribozymes, Rz8 and Rz9 were designed to target accessible CUA and GUU motifs, at positions 234-236 and 190-192 respectively in the 5'- untranslated region of the human peripherin cDNA sequence [Figure 3.1e]. Both ribozymes were cloned into the HindIII and Xba1 sites of pcDNA3 (performed by Gearoid Tuohy). Clones were grown-up in bulk and linearised with Xba1 prior to RNA expression. Rz8 and Rz9 sequences are given below - flanking antisense arms are underlined:

Rz8: 5'-CCAAGUCCUGAUGAGUCCGUGAGGACGAA AGUCCGG-3'
7 bases
8 bases

Rz9: 5'-CAAACCUCUGAUGAGUCCGUGAGGACGAAACGAGCC-3'
8 bases
7 bases
3.2.5 Degeneracy of the genetic code: Wobble-base cleavage and replacement

Ribozyme 30, Rz31 and human peripherin; Wild-type, hPerH2 and hPerH3

(a) Human peripherin cDNA

The human peripherin cDNA/pBluescript clone obtained from Dr. G. Travis was digested with EcoR1. This produced two fragments each of 2.9Kb; the full-length human peripherin cDNA and the remaining vector backbone. The 2.9Kb cDNA fragment was isolated by digesting the vector backbone with AflIII and Pvu1 (only present in vector sequence). The human peripherin cDNA was gel isolated and cloned into the EcoR1 site of pcDNA3 in a 5'→3' orientation. Prior to transcription, the clone was digested with BglII.

(b) Hybrid cDNAs with altered sequence at wobble positions; hPerH2 and hPerH3.

Two human peripherin hybrid cDNAs, hPerH2 and hPerH3, with single nucleotide substitutions at positions 257 and 359 of the peripherin DNA sequence (Accession no. M73531) respectively were generated by primer-directed PCR mutagenesis. The incorporated substitutions were adenine to guanine changes which occurred at silent positions and therefore did not give rise to amino acid changes. However, in both cases (257 and 359) the ribozyme cleavage sites were eliminated; CTA Leu→CTG Leu. hPerH2 and hPerH3 were digested with AvrII and BglII respectively and expressed in vitro.

Substituted bases are underlined and in bold-type:

(1) Flank Forward Primer: 5'-ACCCAAGCTTGGTACCGAGC-3' (885-904 in pcDNA3). Kpn1

(2) Mutation Reverse 257: 5'-TGACTTTCAGCAGCGCCATG-3' (248-267 of H.RDS).

(3) Mutation Forward 257: 5'-CATGGCGCTGCTGAAAGTCA-3' (248-267 of H.RDS).

(4) Flank Reverse Primer: 5'-GTCCACCAGGCTACCGCCCAT-3' (850-869 of H.RDS). Kpn1

(5) Mutation Forward 359: 5'-CATCTTCAGCCTGGGACTGT-3' (347-366 of H.RDS).

(6) Mutation Reverse 359: 5'-ACAGTCCCAGGCTGAAGATG-3' (347-366 of H.RDS).

(7) Seq'97 Primer: 5'-GCTAAGAGAACCACCACACTGC-3' (824-844 in pcDNA3).
A diagrammatic representation of the primer-directed PCR mutagenesis process is given in figure 3.3.

(c) Ribozyme constructs

Two hammerhead ribozymes, Rz30 and Rz31, were designed to target NUX motifs predicted to occur in accessible regions of the human peripherin cDNA sequence. Both target sites were CTA, at positions 255-257 (Rz30) and 357-359 (Rz31). It is of note that the third nucleotide in each triplet motif was degenerative (see above). Both ribozymes were cloned into the HindIII and Xba1 sites of pcDNA3. Xba1 was used to linearise the clones for subsequent transcription. Rz30 and Rz31 sequences are given below - flanking antisense arms are underlined:

Rz30: 5'-'ACUUUCAGCUAGAGUCGUGAGGACGAAAGCGCCA-3'
   8 bases    7 bases

Rz31: 5'-'ACAGUCCUGAUGAGUCGUGAGGACGAAAGGCGUGA-3'
   7 bases    8 bases

Ribozyme 32, Rz33 and mouse rhodopsin: Wild-type and mRhoH3

(a) Mouse rhodopsin cDNA

The mouse rhodopsin cDNA construct was previously described in section 3.2.4. The clone was linearised with Eco47III prior to RNA synthesis.

(b) Hybrid cDNA with altered sequence at wobble position, mRhoH3

A mouse rhodopsin hybrid cDNA with a single nucleotide alteration at position 1460 of the mouse rhodopsin sequence (accession no. M55171) was generated by primer directed mutagenesis, using a HindIII to Eco47III PCR cassette. The sequence alteration was a T→C substitution that occurred at a degenerative site. Once again, the ribozyme cleavage was eliminated; TTT (Phe)→TCT (Phe) but the amino acid sequence remained the same. The clone was digested with Eco47III for expression in vitro.
(C) Ribozyme constructs

Two hammerhead ribozymes, Rz32 and Rz33, were designed to target a predicted open-loop structure in RNA from the coding region of the mouse rhodopsin gene. Both nucleolytic motifs were TTT at positions 1458-1460 (Rz32) and 1459-1461 (Rz33) of the sequence. Rz32 and Rz33 were cloned into the *HindIII* and *XbaI* sites of pcDNA3. *XbaI* was used to linearise both clones. Rz32 and Rz33 sequences are given below - flanking antisense arms are underlined:

Rz32: 5'-GGCACAUACUGAUGAGUCCGUGAGGACGAAAAAUUGG-3’

8 bases

Rz33: 5'-GGCACAUACUGAUGAGUCCGUGAGGACGAAAAAUUGG-3’

7 bases

3.2.6 Determination of molar ratios of template: ribozyme required for maximum cleavage

The levels of ribozyme required to achieve maximum cleavage were assessed for ribozymes, which from initial analysis, appeared efficient (Rz3, Rz9, Rz30 and Rz31) by using varying molar ratios of ribozyme to template RNA in three hour reactions at 37°C. Ratios of template to ribozyme varied between 1:1, 1:5, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500, 1:1000. Molar ratios of ribozyme: template were calculated as follows:

\[
\frac{[A]}{[C/D]} \times \frac{[E/F]}{B} = 1:1 \text{ ratio.}
\]

A = number of disintegrations of template used in reaction.
B = number of disintegrations of ribozyme to be used in reaction.
C = ratio of ‘hot’ rUTP to ‘cold’ rUTP in template reaction.
D = ratio of ‘hot’ rUTP to ‘cold’ rUTP in ribozyme reaction.
E = length of template (bases) and F = length of ribozyme (bases).
3.2.7 Expression of RNA

To obtain optimal yields of RNA, high quality caesium chloride purified DNA templates were used for all transcription reactions. Wild-type, replacement gene and ribozyme constructs were linearised with appropriate restriction enzymes [Tables 3.1, 3.2] prior to expression to obtain transcripts of defined length [Figure 3.4] and enable visual differentiation of RNA products on acrylamide gels. RNAs were obtained from clones expressed *in vitro* using a commercially available Ribomax transcription system and standard protocols (Promega). Radioactively labeled products were obtained by incorporating $[^{32}\text{P}]$ rUTP (10mCi/ml Amersham) into reactions. Transcription products were separated from prematurely terminated transcripts by gel isolation (4% polyacrylamide), and eluted in a solution of 0.3M sodium acetate (pH5.2) and 0.2% SDS. RNAs were purified in equal volumes of phenol x 2, phenol chloroform, chloroform, ethanol precipitated and washed in 70% ethanol. Pellets were dried at 60°C and resuspended in DEPC-treated H$_2$O. Disintegrations used to determine molar ratios and the quantities of RNA electrophoresed (~ 5cpm/lane) were counted by liquid scintillation reading.

3.2.8 Ribozyme Cleavage Reactions

Hammerhead ribozyme cleavage reactions were performed in a solution of 50mM Tris-HCl, pH 8.0 at 37°C, typically for 3 hours, with varying MgCl$_2$ concentrations (0-15mM). Timepoint cleavage reactions (up to 5 hours) were carried out at the pre-determined optimal MgCl$_2$ concentration. In initial experiments, large molar excesses of ribozyme:template were used (approximately 500:1) to examine the feasibility of the ribozyme-based mutation-independent gene suppression and replacement strategies (see Introduction). Radiolabeled templates, hybrids and cleavage products were electrophoresed on 4-8% polyacrylamide gels and analysed by autoradiography. When necessary, cleavage efficiencies were determined using an instant imager (Packard). The expected size of template and cleavage product RNAs are given in tables 3.1 and 3.2.

3.2.9 Size marker for RNAs

*Msp*1 cut pBR322 DNA was end-labeled using standard protocols (see chapter 2.2.15). As the mobility of DNA and RNA differ depending on electrophoretic conditions and percentage of polyacrylamide used, the marker only provides an estimate of transcript size [figure 3.5].
3.2.10 Generation of hammerhead ribozymes

20-25μg of forward and reverse strand oligonucleotides containing ribozyme sequence together with restriction enzyme sites to facilitate cloning were annealed by heating to 94°C for 5 minutes, in the presence of 25mM NaCl, and cooled to room temperature overnight. The annealed primers were digested with appropriate restriction enzymes, purified by phenol chloroform extraction, ethanol precipitated and cloned into suitable expression vectors.
3.3 RESULTS

Mutation-specific approach

3.3.1 Rz447 and human rhodopsin

A hammerhead molecule, Rz447 was designed to target and cleave a known adRP mutation at codon 51 of the human rhodopsin gene. The mutated target rhodopsin clone (GlyGGC51ValGTC), the wild-type rhodopsin clone and Rz447 were transcribed in vitro (by S. Millington-Ward). Resulting RNAs were mixed together in the presence of 0-15mM MgCl₂ for three hours. Uniform cleavage of mutant RNA was attained at all Mg²⁺ concentrations, yielding cleavage products of predicted size (607 + 254 bases; figure 3.6). In contrast, wild-type rhodopsin RNA remained intact under all conditions tested [Figure 3.6]. No cleavage was observed in the absence of MgCl₂, demonstrating that for activity of the ribozyme, the presence of divalent metal ions is a pre-requisite. It is believed that divalent cations aid in structural stabilisation of the folded RNA and are generally required for ribozyme chemistry (Pyle, 1993). Although in vitro cleavage of mutant human rhodopsin RNA by Rz447 was incomplete, such incomplete ribozyme cleavage has been observed with a Pro23His rhodopsin mutant (Drenser et al., 1998) and nevertheless lead to a beneficial effect on photoreceptor degeneration in vivo (Lewin et al., 1998). However, the Gly51Val mutation represents <1% of rhodopsin-linked adRP cases highlighting a need for more general suppression strategies which are independent of the disease mutation. Given the diversity of mutations in retinal genes that can give rise to photoreceptor degenerations in humans, mutation-independent approaches to therapy have been explored in the current study. Hence, a battery of ribozymes targeting retinal transcripts in a mutation-independent manner have been generated and evaluated for cleavage efficiencies.

Mutation-independent approach: 5'-UTR cleavage and replacement

3.3.2 Rz3 and mouse rhodopsin

The mouse rhodopsin and hybrid cDNAs, mRhoH1 and mRhoH2, were transcribed in vitro. Resulting RNAs were purified and mixed separately and together with Rz3 to test for cleavage under various conditions [Figure 3.7a, b, c]. In all cases, the unadapted mouse rhodopsin transcript cleaved into fragments of predicted size in a Mg²⁺-dependent manner [Figure 3.7a, b, c; table 3.1]. Cleavage of the unadapted RNA went to completion with little or no residual target RNA remaining. Notably, mRhoH1 and mRhoH2 transcripts
with altered 5’-UTR sequences (see section 3.2.4) were protected from cleavage by functional Rz3 when combined with Rz3 and unadapted mouse rhodopsin transcript [Figure 3.7b, c]. No cleavage of hybrid RNAs was evident even after three hour incubations. This, when compared to the rate of cleavage of unadapted transcript (immediately following addition of divalent ions) highlights the sequence specificity and efficiency of Rz3. Therefore, subtle sequence modifications, such as the single nucleotide alteration made to mRhoH2, may be all that is required to protect transcripts expressed from replacement genes from cleavage. However, transcripts from the first hybrid cDNA, mRhoH1, would be protected from binding and cleavage by a range of ribozymes or antisense molecules. This could be of particular relevance in situations requiring a spectrum of approaches to achieve complete gene silencing. As the sequences involved in controlling translation of retinal genes have not fully been established, mRhoH1 was designed so that sequence of the mouse rhodopsin cDNA was replaced with 5’-UTR sequence of a gene expressed in the same tissue. This should minimise any possible adverse effects of sequence modifications on the level and specificity of expression.

3.3.3 Rz6, Rz7 and mouse peripherin
The mouse peripherin cDNA, Rz6 and Rz7 were transcribed in vitro. The resulting mouse peripherin transcript was mixed with Rz6 and Rz7 RNAs in the presence of 0, 5, 10 and 15 mM MgCl₂ for three hours at 37°C to test for cleavage. The expressed peripherin transcript was the correct size, however, no cleavage by Rz6 or Rz7 was evident at any MgCl₂ concentration tested [Figure 3.8a-b; table 3.1]. This urged an examination of Rz6 and Rz7 sequence for possible errors introduced during primer synthesis, cloning or initial ribozyme design. However, analysis confirmed that the sequence of the conserved ribozyme catalytic core and antisense flanks, which determine specificity of cleavage were correct. Another possible explanation for non-cleavage of the peripherin transcript by Rz6 and Rz7 may be an inability of the ribozymes to access, bind or cleave their respective targets due to the presence of intermolecular secondary structures protecting the cleavage site on the substrate. This scenario was not immediately apparent from computer-aided RNA folding patterns obtained. However, the open-loops targeted by Rz6 and Rz7 were surrounded by extremely structured RNA which, in an alternate folding pattern may have inhibited binding of both ribozymes [Figure 3.1c]. Alternatively, the small predicted open-loop structures targeted by Rz6 and Rz7 may not have been as robust or conserved as predicted. As the computer program used (RNAPlotFold) only provides predictions of how a
particular RNA folds, it may not in some instances provide accurate information (see Chapter 1; section 1.2.7). Ribozyme binding sites which appear accessible from predicted RNA secondary structure may in fact, in some cases, be absent altogether.

3.3.4 Rz17 and mouse peripherin

The mouse peripherin target, mouse peripherin hybrid (mPerH1) and Rz17 were transcribed in vitro. Resulting target RNAs were mixed separately and together with Rz17 RNA at 37°C to test for cleavage. Experiments were performed at various Mg\(^{2+}\) concentrations over time [Figure 3.9a, b]. All RNAs and cleavage products were of predicted size [Figure 3.9; table 1]. Partial cleavage of unadapted mouse peripherin RNA by Rz17 was obtained which increased significantly over time [Figure 3.9b]. Lack of complete cleavage may be due to inaccessibility of some RNA conformations to ribozyme binding and/or cleavage. Multimeric ribozymes (connected or shotgun) may aid in improving the efficiency of ribozymes at suppressing genes (Chapter 1.3) and could be applicable in situations where complete cleavage, by monomeric ribozymes, cannot be achieved; the design and analysis of a retinal-specific multimeric ribozyme is addressed in chapter 4. In contrast, RNA expressed from the mouse peripherin cDNA construct mPerH1, which contains mouse rhodopsin 5' - UTR sequence remained intact at all MgCl\(_2\) concentrations examined [Figure 3.9c, d]. As with mRhoH2, mPerH1 was constructed with 5'-UTR sequence from a gene expressed in photoreceptor cells thereby minimising any possible effects on tissue-specific expression.

3.3.5 Rz8, Rz9 and human peripherin

Unadapted and adapted human peripherin cDNAs were transcribed in vitro. Resulting RNAs were purified and mixed separately and together with in vitro transcribed Rz8 and Rz9 to test for cleavage. When unadapted human peripherin RNA was mixed with Rz8, in the presence of varying MgCl\(_2\) concentrations, it was efficiently cleaved in to 5' and 3' fragments of predicted size; the intensity of residual uncleaved transcript decreased over time [Figure 3.10a; table 3.1]. In contrast, when adapted hPerH1 hybrid RNA was mixed with Rz8 under similar conditions, no cleavage occurred; the intensity of hPerH1 RNA remained constant (except lane 9 - loading error), [Figure 3.10a]. Timepoint cleavage reactions of adapted and unadapted RNAs with Rz8 and 15mM MgCl\(_2\) demonstrated almost complete ablation of unadapted transcript [Figure 3.10b]. However hPerH1 RNA was not cleaved by Rz8, even after 3 hours, and remained intact. Moreover, the intensity of
hPerH1 remained constant over time and no additional cleavage products were observed. Similar results were obtained after incubation with Rz9 [Figure 3.10c, d]. Although efficient cleavage was obtained with Rz8 and Rz9, the cleavage profiles at various MgCl$_2$ concentrations varied; Rz9 was more active over a broader range of concentrations. Some ribozymes may be preferentially active under certain physiological conditions; combinations of ribozymes targeting different two-dimensional RNA conformations or requiring different intracellular conditions may be required to obtain efficient cleavage in vivo. All in vitro transcription reactions, RNA purifications and cleavage reactions were performed by the author. However, gel electrophoresis was undertaken in conjunction with Sophia M. Ward.

This study clearly demonstrates using multiple examples that by modifying the sequence of non-coding regions, transcripts can be masked from cleavage by ribozymes (and probably antisense molecules) directed to sites in transcribed but untranslated regions. The examples so far provided, employed hammerhead ribozymes directed to 5'-UTR sequences of rhodopsin and peripherin transcripts. However, suppression effectors could and have been directed to non-coding sequence such as 3'-UTRs and intronic regions. Suppression could be achieved using antisense RNA or ribozymes designed to target any one of the aforementioned regions or for that matter, any combination of non-coding sequence. The development of gene therapy approaches based on the use of gene suppressors targeting UTR sequences in the manner described in this chapter addresses many of the shortcomings of previously undertaken investigations which were severely hampered by the immense genetic heterogeneity present in some dominantly inherited diseases - not least of which is retinitis pigmentosa. The UTR-based strategy explored in vitro in this study using retinal transcripts is, in theory, suitable for all mutations associated with rhodopsin- and peripherin-related adRP and thereby circumvents the need to develop over 150 separate mutation-specific therapies.
Degeneracy of the genetic code: Wobble-base cleavage and replacement

3.3.6 Rz30, Rz31 and human peripherin

Human peripherin hybrid cDNAs with single nucleotide alterations at positions 257 and 359 of the peripherin DNA sequence were generated. The alterations were single nucleotide substitutions (adenine to guanine) at degenerative sites which eliminated ribozyme cleavage sites but did not give rise to amino acid changes. This was performed by primer-based PCR mutagenesis comprising of three sets of PCR reactions [see section 3.2.5 for primer information and Figure 3.3 for a diagrammatic representation]. The first involved the amplification of a 320 base pair fragment from human peripherin sequence using primer pairs, flank forward (primer 1) and mutation reverse 257 (primer 2) [Figure 3.11a]. The second involved amplification of a 600 base pair fragment, again using the human peripherin cDNA as a template, but with primer pairs, mutation forward 257 (primer 3) and flank reverse (primer 4) [Figure 3.11b]. During synthesis, all mutation primers had appropriate nucleotide alterations incorporated into the primers. Amplification products from PCR1 and PCR2 were gel-purified and in combination, used as a template for the third PCR. Primer pairs for the third PCR using 15mM MgCl$_2$ and a 52° annealing temperature, were flank forward (primer 1) and flank reverse (primer 4). The resulting 920 base pair PCR product [Figure 3.11c] was sequenced to ensure incorporation of the desired nucleotide change and subsequently digested with Kpn1 to facilitate cloning.

Additionally, the human peripherin cDNA in pcDNA3 clone was digested with Kpn1 to remove a 920 base pair fragment, and purified to enable insertion of the 920bp PCR amplified fragment with the single nucleotide substitution described above into the construct. Similarly, mutation forward and reverse 359 primers [section 3.2.5] in combination with flank forward and reverse primers, were used to generate the human peripherin hybrid cDNA with a single nucleotide substitution at position 359. Several attempts were made at cloning the Kpn1 hybrid fragments but all proved unsuccessful. As Kpn1 demonstrates star-activity, the isoschizomer Acc651 was used, however, cloning was still unsuccessful. Therefore, it was decided to re-design the flank forward primer such that it was upstream of the T7 promoter in pcDNA3 so that the resulting PCR fragments incorporated T7 promoter sequence and hence could possibly be directly expressed in vitro. The new forward primer was denoted Seq'97 [see section 3.2.5]. The resulting hybrid cDNAs were termed hPerH2 (substitution at position 257) and hPerH3 (substitution at position 359).
Wild-type human peripherin and human peripherin hybrids, hPerH2 and hPerH3, were transcribed in vitro. Resulting RNAs were mixed separately and together with in vitro transcribed Rz30 [Figure 3.12a, b, c, d]. When unadapted peripherin RNA was incubated at 37°C with Rz30 at varying MgCl₂ concentrations and for various times, two cleavage products of predicted size were obtained [Figure 3.12a; table 3.2]. Significant cleavage of unadapted RNA occurred with residual amounts of intact RNA remaining at each MgCl₂ concentration. Notably, cleavage of the unadapted transcript was rapid; cleavage products were observed after only 30 seconds incubation with ribozyme [Figure 3.12b]. Residual intact transcript may represent RNA conformations that were inaccessible to ribozyme binding or cleavage. The design of multimeric ribozymes to such transcripts may aid in obtaining complete ablation of the target RNA. In contrast, when adapted RNA with a single base change at position 257 (hPerH2) was mixed with Rz30 under the same conditions, no cleavage was observed; the intensity of hPerH2 remained constant at all MgCl₂ concentrations over the three hour time frame [Figure 3.12c]. Again, when adapted and unadapted RNAs were incubated together with Rz30, the adapted RNA remained intact whereas wild-type peripherin was almost completely digested producing 5' and 3' cleavage products [Figure 3.12d; table 3.2]. The single base change in hPerH2 occurs at a 'wobble' position of the codon and therefore does not alter the amino acid encoded by the original triplet. Similar results were obtained when wild-type peripherin RNA and modified hPerH3 RNA were mixed with in vitro transcribed Rz31 [Figure 3.13a, b, c]. Target RNAs and cleavage products were of predicted sizes [Table 3.2], however, Rz31 was not as efficient as Rz30 at cleaving its target with significant levels of intact human peripherin RNA remaining after three hours. In contrast, transcripts from the replacement cDNA hPerH3, were not digested and remained completely intact after prolonged periods in the presence of active Rz31 [Figure 3.13c]. Hence, Rz30 and Rz31 could potentially be used to down-regulate expression of mutant transcripts in a mutation-independent manner while transcripts from replacement genes would remain intact and therefore, in principle, supply wild-type protein.
3.3.7 Rz32, Rz33 and mouse rhodopsin

Wild-type and hybrid (mRhoH3) mouse rhodopsin cDNAs were transcribed in vitro. Resulting RNAs were incubated with in vitro transcribed Rz32 and Rz33 at varying MgCl₂ concentrations, over time. When unadapted mouse rhodopsin was mixed with Rz32 and Rz33 at 5, 7.5 and 10 mM MgCl₂, cleavage products of predicted size were obtained [Figure 3.14a, c; table 3.2]. No cleavage was observed in the absence of MgCl₂, demonstrating that for successful hammerhead ribozyme cleavage the presence of divalent cations is essential. Even though the UUU triplet motifs targeted by Rz32 and Rz33 were separated by only one nucleotide, Rz32 was significantly more efficient at cleaving mouse rhodopsin RNA. However, even though cleavage with both ribozymes (Rz32 and Rz33) increased significantly over time, substantial levels of target RNA remained intact [Figure 3.14b, c]. This may be due to the specific ribozyme target motifs on the RNA substrate; some studies have suggested that ribozymes directed to UUU sites (Rz32 and Rz33) may be less efficient at cleaving target RNAs when compared to other NUX target site permutations (Shimayama et al., 1995; Chapter 1, section 1.2.7). Notably, the replacement RNA with a sequence modification at a wobble site, mRhoH3, was protected from cleavage by Rz33, remaining completely intact under all conditions tested [Figure 3.14d]. Hence the protected replacement gene could, in principle, supply wild-type protein while mutant transcripts would be cleaved by Rz33 in a manner independent of the disease mutation itself.

3.3.8 Determination of molar ratios of ribozyme to template required for maximum cleavage

The molar ratios of ribozyme to template required to achieve maximum cleavage were assessed for Rz3, Rz9, Rz30 and Rz31. Cleavage profiles can be seen in figures 3.15a-d. Rz3 was extremely efficient at cleaving mouse rhodopsin RNA; as ratios of ribozyme to target increased, the intensity of cleavage products also increased with a concomitant reduction of target RNA. Similar results were obtained for Rz9 and Rz30 when incubated with human peripherin RNA [Figure 3.15b, c]. Rz3, Rz9 and Rz30 were extremely active, cleaving their respective targets almost to completion at ratios of ribozyme to template as low as 5:1. In contrast, Rz31 was inefficient at cleaving human peripherin RNA at all ribozyme to template ratios examined [Figure 3.15d]. Significant levels of target RNA remained intact in all reactions, even when vast excesses of Rz31 were used [Figure 3.15d, lane 9]. All gels were quantified by phosphoimaging and results plotted on graphs of
percentage cleavage obtained versus molar ratios of ribozyme to target used [Figure 3.15e, f, g]. As Rz31 appeared to be so inefficient at cleaving human peripherin RNA in vitro [3.15d], this ribozyme reaction was not quantified. The experiments described above enabled direct comparison of cleavage efficiencies of ribozymes in vitro prior to embarking on cell culture studies. In contrast to Rz31, Rz3, Rz9 and Rz30 cleave over 80% of their respective target RNAs at low ribozyme to template ratios (5:1); moreover as the ratio of ribozyme to template increases, the target RNA is significantly reduced with approximately 7% eventually remaining at a ribozyme to template ratio of 10:1 [Figure 3.15e, f, g].

In summation, two ribozyme-based therapeutic approaches which overcome the extensive genetic heterogeneity associated with adRP were examined in vitro using rhodopsin and peripherin transcripts. The feasibility of each approach was examined using a number of hammerhead ribozymes specific for both UTR sequences and degenerative nucleotides in the coding region of each retinal transcript. In all cases (except Rz6 and Rz7) the ribozymes specifically cleaved their target RNAs while modified replacement transcripts were protected from cleavage. Moreover, a comparative analyses of ribozyme efficiencies demonstrated that three ribozymes in particular, Rz3, Rz9 and Rz30 were extremely efficient in vitro. Given their efficiencies, further experimentation was performed with these ribozymes [Chapters 4 and 5].
3.4 DISCUSSION

Over the past couple of decades there has been a substantial elucidation of the molecular basis of many inherited disorders. In parallel, significant advances have been made in methods of gene delivery. However, translating such developments to the treatment of dominant and polygenic disorders will require methods to suppress the deleterious effects of mutant alleles. Frequently autosomal dominant diseases are heterogeneous in their aetiologies. Indeed, the intragenic heterogeneity associated with rhodopsin and peripherin-linked adRP is mirrored in many other dominant disorders. Osteogenesis imperfecta, Epidermolysis bullosa and Marfan syndrome, induced by various mutations in the collagen 1A1 and 1A2 genes, collagen VII and keratin 14 genes and the fibrillin-2 gene respectively, represent a few of the large number of examples. Therapies for genetically heterogeneous diseases such as RP could be targeted to specific mutations which give rise to disease pathology, to the primary defect in a mutation-independent manner or indeed to a secondary effect such as apoptosis associated with disease pathology. In the current study, the first two of these possible approaches have been explored in vitro. Hammerhead ribozyme, Rz447, directed to a specific rhodopsin mutation known to cause adRP (Dryja et al., 1991), has been generated and evaluated for mutation-specific cleavage of target transcripts in vitro. The mutation, Gly51Val fortuitously creates an NUX target site that occurs in a predicted open-loop structure of the target mutant rhodopsin RNA. Rz447 specifically cleaved the mutant rhodopsin transcript while leaving wild-type transcript intact [Figure 3.6]. However, large excesses of Rz447 were required to obtain significant cleavage and the cleavage regardless of the conditions used was always incomplete.

Similar mutation-specific approaches have successfully been employed for ribozyme-mediated suppression of, for instance, H-ras, N-ras and COL1A1 transcripts (Kashani-Sabet et al., 1994; Feng et al., 1995; Grassi et al., 1997; Scherr et al., 1997). For example, Kashani-Sabet et al generated a hammerhead ribozyme towards a mutation which fortuitously created a GUC ribozyme cleavage motif in codon 12 of the H-ras gene. When a transformed murine NIH3T3 cell line, expressing mutant H-ras and displaying the neoplastic phenotype, was transfected by the H-ras specific ribozyme, the transformed phenotype was abrogated. Specificity of the H-ras ribozyme was demonstrated by showing no reduction in expression of K-ras transcript in transfected cell lines. Subsequently, this ribozyme was shown to successfully repress mutant H-ras gene expression in animals (Feng et al., 1995). Clearly in some instances a mutant-specific approach to gene suppression is appropriate and has been shown to be viable.
However, frequently therapeutic approaches based on targeting specific disease mutations may be problematic for a number of reasons. Individual mutations are often rare and, for example, may be present in a single family. Moreover, discriminating between wild-type and disease alleles requires a high-level of specificity; frequently disease and wild-type alleles differ by a single nucleotide. While such specificity has been achieved, (Grassi et al., 1997; Millington-Ward et al., 1997) in many situations it may be difficult to obtain. The fortuitous creation of hammerhead ribozyme cleavage sites by mutations such as Gly51Val directly facilitates specific targeting of mutant transcripts, however, the probability of such an event is low given the specificity of the target site and the occurrence of open-loop structures in RNA. If antisense RNA alone or antisense flanking a ribozyme core were able to discriminate between normal and mutant alleles, it would extend the number of mutations that could be targeted specifically. However, a number of studies have suggested that in many cases the discriminating power of antisense is not at the level of a single nucleotide. Indeed, these findings are supported by unpublished data from our own laboratory (by Fiona Mansergh). Notably, both normal and mutant transcripts were cleaved by a ribozyme whose antisense arms were complementary to a mutant rhodopsin transcript (Met207Arg). Similarly, the discriminating power of triple helices and peptide nucleic acids based on a single nucleotide difference may frequently not be sufficient to specifically silence the disease allele. Clearly there is a requirement for therapeutic approaches which provide a general suppression strategy that circumvent difficulties associated with developing mutation-specific therapies, such as genetic heterogeneity and discrimination between wild-type and disease alleles. This study addresses two therapeutic strategies for dominantly inherited disorders which are independent of the disease mutation.

Four trans-acting hammerhead ribozymes, based on the Haseloff and Gerlach model (Haseloff and Gerlach, 1988) and targeting predicted open-loop structures in mouse rhodopsin, mouse peripherin and human peripherin transcripts provide examples of how gene suppression and replacement could potentially be undertaken to overcome difficulties associated with genetic heterogeneity. All four ribozymes were directed to 5'-UTR sequence, although 3'-UTR, intronic or any combination of non-coding sequence could potentially be targeted. As UTR sequence is present in both immature and mature message, it may represent a more efficient target for achieving gene silencing. However, if 3'-UTRs were utilised, an unstable full length RNA lacking a poly(A) tail would be generated and
suppression would be dependent on the action of intracellular nucleases (see chapter 5 for
details on the role of 5'- and 3'-UTRs in mRNA stability). Notably, transcripts from all
retinal cDNAs with altered non-coding sequence, generated by primer-directed PCR
mutagenesis, were entirely protected from ribozyme cleavage [Figure 3.7, 3.9 and 3.10].
Indeed, it is worth noting that any apparent decrease in the intensity of modified
replacement transcripts on autoradiographs is due to non-specific RNA degradation and/or
unequal loading and not ribozyme cleavage. In the latter situation, RNA fragments other
than cleavage products of predicted size would be observed on gels. For example, analysis
of figure 3.10d demonstrates that while the wild-type RNA is being cleaved into two
cleavage products of predicted size, the modified RNA (which is more intense than the
wild-type species) remains intact. If ribozyme-mediated cleavage of the modified RNA
was in fact occurring, products would most definitely be visible. In some instances,
protection of replacement RNAs was conferred by the complete obliteration of ribozyme
target motifs and in others because of the absence of sequence complementary to antisense
RNA flanking ribozyme catalytic cores. Even a single nucleotide change protected
modified transcripts from ribozyme cleavage. Notably, in the presence of wild-type and
modified transcripts, cleavage of unadapted transcripts was not compromised.
Modifications to replacement genes included replacement of UTRs with UTR sequence
from a gene expressed in the same tissue or with UTR sequence from the same gene but
from a different mammalian species. These sequence changes were used to limit any
possible subsequent effects on levels and tissue-specific expression of replacement genes.
Other modifications to replacement genes involved subtle single nucleotide sequence
alterations to UTR regions; as with the sequence modifications described above modified
transcripts were masked from ribozyme-mediated suppression (e.g. Rz3 and mRhoH2;
Figure 3.7c). There is considerable evidence suggesting that 5'-UTR and 3'-UTR regions
play a fundamental role in regulating gene expression (Pesole et al., 1994; Tharus et al.,
1997). Moreover, mechanisms involved in regulation of gene expression have not fully
been established for retinal genes such as rhodopsin and peripherin, however, non-coding
sequences may be important in this. Therefore, the use of subtle sequence modifications to
replacement genes to prevent ribozyme cleavage would be preferable. While the strategy
proposed should, in principle, result in gene suppression there is a possibility that transcript
cleavage in UTRs may not affect the ability of cleavage products to generate protein - a
possibility that was eluded to above. Additionally, although lowering RNA levels may lead
to a parallel lowering of protein levels, this is not always the case. Cellular mechanisms
may prevent a significant decrease in protein levels despite a substantial decrease in levels of RNA. However, in many instances ribozyme suppression at the RNA level has proven effective in reducing protein levels (Zhao et al., 1993; Efrat et al., 1994; Little et al., 1995; Zubayko et al., 1997). The ribozymes described in this chapter are all single target ribozymes. It is worth noting that if the efficiency of gene silencing of rhodopsin or peripherin required further optimisation, multi-target ribozymes (connected or shotgun) could be used. Such multi-target ribozymes could be targeted to a single non-coding region (e.g. the 5’-UTR) or indeed to a combination of various non-coding regions (5’-UTR, 3’-UTR or introns). However, it is worth highlighting that the preliminary data obtained in vitro in this study suggests that single target ribozymes directed to the 5’-UTR of rhodopsin and peripherin are efficient and hence the suppression and replacement approach using UTR sequence may be feasible.

The degeneracy of the genetic code has been exploited in this study to test in vitro a second mutation-independent approach also involving suppression and replacement. This second strategy allows for a more flexible choice of target sequence for suppression than the UTR approach and hence the likelihood of obtaining efficient suppression is increased. Notably, in this second approach the target site can be chosen from any region of coding sequence. Hammerhead ribozymes were designed to target suitable cleavage motifs in the coding sequence of mouse rhodopsin and human peripherin, such that replacement genes could be created with a single base change at a wobble position which would eliminate the cleavage site but code for the same amino acid as the wild-type gene. Cleavage of wild-type mouse rhodopsin and human peripherin transcripts was achieved whereas modified transcripts from replacement genes (generated by primer-directed PCR mutagenesis) remained intact [Figure 3.12-3.14]. The single nucleotide alterations protected modified transcripts from ribozyme cleavage and enabled the introduction of replacement genes coding for wild-type protein. Given that the strategy involves alteration of the codon used to code for a given amino acid it is worth noting that codon usage has previously been shown to influence gene expression (Eyre-Walker, 1991). However, altering one or more codons in a gene that are utilised at other sites in the sequence should minimise any possible effects of the modifications. The potential therefore exists to alter DNA and RNA without changing the encoded protein. Again, this approach provides a wider choice of target sequence for suppression, increasing the probability that efficient suppression could be achieved.
Preliminary data obtained from in vitro studies suggests the viability of this wobble-based strategy.

In recent years, due to expansion of sequence databases, it has become apparent that a substantial level of intragenic polymorphism exists. To date polymorphism has been used as a means to generate genetic linkage maps of the human genome. However, an additional novel application of polymorphism would be as a tool to direct therapies to specific alleles. A third approach to achieve mutation-independent gene suppression exists and has been successfully demonstrated in vitro (Millington-Ward et al., 1997). The strategy utilizes polymorphism by directing ribozyme cleavage specifically to transcripts from one allele of a polymorphism while allowing continued expression of the other. Hence, a replacement gene would only be required for dominant disease where pathology was due to haplo-insufficiency. The proportion of patients to whom a given therapeutic agent would be appropriate will depend on the frequencies of polymorphisms in the population as defined by Hardy-Weinberg (2pq). Undoubtedly intragenic polymorphism will be exploited in the future in the development of novel gene-based therapies.

Transferring efficient RNA cleavage obtained in vitro, in this study, to in vivo situations may require modifications to ribozyme constructs to optimise ribozyme efficiency, stability and localisation in cells (Pieken et al., 1991; Chowrika et al., 1994; Heidenreich et al., 1994; Flory et al., 1996; Prasmickaite et al. 1998; Lierdal et al., 1998; Sioud et al., 1998; Samarsky et al., 1999; see Chapter 1, sections 1.2.9-1.2.10,). Efficiency and accuracy of ribozyme cleavage has previously been shown to be enhanced using proteins such as p7 nucleocapsid protein from HIV-1; endogenous proteins may confer similar effects in vivo (Tsukihashi et al., 1993; Bertrand et al., 1994; Muller et al., 1994; Sioud et al., 1996). Despite difficulties, hammerhead ribozymes have been used to elicit sequence specific cleavage of target transcripts in vivo in transgenic animals (Zhao and Pick, 1993; Efrat et al., 1994; Lewin et al., 1998; Chapter 1, section 1.5). Additionally, the strategies outlined in this study are subject to problems of efficient gene delivery and regulation. The level at which a suppression effector is present in a cell is crucial for efficient gene suppression. This is directly dependent on high efficiency of transfer and uptake of gene effectors. Modifications to ribozyme constructs and methods of gene delivery to enhance ribozyme activity in cell-culture are addressed in Chapter 5. Although all mutation-independent strategies have been tested in vitro using hammerhead ribozymes, other
methods for gene silencing, such as antisense RNA or triple helices could be used if ribozymes were inefficient \textit{in vivo} (Chapter 1, see section 1.2).

In summation, one disease-specific hammerhead ribozyme, Rz447, and ten mutation-independent ribozymes, Rz3, Rz6, Rz7, Rz8, Rz9, Rz17, Rz30, Rz31, Rz32 and Rz33, targeting rhodopsin and peripherin transcripts have been designed and analysed \textit{in vitro}. With exception of Rz6 and Rz7, all ribozymes selectively cleaved their target RNAs producing cleavage products of predicted size in a MgCl$_2$ dependent protein independent manner. In contrast, adapted transcripts coding for wild-type protein were masked from cleavage and remained intact. Preliminary kinetic analysis demonstrated that, in particular, Rz3, Rz8, Rz9 and Rz30 were extremely active RNA enzymes, cleaving up to 95\% of target RNA \textit{in vitro}. Hence, these ribozymes will be further analysed both \textit{in vitro} (Chapter 4) and \textit{in vivo} (Chapter 5). Although many modifications may be required to optimise the mutation-independent strategies explored in this study \textit{in vitro}, they should aid in future development of therapeutic approaches for dominant disorders where inherent genetic heterogeneity together with difficulties associated with distinguishing between disease and normal alleles have proven problematic.
Table 3.2  Wobble-base ribozymes: RNA transcript and cleavage product sizes.

<table>
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<tr>
<th>Construct</th>
<th>Restriction enzyme</th>
<th>RNA sizes (bases)</th>
<th>Cleavage products Expected</th>
<th>Cleavage products observed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human peripherin (Fig. 3.12-3.13).</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human peripherin cDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in pcDNA3 + Rz30:</td>
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<td>545</td>
<td>315 + 230</td>
<td>315 + 230</td>
</tr>
<tr>
<td>Human peripherin cDNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in pcDNA3 + Rz31:</td>
<td>BgIII</td>
<td>545</td>
<td>417 + 128</td>
<td>417 + 128</td>
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<tr>
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<td>None</td>
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<td>545</td>
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</tr>
<tr>
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<td>XbaI</td>
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</tr>
<tr>
<td>Rz31</td>
<td>XbaI</td>
<td>55</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Mouse rhodopsin (Fig. 3.14).</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse rhodopsin + Rz32</td>
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<td>774</td>
<td>399 + 375</td>
<td>399 + 375</td>
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<tr>
<td>Mouse rhodopsin + Rz33</td>
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<td>774</td>
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<td>XbaI</td>
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Exploiting the degeneracy of the genetic code:

<table>
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<tr>
<th>Construct</th>
<th>Restriction enzyme</th>
<th>RNA sizes (bases)</th>
<th>Cleavage products Expected</th>
<th>Cleavage products observed</th>
</tr>
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<tr>
<td>Human peripherin</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unadapted sequence</td>
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<td>CTA</td>
<td>CTG AAA GTC</td>
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<tr>
<td></td>
<td></td>
<td>Leu 255-257</td>
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<tr>
<td>Adapted sequence, hPerH2</td>
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<td>CTG</td>
<td>CTG AAA GTC</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unadapted sequence</td>
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<td>CTA</td>
<td>GGA CTG TTC</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Leu 357-359</td>
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<td></td>
</tr>
<tr>
<td>Adapted sequence, hPerH3</td>
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<td>CTG</td>
<td>GGA CTG TTC</td>
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Mouse rhodopsin

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<th>Construct</th>
<th>Restriction enzyme</th>
<th>RNA sizes (bases)</th>
<th>Cleavage products Expected</th>
<th>Cleavage products observed</th>
</tr>
</thead>
<tbody>
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<td>TTT</td>
<td>TAT GTG CCC</td>
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<tr>
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<td></td>
<td>Phe 1459-1461</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adapted sequence, mRhoH3</td>
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<td>TTC</td>
<td>TAT GTC CCC</td>
<td></td>
</tr>
</tbody>
</table>

Ribozyme cleavage sites are underlined. Note the elimination of target motifs in adapted sequences, yet all amino acids remain the same. All ribozymes analysed proved sequence specific cleaving their respective target RNAs into cleavage products of predicted size whereas modified replacement transcripts were protected from ribozyme-mediated suppression and remained intact.
Figure 3.1c
Figure 3.1e
Figure 3.2

(A)-(C) = Presented are the pcDNA3, pZeoSV and pZeoSVLacZ mammalian expression vectors (*Invitrogen*) respectively into which cDNA and ribozyme constructs were cloned. Vectors contain T3 or T7 promoters for *in vitro* expression of constructs placed in the polylinkers and CMV or SV40 promoters for expression in cell culture. Neomycin and Zeocin resistance genes enable selection of positively transfected cells for stable cell line generation. BGH and SV40 poly(A) sequences required for transcript stability are also indicated.
There is an ATG upstream of the XbaI site.

* Bcl I is dam Methylation sensitive.

Figure 3.2a-b
Diagrammatic representation of primer-directed PCR mutagenesis. This process was used to introduce nucleotide alterations into non-coding regions and degenerative sites of retinal cDNA constructs in order to remove ribozyme cleavage sites and hence protect modified replacement genes from ribozyme-mediated suppression. Typically, two complementary primers with a nucleotide alterations(s) are designed to span the targeted ribozyme cleavage site (F2 and R1). Using two flanking primers (F1 and R2) and the mutagenised primers, standard PCR reactions are performed which give rise to products containing a region of sequence homology flanking the incorporated mutation. Both products are subsequently gel isolated and used as a template for a third PCR reaction with flanking primers F1 and R1. The final PCR product has an incorporated sequence alteration such that the modified gene encodes a transcript protected from ribozyme cleavage.
Two PCR reactions using primer pairs F1 & R1 and F2 & R2

PCR product 1

PCR product 2

PCR3 with PCR1&2 products using F1 & R2 primers.

Mutagenised retinal cDNA masked from ribozyme cleavage

Retinal cDNA susceptible to ribozyme cleavage

pcDNA 3

5.4 kb

Figure 3.3
Figure 3.4

Restriction digests of ribozyme and target constructs prior to RNA synthesis.

Lanes 1 and 7 = 1Kb ladder; lane 2 = XhoI digest of Rz3 in pcDNA3; lanes 3-5 = XbaI digests of Rz8, Rz9 and Rz30 in pcDNA3; lane 6 = human peripherin cDNA in pcDNA3 cut by BglII.
Figure 3.4
Radioactively labeled *MspI* cut pBR322 DNA ladder. Fragment sizes are indicated with arrows. As the mobility of DNA and RNA differ, this ladder provided an estimate of transcript size. Indeed, it is of note that the ladder proved extremely accurate for all transcripts sized.
Figure 3.6

MgCl₂ curve of human rhodopsin RNA cleavage by mutation-specific hammerhead ribozyme, Rz447. Lane 1 = MspI cut pBR322; lane 2 = mutated human rhodopsin RNA alone; lane 3 = wild-type human rhodopsin RNA alone; lanes 4-7 = Human rhodopsin RNA and mutated RNA with 0, 2.5, 5 and 10mM MgCl₂ for three hour cleavage reactions. From top to bottom, arrows indicate wild-type rhodopsin RNA, mutated rhodopsin RNA and 5' and 3' cleavage products. A molar ratio of 30:1 of Rz447 to human rhodopsin RNA was used [by Sophia Millington-Ward].
Figure 3.7

(A) MgCl₂ curve of mouse rhodopsin and Rz3. Lanes 1 and 7 = *Msp1* cut pBR322 ladder; lane 2 = mouse rhodopsin RNA alone; lanes 3-6 = mouse rhodopsin RNA and Rz3 subsequent to incubation for 3 hours at 37°C with 0, 5, 10 and 15mM MgCl₂ respectively. From top to bottom, arrows indicate mouse rhodopsin RNA and 5' and 3' cleavage products.

(B) Timepoint cleavage reaction of unadapted mouse rhodopsin RNA, mRhoH1 and Rz3. Lane 1 = intact unadapted and adapted (mRhoH1) mouse rhodopsin RNA alone; lanes 2-6 = unadapted mouse rhodopsin and mRhoH1 RNAs subsequent to incubation with Rz3 and 10mM MgCl₂ for 0, 30, 60, 120 and 180 minutes. No cleavage of mRhoH1 was observed. From top to bottom, arrows indicate residual uncleaved mouse rhodopsin RNA, intact mRhoH1 RNA and two cleavage products from unadapted mouse rhodopsin RNA. The presence of RNA species other than highlighted cleavage products is due to degradation (visible lighter bands are incorrect in size to represent possible adapted RNA cleavage products).

(C) Timepoint cleavage reaction of unadapted mouse rhodopsin RNA, mRhoH2 and Rz3. Lanes 1 and 2 = unadapted and adapted mouse rhodopsin RNAs respectively; lane 3 = DNA ladder; lanes 4-7 = unadapted mouse rhodopsin RNA and mouse rhodopsin hybrid RNA with single base alteration (mRhoH2) subsequent to incubation with Rz3 and 15mM MgCl₂ for 0, 60, 120 and 180 minutes. From top to bottom, arrows indicate residual uncleaved mouse rhodopsin RNA, intact mRhoH2 RNA and 5' and 3' cleavage products form unadapted mouse rhodopsin RNA. Notably, mRhoH2 remained intact ever after 3 hours. Lane 8 = unadapted and adapted RNAs without ribozyme. In all cleavage gels, ratios of Rz3 to mouse rhodopsin/ rhodopsin hybrids were approximately 500:1.
(A) **MgCl₂ curve of mouse peripherin RNA and Rz6.** Lanes 1-4 = mouse peripherin RNA subsequent to incubation with Rz6 for 3 hours at 37°C with 0, 5, 10 and 15 mM MgCl₂. No cleavage of the target RNA occurred. Uncut target RNA is indicated with an arrow. (B) **Timepoint cleavage reaction of mouse peripherin RNA and Rz7.** Lanes 1-6 = mouse peripherin RNA subsequent to incubation with Rz7 and 15 mM MgCl₂ for 0, 30, 60, 120, 180 and 240 minutes at 37°C. Again, no cleavage of target RNA is evident. Arrows from top to bottom indicate mouse peripherin RNA and Rz7. As Rz6 and Rz7 were inactive, these ribozymes were not further analysed.
Figure 3.9

(A) MgCl₂ curve of mouse peripherin RNA and Rz17. Lane 1 = DNA ladder; lane 2 = intact mouse peripherin RNA without ribozyme; lanes 3-6 = mouse peripherin RNA subsequent to incubation with Rz17 at 37°C for 3 hours with 0, 5, 10 and 15mM MgCl₂. From top to bottom, arrows highlight residual uncleaved target RNA and two cleavage products from the unadapted mouse peripherin transcript. (B) Timepoint cleavage reaction of mouse peripherin RNA and Rz17. Lane 1 = DNA ladder; lane 2 = uncleaved mouse peripherin RNA alone; lanes 3-6 = unadapted mouse peripherin RNA subsequent to incubation with Rz17 and 15mM MgCl₂ for 0, 30, 90 and 180 minutes. From top to bottom, arrows highlight residual uncleaved target RNA and 5' and 3' cleavage products. (C) MgCl₂ curve of adapted mouse peripherin RNA (mPerH1) and Rz17. Lane 1 = intact mPerH1 without ribozyme; lanes 2-5 = mPerH1 and Rz17 subsequent to incubation at 37°C for 3 hours with 0, 5, 10 and 15mM MgCl₂; lane 6 = DNA ladder. An arrow highlights the uncleaved adapted RNA. Background smearing is due to RNase degradation. (D) Timepoint cleavage reaction of unadapted mouse peripherin RNA and mPerH1 (adapted RNA) with Rz17. Lanes 1 and 7 = DNA ladder, lane 2 = adapted and unadapted mouse peripherin RNA alone; lanes 3-6 = unadapted mouse peripherin RNA and mPerH1 subsequent to cleavage by Rz17 at 37°C and 15mM MgCl₂ for 0, 30, 90 and 180 minutes. Notably, no cleavage of mPerH1 was observed even after 3 hours. From top to bottom, arrows highlight residual uncleaved mouse peripherin RNA, intact mPerH1 RNA and two cleavage products from unadapted peripherin transcript. Molar ratios of 500:1 of Rz17 to human peripherin/ hybrid RNAs were used for all cleavage reactions.
Figure 3.10

(A) MgCl₂ curve of unadapted human peripherin RNA, adapted peripherin RNA (hPerH1) and Rz8. Lanes 1 and 12 = DNA ladder; lanes 2-5 = unadapted human peripherin RNA and Rz8 after incubation at 37°C with 0, 5, 10 and 15mM MgCl₂ respectively for 3 hours. Notably, efficient cleavage of unadapted peripherin RNA was obtained. Lanes 6-9 = hPerH1 RNA and Rz8 subsequent to incubation with 0, 5, 10 and 15mM MgCl₂ respectively at 37°C for 3 hours. hPerH1 remained intact under all conditions examined (decrease of intensity in lane 9 due to loading error). Lane 10 = unadapted human peripherin RNA alone; lane 11 = hPerH1 RNA alone. Arrows from top to bottom indicate unadapted human peripherin RNA, hPerH1 RNA and two cleavage products from the unadapted RNA. (B) Timepoint cleavage reaction of unadapted and adapted human peripherin RNAs by Rz8. Lanes 1 and 7 = DNA ladder; lanes 2-5 = unadapted and adapted peripherin RNAs and Rz8 after incubation for 0, 1, 2 and 3 hours respectively at 37°C and 15mM MgCl₂; lane 6 = intact unadapted and adapted human peripherin RNA together without ribozyme. Arrows from top to bottom highlight residual uncleaved human peripherin RNA, hPerH1 RNA. (C) MgCl₂ curve of unadapted human peripherin RNA and Rz9. Lanes 1 and 12 = DNA ladder; lanes 2-5 = unadapted peripherin RNA and Rz9 following incubation with 0, 5, 10 and 15mM MgCl₂ respectively at 37°C for 3 hours (efficient cleavage was obtained over most MgCl₂ concentrations); lanes 6-9 = hPerH1 RNA and Rz9 subsequent to incubation with 0, 5, 10 and 15mM MgCl₂ respectively at 37°C for 3 hours (no cleavage obtained); lane 10 = unadapted human peripherin RNA alone; lane 11 = hPerH1 RNA only. Arrows from top to bottom highlight uncleaved human peripherin RNA, hPerH1 RNA and two cleavage products from the unadapted RNA. (D) Timepoint cleavage reaction of unadapted peripherin RNA, adapted RNA (hPerH1) and Rz9. Lane 1 = unadapted human peripherin RNA alone; lane 2 = hPerH1 RNA alone; lanes 3, 4 and 10 = DNA ladder; lanes 5-8 = unadapted peripherin and mPerH1 RNAs following incubation with Rz9 for 0, 1, 2 and 3 hours respectively at 37°C and 15mM MgCl₂; lane 9 = unadapted and adapted RNAs without ribozyme. Arrows from top to bottom indicate uncleaved human peripherin RNA, hPerH1 RNA and 5’ and 3’ cleavage products from unadapted peripherin RNA. Molar ratios of ribozyme to target RNA were 500:1. Notably, both Rz8 and Rz9 appear extremely efficient in vitro.
Figure 3.11

Generation of human peripherin hybrid cDNA, hPerH2, by primer-based PCR mutagenesis. (A) PCR 1 for generation of the human peripherin hybrid, hPerH2, with a single nucleotide substitution at position 257. Lane 1 = 1Kb ladder, lane 2 = Water blank, lanes 3-7 = PCR of 320 bp fragment with flank forward and mutation reverse primers at 10, 12.5, 15, 20 and 25mM MgCl₂ respectively. (B) PCR 2 for hPerH2 generation. Lanes 1-5 = PCR of 600 bp fragment with mutation forward and flank reverse primers at 10, 12.5, 15, 20 and 25mM MgCl₂ respectively. Lane 6 = DNA ladder. (C) PCR 3 for hPerH2 generation. Lane 1 = 1Kb ladder; lane 2 = Water blank; lanes 3-10 = multiple PCRs of 920 bp fragment with flank forward and flank reverse primers at 15mM MgCl₂; lane 11 = 1Kb ladder. Arrows indicate PCR products. Note: the PCR product in lane 3 is barely visible.
Figure 3.11
Figure 3.12

(A) MgCl₂ curve of human peripherin RNA and Rz30. Lanes 1 and 7 = DNA ladder; lane 2 = intact human peripherin RNA without ribozyme; lanes 3-6 human peripherin RNA and Rz30 subsequent to incubation at 37°C for three hours with 0, 5, 7.5 and 10 mM MgCl₂. Arrows from top to bottom indicate residual uncleaved human peripherin RNA and 5’ and 3’ cleavage products.

(B) Timepoint cleavage reaction of human peripherin RNA and Rz30. Lanes 1 and 8 = DNA ladder; lanes 2-6 = human peripherin RNA and Rz30 after incubation at 37°C in the presence of 10 mM MgCl₂ for 0.5, 30, 60, 120 and 180 minutes. Efficient cleavage was obtained which increased over time. Lane 7 = human peripherin RNA without ribozyme. Arrows indicate residual uncleaved peripherin RNA and 5’ and 3’ cleavage products.

(C) MgCl₂ curve of adapted human peripherin RNA and Rz30. Lanes 1 and 7 = DNA ladder; lane 2 = adapted human peripherin RNA, hPerH2, without ribozyme; lanes 3-6 = hPerH2 and Rz30 subsequent to incubation with 0, 5, 7.5 and 10 mM MgCl₂ at 37°C for three hours. No cleavage is evident. An arrow highlights intact hPerH2.

(D) MgCl₂ curve of wild-type and adapted (hPerH2) human peripherin RNA with Rz30. Lanes 1 and 8 = DNA ladder; lane 2 = wild-type human peripherin RNA alone; lane 3 = adapted human peripherin RNA alone (hPerH2); lane 4-7 = wild-type and hPerH2 RNA with Rz30 subsequent to incubation at 37°C for three hours at 0, 5, 7.5 and 10 mM MgCl₂. From top to bottom, arrows highlight uncut human peripherin wild-type RNA, intact hPerH2 and 5’ and 3’ cleavage products from the unadapted RNA. Molar ratios of ribozyme to substrate RNA were 500:1.
Figure 3.12
Figure 3.13

(A) MgCl₂ curve of human peripherin RNA and Rz31. Lanes 1 and 7 = DNA ladder; lane 2 = intact human peripherin RNA without ribozyme; lanes 3-6 = human peripherin RNA and Rz31 following incubation at 37°C for three hours at 0, 5, 7.5 and 10mM MgCl₂. Arrows from top to bottom highlight residual uncleaved human peripherin RNA and 5’ and 3’ cleavage products. (B) Timepoint cleavage reaction of human peripherin RNA and Rz31. Lanes 1-8 = DNA ladder; lane 2 = human peripherin RNA alone; lanes 3-7 = human peripherin RNA and Rz31 subsequent to incubation at 37°C and 10mM MgCl₂ for 0, 30, 60, 120 and 180 minutes. Again, arrows indicate residual uncleaved human peripherin RNA and two cleavage products. (C) MgCl₂ curve of adapted human peripherin RNA, hPerH3, and Rz31. Lanes 1 and 7 = DNA ladder; lane 2 = hPerH3 without ribozyme; lanes 3-6 = adapted RNA and Rz31 following incubation with 0, 5, 7.5 and 10mM MgCl₂ at 37°C for three hours. An arrow highlights the uncut adapted human peripherin RNA.
Figure 3.13
Figure 3.14

(A) MgCl₂ curve of Rz32 and mouse rhodopsin. Lanes 1 and 6 = DNA ladder; lanes 2-5 = mouse rhodopsin RNA subsequent to incubation at 37°C for three hours with 0, 5, 7.5 and 10mM MgCl₂ respectively. Arrows from top to bottom indicate residual uncut mouse rhodopsin RNA and 5’ and 3’ cleavage products. (B) Timepoint cleavage reaction of Rz32 and mouse rhodopsin. Lanes 1 and 7 = DNA ladder; lanes 2-6 = mouse rhodopsin RNA subsequent to incubation with 10mM MgCl₂ at 37°C for 0, 30, 60, 120 and 180 minutes respectively. Arrows from top to bottom highlight uncut target RNA and 5’ and 3’ cleavage products from the mouse rhodopsin RNA. (C) MgCl₂ curve of Rz33 and mouse rhodopsin. Lane 1 = DNA ladder; lane 2 = mouse rhodopsin alone; lanes 3-6 = mouse rhodopsin RNA following incubation with 0, 5, 7.5 and 10mM MgCl₂ for three hours at 37°C. Again, arrows highlight residual intact mouse rhodopsin RNA and 5’ and 3’ cleavage products. (D) MgCl₂ curve of mRhoH3 and Rz33. Lane 1 = DNA ladder; lane 2 = adapted mouse rhodopsin RNA, mRhoH3, alone; lanes 3-6 = mRhoH3 subsequent to incubation with 0, 5, 7.5 and 10mM MgCl₂ at 37°C for three hours. An arrow highlights intact mRhoH3. Molar ratios of Rz32 and Rz33 to mouse rhodopsin RNA were 500:1.
Figure 3.14
Cleavage gels used to determine maximum efficiencies of (A) Rz3 with mouse rhodopsin RNA; (B)-(D) Rz9, Rz30 and Rz31 with human peripherin RNA respectively in vitro. (A)-(D) Lanes 1-9 = 1:1, 1:5, 1:10, 1:25, 1:50, 1:100, 1:250, 1:500 and 1:1000 ratios of target RNA to ribozyme RNA subsequent to incubation at 37°C and 15mM MgCl₂ for 3 hours. Arrows from top to bottom indicate residual uncleaved target RNA and 5’ and 3’ cleavage products. Eventhough the mouse rhodopsin target RNA is not clearly visible on the autoradiograph presented, the sensitivity of instant image analysis enabled the residual substrate RNA to be quantified. (E)-(G) = graphical representations of % cleavage of target RNA versus molar ratios of target RNA to ribozyme RNA for gels (A)-(C).
Figure 3.15a-d
Figure 3.15:  

**G**  
Template: Ribozyme  
Rz30 and human peripherin RNA  
% Cleavage Products  
% Residual Template  
Rz3 and mouse rhodopsin RNA

**F**  
Template: Ribozyme  
Rz2 and human peripherin RNA  
% Cleavage Products  
% Residual Template  
Rz2 and mouse rhodopsin RNA

**E**  
Template: Ribozyme  
% Cleavage Products  
% Residual Template  
Rz2 and mouse rhodopsin RNA
CHAPTER 4

Kinetic evaluation of ribozymes in vitro
tested **in vitro**, when used **in vivo** (Chowrira *et al.*, 1994). It may therefore be instructive to assess kinetic behaviour of ribozymes by developing kinetic models of catalytic activity **in vitro**, prior evaluation in cell-culture and animal systems. This may aid in prioritising which of a battery of ribozymes should be evaluated initially **in vivo**.

Hammerhead ribozymes (Rz3, Rz9, Rz10, Rz30 and Rz40) designed to overcome genetic heterogeneity in rhodopsin and peripherin-linked RP have been kinetically analysed **in vitro**. Moreover, the **in vitro** cleavage characteristics of a connected-type multimeric ribozyme (RzMM) [see Chapter 1, section 1.3] simultaneously targeting four sites in human rhodopsin RNA has been examined. The key advantage of a multi-target ribozyme being that the probability of obtaining efficient target RNA suppression is markedly increased, in that, multiple ribozymes targeting separate regions of the RNA can function simultaneously and therefore if one target site, for example, proved inaccessible to ribozyme cleavage due to secondary structure folding of the target molecule, the remaining accessible sites would still be cleaved by the additional ribozymes present in the multimeric cassette. However, if this scenario pertained when a monomeric ribozyme was being used, the ribozyme may possibly be rendered inactive thus preventing efficient mRNA suppression being achieved. In addition, inhibitory effects on ribozyme cleavage by total RNA and salmon-sperm DNA have been assessed. The $V_{\text{max}}$, $K_m$, $k_2$ and $k_1$ values obtained in the study indicate that some ribozymes tested are extremely efficient **in vitro**, for example, many of the ribozymes cleave complex target RNAs (545-861 bases) with the same efficiency as many reported ribozymes cleaving short unstructured targets (20-40 bases). Hence, these ribozymes may be valuable in the development of mutation-independent therapeutic approaches for adRP.
4.2 MATERIALS AND METHODS

4.2.1 cDNA constructs
Mouse rhodopsin (Accession no. M55171), human peripherin (M62958) and human rhodopsin (K02281) cDNAs were cloned into either the EcoKl or HindIII sites of pcDNA3 (see section 3.2.3). cDNAs were expressed from the T7 promoter in pcDNA3.

4.2.2 Ribozyme constructs
Hammerhead ribozymes were designed to target predicted open-loop structures in target mRNAs. RNA secondary structures were predicted using RNAPlotFold. The integrity of open-loops was evaluated from the 15 most probable two dimensional conformations. Rz10 and Rz40 were designed to an accessible GUC motif at position 475-477 of the human rhodopsin sequence and cloned into the HindIII and XbaI sites of pcDNA3. Rz10 was cloned by Najma al-Jandal. The ribozymes are identical except for a single base mismatch in one antisense arm of Rz10 (highlighted in bold). Antisense flanking sequence is underlined:

Rz10: 5’-GGUCGGUCUGAGAUGAGUCCGUGAGGACGAAACGUAGAG-3’
Rz40: 5’-GGACGGUCUGAGAUGAGUCCGUGAGGACGAAACGUAGAG-3’

Rz3 targets a GUC motif at position 1393-1395 in the 5’-UTR of mouse rhodopsin. Rz9 and Rz30 target GUU and CUA motifs at positions 190-192 (5’-UTR) and 255-257 (degenerative site) respectively of human peripherin RNA. For sequence details see Chapter 3, sections 3.2.4-3.2.6. A connected-type multimeric ribozyme construct, RzMM, comprising of four independent hammerhead molecules (Rz40, 41, 42 and 43) linked in tandem was generated by PCR using flank forward (Rz40-43) and flank reverse (Rz40-43) primers. Overlapping forward and reverse internal oligonucleotides Rz40-41 and Rz42-43 served as a template for PCR. Each ribozyme targeted degenerate sites in human rhodopsin RNA at positions 475-477 (GUCVal), 544-546 (CTCLeu), 577-579 (CTCLeu) and 982-984 (GTCVal). XhoI and XbaI restriction sites were incorporated into forward and reverse flanking primers and used to clone RzMM into pcDNA3.
Flank forward (Rz40-43).

5'-GAATCTCGAGGTT [GGACGGTCTGATGAGTCTGAGGACGAAACGTAGAG]-3'

Xho1 Region complementary to segment of Rz40 in reverse primer, Rz40-41.

Flank reverse (Rz40-43).

5'-TCGACCGTCTGACGTTTCTAGA [AGTTTCACCGTTTCTCGTCC]-3'

Xba1 Region complementary to segment of Rz43 in forward primer, Rz42-43.

Internal oligonucleotides.

Rz40-41 (forward).

5'-GGT [GGACGGTCTGATGAGTCTGAGGACGAAACGTAGAG] TTGAGGCTCTCTAT Rz41 [CCATGAACTGATGAGTCTCCGTGAGGACGA]

AAGTCAGC]CCAGTTTCGTGATGTT-3'

\( \uparrow \) overlapping segment with Rz42-43(F).

Rz40-41 (Reverse). Direct complement of Rz40-41 forward primer.

Rz42-43 (forward).

\( \downarrow \) overlapping segment with Rz40-41(F)

5'-AGCCCAGTTTCGTGATGAGTCTGAGGACGAAACGTAGAG] GACCTGTATC Rz43 [CCCTCCTCTGATGAGTCCGTGAGGACGAAACGGTGAAA]-3'

Rz42-43 (Reverse). Direct complement of Rz42-43 forward.

Multimeric construct sequence (RzMM): antisense flanks are underlined, conserved ribozyme core sequence is regular type and random intervening sequence is italicised.

5'-GGACCGGUCUGAUGAGUCUCGUGCAGAGGACGAAACGUAGAGUCAGAGUUCAG
GCUACCUAUCCAUUGAACUUGACUGAGUCUCGUGAGGACGAAAGGUCAGCCCAGUU
UCGUCGAUGGUGUACUGAUGAGUCUCGUGAGGACGAAAGGUGCGAGCCUGU
AUCCCUCCUCUCUGAUGAGUCUCGUGAGGACGAAACGGUGAA-3'.
4.2.3 Cleavage reactions

Human rhodopsin, mouse rhodopsin and human peripherin cDNA constructs were linearised with BsrEII, Eco47III and BglII respectively. Ribozyme constructs were digested with XbaI. Clones were transcribed in vitro with Ribomax kits (Promega) incorporating [$\alpha$-$^{32}$P] uridine triphosphate and purified as previously described (Millington-Ward et al., 1997). Cleavage reactions were performed in 10mM MgCl$_2$, 50mM Tris-HCl (pH 8.0) at 37°C for up to 3 hours. Timepoints were performed using 10mM MgCl$_2$ quenched with 80% formamide, 1mM EDTA, run on polyacrylamide gels (4-8%) and analysed by autoradiography and phosphoimaging (Packard Instant imager).

4.2.4 Determination of steady-state intervals and saturation rates

Target and ribozyme RNAs were combined in molar ratios of 1:0.2-1:100 respectively. RNAs were heated at 90°C for 3 minutes, cooled on ice and incubated at 37°C in 50mM Tris-HCl. Reactions were initiated with 10mM MgCl$_2$. Aliquots were removed at various times, separated on polyacrylamide gels and analysed by phosphoimaging. Background was accounted for by analysing equal areas of uncleaved and product RNAs. The linear component of the cleavage reaction and the ribozyme saturation rate, were determined from graphs of percentage cleavage versus time.

4.2.5 Single-turnover kinetics; determining $t_{1/2}$, $k_2$ and $k_1$

Substrate and ribozyme RNAs were combined in conditions of ribozyme excess which demonstrated saturation. Ribozyme and substrate RNAs were pre-annealed at 37°C for 5 minutes in the absence of MgCl$_2$. Reactions were initiated with 10mM MgCl$_2$ and aliquots removed at various times and analysed as above, enabling determination of the cleavage step, $k_2$.

\[
\begin{align*}
\text{Kinetic pathway for a hammerhead ribozyme} \\
& Rz + S \leftrightarrow RzS \leftrightarrow RzP1P2 \leftrightarrow Rz + P1 + P2 \\
& k_1 \quad k_2 \\
& k_1 \quad k_2 \\
Rz &= \text{ribozyme} \\
S &= \text{substrate RNA} \\
RzS &= \text{Ribozyme-Substrate complex} \\
P1 &= \text{Cleavage product } 1 \\
P2 &= \text{Cleavage product } 2 \\
t_{1/2}, \text{the half-life of substrate RNA was determined from the slope of a graph of the fraction of uncleaved target RNA versus time. } k_2 \text{ was calculated from } k_2 = \ln 2/t_{1/2} \text{ (Hendry et al., 1997). } k_1, \text{ the dissociation rate was determined using a pulse-chase experiment with a}
\end{align*}
\]
saturating molar excess of ribozyme and a trace of labeled substrate. Reactions were ‘chased’ after 40 seconds with the addition of 100-fold excess of unlabeled substrate (determined by spectrophotometry) and aliquots removed at various times. Labeled substrate that dissociated from ribozyme after addition of ‘chase’, was unable to rebind ribozyme and remained uncleaved. In parallel, a control reaction was performed in which ‘chase’ was added at t = 0 to test the functionality of the unlabeled RNA. $k_1$ was determined using the following equation (Hendry et al., 1997)

$$k_1 = k_2 \times \frac{P_{alone} - P_{\infty \text{ chase}}}{P_{\infty \text{ chase}}}$$

$P_{alone} = \%$ product at $t = \infty$ for reaction without chase.

$P_{\infty \text{ chase}} = \%$ product at $t = \infty$ for reaction with chase.

### 4.2.6 Multiple-turnover kinetics; determination of $V_{max}$ and $K_m$

Molar excesses of target to ribozyme RNAs were incubated in 50mM Tris-HCl (pH 8.0), 10mM MgCl$_2$ at 37°C for times within steady-state intervals. Under these conditions, ribozymes promote cleavage of multiple transcripts enabling determination of $V_{max}$ (maximum velocity of reaction) and $K_m$ (affinity of ribozyme for target RNA). Multiple- and single-turnover data are presented in double reciprocal plots of 1/rate versus 1/substrate concentration. Measurements of $V_{max}$ and $K_m$ were obtained using multiple-turnover readings as outlined below. The equations account for experimental errors commonly associated with kinetic analysis (Cornish-Bowden and Wharton, 1990).

$$V_{max} = \Sigma v^2/a^2 \Sigma v^2 -(\Sigma v^2/a)^2$$

$$K_m = \Sigma v^2 v/a - \Sigma v^2/a \Sigma v$$

$a = \text{substrate concentration} \quad v = \text{rate of the reaction}$

### 4.2.7 Cellular RNA extraction

Total cellular RNA was extracted from 5x10$^6$ Cos-7 cells using the guanidinium isothiocyanate and phenol chloroform isoamyl alcohol extraction process (see Chapter 2.3.4). Preparations typically provided 150-200μg of RNA.
4.2.8 Inhibition of ribozyme cleavage by total RNA and DNA

Reactions were performed under multiple-turnover conditions, in the presence and absence of a 250-fold weight excess of total RNA to ribozyme RNA (determined by spectrophotometry), for times within the steady-state interval. Molar ratios of target RNA to ribozyme RNA varied between 1.25:1 - 5:1. In addition, potential inhibitory effects on ribozyme cleavage were assessed using 0, 10x, 20x, 50x, 100x, 500x and 1000x excesses of sheared (by autoclaving) salmon-sperm DNA to target RNA in reactions. A 5:1 molar ratio of ribozyme to target RNA was used.
4.3 RESULTS

4.3.1 Predicting ribozyme cleavage site accessibility
As was also used in Chapter 3, computer-based predictions of single-stranded regions within RNAs were employed to identify accessible ribozyme cleavage sites. Ribozyme cleavage sites tested were situated in large, robust open-loop structures of transcripts predicted by RNAPlotFold. An example of a ribozyme targeting multiple open-loop structures in human rhodopsin RNA (RzMM) is presented (Figure 4.1). Notably, ribozymes directed towards sites present in richly structured regions of RNAs proved inactive (Chapter 3; section 3.3.3). Rz3, Rz9, Rz10, Rz30, Rz40 and RzMM cleavage motifs appear highly conserved being present in the fifteen most probable two-dimensional conformations of their respective target RNAs - see Chapter 3, section 3.2.1 for further details.

4.3.2 Steady-state intervals and ribozyme saturation rates
The kinetic feature that distinguishes ribozyme catalysed reactions from chemical reactions is that they demonstrate saturation (Cornish-Bowden and Wharton, 1990). Saturating concentrations of ribozyme were determined using graphs of percentage cleavage versus time (see Chapter 1, section 1.4 for details of kinetic definitions; Figure 4.2a-c). Notably, molar excesses of as low as 4:1 of Rz10 to human rhodopsin RNA were sufficient to achieve saturation (Figure 4.2a). Similar results were obtained with Rz9, targeting human peripherin RNA (Figure 4.2b). In contrast, large excesses of Rz3 to mouse rhodopsin RNA were required for saturation (Figure 4.2c). The linear component of cleavage reactions was determined using timepoints with varying ratios of substrate RNA to ribozyme (Figure 4.3a-c). The steady-state interval is the timeframe in which kinetic parameters such as $V_{\text{max}}$ and $K_m$ are determined. Plots of percentage cleavage versus time for Rz40 and human rhodopsin RNA demonstrate an initial ‘burst’ of product formation with approximately 30% of target RNA cleaved in one minute (Figure 4.3a). Experiments were repeated in triplicate under conditions of substrate excess, yielding almost identical results. This rapid initial turnover might indicate that product dissociation was rate-limiting (Fedor and Uhlenbeck, 1990). Moreover, this initial rapid turnover made accurate steady-state determination difficult. As with Rz40, plots of Rz10 and human rhodopsin RNA extrapolated to amounts of product greater than zero at zero time indicating a rapid initial turnover (Figure 4.3b). However, the initial burst of product formation was not as
accentuated, as observed for Rz40, and may be the result of the single-base mismatch introduced into one antisense arm of Rz10. In contrast, no rate inflections were observed for Rz9 during approach to the steady-state (Figure 4.3c). Steady-state intervals for Rz9 and Rz10 were 30 and 6 minutes respectively. As Rz10 and Rz40 were almost identical and the true steady-state for Rz40 was so difficult to ascertain, a six minute steady-state timeframe was taken for Rz40. Subsequent multiple-turnover reactions were performed for times within steady-state intervals, that is, 6, 6 and 20 minutes for Rz40, Rz10 and Rz9 respectively.

4.3.3 Multiple-turnover kinetics

Michaelis-Menten constants \( (V_{\text{max}} \text{ and } K_m) \) for Rz9, Rz10 and Rz40 were determined under conditions of substrate excess in steady-state intervals. Molar ratios of target to ribozyme RNA varied from 1.25:1 - 5:1 respectively. Rz10 despite its long target (861 bases) is extremely active achieving a \( V_{\text{max}} \) of 0.71 min\(^{-1}\), in the same order as many small unstructured RNAs (Figure 4.4a, c). The \( K_m \) value for Rz10 was 8.3 nM. \( V_{\text{max}} \) and \( K_m \) values, similar to Rz10, of 0.55 min\(^{-1}\) and 6.75 nM respectively were obtained for Rz40 (Figure 4.4d). The \( K_m \) value obtained for Rz40 suggests that this ribozyme may have a slightly higher affinity for rhodopsin RNA compared to Rz10. This may be due to the single base mismatch present in one antisense arm of Rz10 which is absent in Rz40. The small reduction in \( V_{\text{max}} \) for Rz40 compared to Rz10 may indicate that a step in the mechanism of hammerhead catalysis, such as product release, may be rate limiting. Alternatively, as the Rz40 and human rhodopsin RNA cleavage reaction was so rapid and the true steady-state interval consequently difficult to define, data may have been collected outside the linear range of the reaction. The results obtained with Rz9 suggest that Rz9 \( (V_{\text{max}} = 0.23 \text{ min}^{-1}, K_m = 13.1 \text{nM}) \) is approximately 3-fold less active than Rz10 or Rz40 and has a reduced affinity for its target (Figure 4.5a, b). Kinetic profiles for Rz10 and Rz40 based on multiple-turnover conditions strongly suggest that these ribozymes are extremely efficient \textit{in vitro} and hence may be valuable as potential therapeutic agents for adRP.

Inhibition experiments were performed under multiple-turnover conditions as above but with a 250-fold excess of cellular RNA to Rz10 (Figure 4.4a, b). Cleavage of target transcripts, although reduced by approximately 25\%, remained specific. \( V_{\text{max}} \) values in the presence and absence of total cellular RNA were similar; 0.8 min\(^{-1}\) and 0.711 min\(^{-1}\)
respectively (Figure 4.4c), whereas $K_m$ values increased from 8.3nM to 26.55nM, suggesting a reduction in affinity of Rz10 for human rhodopsin RNA. Typically, competitive inhibition results in an increased $K_m$ and therefore a reduced affinity of ribozyme for target RNA while $V_{max}$ values remain unaltered. In contrast, uncompetitive inhibition causes reduced $V_{max}$ values with unaltered $K_m$ values (Cornish-Bowden and Wharton, 1990). Therefore, data suggest that cellular RNA results in competitive inhibition of Rz10 cleavage activity. In addition, inhibition experiments were performed with a 50-fold, 100-fold and 500-fold excess of total cellular RNA to Rz9 (Figure 4.6a-d). Results demonstrate that even in the presence of vast excesses of cellular RNA, Rz9 is capable of site-specific cleavage of human peripherin RNA with a maximum of 30% inhibition of cleavage being observed (Figure 4.6d). The data for both Rz9 and Rz10 provides preliminary evidence suggesting that these ribozymes may be effective in mutation-independent gene suppression studies in cell culture systems. In addition, possible inhibitory effects of salmon-sperm DNA on Rz9 and Rz10 activity were investigated briefly. Maximum inhibitory effects of 32% (Rz9) and 25% (Rz10) on ribozyme cleavage rates were observed when extremely large weight excesses of DNA over target RNA were present (Figure 4.7a-c).

### 4.3.4 Single-turnover kinetics

The rates of the cleavage step, $k_2$, substrate dissociation, $k_{1s}$, and half-life of substrate RNA, $t_{1/2}$, were determined for Rz10 and Rz40 under single-turnover conditions (ribozyme excess). $t_{1/2}$ values for human rhodopsin RNA, cleaved by Rz10 and Rz40, were 28.2 and 30 seconds respectively (determined from linear portions of plots of percentage cleavage versus time; Figure 4.8a-d). Previous studies using kinetically well defined hammerhead ribozymes have shown that single base mismatches in the same position and antisense arm as Rz10 (see section 4.2.2) have no effect on the rate of cleavage compared to matched targets (Werner and Uhlenbeck, 1995). As expected, similar $k_2$ values of 1.47min$^{-1}$ and 1.38 min$^{-1}$ for Rz10 and Rz40 respectively were generated from $t_{1/2}$ values (Figure 4.8a-d).

Notably, Rz10 and Rz40 were extremely efficient; over 50% of the 861 base target RNA was cleaved in under 30 seconds (Figure 4.8a, d). The single base mismatch in Rz10 would be predicted to increase the dissociation rate ($k_{1s}$) due to possible destabilisation of substrate and ribozyme binding (Werner and Uhlenbeck, 1995). $k_{1s}$ values for Rz10 and Rz40 which represent the number of molecules of substrate that dissociate from ribozyme per minute and were determined from pulse-chase experiments (see Chapter 1, section 1.4),
indicate that the dissociation rate for Rz10 (0.55min⁻¹) is slightly greater than that for Rz40 (0.4min⁻¹), as predicted. Control experiments indicated that unlabeled chase prevented reassociation of the ribozyme and labeled substrate (Figure 4.8c-d). In these experiments, a large excess of unlabeled target (chase) RNA over labeled target RNA was added at the start (t = 0) of the ribozyme cleavage reaction. If the chase RNA is functional, it outcompetes the labeled RNA in the reaction for ribozyme binding and thus almost completely inhibits ribozyme-mediated cleavage of the labeled target RNA.

While Rz9 was found to cleave human peripherin transcripts in conditions of ribozyme excess (single-turnover conditions), cleavage was significantly less efficient than for either Rz10 or Rz40 with $t_{1/2}$ and $k_2$ values of 18.25min⁻¹ and 0.04min⁻¹ respectively (Figure 4.9a, b). Moreover, Rz9 was less efficient at cleaving its target under multiple turnover conditions (see above). However, the extremely low $k_2$ value generated for Rz9 is atypical, in that cleavage of human peripherin RNA under conditions of ribozyme excess over time (3 hours) almost reached completion (Figure 4.9a). Possible explanations for this kinetic behaviour could include the target RNA initially being in an inactive conformation (preventing ribozyme cleavage) but slowly converting to an active conformation, or the ribozyme and substrate forming an alternate complex which is not part of the normal kinetic pathway of hammerhead ribozyme catalysis (see section 4.2.5) and which did not directly lead to product formation (Stage-Zimmermann and Uhlenbeck, 1998). Comparison of figures 4.2a and 4.2b clearly illustrates the difference in extent of cleavage between a hammerhead ribozyme (Rz10) with a high cleavage rate and a hammerhead ribozyme (Rz9) that cleaves more slowly but nevertheless eventually almost reaches completion.

Examples of ribozymes with atypical cleavage activities similar to that of Rz9 have been reported (Hendry and McCall, 1995; Hertel et al., 1996, 1997). Although in vitro cleavage of human peripherin RNA by Rz9 was incomplete (88%; Figure 4.9a), it is worth noting that somewhat inefficient ribozymes with reduced cleavage activities in vitro compared to Rz9 targeting a P23H rhodopsin mutant (Drenser et al., 1998) nevertheless lead to a beneficial effect on photoreceptor degeneration in vivo (Lewin et al., 1998) suggesting that Rz9 would be worth testing in cell and animal systems. In contrast to Rz9, Rz10 and Rz40, Rz3 and Rz30 were found to elicit significant levels of cleavage of target transcripts but only under conditions of ribozyme excess (Figure 4.2c and 3.15c, g). Indeed, when Rz30 was examined under conditions of substrate excess, cleavage of human peripherin RNA was extremely inefficient with cleavage products barely being visible after 30 minute
incubations (Figure 4.2d-f). Hence as these ribozymes were unable to efficiently function as catalytic molecules they were not further evaluated.

4.3.5 Cleavage profile of multimeric ribozyme, RzMM

A multimeric ribozyme (RzMM) consisting of four hammerhead molecules directed to conserved mutation-independent degenerative sites in human rhodopsin RNA was generated and tested in vitro. Figure 4.10 illustrates the cleavage profile of RzMM. As any single ribozyme or multiple ribozymes within the multimeric cassette can cleave the target transcript, a number of cleavage products were expected and indeed were observed. Moreover, the diverse array of cleavage products prevented in-depth kinetic analysis of RzMM activity. As the labeled ribozyme was suspected of concealing potential cleavage products, non-radioactively labeled RzMM transcript was also tested (Figure 4.10b). Although it cannot be precisely concluded which products result from the action of which ribozyme(s), it is apparent that, using a 10:1 molar ratio of RzMM to human rhodopsin RNA, over 85% of the target transcript was cleaved (Figure 4.10a, lanes 6-9). RzMM may therefore prove valuable in the development of mutation-independent therapeutic approaches for adRP.
4.4 DISCUSSION

Two mutation-independent therapeutic approaches for combating the immense genetic heterogeneity associated with rhodopsin- and peripherin-linked adRP have been adopted in the present study. Both approaches target the primary defect and exploit inherent features of the genome, either the degeneracy of the genetic code or untranslated regions of a transcript. For example, Rz10, Rz40 and RzMM target human rhodopsin transcripts at various wobble sites thereby exploiting the degeneracy of the code and enabling the introduction of a replacement gene with modified sequences around wobble sites (Millington-Ward et al., 1997). For all mutation-independent ribozymes tested, replacement genes coding for wild-type protein but with marginally altered sequences around the ribozyme target site have been shown to escape ribozyme cleavage in vitro (Millington-Ward et al., 1997; Chapter 3). In principle, using a mutation-independent approach, a single therapeutic agent may provide benefit for patients with a range of mutations in a given retinal gene, irrespective of the precise mutation. A connected-type multimeric ribozyme (RzMM) has also been evaluated for cleavage of human rhodopsin transcripts. RzMM simultaneously targets four degenerate sites in open-loop structures of human rhodopsin RNA. Studies have indicated that it may be advantageous to use multimeric ribozymes to achieve efficient cleavage of target RNAs (Chen et al., 1992; Ohkawa et al., 1993; Ramezani and Joshi, 1997; Benedict et al., 1998; Chapter 1, section 1.3). This may be due to the presence of a mixed population of RNA conformations, each of which could be targeted by one or more ribozymes or alternatively efficient cleavage of a predominant RNA conformation at multiple sites. RzMM cleaved human rhodopsin RNA into a range of cleavage products as expected for a multimeric ribozyme, and represents the first such multimeric ribozyme targeting any retinal transcript (Figure 4.10). However, RzMM does not appear, at least in vitro, to be more efficient than either of the rhodopsin-specific monomeric ribozymes, Rz10 or Rz40. This was somewhat surprising given the increased number of cleavage sites in human rhodopsin mRNA targeted simultaneously by the multimeric ribozyme compared to the single sites targeted by the monomeric ribozymes. The absence of an increased cleavage efficiency may be the result of the long concatameric RNA of the multimeric ribozyme adopting an unfavourable secondary structure that may have inhibited the activity of the individual ribozymes in the construct (see Chapter 1, section 1.3). However, the helix disentangling properties of certain intracellular proteins such as GAPDH may enhance the overall catalytic efficiency of RzMM when analysed in vivo (see Chapter 1, section 1.2.9).
Kinetic profiles of ribozymes in vitro can be used as broad predictors of activity in vivo (Birikh et al., 1997a). All ribozymes developed in the study elicited sequence specific cleavage of retinal transcripts, yielding cleavage products of predicted sizes. However, detailed kinetic analyses revealed substantial differences in catalytic activity between ribozymes. Rz3 and Rz30 both demonstrated efficiency cleavage of mouse rhodopsin and human peripherin RNAs but only under conditions of ribozyme excess. In contrast, Rz9, Rz10 and Rz40 were found to be extremely active under all conditions tested. Catalytic efficiency of ribozymes both in vitro and in vivo depend on their ability to turnover. However, product dissociation can often prove rate-limiting (Hendry et al., 1997; Chapter 1). Rz10 and Rz40 were designed such that a single base mismatch was introduced into one antisense arm of Rz10 which would be expected to increase the rate of dissociation but not reduce the overall catalytic efficiency of the ribozyme (Werner and Uhlenbeck, 1995). 

$V_{\text{max}}$ values of 0.71min$^{-1}$ for Rz10 and 0.6min$^{-1}$ for Rz40 obtained using an 861 base target, suggests that Rz10 and Rz40 are extremely efficient ribozymes. Significantly, both ribozymes achieve $V_{\text{max}}$ in the range obtained with short unstructured RNAs (c.20 bases) which at maximum approximate ~1min$^{-1}$ (Zaug et al., 1988). Typically, reductions in $V_{\text{max}}$ of 1000-fold or more have been observed for ribozymes directed to long structured RNAs (Heidenreich and Eckstein, 1992; Hendry et al., 1995; Janowsky and Schwenzer, 1996; Campbell et al., 1997). Small reductions in $V_{\text{max}}$ and $K_m$ (indicating a higher affinity) obtained for Rz40 ($V_{\text{max}} = 0.55\text{min}^{-1}$, $K_m = 6.75\text{nM}$) compared to Rz10 ($V_{\text{max}} = 0.71\text{min}^{-1}$, $K_m = 8.3\text{nM}$) may suggest that product dissociation is possibly rate limiting, in the mechanism of Rz40 cleavage of human rhodopsin RNA. However, both Rz10 and Rz40 have high $V_{\text{max}}$ values despite their long target. Moreover, over 50% of the 861bp target RNA is cleaved in under 30 seconds indicating that these ribozymes are certainly extremely efficient in vitro (Figures 4.8a and 4.11b).

Moreover, the presence of a 250-fold excess of cellular RNA to Rz10, did not significantly inhibit the rate of cleavage of human rhodopsin RNA (25%; Figure 4.4a, b). The reduction in the $K_m$ value while the $V_{\text{max}}$ remained the same suggests that the inhibition was competitive. This was somewhat surprising as it might be expected that cellular RNA could exert an uncompetitive effect by physically interfering with ribozyme and target binding. Similar kinetic studies examining the effects of cellular RNA on ribozyme catalysis have demonstrated either and absence of inhibition or both competitive and uncompetitive inhibition (Grassi et al., 1997; Kisich et al., 1997).
Although Rz9 (targeting the 5'-UTR of human peripherin) was 3-fold less active than either Rz10 or Rz40, ($V_{max} = 0.26\text{min}^{-1}$) and had a slightly reduced substrate affinity ($K_m = 13.1\text{nM}$), Rz9 nevertheless was quite active in vitro when compared to other ribozymes (Figure 4.9a; Stage-Zimmerman and Uhlenbeck, 1998). Notably, when Rz9 was incubated with vast excesses of cellular RNA, it was still able to efficiently locate, bind and cleave human peripherin RNA; cleavage efficiency was reduced by only 30% in the presence of a 500-fold excess (Figure 4.6a-d). Similarly, the presence of large excesses of DNA in Rz9 and Rz10 cleavage reactions, resulted in a mild inhibition of ribozyme cleavage efficiency being observed (32% and 25% respectively; Figure 4.7a-c). Interestingly, a hammerhead ribozyme targeting a proline 23 histidine rhodopsin mutation has previously been generated and kinetically analysed in vitro (Drenser et al., 1998). Results demonstrated that the ribozyme had a reduced catalytic efficiency and target affinity in vitro ($V_{max} = 0.55\text{min}^{-1}$; $K_m = 28\text{nM}$) compared to many of the ribozymes discussed above, in particular Rz9, Rz10 and Rz40. Nevertheless, this ribozyme was subsequently shown to provide a beneficial effect in vivo when tested in a rat model of an inherited photoreceptor degeneration (Lewin et al., 1998). This study, while targeting a specific mutation using an artificially generated ribozyme cleavage site (which is not present in wild-type human rhodopsin sequence) clearly demonstrated the potential utility of hammerhead ribozyme technologies in the development of treatments for disorders such as adRP. Moreover, as the target RNA used in Drenser's kinetic study was a short unstructured RNA of only 39 bases and that a Mg$^{2+}$ concentration of 100mM was found to support optimal catalytic activity (in contrast to ≤ 5mM in this study) offers much promise for the in vivo utility of the mutation-independent ribozymes described. Notably, target RNA length and Mg$^{2+}$ concentration have been shown to significantly influence the catalytic efficiencies of ribozymes both in vitro and in vivo - see above and Chapter 1.

Additionally, single-turnover kinetics was used to determine rates of the cleavage step, $k_2$, and substrate dissociation, $k_{11}$, for Rz9, Rz10 and Rz40. Values for $k_2$ can vary considerably, however, it has been suggested that $k_2$ values typically fall between 0.4-2.5min$^{-1}$ for unstructured RNAs (Stage-Zimmerman and Uhlenbeck, 1998). High $k_2$ values for Rz10 and Rz40 of 1.47min$^{-1}$ and 1.38min$^{-1}$ respectively, given the length of the target, demonstrate the efficiency of both ribozymes. $k_{11}$ values of 0.55min$^{-1}$ and 0.4min$^{-1}$ for Rz10 and Rz40 respectively suggest that, as predicted, the single-base mismatch in Rz10 resulted in an increase in substrate dissociation due to destabilisation of substrate and ribozyme.
binding (Werner and Uhlenbeck, 1995). The relationship between $k_1$ and $k_2$ is extremely important for efficient ribozyme activity. For instance, if $k_1$ is much faster than $k_2$, ribozyme will dissociate before cleavage can occur and a significant reduction in catalytic efficiency will result. In contrast, if $k_1$ is slow compared to $k_2$, every substrate that binds ribozyme will be cleaved. However, if $k_1$ is too slow, the ribozyme will be unable to function catalytically. The defined relationship between $k_1$ and $k_2$ for Rz10 and Rz40, would appear to support the high catalytic efficiencies of both ribozymes \textit{in vitro}. In contrast, an unusually low $k_2$ value of 0.04 min$^{-1}$ was obtained for Rz9, given its ability to cleave up to 88% of human peripherin transcript over time (Figure 4.9a). Many RNA sequences can adopt multiple alternate structures that are as stable as the native structure (Herschlag, 1995; Uhlenbeck, 1995), it is possible that the low $k_2$ and opposing high cleavage efficiency observed for Rz9 could be attributed to either the ribozyme, target or ribozyme-target complex adopting an inactive conformation that, over time converted to an active form (Stage-Zimmerman and Uhlenbeck, 1998).

Ribozyme efficiency \textit{in vitro} can only be used as a broad predictor of potential efficiency \textit{in vivo} (Bertrand \textit{et al.}, 1994; Birikh \textit{et al.}, 1997b). Subsequent experimentation in cell and animal systems is required to assess ribozyme efficiencies \textit{in vivo}. Nevertheless, the kinetic profiles developed above can be used to aid in prioritising which ribozymes should be tested for \textit{in vivo} activity initially. In this regard, a number of mouse models of rhodopsin-linked retinal degenerations some of which closely mirror human adRP, for example, mice carrying human rhodopsin transgenes with Pro23His or Pro347Ser mutations, are available (Olsson \textit{et al.}, 1992; Li \textit{et al.}, 1996; see Chapter 1, sections 1.12-1.13). Moreover, there are now many reports demonstrating that hammerhead ribozymes can be functional in both cell and animal systems (Feng \textit{et al.}, 1995; Lewin \textit{et al.}, 1998; Vaish \textit{et al.}, 1998; Wang \textit{et al.}, 1999a). Indeed one such study discussed above used ribozymes targeting a specific rhodopsin mutation in a transgenic rat with a retinal degeneration (Lewin \textit{et al.}, 1998). Histological examination of rat eyes receiving sub-retinal injections of hammerhead and hairpin ribozymes indicated that the outer nuclear layer thickness was 40-43% and 30-39% greater than that of non-injected controls respectively. Moreover, ERG analyses of ribozyme injected rats demonstrated a functional photoreceptor rescue with a-wave amplitudes being up to 93% greater than in controls. The efficiency of a number of the ribozymes evaluated in the current study such as Rz9, Rz10 and Rz40 strongly suggests that these ribozymes should be evaluated in cell and animal systems (see Chapter 5). Given
efficient cleavage in vivo, these ribozymes should prove valuable in the development of novel therapeutic approaches for inherited photoreceptor degenerations such as adRP.

Ribozymes evaluated in this study target retinal transcripts in a mutation-independent manner and concurrently enable introduction of replacement transcripts coding for wild-type protein but protected from ribozyme cleavage. Such novel mutation-independent approaches will most likely be a pre-requisite to the development of therapies for genetically heterogeneous disorders such as adRP. In this regard, alternative mutation-independent strategies for therapies are being considered including modulating secondary effects associated with the disease pathology such as apoptosis (Hafezi et al., 1997; Travis, 1998; Liu et al., 1999) or administering neurotrophic factors to retinal tissues rather than correcting the primary defect (Cayouette et al., 1998; La Vail et al., 1998; Luthert and Chong, 1998; Chong et al., 1999; Frasson et al., 1999; Chapter 1, section 1.1.6). For example, a transgenically generated rhodopsin-linked retinal degeneration in Drosophila has been ameliorated using the baculovirus p35 gene, the product of which inhibits the pro-apoptotic activity of caspase proteins (Davidson and Steller, 1998). Possibly one of these mutation-independent approaches or indeed a combination of approaches may provide viable future therapies for this group of debilitating eye disorders. Indeed, ribozyme-based therapies may prove useful not only in therapeutic approaches targeting the primary defect but may also be valuable in suppressing expression of pro-apoptotic genes, for example, by targeting transcripts from caspase genes - caspase proteins are known to be central to the execution of the cell death pathway (Chapter 1, section 1.1.6).

In summary, hammerhead ribozymes Rz10, Rz40 and RzMM targeting human rhodopsin transcripts and Rz9 targeting human peripherin transcripts, after detailed kinetic analyses, have been found to be extremely efficient in vitro. Comparative analyses with previously reported ribozymes would suggest that, despite their longer and more structured targets, these retinal ribozymes achieve similar cleavage rates as many ribozymes targeting short unstructured RNAs of 20-40 bases (for a complete summary of the in vitro efficiencies of the ribozymes analysed so far see table 4.1). Moreover, the design of these ribozymes is such that they will enable mutation-independent gene silencing of retinal genes involved in dominantly inherited retinopathies.
<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>Target RNA and length</th>
<th>Target motif</th>
<th>Size of open-loop targeted</th>
<th>% cleavage observed</th>
<th>Kinetic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rz447</td>
<td>Gly51Val mutation in 861 base human rhodopsin RNA</td>
<td>GUC nucleotides 445-447</td>
<td>35 bases</td>
<td>≥ 60%</td>
<td>-</td>
</tr>
<tr>
<td>Rz3</td>
<td>5'-UTR of 778 base mouse rhodopsin RNA</td>
<td>GUC nucleotides 1393-1395</td>
<td>10 bases</td>
<td>≥ 85%</td>
<td>Only efficient under single-turnover conditions</td>
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<tr>
<td>Rz6</td>
<td>5'-UTR of 562 base mouse peripherin RNA</td>
<td>GUA nucleotides 207-209</td>
<td>3 bases</td>
<td>Non-functional</td>
<td>-</td>
</tr>
<tr>
<td>Rz7</td>
<td>5'-UTR of 562 base mouse peripherin RNA</td>
<td>CUC nucleotides 153-155</td>
<td>5 bases</td>
<td>Non-functional</td>
<td>-</td>
</tr>
<tr>
<td>Rz8</td>
<td>5'-UTR of 489 base human peripherin RNA</td>
<td>CUA nucleotides 234-236</td>
<td>18 bases</td>
<td>≥ 90%</td>
<td>-</td>
</tr>
<tr>
<td>Rz9</td>
<td>5'-UTR of 489 base human peripherin RNA</td>
<td>GUU nucleotides 190-192</td>
<td>8 bases</td>
<td>≥ 90%</td>
<td>k_s = 0.04 min^{-1}, V_{max} = 0.26 min^{-1}, K_m = 13.1 nM</td>
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<tr>
<td>Rz10</td>
<td>Degenerative site in coding region of 861 base human rhodopsin RNA</td>
<td>GUC nucleotides 475-477</td>
<td>35 bases</td>
<td>≥ 95%</td>
<td>k_s = 1.47 min^{-1}, k_1 = 0.55 min^{-1}, V_{max} = 0.71 min^{-1}, K_m = 8.3 nM</td>
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<tr>
<td>Rz17</td>
<td>5'-UTR of 488 base mouse peripherin RNA</td>
<td>AUU nucleotides 162-164</td>
<td>9 bases</td>
<td>~ 50%</td>
<td>-</td>
</tr>
<tr>
<td>Rz30</td>
<td>Degenerative site in coding region of 545 base human peripherin RNA</td>
<td>CUA nucleotides 255-257</td>
<td>12 bases</td>
<td>≥ 90%</td>
<td>Only efficient under single-turnover conditions</td>
</tr>
<tr>
<td>Rz31</td>
<td>Degenerative site in coding region of 545 base human peripherin RNA</td>
<td>CUA nucleotides 357-359</td>
<td>15 bases</td>
<td>~ 35%</td>
<td>-</td>
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<tr>
<td>Rz32</td>
<td>Degenerative site in coding region of 774 base mouse rhodopsin RNA</td>
<td>UUU nucleotides 1458-1460</td>
<td>13 bases</td>
<td>~ 45%</td>
<td>-</td>
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<tr>
<td>Rz33</td>
<td>Degenerative site in coding region of 774 base mouse rhodopsin RNA</td>
<td>UUU nucleotides 1459-1461</td>
<td>13 bases</td>
<td>~ 15%</td>
<td>-</td>
</tr>
<tr>
<td>Rz40</td>
<td>Degenerative site in coding region of 861 base human rhodopsin RNA</td>
<td>GUC nucleotides 475-477</td>
<td>35 bases</td>
<td>≥ 95%</td>
<td>k_s = 1.38 min^{-1}, k_1 = 0.40 min^{-1}, V_{max} = 0.55 min^{-1}, K_m = 6.75 nM</td>
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<tr>
<td>RzMM</td>
<td>4 degenerative sites in coding region of 861 base human rhodopsin RNA</td>
<td>GUC: 475-477, CUC: 544-546, CUC: 577-579, GUC: 982-984</td>
<td>35 bases</td>
<td>≥ 85%</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.1

Presented is the most probable secondary structure for human rhodopsin RNA as determined by RNAPlotFold. NUX ribozyme cleavage sites, present in accessible open-loop structures of the RNA, are highlighted with arrows. GTC, CTC, CTC and GTC target motifs at residues 475-477, 544-546, 577-579 and 982-984 respectively to which RzMM was designed are indicated. Hammerhead ribozymes, Rz10 and Rz40 were also directed to the GTC motif at residues 475-477.
Figure 4.2

Graphs (A)-(C) represent timepoint cleavage gels of human rhodopsin RNA and Rz10, human peripherin RNA with Rz9 and mouse rhodopsin and Rz3 respectively. Graphs of % cleavage versus time were used to determine ribozyme saturation rates. Cleavage times were 0, 5, 7.5, 10, 20, 30, 60 and 180 minutes for Rz9 and Rz10. Similarly, the timepoints used for Rz3 were 0, 2.5, 5, 7.5, 10, 20, 30, 90 and 180 minutes. (D)-(F)

Cleavage gels of human peripherin RNA and Rz30 under multiple-turnover conditions - 1:0.2, 1:0.5 and 1:0.8 molar ratios of substrate RNA to ribozyme RNA were used respectively. For each gel, lanes 1-8 = timepoint cleavage reactions of human peripherin RNA by Rz30 for 0, 2.5, 5, 7.5, 10, 20, 30, 90 and 180 minutes respectively. Arrows from top to bottom indicate intact human peripherin RNA and 5' and 3' cleavage products. Fragment sizes are the same on each gel. As is evident, Rz30 proved inefficient under the conditions examined with significant levels of cleavage only being evident following 90 minute incubations. Cleavage reactions were performed with 10mM MgCl₂ @ 37°C. Notably, experiments were performed in triplicate.
Figure 4.2a-c
Figure 4.2d-f
Graphs (A)-(C) represent gels of timepoint cleavage reactions of Rz40, Rz10 and Rz9 with their target RNAs respectively. Graphs of % cleavage versus time were used to identify the linear component of the cleavage reactions, that is, steady-state intervals. Cleavage times of 0, 1, 2, 3, 5, 7, 10 and 15 minutes were used for Rz40 and human rhodopsin RNA with 1:0.5 and 1:0.8 molar ratios of target RNA to ribozyme RNA. Similarly, cleavage times of 0, 1, 2, 3, 4, 5 and 6 minutes were used for Rz10 and human rhodopsin RNA with a molar ratio of 1:0.2 of target RNA to ribozyme RNA. Timepoints used for Rz9 and human peripherin RNA with 1:0.2, 1:0.5, 1:0.8 and 1:2 molar ratios of target RNA to ribozyme RNA were 0, 5, 7.5, 10, 20, and 30 minutes.
Figure 4.3

**A**

Determination of steady-state interval for the Rz40 and human rhodopsin RNA reaction

**B**

Determination of steady-state interval for the Rz10 and human rhodopsin RNA reaction

**C**

Determination of steady-state interval for the Rz9 and human peripherin RNA reaction

Ratios of substrate:Rz9 used.
Figure 4.4

Cleavage gels of human rhodopsin RNA by Rz10 in the absence (A) and presence (B) of a 250-fold excess of total cellular RNA for 6 minute reactions. Lanes 1-8 = molar ratios of target RNA to ribozyme RNA of 1:0.2, 1:0.4, 1:0.5, 1:0.6, 1:0.8, 1:2, 1:5, and 1:10 respectively. Rates of each multiple-turnover reaction were used to determine \( V_{\text{max}} \) and \( K_m \) parameters. From top to bottom, arrows indicate human rhodopsin RNA (861 bases) and 5' and 3' cleavage products (584 and 277 bases). (C) Representative Lineweaver-Burk plot of cleavage gels of human rhodopsin RNA by Rz10, in the presence and absence of cellular RNA. (D) Lineweaver-Burk plot representing cleavage of human rhodopsin RNA by Rz40. Molar ratios of target to ribozyme RNA were 1:0.2, 1:0.4, 1:0.6, 1:2, 1:5 and 1:10 for a 6 minute reaction time.
Figure 4.4a-c
Figure 4.4

Lineweaver-Burk plot of Rz40 and human rhodopsin RNA

Figure 4.4
Figure 4.5

(A) Cleavage gel of human peripherin RNA by Rz9 for determination of $V_{\text{max}}$ and $K_m$ parameters. Molar ratios of target to ribozyme RNA were 1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:2, 1:5 and 1:10 for a 20 minute reaction. Arrows from top to bottom indicate the 545 base human peripherin RNA and two cleavage products (295 and 250 bases). (B) Lineweaver-Burk plot of human peripherin RNA cleavage by Rz9 under multiple- and single-turnover conditions.
Figure 4.5

Lineweaver-Burk plot Rz9 and human peripherin RNA
Figure 4.6

Gels (A)-(D) = human peripherin RNA cleavage by Rz9, under multiple-turnover conditions, alone and in the presence of a 50-fold, 100-fold and 500-fold excess of total cellular RNA respectively. Lanes 1-6 for each gel = 1:0.2, 1:0.4, 1:0.5, 1:0.7, 1:0.8 and 1:0.9 molar ratios of human peripherin RNA to Rz9. Reactions were performed for 20 minutes (within the steady-state). Arrows from top to bottom, highlight intact human peripherin target RNA and 5’ and 3’ cleavage products. Notably, the large excesses of cellular RNA did not significantly reduce Rz9 cleavage efficiency.
Figure 4.6
Figure 4.7

(A)-(B) = cleavage gels of human peripherin RNA by Rz9 and human rhodopsin RNA by Rz10 respectively, in the presence of 0, 10, 20, 50, 100, 500 and 1000-fold excesses of salmon sperm DNA. A 5:1 molar ratio of ribozyme to target RNA was used. Reactions were performed for 20 minute (Rz9) and 6 minute intervals (Rz10). From top to bottom, arrows indicate the peripherin and rhodopsin target RNAs and 5’ and 3’ cleavage products.

(C) = bar-chart illustrating the inhibitory effects of molar excesses of salmon sperm DNA on Rz9 and Rz10 cleavage rates.
Effect of salmon-sperm DNA on Rz9 and Rz10 cleavage rates

Figure 4.7
Figure 4.8

Cleavage gels of human rhodopsin RNA by a 10-fold molar excess of Rz10 alone (A) and in the presence of a 100-fold excess of non-radioactively labeled ‘chase’ rhodopsin RNA (B). Lanes 1-7 = reaction timeframes of 0.5, 1, 3, 5, 10, 15 and 20 minutes respectively. Arrows from top to bottom indicate human rhodopsin RNA and 5’ and 3’ cleavage products. (C)-(D) = graphical representations of % cleavage of human rhodopsin RNA by Rz10 and Rz40 respectively, in the absence and presence of ‘chase’ RNA. 10:1 molar ratios of ribozyme to target RNA were used. Results were used to determine $k_2$ and $k_1$ parameters. Control reactions demonstrate the functionality of the unlabeled ‘chase’ RNA used in these experiments as no additional cleavage of the labeled rhodopsin transcript was observed following addition of the chase.
Figure 4.8
Determination of $k_1$ and $k_{-1}$ for Rz40.

Figure 4.8
Figure 4.9

(A) Cleavage gel of human peripherin RNA under single-turnover conditions, with a 50-fold molar excess of Rz9. Lanes 1-8 = timepoint cleavage reactions at 10mM MgCl$_2$ and 37°C for 10 seconds, 5, 10, 20, 30, 60, 120 and 180 minute timeframes. Arrows from top to bottom highlight human peripherin RNA and two cleavage products. (B) Graph of % cleavage of human peripherin RNA and Rz9. Results of timepoint cleavage gel (A), were used to determine $t_{1/2}$ and $k_2$ values.
Determination of $t_{1/2}$ and $k_2$ for R9 and human peripherin RNA

Figure 4.9
Figure 4.10

(A) and (B) = Cleavage profile of human rhodopsin RNA by the multimeric ribozyme construct. (A) Lane 1 = DNA ladder; lanes 2-9 = A 10-fold molar excess of RzMM and rhodopsin RNA for 0, 5, 10, 20, 30, 40, 60, 90, and 120 minutes. From top to bottom, dark arrows highlight target rhodopsin RNA and labeled ribozyme. Light arrows indicate the complex pattern of cleavage products. (D) Lanes 1-7 = Equimolar ratios of ‘unlabeled’ RzMM and rhodopsin RNA for 0, 10, 20, 40, 60, 90 and 120 minutes. Reactions were performed in the presence of 10mM MgCl₂ at 37°C. From top to bottom, the arrows indicate target RNA and the large number of cleavage products. As any one or more of the monomeric ribozymes within the multimeric construct can function at any given time, it was not possible to determine exactly which cleavage products were the result of a specific ribozyme or combination of ribozymes. Expected cleavage product sizes from Rz40, Rz41, Rz42 and Rz43 in the multimeric construct are 584 + 277, 596 + 265, 582 + 279 and 803 + 58 bases respectively. Cleavage of the target RNA by RzMM was almost complete.
Figure 4.11

(A) Cleavage gel of human rhodopsin RNA and Rz40. Lanes 1-8 = human rhodopsin RNA following incubation with Rz40 for 10 seconds, 5, 10, 20, 30, 60, 120 and 180 minutes respectively. Cleavage reactions were performed with 10mM MgCl₂ @ 37°C. Arrows from top to bottom indicate residual uncleaved rhodopsin RNA (861 bases) and 5' and 3' cleavage products (584 and 277 bases). Notably, Rz40 was extremely efficient in vitro cleaving the rhodopsin RNA to near completion.
Figure 4.11
CHAPTER 5

Evaluation of ribozyme activity in cell culture
5.1 INTRODUCTION
To date, the extensive genetic heterogeneity associated with adRP has severely impaired the development of realistic methods of therapeutic intervention. Three ribozyme-based therapeutic approaches for combating adRP, which are independent of the disease mutation, have been explored \textit{in vitro} (Millington-Ward et al., 1997). All ribozymes were shown to specifically cleave their target RNAs with varying efficiencies producing cleavage products of predicted size while replacement transcripts were masked from cleavage [Chapter 3]. As kinetic profiles of ribozymes \textit{in vitro} can be used as broad predictors of activity \textit{in vivo} (Birikh et al., 1997b), prior to evaluation in cell culture systems ribozymes directed to 5'-UTR non-coding regions and degenerative nucleotides in coding regions of human peripherin and rhodopsin transcripts were kinetically analysed. Three monomeric hammerhead ribozymes and a connected-type multimeric ribozyme, consisting of four \textit{trans}-acting ribozymes linked in tandem with extremely high catalytic efficiencies \textit{in vitro} (Rz9, Rz10, Rz40 and RzmM) have been identified [Chapter 4]. Translating efficiencies observed \textit{in vitro} to \textit{in vivo} situations, primarily requires the availability of suitable model systems to test ribozyme functionality. Cell culture systems offer an ideal semi-natural and therapeutically relevant environment in which to test ribozymes. However, rhodopsin and peripherin are only expressed in non-dividing photoreceptor cells that do not propagate in cell culture. Various retinoblastoma (Rb) cell lines, which are thought to be photoreceptor cell precursors (Griegel et al., 1990), do divide and have been shown to support expression of some photoreceptor-specific proteins (Bogenmann et al., 1988; Fong et al., 1988; Di Polo and Farber, 1995). For instance, expression of the $\alpha$, $\beta$ and $\gamma$ subunits of cGMP phosphodiesterase (PDE), the rod $\alpha$ subunit of transducin, rhodopsin and the cone $\alpha'$ subunit of PDE have been detected in Y79 retinoblastoma cells (Di Polo and Farber, 1995). However, even though Rb cell lines, such as Y79 and WERI-Rb, have been transiently transfected with reporter plasmids (Fong et al., 1993; Di Polo and Farber, 1995), transfection efficiencies have not been as well characterised as, for example, African green monkey kidney cells (COS). The need for an easily transfectable cell line expressing rhodopsin or peripherin together with the ability of COS cells to support high-level expression of functional bovine rhodopsin (Oprian et al., 1987), urged the generation of stable COS-7 cell lines expressing either human rhodopsin or human peripherin. COS-7 cells have previously been used as model systems to assess ribozyme-mediated down regulation of Alzheimer amyloid peptide precursor mRNA ($\beta$APP mRNA). $\beta$APP mRNA and protein levels were reduced by 67-80% and directly
correlated with ribozyme expression levels (Denman et al., 1994). A variety of stable COS-7 cell lines were generated and characterised for expression of rhodopsin and peripherin RNA by RT-PCR and/or Northern blotting. Cell lines were subsequently transfected with Rz10, Rz40, and RzMM and the levels of rhodopsin or peripherin mRNA analysed for possible reductions.

The method chosen for transfection of COS-7 cells with human rhodopsin and peripherin cDNA constructs to generate stable cell lines was electroporation (see Materials and methods). However, as this technique causes considerable cell death it is not suitable for transient transfection assays, particularly when analysing the effects of gene suppressors (e.g. ribozymes) on mRNA expression. Therefore, a milder liposome-mediated delivery system (LipofectAMINE PLUS, Gibco/BRL) has been employed for transfection of cells with ribozyme for transient assays (see Materials and Methods). Liposome-mediated delivery offers a higher transfection efficiency than most chemical and physical delivery systems and therefore is suitable for ribozyme-based studies in vivo. Indeed, liposome-mediated delivery has been employed to deliver ribozymes into various cell-types in culture with the successful ablation of target RNA and lowering of protein levels (Scherr et al., 1997; Gonzalez et al., 1998; Kijima et al., 1998;). Moreover, lipid-based reagents, such as Lipofectin (Gibco/ BRL), have been shown to dramatically enhance the stability of ribozymes in cell-culture presumably by making the ribozyme inaccessible to RNases (Kariko et al., 1994).

In addition, double-stable COS-7 cell lines with integrated target RNA and ribozyme have been generated using a second selectable marker, Zeocin. The presence of selectable marker genes, such as neomycin phosphotransferase, in the mammalian expression vectors employed in this study encode resistance to lethal doses of antibiotic (e.g. G418) and enable selection of positively transfected clones; isolation of resistant colonies ensures the presence of desired cDNA or ribozyme inserts. The generation of stable cell lines can be quite laborious involving selection of stable cell lines with appropriate antibiotic such as G418 for 3-4 weeks and subsequent characterisation of resistant colonies. The vectors used to generate the stable rhodopsin and peripherin expressing COS-7 cell lines contain cytomegalovirus (CMV) or Simian virus (SV40) promoters which allows high-level expression of multiple cloning site (MCS) inserts in a variety of eukaryotic cell-types.
Additionally, the vectors contain bovine growth hormone (BGH) or SV40 polyadenylation signals which polyadenylate transcripts to aid stability (Figures 3.2 and 5.1a).

A major difficulty with analysis of ribozyme studies in vivo, is the ability to isolate intact ribozyme cleavage products. Cleavage products either lack 5' m'G caps or poly(A) tails rendering them vulnerable to rapid degradation by intracellular nucleases. Mechanisms of mRNA stability / turnover have been extensively studied in eukaryotic cells and several distinct but related pathways by which mRNAs are degraded have been identified. mRNA degradation can be initiated by (a) shortening of the poly(A) tail followed by decapping and 5’ to 3’ exonucleolytic degradation (b) 3’ to 5’ degradation subsequent to poly(A) shortening (c) deadenylation-independent decapping followed by 5’ to 3’ degradation or (d) by an endonucleolytic cleavage event within the transcript (Tharun and Parker, 1997). In particular, the polyadenylation of transcript is fundamental to stability of mRNAs in vivo. Classical studies with α-globin mRNAs have shown that the precise removal of the poly(A) tail by oligo(dT)-directed RNase H digestion and injection into Xenopus oocytes had a dramatic effect on transcript stability. While adenylated α-globin mRNAs were stable and translationally competent for 1 week, deadenylated transcripts were degraded within hours (Huez et al., 1974). Extensive research over the last 25 years has shown that 3'-end processing events are promoted by two sequence elements in the 3'-UTR region (Proudfoot and Whitelaw, 1990; Wahle and Keller, 1992; Tharun and Parker, 1997); AU-rich elements (ARE's) consisting of one or multiple copies of the hexanucleotide AAUAAA (termed the polyadenylation signal) or variations thereof (AAUAAA, AAUACA and AAUUAA) and GU- or U-rich downstream elements. A suggested concensus sequence for the GU element is XGUGUUXX where X= any pyrimidine. Analysis of chimeric β-globin mRNAs, with AREs from, for example, c-fos, granulocyte-macrophage colony stimulating factor (GM-CSF), jun B and c-myc have been shown to greatly accelerate deadenylation and decay rates of otherwise stable β-globin transcripts (Chen and Shyu, 1994, 1995; Chen et al., 1995). As some RNAs do not possess convincing GU- or U-rich elements, it has been hypothesised that the presence of these elements relate to the efficiency of polyadenylation and therefore mRNA expression. Studies by Gil and Proudfoot with the rabbit β-globin gene demonstrated that the absence or altered position of both GU- or U-rich elements significantly reduced the levels of mRNA produced by the gene (Gil and Proudfoot, 1987). In addition, many cellular proteins and nucleases such as the poly(A)-binding protein (Pablp) and the Pablp dependent poly(A) nuclease (PAN) have been shown to influence deadenylation of
transcripts (for review see Tharus and Parker, 1997). Therefore, all ribozyme and cDNA constructs used in this study were designed to include poly(A) signals (in pcDNA3) to aid transcript stability in culture. In an effort to circumvent the difficulties of isolating unstable ribozyme cleavage products to demonstrate ribozyme functionality in cells, primer extension assays were performed using Avian Myeloblastosis Virus (AMV) reverse transcriptase. These assays could potentially enable identification of the exact nucleotide (cleavage site) at which Rz10, Rz40 and RzMM cleaves human rhodopsin mRNA.

As the therapeutic application of delivered ribozymes is highly dependent on their stability within cell systems, much effort has been invested in enhancing their stability through chemical modification. Chemical modifications to ribozymes have been demonstrated to increase stability in vivo, often with either minor loss or indeed no loss of catalytic activity (Pieken et al., 1991; Taylor et al., 1992; Heidenreich et al., 1994; Burgin et al., 1996; Lierdal et al., 1998). In particular, the replacement of two uridines in the conserved region of a hammerhead ribozyme by 2'-amino-2'-deoxyuridines and the incorporation of four terminal phosphorothioate linkages was shown to substantially increase ribozyme stability while maintaining a cleavage activity comparable to that of its unmodified counterpart. These modified ribozymes were shown to be stable for at least twenty-four hours in undiluted fetal calf serum (Heidenreich et al., 1994). In contrast, typically unprotected RNA would be degraded in a matter of seconds to minutes. In the current study the influence of uniform 2’-amino uridine modifications on the catalytic activity of Rz40 (targeting human rhodopsin RNA) have been investigated with a view to establishing methods of increasing the stability of Rz40 when used in cell and animal systems.

In addition, hammerhead ribozymes have proven efficient at down-regulating mRNA and protein levels in animal systems. Transgenic mice expressing functional ribozymes directed to β-2-microglobulin (B2M), α-lactalbumin and pancreatic β-cell glucokinase mRNAs represent but a few of a very large number of examples (Larsson et al., 1994; Efrat et al., 1994; L’Huillier et al., 1996). Indeed in one such study, for the generation of an animal model for maturity-onset diabetes of the young (MODY- a disease associated with heterozygous glucokinase mutations which result in decreased β-cell glucokinase activity and a possible impairment of the insulin-secretory response to glucose), a hammerhead ribozyme targeting pancreatic β-cell glucokinase and driven by the insulin promoter was introduced into mice and reduced normal islet activity by 70%. Notably, insulin release in
response to glucose from in situ-perfused pancreas was inhibited. As these mice were expected to be predisposed to type II diabetes (non-insulin dependent) they were proposed as suitable animal models to investigate the interactions of genetic and environmental diabetogenic factors with reduced islet glucokinase activity (Efrat et al., 1994). Moreover, Lewin et al (1998) demonstrated that hammerhead or hairpin ribozymes, targeting an artificially created P23H rhodopsin mutation when delivered in rAAV constructs by intraocular injection into P23H-3 transgenic rats considerably retarded photoreceptor degeneration for at least 3 months. Given that these retinal-specific ribozymes were catalytically less active in vitro when compared to Rz10 and Rz40, it was decided to build a rhodopsin driven Rz40 construct to generate a transgenic mouse carrying Rz40.

Moreover, the on-site availability of mutant and wild-type P23H mice (Olsson et al., 1992) would enable immediate cross-breeding with transgenic Rz40 mice. Any potential beneficial effect(s) could be evaluated directly using a number of readouts, for example, retinal histology and electroretinography.

In summary, Chapter 5 of the thesis describes the generation of stable COS-7 cell lines expressing human rhodopsin and human peripherin, characterisation of these cell lines by RT-PCR and/or Northern blotting techniques and transient transfection of the cells with Rz10, Rz40 and RzMM to determine the efficiency of ribozyme mediated down-regulation of retinal transcripts. Furthermore, the influence of chemical modifications on Rz40 cleavage activity together with the construction of an Rz40 vector for the future generation of a transgenic animal carrying Rz40 are discussed in this chapter.
5.2 MATERIALS AND METHODS

5.2.1 Transfection of mammalian cells: Electroporation and cationic liposomes

Electroporation as a technique for gene transfer studies was first reported in 1982 (Wong and Neumann, 1982) and is based upon perturbation of the cell membrane by a high voltage electrical pulse, which transiently forms pores allowing passage of nucleic acids into the cell. Electroporation is frequently used for cell types that prove recalcitrant to transfection with milder techniques such as calcium phosphate, DEAE-dextran or artificial liposome delivery. However, a critical balance between the strength of the electrical pulse required for efficient delivery and conditions that cause cell death, must be achieved; maximum transfection efficiency occurs when the electrical field causes 50-60% cell death. Therefore, electroporation is not suitable for transient transfection assays. In contrast, stable cell line generation requires only one cell with integrated plasmid to survive and expand into clonal groups for analysis. LipofectAMINE is a polycationic liposome reagent with a highly positively charged spermine headgroup on the cationic component (DOSPA) of the molecule (Hawley-Nelson et al., 1998). It is believed that the positively charged cationic portion of the lipid binds negatively charged nucleic acid, compacting the nucleic acid in a liposome/nucleic acid complex; the higher the positive charge of the complex, the higher the transfection efficiencies. This is presumably because it allows closer association of the liposome/nucleic acid complex with the negatively charged cell membrane. Complexes enter the cell via endocytosis and are transported from endosomes into the cytoplasm and nucleus (Promega transfection guide, 1998). However, the mechanism of nucleic acid release from endosomes and transversion of the nuclear membrane is unclear.

5.2.2 Preparation of DNA for transfections; generation of stable cell-lines

As the quality of DNA used for transfection of mammalian cells is critical, cDNA and ribozyme constructs were purified by two CsCl gradients to remove RNA, protein or chemical contamination. To generate stable cell lines, cDNA constructs were linearised with a suitable restriction enzyme not present in the cDNA sequence or essential eukaryotic plasmid sequence promoting plasmid integration in a manner that does not disrupt the gene of interest. All reagents and solutions used for cell work were sterile and nuclease-free. 5, 10 and 15μg of human rhodopsin and human peripherin cDNAs in
pcDNA3 were digested with *Pvu*I, phenol extracted x 3, EtoH precipitated and resuspended in 50µl PBS for electroporation. The generation of stable cell lines is a very laborious process that takes approximately 8 weeks in total for each cell line (without characterisation) - for a detailed protocol see Chapter 2, section 2.4.6. Briefly, 5, 10 and 15µg of each retinal cDNA (rhodopsin and peripherin) was electroporated into separate batches of COS-7 cells (Chapter 2, section 2.4.4). Following 3-4 weeks selection with 600µg/ml G418, 30-40 antibiotic resistant colonies were individually selected and transferred to 96-well plates. 10-15 of the healthiest rhodopsin and peripherin COS-7 cell lines were propagated to confluency on 150mm tissue culture dishes (a process that takes a further 3 - 4 weeks) and frozen down in liquid nitrogen pending analysis.

5.2.3 Optimisation of COS-7 cell transfection efficiencies

To optimise the transfection efficiency of human rhodopsin and peripherin COS-7 stable cell lines with LipofectAMINE PLUS reagent, levels of DNA, PLUS reagent and Lipofectamine reagent together with cell density and transfection times were varied using the standard protocol detailed in Chapter 2, section 2.4.5. The reporter plasmid pZeoSVLacZ was used for optimisation because of the relatively straightforward β-galactosidase assay (Chapter 2, section 2.4.7). The day before transfection 1.5-8.0 x 10^5 COS-7 / rhodopsin cells were plated in 6 well plates to ensure that the plates were 50-80% confluent on the day of transfection (18-24 hours later). For each well, 2-16µl Lipofectamine reagent and 500ng-5µg pZeoSVLacZ DNA were diluted into separate 100µl aliquots of serum and antibiotic free DMEM. 4-10µl PLUS reagent was added to the DNA solution and incubated at room temperature (R.T.) for 15 minutes. The DNA-PLUS complex was mixed with diluted Lipofectamine, incubated at R.T. for 15 minutes and added to the cells (in 0.8ml serum free DMEM). Incubations proceeded for 3-5 hours at 37°C and 5% CO₂. *In situ* staining was used to demonstrate β-galactosidase expression 24-72 hours post transfection.
5.2.4 Characterisation of stable cell lines

5 X 10^6 cells of each human rhodopsin and peripherin COS-7 cell line were harvested and total RNA isolated by guanidinium isothiocyanate phenol-chloroform extraction (Chapter 2, section 2.3.4). Poly(A) mRNA was subsequently extracted, 500ng to 1μg of each sample electrophoresed on 1.2% denaturing formaldehyde/agarose gels and Northern blotted in 20XSSC (Chapter 2, section 2.3.7). Human rhodopsin and peripherin blots were probed with a HindIII to BstEII human rhodopsin cDNA fragment (850 bp) and a 522 bp human peripherin PCR fragment respectively. 5μg of total RNA was used for RT-PCR reactions. In addition, G3PDH housekeeping gene PCRs were performed to assess the quality of RNA extracted from transfected cells. Primer details for RT-PCRs and generation of the human peripherin probe are as follows:

(I) Human rhodopsin (RT-PCR): Accession number KO2281
(F) - 5'-ATGGTCCTAGGTGGCTTCACC-3' (Exon 3: 550-570)
(R) - 5'-CATGATGGCATGGTCTCCACCC-3' (Exon 5: 2522-2542)

(II) Human G3PDH (RT-PCR): Accession number XO1677
(F) - 5'-ACCACAGTCCATGCACTCAG-3' (Exon 1: 586-605)
(R) - 5'-TCCACACACCTGGTCTGTA-3' (Exon 2: 1018-1037)

(III) PCR for human peripherin / RDS probe: Accession number M73531
(F) - 5'-CATCTTCAGCTGGGGACTGTG-3' (Exon 1: 347-366)
(R) - 5'-GTCCACCGCTGGTACACAT-3' (Exon 2: 850-869)

(IV) PCR for β-actin probe: Accession number M10277
(F) - 5'-CGTACACTGCGCATCGTG-3' (Exon 3: 2104-2121)
(R) - 5'-GTTTTCGTGGGATGCCACAG-3' (Exon 4: 2573-2590)
5.2.5 RNA size standard

A 0.28-6.58 kb RNA ladder (Promega) was used to estimate the sizes of rhodopsin and peripherin transcripts on Northern blots. Typically, 1μg of RNA was mixed with 15μl RNA running dye (Chapter 2, section 2.3.3), denatured at 70°C for 15 minutes and electrophoresed on a 1.2% formaldehyde/agarose gel. Following transfer and fixation to a nylon membrane, the ladder was cut from the blot using a sterile blade and stained with a methylene blue solution (0.03% methylene blue in 0.3% sodium acetate, pH 5.2) for 5 minutes. To remove excess staining solution, the membrane was destained by successive purite H₂O washes (Figure 5.1b). RNA marker sizes are: 6583, 4981, 3638, 2604, 1908, 1383, 955, 623 and 281 bases.

5.2.6 Analysis of mRNA expression

To determine potential reductions in human rhodopsin mRNA expression levels, following transfection of COS-7 cell lines with Rz10, Rz40 and RzMM, Northern blots (Chapter 2, section 2.3.7) were analysed by autoradiography and Instant imaging. Blots were quantified by counting areas showing probe hybridisation. Background was accounted for by measuring equal areas of signal with and without hybridisation and subtracting the latter. To correct for irregular loading which could potentially lead to artifactual results, blots were probed for β-actin and the quantitative rhodopsin mRNA reading for each sample expressed as a ratio of the β-actin reading for the same sample.

5.2.7 Primer extension assays

In an effort to identify physical cleavage of human rhodopsin RNA by Rz10, Rz40 or RzMM in vivo, primers were designed downstream of ribozyme cleavage motifs for extension by AMV-reverse transcriptase. As the distance of primer from the 5'-end of the RNA is critical for effective extension, two primers, OP1 and MP1, were designed approximately 100 bases from the ribozyme target sites which should allow the AMV enzyme to reach the sites and fall off if cleavage has occurred, producing a fragment of defined length. A control primer (CPI) upstream of the ribozyme cleavage sites which should enable identification of the +1 transcriptional start site in pcDNA3 was also generated. 10pmol of each primer and 250ng of dephosphorylated φX174 HinfI DNA ladder was end-labeled with [γ-32P] ATP using T4 polynucleotide kinase (see Chapter 2, section 2.3.8). Poly(A) RNA was extracted from 5X10⁶ cells of each human rhodopsin COS-7 cell line transfected with a control ribozyme (Rz30), Rz10, Rz40 and RzMM.
100 fmol of labeled primer and 100 ng of poly(A) RNA from each transfected line was annealed at 58°C for 20 minutes and extended with AMV-RT (Chapter 2, section 2.3.8). 'No RNA' controls for each primer and a Kanamycin positive control RNA were included. Primer extended samples were denatured at 90°C for 3 minutes and run on 8% polyacrylamide gels. In addition, to identify the precise nucleotides at which the AMV-RT enzyme dissociates from the rhodopsin RNA, manual sequencing reactions of the RNA (Chapter 2, section 2.2.12), using the primer OP1 (see below), were performed and electrophoresed beside AMV-RT samples. Details of human rhodopsin primers are as follows:

(I) Outer reverse primer (OP1): bases 586-606
5'-GACGAAGTATCCATGCAGAG-3'

(II) Middle reverse primer (MP1): bases 564-583
5'-TGTAGAGGGTGCTGGTGAAG-3'

(III) Inner reverse primer (CPI): bases 453-472
5'-AGAGCGTGAGGAAGTTGATG-3'

(IV) φX174 Hinf1 DNA ladder:
Fragment sizes are 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 42, 40 and 24 bases.

5.2.8 Chemical modification of Rz40
To enhance the stability and efficacy of Rz40 in cell culture and animal systems, the ribozyme was modified by incorporating 2'-aminouridines at all uridine positions during automated chemical synthesis (Trilink Biotech, USA). In addition, a CY-5 fluorescent tag was added to the 5' terminus of the ribozyme.

5.2.9 Generation of pZeoSVRz40 and pcDNA3.1(-)Rztrans constructs
Hammerhead ribozyme, Rz40, targeting a GTC motif (with a degenerative third base) present in a large open-loop structure of human rhodopsin RNA was re-synthesised with SpeI & XhoI and XhoI & EcoRI restriction ends for cloning into pZeoSV and pcDNA3.1(-) respectively. The pZeoSVRz40 vector allows transfection and selection of human rhodopsin COS-7 stable cell lines with a second selectable marker, Zeocin, for the generation of double-stable lines expressing both rhodopsin RNA and Rz40. Cloning of
Rz40 into the XhoI and EcoRI sites of pcDNA3.1(-) leaves a number of 5' and 3' restriction sites available for insertion of a tissue-specific promoter and heterologous intron to develop a construct for the generation of an Rz40 expressing transgenic animal (Figure 5.1a).

Rz40trans:

\[ \downarrow \text{XhoI} \]

(F)-5'-'GAACTCGAGGACGGTGCTGATGAGTCCGTGAGGACGAAACGTAGAGGAATTCAGC-3'

\[ \uparrow \text{EcoRI} \]

\[ \downarrow \text{EcoRI} \]

(R) 5'-GCTGAAATTCCTCTACGGTTCCTCACCGAGCTCATCAGGCCGTCCCTCGAGTTCG-3'

\[ \uparrow \text{XhoI} \]

Antisense arms are underlined and positions 2.1, 2.2 and 2.15 of the hammerhead structure are highlighted in bold. Identical primers with SpeI and XhoI restriction sites were also synthesised.
5.3 RESULTS

5.3.1 Detection of photoreceptor-specific messages in stable COS-7 cell lines

The absence of easily transfecatable model photoreceptor cell systems expressing high levels of human rhodopsin or human peripherin mRNAs has severely limited \textit{in vivo} analysis of retinal-specific ribozyme-based studies. To overcome this limitation, the generation of stable rhodopsin and peripherin COS-7 cell lines was undertaken as detailed in section 5.2.1. Expression of rhodopsin and peripherin genes in COS-7 cells was investigated by RT-PCR and/or Northern blotting using sequence-specific primers and probes (section 5.2.3). Total RNA was extracted from 5\times10^6 cells of each stable rhodopsin cell line. Following first-strand cDNA synthesis by MMLV-RT, specific single-band products were qualitatively detected by PCR (Figure 5.2a, b). Amplified products of 210 and 452 bases, for rhodopsin and G3PDH respectively, were of predicted size. To distinguish possible artifactual genomic DNA amplification from true mRNA amplification, primers were designed to span at least one intron. However, as a cDNA clone was used to generate the COS-7 stable lines, this assay was invalid for the rhodopsin primer PCRs. To discount genomic DNA contamination, negative control reactions such as omission of reverse transcriptase and incubation of RNA templates with DNase 1 prior to cDNA synthesis were performed. Moreover, RT-PCRs using RNA obtained from COS-7 cells not transfected with the human rhodopsin cDNA were included. These reactions did not give rise to product (Figure 5.2a) thereby confirming the generation of stable COS-7 cell lines expressing detectable levels of the photoreceptor-specific transcript.

As quantitative RT-PCR technology can give sometimes give misleading and inaccurate results, for example, small variations in the efficiency of PCR can result in substantial differences in product yield (Farber and Di Polo, 1995), it was decided to use Northern blotting techniques for quantifying rhodopsin and peripherin transcripts in COS-7 cells (before and after transfection with ribozyme). However, it was first necessary to investigate if mRNA messages were expressed at high enough levels in COS-7 cells to be detected.
To enhance the possibility of detecting photoreceptor-specific transcripts by Northern analysis, poly(A) mRNA was isolated from bulk total COS-7 cellular RNA preparations from each stable cell lines (Chapter 2, section 2.3.5). 1μg of extracted poly(A) mRNA was electrophoresed on formaldehyde/ agarose gels and transferred to nylon membranes (Chapter 2, section 2.3.7). 25ng of rhodopsin- and peripherin-specific probes (section 5.2.3) were labeled with \[^{32}\text{P}\] dCTP and hybridised to their respective membranes overnight. Probed membranes were subsequently analysed by autoradiography (Figure 5.2 c, d). The presence of a band of predicted size at ~2.2kb in rhodopsin cell line samples and the absence of this band in COS-7 controls confirms expression of human rhodopsin transcript in the stable cell lines generated. It is of note, that with significantly darker exposures of the blot, transcript of the correct size can be seen in the majority of the rhodopsin lines generated (data not shown). In particular, lines G, I and L which appear to express rhodopsin transcript at varying but nevertheless easily detectable levels were chosen for ribozyme transfection studies. Similarly, a band of ~3.0kb corresponding to full length human peripherin transcript can be seen in almost all stable lines analysed (except for controls) (Figure 5.2d). To ensure that all RNA samples were intact, the blots were subsequently probed with β-actin. A strong signal at ~2.0kb is present in all lanes containing RNA (Figure 5.2e). In this experiment it was noted that the peripherin-specific probe labeled inefficiently and thus may possibly account for the appearance of a weak signal in COS-7 peripherin stable cell lines.

### 5.3.2 Transfection optimisation

In order to optimise transfection efficiency, with LipofectAMINE PLUS reagent, it was first necessary to determine the appropriate cell plating density, such that, cells were 50-80% confluent on the day of transfection. 1.5, 2.0, 2.5, 5.0 and 8.0X10^4 COS-7 rhodopsin cells were seeded in 6 well dishes and analysed 18-24 hours later. Even after 18 hours, the higher cell density plating (5.0 and 8.0 X 10^4) were too confluent and were discarded. In contrast the 1.5-2.5X10^5 seedings being 50-80% confluent were transfected with 1μg pZeoSVLacZ DNA, 4μl LipofectAMINE and 6μl PLUS reagent (see section 5.2.2). Considerable cell death occurred on the lowest density plate (1.5X10^5); the observed cell death was found to reduce significantly with increasing cell number (data not shown). This toxicity has been shown to only occur when both lipid and DNA are included in the transfection mixture and can be overcome by optimising cell density (Hawley-Nelson et al., 1998). The optimal 2.5X10^5 cell plating density was kept constant for all subsequent
transfection experiments. Using 1 μg of pZeoSV LacZ and 6 μl PLUS reagent, a dose-response of lipid (2, 4, 6, 8, 10, 12, 14 and 16 μl) was used to identify the most effective concentration for COS-7 cell lines. Cells were fixed, methylene blue (see section 5.2.4) stained and each well photographed (Figure 5.3a1-a8). High levels of β-gal expression can be detected in each well, peaking with 6 μl LipofectAMINE reagent. Similarly, dosage effects of DNA (Figure 5.3b1-b6) and PLUS reagent (Figure 5.3c1-c6) on transfection efficiency were investigated. An optimised transfection efficiency of 50-55% was obtained using pre-determined optimal concentrations of DNA (5 μg), LipofectAMINE (8 μl) and PLUS reagent (7 μl) (Figure 5.3d1); the percent of stained cells in cultures was determined from counts of five fields of view. Moreover, β-gal expression levels peaked at 48 hours with almost no expression evident after 72 hours post-transfection (data not shown). A transfection protocol for wild-type COS-7 cells with LipofectAMINE PLUS was obtained from Life Technologies (2 μg DNA, 6.25-7.5 μl LipofectAMINE and 6-8 μl PLUS reagent) and compared to the optimised stable-cell line protocol. Adhering to manufacturers instructions, similar, if not reduced (45-50%), transfection efficiencies were observed (Figure 5.3d2). Notably, no β-gal expression was seen in ‘mock’ transfected cultures (Figure 5.3d3). With optimal conditions determined, reactions were scaled up for larger tissue culture dishes in proportion to the increase in plate surface area.

5.3.3 Analysis of mutation-independent ribozyme activity in rhodopsin COS-7 cell lines

To investigate the activity of mutation-independent hammerhead ribozymes, Rz10, Rz40 and RzMM, (targeting degenerative sites in human rhodopsin RNA) in cell-culture systems, stable rhodopsin-expressing COS-7 lines G, I and L were transfected (in 100 mm dishes) with 30 μg of each ribozyme construct using the optimal LipofectAMINE PLUS protocol. Rz30, targeting a ‘wobble’ site in human peripherin RNA, was used as a control. 48 hours post-transfection, cells were harvested with GTC, total RNA extracted and poly(A) mRNA isolated. Poly(A) mRNA was quantitated spectrophotometrically and 1 μg of each sample subjected to Northern analysis using a labeled probe specific for the rhodopsin message (Figure 5.4a). Similarly, the membrane was probed for β-actin mRNA which served as an internal loading control for each sample RNA (Figure 5.4b). As expected, no rhodopsin signal was observed in wild-type COS-7 control RNA whereas a strong β-actin signal was present. Levels of rhodopsin and β-actin mRNA were measured
by instant image analysis and expressed as a ratio of one another. However, comparison of rhodopsin: β-actin mRNA ratios for each ribozyme transfected sample to control samples (for lines G, I and L) show no reduction in rhodopsin RNA (Figure 5.4c). In fact, results imply that in samples transfected with ribozyme, rhodopsin expression markedly increased rather than decreased (except for GenePorter samples). The cationic lipid based GenePorter transfection reagent (Apligene-Oncor-Lifescreen, U.K.) was also used to deliver ribozymes into stable COS-7 cells. An optimised COS-7 cell protocol, which is believed (according to the manufacturer) to give a 90% transfection efficiency, was obtained and used for transfection studies. However, in contrast to manufacturers claims, GenePorter studies with pZeoSVLacZ DNA showed extremely low transfection efficiencies (Figure 5.3d4) and therefore, the rhodopsin mRNA reductions observed in line G may have been artifactual. Although the results obtained in lines G, I and L (with LipofectAMINE) are difficult to explain, several mistakes were made during the protocol which may account for some experimental discrepancies. After transfection, rather than incubating cells in complete DMEM, serum-free medium was used which resulted in considerable cell death. It is possible that positively transfected cells were unable to survive, without serum present, due to LipofectAMINE-DNA toxicity and therefore the experiment should probably have been discontinued. In addition, as instant image machinery has an analysis threshold and the rhodopsin signal obtained was very intense, it may be possible that highly inaccurate readings were obtained between samples. For future experimentation, it was decided to load 500ng poly(A) RNA rather than 1μg. In addition, by undertaking a simple curve of RNA concentrations the accuracy of the instant imager could be assessed.

For the reasons stated above, the experiment was repeated. Rhodopsin expressing cell lines (I and L) were again grown-up in bulk and 1.8X10^6 cells of each line plated for transfection. Poly(A) RNA was extracted from transfected lines and 500ng Northern blotted. In parallel, to assess the level of experimental error associated with Northern blotting and instant image analysis, 200, 400, 600, 800 and 1000ng of line G RNA was blotted and probed for β-actin (Figure 5.5a). Notably, the autoradiographic image appeared by eye to accurately reflect the increasing concentrations of RNA (Figure 5.5a). However, quantitative measurements obtained by imaging (753, 1247, 2227, 2784 and 3369 counts respectively) were not completely accurate. Although comparison of the 200 & 600ng readings and 800 & 1000ng readings show a direct correlation with the steady increase in RNA, some experimental error exists which is accentuated by comparing lower (200ng) to
higher (1000ng) concentrations of RNA. Therefore, prior to analysis of mRNA levels in ribozyme transfected lines, the instant imager was professionally re-calibrated.

Surprisingly, in this second experiment, in addition to the 2.2kb human rhodopsin transcript being present in ribozyme transfected samples, a smaller transcript of ~ 1.6 kb was also observed (Figure 5.5b). As the smaller fragment was not present in control samples transfected with a peripherin-specific ribozyme (Rz30), it was possible that the transcript may have represented a stable 3' cleavage product. Eventhough the likelihood of an uncapped cleavage product remaining stable in the presence of cellular nucleases was extremely small, it would explain the appearance of this smaller second band. However, this hypothesis was somewhat weakened as the transcript appeared to be approximately 150 bp too small. The abberant size may have been a function of the difficulty in determining molecular weights with accuracy on Northern blots. Alternatively, given the complex and highly base-paired nature of the rhodopsin mRNA structure (determined by RNAPlotFold), it was possible that following ribozyme cleavage the 3' product was degraded by cellular exonucleases until a stable structure, such as a hairpin, prevented further decay. This could possibly explain a reduction in transcript size and its stability in culture. Studies on 5' and 3' exonuclease degradative pathways in yeast have shown that the insertion of strong RNA secondary structure into mRNAs can impede exonuclease attack and allow the isolation of stable decay intermediates (Vreken and Raue, 1992; Decker and Parker, 1993). Moreover, analysis of these stabilised transcripts demonstrated that accumulation of decay products occurred only after shortening of full-length mRNA poly(A) tails to oligo(A) length (Decker and Parker, 1993; Muhlrad et al., 1994, 1995).

Additional support for this hypothesis came from a recent study in which the 3' cleavage product of a hammerhead ribozyme-mediated digestion of alolipoprotein B (apoB) mRNA was detected in HepG-2 cells by reverse ligation-mediated PCR and the exact cleavage site confirmed by sequencing (Wang et al., 1999b). ApoB mRNA decreased by ~ 80% in vivo, but most notably, the stable ribozyme-directed mRNA cleavage produced a truncated protein of corresponding size. Therefore, in the current study the possibility existed that, the second reduced size transcript observed in Northern blots was a stable 3' ribozyme-mediated rhodopsin mRNA cleavage product. Prior to attempting to confirm the identity of the 1.6kb transcript, the blot was quantified by instant image analysis, rehybridised for β-actin (Figure 5.5c) and rhodopsin: β-actin mRNA ratios for each ribozyme transfected line compared. Figure 5.5d shows a bar-chart of the results obtained. Large reductions in rhodopsin transcript in line I for Rz10, Rz40 and RzMM were observed (62%, 46% and
45% respectively) compared to the control. However, in contrast to line I, as with the first experiment, the levels of rhodopsin mRNA in line L appeared to double following transfection with ribozyme. Given the variability observed between different stable cell lines, it was decided initially to focus on characterisation of the potential cleavage product observed in some of the experiments and in addition to repeat transient transfection assays to establish if consistent results could be obtained.

Because of the problems encountered with quantifying RNA levels using Northern analysis, attention shifted to determining the identity of the second 1.6kb mRNA, with a view that if this second band was indeed a cleavage product it would demonstrate directly that the rhodopsin directed ribozymes were active in cells. Primer extension assays, using rhodopsin-specific primers (section 5.2.6) were performed with ribozyme transfected poly(A) RNA samples. If ribozyme cleavage could be positively identified in transfected cell lines and not in controls, efforts to quantify mRNA reduction would become secondary as ribozyme functionality in vivo would be confirmed. Moreover, this experiment would, in principle, determine if the 1.6kb RNA, (observed only when sequence-specific ribozymes were present) was a stable 3' cleavage product. Using rhodopsin primers OP1, MP1 and CPI, products of 129, 107 and 114 nucleotides respectively were expected if cleavage of the rhodopsin mRNA had in fact occurred (due to the location in the rhodopsin gene at which the primers were designed (see section 5.2.6). Similarly, an 87 nucleotide fragment was expected with the Kanamycin control. However, extension products could not be obtained (except for Kanamycin control RNA) with rhodopsin cell line poly(A) RNAs (Figure 5.6). As rhodopsin mRNA has an extremely complex secondary structure (see Chapter 4, figure 4.1) and no denaturation step was present in the extension protocol (see Chapter 2, section 2.3.8), the experiment was repeated. This time prior to annealing of primers to the RNA template, samples were incubated in a 1X hybridisation buffer (5X=500mM KCL and 250mM TrisHCl, pH8.3) and heated to 90°C for 3 minutes. Again, no extension products were obtained (data not shown). Modifications to enhance the extension protocol such as use of a denaturation buffer (40mM PIPES, 1mM EDTA, 0.4M NaCl and 80% formamide), altering the primer annealing temperature between 58°-62°C and raising the reaction temperature from 42°-48°C were some of a wide range of modifications introduced to attempt to obtain primer extension products. Despite protocol alterations, no products were obtained and hence this line of investigation was discontinued.
As primer extension experiments had been unsuccessful, ribozyme transfection studies were repeated. Even though the concentration of samples used for previous Northern analysis was carefully determined by spectrophotometry, the internal β-actin probe demonstrated unequal loading (Figure 5.5c). This, together with the problems encountered with instant imaging may possibly account for the variations in the results obtained from previous experiments. Therefore, prior to further Northern experimentation, the spectrophotometer was professionally re-calibrated and extensively tested for accuracy. Lines G, I and L were again grown in bulk, transfected with rhodopsin-specific ribozymes (Rz10, Rz40 and RzMM) and poly(A) RNA extracted as previously described (Chapter 2, sections 2.3.4 - 2.3.5). As in previous experiments, Rz30 was used as a control. 500ng poly(A) RNA from each ribozyme transfected sample was blotted and probed for rhodopsin expression (Figure 5.7a, c). Interestingly, two transcripts of ~ 2.2kb and 1.6kb were again present in Northern blots but in contrast to the previous experiment, the smaller mRNA band was also in Rz30 controls thereby clearly discrediting the cleavage product hypothesis discussed above. It is difficult to explain why both transcripts for human rhodopsin were not observed in previous control samples. Possibly the large transcript is more stable than the smaller transcript and therefore may be present in a greater proportion of RNA purifications. Instant image analysis of untransfected line G samples (Figure 5.7a) demonstrated that the smaller transcript (barely visible) represented < 15% of total rhodopsin message. However, in ribozyme transfected samples the average percentage substantially increased to 58%. Similarly, in lines I and L the second transcript accounted for 42% and 32% of overall transcript respectively, but unlike line G, this smaller transcript was not observed in untransfected samples. The reasons for the variability in transcript distribution between each rhodopsin line remains unclear. Blots were stripped, re-probed for β-actin expression (Figure 5.7b, d) and quantified. As is evident, equal loading of sample RNA was attained. Rhodopsin: β-actin mRNA ratios were calculated and graphed (Figure 5.7e). Data indicated an 8, 19 and 22% reduction of human rhodopsin mRNA in line I for RzMM, Rz10 and Rz40 respectively. Moreover, a 30% down-regulation in expression was observed for Rz40 in line G. As only 50-55% of cells can be transfected using LipofectAMINE, these reductions may represent up to 60% ablation of actual message. The results obtained with line L varied somewhat. An 8% reduction was observed for Rz40 in line L, however in contrast line L showed a 9-10% increase in rhodopsin expression with Rz10 and RzMM thereby rendering the results certainly in this stable cell line (L) inconclusive. However, to establish the significance of the results
presented, transfection experiments would need to be repeated several more times and statistical analyses performed. Western blotting to examine the levels of rhodopsin protein expression between cell lines transfected with active and control ribozymes may have been useful at this point to determine the levels of ribozyme activity in cells. However, due to time constraints and the fact that ribozymes function at the RNA level, Western analysis was not considered. If a statistically significant reduction in rhodopsin mRNA expression was positively confirmed, protein analyses to examine if reductions at the RNA level were mirrored at the protein level would then been necessary.

As the 1.6kb rhodopsin transcript was not consistently observed in non-transfected samples, an experiment was designed to identify the agent(s) that, following cell transfection, caused a significant shift in the rhodopsin expression profile or at least in the proportion of small and large rhodopsin transcripts observed via Northern blotting. Human rhodopsin line L was grown in bulk and incubated in the presence of 0, 10, 15, 20, 30, 42 and 50μl LipofectAMINE reagent and 30μl Plus reagent without DNA using the optimised COS-7 transfection protocol detailed in section 5.3.2. Control reactions with PLUS reagent only and LipofectAMINE - PLUS - DNA complexes were also performed. Cells were harvested and 500ng poly(A) sample RNA electrophoresed for Northern hybridisation. Human rhodopsin probing of the membrane demonstrated an extremely low level of expression of the 1.6kb transcript in all samples analysed (Figure 5.8a). However, in cells exposed only to a LipofectAMINE-PLUS reagent - DNA complex, the rhodopsin expression profile was substantially altered with a substantial increase in expression of the 1.6kb RNA [lane 7]. To ensure this observation was not a result of mRNA loading, the membrane was re-probed for β-actin expression (Figure 5.8b). While LipofectAMINE and DNA, in combination, have previously been shown to have toxic effects on eukaryotic cells (Hawley-Nelson et al., 1998), this experiment demonstrates that lipid complexes can also have an effect on expression profiles assessed by Northern blotting thereby questioning the use of LipofectAMINE-based delivery systems in gene inhibition studies.
5.3.4 Characterisation of 2'-modified Rz40 cleavage reactions

To increase the stability of Rz40 against degradation by intracellular nucleases for cell culture and animal studies, the ribozyme was chemically modified by substituting all uridines in the hammerhead structure with 2'-amino uridines. The effect of uniform 2'-amino uridine modifications on Rz40 cleavage activity in vitro was first investigated by performing cleavage reactions with T7 generated human rhodopsin RNA. Human rhodopsin cDNA in pcDNA3 was digested with BstEII, expressed in vitro and gel-isolated using standard protocols (Chapter 3, section 3.2.8). Cleavage reactions were performed with a 10:1 molar ratio of Rz40 to target RNA in the presence of 10mM MgCl₂ for varying times. Unprotected Rz40 was previously shown to cleave over 50% of rhodopsin RNA in under 30 seconds (Chapter 4; Figure 4.10b). In contrast, replacement of all uridines with their 2'-amino analogs completely abolished Rz40 cleavage activity in vitro (Figure 5.9a). As this particular form of chemical protection rendered Rz40 inactive in vitro, more suitable modifications which do not reduce the catalytic activity of Rz40 while increasing the ribozymes stability in cell systems (such as site-specific modifications) will be investigated in future experimentation.

5.3.5 pZeoSVRz40 and pcDNA3.1(-)Rztrans

In order to generate double stable COS-7 cell lines expressing both human rhodopsin and Rz40, the ribozyme firstly needed to be cloned into a mammalian expression containing a selectable marker other than neomycin (originally used to select stable rhodopsin expressing COS-7 cells). The PZeoSV plasmid containing a Zeocin resistance gene was chosen as selection with neomycin and Zeocin would allow cells containing both human rhodopsin and ribozyme constructs to be isolated. The key advantage of double stable cell lines is that, in contrast to transient transfection assays, transfection efficiency is irrelevant as 100% of selected cells will contain stably integrated copies of plasmids containing the rhodopsin target and ribozyme. Thus, 25µg of forward and reverse Rz40 primers were annealed overnight, digested with SpeI and XhoI and cloned into pZeoSV. Positive clones were identified by restriction enzyme analysis using KpnI (Figure 5.9b) and ABI automated sequencing. Miniprep 1 was grown-up in bulk and digested with BspH1. 1.8X10⁶ cells from rhodopsin line I were seeded and subsequently transfected with 30µg of linearised CsCl grade pZeoSVRz40 using the cationic lipid, LipofectAMINE PLUS. pZeoSV plasmid DNA was used as a control. Cells were selected for 4 weeks with 400µg/ml Zeocin antibiotic and 15 positive clonal groups from both ribozyme and plasmid
lines were picked and amplified to confluency on 6-well dishes. However, following communication with the manufacturers of the pZeoSV mammalian expression vector, the author was informed of serious recombinatory problems with the plasmid (see discussion) which resulted in it being withdrawn from production. Therefore, no further work was performed with the double-stable lines generated.

Given the efficiency of Rz40 in vitro (Chapter 4) and the data obtained in cell culture (section 5.3.3), it was decided to build a suitable construct for the generation of a transgenic mouse expressing Rz40. Annealed Rz40 primers with Xho1 and EcoR1 restriction-ends were digested and cloned into pcDNA3.1(-). Positive clones were identified and subsequently digested with Spe1 and Xho1. In addition, an 11.1kb mouse rhodopsin clone (in pBluescript) containing the complete rhodopsin coding sequence and at least 4.5kb of 5' sequence was obtained from a colleague, Marian Humphries and digested with Spe1 and Xho1 liberating a 3.8kb rhodopsin promoter fragment. The 3.8kb fragment was gel-isolated and inserted 5' (Spe1 and Xho1 sites) of Rz40 in pcDNA3.1(-).

Minipreparations were digested with HindIII (Figure 5.9c) and those liberating the expected 1.9kb fragment were sequenced. A heterologous intron, such as SV40, will be inserted immediately 3' of Rz40 and the final pcDNA3.1(-) Rztrans construct used to generate an Rz40 expressing transgenic line. These animals will be extremely useful for examining the in vivo viability of the mutation-independent wobble-base therapeutic approach for adRP addressed in this thesis.
5.4 DISCUSSION

The therapeutic development of mutation-independent ribozyme-based approaches for combating adRP (Millington Ward et al., 1997) has been hindered by the absence of well characterised experimental photoreceptor cell systems that allow assessment of ribozyme activity. While various retinoblastoma-derived cell lines such as Y79 and WERI-Rb have previously been shown to express certain retinal-specific genes, for example, the α, β and γ subunits of cGMP phosphodiesterase, the rod α subunit of transducin, rhodopsin and the red and green opsins (Di Polo and Farber, 1995), the cell lines were unsuitable for rhodopsin- and peripherin-based ribozyme studies. This was owing to the fact that analysis performed by a member of the Ocular Genetics Unit (Sophia Millington-Ward) demonstrated that the level of rhodopsin and peripherin expression in these lines was extremely low (barely detectable by Northern blotting - data not shown). Moreover, the suitability of an alternative transformed photoreceptor cell line, 661W, for retinal-specific ribozyme studies was examined. These cells have been successfully used to investigate the activity of nuclear factor-κB and caspase-1 in photoreceptor cell apoptosis (Krishnamoorthy et al., 1999). However, 661W cells do not express either rhodopsin or peripherin (personal communications, Dr. T. Cotter and Dr. M. Al-Ubaidi) and therefore were not used. In order to address these shortcomings, stable rhodopsin and peripherin expressing COS-7 cell lines (as determined by RT-PCR and/or Northern blotting) have been developed (Figure 5.2). Three stable lines which appeared to express varying levels of human rhodopsin were chosen for subsequent ribozyme transfection studies. Following optimisation of COS-7 cell transfection efficiencies with the cationic lipid, LipofectAMINE PLUS, hammerhead ribozymes Rz10, Rz40 and RzMM targeting degenerative sites in human rhodopsin mRNA were delivered into lines G, I and L. Because of the lability of cleavage products generated by trans-acting ribozymes, ribozyme activity was quantified by comparing the relative abundance of human rhodopsin target RNA in cells transiently transfected with rhodopsin-specific ribozymes to that in cells transfected with non-specific control ribozymes using Northern blotting and instant image analyses. However, significant variations in levels of expression of rhodopsin even between control samples was observed during initial investigations (data not shown). Although transient transfection can offer a more rapid assay than stable cell line generation, only a percentage of cells are transfected thereby substantially reducing the resolution of the experiment in identifying ribozyme-mediated down-regulation of target transcript expression. Given the transfection efficiency attainable for COS-7 cells (~50%;
Figure 5.3d1) and the insensitivity of Northern blot analysis, any experimental error associated with the quantitative techniques employed could potentially 'mask' ribozyme activity and may explain the difficulties encountered with initial analysis. To minimise error and obtain reproducible data, transfection conditions such as LipofectAMINE: DNA ratios, transfection times and cell plating densities were kept constant. In addition, the spectrophotometer and instant image equipment used for quantitation were re-calibrated. When the parameters mentioned above were carefully monitored, between 0-30% reduction in human rhodopsin mRNA expression for Rz10, Rz40 and RzMM was observed in lines G, I and L (determined by Northern blotting and instant image analysis). The variability in degrees of rhodopsin inhibition obtained with different stable COS-7 cell lines possibly originates from the heterogeneous nature of transiently transfected cells, the copy number of the rhodopsin/ribozyme genes and differences in the genetic environment (Lieber and Strauss, 1995; Birikh et al., 1997). As the method used for the generation of stable rhodopsin-expressing cell lines involved random integration of rhodopsin transgene(s), single or concatameric cDNAs could have inserted anywhere in the COS-7 cell genome giving rise to a high variability in levels of rhodopsin expression. Notably, similar variations in reduction of ribozyme-mediated mRNA suppression between stable cell lines was reported by Benedict et al. (1998). In this study, a triple ribozyme construct consisting of two cis-acting ribozymes flanking an internal trans-acting ribozyme directed to retinoblastoma (Rb) mRNA was generated. For in vivo studies, the ribozyme was cloned into a tetracycline-regulated mammalian expression vector and electroporated into 10T1/2 mouse embryo fibroblasts expressing Rb mRNA. A number of stably transfected clones were subsequently isolated by antibiotic selection, grown up in bulk and cellular RNA collected in the absence of tetracycline (conditions allowing expression of the ribozyme). Comparison of Rb mRNA expression levels (by Northern blot and instant image analysis - as above) in a number of the stable cell lines generated indicated that while some cell lines (Rb-10 and Rb11) showed a slight reduction in Rb mRNA compared to cell lines containing inactive ribozyme controls, other cell lines showed up to 70% reduction in Rb expression (Rb-3 and Rb-5). Moreover, analyses of the stable cell lines indicated that the magnitude of reduction observed between each line correlated (reasonably well) with the levels of ribozyme expression in each cell line. Thus, in the context of the transient transfection assays discussed above, the variability in rhodopsin mRNA expression levels observed between lines G, I and L following transfection with ribozymes may be the result of differences in both rhodopsin and ribozyme expression levels in each cell line and
variations in the ability of different cell lines to be transfected. While parameters
determining ribozyme activity in vivo are just beginning to be understood, it has been
demonstrated that ribozyme/target co-localisation is an important determinant of ribozyme
efficacy (Sullenger and Cech, 1993; Bertrand et al., 1997; Hormes et al., 1997; Samarsky
et al., 1999; Chapter 1, section 1.2.10). Eventhough the rhodopsin substrate and ribozyme
constructs were generated such that their respective mRNAs were likely to co-localise
intracellularly (as both genes were cloned into the same mammalian expression vector),
liposome-mediated delivery of ribozymes has been reported in a number of studies not to
be optimal as many molecules can remain trapped in endosomes and are unable to localise
with their target (Birikh et al., 1997a; Vaish et al., 1998). This, together with the inherent
instability and toxicity of liposomes can lead to highly variable results being obtained
(Birikh et al., 1997a; Vaish et al., 1998). The lack of detailed information relating to retinal
mRNA trafficking and the dynamics of ribozyme and target RNA interactions in vivo may
also attribute to the variability in the levels of suppression observed between lines G, I, and
L.

An additional observation derived from transfection studies was the identification of two
rhodopsin-specific transcripts in each stable cell line (Figure 5.7). This in itself was not
surprising as multiple transcripts resulting from the use of alternative polyadenylation
signals have, for instance, been identified for photoreceptor-specific human and mouse
peripherin genes (Travis et al., 1991). Moreover, mouse ops in has been shown to have a
complex transcription pattern producing five major transcripts ranging from 1.7 to 5.1kb
that originate from the selective use of polyadenylation sites in the 3‘-UTR region of the
gene (Al-Ubaidi et al., 1990). Notably, the mRNAs were present at levels that varied over
two orders of magnitude and the shortest RNAs (1.7, 2.2 and 3.1kb) represented more than
80% of the total opsin message. Results also indicated that the presence of multiple opsin
transcripts was not unique to the mouse in that, the rat has four, the human, three, the frog,
two, and the bovine and dog, one major mRNA. There are a number of possibilities that
would explain the multiplicity of processed opsin mRNAs observed in the above
experiment such as alternative splicing, heterogeneous transcription start points, partial
splicing, multiple polyadenylation or any combination of the above (Al-Ubaidi et al.,
1990). However, results demonstrated that, of nine putative polyadenylation sites in the 3’-
UTR of the mouse opsin gene, five were utilised thus explaining the complex five
transcript expression profile observed on Northern blots. As the human rhodopsin clone
used to generate the stable COS-7 cell lines (G, I and L) had two transcription start points (95 and 97bp upstream of translation initiation), at least two putative polyadenylation signals in the 3'-UTR of the human rhodopsin gene at positions 5641-5647 and 6698-6703 respectively (Nathans and Hogness, 1984) and the BGH poly(A) signal in the pcDNA3 expression cassette, it is possible that transcripts of varying sizes (using different polyadenylation signal) could be generated. Although for a given gene, the parameters determining which of a number of poly(A) sites is predominantly utilised in many cases is unknown.

However, it is known that polyadenylation signals can vary in strength. For instance, it has been demonstrated that the AAUACA and AAUUAA variants are considerably less efficient than AAUAAA (Proudfoot and Whitelaw, 1990). Furthermore when two identical sites are present, the upstream site will preferentially be used (Luo and Carmichael, 1991). Indeed, it is of note that both the large and small rhodopin transcripts (~ 2.2kb and 1.6kb) observed in studies performed with lines G, I and L were the same size as two of the three rhodopsin transcripts observed in the study discussed above by Al-Ubaidi et al. (1990) where bands of approximately 1.6, 2.2 and 3.0kb were present on Northern blots for human rhodopsin mRNA. Moreover, the 2.2kb transcript observed in lines G, I and L represented most of the total rhodopsin message (as with Al-Ubaidi’s study) with the smaller 1.6kb transcript being present at extremely low-levels and was in some cases absent altogether (Figures 5.5, 5.7, 5.8). This might possibly suggest an alternative use of polyadenylation signals in the 3'-UTR of the human rhodopsin clone used to generate stable COS-7 cell lines G, I and L. However, when the stable cell lines were transfected with ribozyme, transcript distribution was substantially altered (Figure 5.7a, c). Experiments demonstrated that the LipofectAMINE-PLUS-DNA complex, in combination (but not individually), altered the transcription profile of human rhodopsin mRNA as assessed by Northern blotting - the intensity of the smaller 1.6kb rhodopsin transcript subsequent to ribozyme administration with LipofectAMINE PLUS was estimated to increase approximately 15-fold compared to the intensity of the same transcript obtained from cells transfected without ribozyme (Figure 5.8). While in retrospect it may have been appropriate to include a plasmid only control on Northern blots to discount the possibility that the 1.6kb transcript may represent hybridisation between the probe and transfected plasmid, analysis of the size of transcripts that could potentially be generated from the vector rule out this scenario. As the accuracy in determining ribozyme / gene suppressor efficiency is in many cases
dependent on monitoring potential reductions in the intensity of a particular target mRNA by Northern blotting and instant image analysis, it is extremely important that the transcription profile of the target mRNA is not affected by anything other than the gene suppressor itself. Thus the results obtained in this study pose serious questions as to the use of the LipofectAMINE PLUS transfection reagent in gene inhibition studies.

Given the problems encountered with transient transfection assays, it was proposed to generate double stable cell lines carrying both the target (rhodopsin) and ribozyme (Rz40). Such stable cell lines would overcome many problems associated with transient assays such as optimisation of transient transfections, inability to obtain 100% transfection and heterogeneity of the cell population. Additional difficulties include the number of cells required to obtain sufficient poly(A) mRNA for analysis in that the larger number of cells used, the more LipofectAMINE reagent is required (which in itself is toxic and kills a % of cells) and thus the overall cost of the experiment. Double-stable rhodopsin/ Rz40 cells were selected with G418 and Zeocin and grown up in bulk (taking approximately 6 weeks). However, because of a design flaw in the pZeoSV expression vector which contained two homologous SV40 polyadenylation units flanking the multiple cloning site (MCS) (one down stream of the MCS and the other down stream of the Zeocin gene, see Figure 3.2b), recombination events resulting in splicing and complete removal of the MCS have been observed in cell culture systems (personal communications, Invitrogen) and thus the vector was withdrawn from the market. Therefore, as the Rz40 gene was cloned into the MCS of pZeoSV, double stable cell line work was discontinued. This experiment will be repeated in the future using an alternative mammalian expression vector such as the modified version of pZeoSV now available - pZeoSV2. Moreover, a new quantitative RT-PCR technique (REALTime PCR) will be employed to monitor the levels of rhodopsin expression in double stable COS-7 cell lines generated. The machinery required for this procedure was not available when the experiments described above were being performed. However, as a new REALTime PCR machine has recently been purchased by the department (in the last month or so) it will be utilised in future experiments. REALTime PCR is an extremely accurate procedure that overcomes many of the difficulties and experimental errors commonly associated with conventional RT-PCR technology.
In addition to analysing the activity of mutation-independent rhodopsin ribozymes in stable cell lines, Rz40 was chemically modified to enhance its stability and efficacy in cell culture. However, a uniform 2'-aminouridine modification was found to completely abolish its catalytic activity in vitro (Figure 5.9a). Interestingly, completely contrasting effects on ribozyme catalytic activity have been observed for uniform 2'-pyrimidine substitutions. Whereas Pieken et al demonstrated that a ribozyme with complete 2'-aminouridine modification retained only 1.9% cleavage activity compared to its unmodified counterpart (Pieken et al., 1991), Sioud and Sorensen showed that a ribozyme, PKCα, with uniform 2'-amino pyrimidine modifications directed to protein kinase Ca mRNA, retained a comparable activity to the unmodified ribozyme (~60%). The half-life of modified PKCα was increased 14,000-fold in 10% human serum ($t_{1/2} = 0.25$ min$^{-1}$ for unmodified ribozyme and ~ 55 hours for protected ribozyme) and successfully blocked tumour growth in both glioma cells and rat models of brain gliomas (Sioud and Sorensen, 1998). Similarly, studies performed in the Ocular Genetics Unit have demonstrated that a mutation-independent hammerhead ribozyme (Rzpol1A1), targeting COL1A1 transcripts associated with Osteogenesis imperfecta, with uniform 2'-amino pyrimidine modifications was stable for up to 72 hours in DMEM supplemented with 10% fetal calf serum. Moreover, the protected ribozyme retained approximately 50% of its cleavage activity compared to the unmodified ribozyme control (Millington-Ward et al, 1999). Because of the inconsistency in results reported in the literature, and the observation that all hammerhead ribozymes have virtually the same catalytic core, Leirdal and Sioud hypothesised that inhibition in cleavage must be due to the presence of 2'-amino pyrimidines in helix 1 and/or III (antisense arms). In depth analysis of site-specific 2'-pyrimidine modifications on the activity of a TNFα hammerhead ribozyme have shown that ribozymes with a limited pyrimidine content in the substrate recognition helices and in particular with purines at positions 2.1, 2.2 and 15.2 (using conventional hammerhead nomenclature, Campbell et al., 1997) can be uniformly protected by chemical substitution without loss of catalytic activity (Leirdal and Sioud, 1998). Examination of Rz40 sequence (section 5.2.8) shows that even though 11 of the 15 bases in the hammerheads antisense arms are purines, the most relevant base adjacent to the cleavage site (position 2.1) is a pyrimidine. This may account for the complete abolishment of Rz40 activity in vitro when 2'-amino pyrimidines are incorporated into the ribozyme. With the knowledge that position 2.1 of Rz40 should not be chemically protected (at least with a 2'-amino group) to retain the ribozymes cleavage activity, site-specific chemical modification techniques will be
used in future experiments. Moreover, the feasibility of using chemical modifications such as 2’ modified fluoro, allyl or O-methyl derivatives alone (site-specific or uniform) or in combination with terminal phosphorothioate linkages or an inverted nucleotide will be examined. However, as this experimental work is extremely expensive (approximately 2000 dollars per 60μg of a uniformly modified ribozyme and more for site-specific modifications) the advantages and disadvantages of each particular chemical modification will be carefully considered prior to embarking on further studies.

Given the immense intragenic heterogeneity associated with adRP, it is more likely that methods of therapeutic intervention which are independent of the disease mutation will present a more realistic approach. Rz10, Rz40 and RzMM were designed such that ribozyme-mediated suppression of human rhodopsin mRNA was directed to wobble-bases allowing concurrent introduction of replacement genes with altered third base positions which are masked from cleavage but still code for wild-type rhodopsin protein. The data obtained demonstrates the ability of these ribozymes targeting wobble sites to down-regulate expression of rhodopsin RNA in cell culture systems. Moreover, transcripts from replacement genes have been shown in vitro to be protected from ribozyme cleavage (Chapter 3). In the context of developing a therapy using this approach, it would be important that the ribozyme and replacement genes were administered together and indeed present in the same construct to ensure that they were delivered to the same cell.

Additionally, ribozymes targeting other sequences were analysed in the study (see Chapters 3 and 4). While Rz9, targeting the 5’-UTR of human peripherin RNA, was not examined in a cellular environment previous studies with hammerhead ribozymes targeting both 5’- and 3’-UTRs have proven efficient in vivo (Kilpatrick et al., 1996; L’Huillier et al., 1996). In one such study, transgenic mice carrying a ribozyme specific to the 3’-UTR of bovine α-lactalbumin were cross-bred with animals expressing high levels of a bovine α-lac transgene. Results demonstrated that α-lac mRNA expression in these animals was reduced between 50-78% compared to inactive ribozyme controls. Moreover, reductions in the level of bovine protein were shown to parallel that observed for the mRNA and directly correlated with the level of ribozyme synthesis (L’Huillier et al., 1996).
The observation that a number of the ribozymes tested in cells (Rz10, Rz40 and RzMM) resulted in inhibition of rhodopsin expression together with the high cleavage efficiency of these ribozymes in vitro (Chapter 4) suggests that these ribozymes would be worth evaluating in animals. In this regard, a rhodopsin-specific ribozyme that was somewhat less efficient in vitro than Rz10, Rz40 or RzMM has previously been shown to function efficiently in vivo - the ribozyme when analysed in a P23H transgenic adRP rat model considerably retarded the rate of photoreceptor cell death for at least 3 months post delivery (Lewin et al., 1998). Moreover, this ribozyme as part of an AAV construct has, following a single intraocular injection, continued to provide a beneficial effect in vivo for two and a half years post injection (personal communication, Dr. W. Hauswirth, 1999).

Given the efficiency of this rhodopsin-specific ribozyme in vivo and the numerous examples of successful ribozyme utility in animal systems that have been reported in the literature and discussed throughout the body of this thesis (Zhao and Pick, 1993; Feng et al., 1995; Flory et al., 1996; Lieber and Kay, 1996; L’Huillier et al., 1996; Sioud and Sorensen, 1998; Chapters 3, 4 and 5), a construct has been generated for the development of an Rz40 expressing transgenic mouse. Currently the construct contains 3.8kb of the mouse rhodopsin promoter which should promote tissue specific expression of the ribozyme. Notably, this region of the rhodopsin promoter has been used in the Unit to drive photoreceptor-specific expression of a green fluorescent protein reporter gene (carried in an AAV construct) in mice. Moreover, the Ocular Genetics Unit has a number of appropriate rhodopsin-linked transgenic animals on site to directly assess the activity of any one of the mutation-independent rhodopsin ribozymes discussed above. In particular, a P23H transgenic adRP mouse model carrying both an endogenous mouse rhodopsin gene and a human P23H mutant gene is available (Olsson et al., 1992) and will be initially bred with the Rz40 transgenic animal once generated. If subsequent histopathological, immunocytochemical and ERG analysis (procedures routinely performed in the unit) reveals a rescue of the disease pathology, it will show that Rz40 is active in vivo and has specifically down-regulated expression of the P23H mutant gene leaving the mouse rhodopsin gene intact and functional. Moreover, an alternative adRP mouse model carrying both wild-type and P23H transgenes on a homozygous murine rhodopsin knockout background will be bred with the Rz40 animal and is currently being generated in the unit as follows: wild-type human rhodopsin transgenic mice (Olsson et al., 1992) were first mated with homozygous rhodopsin knockout mice (Humphries et al., 1997). Mice heterozygous for mouse rhodopsin with the human rhodopsin transgene (human Rho^+"\)
were subsequently mated with each other and homozygous murine rhodopsin knockouts containing human rhodopsin were analysed for an amelioration of disease symptoms. Interestingly, the human rhodopsin protein was shown to be capable of substituting for mouse rhodopsin and rescued the disease phenotype thus highlighting the conservation of the rhodopsin protein throughout the evolution of two distant species (McNally et al., 1999). These mice were then bred with the P23H animal discussed above. Mice heterozygous for mouse rhodopsin containing human P23H and wild-type transgenes have recently been mated. Homozygous rhodopsin knockout mice with P23H and human rhodopsin transgenes will be selected and should be available within the next month. These mice will have the equivalent of human autosomal dominant retinitis pigmentosa and thus will be extremely useful for examining the feasibility of the mutation-independent strategies explored in this thesis. Given the flexibility the wobble-base therapeutic approach described in this thesis, the Rz40 animal could also be potentially bred with any number of the rhodopsin-linked adRP transgenic animals currently available such as the Val20Gly, Pro27Leu (Naash et al., 1993), Gln344ter (Sung et al., 1994), Pro296Glu (Li et al., 1995) and Pro347Ser (Li et al., 1996) models. Indeed, these studies would highlight the broad applicability and therapeutic potential of mutation-independent approaches for combating mutational heterogeneity associated with rhodopsin-linked adRP.
Figure 5.1

(A) Presented is the pcDNA3.1(+) mammalian expression vector (Invitrogen). The restriction sites in the multiple cloning site, the CMV promoter and the BGH poly(A) sequence and neomycin phosphotransferase gene for eukaryotic cell studies are shown. (B) 0.28 - 6.58kb methylene blue stained RNA ladder used to determine transcript size on Northern blots. Fragment sizes are indicated with arrows.
Figure 5.1b
Figure 5.2

(A) and (B) RT-PCRs from human rhodopsin COS-7 cell lines with human rhodopsin and G3PDH primers. (A) Lanes 1 and 14 = Msp1 cut pUC19 ladder; lane 2 = RT-PCR using non-transfected COS-7 cell RNA with human rhodopsin primers; lane 3 = RT(-) control using RNA obtained from stable COS-7 cell line G with rhodopsin primers; lanes 4-13 = RT-PCR amplifications from human rhodopsin COS-7 cell lines B, C, D, E and F with human rhodopsin primers & G3PDH primers for each line respectively. (B) Lanes 1 and 12 = Msp1 cut pUC19 ladder; lanes 2-11 = RT-PCRs from human rhodopsin lines G, H, I, K and L with human rhodopsin primers & G3PDH primers for each cell line respectively. From top to bottom, arrows indicate the 452 base G3PDH product and the 210 base human rhodopsin fragment. However, it should be noted that in order to completely discount genomic contamination in RT-PCRs from each cell line examined, RT(-) controls should have been included for each specific cell sample. (C) and (D)

Northern blots of stable COS-7 human rhodopsin and peripherin cell lines. (C) Lanes 1-5 = poly(A) RNA extracted from human rhodopsin lines B, C, D, E, and F respectively; lanes 6 and 12 = untransfected COS-7 cell line control; lanes 7-11 = poly(A) RNA extracted from rhodopsin lines G, H, I, K and L respectively. All lines were probed with an 850 bp rhodopsin-specific cDNA fragment. An arrow indicates the ~ 2.2kb rhodopsin mRNA. (D) Lanes 1 and 2 = untransfected COS-7 RNA controls; lanes 3-12 = poly(A) RNA extracted from human peripherin lines A, B, E, F and H respectively. All samples were loaded in duplicate beside each other. Fragments of the correct size (~ 3.0kb) can be seen in lanes 3-8 and 11-12 and are indicated with an arrow. Notably, the ~ 3.0kb transcript is absent in the control samples (lanes 1-2). (E) Human peripherin cell lines A, B, E, F and H probed with β-actin respectively. Lanes 1-12 = same loading order as the peripherin probed blot above. An arrow indicates the ~ 2.0kb β-actin transcript.
Figure 5.3

(A)-(D) Optimisation of the transient transfection protocol for stable rhodopsin COS-7 cell lines using LipofectAMINE PLUS reagent. 2.5X10⁶ COS-7 cells were plated the day before transfection in 35mm wells. Cells were transfected serum-free with pZeoSVLacZ DNA and LipofectAMINE reagent for 5 hours; medium was then replaced with complete DMEM and the cells stained 24 hours post-transfection. An asterix highlights the most efficient transfection condition for each experiment. (A) 1-8 = cells transfected with 1μg DNA, 6μl PLUS reagent and 2, 4, 6, 8, 10, 12, 14 and 16μl LipofectAMINE respectively. (B) 1-6 = cells transfected with 1μg DNA, 8μl LipofectAMINE and 4, 5, 6, 7, 8 and 10μl PLUS reagent respectively. (C) 1-6 = cells transfected with 8μl LipofectAMINE, 7μl PLUS reagent and 500ng, 1.0, 1.5, 2.0, 3.0 and 5.0μg DNA respectively. (D) D1 = transfection of stable COS-7 cells under pre-determined optimised conditions - 8μl LipofectAMINE, 7μl PLUS reagent and 5μg DNA; D2 = transfection of COS-7 cells using an optimised protocol obtained from Lifetechnologies; D3 = ‘Mock’ transfection without DNA; D4 = transfection of COS-7 cells with the cationic lipid based GenePORTER transfection reagent (Gene Therapy Systems, Appligene-Oncore-Lifescreen, Hertfordshire, U.K.).
Figure 5.3a
Figure 5.4

Northern blot analyses of stable rhodopsin cell lines G, I and L following transfection with mutation-independent ribozymes, Rz10, Rz40 and RzMM. (A) Lines G, I and L probed with a *Hind*III to *Bst*EII rhodopsin-specific fragment. Lane 1 = negative wild-type COS-7 RNA control; lanes 2-5 = rhodopsin line G transfected by GenePorter with Rz30 control, Rz10, Rz40 and RzMM respectively; lanes 6-9 = line G transfected by LipofectAMINE with Rz30, Rz10, Rz40 and RzMM respectively; lane 10-11 = rhodopsin line I transfected by LipofectAMINE with Rz30 control and Rz40 respectively; lanes 12-13 = rhodopsin line L transfected by LipofectAMINE with Rz30 control and RzMM.

(B) Lines G, I and L probed with β-actin fragment. Lane order is the same as above.

(C) Graphical representation of rhodopsin: β-actin mRNA ratios as determined by instant image analysis.
Comparison of ribozyme activity in human rhodopsin cell lines

Figure 5.4
Figure 5.5

(A) Experiment to investigate the accuracy of instant image analysis. Lanes 1-5 = Northern blot of 200, 400, 600, 800 and 1000ng line G poly(A) RNA probed for \( \beta \)-actin expression. An arrow indicates the \( \sim 2.0 \)kb \( \beta \)-actin transcript. (B) Northern analysis of stable rhodopsin cell lines I and L transfected with a control ribozyme, Rz30, and rhodopsin-specific ribozymes, Rz10, Rz40 and RzMM. Lanes 1-8 = rhodopsin probed line L transfected with Rz30, Rz10, Rz40 and RzMM respectively; lanes 9-16 = rhodopsin probed line I transfected with Rz30, Rz10, Rz40 and RzMM respectively. Transfected lines L and I mRNA samples were loaded in duplicate beside one another (i.e. Rz30, Rz30, Rz10 etc.). Arrows from top to bottom indicate two transcripts of \( \sim 2.2 \) and \( 1.6 \)kb respectively. (C) Rz30, Rz10, Rz40 and RzMM transfected rhodopsin lines L and I probed for \( \beta \)-actin expression as an internal loading control. The lane order is the same as the rhodopsin probed blot above. An arrow indicates the \( \sim 2.0 \)kb \( \beta \)-actin transcript. (D) Graphical representation of rhodopsin: \( \beta \)-actin mRNA ratios for ribozyme transfected rhodopsin lines L and I ascertained by instant image analysis.
Figure 5.5a-c
Comparison of ribozyme activity in human rhodopsin cell lines

Figure 5.5d
Figure 5.6

Primer extension assay used to determine ribozyme-mediated cleavage of human rhodopsin mRNA. Lanes 1 and 14 = φX174 HinfI DNA ladder; lane 2 = positive Kanamycin control; lane 3 = negative ‘no RNA’ Kanamycin control; lanes 4-6 = ‘no RNA’ controls with rhodopsin primers OP1, MP1 and CPI respectively; lanes 7-10 = primer extension assays using MP1 primer with Rz30, Rz10, Rz40 and RzMM transfected line L RNA samples respectively; GATC = human rhodopsin dideoxy sequencing reactions using MP1 primer; lanes 11-13 = MP1 extended line L samples transfected by Rz30, Rz40 and RzMM respectively. An arrow indicates the extended 87 base RNA Kanamycin control. Similar reactions were performed with CPI and OP1 primers.
Figure 5.6
Figure 5.7

Northern blot analysis of stable human rhodopsin COS-7 cell lines transfected with Rz30, Rz10, Rz40 and RzMM. (A) Line G RNA samples hybridized with a rhodopsin specific cDNA probe. Lanes 1-4 = 200ng, 400ng, 600ng and 800ng of untransfected line G poly(A) RNA; lanes 5-12 = 500ng poly(A) RNA obtained from line G transfected with the Rz30 control, Rz10, Rz40 and RzMM respectively. Samples were loaded in duplicate beside one another (Rz30, Rz30 etc.). It should be noted that only 390ng of RzMM RNA was blotted. Arrows from top to bottom indicate the ~ 2.2 and 1.6kb rhodopsin transcripts respectively (B) Line G RNA samples hybridised with a β-actin specific probe. The lane order is the same as for the rhodopsin probed blot above. (C) Lines I and L RNA samples probed for rhodopsin expression. Lane 1 = 500ng poly(A) RNA extracted from untransfected line I; lanes 2-9 = 500ng poly(A) RNA from line I transfected with Rz30, Rz10, Rz40 and RzMM respectively. Again samples were loaded in duplicate; lanes 10-11 = 500ng poly(A) RNA extracted from untransfected line L; lanes 12-19 = duplicate 500ng poly(A) RNA samples from line L transfected with Rz30, Rz10, Rz40 and RzMM respectively. In lane 19, only 300ng of RNA was loaded. Arrows from top to bottom indicate the ~ 2.2 and 1.6kb rhodopsin transcripts respectively (D) Line I and L RNA samples probed for β-actin expression. The lane order is the same as in 5.7c. An arrow highlights the ~ 2.0kb β-actin transcript. (E) Graphical representation of the rhodopsin: β-actin mRNA ratios for ribozyme transfected stable cell lines I and L as determined by instant image analysis.
Figure 5.7c-d
Comparison of ribozyme activity in human rhodopsin cell lines

![Bar chart showing ribozyme activity](image)

**Figure 5.7e**
Figure 5.8

Northern blot of poly(A) RNA extracted from stable rhodopsin cell line L transfected with varying concentrations of LipofectAMINE, PLUS reagent and DNA. Lane 1 = untransfected line L RNA; lane 2 = cells transfected with 30μl PLUS reagent only; lanes 3-6 = cells transfected with 10, 15, 20 and 30μl LipofectAMINE, 30μl PLUS reagent and without DNA respectively; lanes 7-8 = cells transfected with 42μl LipofectAMINE, 30μl PLUS reagent both with and without 30μg of Rz40 DNA respectively; lane 9 = cells transfected with 50μl LipofectAMINE and 30μl PLUS reagent without DNA. Arrows from top to bottom indicate the ~ 2.2 and 1.6kb rhodopsin transcripts respectively (B) Line L samples probed for β-actin expression. The lane order is the same as above. An arrow indicates the ~ 2.0kb β-actin mRNA.
Figure 5.8
Figure 5.9

(A) Timepoint cleavage reaction of human rhodopsin RNA and 2'-modified hammerhead ribozyme, Rz40. Lanes 1-8 = human rhodopsin RNA subsequent to incubation with Rz40 and 10mM MgCl₂ for 0, 30 seconds, 5, 10, 15, 20, 25 and 30 minutes respectively. Reactions were performed at 37°C. An arrow indicates the intact human rhodopsin RNA. This experiment was repeated several times and compared with a reaction in which an unmodified Rz40 was used. However, the modified ribozyme consistently proved inactive.

(B) Restriction enzyme analysis of pZeoSVRz40 mini-preparations with KpnI. Lanes 1 and 13 = 1kb DNA ladder; lane 2 = KpnI cut pZeoSV plasmid control; lanes 3-12 = pZeoSVRz40 mini-preparations 1-10 digested with KpnI respectively. As is evident, positive clones were not digested by the enzyme.

(C) Restriction enzyme analysis of pcDNA3.1(-) Rztrans mini-preparations with HindIII. Lanes 1 and 18 = 1kb DNA ladder; lanes 2-16 = mini-preparations 1-15 digested with HindIII respectively; lane 17 = pcDNA3.1(-)/HindIII control. An arrow indicates the 1.9kb insert liberated in positive clones.
Figure 5.9
CHAPTER 6

Conclusion
CONCLUSION

Over the last several years extensive research into degenerative eye disorders, such as Retinitis Pigmentosa (RP), has resulted in a substantial elucidation of the molecular aetiologies of these debilitating conditions. A remarkable feature of many inherited disorders, including RP, is the high-level of genetic heterogeneity inherent in them. Mutations in a range of genes have been implicated in causing human retinopathies. Some of the more frequent causes of autosomal dominant forms of RP (adRP) are mutations in the genes encoding the photoreceptor proteins rhodopsin and peripherin; indeed over 150 mutations have been identified to date in these genes, clearly suggesting a need for mutation-independent approaches for therapy. Therapies for dominant disorders such as adRP could be directed to the primary genetic defect by eliminating or reducing the presence of the mutant protein while maintaining expression of the wild-type protein. Alternatively, therapies could be directed to modulating secondary effects that contribute to disease pathology, for example, by targeting mechanisms of photoreceptor apoptosis (programmed cell death) or administering neurotrophic factors to retinal tissues. The focus of this Ph.D has been the former approach, that is, targeting the primary defect in both a mutation-specific and mutation-independent manner.

The sequence specificity and catalytic activity of hammerhead ribozymes made them attractive as potential therapeutic agents for down-regulating expression of retinal transcripts associated with adRP. To date, ribozyme-based gene therapies for dominant diseases have utilised mutations that fortuitously create NUX motifs present in accessible areas of target transcripts. However, the likelihood of such an event arising is remarkably low given the occurrence of suitable open-loop structures. Moreover, the immense intragenic heterogeneity associated with many dominant diseases, such as rhodopsin- and peripherin-linked adRP, will most likely make mutation-specific therapies impractical. To circumvent these difficulties, ribozyme-based suppression strategies that are independent of the disease mutation have been developed, which in essence involve suppression of wild-type and mutant alleles of a gene using sequences in transcribed but untranslated (UTRs) regions or at degenerate (wobble) sites and concurrent introduction of replacement genes with modified UTRs or degenerative sites which thereby escape suppression but code for wild-type protein (Millington-Ward et al., 1997; Chapter 3). To examine the feasibility of these ribozyme-based mutation independent therapeutic strategies for adRP and indeed highlight the benefits of such technology over for example mutation-specific
strategies, one mutation-specific hammerhead ribozyme targeting a Gly51Val rhodopsin mutation (Rz4447) and twelve mutation-independent hammerhead ribozymes designed to target accessible cleavage sites in 5'-UTR sequence or at degenerative sites in rhodopsin and peripherin transcripts (human and mouse) have been designed, generated and extensively analysed (Chapters 3, 4 and 5). Moreover, a connected-type multimeric ribozyme (RzMM) consisting of four hammerhead ribozymes linked in tandem, each targeting a degenerative site in human rhodopsin mRNA was developed (Chapter 4). All ribozymes cleaved their respective target mRNAs into products of predicted size in vitro. Moreover, replacement retinal transcripts with modified UTR sequence or wobble bases (generated by primer-directed PCR mutagenesis) were shown to escape ribozyme-mediated cleavage in vitro (Chapter 3). Preliminary characterisation of ribozyme activity in vitro revealed substantial differences in ribozyme cleavage rates.

As the ultimate goal of this study was to identify extremely active retinal-specific ribozymes that could function efficiently in vivo, the mutation-independent ribozymes that proved most active in preliminary in vitro studies were kinetically analysed. Indeed, kinetic evaluation of ribozyme activity in vitro can be used as a broad prediction of potential ribozyme activity in vivo. Notably, many of the kinetic values determined for these ribozymes which target highly structured RNAs (545-861 nucleotides) were in the same range as those observed for many ribozymes directed to short unstructured RNAs (20-40 nucleotides) reported in the literature. For example, Rz10 targeting an 861 nucleotide human rhodopsin RNA achieved a $V_{max}$ of 0.71 min$^{-1}$ (O’Neill et al., submitted IOVS, 1999; Chapter 4). Indeed, kinetic studies demonstrated that four ribozymes in particular, Rz9 (targeting 5'-UTR of human peripherin), Rz10, Rz40 and RzMM (each targeting degenerative sites in human rhodopsin) were extremely active in vitro and were initially prioritised for evaluation in vivo. To facilitate in vivo experimentation, stable rhodopsin and peripherin expressing COS-7 cell lines were generated. Following determination of the most optimal COS-7 transfection protocol with the cationic lipid, LipofectAMINE PLUS, three rhodopsin expressing lines (G, I and L) were transfected with Rz10, Rz40 and RzMM. Analysis of rhodopsin mRNA levels by Northern blotting following transfection with ribozymes showed up to 30% reduction in rhodopsin expression compared to control transfections with a peripherin-specific ribozyme (Chapter 5). The activity of Rz40 in particular in vitro and the results obtained in cell culture offered promise for the utility of this ribozyme in animal systems. Moreover, as Rz40 was more active in vitro than another
rhodopsin-specific ribozyme reported in the literature which when examined in a rat model of adRP partially rescued the disease pathology (Drenser et al., 1998; Lewin et al., 1998) an Rz40 construct suitable for the generation of a transgenic mouse was built. However, recent evaluation of the P23H transgenic rats expressing the rhodopsin-specific ribozyme described by Lewin et al. (1998) has shown that inactive ribozyme controls also partially rescue the disease pathology due to antisense suppression caused by the arms of the ribozyme itself (personal communication, W. Hauswirth - Tubingen, Germany). This antisense effect is particularly relevant for ribozyme therapies based on targeting specific mutations associated with dominant diseases, such as adRP, as the ribozymes antisense arms may (as above) suppress expression of both the mutant and wild-type alleles thereby necessitating the introduction of a replacement transcript to prevent disease pathology. However, it is of note that this problem would not arise with the mutation-independent suppression and replacement strategies explored in this thesis as the flexibility of these approaches allows degenerative bases and UTR sequence in the region of ribozyme binding in the replacement transcript to be altered such that the replacement RNA is masked from ribozyme-mediated antisense suppression. Moreover, it is also of interest that the ribozymes generated by Lein et al. were directed to artificially engineered ribozyme cleavage sites in rhodopsin mRNA and thus provide no therapeutic benefit for patients suffering from autosomal dominantly inherited RP. In contrast, the mutation-independent ribozymes analysed in this thesis represent the first therapeutically relevant molecules for combating adRP to be reported in the literature (Millington-Ward et al., 1997).

Indeed, a large number of transgenic mouse models with rhodopsin-related retinal degenerations are readily available for cross breeding with an Rz40 transgenic mouse. For example, the Ocular Genetics Unit currently have onsite a human P23H mouse model of adRP which manifests a severe early onset retinal degeneration. These animals carry a mutant human rhodopsin gene and a wild-type mouse rhodopsin gene. Mating of the P23H animal with the Rz40 animal would enable direct examination of the therapeutic potential of this ribozyme in combating adRP. If subsequent histopathological, immunocytochemical and ERG analysis (techniques routinely performed in the unit) revealed a rescue of disease pathology, it would demonstrate that the ribozyme was active and had selectively reduced expression of the P23H gene, leaving endogenous mouse rhodopsin intact. However, an animal model even more appropriate than the P23H animal for rhodopsin specific ribozyme studies is currently being generated by a member of the
Ocular Genetics Unit. This animal will contain both normal and mutant human rhodopsin genes on a mouse rhodopsin knockout background thus having the direct equivalent of the autosomal dominant human disease. Indeed, if Rz40 proved extremely efficient in the animals discussed above and if sufficient financial resources were available the Rz40 animal could be bred with any number of the many transgenic murine adRP models available such as the Val20Gly, Pro27Leu, Gln344ter, Pro296Glu and Pro347Ser mice. These studies would highlight the broad range applicability of mutation-independent ribozyme-based therapeutic strategies for combating the mutational heterogeneity associated with adRP i.e. one therapeutic agent is, in principle, suitable for all dominant-negative adRP mutations.

However, transgenic ribozyme technology has its limitations in that it is not directly applicable to human therapy. Towards this end, efficient viral or non-viral delivery systems will be required. Ribozymes can be delivered as either pre-formed molecules or as components of suitable vectors allowing the production of ribozyme transcript intracellularly. In the former situation, ribozyme RNA will first need to be protected from intracellular degradation by chemical modification as unprotected RNA would typically be degraded within minutes once administered. Indeed, chemical modification techniques for enhancing the stability of Rz40 in vivo were investigated in this thesis (Millington-Ward et al., 1999; Chapter 4). The uniform 2'-amino uridine modifications incorporated into the Rz40 structure completely abolished the ribozymes activity in vitro and therefore studies were discontinued. However, other forms of protection such as site-specific 2'-amino uridine modifications of Rz40 or uniform 2' modified fluoro, allyl or O-methyl derivatives alone or in combination with terminal phosphorothioate linkages or an inverted nucleotide will be investigated in future experimentation. This technology in combination with advancements in non-viral delivery systems such as artificial liposomes or polymers represent exciting possibilities for enhancing ribozyme efficiency in vivo. Moreover, a novel gene delivery system called iontophoresis (similar to electroporation) which involves applying an electrical current to the eye and the delivery of chemically protected ribozymes to retinal tissue is currently being investigated in collaboration with a research team in Paris (with Dr. Yves Courtois). Alternatively, viral delivery systems such as Adeno-, adeno-associated and lentiviruses which either remain extrachromosomally or integrate into the host genome could potentially be employed to deliver ribozymes to ocular tissue. Given the importance of gene delivery for the development of viable future gene-based
therapies, substantial efforts are currently being invested by vectorologists throughout the world to developing efficient viral delivery systems that generate minimal immune responses from the host. While each system has both advantages and disadvantages, given the intensity of current investigations, it is very likely that highly efficient delivery systems will be developed in the near future.

For any gene therapy, as with drug development, there are strict protocols and regulations which need by law to be adhered to so as to ensure safety of the product. Routinely, research begins in vitro, progresses to cell culture and rodent models and from here to larger animal models, primates and eventually human trials. Clinical trials of experimental drugs or treatments proceed through four phases. In phase I trials, the safety, dosage range and side effects of the treatment is evaluated using a small group of people, typically 20-80. In Phase II trials, the treatment is administered to a larger group of people (100-300) to further evaluate efficiency and safety. Phase III studies are performed using 1000-3000 people in order to confirm the effectiveness of the therapy, to monitor any side effects, compare it to other treatments and analyse the safest route for its implementation. If the therapy progresses through all three phases, it is eventually marketed as a viable product. Phase IV trials proceed when the therapy is on the market and monitor its effect in various populations and any side-effects associated with long-term use. The entire procedure from concept to product takes on average ≥10 years and costs approximately 100-500 million dollars per drug / therapy. Thus, given the capital required it is not surprising that the vast majority of drugs currently on the market have powerful multinational companies backing the research. Therefore, the driving force behind most scientific research changes from pure academic interest to the money-making ability of the particular product. While many ideas are formulated in academic environments, the funding required to develop the idea is in most cases not available. As a result, laboratories in universities require investment initially by venture capitalists who generally make small investments and hope that in the long-term the product will be marketed generating large returns. However, to entice such investment the laboratory / small company developing an idea need strong patent portfolios to protect their idea from ‘poaching’ by other organisations. If an important concept / drug with relevant patent protection in place cannot be developed without a multi-million pound investment (e.g. clinical trials), large multinational companies may, if the concept has extensive market potential, provide the capital required. In other words, once the ground work has been done and the concept looks promising, powerful companies may provide the
capital required to advance the project to clinical trials and beyond. Notably, even though
the investment may at the time appear significant, the returns could potentially be
enormous. Thus, it is apparent from above that intellectual property is becoming a
requirement for the successful development of any drug / therapy. Indeed, it is of note that
a start-up company, Optigen Technologies has been set up around three patents that have
arisen from this Ph.D, a Ph.D performed by a colleague, Sophia Millington-Ward and the
work of the Unit. These patents are based on the concept of mutation-independent gene
silencing and replacement for combating the mutational heterogeneity associated with
autosomal dominant disease - that is the novel use of UTR regions, naturally occurring
polymorphism and degenerative bases in genes. It is evident from the discussion that
mutation-specific strategies for autosomal dominant diseases are unrealistic and that for
therapies to become a reality, methods of therapeutic intervention which are independent of
the disease mutation are required. Even though hammerhead ribozymes have been
employed as therapeutic agents in this Ph.D, other gene suppressors which may or may not
have been discovered to date, could potentially prove more effective and be used as part of
the mutation-independent strategies discussed. Other mutation-independent approaches
such as targeting apoptotic genes, use of neurotrophic factors or retinal transplantation
techniques may also prove viable. However, it is likely that further development of
methods to amend the primary defect or modulating secondary effects associated with the
disease process will most likely unlock the secret to combating autosomal dominant
diseases such as adRP. Given the platform of technologies now available, it is our hope and
indeed our belief that the era of molecular remedies is slowly but surely becoming a
reality.
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