EVALUATION OF DNA ENZYMES TARGETED AGAINST THE RNA COMPONENT OF HUMAN TELOMERASE

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SUMMARY

Glioblastoma Multiforme (GBM) is a highly malignant form of brain cancer for which there is currently no effective cure. Consequently, developing new therapies and elucidating effective targets is crucial for this fatal disease. In recent years, DNA enzymes, deoxyribonucleic acid molecules with enzymatic activity, have emerged. In the same manner as ribozymes, DNA enzymes are able to effect cleavage of RNA in a sequence-specific manner, and operate with catalytic efficiency. In this study, two DNA enzymes were designed to target the template region of human telomerase RNA (hTR), utilising the 10-23 and 8-17 catalytic motifs elucidated by Santoro and Joyce (1997). Telomerase is an RNA-dependent DNA polymerase, which stabilises telomere lengths by adding hexameric repeats (TTAGGG in humans) to chromosome termini, thus preventing the telomere shortening that usually occurs during mitotic cell division. Telomerase activity, whilst absent in normal somatic tissues, is present in almost 90% of all tumours. Thus, there is speculation that telomerase may be the much sought universal target for therapeutic intervention in cancer. *In vitro* cleavage assays showed both DNA enzymes to be catalytically competent. Unmodified phosphodiester (PO) backbone DNA enzymes were rapidly degraded in the presence of serum, with a half-life of 10 minutes. The common approach of introducing phosphorothioate (PS) linkages was used in an effort to overcome this instability. As a result of concurrent activity and stability studies on the DNA enzymes with various numbers of PS linkages, the DNA enzymes with a PO core and PS arms were chosen for use in further cell work The cleavage activity of both was shown to be specific and affected by temperature, pH, $MgCl_2$ concentration and enzyme concentration. Both DNA enzyme motifs reduced telomerase activity in cell lysates, as assessed by the telomerase repeat amplification protocol (TRAP) with an IC_{50} of 100nM. DNA enzymes being polyanionic molecules do not readily cross biological barriers. Cellular association of naked DNA enzyme was inefficient at less than 2%. Cellular delivery of the DNA enzymes was effectively improved using commercial cationic lipid formulations. However, the lipid-mediated delivery of DNA enzymes to U87-MG cells over a 4-hour period did not significantly inhibit cell proliferation compared to controls. This is possibly due to an expected lag period between the inhibition of telomere maintenance and cell death. Therefore, biodegradable polymer microspheres were investigated as a potential delivery option for prolonged and sustained delivery. *In vitro* release profiles showed that after an initial burst, sustained release of DNA enzymes was observed over 35 days. Finally, the efficacy and specificity of the DNA enzymes were demonstrated in a luciferase based reporter assay. Specific inhibition of luciferase expression was displayed at 10nM. Thus DNA enzymes have potential against endogenous cellular targets.

Keywords: nucleic acid enzymes, 10-23, 8-17, hTR, TRAP, luciferase.

"The work of the individual still remains the spark that moves mankind forward"

.....Igor Sigorsky

DEDICATION

For dearest Agha Ji, you inspired me to undertake this PhD and your pride and belief in me gave me the motivation to complete it. I am thankful to you for so much, for encouraging my natural inquisitiveness, teaching me to be more open-minded and giving me an example to try and live up to. You have always been a patient teacher, a special friend and an ever loving father.

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LIST OF ABBREVIATIONS

2'-*O*-Me	2'-*O*-methyl
2'-NH_2	2'-amino
A,G,C,T,U	adenine, guanine, cytidine, thymidine, uridine
ATP	adenosine triphosphate
BBB	blood brain barrier
bp	base pair
BSA	bovine serum albumin
C	degrees Celcius
CHAPS	3'-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate
cpm	counts per minute
ddATP	dideoxy adenosine triphosphate
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DTT	dithiothreitol
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate
DOTAP	N-[1-(2,3-dileoyloxy)propyl)-N,N,N-trimethylammonium methylsulphate
DOTMA	N-[1(-2,3-dioleyloxy)propyl]-N,N,N,-trimethylammonium chloride
ECACC	European collection of animal cell cultures
EDTA	ethylenediamine tetra-acetic acid

EGFR	epidermal growth factor receptor
FBS	foetal bovine serum
Fpmp	1-(2-fluorophenyl)-4-methoxypiperidin-4-yl
g, mg, μg	grams, milligrams, micrograms
GBM	glioblastoma multiforme
HEPES	(N-2-hydroxymethylpiperazine)-N'-2-ethanesulfonic acid
HIV-1	human immunodeficiency virus, type 1
hTR	RNA component of human telomerase
hTERT	catalytic protein subunit of telomerase
i.p.	intra - peritoneal
i.v.	intra – venous
kDa	kilo Daltons
LSC	Liquid scintillation counting
M, mM, μM, nM	molar, millimolar, micromolar, nanomolar
μCi	micro Curies
ml, μl	millilitres, microlitres
MP	methylphosphonate
mRNA	messenger ribonucleic acid
mTR	RNA component of mouse telomerase
Mw	molecular weight
nm	nanometers
OD	optical density
ODN	oligodeoxynucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEI	polyethyleneimine
PKC	protein kinase C
PLA	poly(lactic acid)
PLGA	polylactide-co-glycolide

PMSF	phenylmethylsulphonylamide
pmoles	picomoles
PNA	peptide nucleic acid
PNK	polynucleotide kinase
PO	phosphodiester
PS	phosphorothioate
PVA	polyvinylalcohol
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
RNaseH	ribonuclease H
rpm	revolutions per minute
RT	reverse transcriptase
s.c.	sub - cutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
$t_{1/2}$	half-life
TBE	tris-borate EDTA buffer
TEMED	N,N,N',N'-trimethylethylenediamine
TRAP	telomeric repeat amplification protocol
TRIS	Tris(hydroxymethyl)amino methane
TRF	terminal restriction fragment
u	units
UV	ultra-violet
V	volts
VEGF	vascular endothelial growth factor
v/v, w/v, w/w	volume per volume, weight per volume, weight per weight
w/o, w/o/w	water in oil, water in oil in water

CHAPTER ONE

INTRODUCTION

"Death is a fearful thing"

Claudio, *William Shakespeare*

Since ancient times, man has searched for ways of delaying or even overcoming this fear, death. Cancer cells it seems have succeeded where man has failed. Tumour cells escape the natural controls on proliferation and appear to be immortal. The present treatment of cancer is based on a combination of surgery, radiotherapy and chemotherapy. The complete surgical removal of a tumour is difficult, as even a single cell can re-generate a tumour. Radiotherapy and chemotherapy lack specificity, and thus cause toxic side-effects and further distress.

Hence, the search is on for novel agents for the treatment of this frequently fatal disease. In recent years, antisense therapy has emerged as a promising approach. Antisense therapeutics has come to encompass three different technologies: antigene therapy, antisense oligonucleotides and ribozymes (for review see Curcio *et al.*, 1997). Antisense oligonucleotides are short, synthetic, single-stranded DNA sequences designed as complementary to specific DNA or RNA targets within the cell. Through Watson-Crick base pairing, the oligonucleotide hybridises to the complementary (sense) strand and thus gene expression is inhibited in a highly specific manner.

Nucleic acid enzymes are an alternative of this technology. It was a chance finding that led to the development of ribozymes, a class of oligoribonucleotides with intrinsic RNA cleaving capability. Ribozymes also rely on complementary base pairing for their action. A few years ago, it was found that DNA was also capable of this catalytic activity, and various DNA enzymes have since emerged through *in vitro* selection.

In addition to the development of novel drugs, it is important to pinpoint effective targets for these therapies. Research into telomerase, an enzyme over-expressed in many tumours, indicates this may be a good target for anti-cancer drugs. The discovery of novel agents and identification of targets provides fresh hope in the battle against cancer.

1.1 Glioblastoma multiforme

Malignant gliomas account for the largest number of human malignant brain tumours, and are generally considered incurable (Kondo *et al.,* 1998). In fact, gliomas account for approximately 2.5% of the deaths occurring from cancer each year in the United States (Bruner, 1994). Gliomas are classified according to their degree of malignancy as astrocytomas, anaplastic astrocytomas, or glioblastoma multiforme (Ke *et al.*, 1998). They are severely disabling, producing symptoms of headaches, fits, confusion, personality changes and neurological deficits, which impair the patients' quality of life.

Glioblastoma multiforme (GBM) is the most malignant and frequently occurring subtype, and it accounts for one-third of all primary brain tumours (Wong *et al.*, 1994). GBM tumours are most common in patients who are 50 years of age or older; they do however, occur with some frequency in young children (Bruner, 1994). Clinical features of GBM include a highly invasive nature, an ability to metastasise while avoiding detection by the immune system, and a very high recurrence rate following any method of current treatment.

Despite advances in the diagnosis and treatment of gliomas, the prognosis remains poor, with only 10-15% survival for patients with high-grade gliomas at 30+ months (Brada *et al.*, 1998). Surgery is the mainstay of current treatment. Surgical procedures have been improved due to microsurgical techniques, lasers, and monitoring (Kornblith *et al.*, 1993) but are not curative. Surgical removal will provide temporary relief, but complete eradication is virtually impossible, especially as removal of certain areas of the brain will cause severe detriment to the patient's quality of life.

Malignant gliomas such as GBM show a high resistance to radiotherapy. The doses that can be administered without damaging surrounding tissue are too low to have a tumouricidal effect. Radiotherapy is used post-surgery to limit tumour reoccurrence (Leibel *et al.,* 1994) but it is of modest value till more localised effects can be achieved. Chemotherapy, even as an adjunct to surgery and/or radiotherapy shows only limited effectiveness. The relatively lipid soluble and non-ionised nitrosurea drugs *e.g.*

carmustine have been the most effective drugs to date (Shapiro and Shapiro, 1998). The *in vivo* efficacy of cytotoxic drugs is limited by factors such as the blood brain barrier, resistant tumour cells, and low chemotherapeutic indices. Biodegradable microspheres impregnated with cytotoxic drugs (Brem *et al.*, 1995) could provide an avenue for the successful delivery of chemotherapeutic agents to the brain whilst minimising systemic toxicity.

The development of gliomas is a multistep process involving the accumulation of several genetic events, which involve the activation of oncogenes and loss of tumour suppressor genes (Balesaria *et al.,* 1999). Many detailed cytogenetic studies have been performed in GBM and these reveal common genetic abnormalities amongst patients (Wong e*t al.*, 1994). Losses or mutations on chromosome 10 are the most frequent genetic abnormality observed in high-grade gliomas, and the loss of chromosome 10 has been seen to be an independent prognostic factor in high-grade gliomas (Balesaria *et al.,* 1999). Studies have also shown a number of oncogenes to be amplified in GBM *e.g. c-myc, c-myb* and *c-erbB1* (Wong *et al.,* 1994). The p53 tumour suppressor gene (*TP53*) is the most frequently altered gene in human cancer and is also found mutated in several types of brain tumours (Fulci *et al.,* 1998). The *EGFR* (epidermal growth factor receptor) gene, located on chromosome 7p is amplified in over 40% of human glioblastomas (Sehgal, 1998). It is likely that some of these genes will emerge as targets for the successful gene therapy of GBM.

As traditional therapies are of limited value in GBM, it is essential to explore novel forms of treatment to improve the prognosis of this invariably fatal disease (Kondo *et al.,* 1998). It is widely considered that any cure for GBM will arise from an improved understanding of the molecular genetics of the disease. Therapies that intervene at the level of genetic malfunction are potentially more effective with fewer side effects than traditional drugs. GBM tumours are ideal targets for genetic therapy since metastasis is rare, and they can be precisely monitored. One culprit implicated in cancerous cells overcoming the normal controls on cell growth is the enzyme telomerase. Interest is growing in using telomerase as a target in the development of new therapies for diseases including cancer.

1.2 Telomerase

Four key features of cancer cells distinguish them from their normal counterparts: loss of cell-cycle regulation, loss of control over invasion and metastasis, failure of apoptotic mechanisms and bypass of senescence (Keith *et al.*, 2001). The ultimate goal of cancer therapy is the discovery of a cellular component absolutely required for the growth of cancerous cells in relation to one of these four key features. Such a cellular component would be a universal target for therapeutic intervention. Interest in the enzyme telomerase is growing with speculation that telomerase may be one such target. An understanding of telomerase begins with an understanding of the processes involved in the lifetime of a cell.

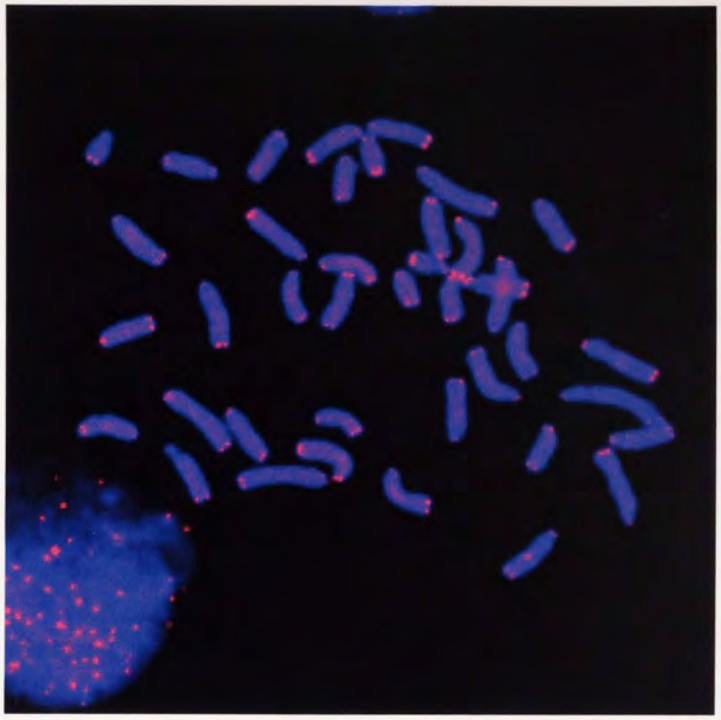
1.2.1 Cellular Senescence and Telomeres

The proliferative lifespan of normal mammalian cells is limited by intrinsic controls. Even in ideal growth conditions, after a given number of cell divisions/population doublings, most cells display a spontaneous decline in growth rates known as the Hayflick limit (Hayflick and Moorhead, 1961), eventually terminating in a quiescent but viable state. Termed cellular senescence (Wynford-Thomas, 1999), there is a gradual decline in the proportion of dividing cells rather than an abrupt arrest of the whole population.

Two major theories have been used to explain limited replicative capacity. The first involves a gradual accumulation of mutations and the second postulates the existence of a molecular clock that counts cell divisions. The latter theory is generally accepted to be correct (Sedivy, 1998). This biological clock appears to depend, at least in part, on telomere length (Campisi, 1997).

The ends of eukaryotic chromosomes are capped with specialised structures known as telomeres (Figure 1.1). Human telomeres, non-coding DNA elements, consist of tandem repeats of the hexameric sequence 5'-TTAGGG-3' (Shay, 1998), with a single

a)



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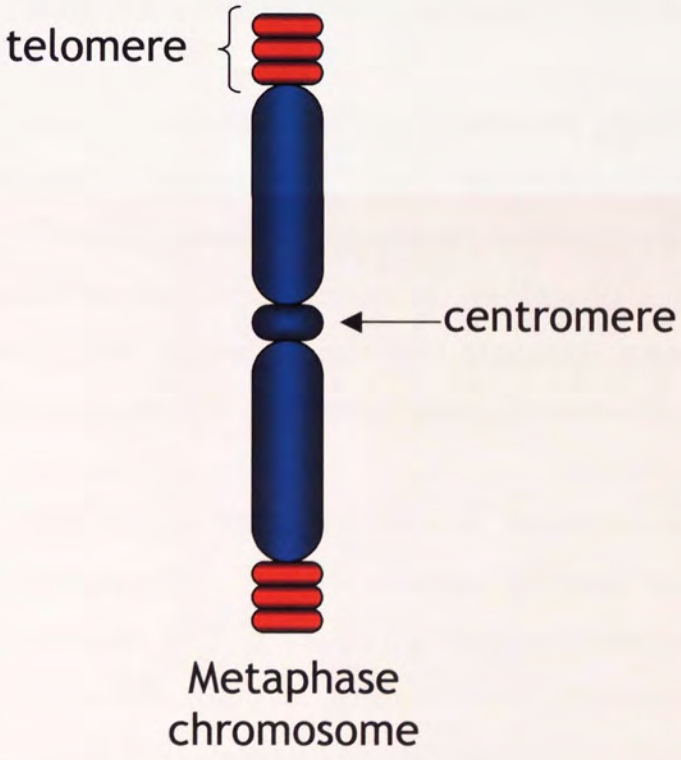


Figure 1.1 Telomeres in human chromosomes.
a) Shows human chromosomes in metaphase stained blue with DAPI and *in situ* hybridised with a fluorescently labelled telomere probe, highlighting the telomeric ends in red.
b) Schematic illustration of a human metaphase chromosome.

stranded 3'-end overhang (Cong *et al.,* 2002). This single-stranded 3'-end overhang is the substrate for telomerase and electron microscopy has revealed that it invades the duplex telomeric DNA repeat array to form a D-loop and T-loop structure *in vitro*. It is thought that this T-loop protects telomeres from degradation or fusion and regulates telomere length (Kim *et al.,* 2002). Telomere binding proteins function to maintain and regulate this unique structure. Several proteins have been identified that associate with mammalian telomeres and most of which regulate one or more aspect of telomere structure or telomere length (described by Kim *et al.,* 2002). Two mammalian proteins, TRF1 and TRF2, bind directly and specifically to double stranded telomeric DNA (Broccoli *et al.,* 1997), and a third protein, POT1, binds specifically to the single stranded 3' overhang (Baumann and Cech, 2001). TRF2 and POT1 appear to be particularly important for stabilizing the telomeric structure and protecting chromosome ends from degradation and fusion (Kim *et al.,* 2002). The other identified proteins have largely indirect interactions with telomeres, mainly by binding TRF1 and TRF2.

The sequence and length of telomeres varies among different species. The telomeric sequence is repeated several thousand times, with human telomeres consising of 5000-15000 base pairs of $\{TTAGGG\}_n$ repeats and telomere binding proteins (McKenzie *et al.,* 1999). Telomeres are non-coding sequences, the functions of which include protecting chromosome ends against exonuclease digestion, preventing abnormal recombination and preventing the loss of essential genes (Sharma *et al.*, 1996).

Due to an inherent flaw in the replication process, chromosomes lose telomeric sequence after each cell division. This phenomenon (depicted in Figure 1.2) first described by James Watson in 1972, is termed the 'end-replication problem' (Greider and Blackburn, 1996). The end-replication problem is well illustrated by McKenzie *et al.* (1999). Briefly, human DNA polymerases only synthesise DNA in the 5' to 3' direction. Thus, the synthesis of each daughter strand occurs continuously on one side of the replication region and discontinuously on the opposite side. In the lagging strand, DNA polymerisation starts from several RNA primers, which are elongated to create DNA fragments termed Okazaki fragments. The RNA primers are then enzymatically removed and replaced with DNA sequences. However, there is no template for the final Okazaki fragments and consequently there is incomplete replication of the lagging

strand. The net result of this incomplete replication is progressive shortening of telomeres at the 3'-end during multiple cell cycles.

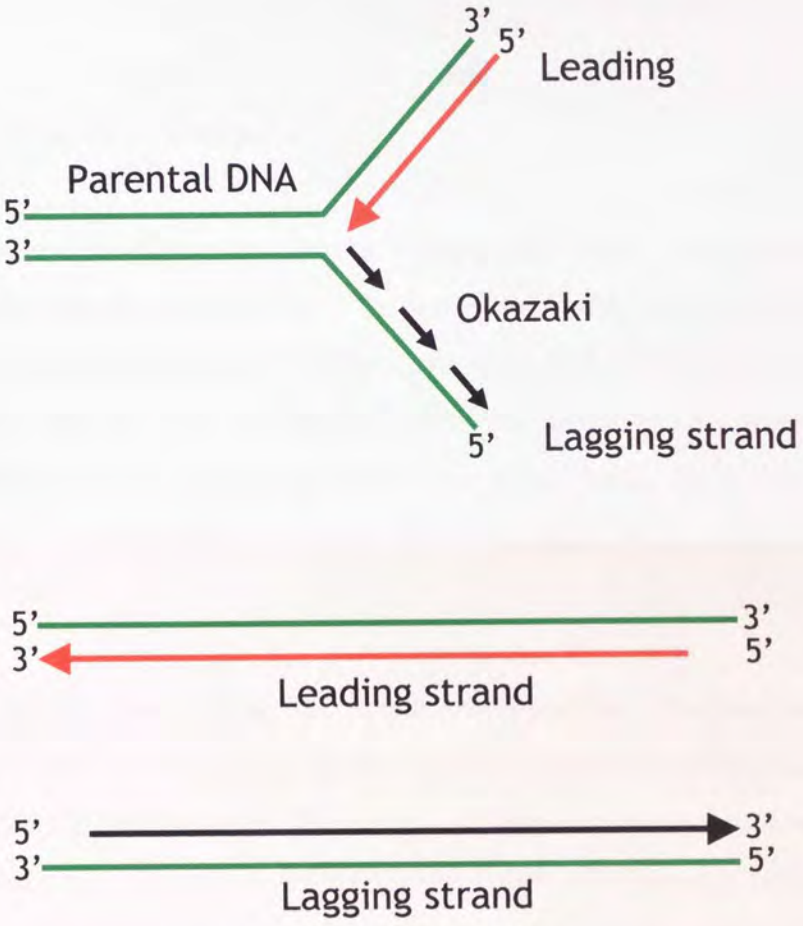


Figure 1.2 The end replication problem.
DNA replication starts by the unwinding of double stranded DNA at the origin of replication. Synthesis of the new strands of DNA proceeds in the 5' to 3' direction. The leading strand is continuous but the lagging strand is synthesised as a series of short segments of DNA (Okazaki fragments) onto the ends of RNA primers. The RNA primers are removed and DNA polymerase fills in the gaps, which are then ligated (not shown). The extreme 5'-end of each daughter strand is not replicated due to polymerase not being able to fill the gaps. This leads to DNA loss and the 'end replication problem'.

It is thought that it is this shortening of telomeres that acts as a mitotic clock, counting cell divisions. After multiple replications, a critically short telomere length is reached which signals the cell to enter senescence (Harley *et al.,* 1992) via a poorly understood mechanism. If cells escape senescence, it results in continued telomere attrition and loss of telomere capping activity, culminating in severe chromosomal instability and massive cell death. This is referred to as 'crisis' and presents an additional barrier to tumour growth (Artandi and DePinho, 2000).

1.2.2 Functions of Telomerase

Telomeric attrition, if it occurred in the human germ line, would have caused the extinction of the human species. Germ cells overcome this progressive shortening by expressing the enzyme telomerase. The basic function of telomerase is to extend the 3'-end of telomeres by *de novo* synthesis of telomeric repeat DNA (Morin, 1997). The hexameric sequences lost during replication are added back, maintaining a dynamic equilibrium, and thus preventing a critical telomere length being reached (Greider and Blackburn, 1996).

The central dogma surrounding the telomere-telomerase involvement in tumour progression has come to be known as the telomere hypothesis (Sharma *et al.*, 1996; Dahse *et al.*, 1997), illustrated in Figure 1.3. This can be summarised as follows: telomerase is quiescent in somatic cells; thus, the length of telomeres declines with each population doubling, resulting in critical shortening. This is recognised, perhaps due to telomeres generating a DNA damage-like signal (Sedivy, 1998), cells are withdrawn from the cell cycle and senesce, entering mortality stage 1 (M1) crisis. If sensing mechanisms are defective, a cell may escape senescence and continue to divide. This results in further telomere loss and instability; with the accumulation of defects, most cells enter a further crisis, M2 stage crisis, and die. A few rare cells survive crisis and reactivate telomerase, which stabilises telomere length, and hence become immortal. Thus there are two barriers- senescence and crisis- that stand in the way of unlimited proliferative potential. In the case of crisis, a close molecular connection can be drawn between telomere shortening, cell death and the avoidance of both by telomerase. On

the other hand, senescence presents a more complex mechanism whose precise connections with telomere shortening remain unclear (explored by Stewart and Weinberg, 2002).

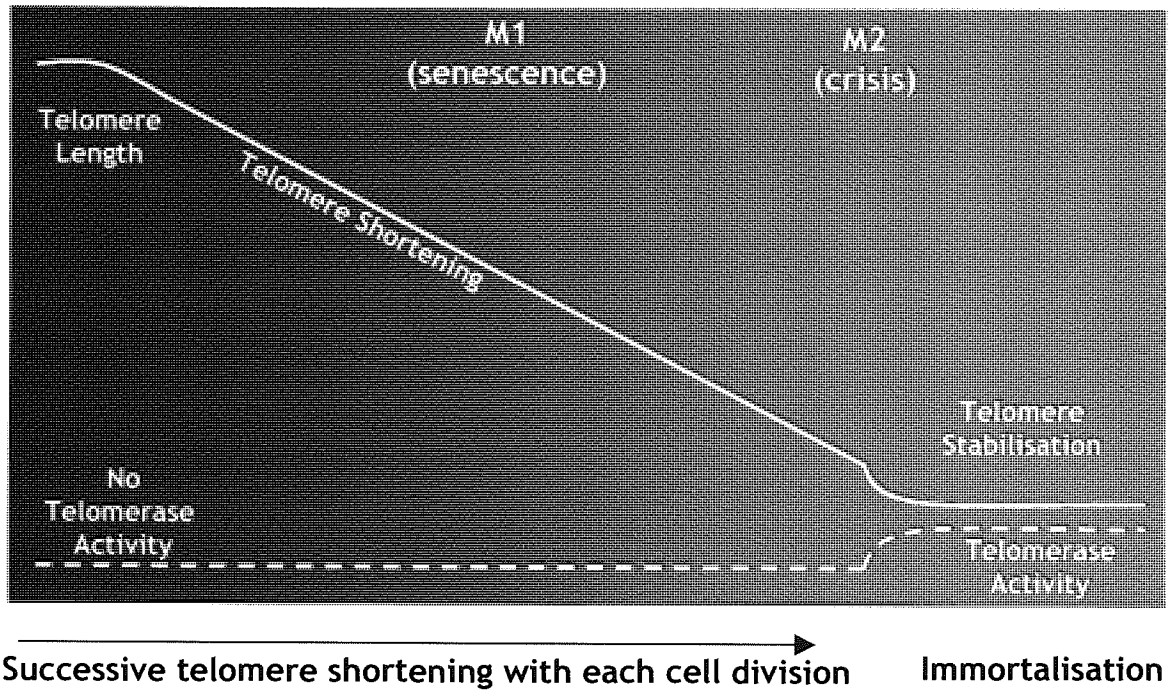


Figure 1.3 The telomere hypothesis.
Telomere length is plotted against the number of cell divisions. Normal somatic cells have progressively shorter telomeres with each cell division. There are two controls on cell division, M1 and M2. Very rarely, a cell will overcome both these proliferation checkpoints, activate telomerase hence stabilising telomere length and become immortal.

To summarise, both telomere dysfunction and telomerase activation are thought to contribute to tumourigenesis. Hackett and Greider (2002) explain that telomere dysfunction probably contributes to relatively early stages of tumorigenesis by inducing genetic instability. Conversely, telomerase activation seems to occur late in tumorigenesis and probably contributes to the growth potential of a tumour once genetic instability has been established.

1.2.3 The Structure of Telomerase

Human telomerase consists of 2 core subunits: the RNA domain (hTR), which contains the template site for replication, and the protein domain (hTERT), which catalyses nucleotide polymerisation (Pitts and Corey, 1999). Various telomerase-associated proteins have now been identified, which may be important in the interaction of the two core subunits (Cong *et al.,* 2002). The human telomerase differs from many other reverse transcriptases in that it remains associated with its template RNA subunit. The telomerase recognises and elongates telomeres through association with the hTR template region and then translocates to the next available position for hTR binding. A schematic representation of telomerase activity is given in Figure 1.4.

1.2.3.1 RNA subunit (hTR) of telomerase

The RNA component of human telomerase was cloned and sequenced by J. Feng and co-workers (Feng *et al.*, 1995). The hTR component is approximately 445 nucleotides long. The template region of hTR encompasses 11 nucleotides 5'-CUAACCCUAAC-3' complementary to the telomeric sequence. This serves as a binding site for telomere ends and as a template for the addition of telomeric repeats. The template region of hTR must be exposed to add new telomeric repeats onto the chromosome, hence making it intrinsically accessible to antisense-based therapies, which can directly inhibit telomerase activity (White *et al.,* 2001). An *in vitro* reconstitution assay has identified a minimal functional region of hTR, 159 nucleotides in length (nt 44-203) (Autexier *et al.,* 1996). Chen *et al.* (2000) established the secondary structure of various vertebrate telomerase, which outlined four highly conserved structural domains; the pseudoknot domain, required for human telomerase function *in vivo*; Box H/ACA domain, likely to be required for proper 3'-end processing and stability of RNA; CR4-CR5 domain and the CR7 domain.

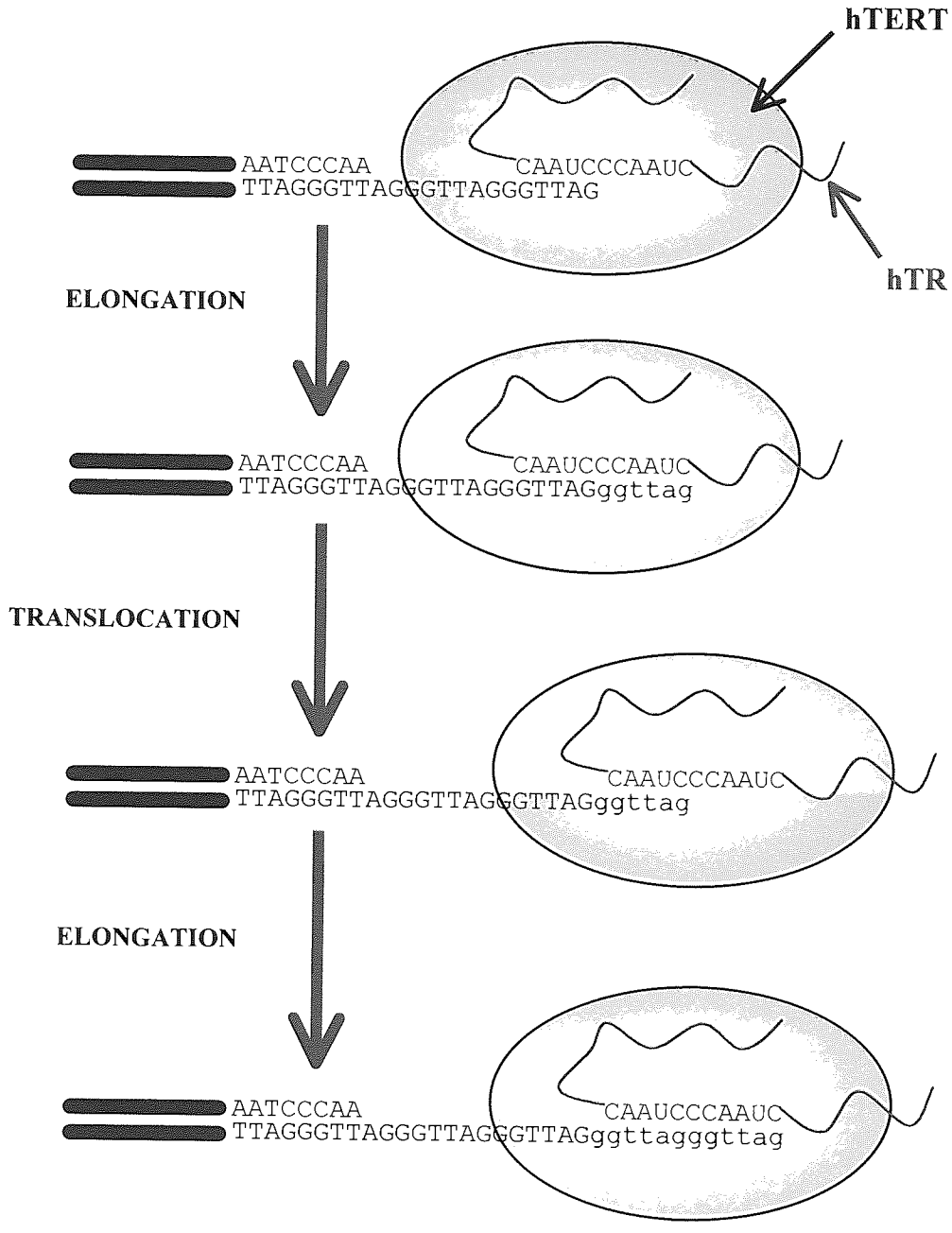


Figure 1.4 Schematic representation of telomerase activity.
Telomerase uses its RNA component as a template to synthesise TTAGGG repeats directly onto telomeric ends preventing telomere shortening. In between the six base pair additions telomerase is thought to pause while it translocates the template RNA for synthesis of the next repeat. This extension of the 3' DNA template end in turn permits additional replication of the 5'-end of the lagging strand, thus compensating for the telomere shortening that occurs in its absence.

1.2.3.2 Catalytic subunit (hTERT) of telomerase

The gene for hTERT has been characterised and the promoter sequenced (Cong *et al.*, 1999; Horikawa *et al.*, 1999). The gene encoding hTERT is located on chromosome 5p15.33 (Bryce *et al.*, 2000) and it consists of 16 exons and 15 introns spanning ~40 kilo-base pairs. The 181-bp promoter region was found to be G-C rich and lacking the typical TATA and CAAT box motifs. It is thought that methylation may be involved in the regulation of hTERT but results are not consistent (Cong *et al.*, 2002). Deletional analysis suggests that the minimum sequence requirement for promoter activity is contained within the 330bp upstream of the ATG *i.e.* the translational start site (Takakura *et al.*, 1999). The promoter region contains binding sites for several transcription factors, suggesting that hTERT expression may be subject to multiple controls. The activity, function, and gene regulation of hTERT is comprehensively reviewed by Poole *et al.* (2001).

1.2.3.3 Telomerase-associated proteins

Both biochemical and genetic studies suggest the existence of additional protein subunits of telomerase that may be involved in the biogenesis or assembly of active telomerase and may mediate or regulate the access of telomerase to its substrates, the telomeres (reviewed in Cong *et al.*, 2002).

1.2.4 Regulation of Telomerase

The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and accessibility and function of the telomerase ribonucleoprotein on telomeres (reviewed in Cong *et al.*, 2002). However, substantial experimental data demonstrate that the transcriptional regulation of hTERT expression represents the primary and rate-limiting step in the activation of telomerase activity in most cells

(Cong *et al.*, 1999; Horikawa *et al.*, 1999; Meyerson *et al.*, 1997; Takakura *et al.*, 1999). The regulation of hTERT is quite complex with a number of transcription factors, tumour suppressors, cell cycle inhibitors, cell-fate determining molecules, hormone receptors and viral proteins implicated in the control of hTERT (reviewed by Ducrest *et al.*, 2002). Examples of some of the most pertinent regulators are discussed below.

The hTERT promoter region was found to contain two binding sites for the myc oncogene (Cong *et al.*, 1999). The myc proto-oncogene encodes a ubiquitous transcription factor c-myc involved in the control of cell proliferation and differentiation (Wu *et al.*, 1999). Studies have shown that c-myc can induce the TERT gene (Wu *et al.*, 1999; Greenberg *et al.*, 1999) and this c-myc-dependent activation of hTERT transcription is likely to be a major mechanism for maintained expression of telomerase (Cerezo *et al.*, 2002). However, the activity of c-myc extends beyond its ability to activate TERT, and multiple signals are likely to act on the TERT promoter and contribute to its activity in cells.

The transcription factor Sp1 is also a key molecule that binds to GC-rich sites on the core promoter and activates hTERT transcription (Kyo *et al.*, 2000), the exact mechanism of which is not known. Cooperative action of c-myc and Sp1 is required for full activation of the hTERT promoter. This cooperation suggests an involvement of other transcription factors in the regulation of hTERT (Kyo and Inoue, 2002).

The tumour suppressor protein p53 inhibits tumour formation by inducing cell cycle arrest or apoptosis in response to a variety of types of cellular damage (Levine, 1997). Studies have provided evidence that p53 is able to inhibit telomerase activity through the transcriptional repression of hTERT (Kanaya *et al.*, 2000; Kusumoto *et al.*, 1999). This repression occurs within hours after induction of p53, before cell cycle arrest or apoptosis takes place. Therefore, the repression of hTERT by p53 is probably independent of these events. Interestingly, repression by p53 requires the transcription factor Sp1 (Xu *et al.*, 2000). Clearly there is a large degree of interaction between activators and repressors for the overall regulation of hTERT.

1.2.5 Telomerase and Cancer

Malignant gliomas are refractory to current forms of treatment (Langford *et al.*, 1995). Therapies targeting telomerase may emerge as novel therapeutic agents for this type of disease. Komata *et al.* (2002) summarise telomerase activity present in various grades of malignant gliomas. Although the ratio of telomerase-positive cells in gliomas varies, most studies show that the incidence of telomerase expression is closely correlated with malignancy, and telomerase is detected in the vast majority of malignant gliomas. Le *et al.* (1998) demonstrated that telomerase activity is absent in normal brain tissues while present in 72% of glioma samples, including 89% of glioblastoma multiforme (GBMs). Also, the incidence of telomerase activity was found to increase with tumour malignancy, suggesting that telomerase may have diagnostic and prognostic value. Indeed, a study of 144 brain tumours (Sano *et al.*, 1998) indicated that positive telomerase activity is strongly associated with malignant tumour types and is rare in benign, non-glial tumours. These mirror previous results obtained by Langford *et al.* (1995). They found 66% of common primary gliomas had detectable telomerase activity. 75% of GBMs contained the enzyme, whereas no telomerase activity was detected in normal brain tissue.

Direct evidence for the involvement of telomerase in oncogenesis is provided by a knockout mouse model lacking the mouse telomerase RNA component (mTR). Telomerase knockout mice exhibit no immediate phenotype, probably due to the long length of their wild-type telomeres. However, by the sixth generation the mice demonstrated evidence of reduced cellular proliferative capacity, with defective spermatogenesis and haematopoiesis (Lee *et al.*, 1998). They also showed relative resistance to multi-stage skin carcinogenesis. Reintroduction of mTR into mTR-deficient mouse cells restored the oncogenic potential of these cells. Herrera *et al.* (1999) later showed that disease states associated with telomerase deficiency appear earlier in mTR-deficient mice with short telomeres.

Feng *et al.* (1995) found that immortal cells with high telomerase activity had 2-7 fold higher concentrations of hTR than telomerase-negative mortal primary cells. Therefore, the presence of hTR may be an indicator of telomerase activity. In another study

(Maitra *et al.*, 1999), hTR expression in childhood neuroblastic tumours (NTs) was correlated with clinical outcome. The authors conclude that hTR expression may be a good indicator of biologic potential and clinical outcome in NTs, but further studies are required to confirm this data.

Telomerase activity and terminal restriction fragment (TRF) length was examined in 170 human brain tumour tissues (Hiraga *et al.*, 1998). Telomerase activity was detected in 61.7 % of tumours, and the mean TRF length was significantly shorter in the telomerase-positive samples. Both these factors varied widely with different grades of tumours. Broccoli *et al.* (1996) studied telomerase activation in mouse mammary glands. Telomerase activity in the tumours was increased 10-20 fold relative to control samples. Telomerase RNA levels were twice as high in the tumour samples. Converse to the results of Hiraga *et al.* (1998), the average telomere array size was not significantly altered during tumorigenesis. This illustrates that the upregulation of telomerase in tumours may have a role other than maintenance of telomere length.

Two similar studies involving the reconstitution of telomerase activity in normal human cells (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998) have added weight to the telomere hypothesis and hence give credence to the use of telomerase as a target for cancer therapy. The results of Vaziri and Benchimol (1998) showed that forced expression of hTERT in normal human cells resulted in telomerase activity, elongation of telomere length, and an extended lifespan of cells. Bodnar *et al.* (1998) obtained similar results after transfection of normal human cells with vectors encoding hTERT. In contrast to telomerase-negative clones, telomerase-expressing clones had elongated telomeres, divided vigorously, and showed a reduction in senescence. The telomerase-expressing clones were phenotypically normal. The authors conclude that their investigations establish a causal relationship between telomere shortening and *in vitro* cellular senescence.

In contrast to the work by Bodnar *et al.* (1998) and Vaziri and Benchimol (1998), a study by Jiang *et al.* (1999) found that forced expression of hTERT *i.e.* reconstitution of telomerase activity, in normal somatic cells did not cause abnormal growth or oncogenic transformation. Similar results were obtained by Ouellette *et al.* (1999), who

found that that hTERT-immortalised cells were normal and lacked cancer-associated changes like those found in most immortal cancer cell lines.

A certain proportion of *in vitro* immortalised cell lines are found to have no detectable telomerase activity, yet have very long and heterogenous telomeres. These cells have presumably acquired one or more novel telomere-lengthening mechanisms, referred to as alternative lengthening of telomeres (ALT) (Bryan and Reddel, 1997). However, ALT is not merely a phenomenon of *in vitro* immortalised cell lines, but has also been found in tumours and tumour-derived cell lines. Various studies have provided evidence for the existence of telomerase-independent mechanisms for cell immortalization, which are involved in the development and/or maintenance of tumours (Yan *et al.*, 1999; Wen *et al.*, 1998; Bryan *et al.*, 1997). Henson *et al.* (2002) provide a comprehensive review of the current knowledge of ALT. Briefly, activation of ALT during immortalisation involves recessive mutations in genes that are as yet unidentified. The exact mechanisms have not been elucidated but telomere length dynamics in ALT cells suggest a recombinatorial mechanism. Significantly, ALT and telomerase activity can co-exist within cultured cells, and within tumours. Thus if ALT were also to be taken into account, the correlation between telomerase activity and cancer may improve. The presence of ALT may pose a challenge to proposals for treating cancer by inhibiting telomerase but combination therapy using ALT and telomerase inhibitors may be a promising avenue to explore.

1.2.6 Inhibition of Telomerase

Initially it was thought that the majority of normal somatic tissues were telomerase negative, whereas it is now clear that sub-populations do sustain a low level of telomerase activity, albeit in insufficient amounts to prevent telomere erosion entirely (Ouellette *et al.*, 1999). The reasons for this expression are poorly understood but it is thought that in normal proliferating tissues, low levels of normally upregulated expression of telomerase could be a tumour suppressive mechanism, protecting cells from telomere erosion and the ensuing genetic instability (Keith *et al.*, 2001). Furthermore, a number of cell lines have been shown to have low levels of telomerase

prior to crisis while telomere shortening is still occurring and the function of these low levels of telomerase activity is unknown (Bryan and Reddel, 1997).

Some normal cells possess functional telomerase activity *e.g.* activated T and B-lymphocytes and germ cells (Dahse *et al.*, 1997). Broccoli *et al.* (1995) found there to be no significant difference in telomerase activity between peripheral leukocytes from normal donors and leukaemia patients. Strong telomerase activity was found in progenitor stem cells and activated lymphocytes both *in vitro* and *in vivo* (Norrback and Roos, 1997), indicating that cells with high growth requirements can readily upregulate telomerase. Hence, inhibition of telomerase could have possible side-effects on the reproductive and immune systems. Another noteworthy fact is that the RNA component of human telomerase, unlike its enzymatic activity is expressed in most somatic tissues. The relevance, if any, of this expression is not known (Sharma *et al.*, 1996).

On the positive side, there now exists compelling cell and animal data for the potential of killing telomerase-positive cancer cells through telomerase inhibition, through therapeutic telomerase vaccines and through suicide genes driven by the telomerase promoter (Harley, 2002).

When contemplating telomerase as a potential target for cancer treatment, it is interesting to note that, in general, telomeres in tumours cells are shorter than those in surrounding normal tissue (Autexier and Greider, 1996). Without telomerase to maintain telomere length in dividing cells, cancer cells should approach critically short telomere length faster than normal cells. Thus, telomerase inhibition has the potential to be selective (Kim, 1997). If the telomere hypothesis holds true and tumour cells are proven to require telomerase function, telomerase inhibitors could be beneficial in the treatment of cancer. However, it is proposed that there would be a lag period of approximately 20 cell divisions before telomerase inhibitors would cause cell death (Herbert *et al.*, 1999). The 11-base template within hTR is intrinsically accessible to binding by telomere ends and this recognition is essential for maintaining telomere length, suggesting that telomerase is an ideal target for inhibition by antisense therapeutics targeting this sequence (Pitts and Corey, 1999).

Agents currently employed in the inhibition of telomerase (reviewed by White *et al.*, 2001; Rowley and Tabler, 2000) can arbitrarily be divided into five groups (Keith *et al.*, 2001). Firstly the telomerase-interactive compounds which destroy RNA or disrupt holoenzyme formation or activity. Examples include ODNs, PNAs, ribozymes, reverse transcriptase inhibitors, isothiazolone derivatives and rhodacyanines. Feng *et al.* (2002) recently investigated the inhibition of telomerase in a gastric cancer cell line, MKN-45, after the introduction of an antisense hTR expression vector. They found this approach to significantly inhibit telomerase activity and proliferation of MKN-45 cells and induce apoptosis.

The second group comprise the telomere-interactive compounds, which disturb the structure of the telomere and block telomere-telomerase interaction. Examples of such compounds are the G quartet interactive agents (such as the 7-deaza nucleotides), amidofluorenone derivatives, cationic porphyrins and amidoanthracene-9,10 diones. Telomeres of human chromosomes contain a G-rich 3' overhang that adopts an intramolecular G-quadruplex structure, which blocks the catalytic reaction of telomerase. Agents that stabilise G-quadruplexes have the potential to interfere with telomere replication by blocking the telomerase elongation step (Sun *et al.*, 1997). A rational approach to drug design led to the development of 3, 6-disubstituted acridine derivatives as telomerase inhibitors (Harrison *et al.*, 1999), which can associate to form guanine quadruplex structures. The authors conclude that these new acridine derivatives are highly active intercalating G-quadruplex stabilisers, with IC_{50} values against telomerase of 1.3-8μM.

Third are the conventional cytotoxics, such as cisplatin, that may target telomerase or the telomeric structure or induce a senescence-like phenotype. Lin *et al.* (2001) used two anti-neoplastic drugs, cisplatin and vincristine, to successfully down-regulate telomerase activity in lymphoma cells.

The fourth group are inhibitors of telomerase signalling pathways, which act by down-regulating hTERT transcription or by inhibiting telomerase function. An example of this group is the protein kinase C (PKC) inhibitors. Ku *et al.*, (1997) found two PKC inhibitors to be effective inhibitors of telomerase activity in cultured nasopharyngeal

carcinoma cells. However, at doses where there was an almost complete inhibition of telomerase activity, treated cells still retained ~75% viability and 20% of protein synthesis. The authors suggest this is due to a selective inhibition of telomerase activity indicating that PKC is involved in the regulation of telomerase activity *in vivo*.

Genetic therapies make up the fifth and final group *i.e.* where therapeutic genes are targeted to cancer cells. For example, one group assessed FADD, the overexpression of which is shown to induce cell apoptosis, gene therapy as a novel tumour-targeting gene therapy (Koga *et al.*, 2001). A plasmid vector expressing FADD gene driven by the hTERT (hTERT/FADD) promoter was constructed, which was investigated for apoptosis induction and whether such apoptosis was restricted to telomerase-positive tumour cells. Transient transfection with the hTERT/FADD construct induced apoptosis in telomerase-positive tumour cells and not in fibroblasts lacking telomerase. Furthermore, the growth of subcutaneous tumours in nude mice was significantly suppressed by the intratumoural injection of the hTERT/FADD construct.

Another idea that is exciting scientists is the development of a universal cancer vaccine, which would theoretically cure cancer by training the patients' immune system to recognise and attack an antigen that is on the surface of tumour cells but not normal cells. Research teams at the Dana Farber Cancer Institute and at the University of California at San Diego have independently identified peptides on the surface of telomerase-positive cells that could be such an antigen. Both research teams propose telomerase could make an ideal vaccine target because it is active in most tumours but silent in normal cells.

At present, the role of telomerase in cancer is the subject of intense research and many debates. Initial work suggests that telomerase may be able to stop aging and cure cancer along with a host of other diseases. Further research is required to confirm the usefulness of telomerase as a target in these diseases and the next few years should clarify just how many promises telomerase is able to keep and whether it is indeed the 'Achilles heel' of cancer.

1.3 Antisense Technology

Antisense technology has now become a real alternative in the treatment of disease states arising from genetic abnormalities such as gene amplification or over-expression. Antisense oligodeoxynucleotides (ODNs) duplexed with the target mRNA can inhibit gene expression by several putative mechanisms including translation arrest mediated by blockade of ribosomal read-through and hydrolysis of mRNA by recruitment of RNaseH. Both antisense ODNs and ribozymes are undergoing clinical trials evaluation for the treatment of a variety of diseases including cancer (Gewirtz, 1999; Holmlund *et al.,* 1999), viral infections (Reding, 2000; Field, 1999) and inflammatory disorders such as Crohns disease (Marcusson *et al.,* 1999). The first antisense ODN drug, Fomivirsen (Vitravene™), developed by Isis Phamaceuticals, has been marketed in the USA and Europe. This ODN is administered by repeated injection into the vitreous of the eye and is used for the treatment of cytomegalovirus retinitis infections in AIDS patients. The Vitravene™ Study Group (2002) has recently published a review of safety data, concluding that intravitreous Fomivirsen is well tolerated with an acceptable safety profile.

1.3.1 Design of Antisense Molecules

The design of antisense ODNs (and ribozymes) at first appears straightforward, since the exact sequence of the target mRNA is known. Using computer programs that predict folding, such as MFOLD (Zuker, 1989), should allow for the identification of hybridisation-accessible sites (*i.e.* single-stranded regions). This approach, however, has proven unsuccessful (Ho *et al.,* 1998) and it is clear that currently available computer algorithms do not accurately predict the complex secondary and tertiary structure of RNA molecules. In the past a strategy of "gene-walking" has been employed, whereby a series of ODNs have been generated against the target mRNA and each one evaluated in cell-culture or *in vivo*. This strategy has been successful in identifying active sequences (Monia *et al.,* 1996) but is far from exhaustive. To obtain a complete picture of RNA accessibility using this method would be very time-consuming

and expensive. More recently two new strategies, RNaseH mapping and scanning combinatorial oligonucleotide arrays, have been adopted both of which have the advantage of being far more exhaustive (for reviews see Sohail and Southern, 2000; Akhtar, 1998).

1.3.2 Stability of Antisense ODNs

Lack of biological stability is a major problem in the use of DNA and RNA ODNs as therapeutic agents. Unmodified phosphodiester (PO) backbone ODNs are rapidly degraded in biological fluids (Wickstrom, 1986; Akhtar, 1991) by a combination of both endo and exonucleases. To overcome this problem, a variety of chemically modified ODNs have been developed that are significantly more stable (reviewed in Kashihara, 1998). The most widely studied of these are the phosphorothioate (PS) ODNs where one of the non-bridging oxygens in the phophodiester backbone is replaced with sulphur. The substitution at phosphorus by sulphur for one of the non-bridging oxygen atoms produces a compound that retains its net charge and aqueous solubility. The substitution also introduces chirality at the phosphorus atom, as each PS linkage can exist as one of two diastereomers, known as Sp and Rp. Eckstein and coworkers (Eckstein, 1985) recognised that different hydrolytic enzymes would accept either the Sp or Rp forms of PS mononucleotides. More recently, other second-generation modifications such as peptide nucleic acids (Hamilton *et al.,* 1999; Shammas *et al.,* 1999), 2'-methoxyethyl (Khatsenko *et al.,* 2000; Knight *et al.,* 2000) and morpholino-based ODNs (Arora *et al.,* 2000; Qin *et al.,* 2000) have also been investigated. These modified chemistries are illustrated in Figure 1.5.

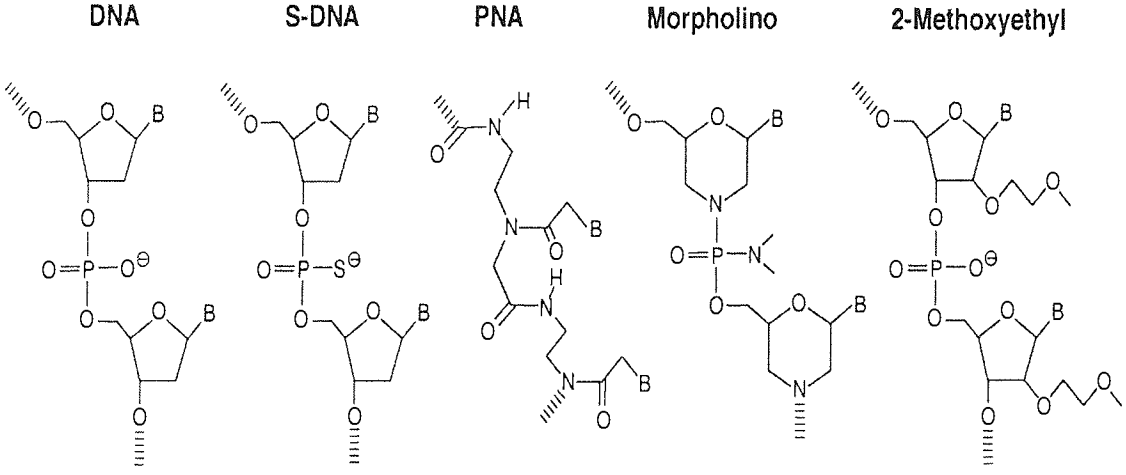


Figure 1.5 Depiction of phosphodiester DNA chemistry and various standard chemical modifications aimed at enhancing stability of ODNs.

1.3.3 Cellular Association of ODNs

Cellular association refers to a combination of both ODN membrane binding and internalisation. In cultured cells, the internalisation of naked ODNs is generally inefficient, with only a small number of ODN molecules actually gaining entry to the cell (Stein and Cheng, 1993). Exogenously administered ODNs enter cells *in vitro* through a combination of fluid-phase (pinocytosis), adsorptive and receptor-mediated endocytosis (reviewed in Akhtar and Juliano, 1992). However, this consequently leads to a trafficking problem in that not all of the internalised ODN will be able to exert its effect by interacting with intended subcellular targets. This is due to the majority of internalised ODN being sequestered into endosome/lysosomal compartments, as characterised by the punctate cytoplasmic distribution and intracellular release of fluorescently labelled ODN following treatment with lysosomatropic agents (Loke *et al.*, 1989). A significant amount of the nucleic acid is also compartmentalised within other cellular organelles such as the golgi complex and the endoplasmic reticulum (Islam *et al.*, 2000). A similar cellular distribution is observed for exogenously administered hammerhead ribozymes (Fell *et al.*, 1997). In both cases little or no fluorescence associated with the ODN is observed "free" in the cytoplasm or nucleus, the sites of action for both ODNs and ribozymes. Microinjection of ODN into the

cytoplasm of cells, bypassing the endocytic pathway, leads to rapid accumulation of the ODN within the nucleus (Leonetti *et al.,* 1991), which suggests that after endosomal exit ODNs are able to migrate to the nucleus. This free mobility of ODNs within the cytosol appears to be a significant advantage of antisense molecules over the sister technology of gene therapy where the cytosol-to-nuclear migration of the larger DNA constructs may be a rate-limiting step to efficacy.

1.3.4 Enhancing ODN Uptake and Delivery

The aims for optimal delivery of ODNs and ribozymes are therefore enhanced cellular uptake, improved exit from sub-cellular compartments and correct targeting (spatial and temporal) to a particular site of action. The latter encompassing the concepts of target matched delivery and pharmacodynamics in which effective concentrations of ODN at the target site need to be maintained for an appropriate length of time. The required time-frame needs to be matched to the biological half-life of the target protein and to the desired level of knockdown. For example, repeated or sustained delivery for a time equivalent to the half-life of target will only result in theoretical maximum effect of 50%. A delivery time period of about 4-half-lives would be required to get greater than 90% inhibition. The requirements for an efficacious nucleic acid based therapy are outlined in Figure 1.6.

Delivery options for nucleic acid therapies are explored below. Most of the *in vitro* delivery systems discussed are equally applicable to antisense ODNs and hence in all probability to DNA enzymes. Thus the requirements for efficacy outlined in Figure 1.6 are likely to be pertinent to both ribozymes and DNA enzymes.

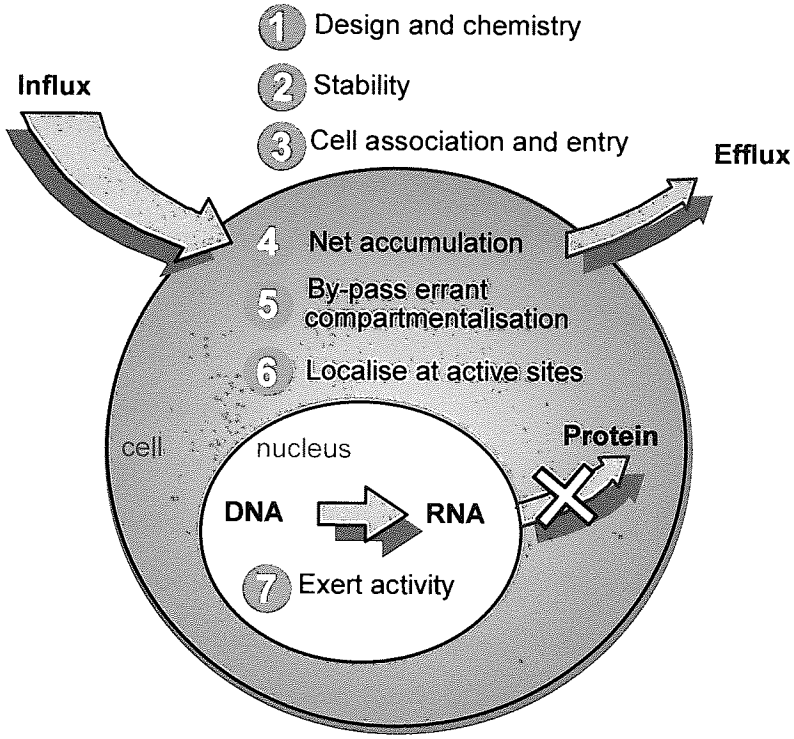


Figure 1.6 Requirements for the cellular delivery and activity of an antisense molecule.

For an oligonucleotide (or nucleic acid enzyme) to be efficacious it has to be:
(1) designed to heteroduplex with the hybridisation-accessible sites within the targeted mRNA and have the appropriate chemistry for effective hybridisation **(2)**, which confers sufficient extra- and intra-cellular biological stability. It needs to have efficient uptake **(3)** usually involving cell association and entry. Once inside the cell **(4)** it needs to have net accumulation, where influx is greater than efflux. However, the cellular oligonucleotide must **(5)** by-pass errant compartmentalisation and remain bioavailable to **(6)** localise at sites of bioactivity within the cell *e.g.* cytoplasm and nucleus where it should **(7)** exert its activity in the intended sequence-specific manner.

1.3.5 Delivery of Nucleic Acids

Critical to the success of nucleic acids as pharmaceuticals will be the development of appropriate delivery systems (Hope *et al.*, 1998). The need of an appropriate delivery system for nucleic acid based therapies has been discussed in section 1.3.3.1. Most of the strategies discussed below have been tested in *in vitro* systems, and where not directly inferred would be applicable to the delivery of both ribozymes and ODNs. Hence in the future they are likely to be extrapolated to the delivery of DNA enzymes.

1.3.5.1 Lipid delivery systems

Liposomes are composed of phospholipids, arranged in a bilayer, which can either encapsulate nucleic acids within an aqueous centre, or can form lipid / nucleic acid complexes due to opposing electrostatic charges (for review see Maurer *et al.,* 1999). They are the most widely used system for *in vitro* delivery of nucleic acids and afford protection to the nucleic acid, whilst enhancing the cellular delivery. Although anionic and neutral liposomes have been studied for ODN delivery, their poor encapsulation efficiencies for nucleic acids has prevented their widespread use (reviewed in Hughes *et al.,* 2000; Tari, 2000).

Cationic liposomes and cationic lipid complexes with ODNs (lipoplexes) have been, by far, the most successful delivery system for achieving biological effects in cell culture for both ODNs (Roh *et al.,* 2000; Abe *et al.,* 1998) and ribozymes (Kisich *et al.,* 1999; Gu *et al.,* 1997). They offer an efficient and simple delivery method that gives reproducible results. In optimal conditions, cationic lipid reagents form small (100-400nm) unilamellar liposomes with a positive surface. By virtue of their positive charge, these delivery systems have a high affinity for most cell membranes, which are negatively charged under physiological conditions, and usually gain entry to cells by adsorptive endocytosis (Juliano *et al.,* 1999). The positive charge also facilitates their binding to the phosphate backbone of DNA. To help facilitate the subsequent release from endosomal/lysosomal compartments, many commercially available lipoplex transfection agents (*i.e.* transfectins or cytofectins) contain a helper lipid, such as

dioelylphosphatidylethanolamine (DOPE). This is an inverted-cone-shaped lipid thought to facilitate cytosolic release through the fusion to and/or the disruption of the endosomal membrane (Farhood *et al.,* 1992) possibly by the formation of hexagonal lipid structures (Zelphati and Szoka, 1996). Studies have also shown that ODNs can readily dissociate from the complexes in a bioavailable form within the cell.

The effectiveness of lipoplexes is dependent on the type and nature of the cationic lipid, cell type, ODN chemistry, ODN length and even the method of formation of the complexes. Studies have shown that only cationic lipoplex formulations optimised for these variables will significantly improve the intracellular bioavailabilty and activity of ODNs (Islam *et al.,* 2000; Williams and Buzby, 2000). Optimisation of the amount of cationic lipid used is also essential to minimise potential toxicity of the lipid to cultured cells (Coulson *et al.,* 1996).

Cationic lipids were used to deliver synthesised ribozymes to peritoneal macrophages *in vivo* (Kisich *et al.,* 1995). Three different lipids were compared: DOTMA, DOSPA and DMRIE. The macrophages incorporated over ten times more ribozyme when delivered in conjunction with DOSPA than with the other two cationic lipids. Later, the same group reported specific inhibition of TNF-α expression *in vivo* following ribozyme treatment (Kisich *et al.,* 1999). Following inter-peritoneal injection of cationic lipid / ribozymes complexes, approximately 6% of the administered ribozymes accumulated in target cells, macrophages. The catalytically active ribozymes suppressed TNF-α secretion by 50% relative to the inactive control.

Despite the few successful uses of cationic lipids *in vivo*, a major pharmaceutical hurdle that remains is the development of a non-toxic, serum-stable, lipoplex formulation that can be routinely used to achieve reproducible biological effects with ODNs and ribozymes.

1.3.5.2 Dendrimers

Dendrimers are a new macromolecular delivery system, which possess a highly branched three-dimensional structure. They are attractive delivery vehicles since they are synthesised by defined polymerisation reactions yielding a monodisperse globular product with a large number of controllable peripheral groups. Several categories of dendrimers have been synthesised, with various functional groups, depending on the initial monomer and the building block monomers used. To date most studies regarding dendrimer delivery of ODNs have been performed using the polyamidoamine (PAMAM) starburst dendrimers (Bielinska *et al.,* 1996). PAMAM starburst dendrimers possess a hydrocarbon core, charged surface amino groups, and have a well-controlled chemistry. The cationic surface charge of PAMAM dendrimers provides a suitable binding surface for anionic DNA (Delong *et al.,* 1997). Newer alternatives to lipid delivery agents on the market include dendrimer-based systems such as SuperFect™, which are not adversely affected by serum.

Stable complexes between ODNs and cationic dendrimers form under a variety of conditions *i.e.* water, PBS and serum (Delong *et al.,* 1997). PAMAM dendrimers have been shown to mediate high efficiency transfection of a number of cultured mammalian cells (Haenslar and Szoka, 1993). Dendrimer-ODN complexes have been shown to cause a 75% reduction in metabolic degradation of phosphodiester ODNs in serum and also to afford protection from the lysosome (Poxon *et al.,* 1996). In one study (Yoo and Juliano, 2000) examining delivery of ODNs with PAMAM dendrimers, it has been shown that the dendrimer remains complexed with the ODN as it enters the cell nucleus. Interestingly, the authors further reported that conjugation of the dendrimer to a small fluorescent dye enhanced its ability to deliver ODNs and increased its effectiveness in serum. This, the authors conclude, opens up the possibility of tailoring the dendrimer surface structure to achieve the desired uptake properties.

1.3.5.3 Biodegradable polymers

The relatively rapid degradation of many nucleic acids in the biological milieu both *in vitro* and *in vivo* suggests that the pharmacological effects of ODNs are likely to be short-lived. Furthermore, the duration of action of ODNs may also be compromised by a relatively rapid redistribution and pharmacokinetic elimination half-life, even the chemically stabilised PS ODNs are cleared rapidly, with a plasma half life of only 1hr (Lewis *et al.,* 1998). Thus, repeated administration of ODNs is often required for efficacy (Akhtar and Agrawal, 1997). This will be especially important for targets with long half-lives. For example, in the case of peripherin, a neurone-specific intermediate filament protein, which has a slow turnover (half-life of about 7 days), repeated administration of antisense ODNs for up to 40 days was required for a reduction of protein levels by 90% in cultured PC12 phaeochromocytoma cells (Troy *et al.,* 1992).

The most widely studied polymers are polylactides (PLA) and co-polymers of lactic acid and glycolic acid (PLGA) and these have been evaluated for the potential use for delivery of antisense ODNs (Putney *et al.,* 1999) and ribozymes (Hudson *et al.,* 1996). These thermoplastic polyesters can be fabricated in any shape as implantable devices for local delivery or even as parenterally administered systemic formulations. Indeed, they have long been used in resorbable surgical sutures (Cutright *et al.,* 1971) and in commercially available sustained release preparations *e.g.* Zoladex, Zeneca Pharmaceuticals, UK.

Polymeric microsphere (or even nanosphere) devices due to their relatively small size could potentially enhance delivery of ODNs directly at the cellular level. They can also provide site-specific delivery to a particular tissue or sub-set of cells and/or provide sustained delivery of the free ODN into the systemic circulation following implantation (either. i.m., s.c., or i.p.). In one cell study (Akhtar and Lewis, 1997), uptake of ODNs by murine macrophages was significantly reduced in the presence of metabolic and phagocytic inhibitors, indicating that the microspheres laden with ODN were entering cells by an endocytic/phagocytic mechanism. However it is not clear, as yet, how the polymer-entrapped ODNs are trafficked through the cells, and how and when they are released from the delivery system. These issues require further study.

Improved efficacy of ODNs as polymer formulations has been reported *in vivo*. Putney *et al.* (Putney *et al.*, 1999) used male CD-1 nude mice injected with a human melanoma cells as an *in vivo* model. They showed that the subcutaneous (s.c.) delivery of a P(LA-GA) microsphere encapsulated antisense ODN, targeted against *c-myc*, led to an enhanced suppression of tumour growth when compared to free ODN administered intravenously. Delivery of ODN s.c. in microspheres (6mg) gave approximately 60% inhibition in tumour weight, whereas the same dose of ODN in solution (administered over 8 days) only gave approximately 20% inhibition. Western blot analysis also revealed a more prolonged reduction in *c-myc* protein levels with microsphere-encapsulated ODN over free ODN. On day 12, 1 day after the final dose of ODN i.v., the *c-myc* levels were reduced to 74% and 51% in the i.v. solution and microsphere treated animals respectively, however, on day 20 there was no reduction in the i.v. solution treated animals whilst the microsphere-treated animals showed 55% reduction.

1.3.5.4 CNS delivery

The major focus of research in our group is towards elucidating novel treatments and delivery systems to be of use in the treatment of glioblastoma multiforme. Thus it is important to consider the potential of nucleic acids to be delivered to, and be active in the brain. Antisense technology has emerged as a useful tool for modulating gene expression in the central nervous system. Successful gene inhibition has been reported for a wide variety of targets, including trophic factors, ion channels, and neurotransmitter receptors (Seidman *et al.*, 1999). However, the large size and often polar nature of nucleic acid drugs prevents these molecules from readily entering the CNS following systemic administration (reviewed by Akhtar and Agrawal, 1997). *In vivo* pharmacokinetic studies have demonstrated that less than 0.01%/cm^3 of a systemic injected dose of a phosphorothioate ODN may reach the brain, where its residence time may be as short as 60 minutes (Tavitian *et al.*, 1998).

Clinical techniques using hyperosmolar agents such as mannitol or arabinose to open the BBB have proven to be unsuitable as they are associated with damage to healthy tissue. An alternative approach to get these compounds across the BBB is to 'parasitise'

or "piggy-back" onto the cells own transport systems to facilitate delivery. The transferrin receptor, which is highly expressed in brain capillary endothelium (Jefferies *et al.,* 1984), has been widely studied as a delivery system. The OX26 Mab is a murine antibody directed against the rat transferrin receptor and undergoes receptor-mediated transport at similar rates to transferrin (Skarlatos *et al.,* 1995). Boado and Pardridge (1994) were able to demonstrate that using a PO-ODN with a single biotin residue at the 3' terminus bound to a streptavidin-OX26 fusion protein they were able to deliver sufficient ODN *in vivo* to effect a complete inactivation of the target mRNA. However, when these experiments were repeated *in vivo* there was rapid degradation of the PO-ODN (Kang *et al.,* 1995). Using a PO-ODN directly conjugated to the OX26 it was also demonstrated that improved cellular uptake could be observed *in vitro* and that this uptake was facilitated by the transferrin receptor (Normand-Sdiqui and Akhtar, 1998).

The most widely used approach in the laboratory is the local introduction (using either direct i.c.v. or localized regio-specific injections) of antisense agents into the region of interest within the CNS, thereby circumventing the BBB (Kathmann *et al.,* 1999; Tremblay *et al.,* 1999). However, repeated administration of some ODN chemistries have led to toxicity within the brain tissue (Lecorre *et al.,* 1997). A promising development is the potential use of biodegradable polymer microspheres, (see above) which may overcome some of the toxicity problems and can also provide regio-specific and sustained delivery of ODNs (Khan *et al.,* 2000).

1.3.5.5 Other methods

Various other methods have been used for the *in vitro* delivery of nucleic acids, two of which are outlined below.

Receptor-mediated endocytosis has the potential for the effective targeting of ODNs and ribozymes to particular sub-sets of cells or to specific organs. Tumour cells show a propensity to this kind of targeted delivery since by their nature they have an increased requirement for essential nutrients compared to benign cells. Kren *et al.* (1999) used polyethyleneimine (PEI) conjugated with lactose to target a chimaeric RNA/DNA ODN

to the ASGP-R receptor. The chimaeric ODN was directed to the rat factor IX gene and was designed to introduce a single point mutation to inactivate the factor IX. Injection of the free chimaeric molecule into male Sprague-Dawley rats resulted in no detectable mutations, but when administered with the PEI-lactose conjugate a dose related genomic DNA conversion was observed up to 40%.

Carrier peptide-mediated delivery makes use of protein transduction domains, small motifs in proteins approximately 10-16 amino acids in length. These can be used as 'carrier' or 'transport' peptides to promote the delivery of active agents across biological membranes in a receptor and transporter-independent pathway. There are a number of examples in the literature of peptides being used for ODN delivery. For example, one group used a chimaeric peptide (MPG) consisting of a hydrophobic fusion domain derived from HIV gp41 and a hydrophilic nuclear localisation signal derived from SV40 T-antigen (Morris *et al.,* 1997). They demonstrated that the MPG peptide was able to efficiently bind to both single stranded and double stranded ODNs (Kd 1-$2x10^{-8}$ M). The MPG-ODN complex improved the stability of the ODNs and no significant degradation was observed after 10 hours in serum containing medium. The MPG-ODN complex was shown to be efficiently delivered with >90% of cells showing uptake of fluorescently labelled MPG-ODN after 1 hour. The cellular distribution of the MPG-ODN was predominantly nuclear whereas free ODN was restricted to subcellular vesicles, presumably endosomal. As previously, treatment at 4°C, which blocks endocytosis, had no effect on cellular uptake or distribution, suggesting a non-endocytic mechanism.

As we learn more about the pharmacokinetics and pharmacodynamics of nucleic acids it will be possible to refine our delivery strategies and make improvements in both the administration and delivery of these molecules. Ultimately, effective delivery will lead to a more widespread use of nucleic acid based therapies as biological tools, drug-target validation agents and, hopefully, as therapeutic agents.

1.3.6 The Future of ODNs

An emerging potential for antisense ODNs is use in combination with conventional therapies such as cytotoxics. Leonetti *et al.* (1999) used two cell lines derived from a human metastatic melanoma inherently resistant to cisplatin. They found that treatment of these two melanoma cell lines with antisense ODNs (of PS chemistry) increased the sensitivity of the cells to cisplatin treatment. Rait *et al.* (1999) demonstrated that antisense HER-2 (HER-2/erbB-2 is a proto-oncogene) ODNs were able to sensitise human breast cancer cells to various chemotherapeutic agents, *e.g.* Taxol, resulting in increased cell death by apoptosis. Similarly, Tian *et al.* (2000) used antisense ODNs targeted to $p21^{WAF1/CIP1}$ to successfully increase sensitisation of colon cancer cells to radiotherapy. The ODNs enhanced tumour cell apoptosis and improved radiocurability.

This approach of combination therapy has also had some success in treating gliobastoma multiforme. Overexpression of the MDM2 oncogene is one of the molecular characteristics of gliomas. Prasad *et al.* (2002) recently determined the therapeutic effects of an antisense anti-MDM2 oligonucleotide administered alone or in combination with the clinically used chemotherapeutic agents Paclitaxel and Irinotecan. In cultured cells, the antisense oligonucleotides were found to produce a dose- and sequence-dependent reduction in MDM2 expression and elevation in p53 and $p21^{WAF1/CIP1}$ levels, resulting in an increase in apoptosis and cytotoxicity. Synergistic effects on p53 and p21 levels between the ODN and chemotherapeutic agents were observed *in vitro*. In *in vivo* studies, the ODN inhibited tumour growth and improved the therapeutic efficacy of paclitaxel and irinotecan 39- and 63-fold, respectively.

This ODN mediated increase of sensitisation to current chemotherapeutic and radiotherapeutic agents has the potential for immense clinical value. It will lower the effective dose and thereby mitigate the adverse side effects associated with chemotherapy. Indeed such an approach has entered the clinic. Genta (Berkeley Heights, NJ) has various ongoing clinical trials of its flagship antisense drug, Genasense™, which blocks the protein Bcl-2, in combination with existing anticancer treatments. Trials include, for example, combining Genasense™ with decarbazine for malignant melanoma and with dexamethasone for multiple myeloma.

1.4 Ribozymes

Ribozymes are small RNA molecules capable of catalytic activity. They were first discovered in the laboratory of Tom Cech, at the University of Colorado in the early 1980s. His research team found the ribosomal RNA precursor from *Tetrahymana thermophila* contained an intron capable of self-cleavage, *in vitro*, without any protein or external energy source (Cech *et al.,* 1981). Shortly afterwards, Sid Altman's group at Yale University, showed that the RNA component of RNase P, M1 RNA, from *Escherichia coli* was likewise able to process tRNA precursors in the absence of any protein factors (Guerrier-Takada *et al.*, 1983). Cech and Altman later shared the Nobel Prize in Chemistry for this pioneering work.

Ribozymes are RNA-based antisense oligonucleotides with an incorporated catalytic core (Akhtar, 1998). Ribozymes combine enzymatic processes with the specificity of antisense base pairing (Putnam, 1996), and as such offer two theoretical advantages over antisense oligonucleotides. Ribozymes are capable of cleaving and hence permanently inactivating their target substrate, and, they are true catalysts in that they have the ability of multiple turnovers (Curcio *et al*, 1997).

Many different classes of ribozyme have now been identified. These include Group I and II introns (Sun *et al.,* 2000), RNase P (Ma *et al.,* 1998), hairpin ribozymes (Yu *et al*, 1995), and hammerhead ribozymes. Of all the various motifs, the hammerhead is the best characterised, possibly due to its small size and simple cleavage mechanism (Scott and Klug, 1996).

1.4.1 The Hammerhead Ribozyme

1.4.1.1 Structure of the hammerhead

Occurring in small pathogenic RNAs, the hammerhead motif is a self-cleaving structure that is thought to process multimeric transcripts into monomers during rolling-circle replication of viroid and virusoid genomes (Doudna, 1998). The ribozyme was so named because its Australian discoverers (Symons, 1992) found the secondary structure, as originally drawn, to be reminiscent of the head of a hammerhead shark.

In the 2-D representation of the *trans* acting form (Haseloff and Gerlach, 1988), there are three Watson-Crick helices and a conserved core. In order to facilitate comparison of data from different laboratories, a uniform numbering system for nucleotides was introduced by Hertel *et al.* (1992), as outlined in Figure 1.7 below.

Figure 1.7 Standard numbering system introduced by Hertel *et al.* (1992) for the Haselhoff and Gerlach (1988) classic hammerhead ribozyme.

3D Structure of the hammerhead

Sophisticated techniques such as X-ray crystallography and FRET (fluorescence resonance energy transfer) have been used to study the three-dimensional structure of the hammerhead ribozyme. The 3-D structure of the hammerhead was first elucidated by Pley *et al.* (1994) using X-ray crystallography. This structure was corroborated by the work of Scott *et al.* (1996). The ribozyme can be visualised as a wishbone, or Y shape, with stems I and II at the arms and stem III at the base (Sigurdsson and Eckstein, 1995; Scott and Klug, 1996). Residues C3-U4-G5-A6 form a sharp turn, the uridine turn, which is the molecule's catalytic pocket (Scott and Klug, 1996).

1.4.1.2 Structure-function relationships

In the Haseloff and Gerlach model (1988), a GUC triplet was required in the substrate for cleavage to occur. As further research was done, the strict requirement for a GUC cleavage triplet in the substrate was relaxed to give the NUX rule, where N is any base and X is any base except G. Nucleotides G5, A6, G8, G12, and A15.1 are deemed conserved nucleotides of the catalytic core and are essential for activity of the hammerhead (Scott and Klug, 1996). Helix II (which comprises a stem and a loop) is the only helix in the hammerhead that is not directly involved in substrate binding (Sigurdsson and Eckstein, 1995), thus it has been targeted in attempts to reduce the size of the hammerhead. Helix II probably promotes folding of the catalytic core and/or acts to stabilise the tertiary structure of the ribozyme (Sigurdsson and Eckstein, 1995). Helices I and III flank the catalytic core and they are the substrate-recognition domains. Sequences 5' to the catalytic domain form helix I, while 3' sequences form helix III when complexed with the target RNA (Tabler *et al.,* 1994). The length and composition of these flanking arms will have an effect on the specificity and turnover of a ribozyme. For efficient association, as well as fast dissociation *in vitro*, the optimal length has been estimated to be 6-8 nucleotides on either side of the catalytic domain (Hormes *et al.,* 1997).

1.4.1.3 Cleavage reaction mechanism

The hammerhead ribozyme catalyses a trans-esterification reaction, cutting the 3'5' phosphodiester bond between nucleotides 17 and 1.1 (see numbering system, Figure 1.7). Nucleophilic attack on the phosphate occurs by the adjacent 2'OH group, generating a 5' hydroxyl and a 2',3' cyclic phosphate (Sigurdsson and Eckstein, 1995). The reaction mechanism is illustrated in the following diagram.

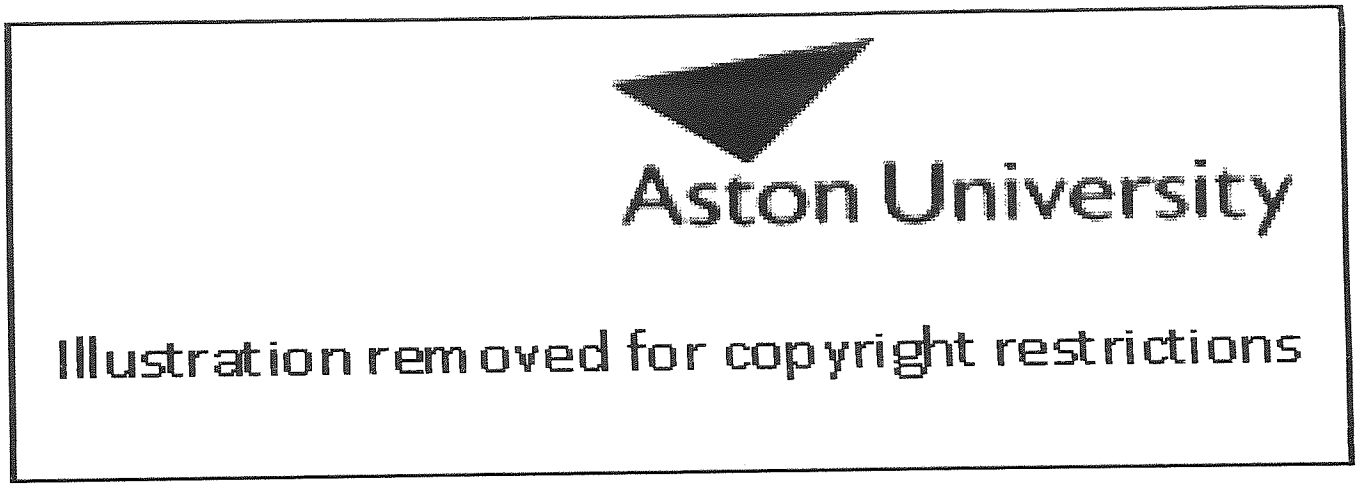


Figure 1.8 Ribozyme-mediated cleavage of a phosphodiester bond.
N represents any base nucleotide, X represents the nucleotides A, U or C. (source: Dahm *et al.*, 1993).

The proposed mechanism of action begins with deprotonation of the 2' sugar at the 3'-side of the cleavage site. This results in nucleophilic attack of the adjacent phosphodiester bond and subsequently protonation of the 5' oxyanion leaving group, generating 2',3' cyclic phosphate and 5' hydroxyl termini. The 2' hydroxyl adjacent to the cleavage site is essential for cleavage (Sullivan, 1994). The principles of Michaelis-Menten kinetics for ribozyme catalysis *in vitro* are outlined by Thomson *et al.* (1996).

The reaction mechanism can be described as:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} E\check{S} \xrightarrow{k_{cat}} E + P1 + P2$$

Where E is ribozyme, S is the substrate, and P1 and P2 are reaction products. Under multiple turnover conditions, ribozyme is in non-saturating concentration over substrate and the catalytic rate constant (k_{cat}) is a measure of the rate-limiting step. As discussed previously, rate of substrate binding (k_1) and dissociation (k_2) will vary with length and composition of the substrate-binding arms. Thus, conditions need to be optimised for different reactions.

1.4.1.4 Role of metal ions in the reaction mechanism

The hammerhead ribozyme, like all other naturally occurring ribozymes is a metalloenzyme, in that it requires a divalent metal ion such as Mg^{2+} to mediate catalytic cleavage (Scott and Klug, 1996). In the absence of divalent metal ions, the hammerhead exists in an open conformation with helices I and II almost co-linear and at a 90° angle to helix III. With increasing concentrations of metal cations, this open configuration converts to the active Y-shaped configuration (Wang and Ruffner, 1998).

There is some dispute over a suitable model to describe the involvement of metal cations in the chemistry of the ribozyme cleavage reaction. In the generally accepted single-metal ion mechanism, as depicted in Figure 1.8, a nucleophilic metal hydroxide acts as a base to deprotonate the 2'OH on the ribose in the cleavage site, initiating nucleophilic attack at the phosphorus (Scott and Klug, 1996).

Murray *et al.* (1998) suggest that it is the presence of a relatively dense positive charge, rather than divalent metal ions, which is the fundamental requirement. Their results illustrate that very high concentrations of monovalent ions could support RNA-cleavage rates similar to those observed in standard concentrations of Mg^{2+}. They conclude that, whether the positive charge plays an active role in the chemistry of catalysis, or a more passive role of aiding the correct folding of the hammerhead is unclear.

1.4.2 Increasing Ribozyme Stability

A major drawback in using RNA is that it is very rapidly degraded in biological fluids. For example, the half-life of an all-RNA hammerhead ribozyme in human serum is less than 0.1 minute (Jarvis *et al.*, 1996). Thus, various chemical modifications are applied in an effort to increase ribozyme stability while retaining catalytic activity. There are several potential sites for modification in the basic nucleotide building block: the phosphodiester internucleotide linker, the ribose sugar moiety, and the nucleotide base (shown in Figure 1.9 below).

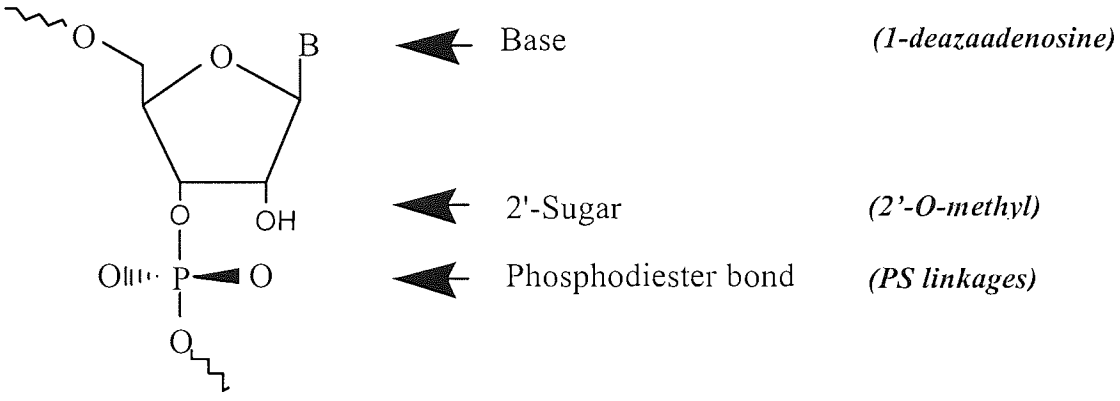


Figure 1.9 Potential sites for chemical modification of ribonucleotides.
Diagram illustrates the sites used to introduce modifications to RNA in an effort to increase its stability. Common examples of modifications at each site are indicated. Introduction of 1-deazaadenosine, a nucleoside analogue; 2-*O*-methyl modification of the 2'-sugar, where the OH is replaced with OCH_3; introduction of phosphorothioate groups at the phosphodiester bond

1.4.3 Applications of Ribozymes

Since their discovery, ribozymes have come to encompass a wide range of applications, including cancer and viral disease. Some of these are briefly outlined below. Also, the success of ribozymes is not confined to the laboratory. Ribozyme Pharmaceuticals Inc. (RPI), currently have Angiozyme™, a ribozyme targeting the vascular endothelial growth factor (VEGF), in clinical trials (Sandberg *et al.*, 2000).

1.4.3.1 Applications in cancer

In search for a novel treatment for glioblastoma multiforme (GBM), Ke *et al.* (1998) constructed two hammerhead ribozymes to target the 5' common region of VEGF (vascular endothelial growth factor) mRNA. Studies of glioblastoma angiogenesis have shown that the expression of VEGF is up regulated in these tumours. Both ribozymes displayed 65-95% efficiency *in vitro*. U251-MG and U87-MG cells were both transfected with the ribozymes in a transient and then more stable manner. Transient transfection (LipofectAMINE™) resulted in ≈20% reduction of VEGF secretion. Stable transfected (using plasmids) clones had VEGF reduced by up to 70%. These results were confirmed at the protein level.

Expression of the proto-oncogene *c-myb* is necessary for proliferation of vascular smooth muscle. *c-myb* has been implicated in various cancers including melanoma and leukaemia. Jarvis and co-workers (Jarvis *et al.*, 1996) developed synthetic hammerhead ribozymes that recognise and cleave *c-myb* RNA, thereby inhibiting cell proliferation. Efficacy was measured in the aortic smooth muscle of rats. Ribozymes produced a dose-dependant inhibition of smooth muscle proliferation by targeting *c-myb*. Various chemical modifications were subsequently made to improve the ribozyme's resistance to degradation.

1.4.3.2 Applications in viral diseases

HIV is a common target for ribozyme therapeutics, investigated by many groups. The *tat* RNA sequence is conserved in various RNA isolates and its product is essential for viral replication (Kijima *et al.,* 1995). Hormes *et al.* (1997) assessed the efficacy in living cells of the HIV-1 *tat*-directed asymmetric hammerhead ribozyme αRz195, and various modified versions of this ribozyme. The ribozymes displayed effective *in vitro* cleavage of the mRNA target, and were effective upon transfection into cells. Length of the antisense arms, the mode of delivery and the subcellular localisation of ribozyme affected efficacy in HIV-1 producing cells. Ribozymes were either microinjected into cells or were transfected with infectious pro-viral HIV-1 DNA.

With an aim of developing gene therapy for HIV-1 infection, Michienzi *et al.* (1998) tested in a human T lymphoblastoid cell line, the antiviral activity of ribozymes specifically designed to co-localise inside the nucleus with the Rev pre-mRNA before it is spliced and transported into the cytoplasm. The ribozyme was inserted in the spliceosomal U1 small nuclear RNA (snRNA), with complementarity to the 5' splice site of the Rev pre-mRNA. The ribozymes displayed specific and efficient inhibition of viral replication in human T cell clones. The ribozyme reduced both protein levels and intracellular HIV transcripts.

1.4.3.3 Applications against telomerase

Various groups have designed effective ribozymes targeted to the enzyme telomerase. Telomerase is an enzyme that is expressed in many tumours (see section 1.4). Wan *et al.* (1998) demonstrated activity of a 36-mer phosphorothioate-modified ribozyme targeted to the RNA component of human telomerase (hTR). 4μM of ribozyme inhibited telomerase activity, as measured by the TRAP (telomeric repeat amplification protocol) assay, in U87-MG cell lysates. Inhibition was due to a catalytic cleavage mechanism, as confirmed by *in vitro* cleavage reactions and RT-PCR analyses.

Yokoyama *et al.* (1998) designed three ribozymes targeting various sequences along the hTR. These were 44-base pair ribozymes, with no chemical modifications outlined. All three were able to cleave the 601-base telomerase RNA substrate *in vitro*. When these ribozymes were introduced into Ishikawa cells (endometrial carcinoma cells), only the ribozyme targeting the RNA template region was effective. This effective ribozyme sequence was subcloned into the expression vector, pHβAPr-1-neo, and transfected to AN3CA cells (another endometrial carcinoma cell line). A reduction of telomerase activity and telomerase RNA was observed with expression of the ribozyme.

Thus ribozymes have shown some promise in the clinic as well as the laboratory. It remains to be seen whether DNA enzymes will equal or even surpass their success.

1.5 DNA Enzymes

1.5.1 DNA as an Enzyme

Enzymes are traditionally thought of as being proteins, and this is true for the vast majority of enzymes found in nature. The discovery of catalytic RNA or ribozymes, showed that nucleic acids were not merely informational molecules (Bashkin, 1997) but capable of catalysing specific chemical reactions, often with an efficiency comparable to that of protein enzymes (Santoro and Joyce, 1997). It followed that it may also be possible for DNA to possess catalytic activity. DNA is generally regarded as a passive molecule, well suited to carrying genetic information. The concept that DNA could act as an enzyme seems strange not least due to the traditional view that DNA exists only as a Watson-Crick base-paired duplex. However, if liberated from this double helix, single-stranded DNA can adopt a variety of defined secondary and tertiary structures, including non-standard base pairs, stabilizing hairpin loops, internal bulges, multistem junctions, pseudoknots, and four-stranded G quartet structures. Some of these structures may be capable of catalytic function (Breaker, 2000). Unfortunately, the single-

stranded form of DNA is rarely seen under typical biological conditions and double stranded DNA is relatively rigid and structurally homogenous (Bashkin, 1997).

Protein and RNA enzymes must fold into stable structures that create active sites using precise spatial positioning of functional groups (Breaker, 1997). The specific recognition of ligands by DNA aptamers (Mishra *et al.,* 1996) demonstrated that DNA too could create binding pockets with similar precision. This led to the notion that DNA should be able to catalyse chemical reactions, which served as the basis of experiments trying to create single-stranded DNAs that function like enzymes.

To date, no naturally occurring DNA enzymes have been identified. However, various synthetic DNA enzymes, also known as DNAzymes or deoxyribozymes, have been obtained by *in vitro* selection (Carola and Eckstein, 1999). *In vitro* selection of nucleic acids is a combinatorial method, which allows the rapid screening of vast combinatorial libraries of RNA, or single stranded DNA with specific properties (Faulhammer and Famulok, 1997). By using such combinatorial strategies, enzyme engineers have created approximately 100 classes of DNA enzymes that catalyse nearly a dozen different types of reactions including RNA cleavage and DNA modification (Breaker, 2000).

A DNA enzyme can be described as an oligonucleotide sequence incorporating a non-complementary catalytic loop. Antisense oligonucleotides recognise a target through Watson-Crick base pairing and rely on cellular factors such as ribonuclease H to inactivate the target (Dash *et al.*, 1987). In contrast, a DNA enzyme is able to carry out both target recognition and cleavage, and it operates with catalytic efficiency (Santoro and Joyce, 1997).

Although an exciting prospect, there appear to be several disadvantages of DNA acting as an enzyme. In contrast to proteins, nucleic acids have limited diversity as catalytic agents. Amino acids provide a much wider array of functional groups than the four major nucleotides of DNA/RNA (Bashkin, 1997; Breaker, 1997; Santoro and Joyce, 1997). However, proteins lack side chains with electrophilic groups and hence commonly require co-enzymes for catalytic activity (Breaker, 1997). Nucleic acids

have negatively charged backbones, which, in combination with limited functional groups, may restrict the formation of rigid structures. Structural rigidity is important for the positioning of functional groups needed for catalysis (Sen and Geyer, 1998). Additionally, DNA lacks a 2' hydroxyl group on the ribose moiety, which is an important donor, and acceptor group for hydrogen bonding in RNA (Breaker, 1997). However, it is the absence of this 2' hydroxyl group which makes DNA approximately 100,000 fold more stable than RNA under physiological conditions (Breaker, 2000).

As a catalytic agent, DNA does have some advantages over RNA (Breaker, 1997; Santoro and Joyce, 1997). DNA is amenable, more so than RNA to rational and combinatorial methods for molecular design which can be used to engineer novel compounds. DNA is easier to synthesise and is more stable to chemical and enzymatic degradation. Also, as DNA enzymes are engineered entities, greater catalytic power could be conferred upon DNA simply by grafting more powerful and diverse chemistries directly onto its nucleotides (Breaker, 2000). Sen and Geyer (1998) outline the three major factors driving the research. Firstly, the need to define the limits of catalytic activity by nucleic acids, for example, the existence of catalytic DNA is a setback to the 'RNA World' theory (Breaker, 1997). Second, to investigate alternative catalytic mechanisms for different reactions. Third, the design of therapeutically active DNA enzymes for a variety of cancers, and this is where our interest lies.

1.5.2 An RNA-Cleaving DNA Enzyme

Early work in the development of DNA enzymes found DNA able to catalyse the Mg^{2+} dependent cleavage of an RNA phosphoester embedded within an otherwise all-DNA substrate (Breaker and Joyce, 1995). In fact, Breaker and Joyce (1995) used combinatorial methods to isolate four different populations of DNA enzymes able to cleave RNA phosphodiester bonds, with a requirement of Pb^{2+}, Zn^{2+}, Mn^{2+} or Mg^{2+} for catalytic activity. Elsewhere, the isolation by *in vitro* selection of two distinct classes of self-cleaving DNAs, which used Cu^{2+} as a cofactor, was reported (Carmi *et al.*, 1996). These innovations suggested that a DNA enzyme with general purpose RNA cleavage activity might be attainable.

Santoro and Joyce (1997) sought to develop a DNA enzyme that could be made to cleave almost any RNA substrate, efficiently and specifically under physiological conditions. Such a molecule could be used to inactivate a target RNA, probe a structured RNA or assist in the manipulation of recombinant RNA. Using a novel strategy known as catalytic elution, they first developed a general RNA-cleaving DNA enzyme.

Santoro and Joyce (1997) used *in vitro* selection to search for DNA molecules which best met the following criteria: multiple turnover cleavage of RNA under simulated physiological conditions; recognition of target RNA substrate via Watson-Crick base pairing; potential for substrate-binding domains to be generalised to other targets; activity equal or greater than comparable RNA enzymes and total size of 50 deoxynucleotides or less.

Two motifs were elucidated which best met all of the above criteria. These were the 8-17 and the 10-23 motifs illustrated below. The two DNA enzymes used in this study are based on these two motifs.

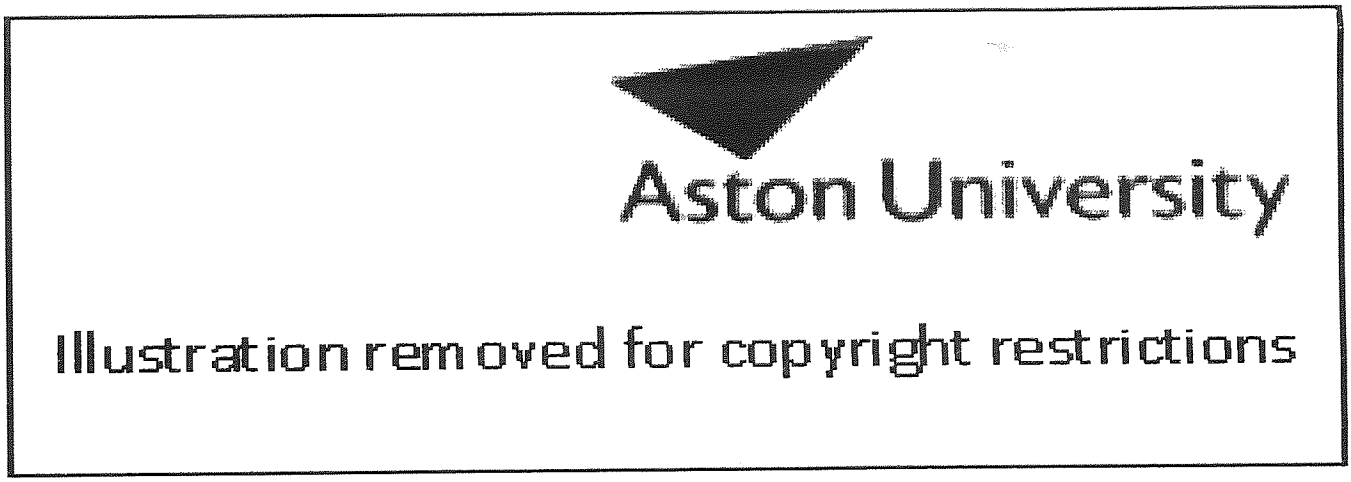


Figure 1.10 The 8-17 and 10-23 motifs as elucidated by Santoro and Joyce.
The DNA enzyme (bottom strand) binds the RNA substrate (top strand) by complementary base pairing and cleavage occurs at the positions indicated by arrows. R = A or G; Y = U or C.

The DNA enzymes consist of a catalytic domain flanked by two substrate-recognition domains of 7 or 8 deoxynucleotides. The catalytic cores of the 8-17 and 10-23 enzymes contain 13 and 15 deoxynucleotides respectively. The RNA substrate is bound through Watson-Crick base pairing. It was found that both motifs could be applied to various substrates, without loss of catalytic activity, provided the substrate-binding arms were changed in a complementary manner (Santoro and Joyce, 1997). Also, both catalysts directed the site-specific cleavage of the RNA substrate in a reaction that proceeded with multiple turnover under simulated physiological conditions.

The 8-17 motif cleaves its RNA sequence at a phosphodiester bond between an A and a G residue. The 10-23 motif was shown to cleave on the 3' side of a purine followed by a pyrimidine, with target sites surrounded by A and U cleaved most efficiently. The reaction mechanism of the cleavage reaction is thought to involve attack by a 2' hydroxyl on an adjacent phosphate (Santoro and Joyce, 1997).

There has been an attempt to solve the 3D structure of the 10-23 motif. Nowakowski *et al.* (1999) reported an attempt to solve the atomic resolution structure of the entire 10-23 DNA enzyme motif using X-ray crystallographic techniques. Unfortunately, in the crystal form, the complex that was formed displaced the active structure of the 10-23. Thus there is no atomic resolution yet available for the 10-23 but its simple structure permits easy alteration to target almost any RNA sequence (Santoro and Joyce, 1997).

The 10-23 and 8-17 remain the most common motifs for DNA enzymes to date. However, recently a novel DNA enzyme for the sequence-specific cleavage of RNA has been described (Feldman and Sen, 2001). The authors report the selection of a new and general RNA-cleaving DNA enzyme, the "bipartite DNA enzyme", so called because of the segregation of purine and pyrimidine bases in its conserved catalytic core. The bipartite DNA enzyme was obtained from a random DNA library by *in vitro* selection. The investigators found this DNA enzyme to display some novel biochemical properties, including a rate versus pH profile resembling that of the hepatitis delta virus (HDV) ribozyme. The authors suggest this property raises the possibility that, as with the HDV ribozyme, the bipartite DNA enzyme may display acid-base catalysis involving its constituent bases. This property is different to all reported metal-

dependent DNA enzymes and the hammerhead ribozyme, being more commonly associated with protein enzymes, *e.g.* ribonuclease A. The addition of bipartite DNA enzyme extends the repertoire of DNA enzymes, but much work will be required to fully characterise the usefulness of this novel DNA enzyme.

Santoro *et al.* (2000) also describe a novel RNA-cleaving DNA enzyme, 16.2-11. *In vitro* selection techniques were utilised resulting in the development of a DNA enzyme containing three catalytically essential imidazole groups, able to catalyse the cleavage of RNA substrates. This novel imidazole-functionalised DNA enzyme has a minimised catalytic domain composed of only 12 residues, which forms a compact hairpin structured that displays the three imidazole–containing residues. The 16.2-11 enzyme was found to be adaptable to almost any target RNA by alteration of the two substrate-recognition domains flanking the catalytic domain. The enzyme operates with multiple turnover in the presence of micromolar concentrations of Zn^{2+}. The authors suggest this imidazole-containing DNA enzyme may be the future of DNA enzymes, combining the substrate-recognition properties of nucleic acid enzymes and the chemical functionality of protein enzymes in a small, versatile and efficient molecule.

The main advantage that DNA enzymes have over RNA enzymes is their superior stability. They are well suited for use in a drug-delivery system as DNA molecules are more stable than RNA molecules *in vivo* (Kuwabara *et al.*, 1997). The success of DNA enzymes will largely depend on whether their catalytic activity equals or even surpasses that of their RNA counterparts.

1.5.3 Design of DNA Enzymes

1.5.3.1 Target site selection

As discussed earlier, DNA enzymes seemingly provide ample choice of target site compared to the hammerhead ribozyme, which is restricted to the NUX triplet described in section 1.4.1.2. With the aim of developing a treatment for chronic myelogenous leukaemia (CML), Kuwabara *et al.* (1997) designed versions of the 10-23 and 8-17

DNA enzyme motifs to target *BCR-ABL* gene fusion mRNA. The protein derived from this RNA fusion is a negative regulator of apoptosis, consequently its expression permits uncontrolled cell growth leading to CML. Due to restricted potential cleavage sites for hammerhead ribozymes near the *BCR-ABL* junction, non-specific cleavage of normal *ABL* mRNA could not be avoided when using conventionally designed ribozymes. The DNA enzymes however, having a wider range of potential cleavage sites, were designed to within 3 nucleotides of the *BCR-ABL* junction. No cleavage of the normal, unfused mRNA occurred, demonstrating the high substrate-specificity of the DNA enzymes.

One of the greatest obstacles for DNA enzymes and other agents that bind their target by Watson-Crick base pairing is the underlying RNA secondary structure. A new multiplex approach to target site selection for DNA enzymes has been demonstrated in the literature, which provides a rapid assessment of different anti-mRNA DNA enzymes. Cairns *et al.* (1999) have documented the development of a multiplex cleavage assay for screening the entire length of a target RNA molecule for DNA enzyme cleavage sites that are efficient, both in terms of kinetics and accessibility. More than 100 versions of the 10-23 DNA enzyme with different substrate binding domains were made. Mixtures of these DNAs were used to probe for cleavage sites within structured E6/E7 transcripts of a human papillomavirus (HPV16) and within the rat c-myc RNA transcript. Significantly, it was found that the most efficient DNA enzymes revealed by the target selection assay were also efficacious in cell-free expression assays as well as inside cells. If all the possible target sites on a particular mRNA were to be tested empirically, it would be a very time-consuming process. The streamlined approach used in this study (Cairns *et al.,* 1999) offers the possibility of direct comparison of all sites for a given target in a single reaction.

A similar strategy to identify accessible sites has been described by Sriram and Banerjea (2000). They used a pool of random sequences containing the 10-23 motif and tested their ability to cleave the target HIV-1 *gag* RNA. Briefly, a 740-base *in vitro* transcript of HIV-1 *gag* was prepared and mixed with a random pool of DNA enzymes. The cleaved products were subjected to primer extension and then analysed alongside a sequencing gel to map the cleavage site in the target RNA. Two DNA enzymes were

chosen by this strategy for further characterisation. Both were Mg^{2+}-dependent and showed interference with HIV-1-specific expression in cells.

1.5.3.2 Length of substrate binding arms

There are conflicting reports in the literature as to the effect of substrate recognition domain length. Elongation of the stem sequences has been described to both increase (Oketani *et al.,* 1999) and decrease (Warashina *et al.,* 1999) catalytic efficiency of DNA enzymes. There also appears to be differences in the effect of length of binding arms depending on whether the target RNA is long or short. Results obtained by Kurreck *et al.,* (2002) with a long RNA transcript as a target, show the DNA enzymes they tested to be more active with longer than with shorter antisense arms even under multiple turnover conditions. In contrast, when using short RNA targets, no difference in catalytic activity was recorded for substrate recognition domains from 7 to 9 deoxynucleotides (Santoro and Joyce, 1998). However, it is clear that the length of the DNA enzyme stem sequences is critical for catalytic efficiency.

One interesting possibility arising from this is the investigation of oligonucleotide facilitators. These facilitators are oligonucleotides, which have previously been shown to enhance hammerhead ribozyme activity by interacting with the substrate at the termini of the ribozyme (Jankowsky and Schwenzer, 1996). It followed that oligonucleotide facilitators may similarly enhance activity of DNA enzymes. Horn and Schwenzer (1999) analysed the influence of facilitators and elongated substrate recognition sequences on the DNA enzyme mediated cleavage of short model substrates under multiple turnover conditions *in vitro*. They found that oligonucleotide facilitators strongly enhanced the multiple turnover activity of the DNA enzyme reaction, in one system displaying a more than 200-fold enhancement of the catalytic efficiency.

1.5.3.3 The role of metal ions

Overall, metal ions feature highly in the activity of DNA enzymes. A recent study used DNA phosphorylation as a model reaction and Ca^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+} as representative metal ions to demonstrate that divalent metal ions can have a profound influence on the outcome of DNA enzyme selection (Wang *et al.*, 2002). It was shown that the choice of metal ions affected the sequence diversity, metal-ion specificity and catalytic proficiency of DNA enzymes to be isolated and optimised from a random sequence DNA library. The authors surmise that DNA has sufficient structural diversity to facilitate efficient catalysis using a broad scope of metal cofactor utilizing mechanisms.

The activity of the general purpose RNA-cleaving DNA enzyme developed by Santoro and Joyce (1997) was dependent on the presence of Mg^{2+} ions. These and other DNA enzymes are dependent on divalent metal cations *e.g.* Mg^{2+} for activity (Breaker, 1997; Carola and Eckstein, 1999). These metal co-factors possibly act to help DNA enzymes overcome functional limitations in two ways. Firstly, they neutralise the negatively charged backbone. Second, water molecules co-ordinated to divalent cations can carry out acid/base catalysis, which is useful for RNA phosphodiester cleavage (Sen and Geyer, 1998).

A group of highly efficient Zn^{2+}-dependent RNA-cleaving DNA enzymes have been isolated using *in vitro* selection (Li *et al.*, 2000). These enzymes, named 17E show high activity, requiring 10-100 fold less metal ion than most ribozymes, in cleaving both chimeric DNA/RNA and all-RNA substrates. Surprisingly, the catalytic core sequence of these Zn^{2+}-dependent DNA enzymes is similar to the 8-17 motif despite being selected under different conditions. This made the study of the 17E DNA enzymes more interesting as most studies have focused on the 10-23 motif obtained at the same time as the 8-17 (Santoro and Joyce, 1997). The target site of 17E was defined as 5'-rNG-3', where rN is any ribonucleotide and G can be either ribo- or deoxyribo-G. The 17E displays similar pH profile and reaction products to the hammerhead ribozyme and the authors propose they may have a similar chemical mechanism. This type of novel

work will serve to broaden the scope and perhaps increase the efficiency of nucleic acid enzyme catalysis.

The 10-23 and 8-17 motifs are the most successful RNA-cleaving DNA enzymes elucidated to date. However, research on these two DNA enzymes is still in its infancy, in particular our knowledge of the 8-17 remains very limited. To address this, a comprehensive analysis of cleavage rates of variants of the 8-17 DNA enzyme was undertaken in the presence of different divalent metal ions (Peracchi, 2000). Despite the fact that 8-17 was originally selected *in vitro* for activity in the presence of Mg^{2+} (Santoro and Joyce, 1997), nearly all the 8-17 variants exhibited substantially higher (up to 20 fold) reaction rates in Ca^{2+} as compared to Mg^{2+}. This unexpected finding is not easily explained as compared to Mg^{2+}, Ca^{2+} is a weaker Lewis acid and does not show a higher affinity for nucleotide monophosphates and oligonucleotides (Peracchi, 2000). This indicates the need to test the activity of *in vitro* selected nucleic acid enzymes with all the common metal ions, in addition to the metal ion used in selection, to maximise activity potential.

This preference of calcium over other metal ions was similar to another DNA enzyme, termed Mg5, originally described by Faulhammer and Famulok (1996). The catalytic efficiency of the *in vitro* selected Mg5 was found to strongly depend on the concentration of Mg^{2+} and Ca^{2+} ions. However, the extent of cleavage was significantly higher in the presence of Ca^{2+} than with Mg^{2+}. Later, the authors presented a detailed characterisation of their selected DNA enzymes (Faulhammer and Famulok, 1997). They performed a comparison of the secondary structure and reactivity of the Mg5 DNA enzyme with Ca^{2+} and Mg^{2+}, concluding that the higher reactivity with Ca^{2+} results from the existence of a specific binding pocket for Ca^{2+}, which allows the optimal positioning of the functional groups participating in the cleavage reaction.

One strategy to enhance the catalytic diversity of a DNA enzyme is to benefit from a particular property of a metal ion. This has been done for a DNA enzyme to catalyse the oxidative cleavage of a single-stranded DNA in the presence of Cu^{2+} (Carmi *et al.*, 1998). The investigators had isolated two distinct types of DNA enzymes (classes I and II) that undergo oxidative self-cleavage in the presence of copper ions. Using *in vitro*

selection, the class II self-cleaving DNAs were further optimised for catalytic function, to isolate the most active structure. This class II, copper-dependent DNA enzyme was minimised to its smallest active domain, and was engineered into a 'restriction endonuclease' for use in cleaving single stranded DNA substrates. The structural, kinetic and mechanistic characteristics of this novel class of Cu^{2+}-dependent DNA-cleaving deoxyribozymes are described in detail by Carmi and Breaker (2001).

A possible alternative to metal ions is the use of amino acids as an additional catalyst. Indeed a DNA enzyme with histidine as a catalyst has been isolated for the cleavage of RNA (Roth and Breaker, 1998). Here the imidazole moiety was shown to act as a base for activating the 2'-hydroxyl nucleophile. It was shown that histidine does not act as an inert cofactor but that it is directly involved in catalysis (Carola and Eckstein, 1999). This report of histidine is encouraging for the development of DNA enzymes more closely resembling protein enzymes in their repertoire.

It may be that some DNA enzymes do not require metal ion cofactors for catalysis as they fold in such a way as to force the nucleophilic 2' hydroxyl group into a position for a favourable in-line attack. Geyer and Sen (1997) used catalytic elution to isolate an RNA-cleaving DNA enzyme, termed G3, which functions efficiently in the absence of either divalent cations or any other cofactor. This suggests that DNA has a significant intrinsic ability to catalyse RNA cleavage. Rigorous steps were taken by the authors to rule out the participation of minute traces of divalent cations. Other groups have also described cofactor independent DNA enzymes that mediate RNA cleavage (Roth and Breaker, 1998; Faulhammer and Famulok, 1997).

1.5.3.4 Mechanism of cleavage

Compared to the extensive works published on ribozymes, there is limited information regarding the mechanism of cleavage by DNA enzymes and structure-activity relationships.

Ota *et al.* (1998) suggest that the 10-23 motif appears to have a mechanism of action very similar to that of the hammerhead ribozyme. Various observations (*e.g.* the identical dependence on pH) lend their support to the involvement of the double-metal ion mechanism of catalysis in both reactions. Also, Ota *et al.* (1998) analysed the cleavage reaction kinetics of various chimeric DNA enzymes. They generated helices composed of binding arms and substrates, consisting of RNA and RNA, of RNA and DNA or of DNA and DNA. In all cases, they found the introduction of DNA into the binding helix to enhance the rate of chemical cleavage. This observation is similar to the hammerhead ribozyme, where DNA substitutions in the binding arms (stems I and II) have enhanced the rate of hammerhead ribozyme cleavage (Sawata *et al.,* 1993).

1.5.4 Biological Applications of DNA Enzymes

The first antiviral application of DNA enzymes was in the inhibition of HIV. Santoro and Joyce (1997) used the 10-23 motif they developed to cleave synthetic HIV RNAs. Banerjea and coworkers (Dash *et al.,* 1998) designed a DNA enzyme possessing the 10-23 motif, to target the HIV-1 envelope gene. More than 50% *in vitro* cleavage of the 180bp HIV-1 Env transcript by the DNA enzyme was seen 5 minutes after mixing equimolar concentrations of DNA enzyme and RNA substrate, and cleavage increased steadily over a period of 4 hours. Cleavage activity was dependent on Mg^{2+} concentration. As well as *in vitro* cleavage, the DNA enzyme was able to demonstrate its ability to interfere with the functional expression of the HIV-1 envelope gene in a cell fusion assay performed in HeLa cells.

There has been much work in the Banerjea group on DNA enzymes targeting various regions of the HIV-1 genome. After the work on HIV-I Env described above, the group developed a DNA enzyme and hammerhead ribozyme targeting the chemokine receptor CCR5 (Goila and Banerjea, 1998). CCR5 is a major coreceptor required by HIV-1 for entry and fusion into target cells. Although direct comparisons between the hammerhead ribozyme and DNA enzyme were difficult in this case as they had different targets, nevertheless the DNA enzyme was found to be more efficient. Also, when the DNA enzyme was introduced into HeLa cells, there was a significant decrease

in the membrane fusion activity. This interference with the functional expression of CCR5 was sequence specific, and significantly higher than interference with antisense alone.

More recently, this group reported a functional DNA enzyme against the second most important HIV-1 co-receptor, CXCR-4 (Basu *et al.,* 2000), which showed sequence-specific cleavage activity. When CXCR-4 DNA enzyme was placed in tandem with the earlier identified CCR-5 DNA enzyme, specific cleavage of their respective target sites was observed against a 60-base synthetic target RNA, which possessed the target sites for both the DNA enzymes. This novel di-DNA enzyme was able to cleave substrate RNA to completion and also interfered specifically with the HIV-1 co-receptor functions in mammalian cells. Specificity and efficiency of cleavage of the mono-DNA enzymes were not lost in the di-DNA enzyme. It has been argued that any strategy directed against only one receptor, especially CCR5, may allow HIV to adapt quickly to use other co-receptors, namely CXCR-4 or others. This di-DNA enzyme approach targets co-receptors simultaneously and thus could be exploited for future therapeutic purposes.

Banerjea and coworkers have effectively used the novel di-DNA enzyme approach in other instances also. Unwalla and Banerjea (2001) report the targeting of *TAT* and *TAT-REV* RNA separately with mono-DNA enzymes and simultaneously with di-DNA enzymes. The regulatory proteins TAT and REV play an important role in the transcription and replication of HIV-1. The di-DNA enzyme was able to cleave the target RNA at multiple sites and showed approximately 80% inhibition of HIV-1 gene expression when introduced into cells.

Goila and Banerjea (2001) used a similar approach to target the X gene of the hepatitis B virus. The X gene is responsible for increasing the expression levels of other hepatitis B virus genes, and it has also been implicated in hepatocellular carcinoma. Two well-conserved sites were effectively targeted, individually with two mono-DNA enzymes, as well as simultaneously with a di-DNA enzyme. All DNA enzymes showed efficient, Mg^{2+} dependent cleavage of *in vitro* synthesised full length X RNA. The di-DNA enzyme retained cleavage specificity under simulated physiological conditions, and was

able to down-regulate the expression of the X gene in a liver-specific mammalian cell line. It is important to note that in all the reports of effective di-DNA enzymes discussed above, it is unclear whether cleavage at multiple sites is simultaneous or sequential.

Toyoda *et al.* (2000) designed RNA-cleaving DNA enzymes from the 10-23 catalytic motif for the inhibition of influenza virus replication. They report that although the RNA cleavage activity was not optimal under physiological conditions, the DNA enzymes inhibited viral replication in cultured cells more effectively than comparable antisense oligonucleotides.

Sioud and Leirdal (2000) explored the possibility of using the 10-23 catalytic motif to suppress the expression of the protein kinase Cα (PKCα) isoform in malignant cells. By selective modification of the nucleotides, they designed DNA enzymes that were relatively stable in serum and demonstrated sequence-specific inhibition of *in vitro* cell growth at nanomolar concentrations. They also demonstrated the therapeutic potential of DNA enzymes as most of the PKCα DNA enzyme treated cells underwent apoptosis.

DNA enzymes may have applications as research tools in delineating the specific role(s) of individual genes. Santiago *et al.* (1999) designed a 10-23 based DNA enzyme to target the early growth response factor-1 (Egr-1), which binds to the promoters of many genes whose products influence cell movement and replication in the artery wall. The enzyme (capped at the 3'-end with an inverted 3'-3'-linked thymidine) specifically cleaved Egr-1 mRNA, blocked induction of Egr-1 protein, and inhibited cell proliferation and wound repair in culture. In contrast, a control DNA enzyme with nonsense arms, was unable to cleave Egr-1 mRNA, had no effect on induction of the protein and failed to influence cell growth.

Taira and co-workers designed DNA enzymes to successfully target the aberrant mRNA produced by *BCR-ABL* gene fusion in humans (Kuwabara *et al.*, 1997). These *BCR-ABL* fusion genes are created from chromosome 22 (the Philadelphia chromosome) translocations, which are implicated in chronic myelogenous leukaemia. Having designed highly specific DNA enzymes, the investigators chose their 10-23 based DNA

enzyme to test further in mammalian cells (Warashina *et al.,* 1999). When introduced into cultured HeLa cells expressing the *BCR-ABL* mRNA, both the 10-23 enzyme and a nuclease-resistant version with several 2-methoxy modifications were found to induce apoptosis as a result of DNA enzyme-mediated cleavage of the aberrant transcripts.

Early work to create DNA enzymes from single stranded DNA using *in vitro* selection methods has met with significant success (for reviews see Sen and Geyer, 1998; Bashkin, 1997). Papers have described DNA molecules capable of catalysing DNA ligation (Cuenoud and Szostak, 1995), porphyrin metallation (Li and Sen, 1996), peroxidation (Travascio and Sen, 1998), DNA self-cleavage (Carmi *et al.,* 1996) and of course RNA cleavage.

The problems of delivery, biostability and cellular localisation facing antisense and ribozyme technologies also face DNA enzymes. There may be other applications *e.g.* biotechnology prior to fully developing biological applications.

1.5.5 The Future of DNA Enzymes

Over 3 billion years of evolution, nature has chosen not to use DNA as an enzyme, which may imply it is not suited for such a function. However, the lack of naturally occurring DNA enzymes may simply be because nature has never had any real need of them and thus it remains for the enzyme engineers to unveil and put to use DNA's true catalytic potential.

To summarise, there are many reasons why the prospect of DNA enzymes is exciting despite the absence of any naturally occurring examples. Catalytic molecules offer advantages over standard oligonucleotides such as the ability to both recognise and cleave target mRNA. DNA enzymes have advantages over their closest cousins ribozymes, of increased stability and greater adaptability. The main obstacle facing DNA enzymes in the future is demonstrating that their activity can surpass or at least equal that of ribozymes. To this end, a recent publication reports a systematic comparative study of the efficiencies of DNA enzymes and ribozymes to bind and

cleave the same full-length mRNA of the vanilloid receptor subtype I (Kurreck *et al.*, 2002). Messenger walk screening with antisense ODNs was used to identify accessible sites on the mRNA to use as targets for DNA enzymes and ribozymes. Though the authors stipulate the difficulty in directly comparing the activity of DNA enzymes and ribozymes against the same target sites, they do draw some interesting conclusions. Firstly the most efficient DNA enzyme found in the study had an approximately 15-fold higher reaction rate than the fastest ribozyme under single turnover conditions. Both ribozymes and DNA enzymes showed significant activity under multiple turnover conditions, the DNA enzymes again showing higher efficiencies. DNA enzymes also have fewer restrictions on potential cleavage sites than ribozymes. Considering all these factors, the authors conclude that DNA enzymes are an inexpensive, very stable and active alternative to ribozymes and show much therapeutic potential.

Another recent strategy to emerge was for effector-mediated control of RNA-cleaving ribozymes and DNA enzymes (Wang *et al.*, 2002). The authors describe the creation of variants of 10-23 and 8-17, whose catalysis was activated by up to 35 fold by the binding of the effector adenosine. The 'Ado-DNA enzyme' is inefficient on its own, but the presence of adenosine promotes the stable assembly of the enzyme-substrate-regulator complex and allows catalysis to proceed efficiently. Effector-mediated regulation has been used with ribozymes with an allosteric approach. Whereas in conventional allosteric ribozymes, effector binding modulates the chemical step of catalysis, in this new approach effectors exercise their effect upon the substrate-binding step and stabilise the enzyme-substrate complex. Also, in contrast to the allosteric approach, this new strategy requires no prior knowledge of the enzymes's secondary or tertiary folding. Thus this regulatory strategy should be generally applicable to any RNA-cleaving ribozyme or DNA enzyme, providing substrate recognition is achieved by Watson-Crick base pairing. One of the advantages of effector-controlled enzymes is their *in vivo* catalytic activity would be contingent on sensing an appropriate cellular environment, defined by the presence of the effector molecule.

The arrival of RNA-cleaving DNA enzymes has been an exciting development with regards to oligonucleotide-based catalysts. These 'deoxyribozymes' essentially combine the benefits of highly sequence-specific, ribonuclease-independent RNA

destruction, with the relative stability of ODN-based antisense reagents. The major obstacle to the development of these technologies as gene suppression agents is the difficulty involved in effective cellular delivery and target co-localisation. These challenges are being met with a multidisciplinary approach with the hope that a greater understanding of each facet of this problem will enable a more optimal utilization of this technology.

1.6 AIMS

The overall aim of this Ph.D. project was to evaluate two DNA enzymes, the 10-23 and 8-17, targeted against the RNA component of human telomerase (hTR). As discussed previously, GBM is a highly malignant tumour, with no current effective form of treatment. Novel strategies such as nucleic acid enzymes need to be investigated as a possible cure for this frequently fatal disease. DNA enzymes are a relatively recent development, which can be expected to be more stable than their RNA counterparts, but their biological efficacy must be ascertained. Many factors will determine their success as therapeutic agents, including their catalytic efficiency and the extent to which they are taken up into cell. Thus here we aimed to investigate the efficacy of chemically modified DNA enzymes targeted against telomerase *in vitro* and in cell culture models.

More specifically the aims were to:

1 Design active DNA enzymes against suitable sites in the template region of hTR.
2 Assess the *in vitro* cleavage activities of these DNA enzymes.
3 Investigate the activity of the DNA enzymes in effectively inhibiting telomerase in glioma cell extracts.
4 Examine the uptake mechanism of the DNA enzymes and evaluate methods of improving delivery of DNA enzymes to glioma cells.
5 Determine the biological efficacy of the DNA enzymes in targeting telomerase in a cellular environment.

CHAPTER TWO

MATERIALS AND METHODS

General materials and methods are outlined below; details of any variations to these procedures are highlighted in the relevant sections. Unless otherwise stated, chemicals were purchased from Sigma and were of analytical grade or higher.

2.1 DNA and RNA Synthesis

The synthesis of DNA enzymes and their substrates, as well as primer sequences was carried out on an ABI 392 automated DNA/RNA synthesiser (Applied Biosystems). This automated method of solid-phase synthesis has been developed from the technique devised by Merrifield (1963) and has become widely used in the synthesis of oligonucleotides (Brown and Brown, 1991).

Standard phosphoramadite reagents (Cruachem Ltd.) were used. All syntheses were carried out to a 0.2μM scale.

2.1.1 RNA Synthesis

Unmodified RNA substrates were synthesised as outlined above, on a 0.2μM scale. After synthesis of oligoribonucleotides, the sequences were manually cleaved from the solid silica support, deprotected, and purified. An RNA deblocking kit (Cruachem Ltd) was used for this purpose, as per manufacturers instructions. Briefly, 500μl of acidic buffer (0.5M sodium acetate, pH 3.25) was added to the solid support, vortexed and the solution incubated at 30°C for 36 hours. The acidic solution was neutralised by the addition of 100μL of neutralising buffer (3.0M Tris-base). After mixing and centrifugation, the resulting supernatant was purified by ethanol precipitation. The final pellet was dried, resuspended in RNase-free water (section 2.1.1.1), aliquoted and quantified (section 2.1.4).

2.1.1.1 Precautions taken in the handling of RNA

RNA is susceptible to degradation by 2'-hydroxyl dependent ribonucleases. Thus, great care is needed when working with RNA to avoid introducing ribonuclease (RNase) activity, two of the most common sources of which are the operator's hands and bacteria or moulds present on some airborne dust particles. Sterile technique was used whenever handling RNA, including wiping of all surfaces and equipment with ethanol prior to use.

A separate set of pipettes was set aside for RNA work. Sterile, disposable plasticware is essentially free of RNases and was used where possible. Non-disposable glassware and plasticware was treated with 0.1% v/v diethyl pyrocarbonate (DEPC) water prior to use. DEPC is a strong inhibitor of most RNases. Beakers, flasks etc were filled with DEPC treated water and allowed to stand at 37°C for at least 4 hours, rinsed with sterile water and then autoclaved.

RNase-free water was prepared by DEPC treating double distilled water to 0.1% for 12 hours, and then autoclaving to remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation. Solutions for use with RNA were similarly treated with DEPC. Tris buffers could not be treated in this manner because DEPC reacts rapidly with amines; hence, sterile DEPC treated, double distilled water was used in the preparation of all Tris containing solutions.

2.1.2 DNA Enzyme Synthesis

The two DNA enzymes used were synthesised using standard DNA bases as outlined previously. Synthesis was carried out to a 0.2μM scale. Phosphorothioates were introduced with tetraethyl disulphide (Applied Biosystems) as the sulfurizing reagent. Synthesised DNA enzymes were automatically cleaved from the column by concentrated ammonium hydroxide. These were then incubated at 55°C for 8-15 hours to remove the cyanoethyl phosphorus protecting groups on the exocyclic amines of adenine, cytosine, and guanine.

2.1.2.1 Purification of synthesised DNA enzymes

After deprotection, the synthesised products were lyophilised (Savant DNA Speed Vac). The dried products were resuspended in 50µl of RNase-free water. Purification of the DNA enzymes was carried out using a mini Quick Spin Oligo column (Roche) as per manufacturers instructions. Briefly, the column was prepared by centrifugation at 1000*g* for 1 minute to remove the residual buffer. After this, the sample was applied to the centre of the column bed in a volume of 20-50µl and eluted by centrifugation at 1000*g* for 4 minutes.

Eluted samples were diluted to 100µl with RNase-free water, aliquoted and stored at -20°C. This purification procedure removes any salts and short failed sequences (less than 10-mer) present in the sample.

2.1.3 Coupling Efficiency

During DNA/RNA synthesis, trityl cations are released upon addition of each base to the next. These provide an effective method of determining coupling efficiency. The trityls are a bright orange colour and can be analysed spectrophotometrically. The trityl fractions collected were diluted to 25ml with 0.1M p-toluene sulphonic acid in acetonitrile. Absorbance was measured at 498nm and the yield determined by the following formula:

Overall % yield = last or lowest fraction / second or highest fraction x 100
% stepwise yield = overall yield $^{1/\text{number of couplings}}$ x 100

The first trityl fraction is ignored as this represents the base that was attached to the column. A stepwise yield of 97-99% is taken as acceptable.

2.1.4 Quantification of Nucleic Acids

The concentration of the synthesised nucleic acids was determined by UV spectroscopy at 260nm. The purine and pyrimidine bases of DNA and RNA strongly absorb light with a maximum near 260nm.

The absorbance readings can be translated to concentration according to the Beer-Lambert Law:

$A = \varepsilon Cl$ where A = absorbance

ε = molar extinction coefficient

C = concentration

l = path length (usually 1cm)

One optical density (OD) unit (amount of DNA in 1ml volume in a 1cm path-length quartz cuvette) of single stranded DNA approximates to 33μg (Sambrook *et al.*, 1989). A more accurate method (Brown and Brown, 1991) uses the molecular weight of nucleic acid sequences to calculate their concentration.

Molecular weight of an unmodified oligodeoxynucleotide sequence:

= (249 x nA) + (240 x nT) + (265 x nG) + (225 x nC) + (64 x n-1) + 2

where nA = number of adenine bases in the sequence etc

n = total number of bases

(64 x n-1) accounts for the molecular weight of the phosphate groups

Calculation of the micromolar extinction coefficient:

ε = { (15.4 x nA) + (8.8 x nT) + (11.7 x nG) + (7.3 x nC) } x 0.9

Multiplication by 0.9 is necessary to account for the base stacking interactions in the single strand, which suppress the absorbance of DNA.

Conversion of O.D. units to micrograms

1mg = ε/ (mol wt/1000) = x OD units

Thus, 1 OD unit = 1/x = y micrograms

However, this method does not allow for changes in the molecular weight of DNA after backbone modifications and base additions *e.g.* 2'-*O*-methyl. The following adjustments are made to account for such differences:

Phosphorothioates:

Here, a sulphur atom replaces oxygen on the phosphodiester side chain. To account for the difference in molecular weights of oxygen (Mw=16) and sulphur (Mw=32), an adjustment of +16 is made for n-1 bases.

Thus, Mw = (249 x nA) + (240 x nT) + (265 x nG) + (225 x nC) + (**80** x n-1) + 2

Unmodified RNA sequences:

In this case, adjustment is required to account for an additional oxygen on the 2' site of each nucleotide. Thus, an adjustment of +16 is made for each base and an allowance made for the difference in molecular weight between thymine and uracil.

Thus, Mw = (**265** x nA) + (**242** x nU) + (**281** x nG) + (**241** x nC) + (64 x n-1) + 2

Absorbance readings were also used to ascertain the purity of the synthesised nucleic acids. Measurements were taken at 260nm and 280nm, with the ratio of these values (A_{260}/A_{280}) giving an indication of purity. Pure preparations have values of between 1.7 and 2.0.

2.2 Radiolabelling of Nucleic Acids

2.2.1 5'-End Labelling

Nucleic acid enzymes can be radiolabelled at their 5' termini, by the enzymatic transfer of $\gamma^{32}P$ from [$\gamma^{32}P$]-ATP using bacteriophage T4 polynucleotide kinase. The required nucleic acid sequences were radiolabelled at the 5'-end with ^{32}P-γ-ATP (ICN), using bacteriophage T4 kinase (Biolabs) in a 1x forward reaction buffer (100mM Tris pH 7.5, 20mM $MgCl_2$, 10mM DTT, 0.2mM spermidine and 0.2mM EDTA).

Reaction mixture:

Nucleic acid solution (typically 100 pmoles)	2µl*
10x reaction buffer	2µl
T4 kinase (5units/ µl)	1µl
[$\gamma^{32}P$] ATP	1µl*
Sterile DEPC treated ddH_2O	to 20µl

*These values may vary: nucleic acid solution according to the concentration to be labelled and amount of [$\gamma^{32}P$] ATP (Specific activity > 4000Ci/mmol) according to the activity of the label.

The reaction mixture was incubated at 37°C for 30-45 minutes.

2.2.2 3'-End Labelling

Labelling at the 3'-end is achieved by using terminal transferase, a template-independent polymerase which catalyses the addition of deoxynucleotides to the 3' hydroxyl terminus of DNA molecules.

The desired nucleic acids were radiolabelled with α-^{32}P[ddATP] (Amersham) using calf thymus terminal transferase (BioLabs) in 1x reaction buffer (50mM potassium acetate, 20mM Tris acetate, 10mM magnesium acetate and 1mM dithiothreitol @ pH 7.9), supplemented with 0.25mM $CoCl_2$.

Reaction mixture:

Nucleic acid solution (typically 100 pmoles)	2μl*
10x reaction buffer	5μl
10x $CoCl_2$ (2.5mM)	5μl
terminal transferase (20units/ μl)	1μl
α-^{32}P[ddATP]	1μl*
Sterile DEPC treated ddH_2O	to 50μl

*These values may vary: nucleic acid solution according to the concentration to be labelled and amount of α-^{32}P[ddATP] (Specific activity > 5000Ci/mmol) according to the activity of the label.

This reaction mixture was incubated at 37°C for 60 minutes. The mixture was then passed through a Quick Spin Column (see section 2.1.2.1) for the removal of cocadylate salts. The radiolabelled products were then purified by PAGE, as described below.

2.2.3 Purification of Labelled Nucleic Acids

The radiolabelled nucleic acids were purified by PAGE, as detailed below in section 2.3.1. The labelled samples were mixed with an equal volume of loading buffer (5% glycerol in 1x TBE) and run on a gel with 1x TBE as the running buffer, at 300V for approximately 2 hours. After electrophoresis, the position of the required bands was visualised by autoradiography (section 2.3.2). The visualised products were extracted from the gel using the 'crush and soak' method (Sambrook *et al.*, 1989). Following excision from the gel, the bands were eluted in sterile water and lyophilised. The dried, radiolabelled nucleic acids were re-suspended in RNase-free water prior to use and desalted using a Quick Spin Column if required (see section 2.1.2.1).

2.3 General Analytical Methods

2.3.1 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE can separate short chain nucleic acids according to differences in size. Polyacrylamide gels are formed by polymerisation of acrylamide monomers into chains of polyacrylamide, cross-linked by the inclusion of a co-monomer, *N-N'*-methylene-bis-acrylamide. The resulting cross-linked chains form a gel structure, the pore size of which is determined by the concentrations of both acrylamide and bis-acrylamide. A 30 % stock solution (Severn Biotech) of acrylamide: bis-acrylamide was used. For the separation of TRAP products (Chapter 4) 8% polyacrylamide gel solutions were used, with a acrylamide:bis-acrylamide ratio of 19:1. In all other instances, 20% polyacrylamide gels were used, with an acrylamide:bis-acrylamide ratio of 29:1. For the purposes of some experiments, urea was added to the 20% gels to obtain a denaturing gel mix.

Preparation of polyacrylamide gel mix:

STOCK REAGENT	20% GEL	8% GEL
30% acrylamide:bisacrylamide mix*	33.3ml	13.3ml
10 x TBE	5ml	5ml
Urea (denaturing gels only)	42g	n/a
ddH2O	to 50ml	to 50ml

*29:1 or 19:1 mix, as explained above

Polymerisation was initiated by the addition of 10%w/v ammonium persulphate (600μL) and N, N, N', N'-tetramethylethylenediamine (TEMED) (40μl) to 50ml of the polyacrylamide gel mix. This mixture was then poured quickly between two glass plates, held apart by spacers to form a gel of dimension: 20 x 20 x 0.03 cm. A 15 well comb was inserted at the top of the plates to form wells, and the gel allowed to set.

Preparation of 1 litre stock solution of 10x Tris borate buffer (TBE):

STOCK REAGENT	AMOUNT
Tris base	108g
Boric acid	55g
EDTA	5.84g
ddH_2O	to 1 litre

The cast gels were then pre-run at 300V for 15 minutes, and the formed wells washed well with 1x TBE before the loading of samples (10-50µl). Samples were diluted with native (5% glycerol in 1x TBE) or denaturing (9:1 v/v formamide:1x TBE) loading buffer prior to loading on the gel. Gel electrophoresis was carried out at 250-300V for 2-3 hours in 1x TBE (0.5x TBE for TRAP gels), using a BioRad power pack. Water was run through the tank as a coolant because the high voltages used in electrophoresis generate heat, which could distort the product bands. Marker dye (0.25% bromophenol blue and 0.25% xylene cyanol in 1x TBE) was used to estimate the migration of the samples through the gel.

2.3.2 Autoradiography

2.3.2.1 Visualisation of samples

Upon completion of electrophoresis of the radioactive samples, the plates were separated, with the gel remaining attached to one of the plates. The gel was covered with cling film and, in dark room conditions exposed to photographic film (Kodak film, Sigma) in a Hypercassette fitted with an intensifying screen. The exposure time was varied according to the activity of the samples; for periods longer than 5 minutes, the gels were kept at –70°C to prevent migration of the samples.

The film was developed and fixed under safelight conditions using Kodak reagents. The film was firstly placed in developer/replenisher solution for approximately one

minute, or until bands were seen. The film was then rinsed in water and transferred to fixer/replenisher solution for a further minute. After fixing, the film was rinsed under running water and then allowed to dry.

2.3.2.2 Densitometric analysis of autoradiographs

For analysis of autoradiographs, the images were scanned using a Canon scanner, and then analysed using a Phoretix 1D Advanced programme (v4.01, Phoretix International, Non Linear Dynamics Ltd.). This gave the relative intensities of the bands on the autoradiographs, which were then depicted graphically.

2.3.3 Liquid Scintillation Counting (LSC)

The specific activity of radiolabelled (see section 2.2) nucleic acids was determined by LSC. A small volume (5µl) of the purified sample was added to 5ml Optiphase Hi-Safe 3 (Wallac, Fisher) and counted in a Packard 1900TR scintillation counter. The appropriate program was used for detecting ^{32}P activity against a background. ^{32}P is a β emitter, with a half-life of only 14.3 days, thus decay in the experimental period has to be accounted for. Specific activities were thence calculated as cpm (counts per minute) per µl of sample.

2.4 *In Vitro* Activity Studies

Cleavage reactions were carried out to assess the *in vitro* catalytic activity of DNA enzymes. Reactions were carried out under enzyme saturating (single turnover) conditions, *i.e.* with enzymes in excess over substrate (2μM enzyme : 2nM substrate).

Short target sequences from a region of the telomerase RNA substrate were synthesised and purified as described previously (section 2.1). The synthetic RNA substrates were end-labelled at the 5' position using [γ^{32}P] ATP (section 2.2.1), and then purified by native 20% PAGE (section 2.2.3).

In vitro cleavage reactions were carried out in 50mM Tris-HCl, pH 8.0, and 100mM $MgCl_2$ at 37°C. In order to disrupt aggregates that can form during storage, labelled substrate and unlabelled DNA enzyme were denatured and renatured separately by heating to 90°C for 2-3 minutes and then cooling to the reaction temperature of 37°C over 10 minutes. The solutions then received an addition of $MgCl_2$ to a final concentration of 100mM and were incubated at 37°C for a further 10 minutes.

Reactions were initiated by combining the DNA enzyme and the substrate samples to the required concentrations in a final volume of 100μl. Reactions were incubated at 37°C and aliquots of 10μl were removed at appropriate times between 0 and 120 minutes and quenched by adding an equal volume of cold formamide loading buffer (formamide: 1x TBE, 9:1), and kept on ice.

Cleavage products and substrate were separated on a 20% denaturing polyacrylamide gel (section 2.3.1), and visualised using autoradiography (section 2.3.2). The resultant autoradiographs were analysed using densitometry as detailed in section 2.3.2.2. Reaction rate constants were obtained from the slope of semi-logarithmic plots of the amount of remaining intact substrate versus time. Plots tended to fit a double exponential curve where the fast portion of the curve was generally 60-90% of the total reaction. The cleavage rates and activity half times were calculated from the first exponential.

2.5 Cell Culture Techniques

All cell culture work was undertaken in a Gelaire, biohazard level II, laminar flow cabinet under strict aseptic conditions. All disposable plasticware used for cell culture was obtained from Corning Costar unless otherwise specified.

2.5.1 Cell Lines

Three adherent cell lines (purchased from ECACC) were used in various studies presented in this thesis. The U87-MG cell line was predominantly used for most studies. These human glioblastoma astrocytoma cells were originally derived from a grade 3 malignant glioma by explant technique. These cells express high levels of telomerase (Kiaris and Schally, 1999). HeLa cells, human cervix carcinoma cells, were used in the luciferase studies (Chapter 6). HeLa was the first aneploid, epithelial-like cell line derived from human tissue and maintained by serial sub-culture. T98G cells, derived from a human glioblastoma multiform tumour, were also used in some experiments.

2.5.2 Culture Media

All cells were maintained in Dulbecco's Modified Eagles Media (DMEM) supplemented with 10%v/v foetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin (all supplied by GIBCO BRL). A transfection medium was also prepared comprising DMEM supplemented with 1% L-glutamine only. Culture media was stored at 4°C, and warmed to 37°C prior to use.

2.5.3 Maintenance of Stock Cultures

Cells were grown in $75cm^2$ tissue culture vented cap flasks in 20ml of complete DMEM media. Flasks were kept at 37°C in a 95% humidified incubator with an atmosphere of 5% CO_2 in air.

Stock cultures of cells were maintained by passaging when confluent, generally every 3-4 days. Cultures were assigned a passage number of 1 upon their receipt in the lab and thus passage number is an arbitrary indication of age.

The standard aseptic procedure used for passaging was as follows:

The original medium was removed and the adhered cells washed with 5ml of sterile phosphate-buffered saline (1x PBS). Following aspiration of the excess PBS, the cells were covered with 3ml of 1x Trypsin (0.25% w/v trypsin in PBS, pH 7.2) (GIBCO BRL) and incubated at 37°C for 3-5 minutes.

Upon detachment of the cells from the flask, fresh medium was added in excess to neutralise the trypsin and this mixture was agitated to ensure uniform dispersion of cells. A small volume of cell suspension, to give the required density was transferred to a fresh flask and media added to a final volume of 20ml. For specific experiments, the cells were counted using a haemocytometer and used at the required cell number.

2.5.4 Freezing and Thawing of Cell Lines

2.5.4.1 Freezing of cells

Cells were trypsinised as above and then pelleted by centrifuging at 1000*g* for 5 minutes. The cell pellet was re-suspended in freezing media (90% FBS and 10% DMSO) to give a concentration of $1\text{-}5x10^6$ cells ml^{-1}. A 1ml aliquot of cell suspension was transferred to a 2ml screw capped cryovial, kept at –70°C overnight and subsequently transferred to storage in the liquid nitrogen cell bank.

2.5.4.2 Thawing of cells

The cells were recovered from liquid nitrogen by rapid thawing at 37°C and gradual dilution with DMEM media. Cells are subsequently re-seeded into a small 25cm^2 tissue culture flask. Cells were transferred to the standard 75cm^2 flasks when they reached confluence, and then passaged as usual.

2.5.5 Determination of Cell Number/Viability

The number of viable cells in a cell suspension can be assayed using the trypan blue exclusion test. To perform this test, 100µl of trypan blue (4mg ml^{-1}) was mixed with 400µl of cell suspension *i.e.* a 1 in 5 dilution. A small amount of the trypan blue-cell suspension was introduced into the counting chamber of a Neubauer haemocytometer, (Weber Scientific International Ltd.). The cells in the five large squares of the haemocytometer were counted under a light microscope. Viable cells can be distinguished due to the fact that only dead cells take up the blue dye, whilst live cells do not; hence viable cells are unstained.

The cell density is determined using the following equation:
Cells ml^{-1} = average count per square x 10^4 x dilution factor (1.25)
The percentage viability = viable cells / total number of cells x 100

2.6 Cell Growth Assays

The Trypan blue exclusion assay as outlined above, was the main method used to determine cell growth.

To establish standard growth curves, U87-MG and T98G cells were seeded in 24-well plates at a starting density of $2.5x10^4$ cells $well^{-1}$. The cells were incubated at 37°C and at specified times plates were removed from the incubator and cells trypsinised and counted as described in section 2.5.5.

To assess the effect of treatments on cell growth, U87-MG cells were seeded into 24 well plates at a density of $5x10^4$ cells $well^{-1}$. Following a 24-hour incubation, cells were inspected under the microscope to ensure a healthy appearance and the formation of a uniform monolayer. Apical media was removed and cell monolayers were rinsed with transfection medium, following which the test substances (*e.g.* lipid, DNA enzyme, lipid-DNA complexes etc) were added to the wells in transfection medium to a final volume of 200µl, and incubated at 37°C. Following an incubation period of 4 hours, treatments were removed and the monolayers rinsed with transfection medium. At this point, a zero hour time point was taken. The remaining cells received 1ml fresh media supplemented with FBS, and were incubated at 37°C for a further 24 hours. After incubation, the cells were rinsed with transfection medium, trypsinised and the number of viable cells determined by Trypan blue exclusion assay (section 2.5.5)

2.7 Cell Association Studies

These experiments were conducted to examine the cellular association/uptake of DNA enzymes; most of the data presented is in U87-MG cells.

2.7.1 Labelling of the Nucleic Acid Enzymes

The DNA enzymes were radiolabelled at the 3'-end (section 2.2.2) and purified by PAGE (section 2.2.3). Typically, 100 picomoles of the required nucleic acid were labelled.

2.7.2 Evaluating Cellular Association/Uptake

Confluent stock cultures of U87-MG cells were trypsinised and counted as detailed previously. The pelleted cells were resuspended in DMEM to give a resultant cell suspension concentration of $5x10^4$ cells ml^{-1}. Plastic 24 well tissue culture plates were used to culture the cells and each well was seeded with 1ml of cell suspension to give a final concentration of $5x10^4$ cells $well^{-1}$. The plates were incubated at 37°C in a humidified (95%) atmosphere of 5% CO_2 in air. Following a 48 hour incubation, the cells were approximately 80% confluent and had formed a uniform monolayer, ready to be used for uptake experiments. For the various parameters examined n (*i.e.* no. of wells) = 4 unless otherwise specified. Furthermore, each plate was seeded with four extra wells for the purpose of determination of viable cell number (section 2.5.5), thus enabling normalisation of cellular association to cell number.

At the start of an uptake experiment, the media was then removed from the wells and the monolayers washed with transfection media to remove any traces of serum. The required concentration of radiolabelled DNA enzyme was suspended in transfection media and added to the wells to give a final volume of 200µl per well. Activity in each well should be no less than 100,000cpm to ensure accurate LSC analysis of radioactive

content. The plates were then incubated at a specified temperature for the duration of the experiment, typically 4 hours at 37°C.

After incubation for the desired time, the apical media was carefully removed and collected in scintillation vials to be analysed by LSC as detailed earlier. The cell monolayer was then washed 3 times with 0.5ml of ice-cold PBS-azide (0.05% w/v sodium azide in PBS) to remove any loosely bound DNA enzyme and to prevent any further cellular metabolism. These washings were also collected for analysis by LSC. Cells were detached from the wells by shaking with 0.5ml of 1% Triton X-100 for 30 minutes at room temperature. Following removal of the solubilised cells, the wells were rinsed with fresh Triton X-100 to ensure removal of all the cells. The radioactive content of the cellular fraction was determined by LSC.

The cellular association/uptake is determined by the ratio of radioactivity present in the various collected fractions, as determined by LSC.

The following equation was used:

$$\% \text{ cellular association / uptake} = \frac{\text{Activity of cellular fraction}}{\text{Activity (cellular fraction + washes + apical media)}} \times 100$$

2.8 Stability Studies

The stability of DNA enzymes was assessed in transfection medium and media containing foetal bovine serum (FBS) to 10%. 3'-end labelled DNA enzymes were incubated in 500µl of either media at 37°C for 24 hours. At set time intervals, a 10µl aliquot was removed, mixed with an equal volume of denaturing loading buffer (9:1 v/v formamide: 1x TBE) and kept at –20°C. Degradation profiles were analysed by PAGE using a 20% denaturing (containing 7M urea) gel mix, and autoradiography of the gels (sections 2.3.1-2.3.2).

2.9 TRAP Assays

Telomerase activity in U87-MG and T98G cell extracts was measured using the PCR-based telomeric repeat amplification protocol (TRAP). The protocol used is essentially the same as described previously by Kim *et al.* (1995).

2.9.1 Preparation of Cell Lysates

Firstly, the following buffers were prepared:

Wash buffer:
10mM HEPES-KOH pH7.5
1.5mM $MgCl_2$
10mM KCl
1mM dithiothreitol

Lysis buffer:
10mM Tris-HCl pH 7.5
1mM $MgCl_2$
1mM EGTA
0.1mM phenylmethylsulfonylamide (PMSF)
5mM β-mercaptoethanol
0.5% CHAPS
10% glycerol

The individual components of the buffers were made as stock solutions and DEPC treated where possible. 10ml of each buffer was freshly prepared when needed. These precautions are necessary to reduce the risk of RNase contamination of cell samples. Aseptic technique was used throughout the preparation of lysates.

A confluent stock flask of cells was trypsinised and counted (see section 2.5.5). Cells were kept on ice whilst counting. The cell suspension was centrifuged at 1000*g* at 4°C for 5 minutes. The media was aspirated and the cell pellet washed and re-suspended in

PBS (Ca^{2+} and Mg^{2+} free). The re-suspended cells were transferred to sterile eppendorf tubes at approximately $1x10^6$ cells ml^{-1} in each tube. The harvested cells were then centrifuged at 13,000*g* at 4°C for 5 minutes. Supernatant was discarded and the cell pellet resuspended in 1ml ice-cold wash buffer (see above). Cell suspension was again centrifuged at 13,000*g* at 4°C for 5 minutes. The resulting cell pellet was suspended in 80µl of ice-cold lysis buffer (see above). Cells were incubated on ice for 30 minutes and then centrifuged at 13,000*g* at 4°C for 30 minutes to remove the membrane fraction. The supernatant was pooled, aliquoted, and stored at –70°C.

2.9.2 Protein Determination of Cell Lysates

The Bio-Rad protein assay, which is based on the Bradford method (Bradford, 1976), was used to measure the protein concentration of the cell extract. This is a dye-binding assay based on the differential colour change of a dye in response to various protein concentrations.

Firstly, a series of protein standards were prepared using BSA (1mg ml^{-1}). Concentrations from 0-20µg μl^{-1} were made by diluting stock BSA with distilled water to a final volume of 800 µl. Volumes from 0-20µl of the test samples (*i.e.* U87-MG cell lysates) were similarly diluted to 800µl. 200µl of Bio-Rad reagent was then added to all tubes, including one of 800µl distilled water as a blank. The tubes were vortexed and left to stand for a minimum of 5 minutes. The absorbance of the standards and test samples was read at 595nm. A standard curve was constructed and its equation used to calculate the concentrations of the samples.

2.9.3 TRAP Assay Procedure

Firstly, the upstream (TS) and reverse (CX) primers were synthesised and purified (see section 2.1.2). The TRAP assay is based on the recognition and elongation of an upstream primer, TS of sequence 5'-AATCCGTCGAGCAGAGTT-3'. Initially, activity was monitored by incorporation of [α^{32}P] dCTP into the reaction mixture;

however, better results were obtained by 5'-end radiolabelling the TS primer (see section 2.2.1).

Trap Reaction Mixture:

In a final volume of 50µl each TRAP reaction mixture contained:

Deoxynucleoside triphosphates	50µM
Upstream TS primer (labelled)	0.1µg
T4 gene 32 protein (Boehringer)	5ng
Taq DNA polymerase (Promega)	3 U
Tris-HCl pH 8.3	20mM
$MgCl_2$	1.5mM
KCl	63mM
EGTA pH 8.0	1mM
BSA	0.1mg ml^{-1}

For convenience, a 10x TRAP reaction buffer was prepared containing 200mM Tris-HCl, pH 8.3, 15mM $MgCl_2$, 630mM KCl, 10mM EGTA and 1mg ml^{-1} BSA. All the constituents of the buffer were prepared as separate stock solutions and except Tris were DEPC treated. A small amount of 10x buffer was made up and stored at –20°C.

The required amount of cell extract (as prepared in section 2.7.1) was incubated with the TRAP reaction mixture (final volume of 50µl) for 30 minutes at 20°C. Following incubation, 0.1µg of reverse primer CX, (5'-GCGCGGCTTACCCTTACCCT-TACCCTAA-3') was added to each reaction tube. Reactions were placed in a thermal cycler (touchdown, Hybaid) for 29 cycles. Each cycle consisted of 3 steps: 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds.

Upon completion of thermal cycling, the PCR products were electrophoresed on an 8% polyacylamide (19:1 acrylamide: bisacrylamide) gel (section 2.2.1). The gel was run for 3 hours at 250V in 0.5x TBE. Telomerase activity was visualised by autoradiography (section 2.2.2), usually following overnight exposure. TRAP products are of heterogenous length, and appear on the gel as a six base pair nucleotide ladder.

2.10 Microsphere Methods

2.10.1 Microsphere Preparation

Poly-D,L-lactide-co-glycolide (PLGA) 50:50 polymer with an inherent viscosity of 0.2 (Polysciences Inc) was used for microsphere fabrication.

A double-emulsion method was employed for the preparation of microspheres, as outlined below. An aqueous solution of the DNA enzyme to be incorporated into microspheres was prepared using 10 picomoles of 3'-end labelled DNA enzyme mixed with unlabelled DNA enzyme to give the desired concentration. An emulsifying agent of 0.4% w/v polyvinylalcohol (PVA) (87-89% hydrolysed, Mw 13,000-23,000 kDA) was added to the DNA solution in a final volume of 100μl. The aqueous solution was mixed with organic solvent, 7.5ml dichloromethane containing 500mg of dissolved PLGA polymer, to form the primary emulsion. The aqueous and organic solutions were mixed at 4,000 rpm for 4 minutes using a Silverson homogeniser STD2 (Silverson Machines) with a 3/8" mini microprobe (Silverson Machines). The resultant water-in-oil (w/o) emulsion was then emulsified into 50ml of aqueous external phase (0.9%w/v NaCl and 4% w/v PVA) at 6,000 rpm for 6 minutes using a 1" tubular probe (Silverson Machines). The w/o/w emulsion was stirred on a magnetic stirring plate overnight to allow for the evaporation of solvent.

The resulting microspheres were harvested by centrifugation (LC1 centrifuge, Sarstedt) at 4,000 rpm for 15 minutes, and then washed three times in sterile distilled water to remove any non-encapsulated ODN and emulsifying agent. At each washing, microspheres were pelleted by centifugation, supernatants discarded and the pellet re-suspended in sterile distilled water. Finally, the microspheres were suspended in 1ml sterile distilled water, frozen at –70°C, then freeze-dried (Modulyo, Edwards) for 48 hours (BOC Ltd, Sussex, UK) and subsequently stored in a dessicator at room temperature.

2.10.2 Characterisation of Microspheres

The yield was calculated from the ratio of the weight of freeze-dried microspheres obtained to the total amount of polymer used in the preparation.

2.10.2.1 Determination of encapsulation efficiency

5μl of radiolabelled DNA enzyme and 30mg of spheres were each added to 5ml of Optiphase Hi-Safe 3 scintillation cocktail (Wallac, Fisons). The radioactivity of these samples was determined in triplicate by LSC (section 2.3.3). From the LSC, the % w/v DNA enzyme encapsulated per dry weight of microspheres could be determined and the encapsulaton efficiency calculated.

$$\text{Encapsulation efficiency} = \frac{\text{Actual loading \% w/v}}{\text{Theoretical loading \%w/v}} \times 100$$

2.10.2.2 Scanning electron microscopy (SEM)

SEM was used to examine the surface morphology of the microspheres. Dry microsphere samples were mounted on aluminium stubs using carbon discs, and coated with gold under an argon atmosphere (Emscope SC500). The surface morphology was examined using a Cambridge Instruments Stereoscan 90B and captured with a PIXIE Image Processing and Archiving software package.

2.10.2.3 Particle-size determination

Approximately 10mg of dry microspheres were dispersed in 0.2μm filtered water, and sized by laser diffractometry using a Malvern Mastersizer X particle sizer (Malvern Instruments Ltd). The instrument was fitted with a 45mm angle lens and a flow cell and the presentation was for polystyrene in water. Size data were plotted as a spread of microspheres over a size range.

2.10.3 *In-Vitro* Release Profiles

The release of DNA enzymes from microspheres was performed by incubation of 30mg of spheres in 1.5ml phosphate buffered saline (PBS) as the release media. Release from microspheres was monitored over a 35-day period with incubation at 37°C in a shaking water bath (OLS 200, Grant) at 60 strokes minute^{-1}. Samples were taken hourly on the first day of the release, then daily for seven days and then every seventh day. At each sampling time, the release media was removed and centrifuged at 13,000 rpm for 5 minutes to remove any suspended microspheres, and the supernatant was analysed by LSC (section 2.2.3). The microsphere pellet was resuspended in fresh release media, replaced into the release vial and returned to the water bath until the next sampling time.

2.11 RT-PCR Reactions

RT-PCR analyses of the full length RNA substrate in cells were performed. A 23-mer forward (5'-TTTGTCTAACCCTAACTGAGAAG-3') and a 22-mer reverse (5'-TTGCTCTAGAATGAACGGTGGAA-3') primer were synthesised and purified (see section 2.2.2).

The total RNA was isolated from the cell lysates using a commercially available kit (SV Total RNA Isolation System, Promega), and RT-PCR was performed to amplify a portion of telomerase RNA (128 bp) that included the GUC cleavage site. Amplification of β-actin mRNA (323 bp) was used as a positive control for the PCR reaction. A 20-mer forward (5'-AAGTACTCCGTGTGGATCGG-3') and a 22-mer reverse (5'-CTGCTGTCACCTTCACCGTTCC-3') primer for were synthesised for actin amplification.

Amplification was carried with the one tube RT-PCR System (Promega), using Avian Myeloblastosis Virus reverse transcriptase and the thermostable T*fl* DNA polymerase

from *Thermus flavus*. T*fl* catalyses the polymerisation of nucleotides into duplex DNA in a 5' to 3' direction in the presence of magnesium.

In a final volume of 50µl per reaction tube, the extracted total RNA was mixed with: 1x reaction buffer, 0.2mM dNTPs, 1mM $MgSO_4$, 0.1u μl^{-1} of each of the two enzymes and 50µM of the forward and reverse primers. The reaction was incubated at 48°C for 45 minutes, then 94°C for 2 minutes. Amplification was carried out in a thermal cycler (Touchdown, Hybaid) for 40 cycles of 3 steps each: 94°C for 30 seconds, 60°C for 1 minute, and 68°C for 2 minutes. The PCR products were added to loading buffer and separated by electrophoresis in 1x TAE (0.04M Tris-acetate, 1mM EDTA, pH 8.3) buffer on a 2% agarose gel (run at 80V for 2-3 hours). Products on the gel were visualised by ethidium bromide staining.

2.12 Luciferase Assay

2.12.1 Plasmid Preparation

For the purposes of the luciferase assay, a commercially available plasmid, pGL3-Control (Promega) constructed from the pUC series of plasmids, was modified using a PCR protocol (as detailed in Chapter 6). The modified and unmodified plasmids were introduced into a strain of *E. coli* (DH5α) for the purposes of cloning and the bacteria were grown in Luria-Bertani Broth. Subsequently, the plasmid was extracted from the bacteria by alkaline lysis using a Mobius 1000 Plasmid Kit (Novagen) following the manufacturers instructions. Briefly, cells were harvested by centrifugation and then resuspended in buffer. The resuspended bacteria were lysed, neutralised, and then clarified by a brief centrifugation and filtration. The clarified lysate was eluted through a washed Mobius column (an anion exchange resin). Plasmid DNA was obtained from the eluted sample by isopropanol precipitation. The extracted plasmid was analysed by agarose gel electrophoresis, and quantified by densitometry using a Phoretix 1D advanced programme (v4.01, Phoretix International, Non Linear Dynamics Ltd.).

2.12.2 Luciferase Activity in Cells

The extracted plasmids were transfected into HeLa cells using SuperFect™ (Qiagen). Optimisation of delivery is detailed in chapter 6. Briefly, HeLa cells were plated at a starting density of $1x10^5$ cells $well^{-1}$ in 24 well plates. Plated cells were incubated at 37°C for 24 hours and then transfected. The DNA-SuperFect™ complexes were delivered to cells over four hours at 37°C. After this time, treatment media was removed, cells rinsed with PBS, and 1ml full growth media added per well. 20-24 hours following transfection of cells, media was removed and cells were lysed by shaking in 500µl of 1x Glo-Lysis buffer (Promega, UK) as per manufacturers protocol. Luciferase Activity was then detected using the Bright-Glo™ Luciferase Assay System (Promega, UK). Samples were read in luminometer tubes (Thermo Life Sciences) using 400µl of cell lysate mixed with 100µl of luciferase reagent for 60 seconds and then read in a luminometer (Jade, Labtech International). N.B. cells, buffer and reagent were all equilibrated to 25°C for 15-20 minutes prior to use, to maximise luciferase activity.

2.13 Statistical Analysis

Significance testing between mean values was performed with an unpaired students t-test assuming equal variance (Gaussian population) using the Microsoft Excel statistical software package. Unpaired t-tests assume random sampling of data, that each value was obtained independently of the others and that the populations follow a Gaussian distribution. The SPSS software package (version 10.0.7) was used to perform Fisher (F) tests, which indicated whether the variance of the two populations tested was significantly different. For studies involving more than two experimental groups, the one-way ANOVA was used to determine significant differences between groups using the SPSS package with different post hoc tests depending on the design of the experiment. Low P values indicated that experimental populations were unlikely to be sampled from populations with equal mean values. Data sets were assumed significantly different when the two-sided P values were calculated below 0.05.

CHAPTER THREE

DESIGN AND *IN VITRO* ACTIVITY OF DNA ENZYMES

3.1 DNA Enzymes

The field of nucleic acid enzymes is an expanding area (reviewed in Carola and Eckstein, 1999). Originally the exclusive domain of ribozymes, DNA enzymes are now attracting increasing attention. These DNA enzymes (DNAzymes or deoxyribozymes) have not been reported in nature, but rather have been obtained by a method known as *in vitro* selection (reviewed in Li and Breaker, 2001). In the literature to date, the only widely reported generalised RNA-cleaving DNA enzymes are the '10-23' and '8-17' catalytic motifs identified by Santoro and Joyce (Santoro and Joyce, 1997).

The reasons why the idea of DNA enzymes is appealing, despite the absence of any naturally occurring examples, have previously been discussed in Section 1.5. There is excitement over the prospect of DNA enzymes as they may offer more therapeutic potential than ribozymes and antisense oligonucleotides. Ribozymes are susceptible to chemical and enzymatic degradation and have restricted target site specificity. Chimeric ribozymes containing DNA or phosphorothioate (PS) linkages are less sensitive to degradation but they are expensive to synthesise and tend to still degrade in serum. Antisense ODNs with PS modifications offer improved resistance to nucleolytic cleavage but lack the catalytic activity of DNA enzymes. Also, PS modified antisense ODNs generally tend to result in decreased activity. Catalytic DNA molecules are more stable than ribozymes and have greater flexibility for selection of cleavage sites than hammerhead ribozymes (Santiago *et al.*, 1999). Thus DNA enzymes are promising molecules and there is a real need for contributions to the area.

The aim here was to test the activity of two novel DNA enzymes based on the 10-23 and 8-17 catalytic motifs. The enzymes were engineered to target telomerase, an enzyme emerging as a promising target for cancer therapies (discussed in section 1.2, see also Chapter 4). For the purposes of these studies, telomerase also provided a useful marker as its activity can be assayed directly.

3.2 Design of the DNA Enzymes

In order for a nucleic acid enzyme to exert its effect, it needs first to hybridise to its target RNA via complementary base pairing. However, designing an appropriate enzyme for a specific target is not as straightforward as it may appear at first. This is due to the fact that RNA molecules exhibit a high degree of folding and have complex secondary and tertiary structures, which are poorly predicted by currently available computer algorithms. The situation is further complicated by the presence of RNA-binding proteins inside the cell (Akhtar, 1998). Various methods of identifying accessible target sites have been used. In addition to the traditional empirical technique, new strategies such as RNaseH mapping and scanning combinatorial arrays have emerged (Akhtar, 1998).

For the purposes of these studies, the RNA component of human telomerase (hTR) is the target to be used. Previous work in our laboratory has shown inhibition of telomerase using a hammerhead ribozyme to target a GUC triplet in the template region of hTR (Wan *et al.*, 1998). The gene encoding hTR has been cloned and sequenced (Feng *et al.*, 1995), and the template region can be assumed to be accessible, as it serves as the binding site for telomere ends and subsequently as a template for the addition of telomeric repeats.

The two DNA enzymes used in these studies were also designed to target the template region of hTR. The designs incorporate the 8-17 and 10-23 motifs elucidated previously (Santoro and Joyce, 1997). Flanking the conserved catalytic domain are two substrate recognition arms, which provide both the sequence information necessary to specify a particular RNA substrate and the binding energy to hold said substrate within the active site of the enzyme (Santoro and Joyce, 1998). It was found that the sequence of the substrate could be changed without loss of catalytic activity provided the substrate-binding arms of the DNA enzyme were changed in a complementary manner (Santoro and Joyce, 1997). Thus the two DNA enzymes were designed against complementary regions in the template region of hTR as illustrated in Figure 3.1.

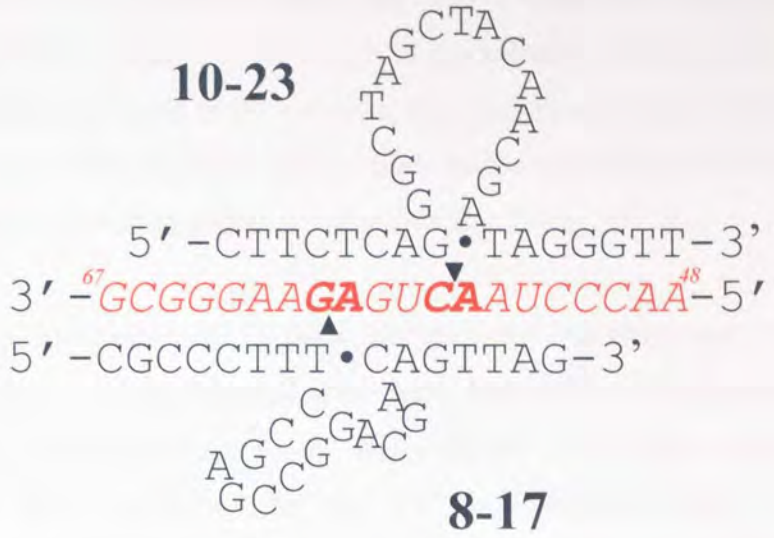


Figure 3.1 Design of the two DNA enzymes.
The two DNA enzymes have two different catalytic cores, 10-23 and 8-17. The complementary arms were designed to target the template region of hTR. Arrows show point of cleavage and the target RNA is denoted in *italics*. In the 5'-3' orientation, the 8-17 is designed to cleave between the emboldened GA doublet in the hTR sequence and the 10-23 to cleave between the emboldened CA doublet.

The human telomerase RNA (hTR) sequence is 451 nucleotides long, with an 11 nucleotide template region (CUA ACC CUA AC) numbering from 46-56 relative to the 5'-end of the transcript (Feng *et al.*, 1995).

The catalytic motifs, denoted as 10-23 and 8-17, were elucidated by Santoro and Joyce (1997) and consist of 15 and 13 deoxynucleotides respectively. Santoro and Joyce found that both these DNA enzymes were adaptable to any substrate sequence as long as the substrate-binding arms were complementary. Thus, the DNA enzymes can be adapted for use against almost any target sequence with only a couple of restrictions. The 10-23 motif was shown to cleave on the 3' side of a purine followed by a pyrimidine, whilst the 8-17 motif cleaves its RNA sequence at a phosphodiester bond between an A and a G residue. In addition, the 8-17 has a special requirement for an rG-dT 'wobble' pair located immediately downstream from the cleavage site.

Substitution of a Watson-Crick pair at this position eliminates catalytic activity (Santoro and Joyce, 1997). Santoro and Joyce (1997) found the 10-23 and 8-17 based DNA enzymes to be sensitive to the length of the substrate binding arms and a length of 7-8 nucleotides was found to be optimum for catalytic activity. The substrate recognition domains are highly specific, with a single mismatch resulting in substantial reduction of the enzyme's catalytic efficiency (Santoro and Joyce, 1998).

The DNA enzymes used in these studies were designed with these requirements in mind. The substrate binding arms were kept to 7 or 8 nucleotides in length with sequences complementary to the target RNA. The only exception to this was the 'wobble' pair requirement for the 8-17 as described earlier. It is estimated that approximately 14 nucleotides are necessary to define a unique RNA sequence within human cells (Santoro and Joyce, 1998).

The cleavage sites of the DNA enzymes designed here were chosen within or very close to the template region of hTR. In the 5'-3' orientation, the 8-17 was designed to cut between nucleotides 59 and 60 *i.e.* between an A and a G residue. The 10-23 was designed to cut between nucleotides 55 and 56 *i.e.* between an A and C residue. The 10-23 based DNA enzyme was originally designed to cleave between residues 65 and 66 but this enzyme was found to be inactive against the target substrate over the experimental period of *in vitro* cleavage assays. Thus it was re-designed to be closer to the template region as shown in Figure 3.1

As discussed in Chapter 1, stability of nucleic acid enzymes is a prime factor in considering their therapeutic applications. As outlined, efficacy is usually a compromise between activity and stability. Thus in an effort to obtain the optimal balance between stability and activity, various chemistries of the two DNA enzymes were synthesised, as outlined in Table 3.1. The various chemistries were then compared for their activity *in vitro* and their stability in serum. Phosphorothioate (PS) modifications were introduced, one of the most commonly used in the nucleic acids field to increase stability of DNA in serum.

Four different versions of each DNA enzyme were synthesised as follows:

1) unmodified phosphodiester (PO) DNA enzyme (HR1 and HR13),
2) DNA enzyme with total PS modification (HR2 and HR14),
3) DNA enzyme with PS groups at the antisense arms (HR3 and HR15) and
4) DNA enzyme with 3 PS groups at its 3'-end (HR4 and HR16).

Synthesis of these various chemistries was carried out as described in section 2.1.2.

Various control DNA enzymes were also synthesised, these are also outlined in Table 3.1 and they are elaborated on further in Chapter 4. The rigorous use of controls is very important in any antisense experiments to prevent mistaking artefact for antisense activity. This is especially pertinent whilst using the PS modification, which is very popular for increasing nuclease stability but has some inherent problems including non-specific protein binding (Lebedeva and Stein, 2001). Various recommendations now exist that should be considered before undertaking any antisense experiments (reviewed by Stein, 1999), *e.g.* the use of appropriate controls and the avoidance of four contiguous guanosine residues in antisense sequences.

The three controls for DNA enzyme activity implemented here were:

1) an equivalent antisense sequence *i.e.* an ODN of identical sequence to the substrate binding arms of the DNA enzymes but lacking the catalytic core,
2) a sense control *i.e.* a DNA enzyme where the catalytic core was kept intact but the arms were of sense sequence, and
3) a scrambled control *i.e.* where the sequence of the binding arms was unchanged but the sequence of the catalytic core was scrambled. In this case the composition of the bases was kept the same as the active motif but the bases were randomly scrambled.

Controls 1 and 3, *i.e.* the equivalent antisense sequence and DNA enzyme with inactivated core, will help indicate the extent of DNA enzyme activity that can be ascribed to a purely antisense mechanism of action. Control 2, *i.e.* active core with arms of sense sequence, will demonstrate the importance of base pairing and the specificity of the substrate recognition domains.

The sequence and chemistry of all DNA enzymes and RNA substrates employed throughout his thesis are summarised in Table 3.1, below.

Design	Sequence 5'-3'	Name
Unmodified PO 10-23	CT TCT CAG *GGC TAG CTA CAA CGA* TAG GGT T	HR1
Fully PS modified	ct tct cag *ggc tag cta caa cga* tag ggt t	HR2
10-23, PO center and PS arms	ct tct cag *GGC TAG CTA CAA CGA* tag ggt t	HR3
PO with 3 PS linkages at 3' end	CT TCT CAG *GGC TAG CTA CAA CGA* TAG Ggt t	HR4
Equivalent antisense sequence	ctt ctc agt tag ggt t	HR5
10-23 with sense arms	ga aga gtc *GGC TAG CTA CAA CGA* aat ccc aa	HR6
10-23 with scrambled core	ct tct cag *ATG AGC AGA CTC GAC* tag ggt t	HR7
Target RNA substrate of 10-23	UAA CCC UAA * CUG AGA AGG	HR8
Unmodified PO 8-17	CG CCC TTT *CCG AGC CGG ACG A* CAG TTA G	HR13
Fully PS modified 8-17	cg ccc ttt *ccg agc cgg acg a* cag tta g	HR14
8-17, PO center and PS arms	cg ccc ttt *CCG AGC CGG ACG A* cag tta g	HR15
PO with 3 PS linkages at 3' end	CG CCC TTT *CCG AGC CGG ACG A* CAG Tta g	HR16
Equivalent antisense sequence	cgc cct tct cag tta g	HR17
8-17 with sense arms	gc ggg aag *CCG AGC CGG ACG A* agt caa tc	HR18
8-17 with scrambled core	cg ccc ttt *AGA CCA GCC GGC G* cag tta g	HR19
Target RNA substrate of 8-17	CUA ACU GA*G AAG GGC GUA GGC GCC	HR20

BASE = bases with phosphodiester linkages
base = bases with phosphorothioate linkages
Base = bases incorporating the catalytic core of the DNA enzymes
BACB = unmodified RNA bases, with * indicating DNA enzyme cut site

Table 3.1 Table of the various DNA enzymes and their target RNA sequences. Table summarises the sequences and the different chemistries of the two DNA enzymes. The respective target RNA sequences are also indicated. The assigned names of the various molecules are referred to throughout the thesis.

3.3 Activity of the DNA Enzymes

Cleavage reactions were performed to assess the *in vitro* activity of enzymes. The procedure used for cleavage reactions is outlined in section 2.4. Briefly, this involved the synthesis of a short segment of the target substrate incorporating the cleavage site and incubation with the appropriate enzyme at 37°C for a specified time. Reactions were performed under single turnover conditions *i.e.* with enzyme in excess over substrate. In such conditions, reactions ensue with first-order kinetics and activity can be measured independent of substrate concentration. All four chemistries were assessed for catalytic activity and representative autoradiographs are given in Figures 3.2 and 3.3. The sizes of the full length and cleaved substrate were checked initially by PAGE concomitantly with radiolabelled ODNs of known lengths.

The appearance of radiolabelled cleavage products in the autoradiographs of all chemistries except the fully phosphorothioated indicates that the DNA enzymes retained catalytic activity unless indiscriminately modified. In order to compare the rate and extent of cleavage of the various enzymes, the intensity of the autoradiograph bands were quantified as detailed in section 2.3.2, and the activity half time ($t_{1/2}$) calculated from semi-logarithmic plots of the amount of substrate remaining versus time (Figure 3.4), as described in section 2.4. Plots tended to fit a double exponential curve, where the initial fast portion of the curve was generally 60-90% of the reaction. The activity half times were calculated from fits of the first exponential. Consequently, the values calculated will be more pertinent to the initial rate of reaction and less so to the ultimate extent of cleavage. This is suitable as the aim here is to compare activities of different catalytic motifs with various chemistries, and not to produce a comprehensive analysis of cleavage kinetics.

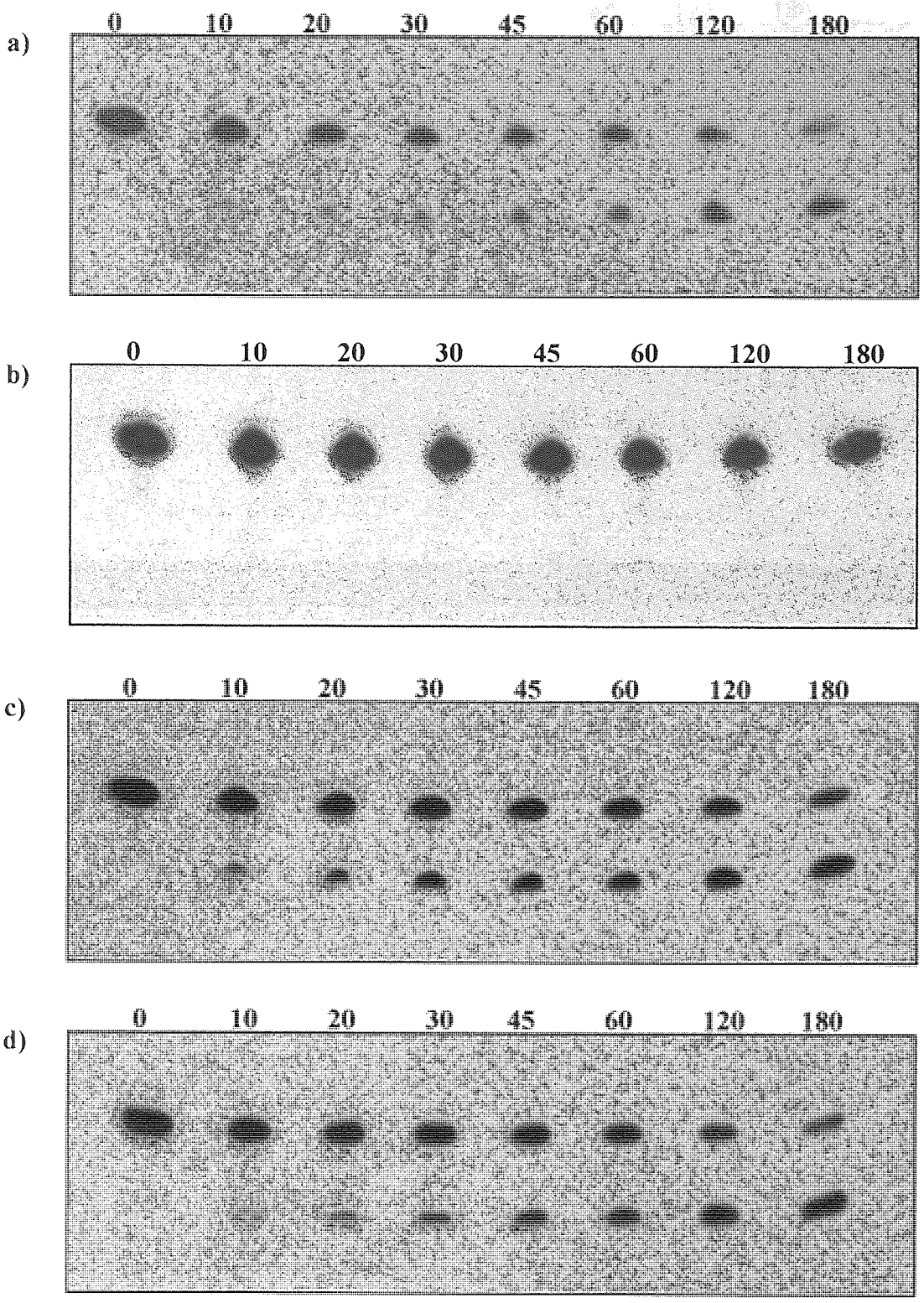


Figure 3.2 Representative autoradiographs of single turnover cleavage reactions of the 10-23 based DNA enzymes.

In vitro cleavage activity of the 10-23 based DNA enzymes, **a)** unmodified, **b)** fully phosphorothioated, **c)** PS arms, **d)** 3 PS linkages at the 3'-end.

Reactions were performed in 50mM Tris-HCl (pH 8.0), 100mM $MgCl_2$ at 37°C (section 2.4). Reaction times, in minutes are given above the lanes. Reactions were separated using denaturing PAGE (section 2.3.1) and quantified using densitometric analysis of the autoradiographs (section 2.3.2.2).

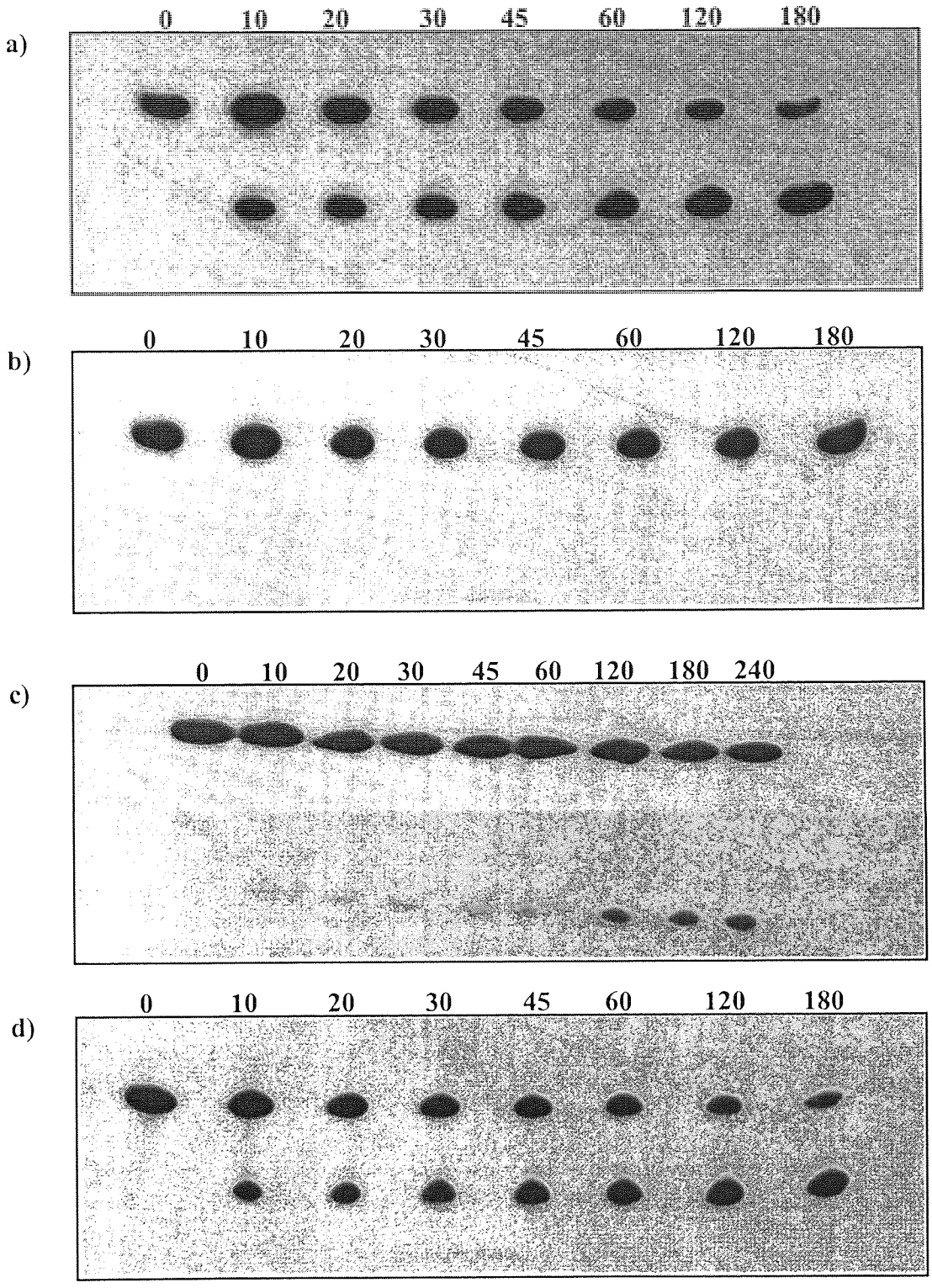


Figure 3.3 Representative autoradiographs of single turnover cleavage reactions of the 8-17 based DNA enzymes.

In vitro cleavage activity of the 8-17 based DNA enzymes, **a)** unmodified, **b)** fully phosphorothioated, **c)** PS arms, **d)** 3 PS linkages at the 3'-end.
Reactions were performed in 50mM Tris-HCl (pH 8.0), 100mM $MgCl_2$ at 37°C (section 2.4). Reaction times, in minutes are given above the lanes. Reactions were separated using denaturing PAGE (section 2.3.1) and quantified using densitometric analysis of the autoradiographs (section 2.3.2.2).

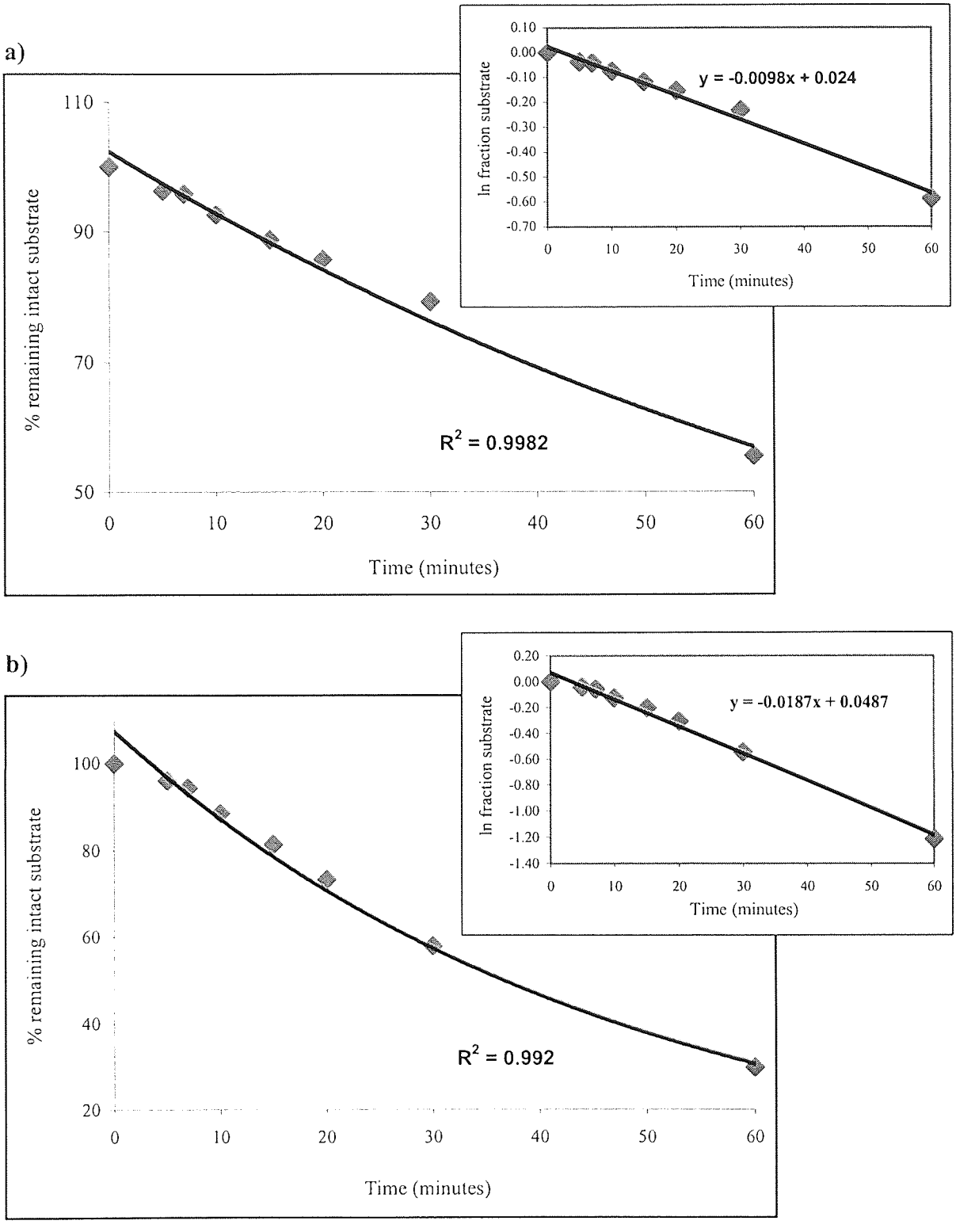


Figure 3.4 ***In vitro*** **activity profiles of the unmodified a) 10-23 and b) 8-17 DNA enzymes.**

To quantify the extent of substrate cleavage and to allow the activity half-life to be determined, the intensity of autoradiograph bands was measured. The percentage of remaining intact substrate, under single turnover conditions, was plotted as a function of time. Inset graphs show semi-natural (ln) log plot of the same data.

Activity profiles such as in Figure 3.4 were used to calculate estimated activity half times for the two DNA enzymes of various chemistries, (refer to Table 3.1) targeted against short complementary substrates under single turnover conditions. The results of this are summarised in Table 3.2 below.

DNA enzyme	Activity half-time ($t_{1/2}$) *in vitro* (minutes)
HR 1 (PO)	81.0 ± 7.2
HR 2 (PS)	No measurable cleavage
HR 3 (PO with PS arms)	157.9 ± 9.8
HR 4 (3 PS at 3'-end)	96.0 ± 9.4
HR 13 (PO)	36.5 ± 3.4
HR 14 (PS)	No measurable cleavage
HR 15 (PO with PS arms)	2007.9 ± 289.8
HR 16 (3 PS at 3'-end)	40.2 ± 7.5

Table 3.2 Cleavage activities of DNA enzymes targeted against short complementary substrates under single turnover conditions.
Activity is expressed as cleavage half time against short complementary substrates. Values represent the mean of three independent experiments ± SD.

As illustrated by these results, introducing modifications impairs cleavage activity and if the catalytic core is PS modified, activity diminishes to the extent of being undetectable with the experimental procedures utilised. Thus modification needs to be selective. In the unmodified versions of the DNA enzymes, the 8-17 based HR13 has a shorter $t_{1/2}$ and thus a quicker initial reaction rate than the 10-23 based HR1. However, from the autoradiographs it appears that after a period of two hours, a similar amount of cleavage is achieved. The activity of both motifs is adversely affected by increasing numbers of PS linkages, but it is interesting to note that the 10-23 motif seems more

resistant to PS modification of the binding arms than the 8-17. Activity is reduced to a far lesser extent from HR1 to HR3 than from HR13 to HR15.

These results can be compared to those obtained by Sioud and Leirdal (2000). The authors designed 10-23 based DNA enzymes to target protein kinase Cα in malignant cells. They synthesised various chemistries in an effort to increase stability. The results of the *in vitro* activity assays are not directly comparable as the investigators used different reaction conditions, in that they assessed activity under multiple turnover conditions with reactions performed at lower pH and lower $MgCl_2$ concentration to those used here. However, parallels can be drawn. The authors (Sioud and Leirdal, 2000) report of a completely PS modified DNA enzyme exhibiting very little cleavage activity. They also report that the introduction of three PS links at the 3'-end had no significant effect on activity. Sioud and Leirdal (2000) found their DNA enzyme modified with PS links in the antisense arms and within the pyrimidine residues of the catalytic core retained cleavage activity albeit with an approximately 10-fold decrease compared to their unmodified DNA enzyme. These results show, as here, that uniform modification cannot be applied to the catalytic domain of these *in vitro* selected catalytic motifs. Also, it can be concluded that a selectively modified DNA enzyme will retain substantial catalytic activity.

These *in vitro* cleavage assays demonstrate that the DNA enzymes designed here are successful in recognising, binding and cleaving their target RNA. The next step towards efficacy of a potential therapeutic molecule is the possession of sufficient extra- and intra-cellular biological stability. Accordingly, the next step in these studies was the assessment of stability of the DNA enzymes.

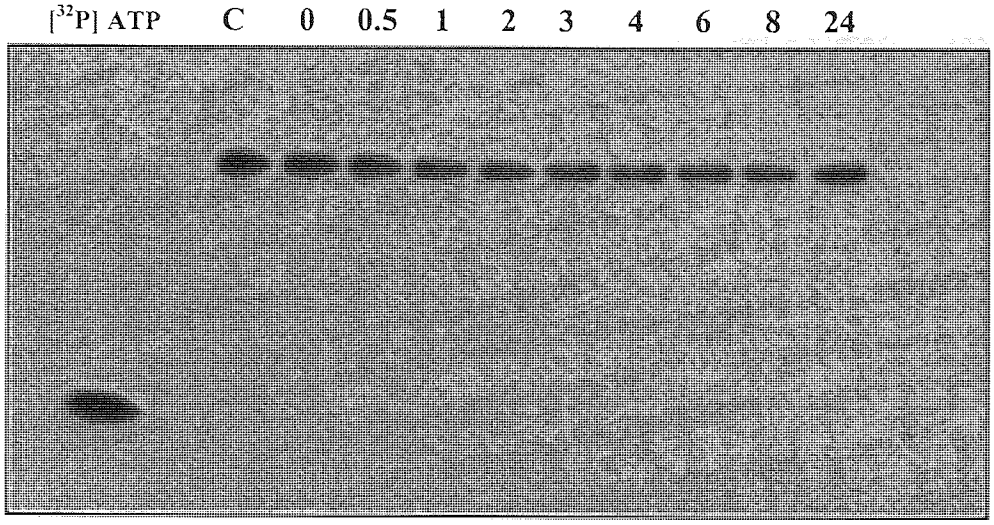
3.4 Stability of the DNA Enzymes

As discussed previously, the efficacy of a drug is usually a balance or compromise between activity and stability. A drug molecule must be sufficiently stable to resist the harsh environment of the biological milieu and remain intact long enough to reach its target site.

The activity of DNA enzymes of various chemistries has been tested *in vitro*. An assessment of stability will complement these data and enable a better evaluation of which chemistry warrants further investigation. Thus the stabilities of both the 10-23 and 8-17 based DNA enzymes of all chemistries were assessed in serum. Serum displays a substantial nuclease activity and serum stability is frequently used in the antisense field as a general indication of the extra cellular stability of molecules *in vivo*. The nuclease activity of sera derived from various species varies, but foetal bovine serum (FBS) is reported to have higher nuclease activity than mouse or even human serum (Crooke, 1992). Hence FBS was used in these studies as a stringent test of stability.

In subsequent analysis of the two DNA enzymes, activity in cells will be assessed. Cell work such as efficacy experiments or uptake assessments will be carried out either in serum-free or 10% serum-containing DMEM. Hence stability experiments were carried out in both of these media as described in section 2.8. Briefly, 3'-end labelled DNA enzymes were incubated with the appropriate media at 37°C for 24 hours. At specific time points, aliquots were removed and quenched by adding to an equal volume of ice-cold formamide buffer. The samples were visualised using denaturing PAGE and autoradiography (sections 2.3.1-2.3.2). Autoradiographs were subjected to densitometric analysis for quantification of bands (section 2.3.2.2). The following figures show representative autoradiographs depicting the degradation profiles of the DNA enzymes of various chemistries. There appears to be extra banding in some of the serum profiles. In fact this was a feature of the modified DNA enzymes, and may be due to a lower labelling efficiency of PS modified nucleotides.

a)



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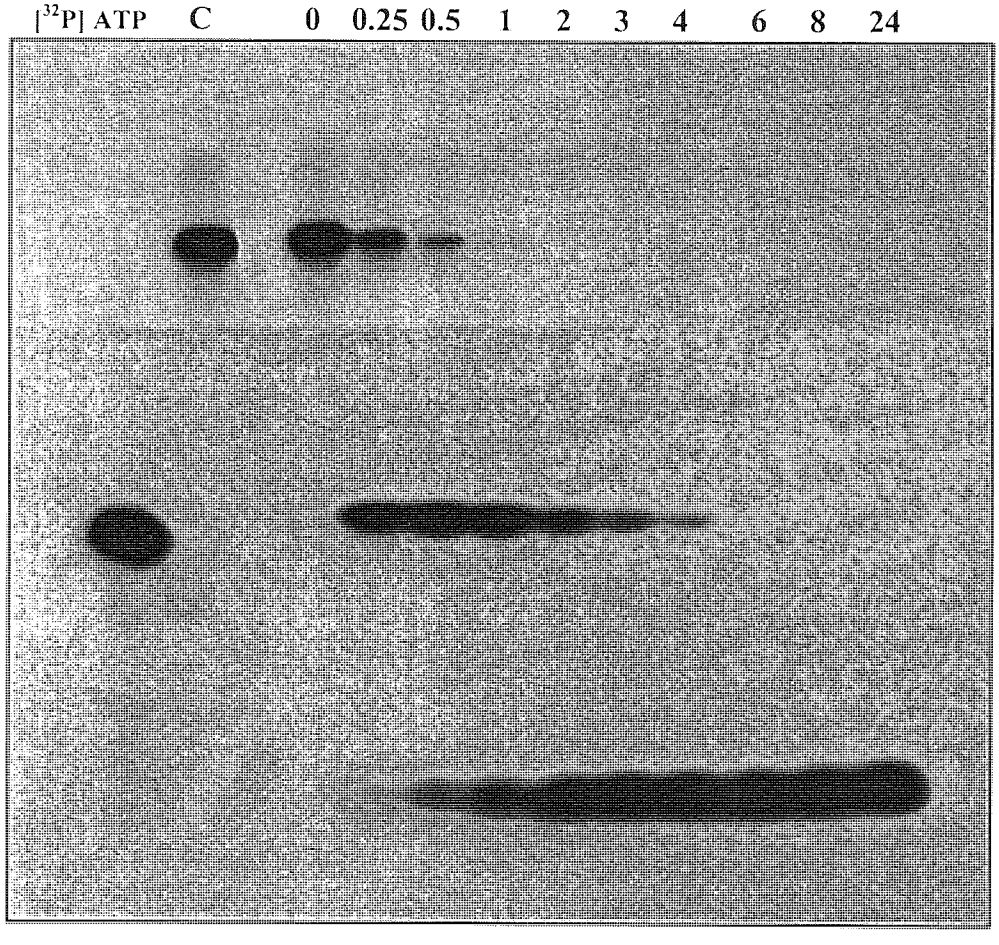
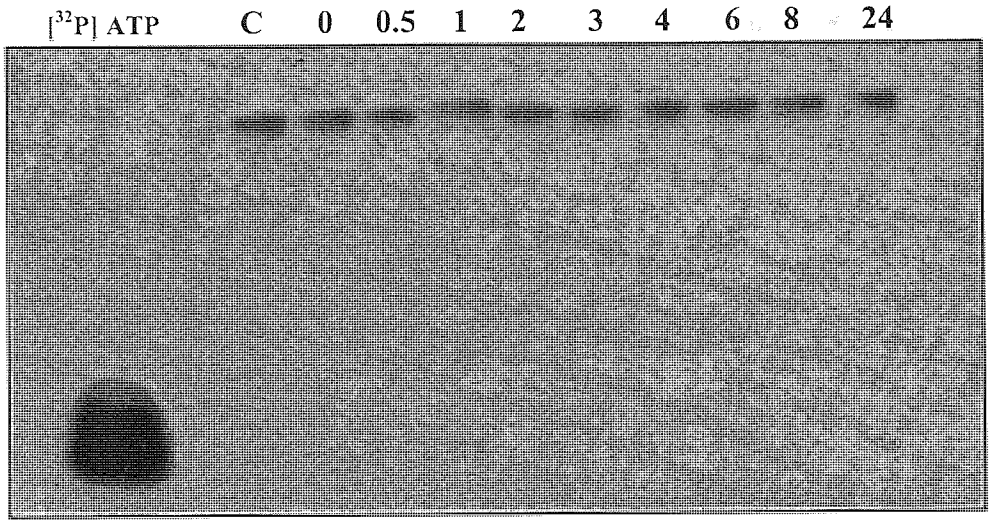


Figure 3.5 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR1.
Representative autoradiographs illustrating stability of 3'-end labelled HR1 *i.e. 10-23 of PO chemistry* in media containing **a)** no serum and **b)** 10% serum. 500nM DNAzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents DNA enzyme not exposed to any media. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

a)



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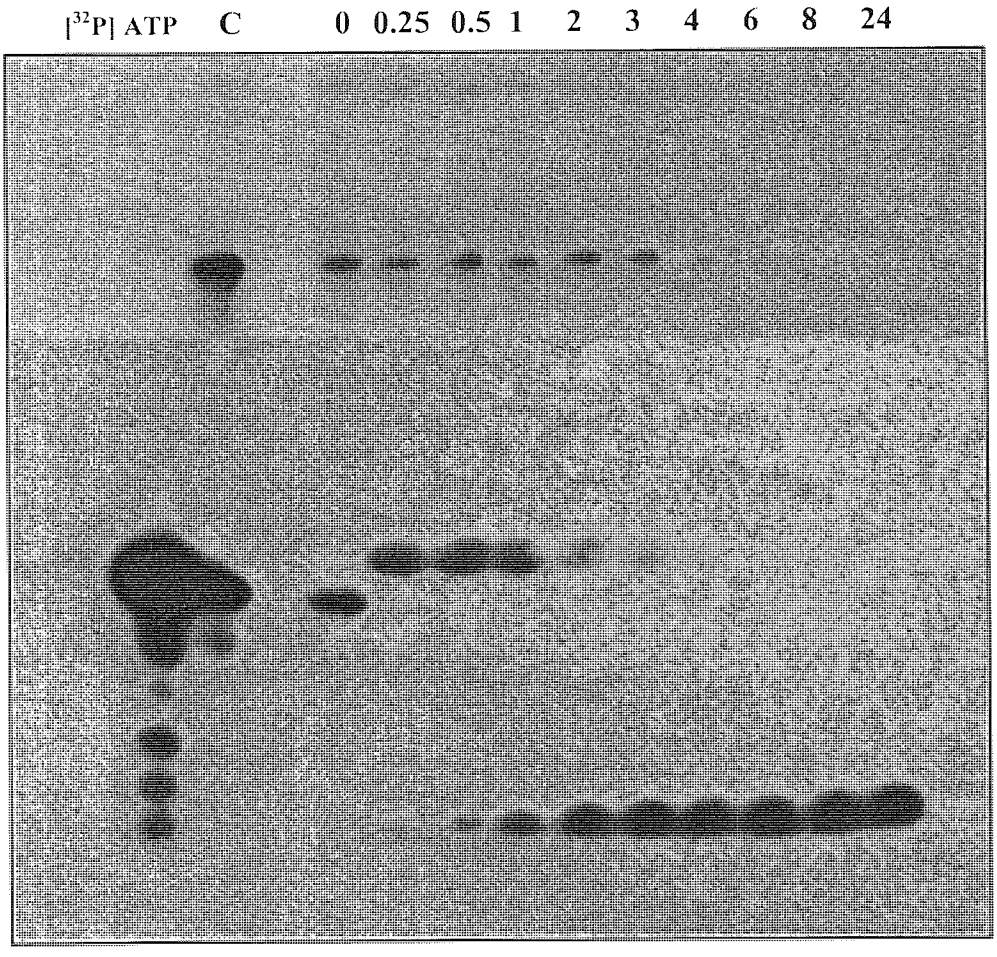
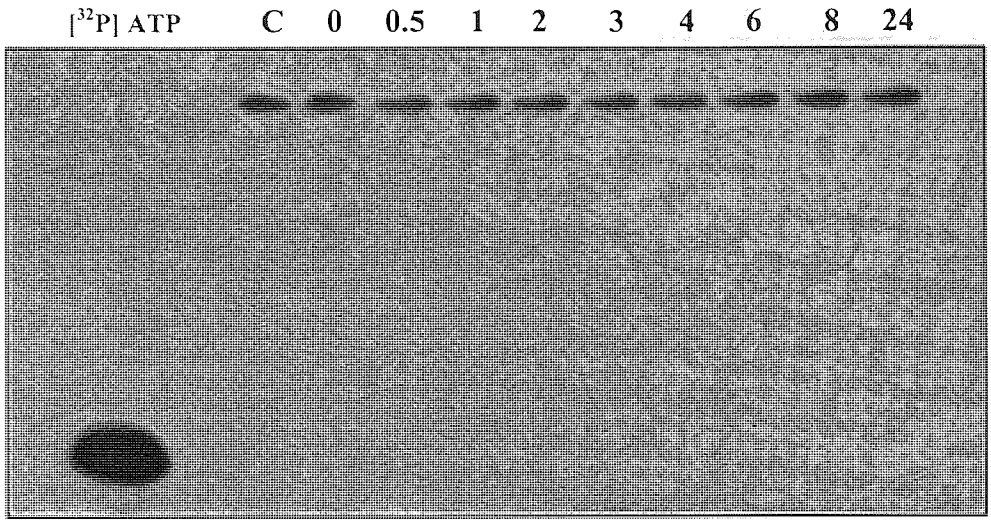


Figure 3.6 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR2.
Representative autoradiographs illustrating stability of 3'-end labelled HR2 *i.e. 10-23 of PS chemistry* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed DNA enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

a)



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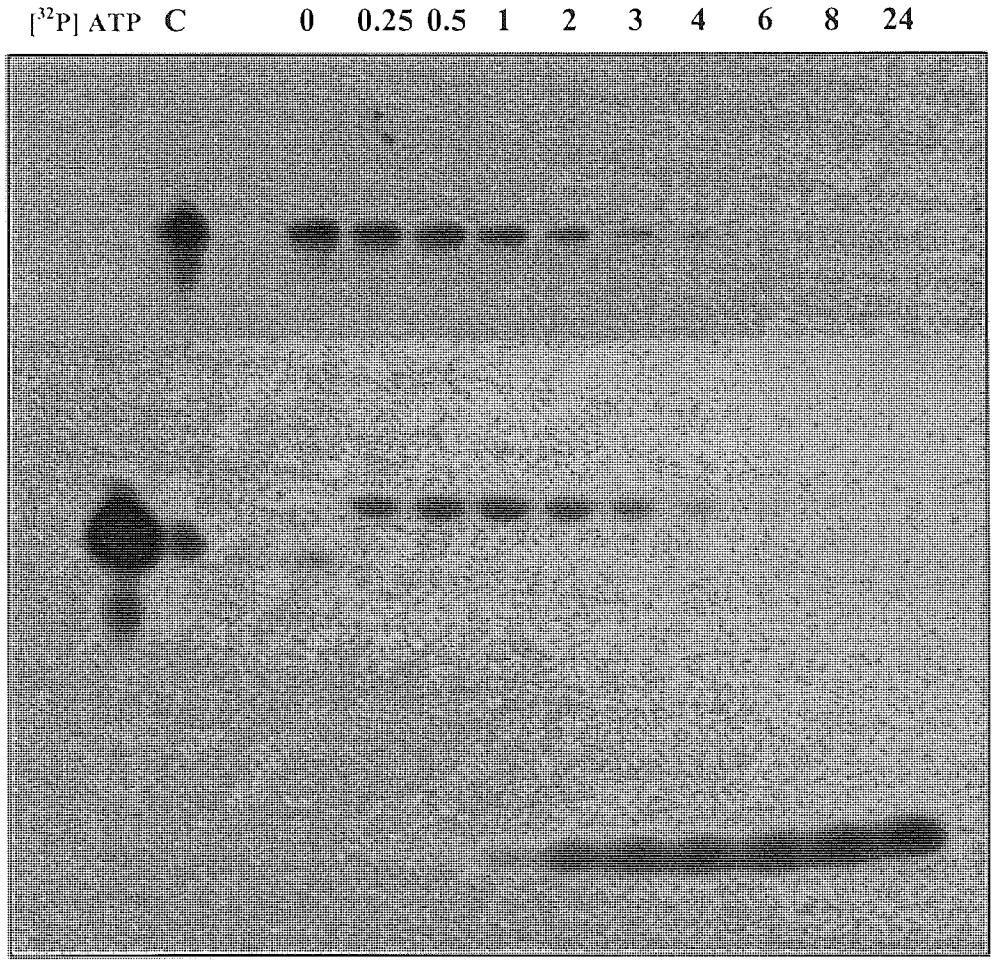


Figure 3.7 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR3.
Representative autoradiographs illustrating stability of 3'-end labelled HR3 *i.e. 10-23 of PO chemistry with PS arms* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed DNA enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

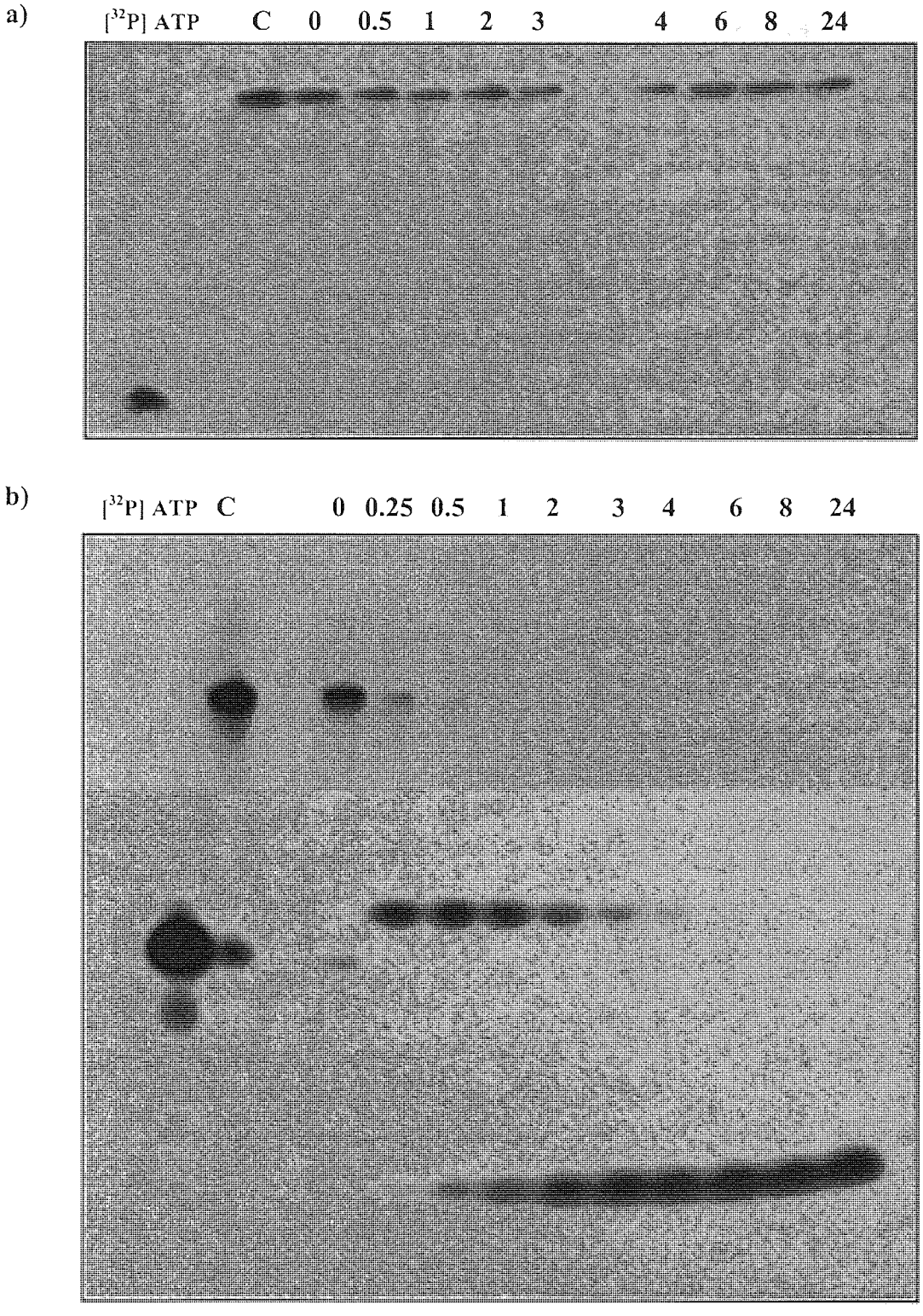
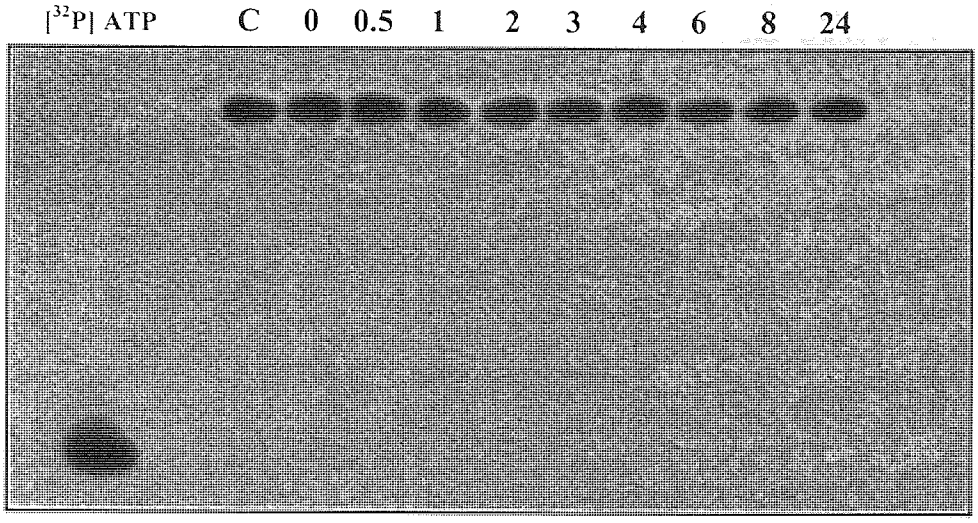


Figure 3.8 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR4.
Representative autoradiographs illustrating stability of 3'-end labelled HR4 *i.e. 10-23 of PO chemistry with 3 PS links at the 3'-end* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed DNA enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

a)



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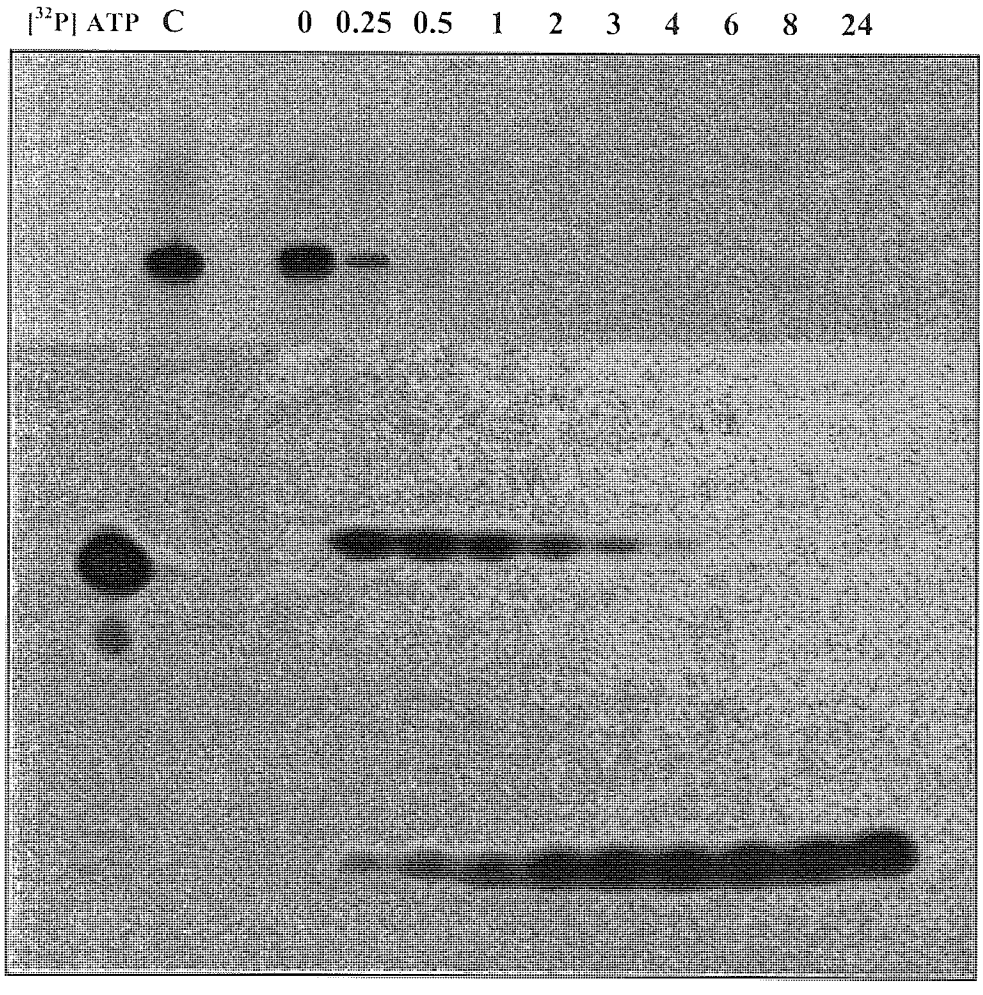


Figure 3.9 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR13.
Representative autoradiographs illustrating stability of 3'-end labelled HR13 *i.e. 8-17 of PO chemistry* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

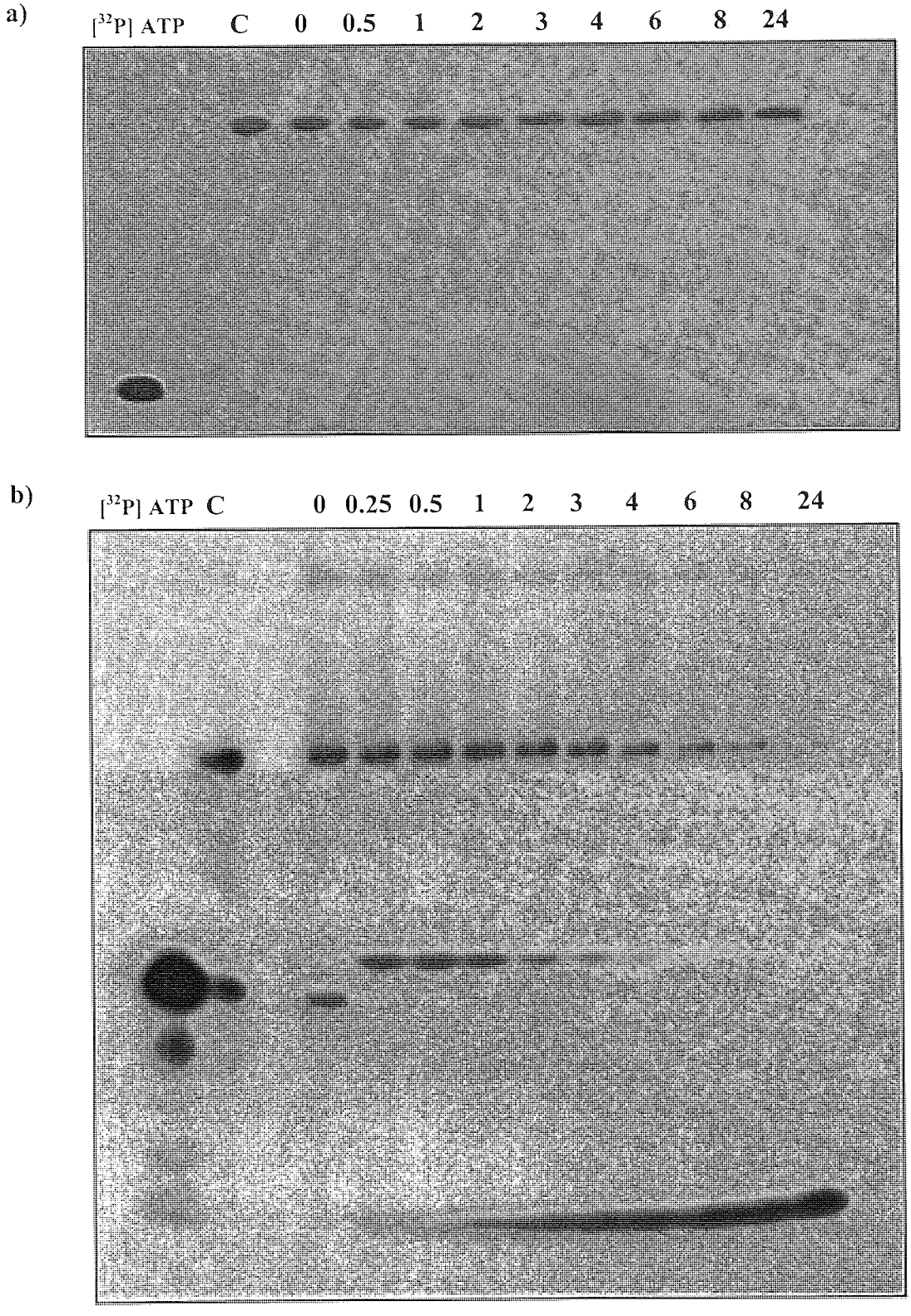
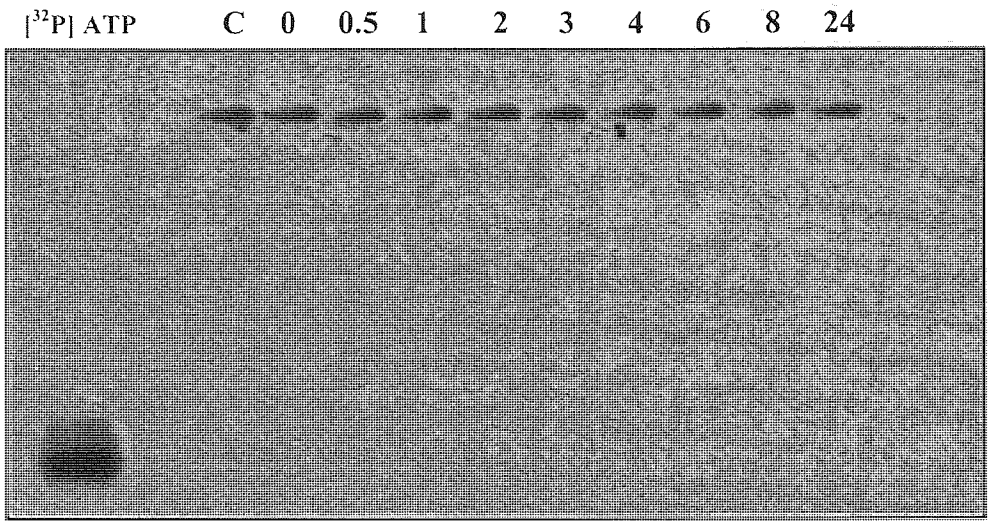


Figure 3.10 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR14.
Representative autoradiographs illustrating stability of 3'-end labelled HR14 *i.e. 8-17 of PS chemistry* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

a)



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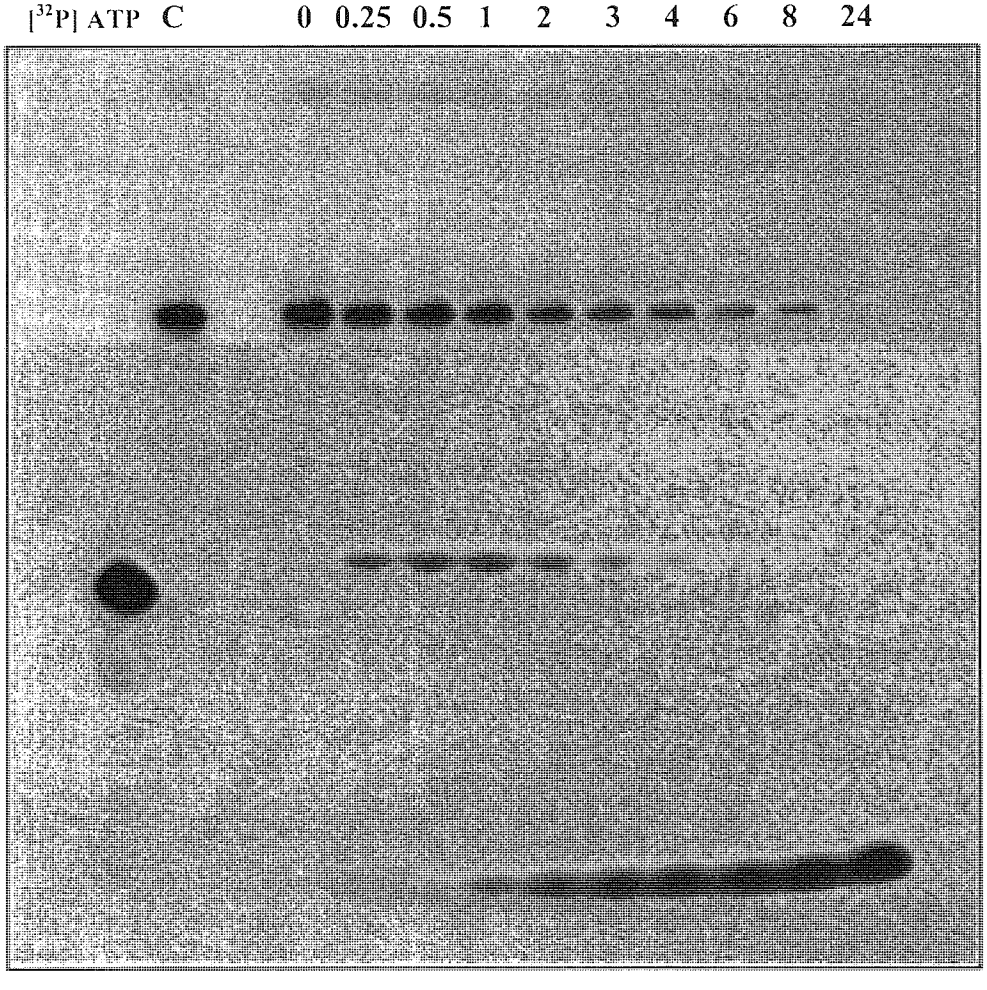
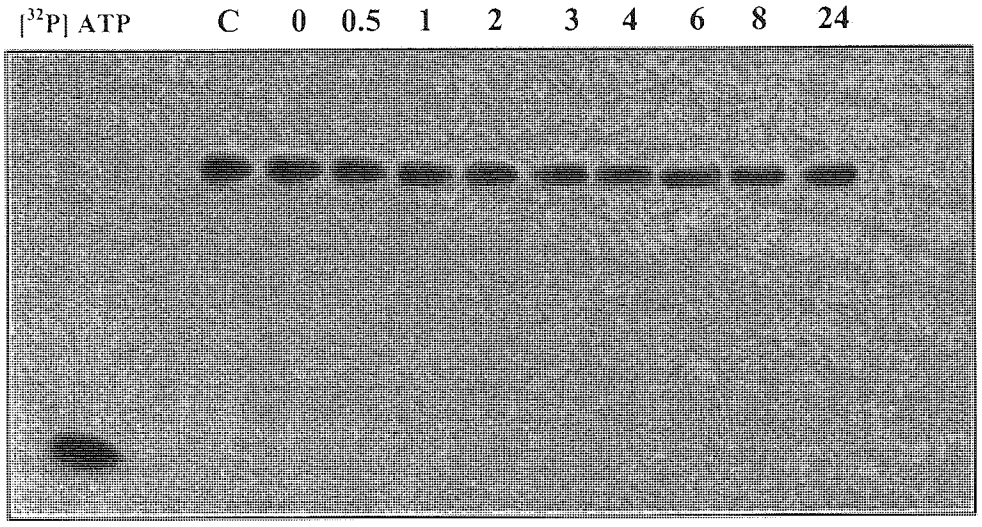


Figure 3.11 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR15.
Representative autoradiographs illustrating stability of 3'-end labelled HR15 *i.e. 8-17 of PO chemistry with PS arms* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

a)



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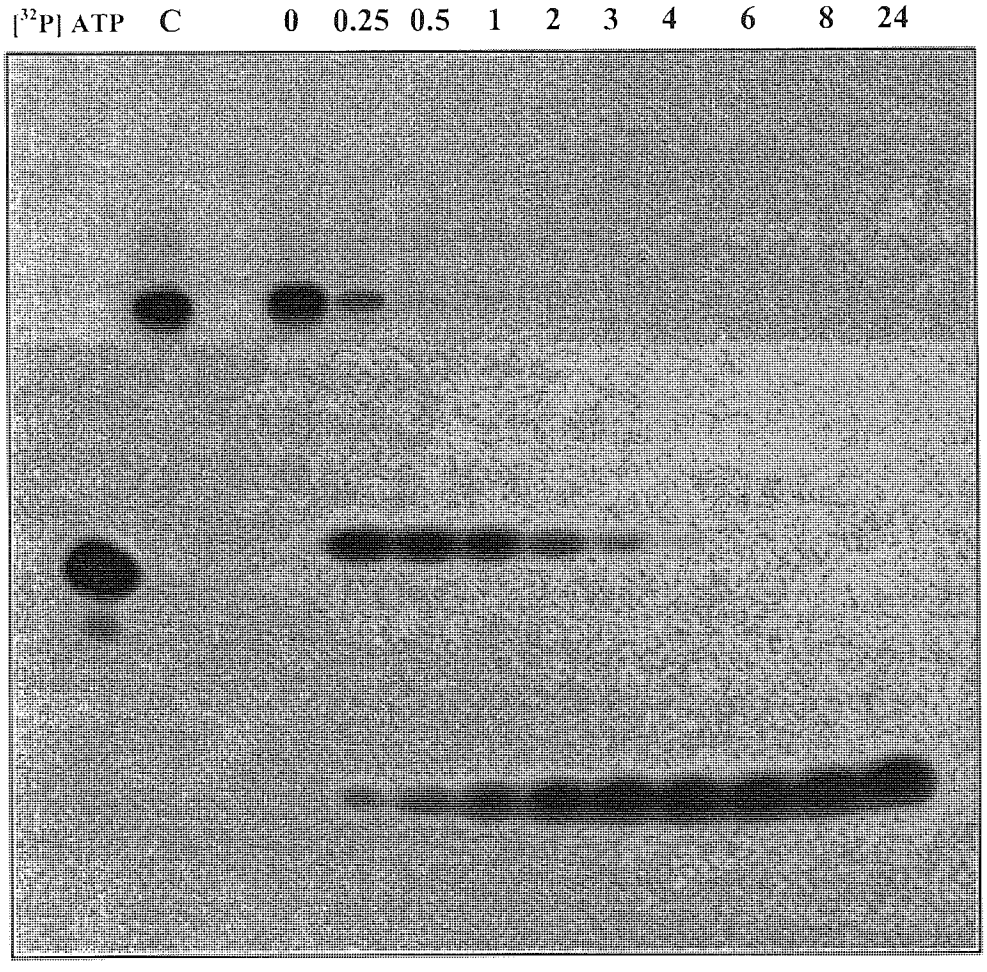


Figure 3.12 Stability of 3'-end [^{32}P]-labelled DNA enzyme-HR16.
Representative autoradiographs illustrating stability of 3'-end labelled HR16 *i.e. 8-17 of PO chemistry with 3 PS links at the 3'-end* in media containing **a)** no serum and **b)** 10% serum. 500nM DNA enzyme was added to media and incubated at 37°C for 24 hours. Aliquots were removed at the times (hours) indicated and quenched with ice-cold formamide buffer. The control C represents unexposed DNA enzyme. Degradation profiles were obtained using denaturing PAGE, and autoradiographs analysed using densitometry.

It is clear from the degradation profiles that PS modifications substantially increase the stability of DNA enzymes. To summarise, in serum-free media all chemistries were found to be stable at 37°C from 0-24 hours. In 10% FBS-containing media, there was no appreciable difference of stability between the two DNA enzymes, and approximate stability half-lives (determined in at least two independent experiments) were found to be:

unmodified PO chemistry (HR1 and 13):	**10 minutes**
fully modified PS chemistry (HR2 and 14):	**5 hours**
PO with PS arms (HR3 and 15):	**2.5 hours**
PO with 3 PS links at the 3'-end (HR4 and 16):	**12 minutes**

Considering the activity and stability data together, several inferences can be made. It is apparent that introducing a few PS links at the 3'-end offers no substantial protection against nuclease degradation but does, as demonstrated earlier, inhibit cleavage activity to a small extent. Fully modifying the DNA enzymes with PS links greatly increases serum stability but at the expense of cleavage activity. Introducing PS modifications in the antisense arms increases the serum stability of the DNA enzymes whilst allowing them to retain considerable activity. HR3 and HR15 suffer some loss of activity with respect to the wild-type DNA enzymes, but higher/faster activity is of little value if the drug is not stable enough to reach its target. Thus, PO chemistry with PS linkages in the antisense arms was chosen as the best compromise between activity and stability.

The stabilities found here are inferior to some reports in the literature. One study (Sioud and Leirdal, 2000) estimated the stability half-life of a fully PS modified DNA enzyme in 10% FBS to be in excess of 24 hours. The discrepancy between the stability data obtained with other reports in the literature may be attributable to variation in sera. It is generally acknowledged that nuclease activity in FBS can vary considerably between batches hence making direct comparisons between studies difficult. Notably however, the authors came to similar conclusions regarding the balance between activity and stability. They chose a partially PS modified DNA enzyme to study further and concluded that the deleterious effect of selective PS modification on *in vitro* activity did

not translate to efficacy in cells. Also, they reported higher stability in human serum than FBS for all DNA enzyme chemistries, which is encouraging for future potential *in vivo* applications.

For the purposes of these studies, the PO core with PS arms of the 10-23 and 8-17 DNA enzymes, HR3 and HR15 respectively, was chosen as the chemistry with the best balance between activity and stability. Hence the HR3 and HR15 enzymes were further characterised and studied.

3.5 Specificity of Cleavage Activity

Various control DNA enzymes were synthesised for activity comparisons of HR3 and HR15. To reiterate, these controls (see Table 3.1 for details), with respect to both DNA enzyme motifs were:

1. Equivalent antisense sequence *i.e.* complementary base-pairing arms without the catalytic core. This was of full PS chemistry.
2. Sense control *i.e.* active catalytic core with binding arms of a sense orientation. This, similar to HR3 and HR15, was of PO chemistry with PS arms.
3. Scrambled control *i.e.* complementary arms with a catalytic core that has been inactivated due to scrambling of the nucleotide sequence. Again, this was PO chemistry with PS arms.

These represent the usual controls used in the literature for antisense applications. Appropriate controls are vital in the design of antisense experiments to differentiate between real and non-specific activities. This is particularly true when using PS modified deoxynucleotides as these have been shown to result in lower specificity of DNA enzymes despite higher nuclease resistance (Warashina *et al.*, 1999). The controls will also help discern the mechanism of action of DNA enzymes as cleavage, antisense inhibition or both. These control molecules were tested using the *in vitro* cleavage reactions to establish specificity of action of HR3 and HR15. The standard

methodology of cleavage assays was used as described in section 2.9. Briefly, *in vitro* activity assays were carried out with excess of enzyme over substrate (2μM:2nM) with 100mM $MgCl_2$, at 37°C for 1 hour. Reactions were stopped by addition of formamide buffer and analysed using denaturing PAGE. The representative autoradiographs from these reactions are illustrated in Figure 3.13.

Cleavage is only observed (see Figure 3.13) with the active DNA enzymes of PO chemistry with PS links in the antisense arms. The three controls, antisense sequence, sense and scrambled showed no cleavage activity. Also, the active DNA enzymes had no effect when reacted with non-target RNA substrate of a similar length. These experiments clearly demonstrate the sequence specific action of the active DNA enzymes. Similarly, Toyoda *et al.* (2000) recorded no cleavage activity associated with an equivalent antisense sequence without the DNA enzyme catalytic core. Also, Dash *et al.* (1998) documented similar results when testing the *in vitro* cleavage of a 178bp HIV-1 Env transcript by DNA enzymes. No cleavage was seen when using an antisense construct lacking the catalytic motif at equivalent concentrations to the active DNA enzyme. Also, when the active 10-23 based DNA enzyme was used with non-target regions of HIV RNA, no cleavage was observed. The investigators reported similar results at a later date, obtained when using a comparable DNA enzyme targeted to a different region of HIV-1 (Goila and Banerjea, 1998).

Thus far it has been shown that HR3 and HR15 are active *in vitro* against a short RNA substrate, remain stable in serum for a few hours, and their activity is dependent on sequence specificity of the substrate binding arms as well as the presence of their respective catalytic domains. Before the next stage of these studies, factors affecting the cleavage activity of HR3 and HR15 were investigated.

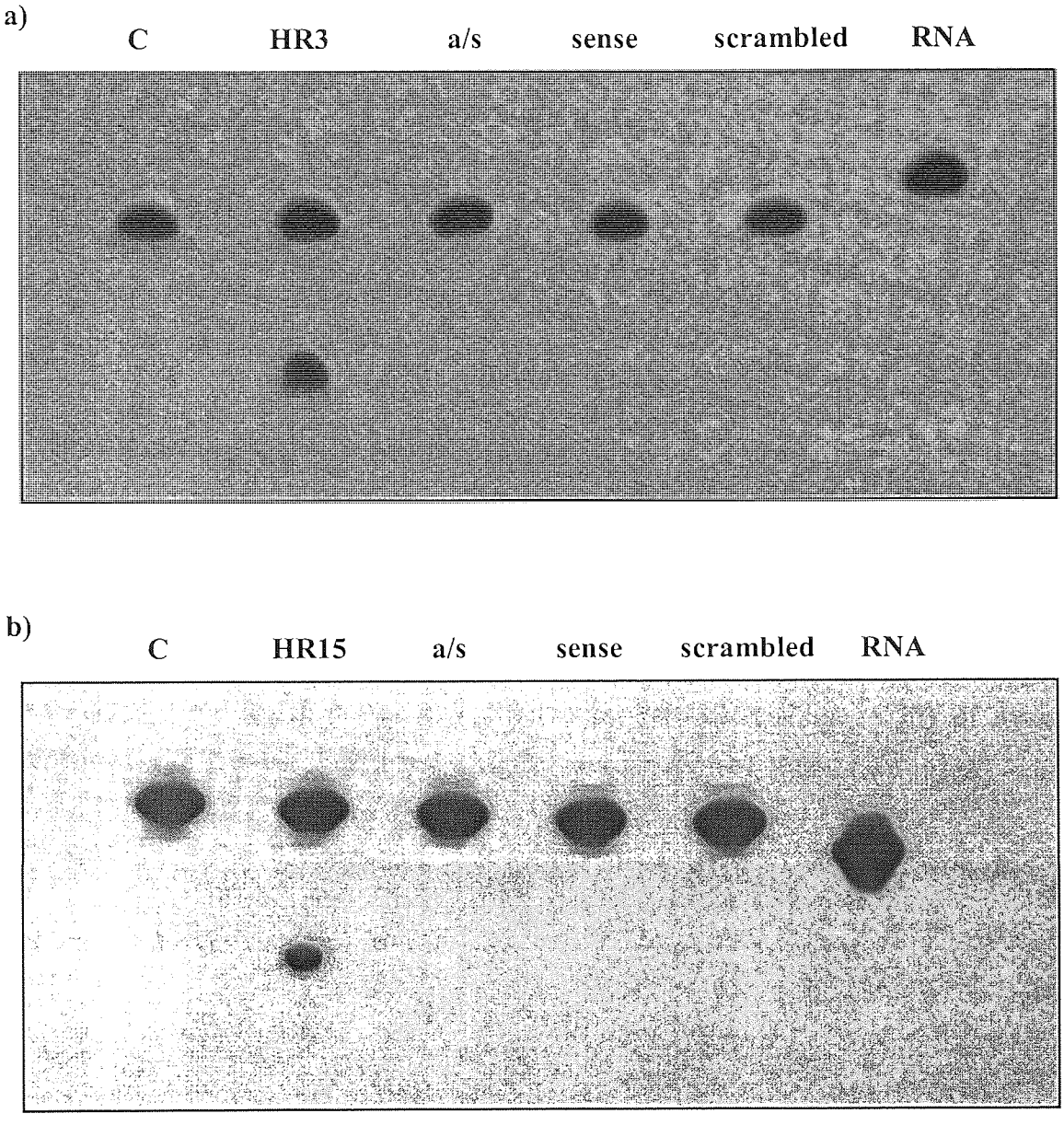


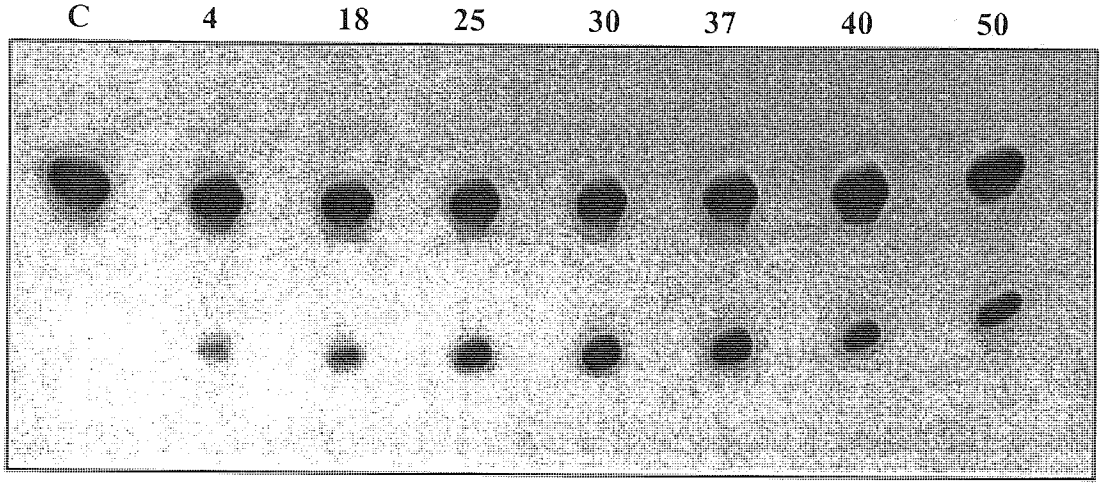
Figure 3.13 Autoradiographs showing specificity of catalytic cleavage by HR3 and HR15.

The sequence-specific cleavage by DNA enzymes of the chosen PO/PS chemistry and control DNA enzymes was assessed using short RNA targets. *In vitro* activity assays were carried out with excess of enzyme over substrate (2μM:2nM) with 100mM $MgCl_2$, at 37°C for 1 hour. Reactions were stopped by addition of formamide buffer and analysed using denaturing PAGE. Lane **C** indicates control RNA, not exposed to DNA enzyme. HR3 and HR15 are the active DNA enzymes containing the 10-23 and 8-17 motifs respectively of the PO/PS chemistry. The control DNA enzymes are: **a/s** *i.e.* the equivalent antisense sequence; **sense** *i.e.* preserved core with sense arms; **scrambled** *i.e.* correct binding arms with scrambled core. **RNA** indicates the active DNA enzyme reacted with non-target RNA of a similar length.

3.6 Factors Affecting Cleavage

Once the specificity of the DNA enzymes was established, cleavage of the chosen PO/PS chemistry was analysed under various conditions, including temperature, pH, $MgCl_2$ concentration and enzyme concentration. Figures 3.14 to 3.21 show autoradiographs and graphs of percentage cleavage with respect to these various factors.

A set of standard conditions was used for the *in vitro* cleavage assays, with a single factor being varied for each experiment to examine the effect on cleavage activity. The standard conditions were single turnover conditions, with enzyme in excess over substrate *i.e.* 2nM substrate:2μM enzyme. The reactions were performed at 37°C over one or two hours. HR15 had quite slow cleavage so the activity was compared after two hours rather than one to ensure any differences were detectable. A pH of 8 was maintained with Tris-HCl buffer and a $MgCl_2$ concentration of 100mM was used.



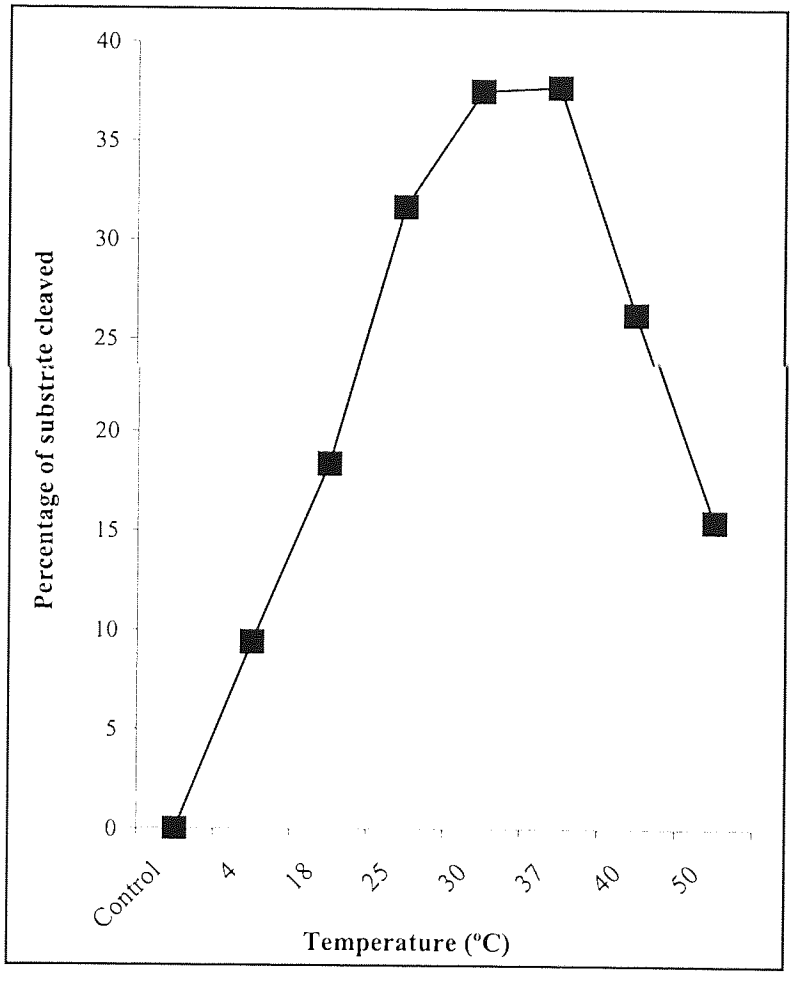
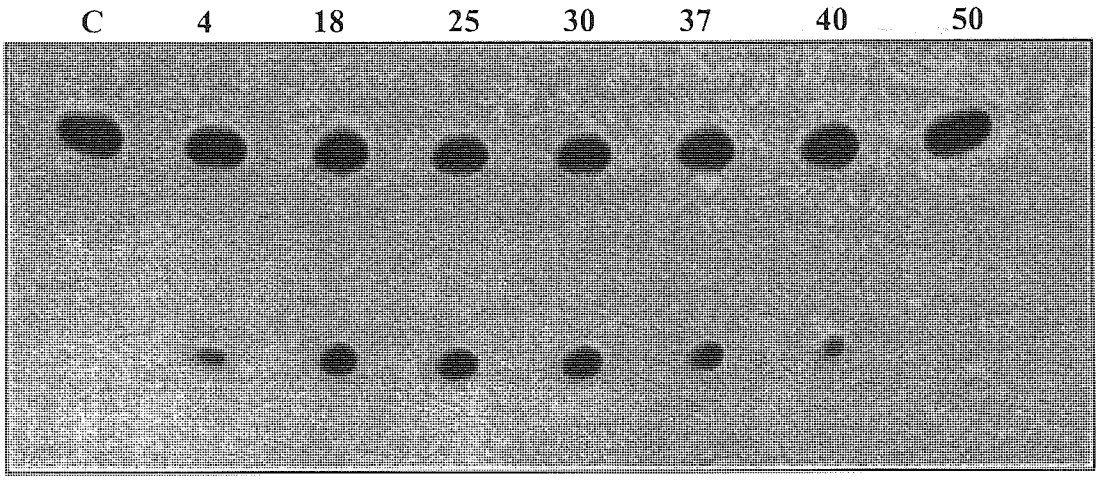


Figure 3.14 Effect of temperature on cleavage activity of HR3.
Autoradiograph showing the effect of various temperatures (given in °C) on the *in vitro* cleavage activity of HR3 (10-23 of PO/PS). Activity was assessed in 50mM Tris-HCl (pH 8.0) with enzyme in excess over substrate (2μM:2nM), 100mM $MgCl_2$ at various temperatures over one hour. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



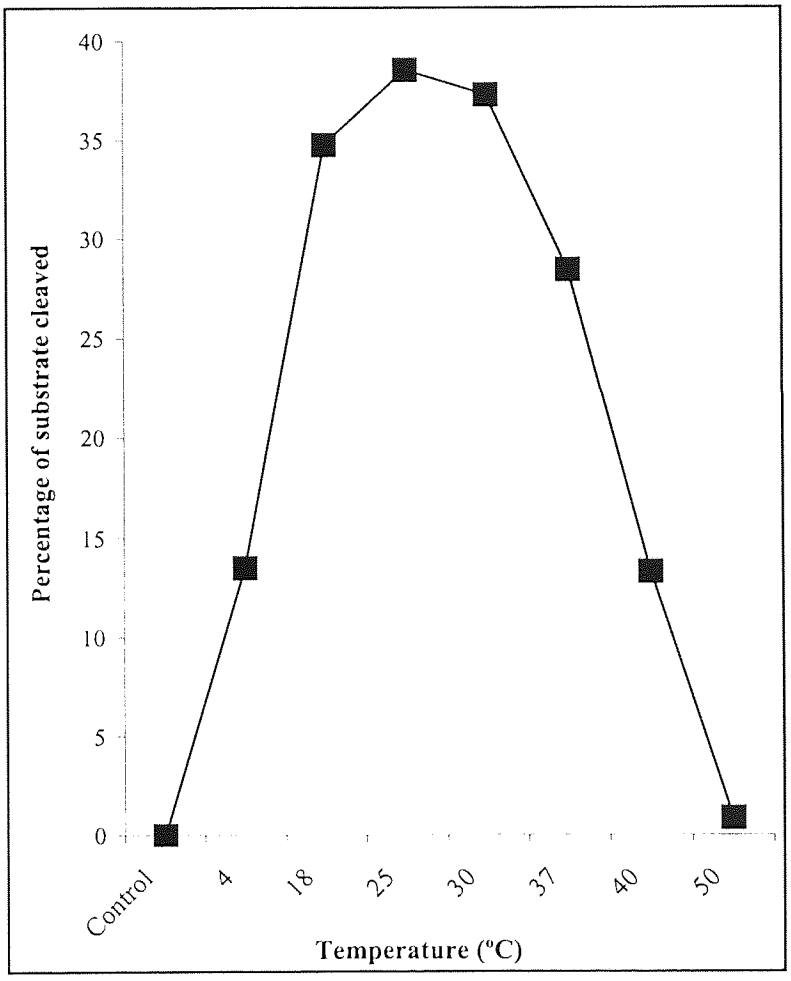
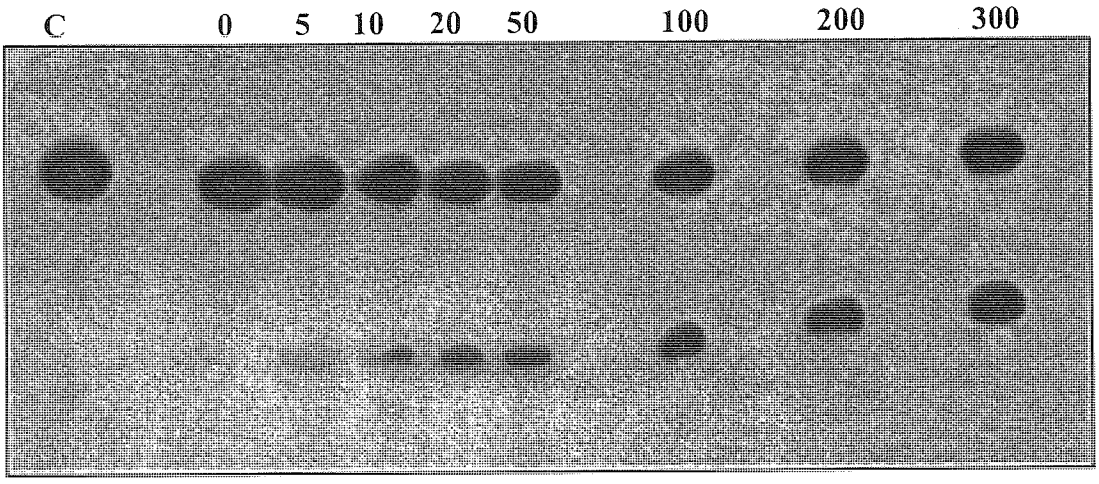


Figure 3.15 Effect of temperature on cleavage activity of HR15.
Autoradiograph showing the effect of various temperatures (given in °C) on the *in vitro* cleavage activity of HR15 (8-17 of PO/PS). Activity was assessed in 50mM Tris-HCl (pH 8.0) with enzyme in excess over substrate (2μM:2nM), 100mM $MgCl_2$ at various temperatures over two hours. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



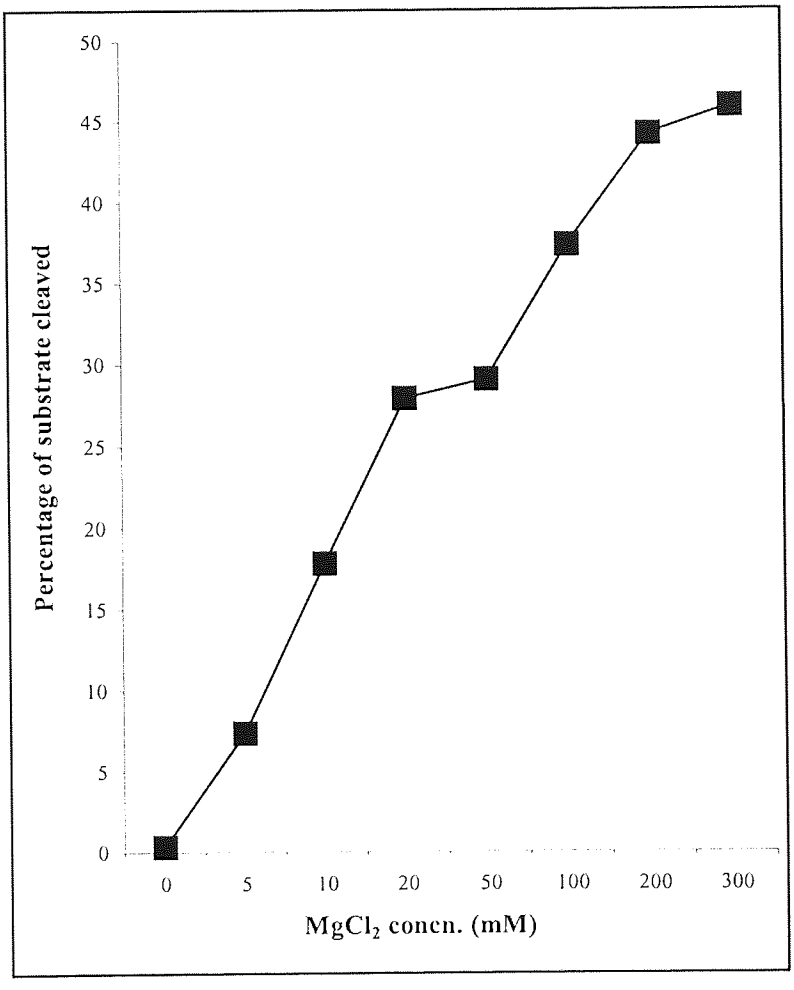
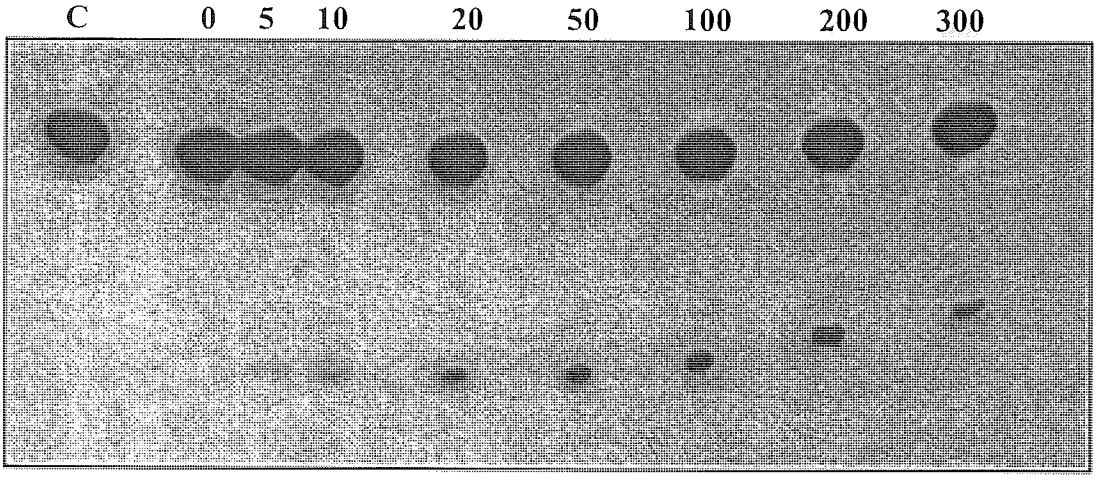


Figure 3.16 Effect of $MgCl_2$ concentration on cleavage activity of HR3.
Autoradiograph showing the effect of various $MgCl_2$ concentrations (given in mM) on the *in vitro* cleavage activity of HR3 (10-23 of PO/PS). Activity was assessed in 50mM Tris-HCl (pH 8.0) with enzyme in excess over substrate (2μM:2nM), at 37°C with various $MgCl_2$ concentrations over one hour. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



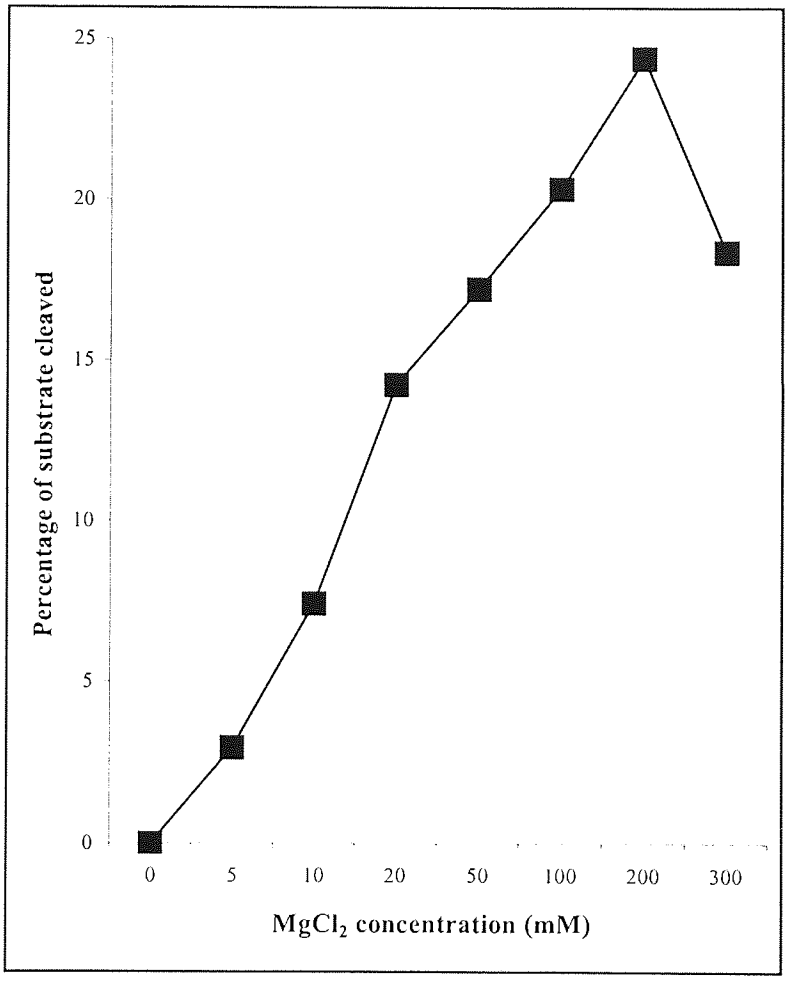


Figure 3.17 Effect of $MgCl_2$ concentration on cleavage activity of HR15. Autoradiograph showing the effect of various $MgCl_2$ concentrations (given in mM) on the *in vitro* cleavage activity of HR15 (8-17 of PO/PS). Activity was assessed in 50mM Tris-HCl (pH 8.0) with enzyme in excess over substrate (2μM:2nM), at 37°C with various $MgCl_2$ concentrations over two hours. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.

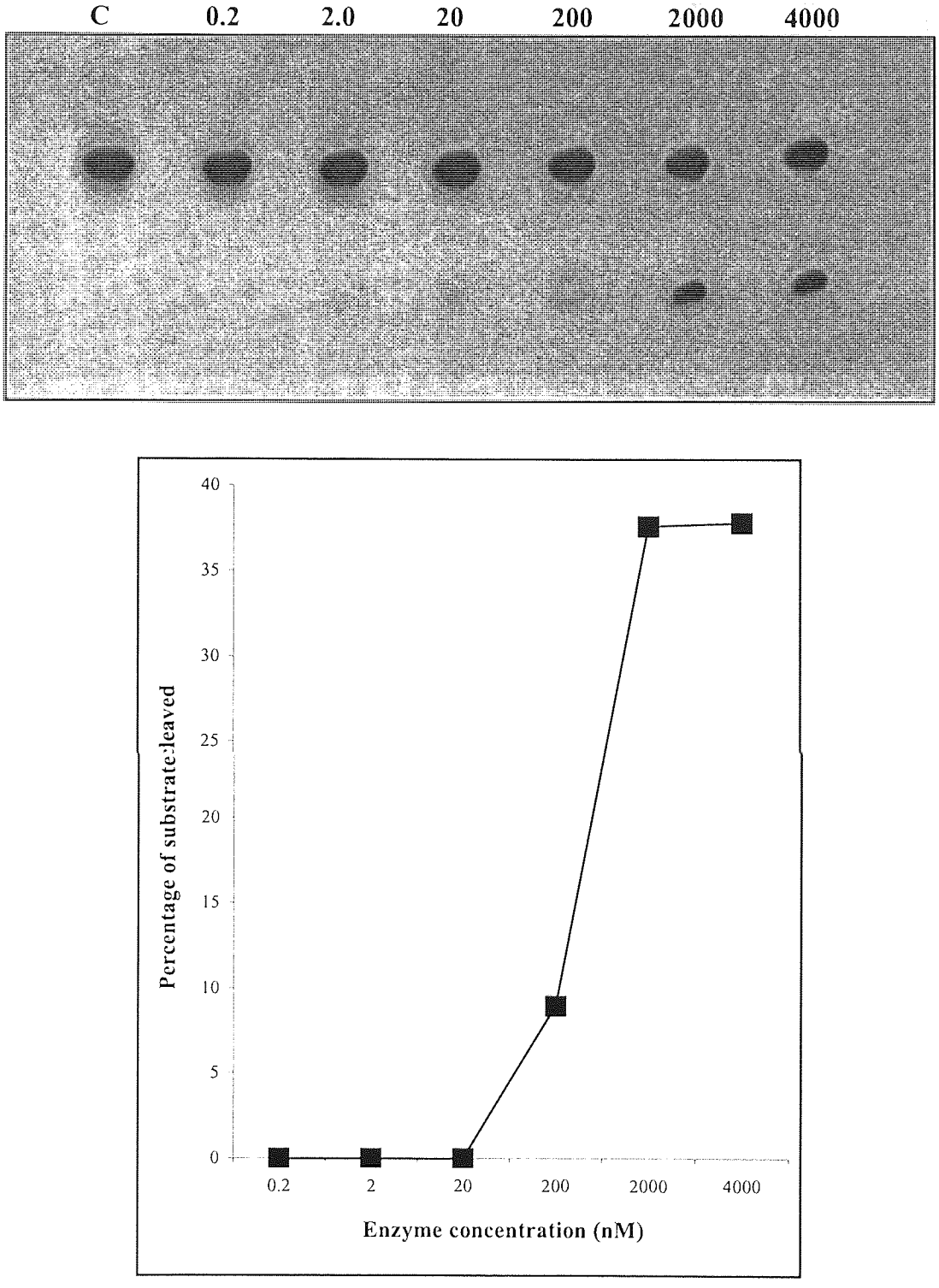
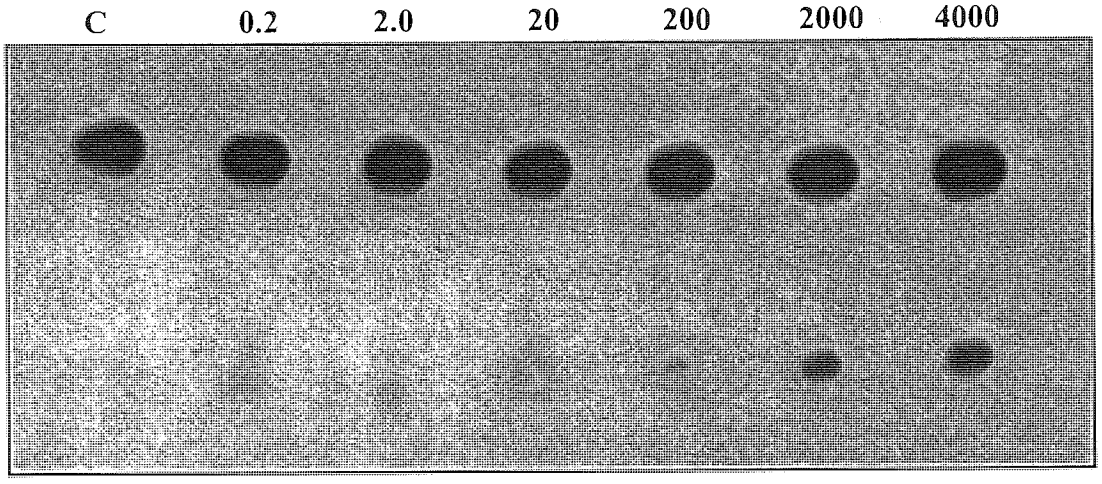


Figure 3.18 Effect of enzyme concentration on cleavage activity of HR3. Autoradiograph showing the effect of various enzyme concentrations (given in nM) on the *in vitro* cleavage activity of HR3 (10-23 of PO/PS). Activity was assessed against 2nM substrate in 50mM Tris-HCl (pH 8.0) with 100mM $MgCl_2$, at 37°C with various concentrations of DNA enzyme, over one hour. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



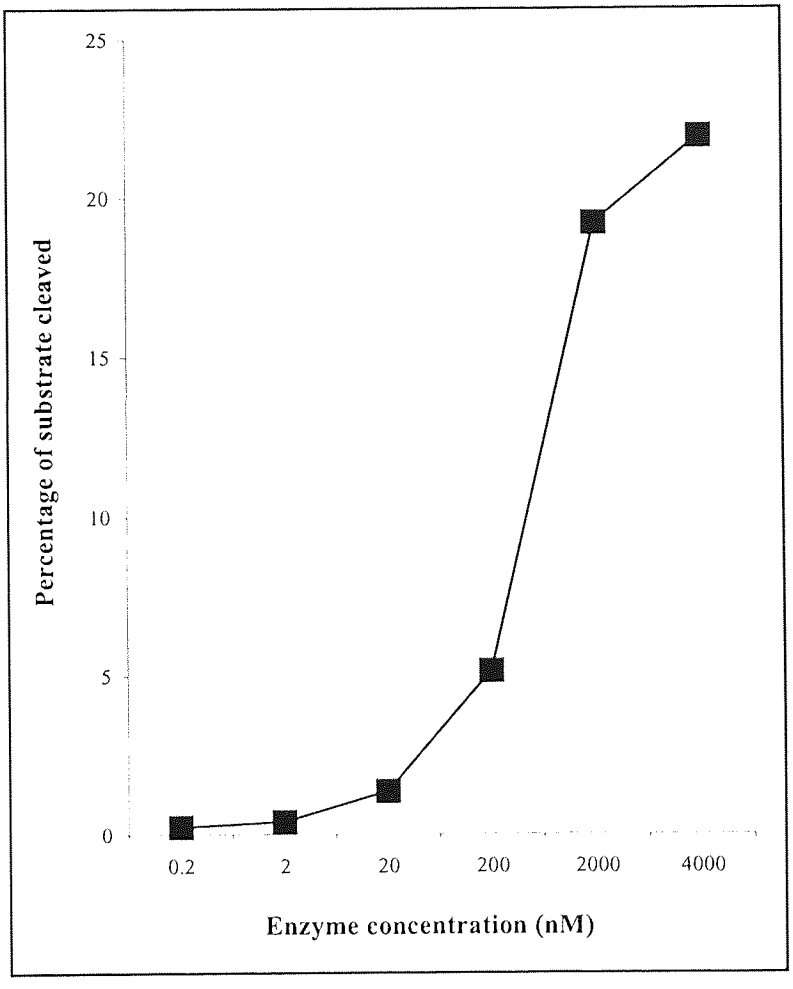
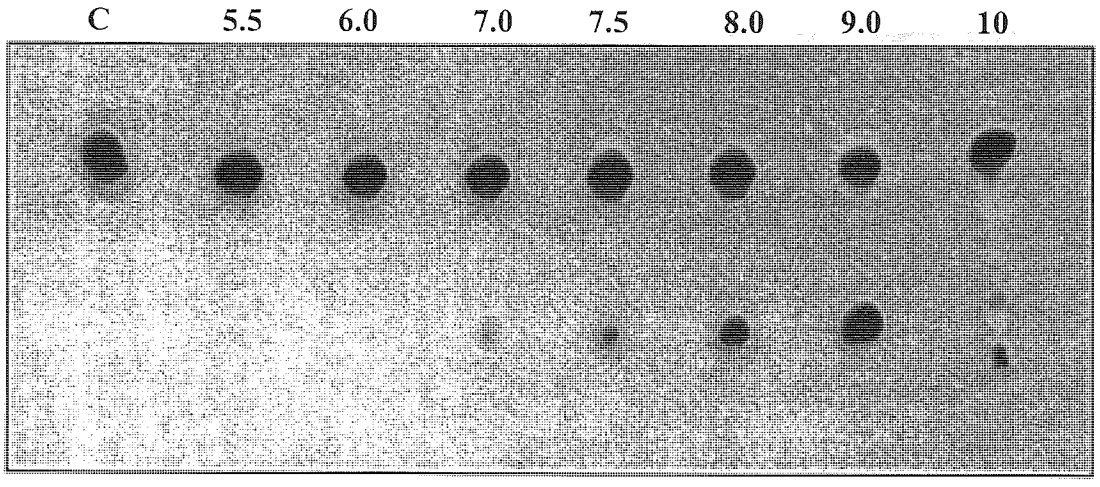


Figure 3.19 Effect of enzyme concentration on cleavage activity of HR15. Autoradiograph showing the effect of various enzyme concentrations (given in nM) on the *in vitro* cleavage activity of HR15 (8-17 of PO/PS). Activity was assessed against 2nM substrate in 50mM Tris-HCl (pH 8.0) with 100mM $MgCl_2$, at 37°C with various concentrations of DNA enzyme, over two hours. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



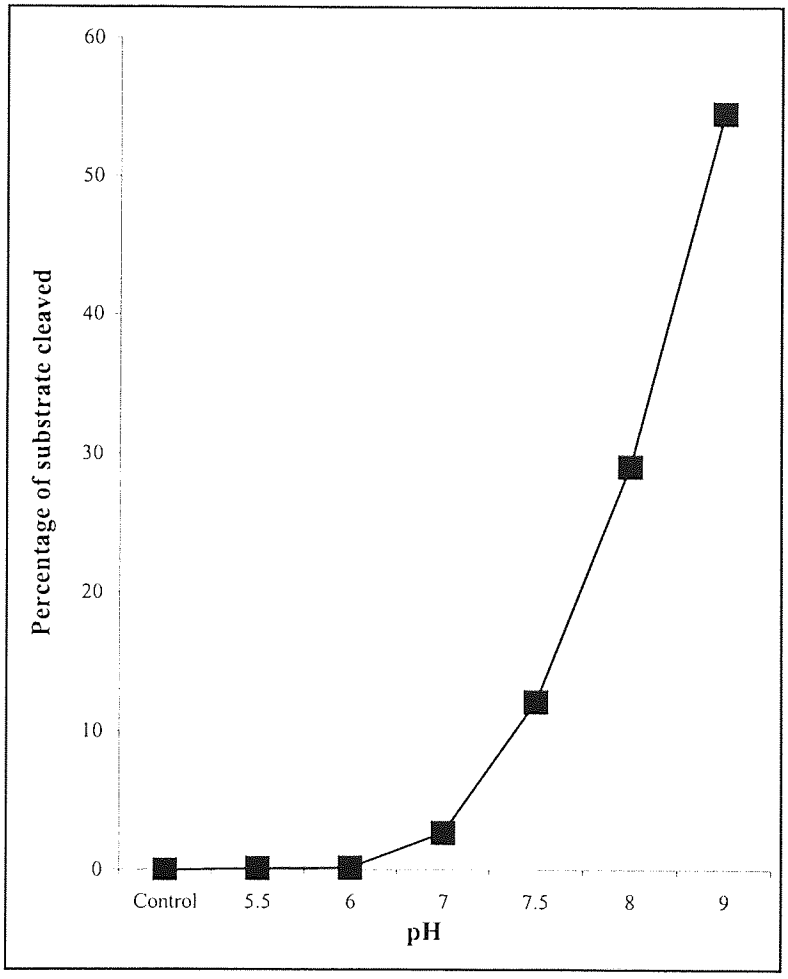
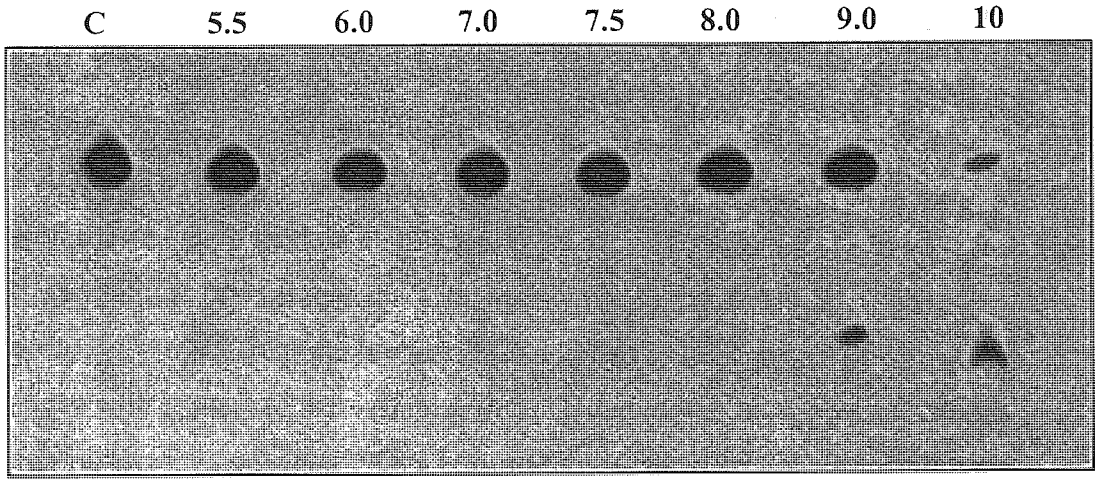


Figure 3.20 Effect of pH on cleavage activity of HR3.
Autoradiograph showing the effect of various pH levels on the *in vitro* cleavage activity of HR3 (10-23 of PO/PS). Activity was assessed with enzyme in excess over substrate (2μM:2nM) substrate in various buffers (pH 5.5-10) with 100mM $MgCl_2$, at 37°C over one hour. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.



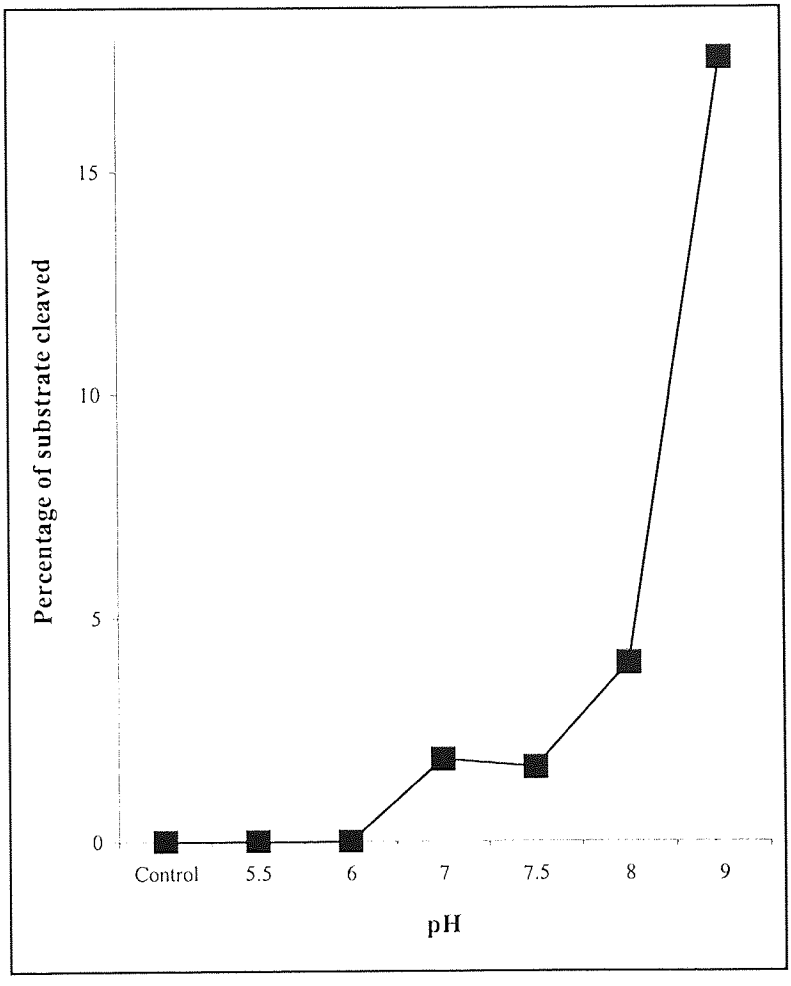


Figure 3.21 Effect of pH on cleavage activity of HR15.
Autoradiograph showing the effect of various pH levels on the *in vitro* cleavage activity of HR15 (8-17 of PO/PS). Activity was assessed with enzyme in excess over substrate (2μM:2nM) substrate in various buffers (pH 5.5-10) with 100mM $MgCl_2$, at 37°C over two hours. C indicates control RNA, not exposed to DNA enzyme. The average percentage cleavage from two independent experiments is plotted in the graph.

The effect of each of these factors on cleavage activity is indicative of potentially how successful cleavage would be under physiological conditions. In experiments with DNA enzymes, simulated physiological conditions are quoted as: 2mM $MgCl_2$ and 150 mM KCl at 37°C with a pH of 7.5 (Santoro and Joyce, 1997). It is plain that all the variables tested have marked effects on the cleavage activity of both the DNA enzymes, HR3 and HR15. Seemingly the cleavage activity of both motifs is similar under the influence of the different parameters. Since the 10-23 and 8-17 DNA enzymes behave similarly with respect to the variables investigated, it can be suggested that they have a similar catalytic mechanism. This is fortuitous as most of the comparable work in the literature is performed only on the more popular 10-23 motif.

The graphs of temperature versus catalytic activity are almost a classic bell shape. Minimal activity occurs at 4°C, the lowest temperature studied. The amount of cleavage increases with increasing temperatures, peaking near 30°C and then falling back to a minimum at the high of 50°C. Reassuringly though activity at the physiological temperature of 37°C is not appreciably inferior to the maximal level. Toyoda *et al.* (2000) looked at the effect of temperature on the *in vitro* cleavage activity of a 10-23 based DNA enzyme, obtaining results similar to those outlined here. From a low of 4°C, the amount of cleavage increased with increasing temperature, with peak efficiency achieved at 30°C. Above 30°C however, the amount of cleavage steadily decreased and no specific cleavage was observed at the highest test temperature of 55°C. The effect of temperature on cleavage is likely to be due to maintenance of an active conformation of the DNA enzymes at higher temperatures; whilst at lower temperatures there is insufficient energy to enable the reaction to proceed.

Ribozymes are widely regarded as metalloenzymes. Metal ions are also of prime importance in DNA enzyme selection and subsequent activity. Santoro and Joyce (1998) outline the 3 possible roles of the divalent metal ion in the cleavage mechanism of the 10-23 DNA enzyme. Firstly, the metal may participate in the chemical mechanism of the DNA enzyme as a metal hydroxide that functions as a general base to assist in deprotonation of the 2'-hydroxyl. Secondly, the divalent metal cation may function as a Lewis acid that coordinates directly to the 2'-hydroxyl and enhances its

acidity. Thirdly, the metal may play a purely structural role, helping to organise the enzyme into its active conformation.

Figures 3.16 and 3.17 illustrate the effect of $MgCl_2$ concentration on the amount of cleavage by HR3 and HR15. No cleavage occurs in the absence of $MgCl_2$, and subsequently the amount of cleavage increases with increasing concentrations of $MgCl_2$. This progressive increase levels off above a concentration of 200mM, and in the case of HR15, there is a slight fall in the amount of cleavage observed above 200mM. This is comparable to the results obtained by Toyoda *et al.* (2000), who looked at the effect of $MgCl_2$ on the *in vitro* cleavage activity of a 10-23 based DNA enzyme. They report no cleavage occurred in the absence of $MgCl_2$, otherwise cleavage increased with increasing $MgCl_2$ levels with the most efficient cleavage observed in the presence of 100mM $MgCl_2$. Increasing the $MgCl_2$ concentration above 100mM did not increase the amount of cleavage that occurred, in fact activity slightly decreased. Similarly, Dash *et al.* (1998) studied the cleavage activity of a 10-23 based DNA enzyme under various $MgCl_2$ concentrations. No cleavage was observed in the absence of $MgCl_2$, but the cleavage efficiency increased significantly at concentrations of 5, 10, 15 and 20mM $MgCl_2$. Later, this group reported a related DNA enzyme achieving 85% cleavage in the presence of 100mM $MgCl_2$ (Goila and Banerjea, 1998). Here again a direct correlation was seen between $MgCl_2$ concentration and cleavage efficiency.

The *in vitro* cleavage assays were performed under single turnover conditions, with enzyme in excess over substrate. The graphs of enzyme concentration versus percentage cleavage illustrate that a quantifiable level of cleavage is only obtained when enzyme is present at 10-fold excess of substrate concentration. There is a steep increase in percentage cleavage with each 10-fold increase in enzyme concentration. At 2μM, there appears to be a maximal level of cleavage, as a further increase of enzyme to 4μM has no substantial effect on the percentage of substrate cleaved. This result would suggest that cleavage of our DNA enzymes would not be favourable in multiple turnover conditions, where substrate is in excess over enzyme. However, DNA enzyme cleavage has been documented to proceed under multiple turnover conditions with a high catalytic rate (Sioud and Leirdal, 2000; Santoro and Joyce, 1998). This is

encouraging for future therapeutic potential as multiple turnover conditions simulate *in vivo* conditions where an excess of target would be present.

The effect of pH on cleavage was assessed over the range of 5.5 to 10. A series of HEPES buffers was used for this purpose. As Figures 3.20 and 3.21 illustrate, there was minimal cleavage at pH 5 and 6, after which there was a rapid increase in percentage cleavage with each increase in pH unit. At pH 10 cleavage could not be assessed as the RNA substrate degraded. Some cleavage does occur at the physiological pH of 7.5. Toyoda *et al.* (2000) looked at the effect of pH (4-10) on the *in vitro* cleavage activity of a 10-23 based DNA enzyme and documented similar results to those attained here. Very little cleavage occurred at pH 4 and 5, the amount of cleavage increased with increasing pH and the highest cleavage was achieved between pH 8 and 9. At pH 10, the RNA substrate degraded, as found in these studies.

Santoro and Joyce (1997) found that the catalytic rate of the 10-23 motif increased in a roughly log-linear fashion with increasing pH over the range of 7.0 to 8.5. The authors emphasise that this increase is consistent with a reaction mechanism involving rate-limiting deprotonation of the 2'hydroxyl that lies adjacent to the cleaved phosphoester. Okumoto *et al.* (2000) explain that a log-linear correlation between catalytic rate and pH is common to the hammerhead ribozyme and indicates the rate limiting step is the chemical cleavage step rather than the DNA enzyme-RNA substrate binding step.

The effect of these various factors on the cleavage activity of the DNA enzymes indicates that the annealing condition of the DNA enzyme and the substrate RNA impinges on cleavage activity (Toyoda *et al.*, 2000). To conclude, cleavage by the 10-23 and 8-17 motifs does occur in simulated physiological conditions albeit it is not optimal. However, as DNA enzymes are synthetically engineered, once their limitations have been recognised steps can be taken to improve their design.

3.7 Concluding Remarks

DNA enzymes, an extension of ribozyme technology, represent a rapidly expanding area of research. DNA enzymes are a relatively new phenomenon and as such our knowledge of them remains limited. Despite this, there is growing interest in the field and there are preliminary reports of DNA enzyme efficacy in a cellular environment (Wu *et al.*, 1999; Basu *et al.*, 2000; Goila and Banerjea, 2001). As prospective therapeutic agents, DNA enzymes face many of the same challenges that confront other nucleic acid based gene-inactivation agents. Efficient cellular uptake, appropriate subcellular localisation, resistance to nuclease degradation, and favourable pharmacokinetic properties are some of the key issues that will determine the efficacy of the DNA enzyme *in vivo*.

Thus far the successful design of two DNA enzymes containing the 10-23 and 8-17 catalytic motifs has been described. The *in vitro* activity of these DNA enzymes against RNA substrates has been illustrated. There does not appear to be a strict correlation between *in vitro* catalytic activity and the level of activity in cells (Oketani *et al.*, 1999). Thus the next pertinent question was whether these molecules are successful against their target in a cellular environment and this formed the next stage of these studies.

CHAPTER FOUR

ACTIVITY OF DNA ENZYMES IN CELL LYSATES

4.1 Telomeres and Telomerase

Telomerase is a specialized ribonucleoprotein enzyme complex that stabilizes telomere lengths by adding hexameric $(TTAGGG)_n$ repeats to the telomeric ends of chromosomes, thereby circumventing the cumulative damage that normally occurs during mitotic cell division. That is, telomerase compensates for the end-replication problem.

As discussed earlier, (section 1.2), telomerase activity has been shown to be present in most foetal tissues and germ cells as well as in a variety of tumours, including a high proportion of GBMs, but absent in normal somatic tissues (Le *et al.*, 1998). Thus telomerase could prove to be an important target in cancer therapy. Telomerase is an ideal target for oligonucleotides because the RNA template region is essential for its activity and it is also intrinsically accessible to binding by nucleic acids (Herbert *et al.*, 1999). Thus the experimental set-up being used in these studies provides a useful model marker for the evaluation of novel DNA enzymes' activity

Telomeres are repetitive DNA sequences at the ends of chromosomes. They protect chromosome ends and are also thought to act as a mitotic clock regulating cell division. The role of telomere length and telomerase expression in cancer has been discussed in length previously. Telomerase recognizes the G-rich strand of an existing telomere repeat sequence and elongates it in the 5' to 3' direction. Briefly, telomerase binds a telomeric end of a DNA chromosome and aligns it by recognition of the RNA template. The 3'-end of the chromosome is elongated with six nucleotides complementary to the RNA to create a telomere repeat. A translocation step then repositions the telomerase to repeat the polymerisation step.

4.2 Measurement of Telomerase Activity

The detection of telomerase using the conventional assay, which requires a large amount of cells or tissues, has largely been replaced by the PCR-based telomeric repeat amplification protocol (TRAP) assay (Kim *et al.*, 1994). The TRAP is a very sensitive assay, and can detect one telomerase positive cell in 10,000. The TRAP is theoretically the most sensitive method for detecting telomerase expression, but it is sensitive to heat, RNase and protease contamination (Kim, 1997). It measures the biochemical activity of telomerase in cells and thus provides the most biologically relevant information. There are however, other options to detect telomerase levels *e.g.* Northern blots and RT-PCR analysis.

Telomerase activity in U87-MG cells was measured using the PCR-based TRAP based on the method described by Kim *et al.* (1994). The first step is the preparation of cell lysates, which in this case were U87-MG or T98G cells. A telomere-specific oligonucleotide primer is introduced as a substrate. If telomerase is active in the cell extract, it adds on telomeric repeats (TTAGGG), hence elongating the substrate. The amount of reaction product is then amplified many thousand fold by PCR (polymerase chain reaction) cycling. Telomerase pauses after synthesis of a set of six nucleotides, thus the products on a gel consist of a 6 base pair nucleotide ladder (Mckenzie *et al.*, 1999).

Previous workers in our laboratory (Wan *et al.*, 1998) have used the TRAP protocol that, as in the standard TRAP, involved incorporation of [α-^{32}P] dCTP into the assay for the purposes of detection. However, experiments in this study were modified and usage of a [γ-^{32}P] ATP end-labelled TS primer instead was found to give better sensitivity and clearer results (data not shown).

The TRAP assay was performed using lysates of two glioma cell lines, U87-MG cells, which have demonstrable telomerase activity (Wan *et al.*, 1998) and also T98G cells. The cell lysates were prepared as described in section 2.9.1. Prior to use, the protein concentrations of the resultant lysates were estimated (see section 2.9.2.) using a BSA based protein assay. An example of a BSA calibration curve is illustrated in Figure 4.1. From the equation of such a curve and the absorbance readings of the test samples, the protein concentrations of the cell lysates were calculated.

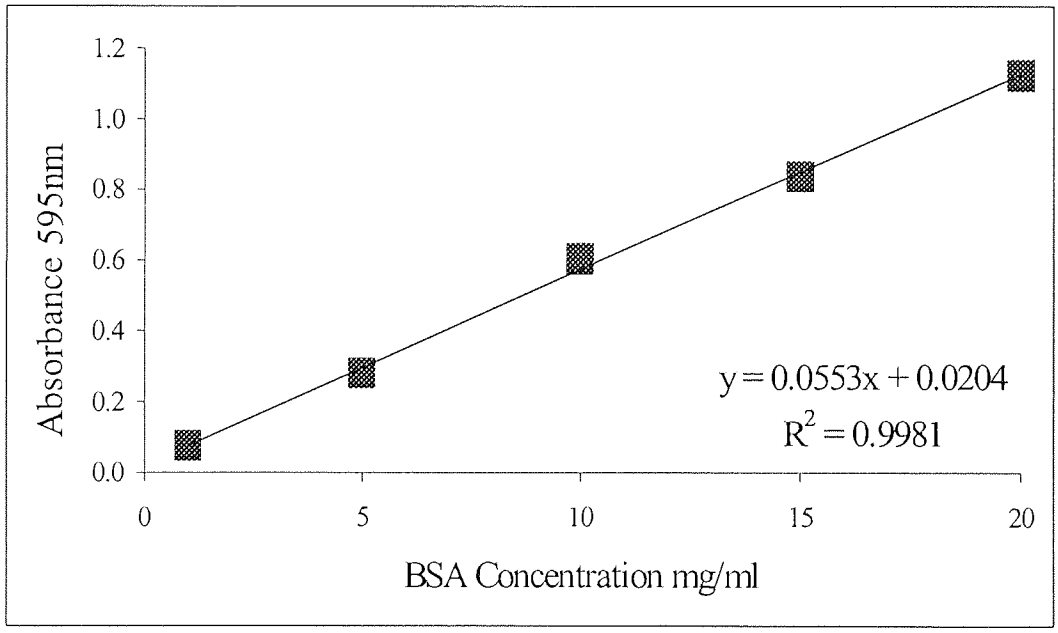


Figure 4.1 Standard BSA curve for the protein estimation of TRAP cell lysates.

Each reading is an average of two independently prepared standards.

As aforementioned, the TRAP is a very sensitive assay and is affected by various factors. The first important consideration was the amount of protein to be used in the assays. Only a very small amount, 100 to 1000, of cell equivalents are needed for adequate detection (Holt *et al.*, 1996). Ideally the lowest concentration of protein that will give a clear ladder would be used, hence making small amounts of down-regulation clearly apparent. A standard TRAP reaction was performed with a range of protein concentrations. Figure 4.2 shows a representative autoradiograph of a TRAP performed with various protein concentrations to determine the optimum.

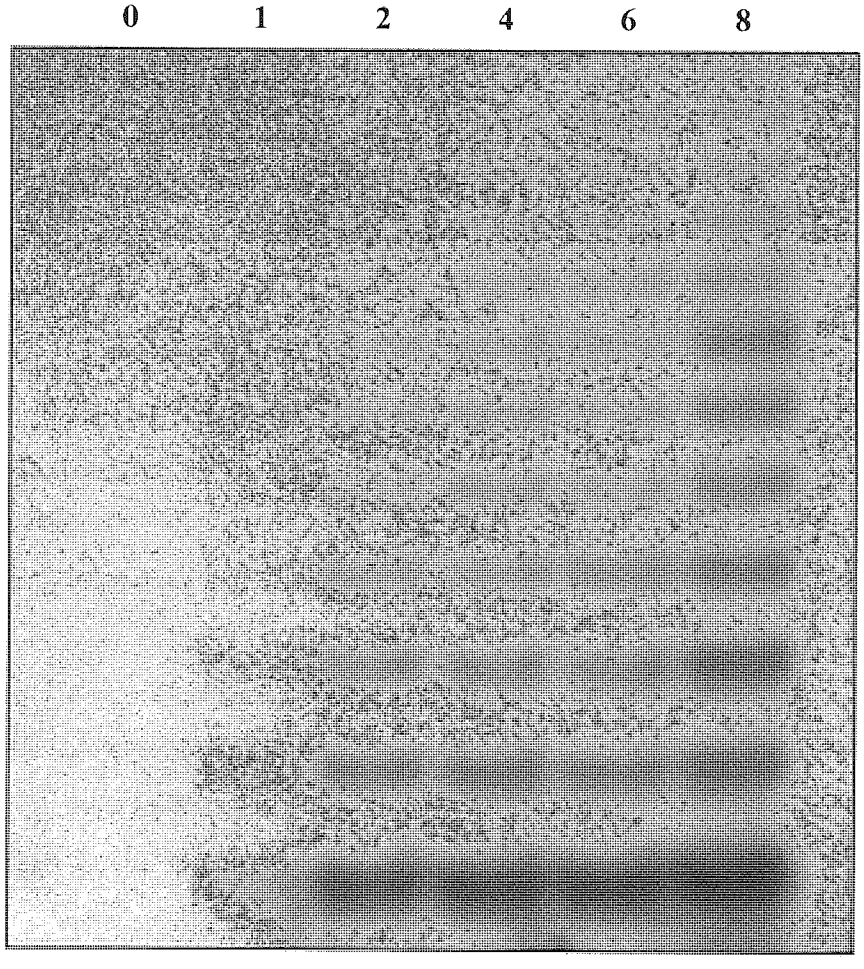


Figure 4.2 **TRAP performed using various amounts of U87-MG cell lysate protein (μg).**

From this result it was determined that the optimum protein concentration of U87 cell lysate for the TRAP was 2μg and this was used for subsequent experiments. At this protein concentration, consistently clear ladders were obtained. This was different to earlier findings, where 4μg was reported to be the optimum protein concentration (Wan *et al.*, 1998).

Detection of telomerase activity via the TRAP assay is based on the recognition and amplification of a synthetic oligonucleotide primer (TS). The basic function of telomerase is to extend the 3'-end of telomeres by de novo synthesis of telomeric repeat DNA (Morin, 1997). Thus the sequence of the TS primer is of extreme importance as telomerase binds to the TS primer and adds hexanucleotide TTAGGG repeats to its 3'-end to elongate it. Hence it was surprising when the serendipitous finding was made that the TS primer worked with two deoxyadenosine residues at the 3'-end instead of two thymidine residues, at identical concentrations. The TS primer, used throughout this work, ends with the sequence GTT. However, on one occasion the TS primer was erroneously synthesised ending with the sequence GAA. Surprisingly, this error in the sequence did not affect the TRAP assay, especially since the mismatch was at the 3'-end, which is more important for binding and recognition. This was not restricted to U87-MG cells, and also worked in T98G cells with similar efficiency to the GTT ending primer, albeit the pattern of the ladders does seem different. The representative autoradiographs in Figure 4.3 illustrate this across a range of protein concentrations.

The reasons for this finding are unclear and there does not appear to be any similar reports in the literature. This was a chance-interesting finding but nevertheless has no bearing on the overall aims of the study. The TS primer of correct sequence was used as before for further work.

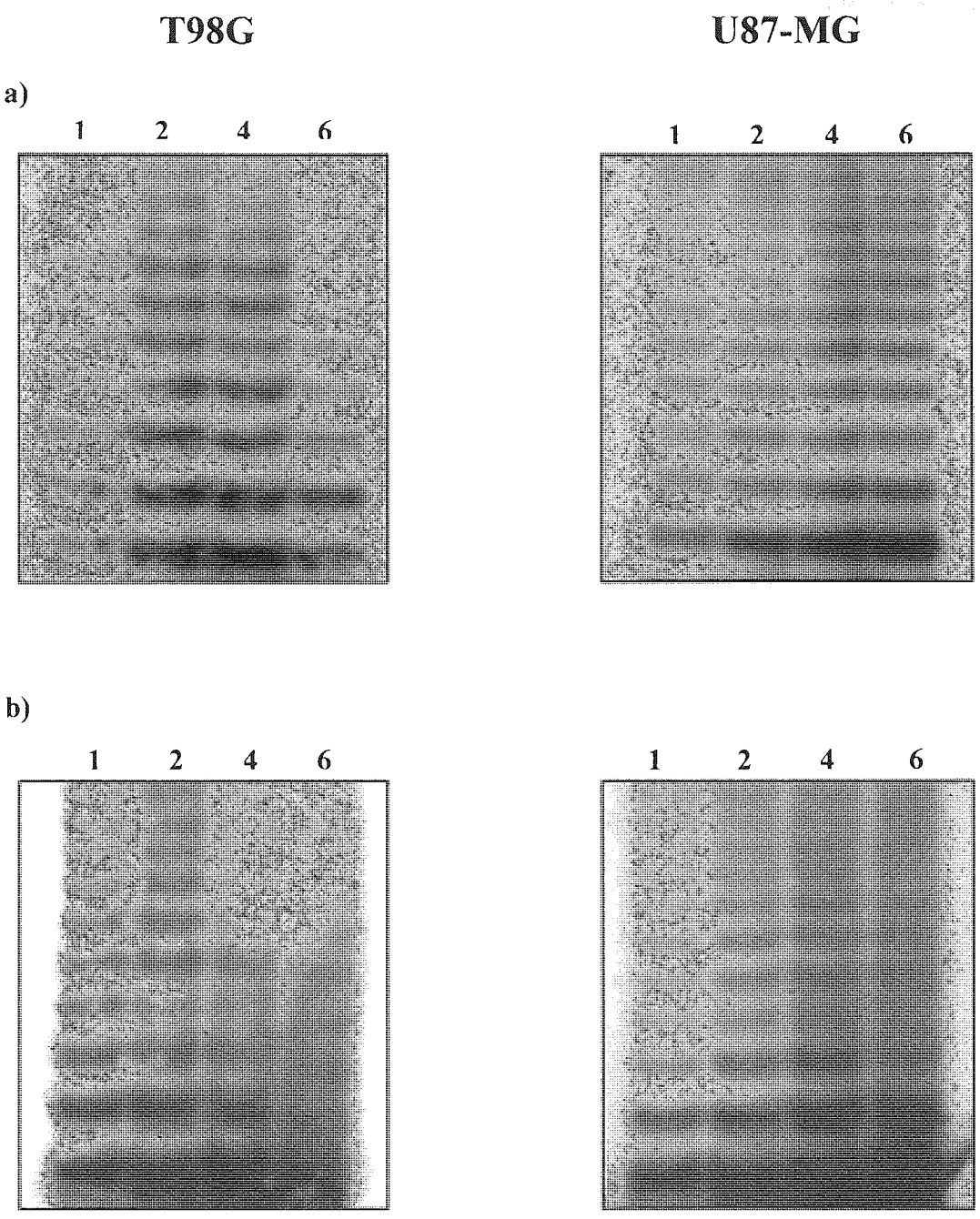


Figure 4.3 Telomerase activity in T98G and U87-MG cells.
TRAP reactions were performed with a range of protein concentrations (1-6 μg per reaction as indicated) of U87-MG cells using the **a)** incorrect GAA ending and **b)** correct GTT ending TS primers.

A further consideration before assessing the activity of DNA enzymes against telomerase was to determine which chemistry should be used in the TRAP reactions. As discussed in Chapter 3, chemical modifications can have a marked effect on DNA enzyme stability and cleavage activity. It was shown that introducing phosphorothioate (PS) linkages into DNA enzymes increased their stability in serum containing media but at a cost to catalytic activity. Indiscriminate modification resulted in total loss of activity, and it has been reported that modification of the catalytic core of DNA enzymes should be undertaken very carefully (Oketani *et al.*, 1999). The conclusion of the data presented in Chapter 3 was that DNA enzymes of mixed chemistry, *i.e.* with a phosphodiester (PO) core and PS arms would be the optimal compromise between stability and activity.

On the other hand, as the TRAP reactions are being done in cell lysates rather than intact cells, they are mainly a theoretical proof of principle. Thus ideally, an unmodified version of the DNA enzymes would be used, as unmodified DNA is likely to have minimal interference in the reactions. Phosphorothioate modifications to DNA are the most widely reported modification (Roh *et al.*, 2000) but they can bind non-specifically to cellular proteins (Sohail, 2000).

A study was undertaken to determine the stability of unmodified (PO chemistry) DNA enzymes in the TRAP reaction mix. A mock reaction mix was made up, with the standard 2μg protein of cellular lysate. To this mixture was added a quantity of 5'-end labelled DNA enzyme, and incubated as usual for 30 minutes at 25°C (see section 2.9). Aliquots were taken from this mixture at regular intervals and visualised using denaturing gel electrophoresis (section 2.3.1).

As can be seen in Figure 4.4, both of the unmodified DNA enzymes, 10-23 and 8-17 remained stable in the TRAP mixture for the entirety of the 30-minute experimental period. The stability of the DNA enzymes of PO chemistry in the cellular extracts meant they could be used in the TRAP reactions with no chemical modification.

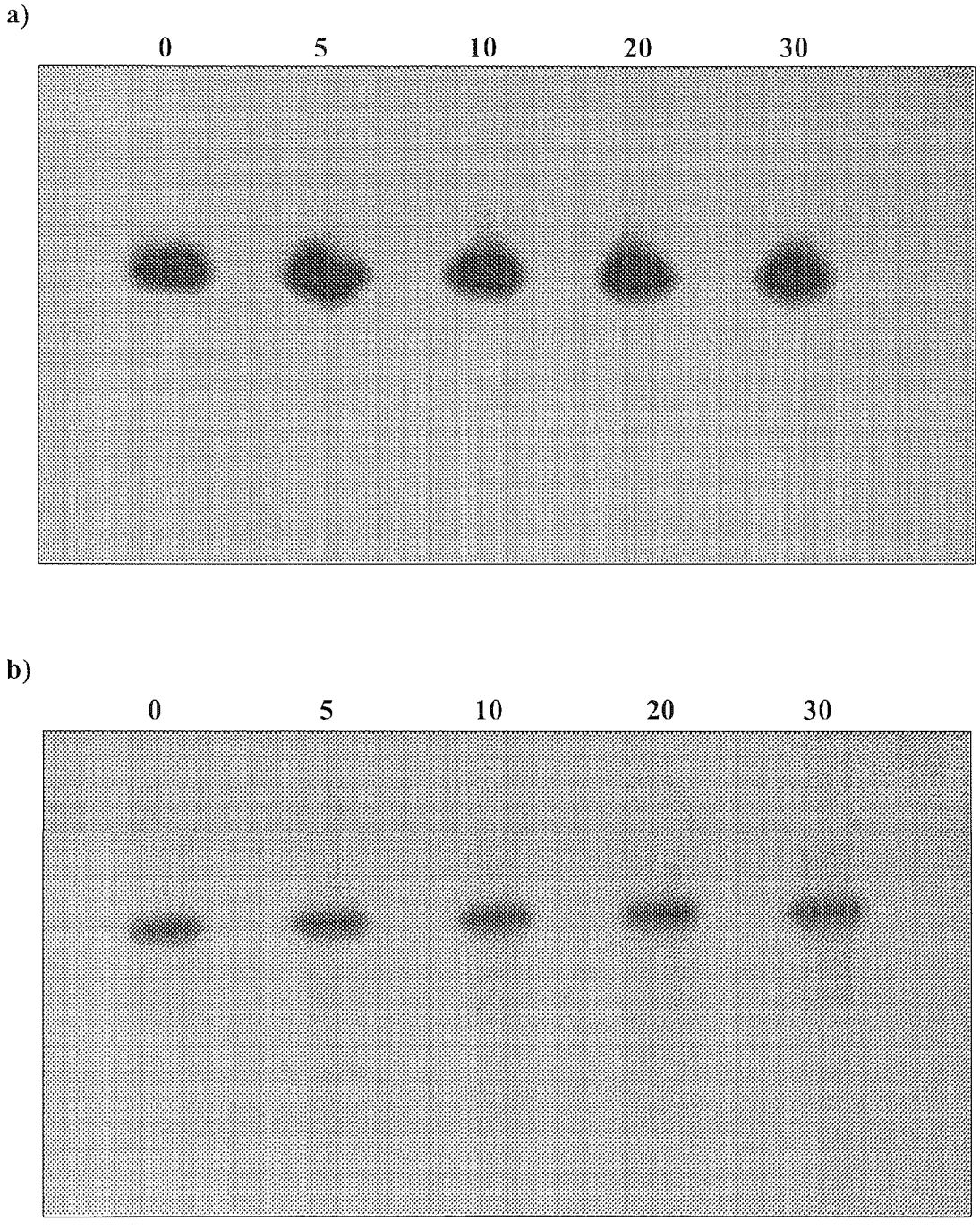


Figure 4.4 Stability of 5'-end ^{32}P [ATP] labelled DNA enzymes in TRAP reaction mix.

DNA enzymes, **a)** 10-23 and **b)** 8-17, of unmodified chemistry were incubated in a TRAP reaction mix at 25°C for 30 minutes. Aliquots were taken at the time intervals indicated in minutes and quenched in formamide buffer. Degradation profiles were analysed by 20% denaturing PAGE.

4.3 Telomerase Inhibition by DNA Enzymes

There is a strong correlation between telomerase activity and malignant brain tumours, thus glioma cell lines such as the U87-MG have generally been shown to have demonstrable telomerase activity (Kiaris and Schally, 1999). The DNA enzymes have been designed against the template region of hTR, which is theoretically intrinsically accessible and targeting this region has been effective in reducing telomerase. DNA enzyme inhibition of telomerase activity has not been studied before but inhibition by oligonucleotides (ODNs) has been demonstrated. Glukhov *et al.* (1998) reported inhibition of the telomerase activity of melanoma cells *in vitro* by antisense ODNs of PO chemistry. The investigators used various ODNs complementary to a range of sites on hTR. Antisense sequences showed higher activity than sense controls. Interestingly and most pertinently to this research was that highest inhibition activity was seen with the ODNs complementary to the template region of hTR.

Ribozyme activity against telomerase has also been documented in research carried out in our laboratory (Wan *et al.*, 1998). Here a hammerhead ribozyme with various chemical modifications was shown to inhibit telomerase activity with an IC_{50} of 0.4μM. These results suggest that inhibition of telomerase by DNA enzymes may also be successful.

The ability of the two DNA enzymes under investigation to inhibit telomerase activity was determined by treating 2μg of cell extract with a range of DNA enzyme concentrations at 25°C for 30 minutes. The reaction products were amplified by PCR and visualised using gel electrophoresis, then quantified using densitometry (see section 2.9 for detailed methodology).

The DNA enzymes were shown to successfully inhibit telomerase activity as measured by the TRAP assay. Initially a range of DNA enzyme concentration from 5nM to 50 μM was used, as illustrated in Figure 4.5.

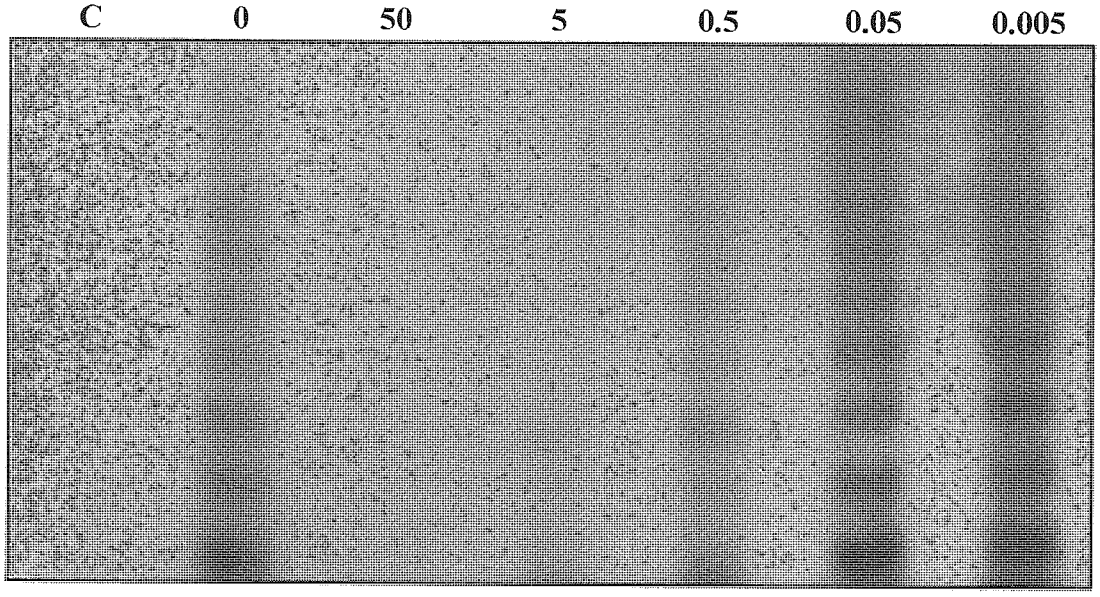


Figure 4.5 Representative autoradiograph of telomerase activity inhibition in U87-MG cell lysates by HR1 in the range 0.005-50μM.

Cell lysates (2μg) were incubated with increasing amounts of DNA enzyme 1 (10-23) at 25°C for 30 minutes. Telomerase activity was analysed using the TRAP assay as described in section 2.9.3. The resultant products were then visualised via gel electrophoresis and autoradiography. Concentrations of HR1 used are indicated in μM quantities. The lane denoted as C is the negative control and was performed in the absence of any cell lysate protein.

This initial range was too large to enable a determination of a dose-response effect, as it produced an all or nothing response. At concentrations above 0.5μM, telomerase activity was undetectable. Thus inhibition experiments were repeated using a narrower range of DNA enzyme concentration, *i.e.* from 10nM to 10μM DNA enzyme. The resulting inhibition of telomerase activity using this narrower range of concentrations is shown in representative autoradiographs in Figure 4.6.

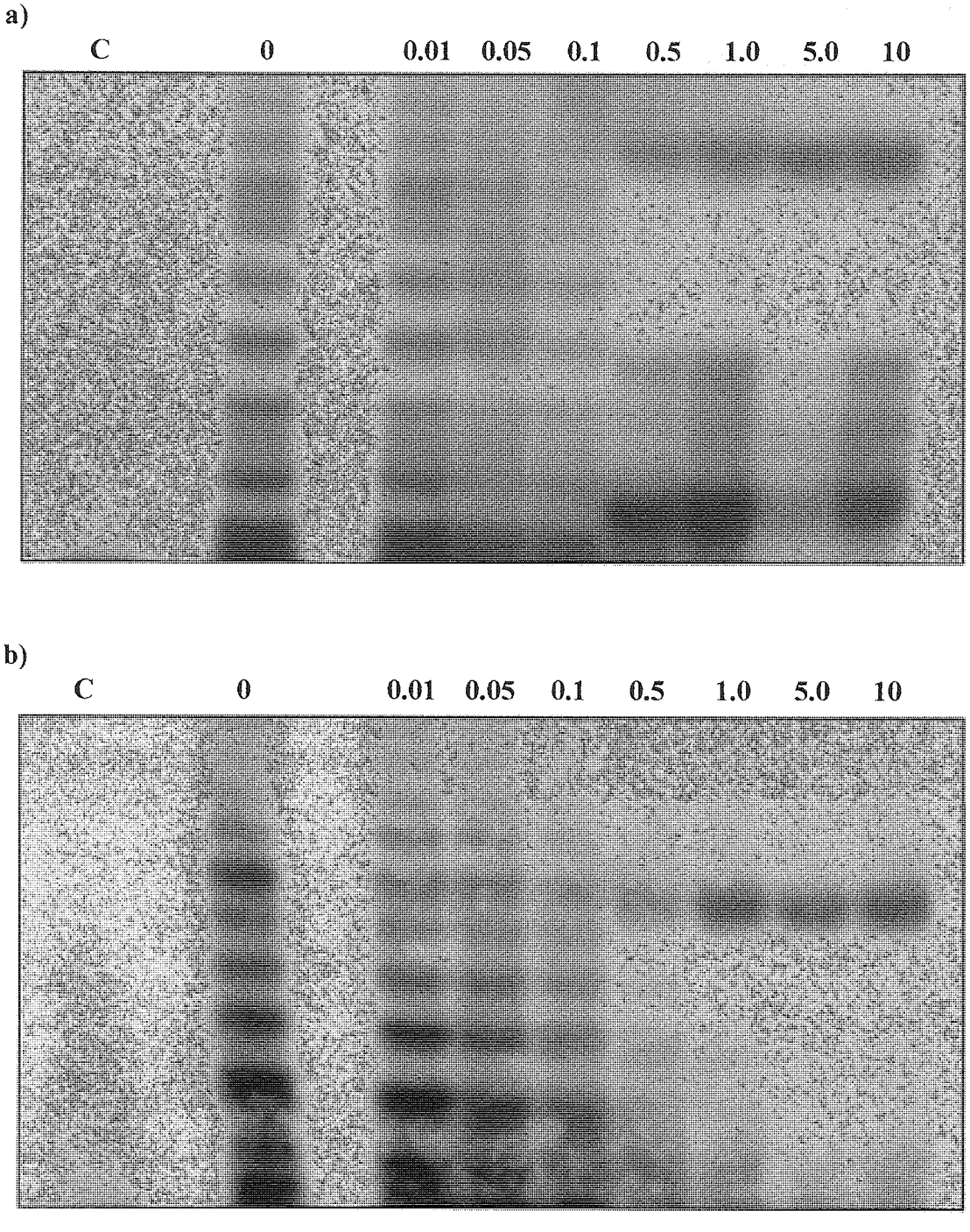


Figure 4.6 Representative autoradiographs of telomerase activity inhibition in U87-MG cell lysates by HR1 and HR13 (10nM-10μM).

Cell lysates (2μg) were incubated with increasing amounts of **a)** DNA enzyme HR1 (10-23) and **b)** DNA enzyme HR13 (8-17) at 25°C for 30 minutes. Telomerase activity was analysed using the TRAP assay as described in section 2.9.3. The resultant products were then visualised via gel electrophoresis and autoradiography. Concentrations of HR1 used are indicated in μM quantities. The lane denoted as C is the negative control and was performed in the absence of any cell lysate protein.

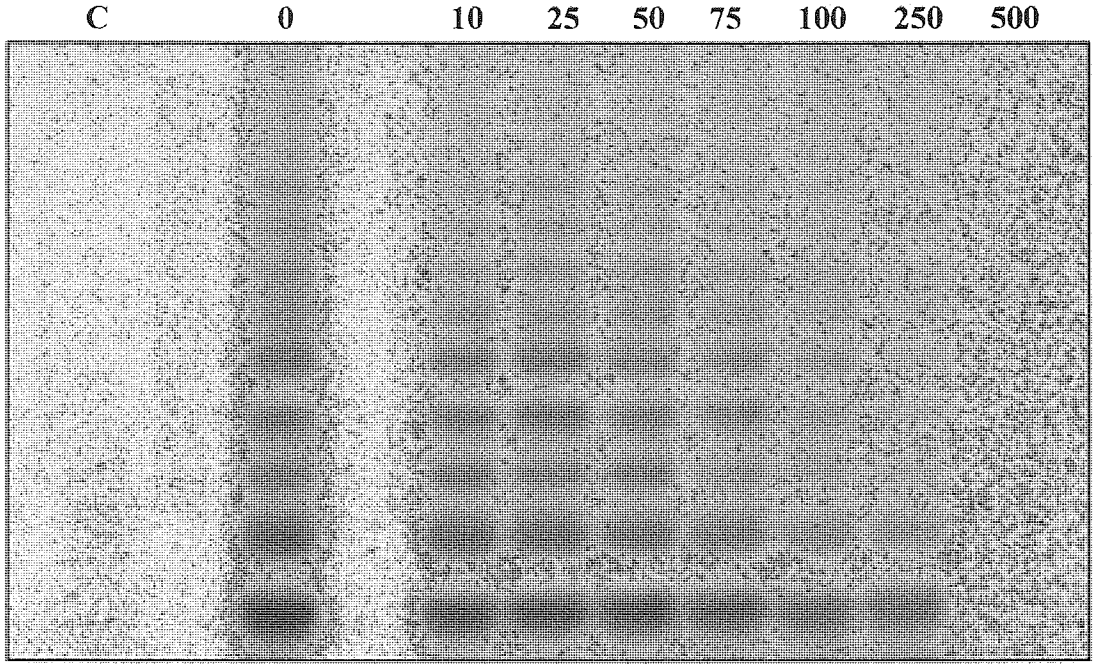
In Figure 4.6, some banding is seen with the higher concentrations of DNA enzymes even when telomerase activity has been diminished, an effect commented on in the literature also (Burger *et al.*, 1997). Glukhov *et al.* (1998) report banding in their TRAP assays when ODN concentrations of 0.4μM or higher were used and conclude that it is due to amplification of an ODN-CX primer complex. Falchetti *et al.* (1998) support this and assert that artefacts may arise from primer-dimer formation and subsequent slippage during amplification.

As illustrated in Figures 4.5 and 4.6, complete inhibition of telomerase activity was seen at concentrations above 0.5μM. Thus the concentrations were further amended to a lower range in to enable quantification of inhibition. The maximum concentration used was 0.5μM.

Figure 4.7 shows the representative autoradiographs of telomerase inhibition by the two DNA enzymes in the concentration range of 10-500nM. The experimental autoradiographs were analysed by densitometry (section 2.3.2.2) to enable quantification. Figure 4.8 shows the equivalent graphical representation of telomerase activity at these concentrations of both DNA enzymes. Results are based on at least four independent experiments performed with independently prepared cell lysates.

As depicted in Figure 4.7 and illustrated by the graphs in Figure 4.8, inhibition of telomerase activity was clearly dose-dependent with 100nM of DNA enzyme producing approximately 50% inhibition. This is more favourable than previous findings of ribozyme-mediated inhibition of telomerase (Wan *et al.*, 1998) where an IC_{50} of 0.4μM was reported. The results are comparable with ODN mediated activity against telomerase (Glukhov *et al.*, 1998) where inhibition was reported in the nanomolar range (5-60nM).

a)



b)

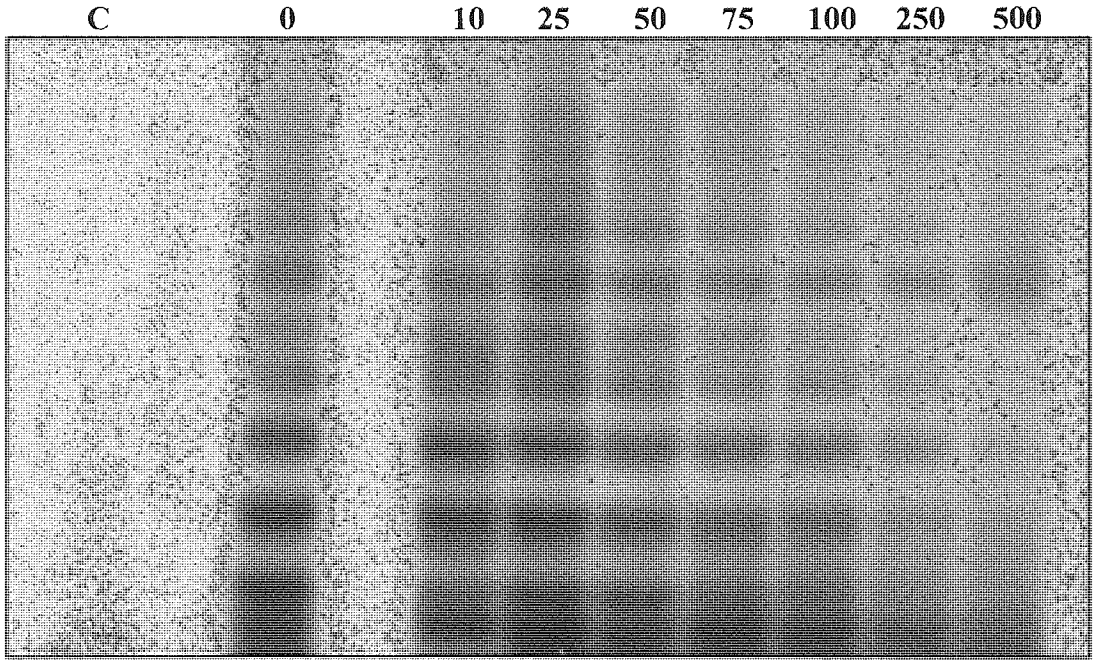
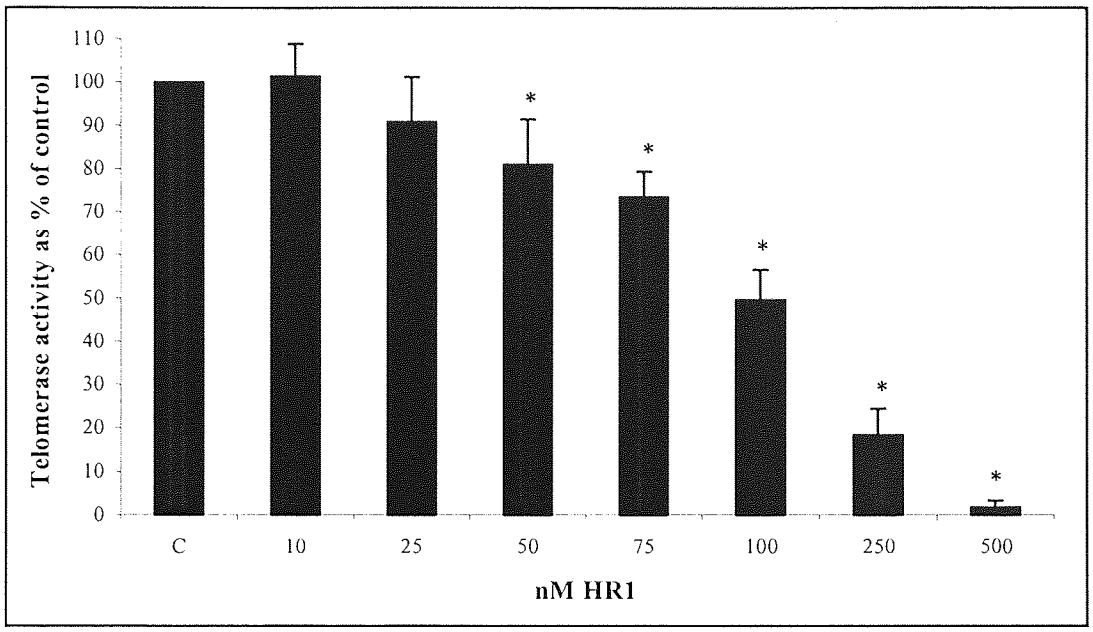


Figure 4.7 Representative autoradiographs of telomerase activity inhibition in U87-MG cell lysates by HR1 and HR13 (10nM-500nM).

Cell lysates (2μg) were incubated with increasing amounts of **a)** HR1 (10-23) and **b)** HR13 (8-17) at 25°C for 30 minutes. Telomerase activity was analysed using the TRAP assay as described in section 2.9.3. The resultant products were then visualised via gel electrophoresis and autoradiography. Concentrations of DNA enzyme used are indicated in nM quantities. The lane denoted as C is the negative control and was performed in the absence of any cell lysate protein.

a)



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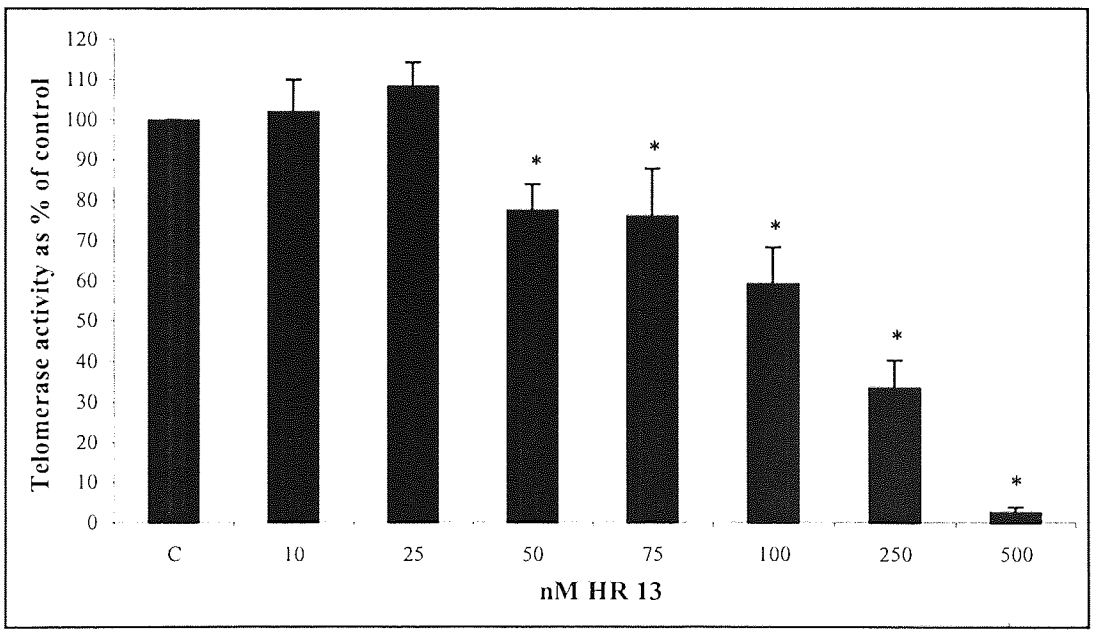


Figure 4.8 Graphical representation of telomerase inhibition data with HR1 and HR13

Results of inhibition of telomerase activity in U87-MG cell lysates over a range of concentrations of **a)** HR1 (10-23) and **b)** HR13 (8-17). Results are based on densitometric analysis of autoradiographs from at least four independent TRAP assay experiments, performed with independently prepared cell lysates. Telomerase activity is given as a percentage of the control, *i.e.* TRAP performed in the absence of DNA enzyme. * denotes significant ($p<0.05$) difference from control.

Several successful approaches for inhibiting telomerase have been described, including inhibitors of retroviral reverse transcriptase, peptide nucleic acids, cisplatin, hammerhead ribozymes, hTR antisense RNA and hTR gene deletion. Biological consequences of this inhibition have been varied (Bisoffi *et al.*, 1998), and the consequences of telomerase inhibition by DNA enzymes will be assessed later.

This is the first instance of DNA enzyme-mediated inhibition of telomerase activity, but as a therapeutic target, interest in telomerase is growing and the enthusiasm has sparked much research in the area. Feng *et al.* (1995) found that an antisense human telomerase RNA expression construct caused loss of telomeric DNA and death of tumour cells (HeLa cells) *in vitro*. Bisoffi *et al.* (1998) illustrated that the retroviral expression of RNA complementary to the template region of hTR was sufficient to inhibit telomerase both *in vitro* and *in vivo*. The inhibition also reduced the viability of cells, illustrated by the appearance of multinucleated, giant cells.

As aforementioned, previous work in our laboratory has shown ribozyme-mediated telomerase inhibition, and there are other reports of ribozyme activity in this regard. One of the earliest (Kanazawa *et al.* 1996) designed a hammerhead ribozyme to cleave a site located at the end of the hTR template element. The ribozyme showed *in vitro* cleavage activity and was able to inhibit telomerase in human carcinoma cell extracts. Here, 2μg of protein equivalent was treated, and telomerase activity was reduced to 50% by 1μM ribozyme.

It has been determined that DNA enzymes are able to inhibit telomerase in a dose-dependent manner. In order to try and elucidate the mechanism of inhibition, various controls were tested in the TRAP assays to determine the importance of base complementarity and hybridisation and whether the DNA enzymes are acting through a catalytic mechanism. The catalytic competence has been shown *in vitro* but needs to be demonstrated in cell lysates. As discussed previously, telomerase activity was reduced to 50% with 100nM of active DNA enzyme. Thus TRAP assays were repeated using four control DNA enzymes all at 100nM. The controls used are representative of the controls used generally in the antisense field.

When an antisense-based molecule causes a biological effect, it can be extremely difficult to determine whether the change occurred because the reagent interacted specifically with its target RNA, or because some non-antisense reaction, involving other nucleic acids and proteins, was set in motion (Branch, 1998). Thus controls are vital in such experiments to distinguish between antisense and non-antisense effects. Stein and Kreig (1994) suggest the four types of controls that should be considered in any antisense-based experiments. A sense control, which maintains structural feature but not composition; a scrambled control, which maintains composition but not structural features; a mismatched control, which demonstrates target hybridisation selectivity; and a mismatched target control, where a mutated or different target is used. The controls designed here are reflective of these suggestions.

Firstly, as seen in Chapter 3, introducing phosphorothioate linkages to the DNA enzyme sequence affects both cleavage and stability. Thus, the first control used was the mixed PO/PS chemistry, which was chosen as the best compromise between activity and stability. This would help the assessment of the effect of chemistry on telomerase inhibition. PS ODNs have therapeutic potential because of their ability to resist nuclease digestion, gain entry into cells and the nucleus, and have favourable pharmacokinetics and minimal toxicity (Mata *et al.*, 1997). Promisingly, it has been demonstrated that although catalytic activity may decrease with PS modifications under cell-free conditions, this detrimental effect was not observed when the PS modified DNA enzymes were used in cells (Oketani *et al.*, 1999). Newer chemistries, for example peptide nucleic acids have emerged, but the reports on their efficacy are mixed. In one case they have been shown to be 10-50 times more efficient than PS ODNs in inhibiting telomerase *in vitro* (Norton *et al.*, 1996), whilst in another study they were less efficient inhibitors of telomerase (Matthes and Lehman, 1999).

To determine whether inhibition was purely an antisense mechanism, the second control used was an oligonucleotide sequence identical to the hybridisation arms of the DNA enzyme minus the catalytic core. This antisense sequence was synthesised as PS chemistry.

The third control was an inactivated DNA enzyme, where the binding arms were unchanged but the catalytic core had been scrambled. This is essentially another control for antisense effect as complementary base binding will take place but catalytic activity has been disabled. The chemistry was mixed *i.e.* the scrambled core was PO but the arms were PS to be comparable to the active PO/PS enzymes.

The fourth and final control retained the active core but the binding arms were not complementary to the target, *i.e.* they were of sense rather than antisense orientation. This control will assess the importance of base complementarity for DNA enzymes. As the scrambled control, this was also of mixed chemistry with a PO core and PS arms.

Figure 4.9 shows the representative autoradiographs of telomerase inhibition by the various control DNA enzymes at a concentration 100nM. The experimental autoradiographs were analysed by densitometry (section 2.3.2.2) to enable quantification. Figure 4.10 shows the graphical representation of telomerase activity at various concentrations of the active and control DNA enzymes. Results are based on at least four independent experiments performed with independently prepared cell lysates.

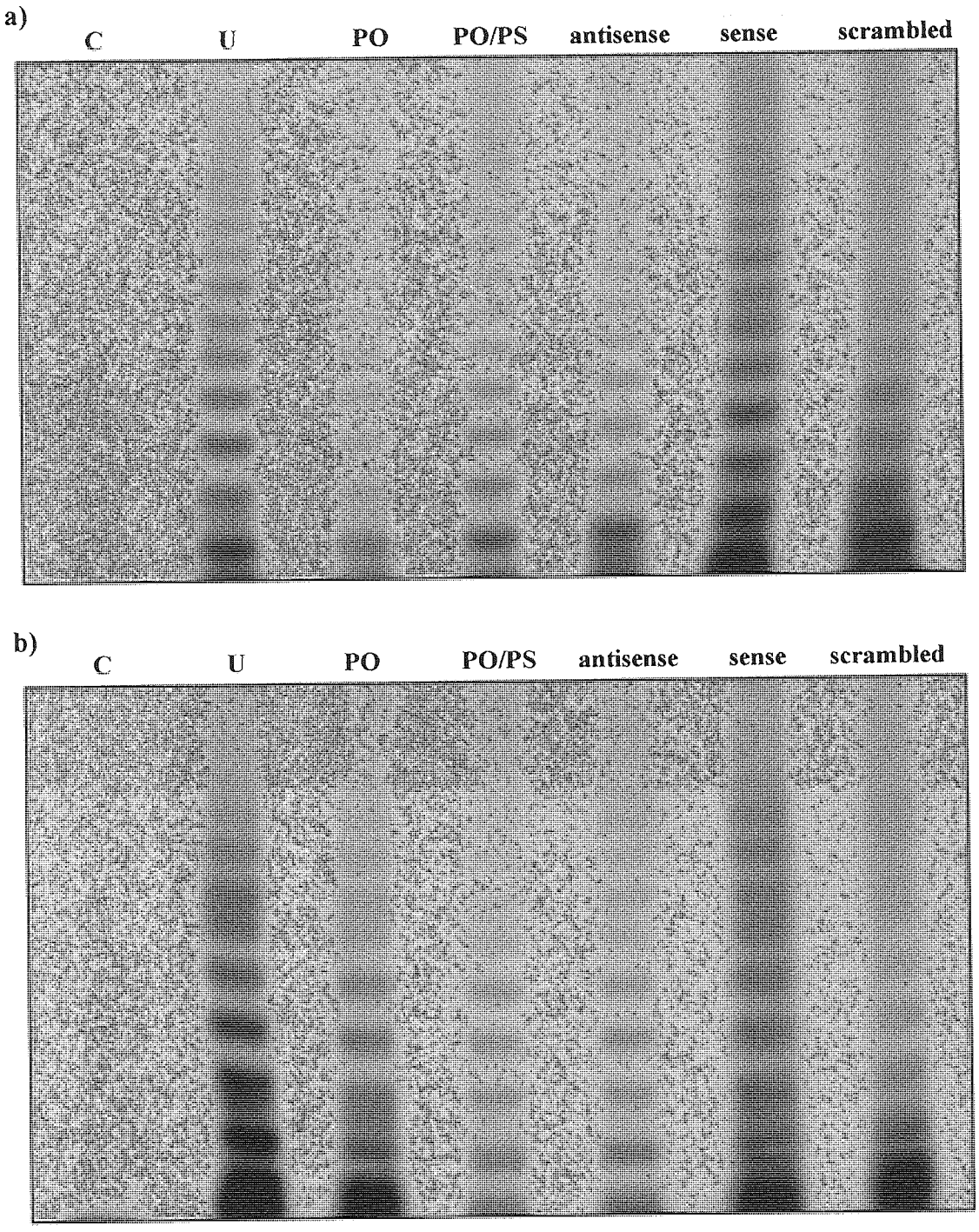
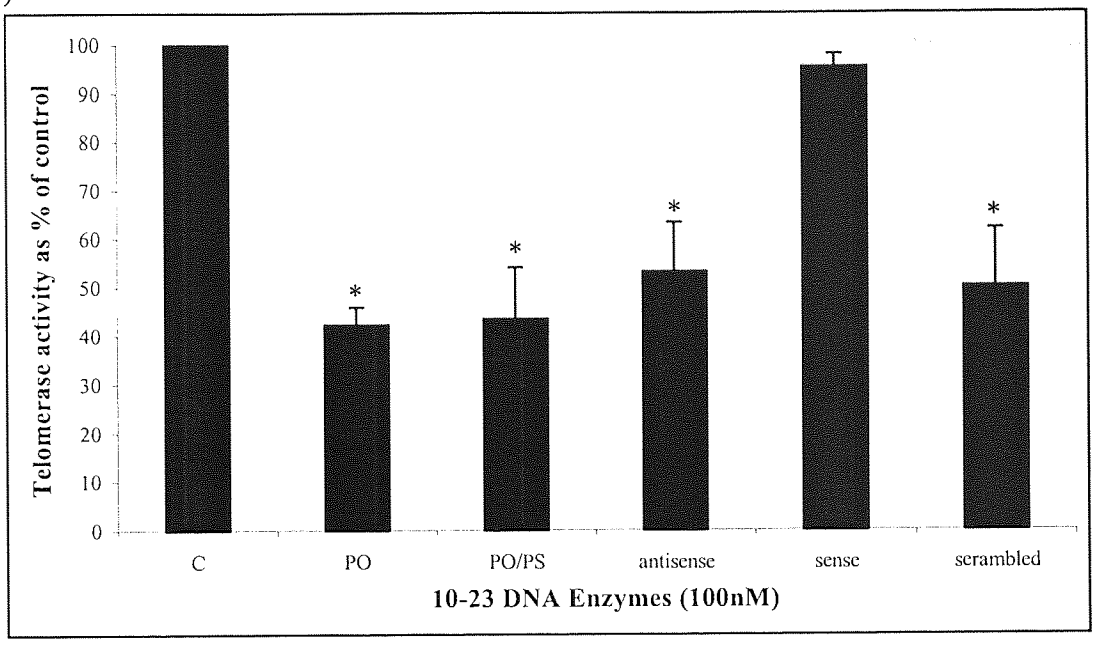


Figure 4.9 Effect of control DNA enzymes of a) 10-23 and b) 8-17 on telomerase activity in U87-MG cell lysates.

Cell lysates (2μg) were incubated with 100nM of DNA enzymes as indicated at 25°C for 30 minutes. The lane denoted as U is an untreated TRAP reaction (positive control); the lane C is the negative control and was performed in the absence of any cell lysate protein; **PO**=active unmodified DNA enzyme; **PO/PS**=active DNA enzyme with phosphorothioate arms; **antisense**=complementary oligonucleotide sequence minus the catalytic core; **sense**=active core with non-complementary/sense arms; **scrambled**=scrambled core. Telomerase activity was analysed using the TRAP assay as described in section 2.9.3. The resultant products were then visualised via gel electrophoresis and autoradiography.

a)



b)

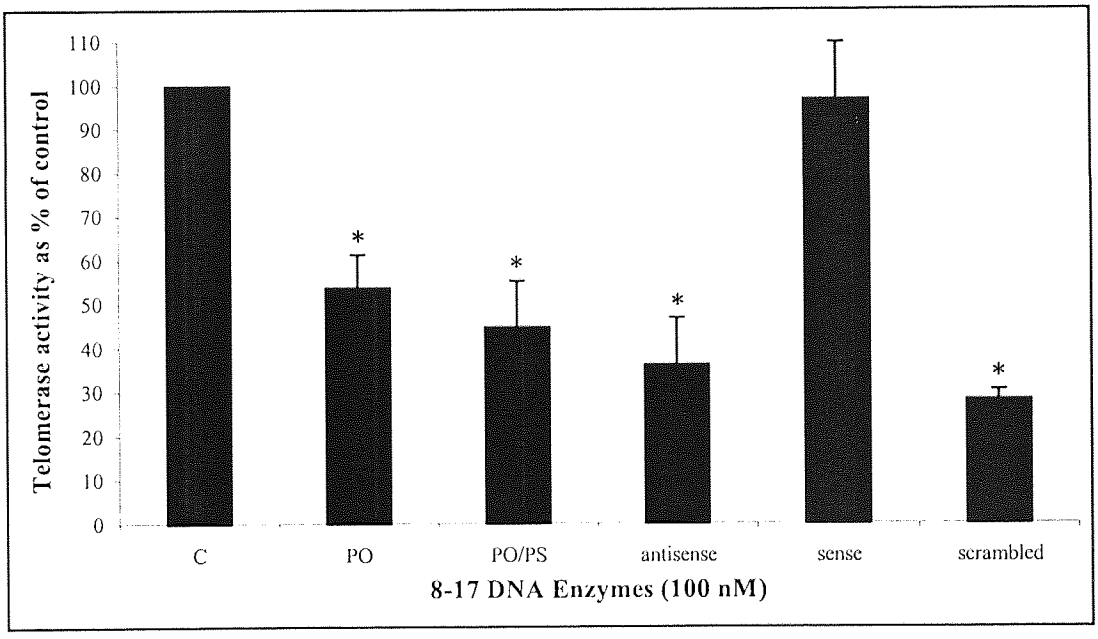


Figure 4.10 Graphical representation of telomerase inhibition with DNA enzyme controls of a) 10-23 and b) 8-17.

Results are based on densitometric analysis of autoradiographs from at least four independent TRAP assay experiments, performed with independently prepared cell lysates. Telomerase activity is given as a percentage of the control (C), *i.e.* TRAP in the absence of DNA enzyme. **PO**=active unmodified DNA enzyme; **PO/PS**=active DNA enzyme with phosphorothioate arms; **antisense**=complementary oligonucleotide sequence minus the catalytic core; **sense**=active core with non-complementary/sense arms; **scrambled**=scrambled core. * denotes significant ($p<0.05$) difference from control.

Results from the controls seem to indicate a mainly antisense mechanism of action in cell lysates. There is no significant difference between the active DNA enzymes, both unmodified and those with phosphorothioate arms; the antisense and scrambled controls inhibited telomerase activity with similar efficiency to the active enzymes; the DNA enzymes with non-complementary, sense arms did not inhibit telomerase activity to any significant effect. Thus base complementarity is very important but the catalytic competence of the DNA enzymes does not appear to have any substantial benefit in cell lysates.

Results on activity with controls vary in the literature. Mata *et al.* (1997) observed no activity with scrambled controls when using a short PS ODN telomere mimic as a telomerase inhibitor in lymphoma cells and xenograft models. The activity seen here with the scrambled controls may be due to the non-specific binding of PS to non-template RNA or protein components (Tao *et al.*, 1999). However, since minimal activity is seen with the sense control of identical chemistry, this explanation is less likely than an antisense mechanism of action.

Sioud and Leirdal (2000) found a catalytically incompetent DNA retained some activity, albeit less than the active enzyme, against protein kinase Cα. This suggested that part of the DNA enzyme activity was due to an antisense mechanism. Also, similar to the results seen in the TRAPs, the authors found a DNA enzyme with sense arms to lack any activity. Oketani *et al.* (1999) established that although a DNA enzyme with a mutated catalytic core had significantly lower inhibitory effects compared to the wild-type DNA enzyme, activity was not eliminated, hence suggesting that some antisense contribution was present.

There have been many reports of successful inhibition of cellular gene products by PS ODNs but this activity is attributed in part to the polyanionic nature of PS ODNs (Agrawal, 1999). The reports of telomerase inhibition by PS ODNs are variable. Norton *et al.* (1996) describe IC_{50} values of between 50-200nM, which is comparable to what has been achieved here with DNA enzymes. Matthes and Lehmann (1999) report much lower IC_{50}values (1nM) for PS ODNs, but contrastingly document very high IC_{50} values (430-460nM) for PO ODNs.

To support the inhibition data presented thus far, it was desirable to show that inhibition of telomerase activity by DNA enzymes was not restricted to U87 cells. Thus initial work was done to illustrate DNA enzyme-mediated telomerase inhibition in T98G cell lysates. The results of this preliminary work are shown in Figure 4.11.

As illustrated in Figure 4.11, there is inhibition of telomerase activity in T98G lysates by both DNA enzymes of umodified chemistry. The inhibition profile seems different to that obtained in U87MG cell lysates and ideally the inhibition profile in T98G cells would have been characterised as thoroughly as in U87-MG cells. Unfortunately however, time did not allow for a more thorough analysis.

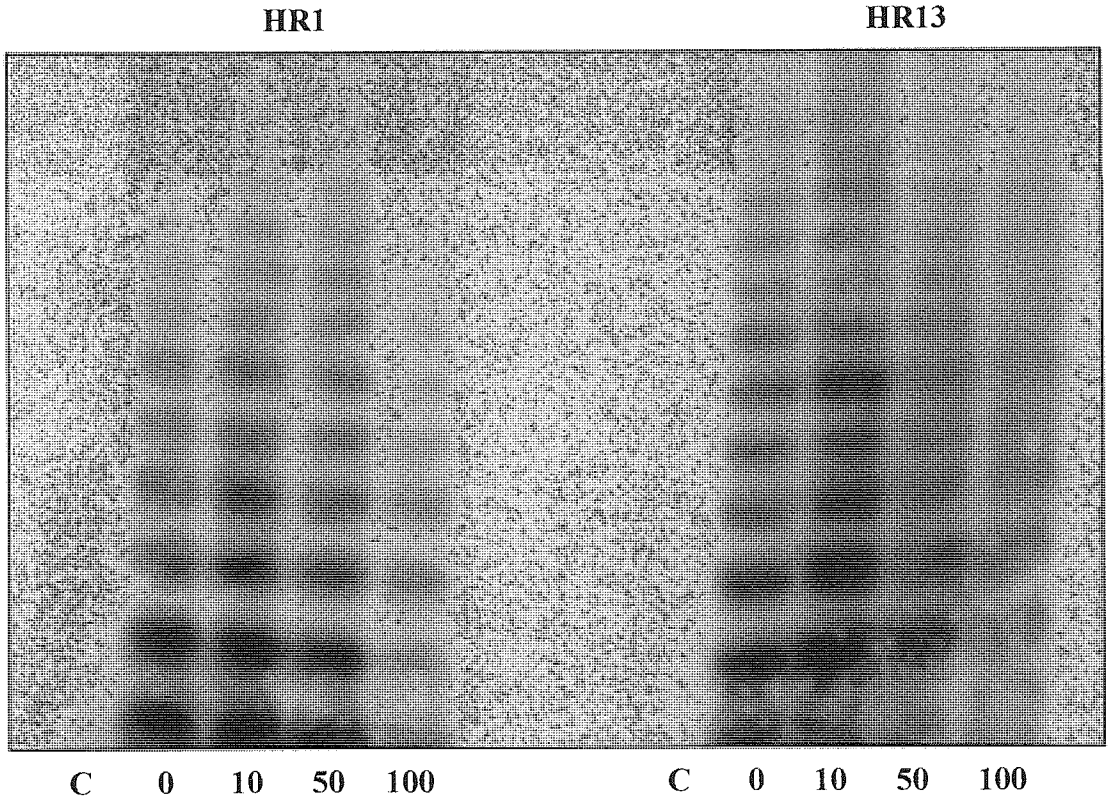


Figure 4.11 Inhibition of telomerase activity in T98G cell lysates.
2μg lysates were incubated at 25°C for 30 minutes in the presence of a range of concentrations of HR1 (10-23) and HR13 (8-17), indicated in nM concentrations. Telomerase activity was analysed using the TRAP assay as described in section 2.9.3. The PCR products were analysed by gel electrophoresis and visualised by autoradiography.

4.4 Development of an Internal Control

As a final consideration of the TRAP assays, it was endeavoured to develop an internal control. This would discount the possibility that telomerase inhibition in the TRAP assays was due to the presence of *Taq* polymerase inhibitors. Inclusion of a short oligonucleotide sequence to be amplified independently of the other assays would make it easier to quantitate telomerase activity and, in addition, would identify false negative samples that contain *Taq* polymerase inhibitors.

Thus attempts were made to develop such an internal control for inclusion in the TRAP assay that would be amplified independently of the assay. An example in the literature (Lucas-Vaquero *et al.*, 1999) was used as the basis of designing an 180bp internal telomerase amplification standard (ITAS). This was done using a fragment of the EGFR sequence (NCBI Genbank, X00663 = human mRNA fragment for the EGF receptor).

A segment of the EGFR sequence was chosen meeting various requirements *i.e.* a sequence of approx. 130-140 bp length, avoiding long runs (4 or more) of the same nucleotide and keeping the AT:GC ratio at approximately 1:1. A 135 bp fragment was chosen as matching these requirements, nucleotide #1891-2026.

This chosen EGFR fragment was amplified from a plasmid using PCR. Primers were designed, 18-20 bp fragments on each end of the sequence to amplify the desired portion from the plasmid containing the EGFR sequence. The TS and CX, standard TRAP primer sequences were synthesised onto the end of these primer sequences.

The EGFR sequence amplified is shown below, with highlighted nucleotides representing the nucleotides for the design of the primer sequences.

5'- ACG TGT GCC GCC TGC TGG GCA TCT GCC TCA CCT CCA CCG TGC AAC TCA TCA CGC AGC TCA TGC CCT TCG GCT GCC TCC TGG ACT ATG TCC GGG AAC ACA AAG ACA ATA TTG GCT CCC AGT ACC TGC TCA ACT GGT –3'.

Using the highlighted sequences, the following forward and reverse primer sequences were synthesised:

Forward: (*Italics* = TS primer sequence)
5'- *AAT CCG TCG AGC AGA GTT* ACG TGT GCC GCC TGC TGG –3'.

Reverse: (*Italics* = CX primer sequence)
5' – *GCG CGG CTT ACC CTT ACC CTT ACC CTA* AAC CAG TTG AGC AGG TAC TG –3'.

In this way, after the PCR process, the TS and CX primers were incorporated into the final construct. The final construct is illustrated in Figure 4.12 below.

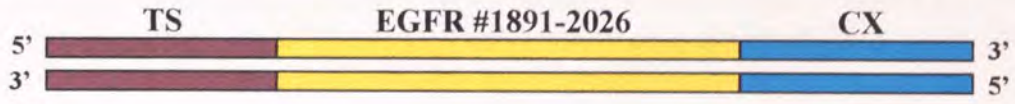


Figure 4.12 Composition of the internal telomerase amplification standard. The ITAS construct contained a 135-bp segment from the human EGFR sequence. This is flanked by sequences complementary to both the TS and CX primers used in the TRAP, thus allowing co-amplification during PCR.

Unfortunately, this could not be optimised to work within the TRAP assay, the amplification was too competitive with the telomerase amplification reaction. Nonetheless, this 180bp standard was used in a PCR process using primers (TS and

CX) as used in the TRAP reaction. That is, the 180bp fragment was amplified in a false TRAP reaction mix, without the presence of cell lysate. The mixture was made up and amplified by PCR without the 30 minute telomerase extension step. This would serve to illustrate that the DNA enzymes were actually inhibiting telomerase activity and not merely inhibiting *Taq* polymerase activity. A representative result of such a PCR is illustrated in Figure 4.13.

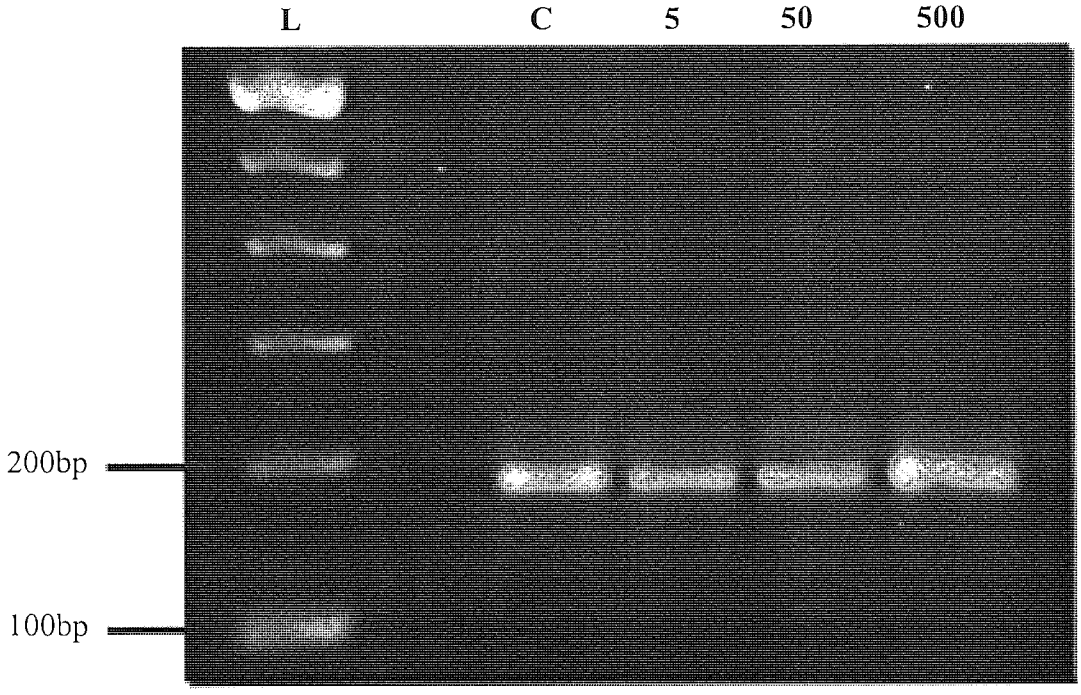


Figure 4.13 Representative gel showing successful PCR of 180bp ITAS. PCR was performed in the presence of increasing amount of DNA enzyme HR1 (10-23). Concentrations of DNA enzyme are indicated in nM quantities. L= 100bp ladder; C= control, performed in the absence of any DNA enzyme.

The amplification of the developed ITAS in the presence of DNA enzyme illustrates that telomerase inhibition as measured by the TRAP assays was not due to inhibition of PCR. Successful amplification was achieved in the presence of 500nM DNA enzyme, the maximum concentration used for the determination of the dose response curve. This experiment does not of course discount the possibility of the presence of PCR inhibitors in the cell lysate. However, such PCR inhibitors can be assumed to

be constant within each individual TRAP experiment as the same lysate preparation was used within a set of assays.

4.5 Concluding Remarks

Thus far it has been shown that DNA enzymes are catalytically competent and inhibit telomerase activity in glioma cell extracts, albeit perhaps not by a mechanism of catalytic cleavage. Studies have shown that activity of test substances may vary in cell lysates and in intact cells (Tamura *et al.*, 2000; Tao *et al.*, 1999). Thus it is important to study the activity of DNA enzymes in cells before drawing conclusions about them.

There is hope in the literature of using telomerase inhibitors to halt progression of tumours. Naka *et al.* (1999) examined the effects of antisense hTR expression on the growth of human gastric cancer cell lines through transfection via an expression vector. They observed telomere shortening in the transfected cells, followed by morphological changes into multinucleate giant cells and ultimately cell death after 10-40 days.

Feng *et al.* (1995) were the first to clone the hTR complex and describe the template region. The authors also report that HeLa cells transfected with an antisense hTR lost telomeric DNA and began to die after 23-26 doublings.

The mechanism of cytotoxicity as a result of telomerase inhibition is unclear and quick cell death (Kondo *et al.*, 1998) may indicate that telomerase inhibitors function by a mechanism other than gradual telomere erosion. Page *et al.* (1999) also reported cytotoxic effects of telomerase inhibition by single-stranded telomere mimics but could not elucidate the mechanism of cytotoxicity.

There are also contrasting reports of telomerase inhibition resulting in no substantial effects on cell viability. Villa *et al.* (2000) found a PNA construct to induce a dose-dependent inhibition of telomerase activity. However, this inhibition failed to induce telomere shortening and caused only a slight increase in the melanoma cell doubling time.

It may emerge that hTERT is the rate limiting step to telomerase activity (Nakamura, *et al.*, 1997) and should be targeted instead of the hTR component. In fact there are reports of hammerhead ribozymes targeting hTERT mRNA with the aim of regulating telomerase activity (Yokoyama *et al.*, 2000). It has even been suggested that whilst the catalytic activity of hTERT is required for cellular immortalisation, the presence of active telomerase does not necessarily imply telomere maintenance and immortality (Ouellette *et al.*, 1999), thus targeting it would not be beneficial in tumour therapy.

The components of telomerase, their functions and relative importance to enzyme function have been discussed at length in section 1.2. However the aim here was to have a good model to evaluate DNA enzyme activity. More research is needed before the therapeutic implications of telomerase are fully appreciated. The main conclusions from these studies thus far are that the novel DNA enzymes have been shown to be catalytically component *in vitro,* and they retain activity in cell lysates. If DNA enzymes are to have therapeutic potential, they must first demonstrate activity in a cellular environment. Thus the next pertinent area of study is activity in and delivery to cells.

CHAPTER FIVE

CELLULAR DELIVERY & ACTIVITY OF DNA ENZYMES

5.1 Cell Culture Systems

Thus far the DNA enzymes have been shown to be catalytically competent *in vitro* and retain activity in cell lysates. The work presented in this chapter assesses the potential for delivering these DNA enzymes into cells and resultant activity therein. Work in previous chapters found HR3 and HR15, DNA enzymes of PO/PS chemistry, to be the optimal compromise between stability and activity. Stability is of prime importance in cell experiments hence suitably modified molecules must be used. Unmodified ODNs have in the past been documented to have antisense effects but it is now known that they rapidly degraded to monomeric nucleotides which in turn are toxic to the cell and are probably responsible for the assumed antisense effect observed (Vaerman *et al.*, 1997).

Delivery of DNA to cells is an inefficient process, with only a fraction of applied ODNs becoming bound to the cell membrane and internalised (Akhtar *et al.*, 1996), due to rapid degradation by serum and cytoplasmic nucleases. It has been reported that only 1-2% of naked ODN applied is actually taken up by cells (Miller and Das, 1998). Various methods are under development for the improvement of delivery of charged macromolecules, such as oligonucleotides, to cells and some of these have previously been discussed in section 1.4.3. Examples include calcium phosphate co-precipitation, DEAE-dextran, cationic lipid reagents, dendrimers, biodegradable polymers and mechanical methods such as microinjection.

The 10-23 and 8-17 DNA enzymes used in these studies have been designed to target the RNA component of human telomerase. Both DNA enzymes were shown to inhibit telomerase activity in glioma cell lysates (Chapter 4). In this chapter, the ability of these DNA enzymes to act in a cellular environment was assessed by testing their anti-proliferative capacity. Cell growth assays *e.g.* Trypan blue assay are commonly used for this purpose in the cancer research field. The premise of such assays being that when a factor such as telomerase, which is required for tumour growth, is inhibited this will lead to detrimental effects on cell growth.

5.2 Characterisation of Cell Lines

Two glioma cell lines, U87-MG and T98G, were to be used to assess the delivery of DNA enzymes to cells and the activity thereof. Unfortunately, due to time constraints, the work presented in this chapter has mainly been performed in the U87-MG cell line only. The appearance and morphology of these cells was assessed by simple microscopy. Changes in appearance and morphology are important indicators of cell health and thus should be closely monitored when undertaking any work on cells. Figure 5.1 shows colour images of the two cell lines depicting normal, healthy monolayers in full serum media.

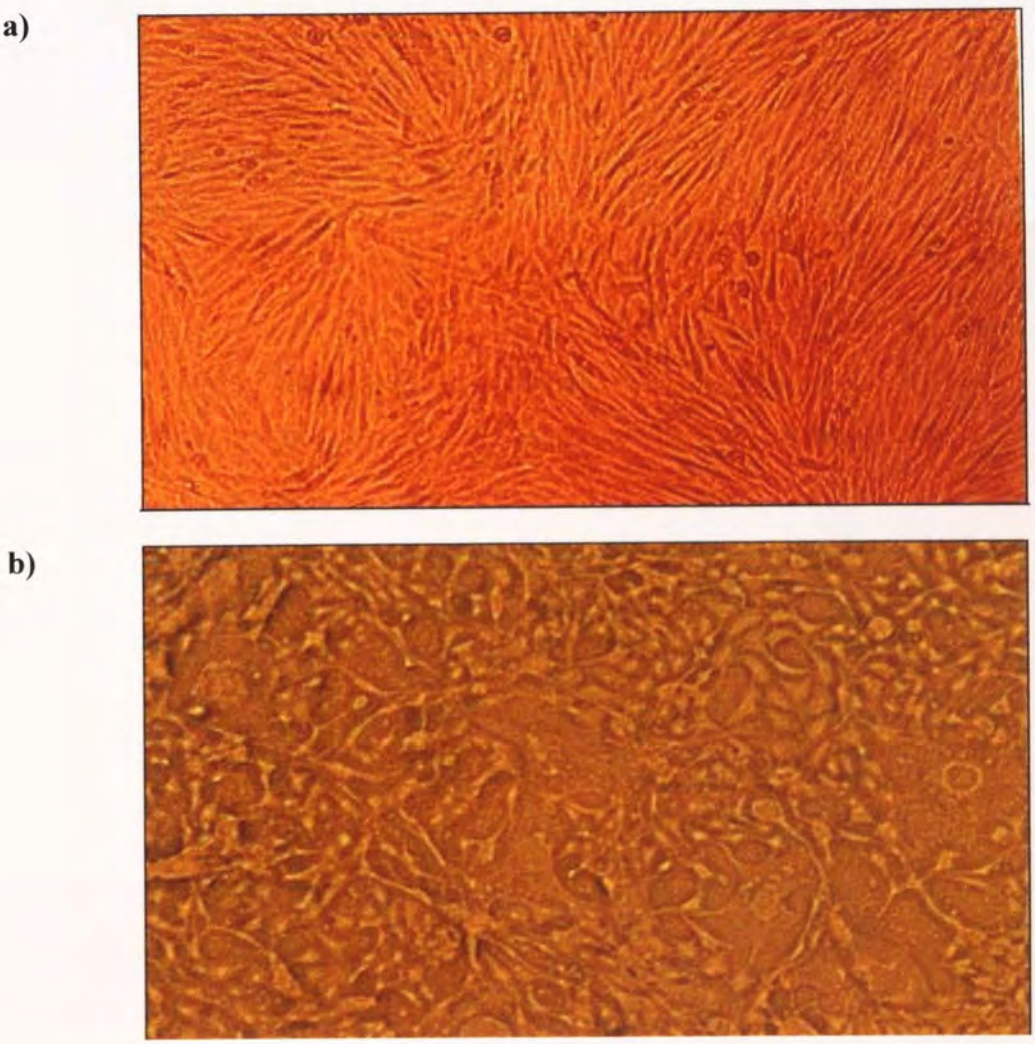


Figure 5.1 Phase contrast photographs of the two glioma cell lines.
a) U87-MG cells and **b)** T98G cells. Cells were plated onto plastic 24 well plates and photographed at confluence (magnification x 100).

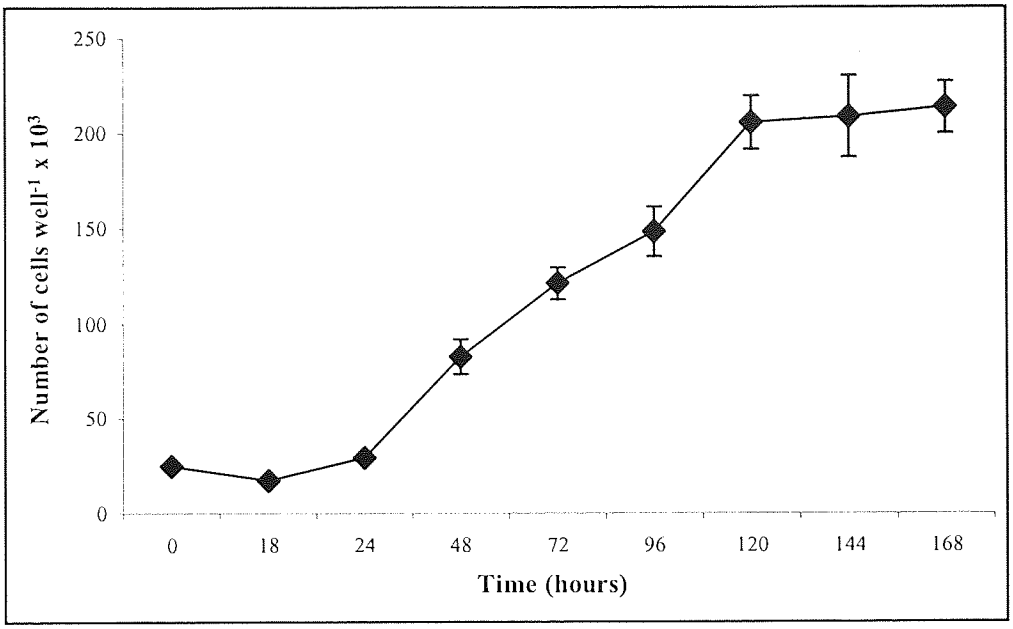
Prior to any experiments being conducted on these cells, it was essential to characterise optimal growth conditions in order to assess plating conditions. Both cell lines were seeded into 24-well plates at a density of $2.5x10^4$ cells $well^{-1}$ (time 0) in full serum media. Cells were then incubated at 37°C for the time periods indicated, at which point the cell numbers were determined by Trypan blue exclusion assay (section 2.5.5). The resultant growth curves are shown in Figure 5.2. The graphs illustrate the lag in growth of both cell lines over the initial 24 hours. Cells then enter the faster, exponential phase of growth from 24-120 hours post seeding. At this point, growth is observed to plateau, with U87-MG cells achieving densities of approximately $200x10^3$ cells $well^{-1}$. T98G cells reach a plateau in growth at approximately $140x10^3$ cells $well^{-1}$, the lower cell density attributable to the larger size of T98G cells, as shown by microscopic examination (Figure 5.1).

5.3 Comparison of Cell Proliferation Assays

Trypan blue exclusion assay is a standard assay to determine cell number. It is however, somewhat labour-intensive. Thus two alternative methods for assessing cell number were investigated for their suitability as cell proliferation assays.

The first of these was the MTT assay. This measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by the mitochondrial dehydrogenase of viable cells. The amount of formazan produced (maximum absorbance at 562nm) is proportional to the number of living cells present in the test culture (Mossmann, 1993). Before such an assay can be used, it is important to test whether it will yield linear results. Thus a range of U87-MG cell concentrations were seeded into 24-well plates in triplicate. The cells were incubated at 37°C for a short while (2 hours) to allow them to attach to the plate and assayed before the cells had a chance to grow. After this time, each well was incubated with 1ml of 5μg ml^{-1} MTT at 37°C for 4 hours. Subsequently a stop solution of 1ml 10% SDS/0.01M HCl was added

a)



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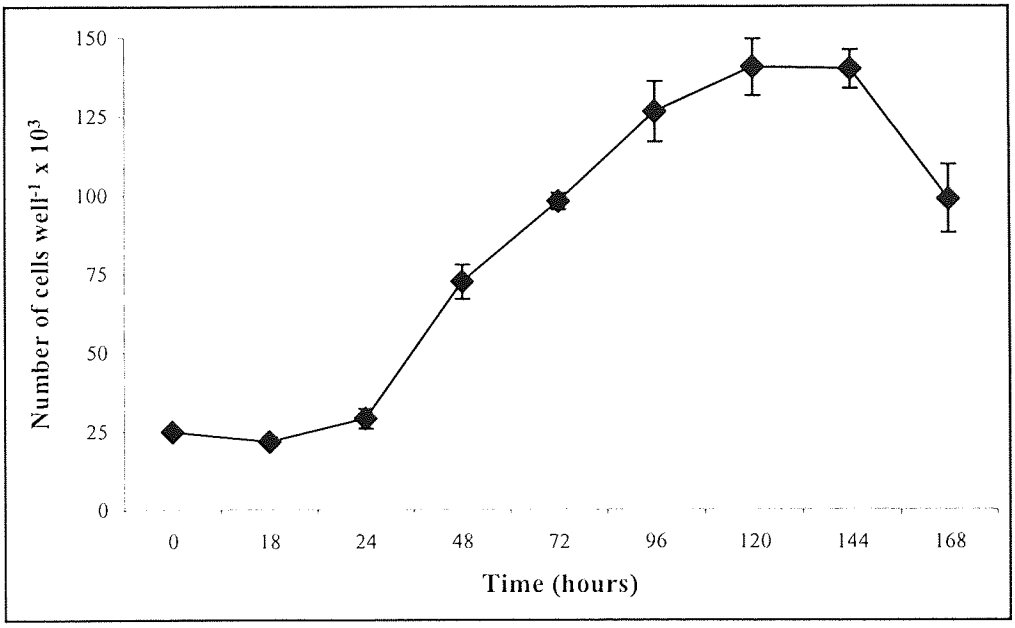


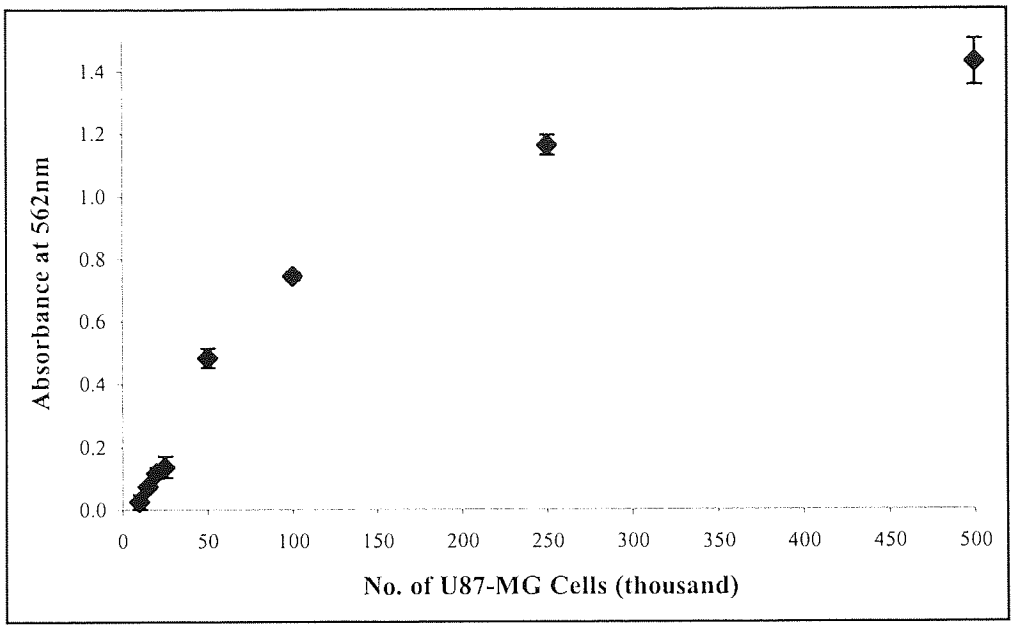
Figure 5.2 Growth curves of the two glioma cell lines.
Standard growth curves for **a)** U87-MG and **b)** T98G cells from an initial seeding density of $2.5x10^4$ cells $well^{-1}$ at 37°C in 24 well plates. Results (n=4±SD) are given as the number of cells $well^{-1}$ x 10^3 at each time point as determined by Trypan blue exclusion assay (section 2.5.5).

to each well. The cells were then returned to 37°C overnight to allow the cells to solubilise and the colour to develop overnight. The absorbance of the resultant solution was measured at 562nm. The results of this trial MTT assay are shown in Figure 5.3a. As can be seen from the graph, the results produced are not linear over the entire range studied. Hence the cell number cannot be correlated with the absorbance measured and the MTT assay was thus unsuitable to use for investigation of U87-MG cell number.

The second alternative assay tested for the determination of cell number was the CellTitre-Glo™ Assay (Promega). This is a luminescent cell viability assay, which determines the number of viable cells in culture based on quantitation of the ATP present, which is a marker for the presence of metabolically active cells. CellTitre-Glo™ is marketed as 'ideal for automated high-throughput screening, cell proliferation and cytotoxicity assays'. A supplied single reagent is added to cells in full serum media that generates a luminescent signal proportional to the amount of ATP present, which in turn is directly proportional to the number of cells present. To validate the use of this assay with U87-MG cells, a range of cell concentrations were seeded in triplicate in a 96-well plate in a volume of 100µl. Control wells were also included, which contained medium without cells to account for background luminescence. The cells were incubated at 37°C for 2 hours to allow adherence to the plates, following which the plate and its contents were removed from the incubator and allowed to equilibrate to room temperature for approximately 10 minutes to stabilise the luminescence signal. Then, as per manufacturers instructions, Cell-Glo™ reagent, in equal volume to media *i.e.* 100µl, was added to the cultures and mixed to induce cell lysis shortly before reading plates in a luminometer. As shown in Figure 5.3b, linear values are achieved for values up to 50,000 cells $well^{-1}$. Product information also indicates that a linear relationship will be achieved between the luminescent signal and the number of cells from 0 to 50,000 cells. This is quite an expensive assay as compared to other methods. Also, the limits of accuracy are confined to cell numbers that are quite low compared to what would normally be used for our studies.

Whilst these alternative assays are attractive for their speed and convenience, for our purposes the Trypan blue assay was most appropriate.

a)



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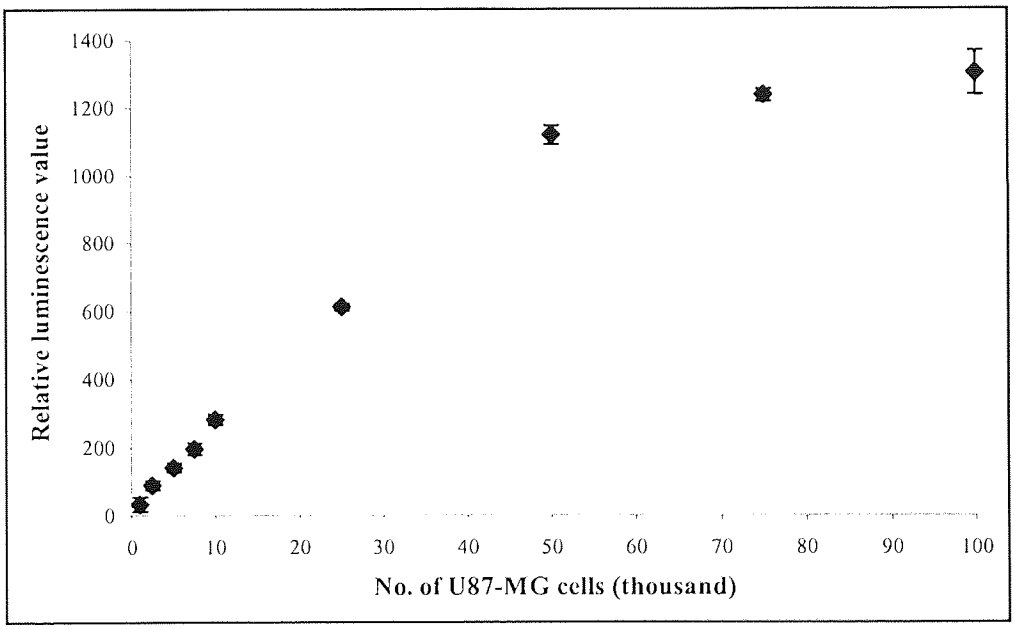


Figure 5.3 Alternative cell number assays.
Graphs of cell number assays: **a)** MTT assay and **b)** ATP based luminescence assay, used to ascertain number of U87-MG cells as alternatives to Trypan blue exclusion assay. (n=3±SD)

5.4 Cellular Association of DNA Enzymes

5.4.1 Optimisation of Cellular Association Assay Protocol

Three main points were considered when optimising the protocol for cellular association studies:

1) The initial seeding density of cells.
2) Time period over which to assess uptake.
3) Stability of DNA enzymes during the experimental period.

The initial seeding density is important for any cell-based assay, as cell density at the time of the experiment will affect results. Cells need to be in the exponential growing, *i.e.* log phase, when they are used to ensure they are performing normal cellular processes. Thus in order for valid comparisons to be made between different experiments, the range of linear growth needed to be established.

To further assess the effect of initial seeding density on the pattern of cell growth, growth curves were established following cells plated at either 50,000 or 100,000 cells well^{-1} in 24-well plates (see section 2.7). Figure 5.4 shows the difference in time that U87-MG cells will take to achieve linear growth depending on their initial seeding density. When seeded at 100,000 cells well^{-1}, there is an initial fall in cell number following which linear growth is achieved fairly rapidly with no observable lag in growth. Following seeding at 50,000 cells well^{-1}, linear growth is only achieved after 48 hours following an initial period of slower growth. With both initial seeding densities, cell growth eventually plateaus and even begins to fall slightly. As transformed cells do not undergo normal contact inhibition (Freshney, 2000), this is possibly due to an increase in toxins and/ or a decrease in available nutrients. Based on these experiments, uptake experiments were performed after 48 hours with cells plated at an initial density of 50,000 cells well^{-1}. Compared to seeding at 100,000 cells, as assessed by microscopy, a more even monolayer was achieved at this density.

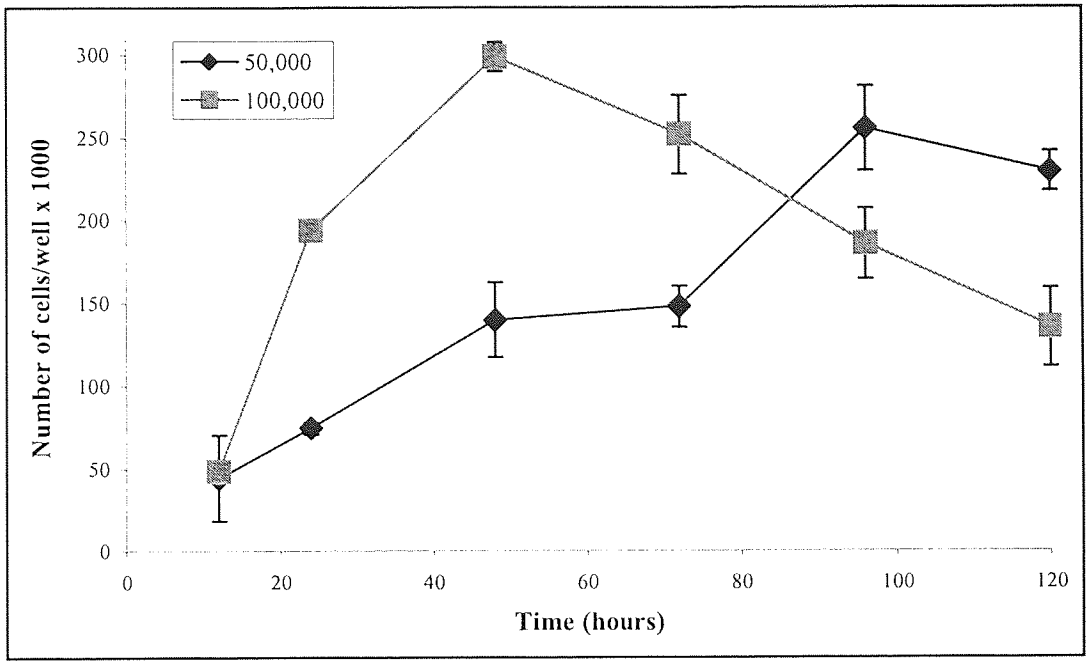


Figure 5.4 Growth curves of U87-MG cells following plating at two different initial densities.

U87-MG cells were seeded in 24-well plates at either $5x10^4$ or $1x10^5$ cells $well^{-1}$ and incubated at 37°C. Results (n=4±SD) are given as the number of cells $well^{-1}$ x 10^3 at each time point as determined by Trypan blue exclusion assay (section 2.5.5).

After determining the initial seeding density to be used, the next factor to be considered was the time over which uptake experiments should be performed. Thus, uptake of a naked DNA enzyme was characterised over 12 hours in serum-free media. DNA enzymes, labelled at the 3'-end, were incubated with U87-MG cells and uptake analysed at various time points over a 12-hour period (see section 2.7).

From the profile in Figure 5.5 and subsequent statistical analysis, it is apparent that there is no significant increase in uptake after 4 hours. This is consistent with the pattern of cellular uptake of DNA oligonucleotides where cellular uptake has been shown to plateau after a few hours (Yakubov *et al.*, 1989). Thus, four hours was taken as the appropriate time over which cellular association/uptake experiments should be performed.

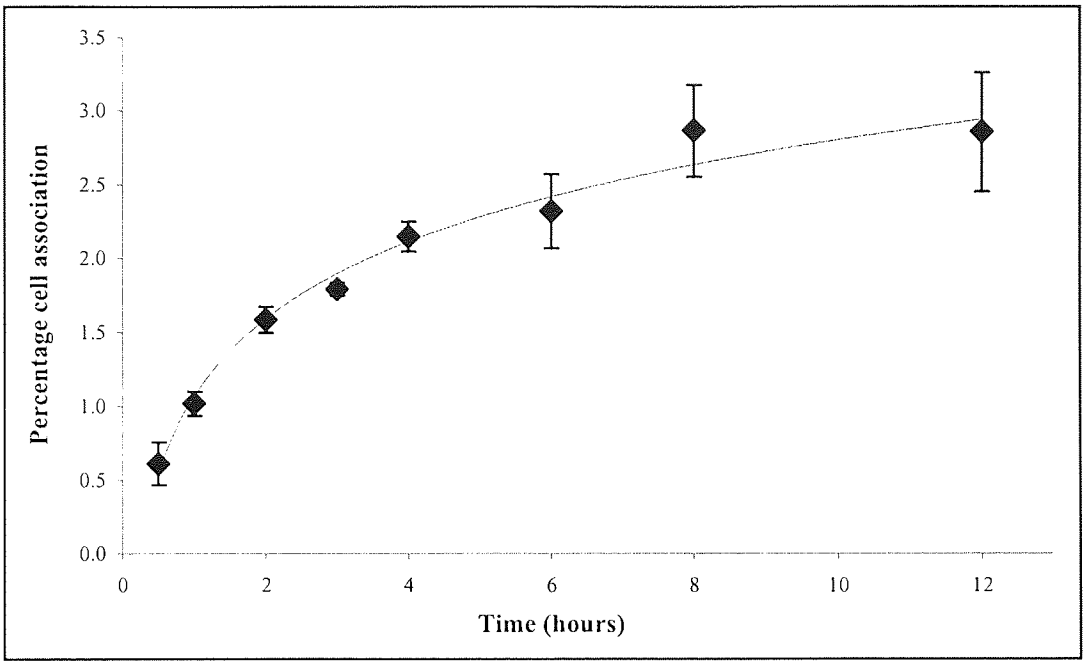


Figure 5.5 Effect of time on cellular association of DNA enzyme with U87-MG cells.

Cell monolayers were incubated with 3'-end labelled HR15 in serum-free media at 37°C for various times over a 12-hour period and cellular association assessed as described in section 2.7.2. (n=3±SD)

In conjunction with the time profile of cell association, it was important to determine the stability of the DNA enzyme during this time. In Chapter 3, it was seen that DNA enzymes in serum-free media were stable for extended periods. However, the stability profile may be altered in the presence of cells. It was important to determine that the cell association recorded was representative of intact DNA enzyme and not of shorter degraded fragments or indeed of free label. Thus, during the time profile of cellular association performed above, a small (100µl) sample of apical media was removed at each time point and quenched in cold formamide buffer. Samples were then visualised by electrophoresis on a 20% denaturing PAGE gel (Figure 5.6). The DNA enzyme (HR15 was used as representative), as is apparent in the autoradiograph in Figure 5.6, is stable up to 8 hours and some degradation is apparent only after 12 hours of incubation in apical media on U87-MG cell monolayers at 37°C. Thus, uptake assessed after a four-hour incubation will be representative of intact DNA enzyme.

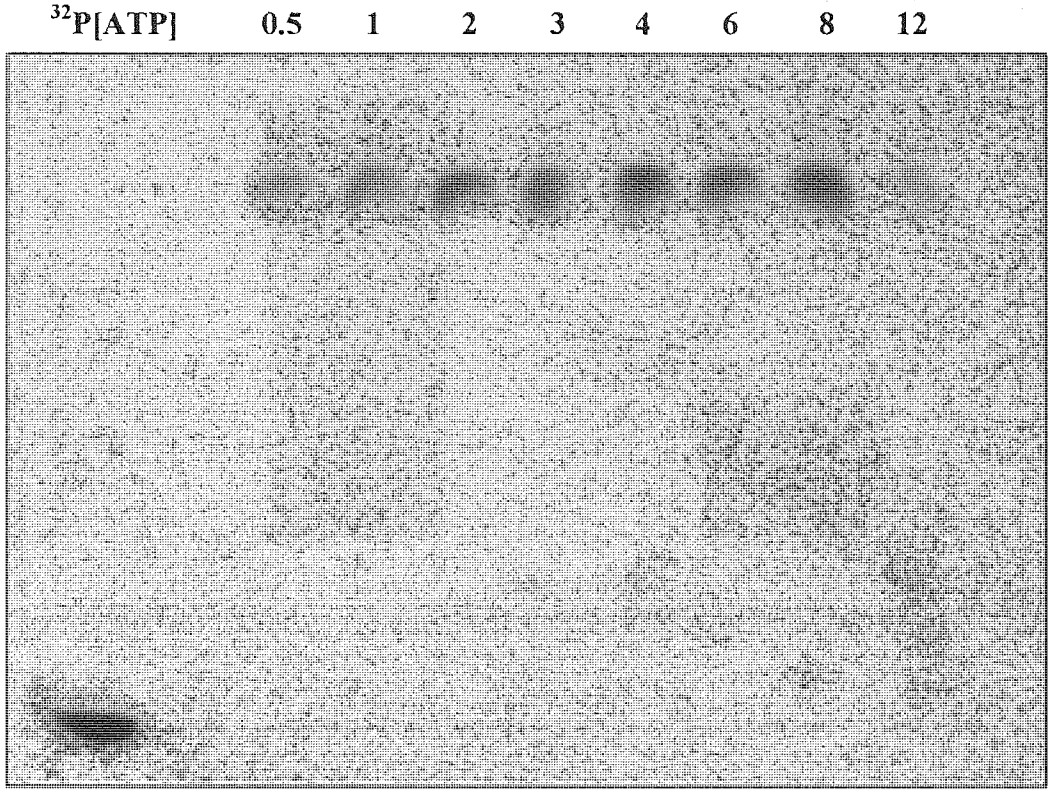


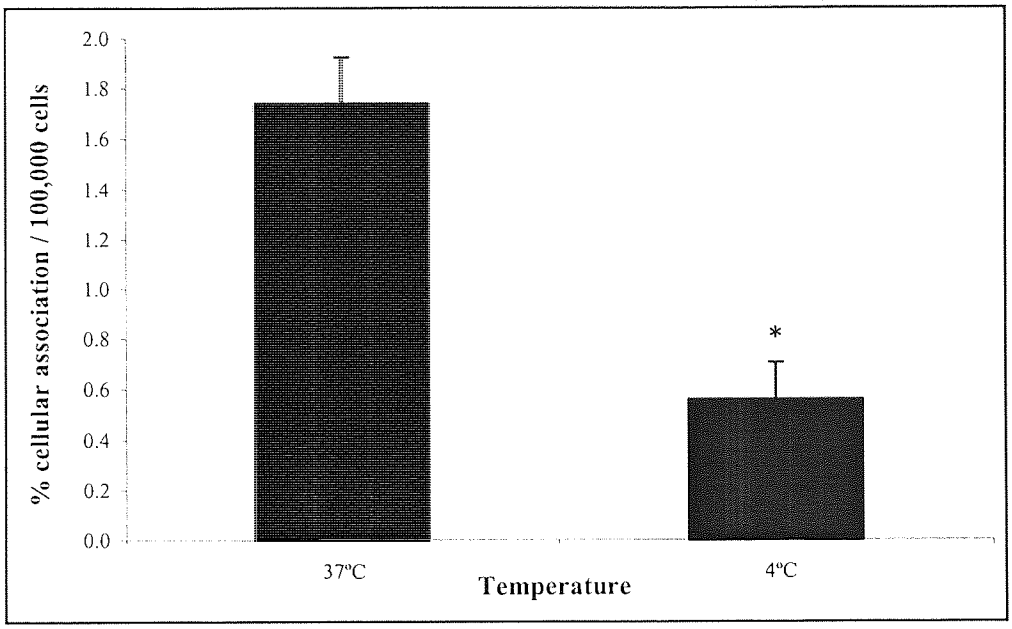
Figure 5.6 Autoradiograph demonstrating stability of DNA enzyme in apical media over time.

3'-end labelled DNA enzyme of mixed PO/PS chemistry (HR15) was incubated with U87-MG cell monolayers in serum-free media at 37°C. At various intervals (times indicated in hours) samples were removed and quenched in formamide buffer (see section 2.8). Samples were analysed by 20% denaturing PAGE.

5.4.2 Temperature Dependence of Cellular Association

Uptake of oligonucleotides is well characterised and it is likely that DNA enzymes will have similar uptake properties. Extensive studies were not carried out on DNA enzyme uptake but a further selected experiment was chosen to establish parallels in the mode of uptake. A study was performed characterising the uptake of naked DNA enzymes at two temperatures, namely 4 and 37 degrees Celsius. A significant decrease in uptake is observed at 4°C compared to 37°C (see Figure 5.7). This strong dependence on temperature is indicative of uptake being an active, energy-dependent process. This suggests uptake is mediated via endocytosis as has previously been cited for ODNs (Akhtar and Juliano, 1992) and largely discounts the possibility of a passive-type mechanism.

a)



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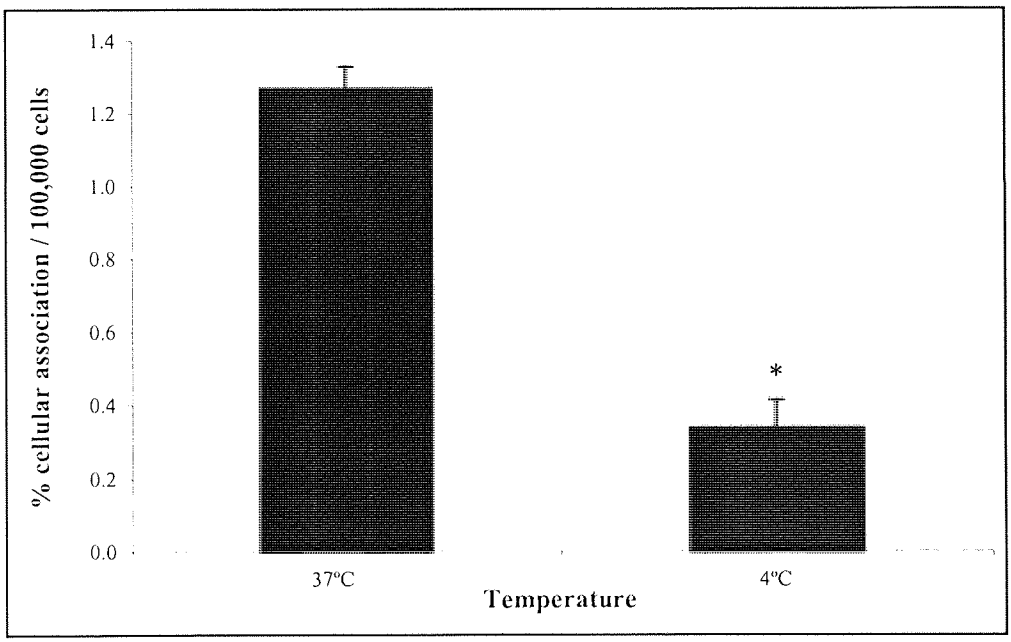


Figure 5.7 Effect of temperature on the cellular association of naked DNA enzymes with U87-MG cells.

Cell monolayers were incubated with 3'-end labelled DNA enzyme: **a)** HR3 and **b)** HR15 at different temperatures. Cells were assessed for association (section 2.7.2) after 4 hours at either temperature. Results (n=4+S.D) are expressed as a percentage of the initial amount of radioactivity applied to the cells, adjusted to 100,000 cells. * denotes a significant ($p<0.05$) difference from association at 37°C.

5.5 Enhancing Delivery of DNA Enzymes to Glioma Cells Using Cationic Lipids

The work presented in this section concentrates on the effort to improve the delivery of DNA enzymes to cells. Delivery methods have to be optimised with respect to various factors, *e.g.* the cell line being used, to ensure maximal delivery. The aim here was to evaluate the cellular delivery of the two novel DNA enzymes and not an exhaustive comparison of the myriad of delivery options available.

As outlined previously, it can be assumed that the uptake properties of DNA enzymes will be similar to those of antisense DNA oligonucleotides (ODNs). The role of cationic lipids in enhancing ODN uptake is described by Cooper *et al.* (1999). Cationic lipids appear not just to increase oligonucleotide accumulation in treated cells, but also to change ODN intracellular distribution. This is characterised by increased ODN accumulation in the nucleus, which may be required for antisense activity to reduce target mRNA. ODN-cationic lipid complexes dissociate subsequent to transfection with the oligonucleotide trafficking to the nucleus and the cationic lipid remaining in a cytoplasmic compartment. Studies suggest that the ODN-lipid complex is internalised by endocytosis. The complex induces a "flip-flop" of anionic lipids normally present on the cytoplasmic face of the membrane bilayer. The anionic lipids then diffuse laterally in the monolayer, displace the ODN from the cationic lipid, resulting in release of free ODN into the cytoplasm of the cell (Zelphati and Szoka, 1996). Charge-mediated uptake is not tissue specific as virtually all cellular membranes are negatively charged from the presence of glycosylated integral membrane proteins and phospholipids (Lee and Huang, 1997).

Two commercially available cationic lipids, Lipofectin™ and LipofectAMINE™ were assessed. Studies have shown that the efficiency of DNA oligonucleotide uptake is dependent on the ratio of DNA to lipid, which can differ between cell types and thus needs to be optimised in each system (Islam *et al.*, 2000). Many studies optimise the amount of lipid to be used by evaluating cellular association at varying ratios of DNA to lipid. However, the problem with this approach when looking at the effect of a range of

DNA concentrations is that amount of lipid may reach toxic concentrations. It would also be difficult to discern activity as in some biological systems, toxicity of a cationic lipid-nucleic acid complex may mimic the nucleic acid activity (Freedland *et al.,* 1996). Thus, a range of lipid concentrations was assessed for potential toxicity using standard Trypan blue cell viability assays. When evaluating lipid-assisted uptake it is vital to perform parallel toxicity assessments, as cellular uptake becomes more pronounced in dead, non-viable cells due to the membrane losing its selective properties (Freshney, 1992).

Manufacturers of cationic lipid reagents recommend that prior to use, careful optimisation is undertaken with regards to lipid and DNA concentrations, cell number, time of exposure to DNA-lipid complexes. When using cationic lipids, DMEM media was supplemented with glutamine only (henceforth known as transfection media). Serum was omitted as it can cause problems with DNA stability and its presence during lipid-DNA complex formation has been shown to reduce transfection (Felgner *et al.,* 1987). It is thought that this effect is a result of negatively charged serum components attaching to the complex and preventing cellular interaction (Litzinger *et al.,* 1996). Antibiotics were omitted as lipids increase cell permeability and allow influx of antibiotics into cells, adversely effecting cell health and consequently reducing apparent transfection efficiency (Fell, 1999).

The uptake of a fixed amount of DNA enzyme (10pmoles labelled and 10nM unlabelled), reflecting the concentration range of ODN found to be effective in cells, was studied in the absence and presence of various concentrations of cationic lipid. The DNA enzymes and lipids were allowed to complex for 15 minutes at room temperature before applying to washed cell monolayers in a final volume of 200μl (see section 2.6). Cellular association was measured after a 4 hour incubation at 37°C as outlined in section 2.7).

5.5.1 Lipofectin™-aided Delivery

A highly efficient DNA transfection technique, 'lipofection' was introduced in 1987 (Felgner *et al.*, 1987). Lipofectin™ is a 1:1 w/w liposome formulation of the cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) and the neutral lipid DOPE (dioleoyl phosphotidylethanolamine). The fusogenic DOPE acts as a helper lipid to destabilise the endosomal membrane, thus permitting release of nucleic acids once inside the cell (Jaaskelainen *et al.*, 1998). Lipofectin™ is a commercially available lipid, widely used in the literature. It has been reported to increase the biological effect of an antisense ODN of PS chemistry 1000-fold (Chiang *et al.*, 1991).

A range of Lipofectin™ concentrations was assessed for toxic effects on U87-MG cells as described in Section 2.6. Cells were plated at $5x10^4$ cells $well^{-1}$ in a 24-well plate and incubated at 37°C for 24 hours. This seeding density has previously been shown to ensure U87-MG cells are in an exponential phase of growth for 48 hours (Figure 5.4). Cell monolayers were then incubated at 37°C with a range of Lipofectin™ concentrations, 0-20μM, diluted in transfection media. After a period of four hours, the lipid-containing medium was removed and replaced with fresh, full serum media. Cells were incubated at 37°C for a further 24 hours before assessment of cell number using the Trypan blue exclusion assay.

The results of this Lipofectin™ toxicity assay are shown in Figure 5.8. As a result of this study, 10μM was chosen as the optimum Lipofectin™ concentration to use in enhancing the uptake of DNA enzymes, as this represents the maximum amount of lipid that can be used without significant toxicity being observed. Some studies have adopted the approach of keeping a constant lipid concentration to ensure toxic effects of the lipid do not confound results (Roh *et al.*, 2000; Monia *et al.*, 1996). Whichever method of optimisation is used, it is clear that DNA-lipid complexes need to be optimised empirically for each experimental setup and will be a balance between DNA uptake and cytotoxicity of the lipid (Holmes *et al.*, 1999).

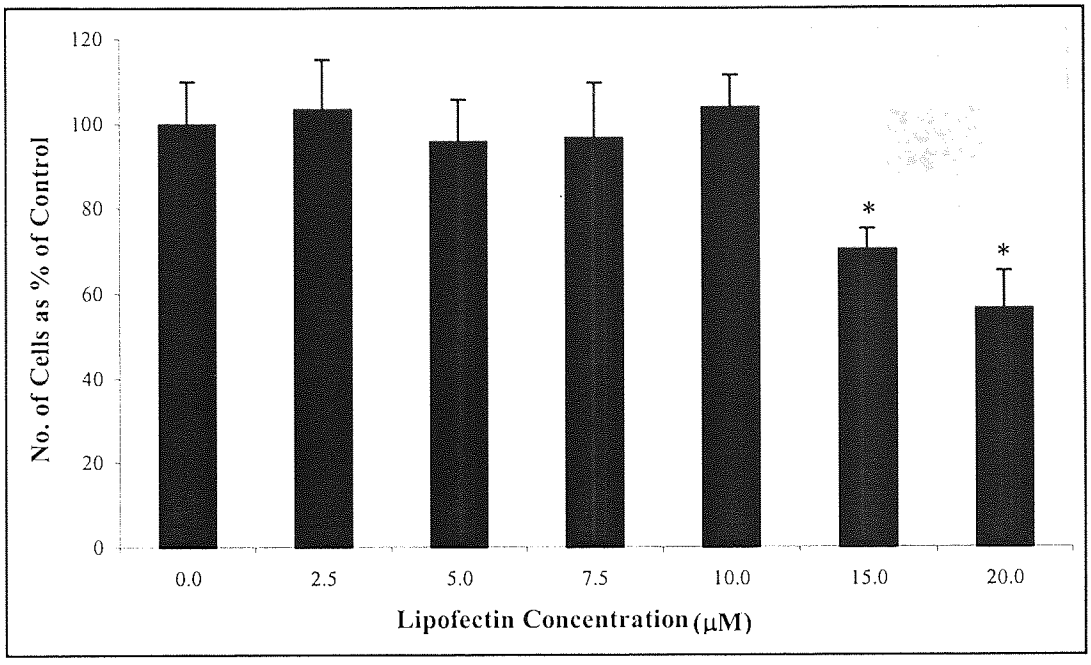
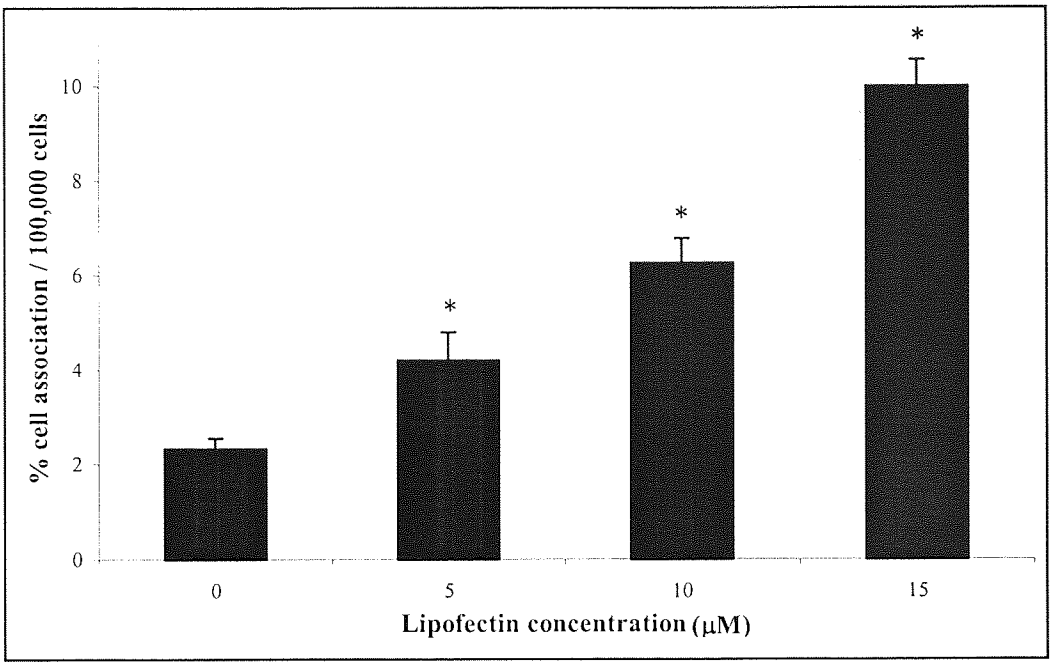


Figure 5.8 Toxicity of Lipofectin™ on U87-MG cells.
Lipofectin™, diluted in transfection medium, was added to U87-MG cells at concentrations ranging from 2.5 to 20μM as described in section 2.6. Mean cell numbers (n=3±S.D.) as determined by Trypan blue assay are expressed as a percentage of the untreated cell number. * denotes significant ($p<0.05$) difference from control.

After establishing the maximum non-toxic concentration, the improvement in cellular association offered by Lipofectin™ was assessed. The uptake of a fixed amount of DNA enzyme (10pmoles labelled and 10nM unlabelled) was studied with 0, 5, 10 and 15μM Lipofectin™ *i.e.* the established maximum non-toxic concentration and two values on either side of this. The graphs in Figure 5.9 show the Lipofectin™ aided uptake of HR3 and HR15. Clearly, Lipofectin™ similarly increases the transfection of both DNA enzymes, to approximately 8% at the optimum concentration of 10μM. The importance of doing parallel toxicity studies with uptake studies is evident. Cellular association/uptake of DNA enzyme apparently increases with 15μM Lipofectin™, but the toxicity study identified this concentration to be toxic to cells. As aforementioned, cellular uptake becomes more pronounced in dead, non-viable cells, due to the membrane losing its selective properties. The toxic effects of high lipid concentrations may be attributable to the lipid puncturing holes in the cell membrane.

a)



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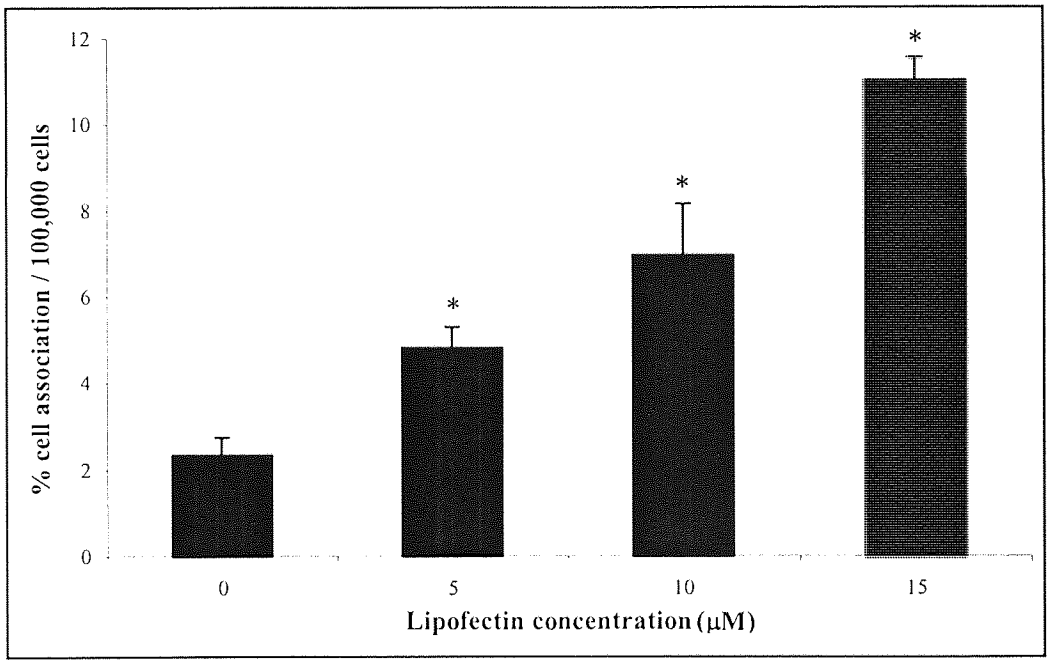


Figure 5.9 Enhancement of cellular association of DNA enzymes using various concentrations of Lipofectin™.

Cellular association of **a)** HR3 and **b)** HR15 (each using 10pmoles 3'-end labelled DNA enzyme and 10nM unlabelled) was studied with different concentrations of Lipofectin™. Uptake is normalised to every 100,000 cells. Three different concentrations of Lipofectin™ were used, the optimum as determined by cell viability assay and concentrations on either side of this optimum. * denotes significant ($p<0.05$) difference from association of naked DNA. (n=4±SD)

5.5.2 LipofectAMINE™-aided Delivery

LipofectAMINE™ is a 3:1 liposomal formulation of the polycationic DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxaimdo)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate) and the neutral DOPE. It has been used successfully for the delivery of DNA oligonucleotides to cells (Holmes *et al.*, 1999). Akin to Lipofectin™, the charged lipid (DOSPA) helps the liposome to bind to the negatively charged cell membrane by charge interactions (Zhou and Huang, 1994). The neutral DOPE, an inverted-cone-shaped lipid, is included as it is thought to facilitate cytosolic release through the fusion to and/or the disruption of the endosomal membrane (Farhood *et al.*, 1992).

As for Lipofectin™, toxicity of lipid was assessed using standard Trypan blue cell viability assays. LipofectAMINE™ has more positive charges per molecule than Lipofectin™, thus a slightly lower range of concentrations, 1 to 15μM, was used. Maximal cell association of DNA is generally reported near the point at which a neutral charge is achieved (Bally *et al.*, 1999; Hope *et al.*, 1998) *i.e.* the negative charges on the DNA are neutralised from positive charges on the cationic lipid (Bennett *et al.*, 1997); or where there is a slightly positive net charge (Hartmann *et al.*, 1998). It is postulated that lipid-DNA complexes exhibiting a net positive charge are more readily endocytosed by cells in culture. Lappalainen *et al.* (1997) demonstrated that release from vesicles and transport into the nuclear area was faster when complexes had a positive net charge. In addition, Jaaskelainen *et al.* (1994) have reported that complexes where there is a net negative charge do not result in release of vesicle contents.

The toxicity assay was performed as done previously for Lipofectin™. In the case of LipofectAMINE™ as shown below (Figure 5.10), concentrations above 2.5μM are toxic to U87-MG cells. Thus 2.5μM was chosen as the optimum concentration to use to enhance uptake of DNA enzymes, as it is the maximum concentration that can be applied to cells with no significant deleterious effect on cell health. The much lower concentration, as compared to Lipofectin™, at which toxicity is observed, reflects the fact that LipofectAMINE™ has more positive charges per molecule.

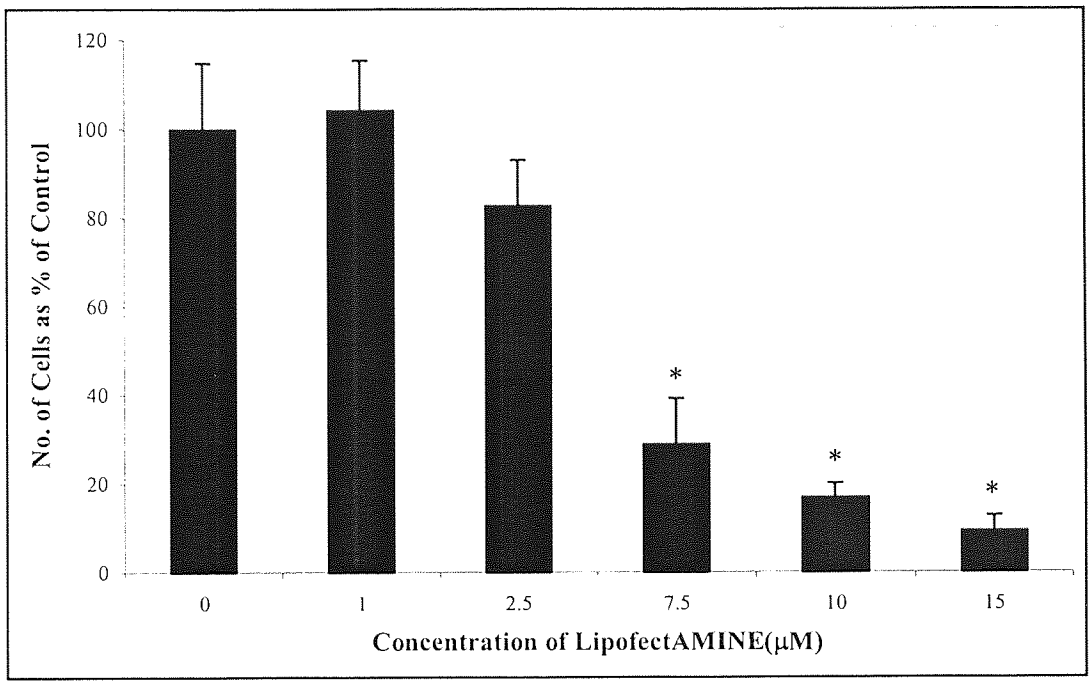
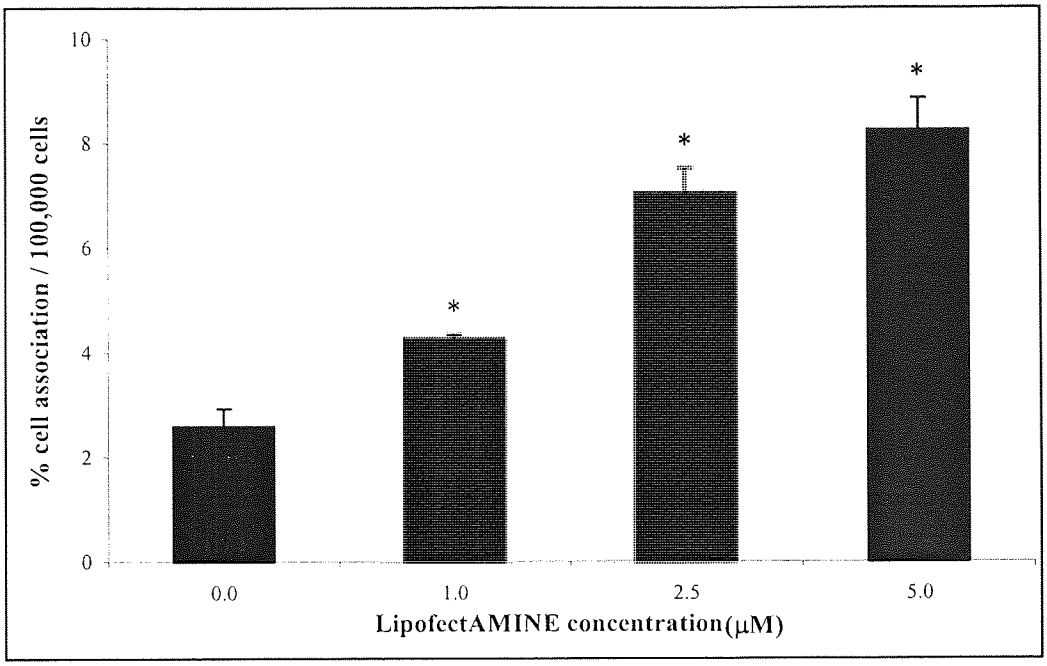


Figure 5.10 Toxicity of LipofectAMINE™ on U87-MG cells.
LipofectAMINE™, diluted in transfection medium, was added to U87-MG cells at concentrations ranging from 1.0 to 15μM as described in section 2.6. Mean cell numbers (n=3±S.D.) as determined by Trypan blue assay are expressed as a percentage of the untreated cell number. * denotes significant ($p<0.05$) difference from control.

After establishing the maximum non-toxic concentration, the improvement in cellular association offered by LipofectAMINE™ was assessed. The uptake of a fixed amount of DNA enzyme (10pmoles labelled and 10nM unlabelled) was studied with 0, 1.0, 2.5 and 5.0μM LipofectAMINE™ *i.e.* the established maximum non-toxic concentration and two values on either side of this. The graphs in Figure 5.11 depict the LipofectAMINE™ assisted uptake of HR3 and HR15. Clearly, LipofectAMINE™ also effectively increases the transfection of both DNA enzymes, to approximately 10% at the optimum concentration of 2.5μM. The importance of doing parallel toxicity studies with uptake studies is again apparent. Cellular association/uptake of both DNA enzymes increases when LipofectAMINE™ concentration is increased to 5.0μM, which was identified to be toxic to cells. Both cationic lipids increase the cellular association/ uptake of DNA enzymes with similar efficiencies. There is also no observable difference in the amount of association of HR3 and HR15.

a)



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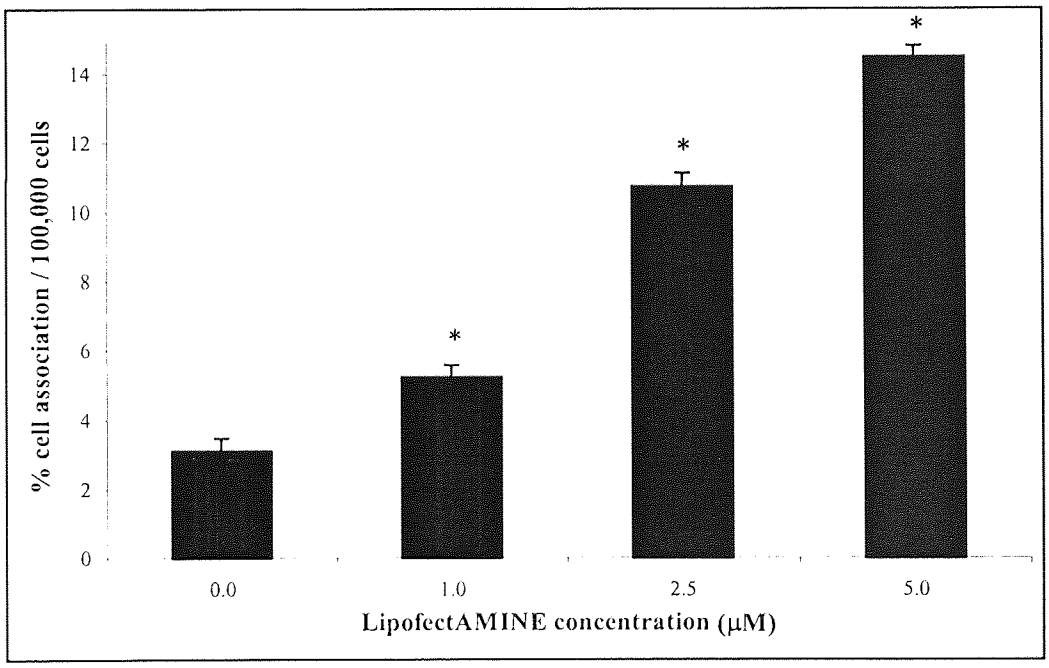


Figure 5.11 Enhancement of cellular association of DNA enzymes using various concentrations of LipofectAMINE™.

Cellular association of **a)** HR3 and **b)** HR15 (each using 10pmoles 3'-end labelled DNA enzyme and 10nM unlabelled) was studied with different concentrations of LipofectAMINE™. Uptake is normalised to every 100,000 cells. Three different concentrations of LipofectAMINE™ were used, the optimum as determined by cell viability assay and concentrations on either side of this optimum. * denotes significant ($p<0.05$) difference from association of naked DNA. (n=4±SD)

5.6 Cellular Efficacy of DNA Enzymes

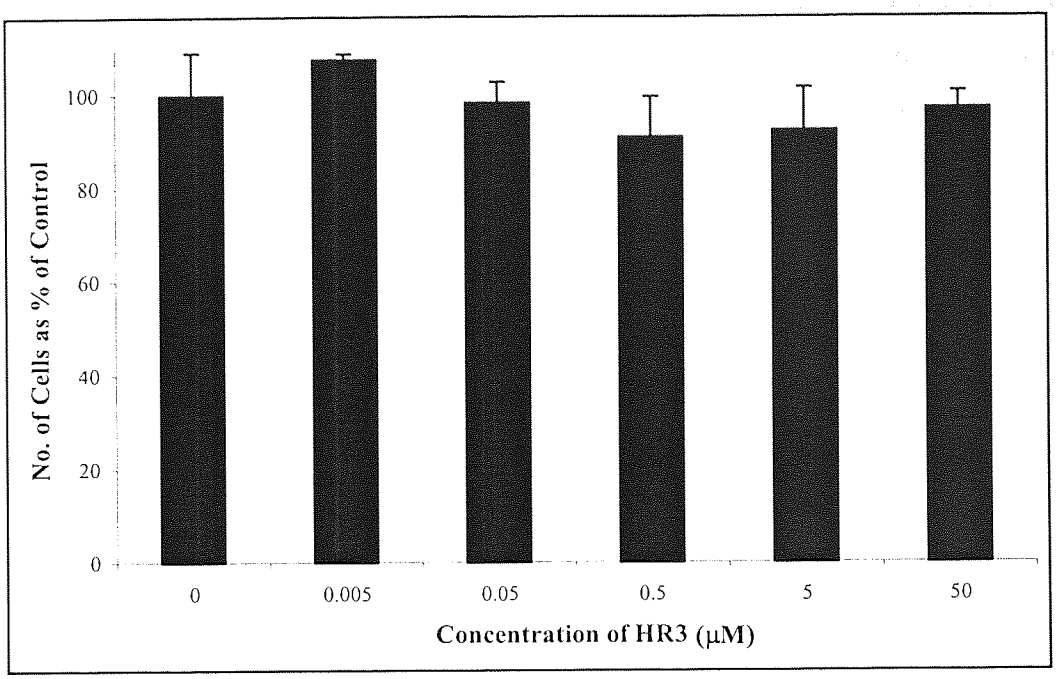
DNA enzymes, like antisense oligonucleotides, are polyanionic molecules, which do not readily cross biological barriers. Earlier, it was demonstrated that uptake of naked DNA enzymes, similar to ODNs, is very inefficient. The two commercially available cationic lipids, Lipofectin™ and LipofectAMINE™, have successfully improved the transfection of DNA enzymes into glioma cells.

Having optimised the lipid-mediated delivery of DNA enzymes, the next step was to assess the DNA enzymes for anti-proliferative effects on the U87-MG glioma cell line. As the two lipids displayed similar efficiencies in improving transfection, Lipofectin™ was chosen as the delivery agent of choice on the basis of lower apparent toxicity on U87-MG cells.

The first step was to assess the potential toxicity of DNA enzymes to U87-MG cells. The DNA enzymes being tested *i.e.* HR3 and HR15, are of mixed PO/PS chemistry and PS ODNs have been known to have non-specific toxic effects on cells (Coulson *et al.*, 1996). ODNs in cell culture are generally used in the nM to μM range (Coulis *et al.*, 2000; Tian *et al.*, 2000). Thus, a wide range of DNA enzyme concentrations, from 5nM to 50μM, were tested for potential toxicity. HR3 and HR15 were diluted to various concentrations with transfection medium, administered to U87-MG cell monolayers and incubated at 37°C (see section 2.6). After a period of 4 hours, the treatment media was removed and replaced with full media. The cells were assayed using the Trypan blue assay (section 2.5.5) 24 hours post-transfection.

The results of the DNA enzyme toxicity tests (Figure 5.12) show that even at a concentration of 50μM, neither DNA enzyme displayed any significant (*i.e.* $p>0.05$) toxic effect on U87-MG cells.

a)



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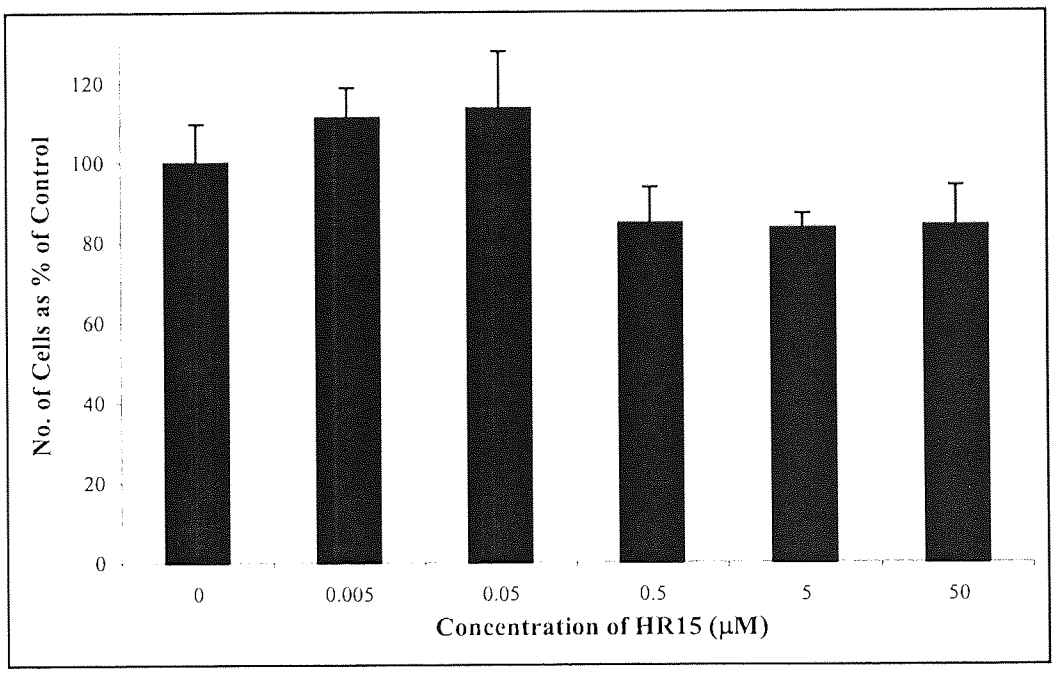


Figure 5.12 Effect of DNA enzyme concentration on U87-MG cell number. DNA enzymes, **a)** HR3 and **b)** HR15, were applied to U87-MG cells at a range of concentrations in transfection media (section 2.6). Results (n=3±S.D.) are expressed as a percentage of untreated cell number. No significant ($p<0.05$) difference to control values was observed. (n=3±SD)

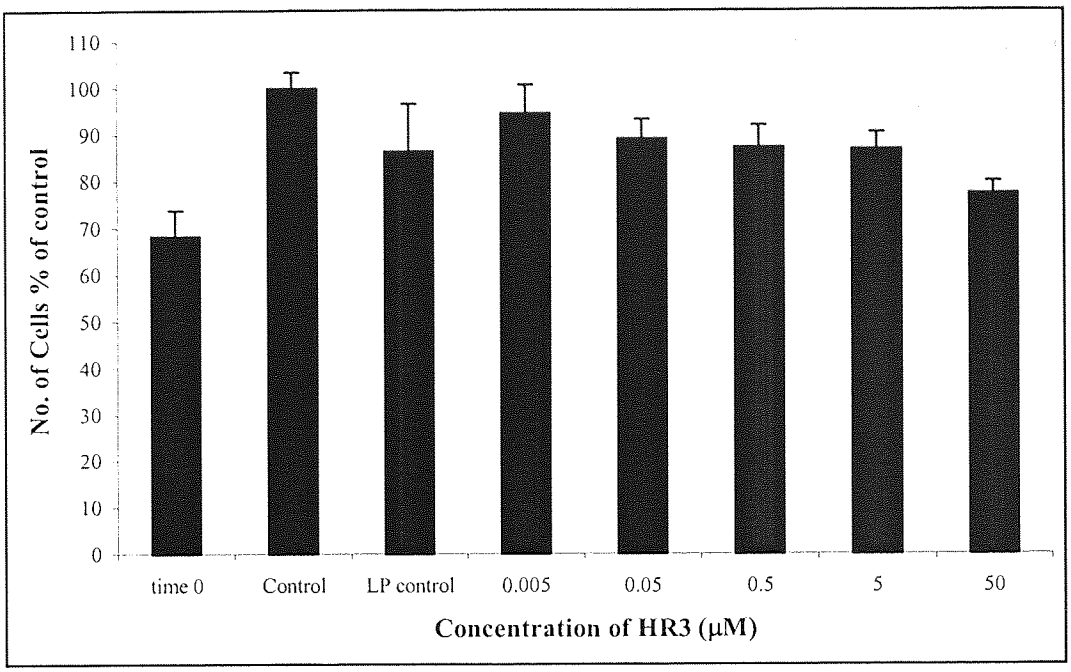
The basis of using telomerase as a target in cancer therapy is the telomere hypothesis (Harley, 1991), which postulates that in the absence of telomerase, loss of telomere DNA eventually triggers a cellular response, perhaps via a DNA damage response, that leads to growth arrest or apoptosis. In addition there now exists a large body of evidence implicating telomerase in cancer (as discussed in section 1.2.5).

From the data presented in Figure 5.12, it was ascertained that the two DNA enzymes did not have any significant toxic effects on U87-MG cells over a wide range of concentrations. Thus to assess the anti-proliferative capacity of HR3 and HR15, the two DNA enzymes were delivered to U87-MG cells using Lipofectin™ at the optimised concentration of 10μM (section 2.6).

Cells were seeded in 24-well plates and used 48 hours post-seeding at approximately 80% confluency. Various concentrations (5nM-50μM) of DNA enzyme were mixed with 10μM Lipofectin™ in transfection media. DNA-lipid complexes were allowed to form during a 15 minute incubation at room temperature. The U87-MG cells were incubated with the DNA-lipid complexes, in a final volume of 200μl, for 4 hours at 37°C. After the transfection period, treatment media was removed and cells supplemented with fresh full serum media. The effect on cellular viability was determined using the Trypan blue exclusion assay 24 hours post transfection.

The results of the cellular activity of DNA enzymes are shown in Figure 5.13. It is clear that lipid-mediated delivery of the DNA enzymes over a 4 hour period did not significantly (*i.e.* $p>0.05$) inhibit cell proliferation compared to controls.

a)



b)

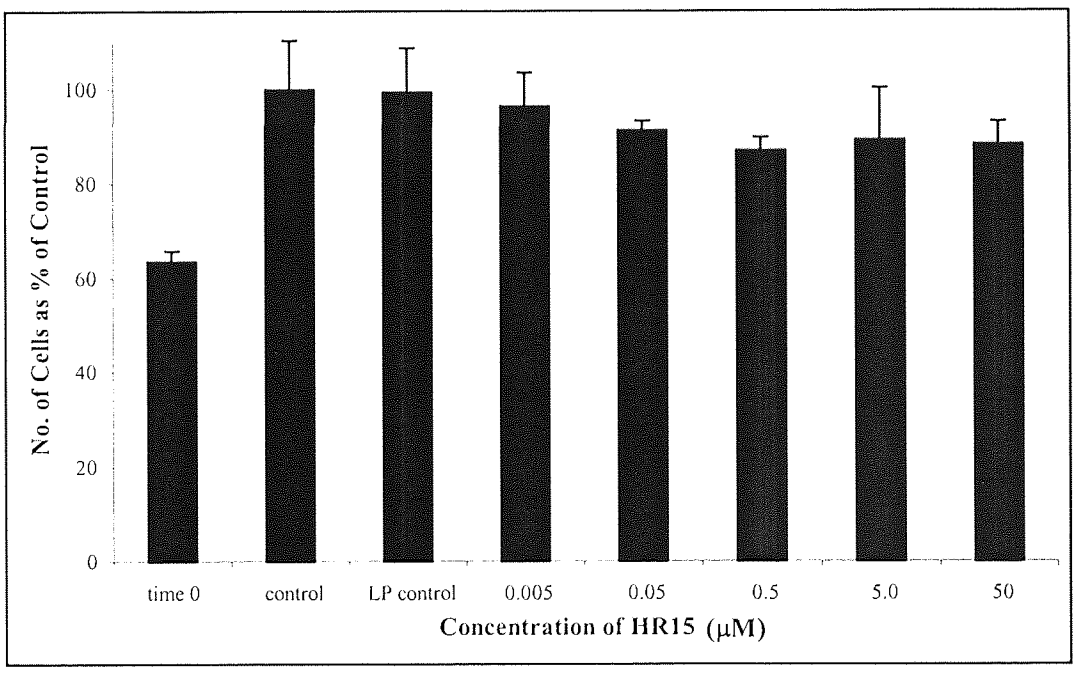


Figure 5.13 Effect of lipid-mediated DNA enzyme treatment on the proliferation of U87-MG cells over 24 hours.

DNA enzymes, **a)** HR3 and **b)** HR15, were complexed with 10μM Lipofectin™ in serum-free DMEM and delivered to U87-MG cells at a range of concentrations. Results (n=4±S.D.) are a percentage of untreated cell number. Control represents untreated cells, and the LP control was treated with Lipofectin™ alone. No significant ($p<0.05$) difference to control values was observed.

Telomerase is a critical enzyme for the long-term proliferation of immortal tumour cells. The role of telomerase in cancer and subsequent approaches to inhibiting telomerase has been discussed at length previously. Here, after a 4 hour Lipofectin™ assisted treatment, no effect on cell number was observed

Expression of an antisense RNA complementary to hTR caused a decrease in the proliferation of HeLa cells (Feng *et al.,* 1995). However, this was after 23-26 population doublings. It is due to this long lag period between telomerase inhibition and telomeres shortening sufficiently to produce detrimental effects on cells that telomerase poses such an unusual challenge for drug development. Similarly, Herbert *et al.* (1999) showed 2'-*O*-methyl RNA targeted to the template region of hTR to be effective in reducing telomerase activity and telomere length. However, to achieve detrimental effects on cell growth took a long time, a minimum of 15 days before a slowing of growth was seen and 110 days before 100% apoptosis was observed.

However, although it is generally proposed that telomerase inhibitors would require approximately 20 rounds of cell division in target cells before their telomeres become critically shortened, there is evidence to suggest that telomerase activity in some cell types can be dissociated from telomere maintenance, which is indicative of a regulatory function for telomerase involving telomere capping (Ouelette *et al.,* 1999). Consequently telomerase inhibition may result in anti-proliferative effects after a much smaller number of generations of cell growth.

Assuming the inhibition of telomerase will have detrimental effects on telomere length, a method for prolonged and sustained delivery is required to overcome the lag period between inhibition of telomere maintenance and cell death

5.7 Delivery of DNA Enzymes Using PLGA Microspheres

DNA enzymes or indeed antisense oligonucleotides are relatively short-lived in circulation. When targeting telomerase, which will take many generations to have an effect on cell growth, repeated or sustained delivery would be needed. As aforementioned, the aim of these studies is not to evaluate delivery options, rather study DNA enzyme characteristics using telomerase as a model target. However, it is worth thinking of possible options for using antisense therapy to target telomerase as a possible therapy for GBM or other cancers.

Poly (lactide-co-glycolide) (PLGA) microspheres have been widely studied as a biodegradable sustained release formulation for the delivery of nucleic acid based therapies. These particulate delivery systems offer several benefits including the potential for localised, site specific or organ specific delivery, protection from nucleases present in the biological milieu and the ability to tailor release characteristics to control pharmacokinetic and pharmacodynamic parameters of the entrapped drug (Khan *et al.*, 2000). PLGA formulations are biocompatible as degradation produces lactic and glycolic acid monomers and carboxylic acids, which are finally metabolised to carbon dioxide and water and excreted via the kidneys (Gilding *et al.*, 1979).

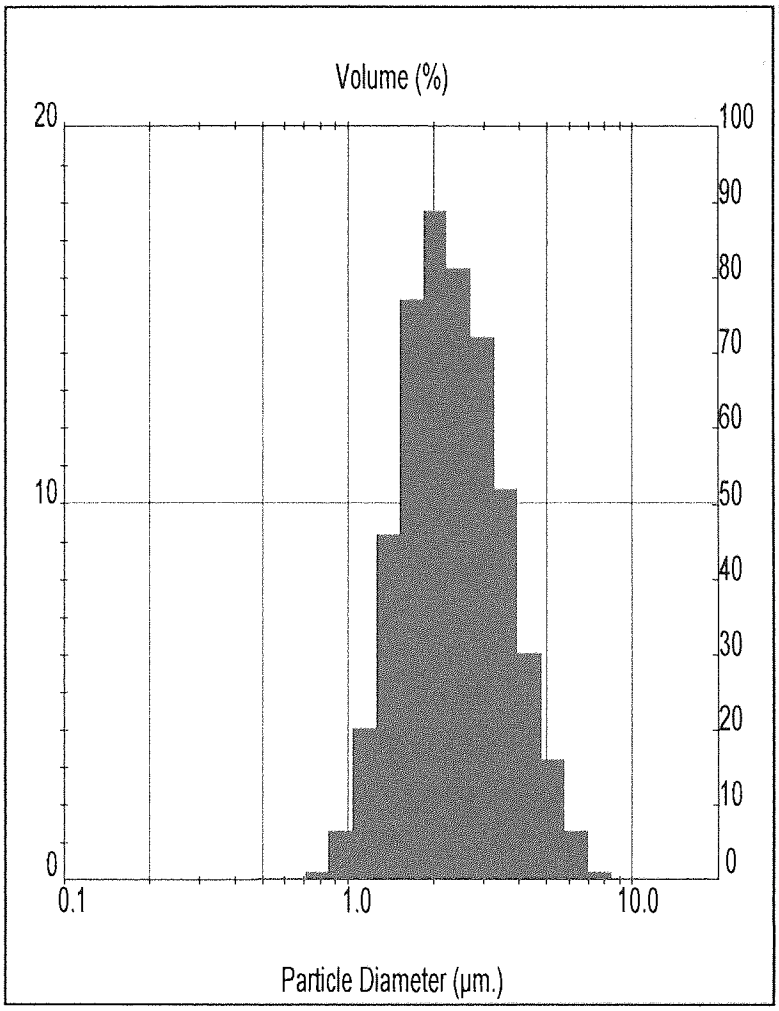
Microspheres are microporous spherical matrix systems where the drug is uniformly dispersed within the polymer. They have been fabricated using various methods, the most popular being the w/o/w solvent evaporation (*i.e.* double emulsion) method (O'Donnell and M^{c}Ginity, 1997) used here. Briefly, this method entails dissolving polymer in an organic solvent *e.g.* methylene chloride and emulsifying into an aqueous drug solution to form a w/o emulsion. This primary emulsion is then re-emulsified into aqueous solution containing an emulsifier, usually polyvinyl alcohol, to produce the final w/o/w emulsion. The common process parameters that have to be considered when developing such a procedure are the selection of solvents, the rate of solvent removal, polymer type and concentration, ratio of solvent to aqueous drug solution, emulsifier type, emulsion stability and mixing conditions (Nihant *et al.*, 1994). A standard method, previously optimised in our laboratory was used here but if this work

was carried further the formulation characteristics could be changed to achieve a variety of microsphere characteristics. *In vitro* release profiles can also be tailored for different uses under the influence of factors such as type of polymer used, size of fabricated microspheres and release conditions.

A small (pM) amount of radiolabelled DNA enzymes was entrapped, as the aim is to establish a proof of concept of sustained delivery of DNA enzymes. PLGA microspheres have been shown to allow entrapment of therapeutic drug concentrations (Ciftci *et al.*, 1996). After formulation of the microspheres, their surface morphology and size were characterised (section 2.10.2). Particle size, an important factor in predicting microsphere behaviour, was determined using a Malvern Mastersizer. Microspheres were also visualised using SEM. The results of this characterisation are shown in Figure 5.14. Each time a batch of microspheres was prepared, an equivalent batch without radiolabelled DNA enzyme was prepared for the purposes of characterisation. Microsphere characteristics can have a major influence on the potential use of these systems (Alonso *et al.*, 1993).

As can be seen in Figure 5.14b, the DNA-enzyme loaded microspheres exhibited a spherical, smooth surface morphology. Figure 5.14a demonstrates a normal size distribution was evident, with an average particle diameter of 2.55μm achieved and 90% below 4μm. High yields (>90%) and encapsulation efficiencies (>50%) were achieved (data not shown) with the method of fabrication employed.

a)



b)

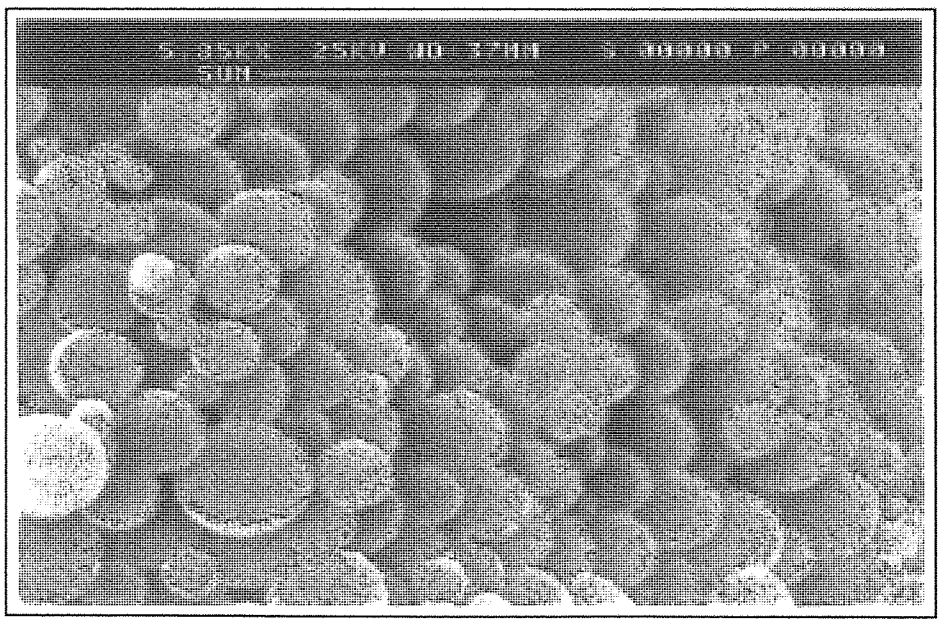


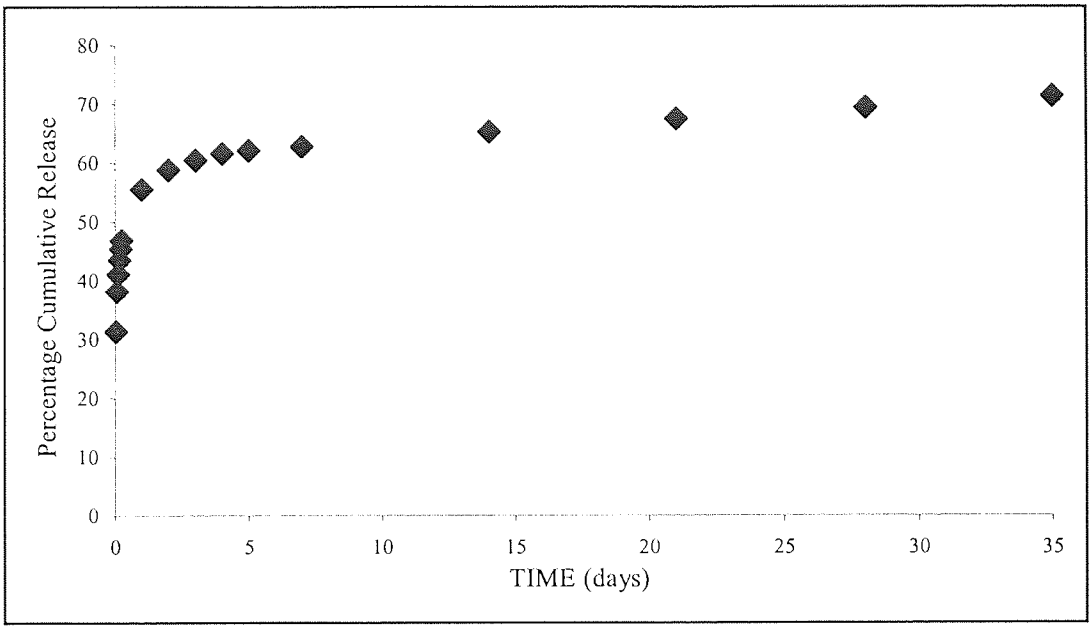
Figure 5.14 Microsphere characterisation data.
a) Particle size distribution of DNA enzyme-loaded microspheres prepared, expressed as particle diameter (log scale) versus percentage frequency.
b) Scanning electron micrograph showing the surface morphology of the microspheres.

Release of DNA enzymes from the formulated PLGA microspheres was studied in physiological buffer at 37°C over a period of 35 days as outlined in section 2.10.3. The resultant release profiles (as shown in Figure 5.15) were similar to those reported for antisense ODNs (Lewis *et al.,* 1998). The profiles for both DNA enzymes are essentially equivalent. Release appears biphasic, which is characterised by an initial 'burst effect' (phase 1) followed by sustained release (phase 2). Phase 1 is usually attributed to the drug being present at or near the surface of the microspheres whereas the slower Phase 2 is thought to represent the movement of drug entrapped deeper in the polymer matrix through pores or aqueous channel networks formed within the polymer matrix upon hydration (Khan *et al.,* 2000).

Numerous studies using PLGA microsphere devices for entrapping ODNs have documented advantages of using biodegradable polymer delivery systems (Putney *et al.,* 1999; Akhtar and Lewis, 1997). Lewis *et al.* (1995) prepared PLGA microspheres containing ODN using a double-emulsion method and evaluated the release of the encapsulated ODN. The authors showed that release profiles could be controlled, and ultimately tailored to specific requirements, by altering the size of the microspheres, the amount of ODN loading and the length of the ODN. In many cases *in vitro* release profiles of antisense ODNs from PLGA matrices have been shown to be triphasic (Cleek *et al.,* 1997; Lewis *et al.,* 1995). Typically, profiles were characterised by an initial "burst effect" during the first 48 hours (phase 1) of release, followed by a more sustained release (phase 2) with a subsequent additional release (phase3) resulting from bulk degradation of the microspheres.

The use of biodegradable polymeric delivery systems, that provide sustained release of the active compound, may obviate the need for repeated administration by improving both the pharmacokinetics and the pharmacodynamics of ODNs and ribozymes. The entrapment of ODNs within such polymeric matrix systems may improve ODN stability, reduce the ODN dose required for efficacy and further reduce toxicity or non-specific activities associated with ODNs.

a)



b)

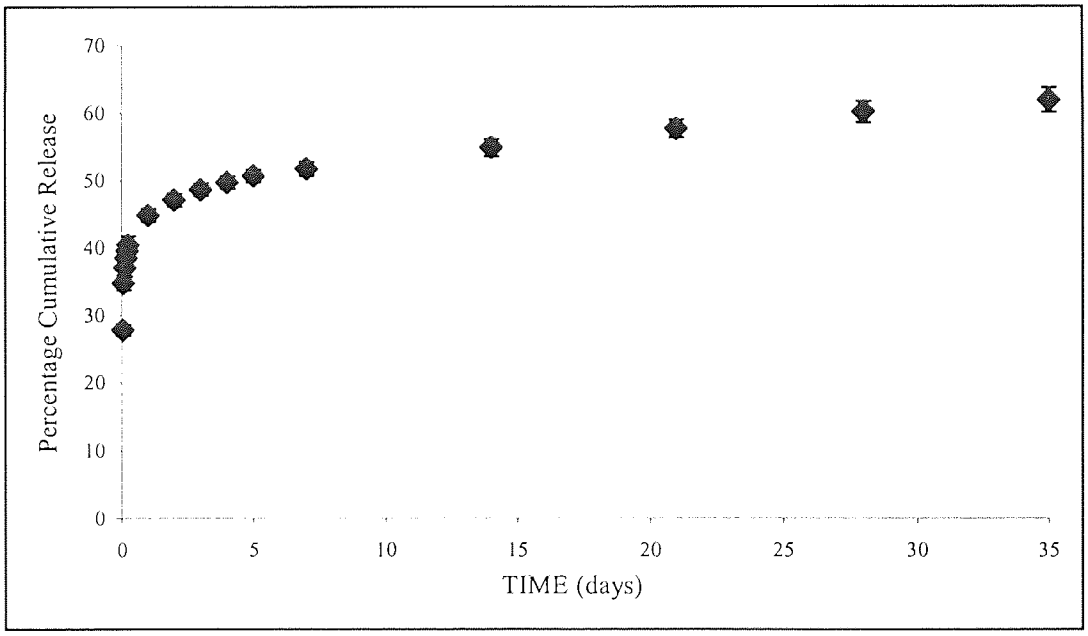


Figure 5.15 ***In vitro*** **release profiles of DNA enzymes from PLGA microspheres.** Cumulative release of **a)** HR3 and **b)** HR15 from 100mg microspheres over 35 days (loading ~ 10pmoles/mg polymer). Results (n=3±S.D.) are expressed as average percentage cumulative release.

5.8 Concluding Remarks

Having previously established HR3 and HR15 as catalytically competent (Chapter 3) and active in glioma cell lysates (Chapter 4), the work outlined in this chapter investigated the delivery and activity of DNA enzymes in a cellular environment.

DNA enzymes are polyanionic molecules, which do not readily cross biological barriers. The cellular association of DNA enzymes is likely to proceed in a similar mechanism to antisense ODNs *i.e.* an active process probably endocytosis. Lipid-mediated delivery was used in an effort to improve the efficiency of delivering naked DNA enzyme to cells. Both Lipofectin™ and LipofectAMINE™ improved the cellular association of the DNA enzymes to U87-MG cells. However, lipid-mediated delivery of DNA enzymes to U87-MG cells over a 4 hour period did not significantly inhibit cell proliferation.

This lack of anti-proliferative effect is likely to be due to the lag period between inhibition of telomere maintenance and cell death. If inhibitors of telomerase are to be of use in the clinic, a method for prolonged and sustained delivery will be required to compensate for this lag. To this end, PLGA microspheres were investigated as a possible device for providing sustained delivery of our DNA enzymes. Small, spherical particles of a narrow size range were achieved and these microspheres demonstrated sustained release over a period of 35 days. ODNs entrapped in polymeric systems have been used successfully *in vivo* (Khan *et al.*, 2000; Putney *et al.*, 1999). Here we have shown the possibility of delivering DNA enzymes by this method, which may prove a promising way forward.

CHAPTER SIX

DNA ENZYME ACTIVITY IN A LUCIFERASE ASSAY

6.1 Development of the Luciferase Assay

The aim of the studies undertaken in this chapter was to develop an assay that would allow the assessment of DNA enzyme activity in cells. As discussed in the previous chapter, when targeting telomerase there is a long lag period before telomeres shorten sufficiently to produce detrimental effects on cell growth. Hence we wanted to develop a reporter assay to allow the activity of DNA enzymes to be studied in a cellular environment. Northern blotting and RT-PCR are possible methods to look at *in situ* effects on cells. However, such techniques are time-consuming, difficult to quantitate and inconvenient when multiple samples are involved. An alternative approach is to fuse sequences from the gene of interest to the coding sequence of an unrelated reporter gene, which allows levels of gene expression to be quantified. Several reporter genes have been developed specifically for this purpose, including green fluorescent protein (GFP), luciferase and chloramphenicol acetyl transferase (CAT).

These studies used firefly (*Photinus pyralis*) luciferase, which was introduced as a reporter of gene expression in 1986 (DeWet *et al.*, 1987). Luciferase is a popular choice as a reporter gene as functional enzyme is created immediately upon translation, and the assay is rapid, reliable and easy to perform. Firefly luciferase catalyses a bioluminescent reaction involving its substrate luciferin, ATP, Mg^{2+} and molecular oxygen. When these components are mixed with cell lysates containing luciferase, a flash of light is emitted, which can be detected using a luminometer or liquid scintillation counter. Light emission is proportional to the luciferase activity of the sample, thereby providing an indirect measurement of the expression level.

There are numerous examples in the literature of luciferase-based reporter gene assays being used to assess DNA enzyme activity (Dash *et al.*, 1998; Goila and Banerjea, 1998; Basu *et al.*, 2000; Unwalla and Banerjea, 2001). In these studies, the reporter gene will be modified to assess the ability of DNA enzymes to cleave their target in a cellular environment.

The commercially available pGL3-Control (pGL3-con) vector was to be modified in two different ways hence producing two slightly different plasmids. The modified plasmids would be created by the insertion of the target (complementary) sequences of the two DNA enzymes. The theoretical premise is that the respective DNA enzymes will cleave their target RNA produced by the modified plasmids. This cleavage will lead to the removal of the 5' cap on the mRNA and subsequently reduce luciferase activity many thousand fold (Weaver, 1999). The cap is attached to the RNA via a triphosphate linkage, which affords protection from degradation at the 5'-end. Hence removal of the cap leads to enhanced degradation (Furuichi *et al.*, 1977). Eukaryotic mRNA gains access to the ribosome for translation through a cap-binding protein. If there is no cap, this protein cannot bind and the mRNA is very poorly translated (Gallie, 1991).

6.1.1 pGL3-con

This vector contains a modified coding region for firefly (*Photinus pyralis*) luciferase. Firefly luciferase is a 61kDa monomer that catalyses the mono-oxygenation of beetle luciferin. Beetle luciferin is a relatively stable molecule found only in luminous beetles. The enzyme uses ATP as a cofactor, although most of the energy for photon production comes from molecular oxygen. The quantum yield is about 0.9, the highest of any known luminescent reactions (Wood, 1990). The gene encoding firefly luciferase (*luc*) is a cDNA clone that has been incorporated into a number of reporter vectors.

The pGL3-con vector has been optimised for monitoring of transcriptional activity in transfected eukaryotic cells. The vector contains an SV40 promoter and enhancer sequences resulting in strong expression of luc^+ in mammalian cell lines. The pGL3-con vector also contains a high copy number prokaryotic origin of replication for maintenance in *Escherichia coli* (*E. coli.*), and an ampicillin resistance gene to aid selection. A Kozak consensus sequence has been engineered immediately 5' of the luciferase gene, which provides optimal translational efficiency.

6.1.2 Construction of the Modified Plasmids

The target sequences were introduced into the vector using a PCR protocol with a proof reading DNA polymerase (*Pwo*). *Pwo* is a highly processive 5' to 3' DNA polymerase possessing a 3' to 5' exonuclease activity also known as proofreading activity, which results in an over 10-fold increased fidelity of DNA synthesis compared to Taq DNA polymerase. Sequences were inserted immediately 5' (upstream) of the Kozak sequence and thus do not alter the sequence of the expressed luciferase protein. The inserted sequences do not contain an ATG codon in any of the three open reading frames and should therefore not initiate aberrant translation.

Figure 6.1 illustrates the plasmid map of the pGL3-con vector. The target sequences were inserted between the SV40 promoter and the luciferase gene, elaborated below.

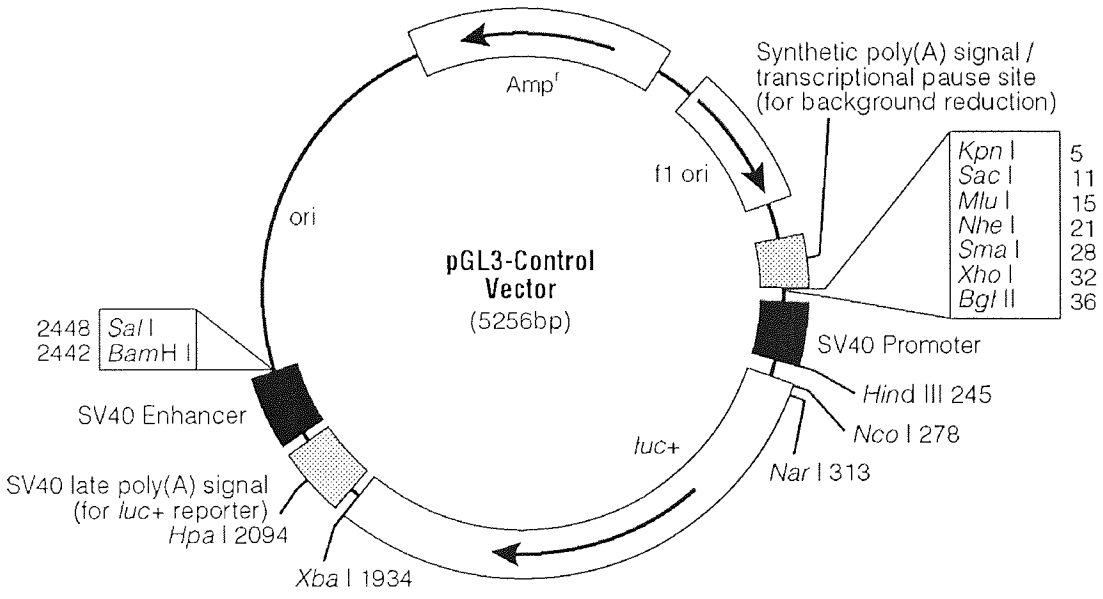
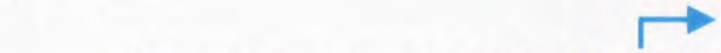


Figure 6.1 Plasmid map the of pGL3-Control vector.

The primer sequences used in the PCR process and the resultant modified sequences are outlined below in Figure 6.2. The vector sequence around the insertion site is shown in detail, with the start codon of the luciferase gene highlighted. The target sites for both DNA enzyme motifs are indicated with the sequence of the designed primers. The reverse primer is the same in both cases, whilst the forward primer contains the inserted DNA enzyme' target sequences, shown in red. The primers were used in a PCR process to introduce the target sequences into pGL3-con vector. Figure 6.3 illustrates a schematic representation of the insertion sites and PCR process.

Vector Sequence:



.....CCGGTACTGTTGGTAAAGCCACCATGGAAGACGCCA.....

.....GGCCATGACAACCATTTCGGTGGTACCTTCTGCGGT.....

10-23 DNA Enzyme Substrate: 5'-GAAGAGUCAAUCCCAAU-3'

Primers:

Forward 5'-GAAGAGTCAATCCCAATCCACCATGGAAGACGCCA-3'

Reverse 3'-GGCCATGACAACCATTTC-5'

8-17 DNA Enzyme Substrate: 5'-GCGGGAAGAGUCAAUC-3'

Primers:

Forward: 5'-GCGGGAAGAGTCAATCCCACCATGGAAGACGCCA-3'

Reverse: 3'-GGCCATGACAACCATTTC-5'

Figure 6.2 Design of primers for the introduction of DNA enzyme target sequences into pGL3-con.

The inserted target sequences for the DNA enzymes are indicated in red. The Kozak sequence is highlighted in yellow. Arrow indicates the start codon of the luciferase gene.

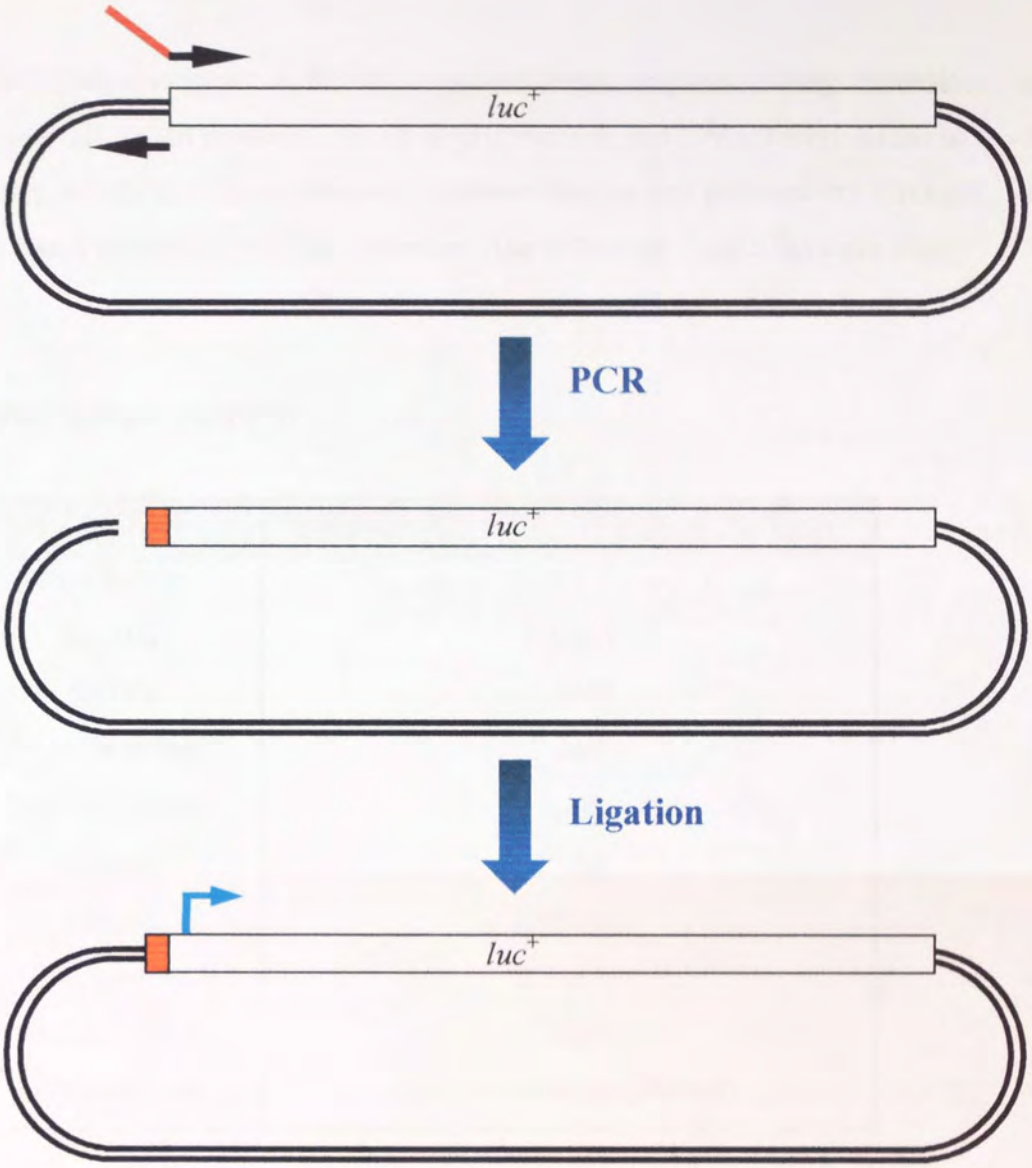


Figure 6.3 Schematic representation of plasmid and insertion sites.
Diagram illustrates the insertion of target sequences, via PCR, into the pGL3-con plasmid upstream of the luciferase gene, and subsequent ligation. Arrow indicates the start codon of the luciferase gene.

6.1.3 Optimisation of the PCR Protocol

The pGL3-con plasmid is 5.5 kb long and hence requires a long extension step to generate full length product. To aid in this, betaine and DMSO were added to the PCR reaction, which have been shown to improve fidelity and processivity (Hengen, 1997). After much optimisation in the laboratory, the following conditions were used:

Reaction mixture for PCR:

REAGENT	FINAL AMOUNT/CONCENTRATION
Pwo buffer	1X
$MgSO_4$	1.5mM
dNTPs	0.2mM
Reverse primer	1μM
Forward primer	1μM
Betaine	1.3M
DMSO	1.3% v/v
Pwo enzyme	2.5units
Plasmid DNA	1ng
DEPC ddH_2O	To final volume of 50μl

Table 6.1 PCR reagents for reaction of pGL3-con.

Conditions of the PCR were as follows:

94°C 1 minute
94°C 30 seconds
55°C 1 minute } 35 cycles
72°C 5.5 minutes
72°C 7 minutes
26°C 5 minutes

Figure 6.4 illustrates a representative agarose gel of a successful PCR reaction.

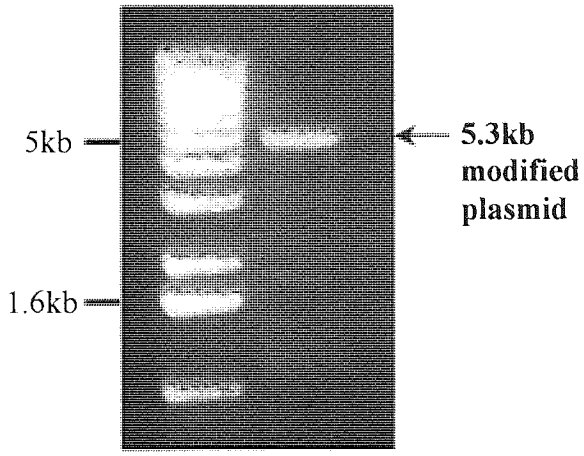


Figure 6.4 PCR of the modified plasmid.
Representative agarose gel showing amplified plasmid with an introduced substrate for the 10-23 DNA enzyme.

Following the PCR, generated products were phosphorylated and subsequently ligated prior to transformation into chemically competent *E. coli* (DH5α).

6.1.4 Preparation of Plasmid DNA

The plasmids were extracted from the bacteria using alkaline lysis (see section 2.12.1). Figure 6.5 shows the extracted plasmids, the so named pGL10-23 and pGL8-17.

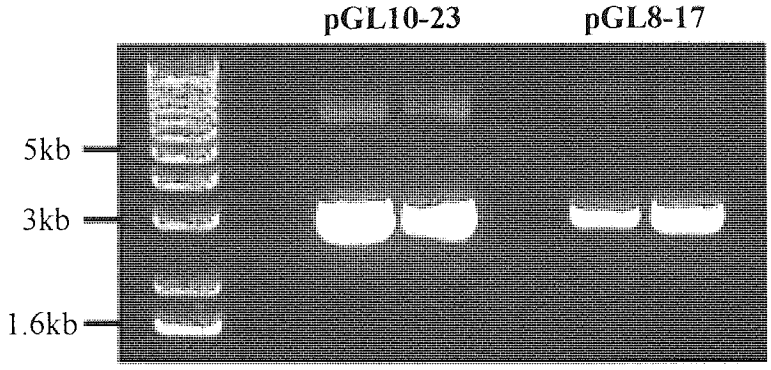


Figure 6.5 Purified plasmids: pGL10-23 and pGL8-17.
Agarose gel showing the two modified plasmids after the extraction process. The plasmids named pGL10-23 and pGL8-17 contain the target substrates for HR3 and HR15 respectively.

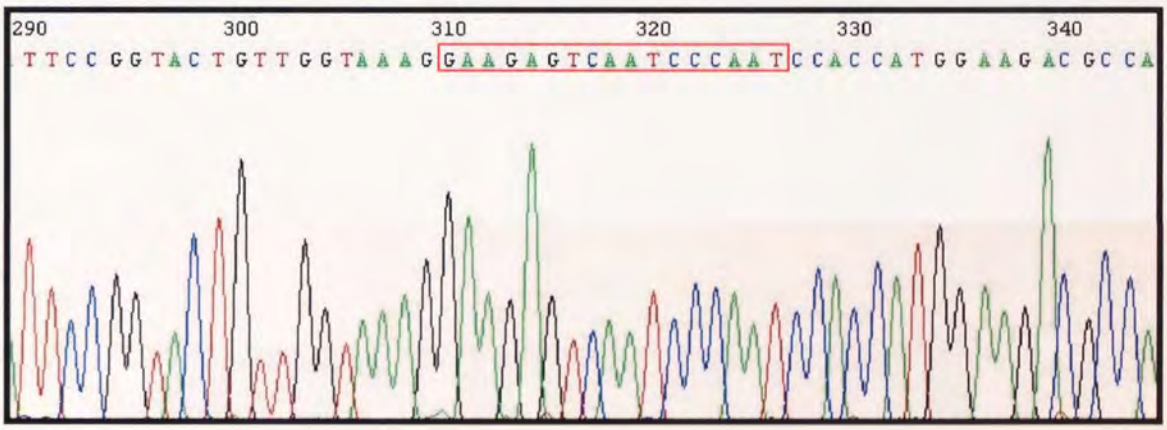
6.1.5 Sequencing of the Modified Plasmids

Sequencing of the extracted plasmids showed only the 10-23 modification had been successful. The 8-17 DNA enzyme target sequence did not insert correctly and actually resulted in deletion of sequence around the translational start site. Unfortunately time did not allow the plasmid construction to be repeated and thus the luciferase assay was performed only with pGL10-23. Figure 6.6 illustrates the sequencing data.

a)

TTCCGGTACTGTTGGTAAAG----------CCACCATGGAAGACGCCA

b)



c)

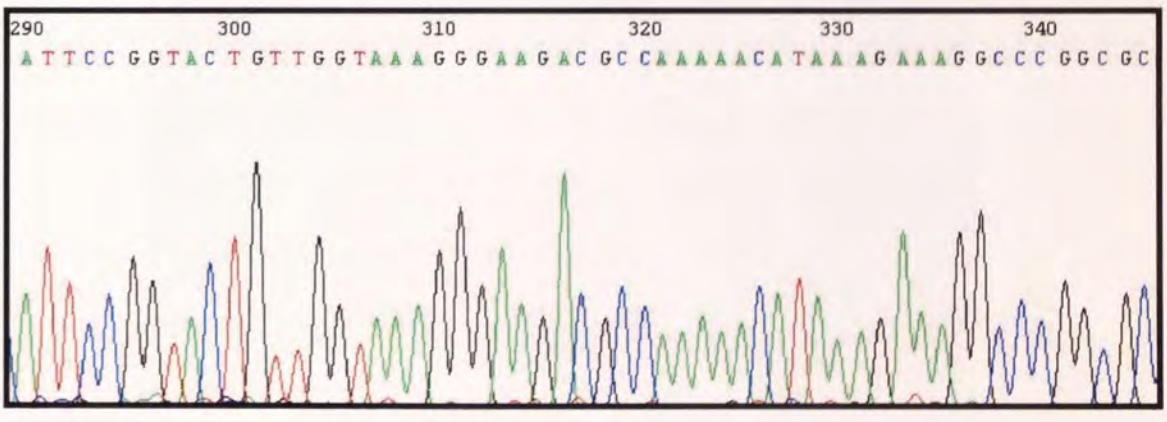


Figure 6.6 Sequencing chromatograms showing the inserted DNA enzyme target sequences.

Panel **a** shows the original plasmid sequence indicating where the target sequences were to be inserted; **b** shows the sequencing data for pGL10-23 and **c** for pGL8-17.

6.2 RT-PCR Analysis of Cell Lines

Most of the work thus far has been done on U87-MG cells. However for the purposes of this assay, U87-MG cells would be unsuitable as they have a high level of intrinsic telomerase RNA. The inserted target sequence is from hTR, and high natural levels of this RNA sequence is to be avoided as it may provide an undesirable target for the DNA enzyme. An RT-PCR reaction of U87-MG and HeLa cells was performed to amplify a 128bp region of the hTR sequence containing the target sequence of the DNA enzyme.

Figure 6.7 shows the absence of telomerase RNA amplification from HeLa cells. This was found consistently to be the case, with independently prepared RNA from different batches of cells. HeLa cells have been shown to possess telomerase activity in some studies but here no detectable levels of hTR were found.

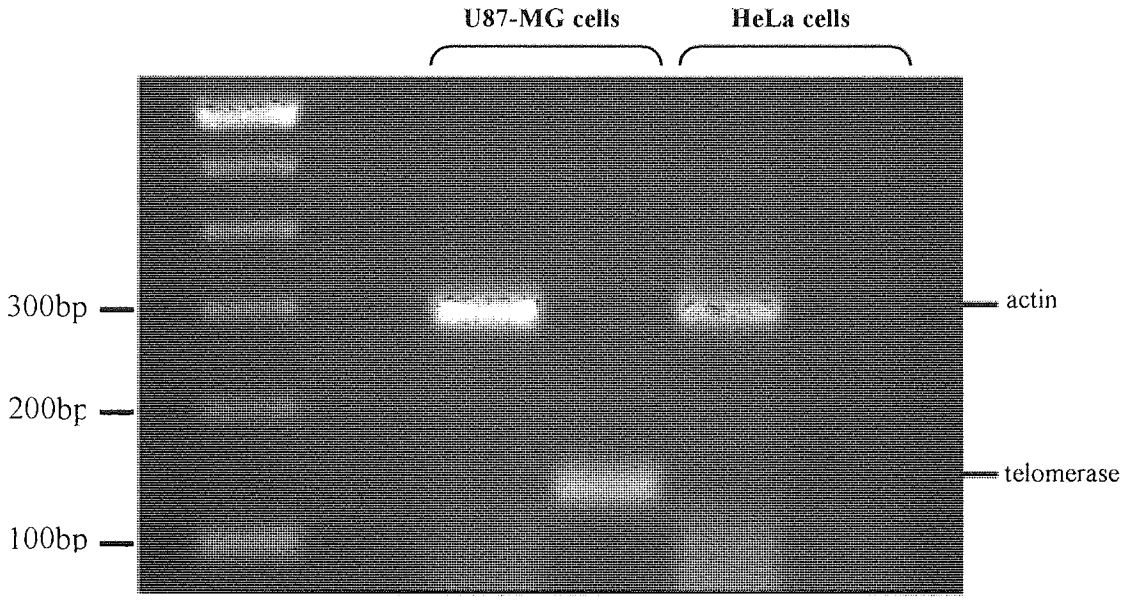


Figure 6.7 Comparative RT-PCR of HeLa and U87-MG cells.
Representative agarose gel showing expression of actin (323 bp fragment) and hTR (128 bp fragment) in U87-MG and HeLa cells as detected by RT-PCR.

6.3 Detection of Luciferase

The Bright-Glo™ Luciferase Assay System (Promega) was used for the detection of luciferase activity (section 2.12.2). This assay system has been developed to maximise the sensitivity of the assay whilst providing a signal half-life of approximately 30 minutes. Bright-Glo™ has a shorter half-life than some other commercially available luciferase reagents (*e.g.* Steady-Glo™ from Promega) with extended half-lives. However, it gives a higher sensitivity of reagent and thus was chosen as the reagent of choice as expression levels may be quite low and hence it was important to maximise the luminescent signal. This assay system is also tolerant of deviations in luminescence between samples caused by, for example, incomplete mixing or pipetting errors.

The manufacturers instructions outline various factors that may influence assay performance. These include the culture medium of the cells, DMEM containing phenol red used here is compatible; the temperature, which should be kept at room temperature (25°C) for optimum activity; and reagent mixing, complete mixing needs to be ensured. Before starting to optimise the transfection of plasmids to cells and the detection of luciferase activity, it was important to establish a positive calibration curve. It is important to establish the range of the instrument to be used and establish the linear range of results.

Using the manufacturer's positive control, QuantiLum® Recombinant Luciferase enzyme (Promega), a calibration curve was established. Serial dilutions of the enzyme were made and tested with the luciferase reagent. Recombinant luciferase is a 61kDa monomeric protein which catalyses the oxidation of beetle luciferin with concomitant production of light. As shown in Figure 6.8, linear results are obtained through six orders of magnitude. The graph establishes that in this experimental set-up, relative light values of 0.01 to 1000 are directly proportional to the amount of luciferase activity present.

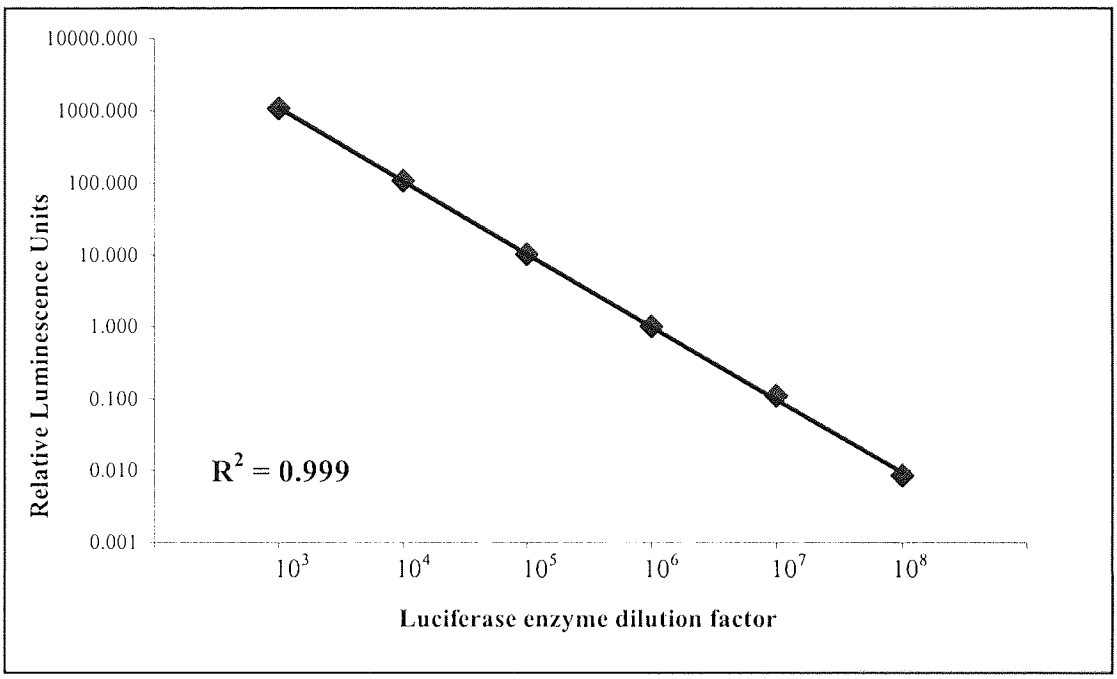


Figure 6.8 Establishing a linear relationship of light emission with respect to luciferase enzyme concentration.

Graph showing relative light emission with various dilutions of the positive control, QuantiLum® Recombinant Luciferase enzyme.

6.4 Optimisation of Transfection

When setting up a new experimental format for transfection, careful consideration needs to be given to a variety of parameters. Optimisation of these parameters will ensure maximum transfection and also improve reproducibility if kept constant. These parameters include cell density at the time of complex addition, amount of DNA, amount of transfection reagent and incubation period with DNA-reagent complexes.

Initially the unmodified pGL3-con was used to optimise transfection i.e. attain maximal light units. SuperFect™, an activated dendrimer reagent was chosen as the transfection agent (described in section 1.3.5.2). This is a branched dendrimer formulation shown to be highly efficient, more so than cationic lipids, in delivering plasmid DNA to HeLa cells (Kang *et al.,* 1999). Cells were plated at $7.5x10^4$ $well^{-1}$ in a 24 well plate. 24 hours post transfection; cells were treated with complexes of 5µl SuperFect™ and 1µg plasmid DNA (recommended start points by SuperFect™ manufacturers protocol). Cells were incubated with the DNA complexes for 4 hours at 37°C, at which point complexes were removed and replaced with fresh full serum DMEM. Cells were then incubated at 37°C for a further 24 hours before being lysed and tested for luminescence (see section 2.12 for further details).

The initial experiment involved the comparison of firstly the volume in which the DNA-SuperFect™ complexes should be applied to the cells and secondly the volume of lysis buffer the cells should be lysed in. The volume of SuperFect™-DNA complexes was either 200µl or 410µl. The second variable was the volume of lysis buffer used and this was either 200µl or 500µl. Cells lysed in 200µl were read by mixing together 100µl each of lysate and reagent. Cells lysed in 500µl were read with 400µl of lysate mixed with 100µl reagent. Figure 6.9 shows the results of the comparative transfections. Consequent to this experiment, it was decided that the optimum was to treat the cells in a volume of 200µl and lyse in 500µl as these conditions gave the maximum light output.

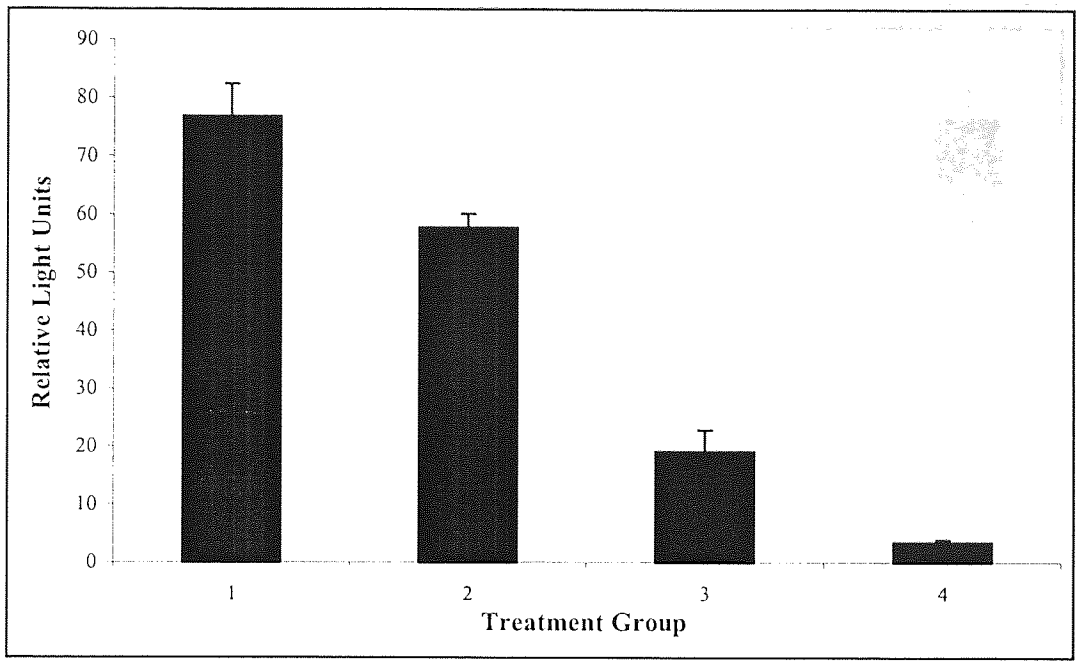
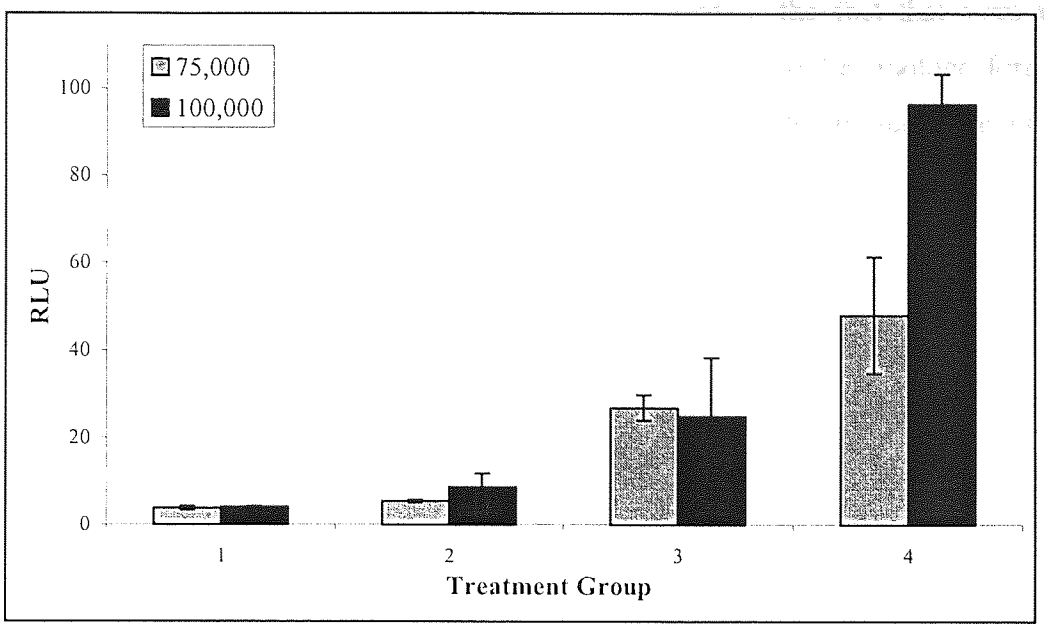


Figure 6.9 Optimisation of pGL3-con transfection: comparison of treatments. Luciferase light emission from HeLa cells transfected with 1μg pGL3-con using 5μl SuperFect™. Results (n=3±S.D.) are expressed as relative light units. Treatment groups: **1**= cells were treated in 200μl and lysed in 500μl; **2**= cells treated in 200μl and lysed in 200μl; **3**= treated in 410μl and lysed in 500μl; **4**= treated in 410μl and lysed in 200μl. A control group, DNA with no transfection reagent produced no detectable light emission.

The next step in optimisation was to compare three factors simultaneously: initial seeding density of cells, time between transfection and assay, and the relative amounts of DNA and transfection reagent.

Two different plasmid DNA concentrations (0.5μg and 1μg) were transfected using two different amounts of SuperFect™ (2.5μl or 5μl DNA). Cells were seeded at an initial density of either $7.5x10^4$ or $1x10^5$ $well^{-1}$ in 24-well plates. Assay times of 24 and 36 hours post transfection were also compared. Figure 6.9 illustrates the results of these experiments. As a conclusion from these, the conditions adopted for maximum light emission were cells seeded at an initial density of $1x10^5$ $well^{-1}$ and assayed 24 hours post- transfection. The same density 36 hours post-transfection still gave high light emission but the standard deviation between results was much greater. The optimum complex constituents appeared to be 1μg DNA and 5μl SuperFect™.

a)



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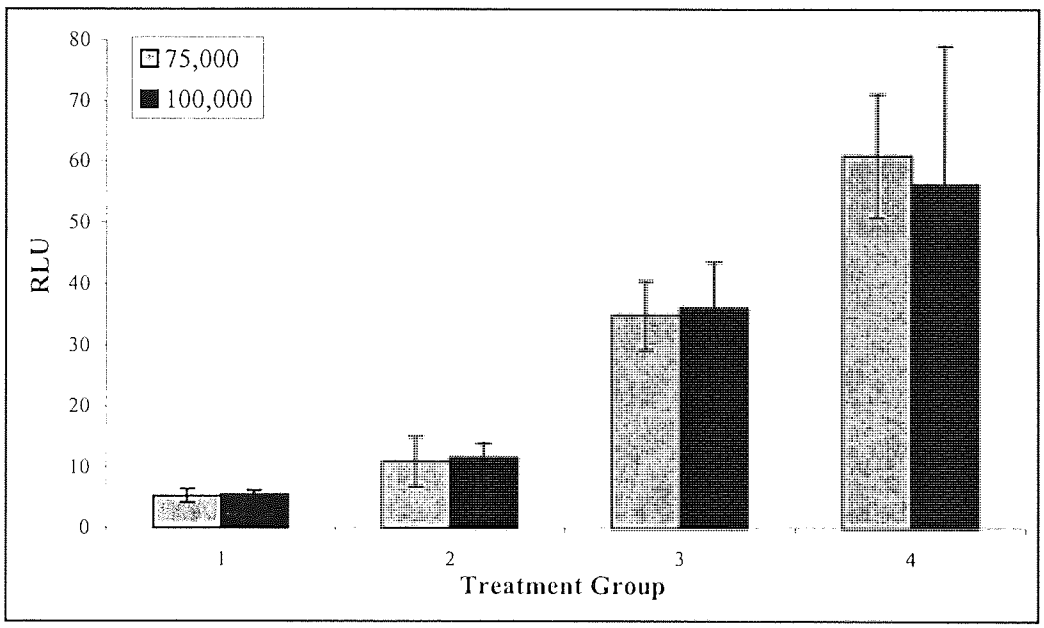


Figure 6.10 Optimisation of pGL3-con transfection with respect to different variables.

Luciferase light emission from HeLa cells assayed **a)** 24 hours and **b)** 36 hours post-transfection. Results (n=3±S.D.) are expressed as relative light units. Cells were seeded at two different initial densities as indicated. Treatment groups: **1**= cells were treated with 0.5μg DNA + 2.5μl SuperFect™; **2**= 0.5μg DNA + 5.0μl SuperFect™; **3**= 1.0μg DNA + 2.5μl SuperFect™; **4**= 1.0μg DNA + 5.0μl SuperFect™. A control group, DNA with no transfection reagent produced no detectable light emission.

The first few optimisation experiments brought to attention the fact that even with keeping all transfection parameters constant, there needed to be another form of normalising the data. Thus it was decided to assay samples for protein content and express results as relative light units per mg of protein. This will not eliminate variability, a factor intrinsic to cell work. However, results will be made more comparable. Protein assays were performed after the luminescence assay, using the method of BSA protein assay as described previously (section 2.9.2). Equal volumes of Glo-Lysis™ buffer and luciferase reagent were used in the blank. Henceforth results are expressed as relative light units (RLU) per mg of protein.

The final optimisation experiment involved the comparison of SuperFect™ with a lipid based transfection reagent, Lipofectin™. Successful transfection, using a lipid-based transfection agent, into mammalian cells of a plasmid containing the luciferase gene has been reported in the literature (Basu *et al.*, 2000; Unwalla and Banerjea, 2001). In this final experiment, variation in the amounts of DNA and transfection reagent was also studied further. 1μg or 2μg plasmid DNA were transfected using either transfection reagent.

Figure 6.11 illustrates the results of this experiment and as can be clearly seen, SuperFect™ is a far superior transfection reagent for plasmid DNA. This may be due to the fact that serum can be used with SuperFect™ hence increasing transfection efficiency. Higher light output was seen with 1μg DNA than with 2μg DNA. There appears to be no benefit in further increasing the amount of SuperFect™ to 7.5μl, thus it was kept at 5μl to minimise toxicity. Once again, the ideal complex constituents were shown to be 1μg DNA and 5μl SuperFect™.

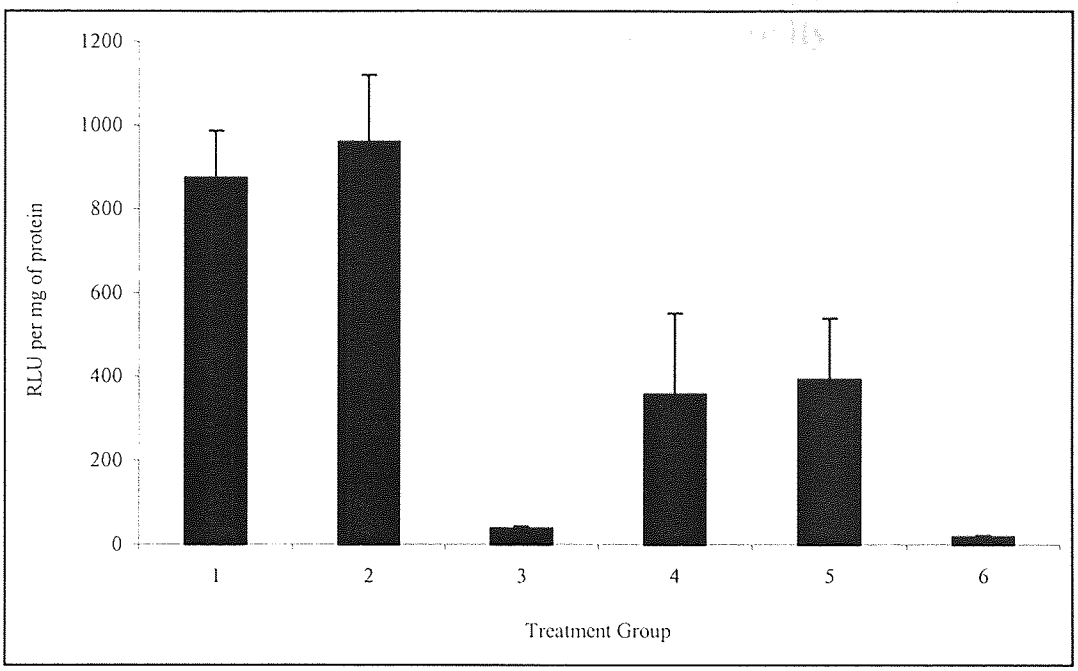


Figure 6.11 Optimisation of pGL3-con transfection: comparison of transfection agents.

Luciferase light emission from HeLa cells transfected with pGL3-con plasmid using either Lipofectin™ or SuperFect™. Results (n=3±S.D.) are expressed as relative light units per mg of protein. Cells were seeded at an initial density of $1x10^5$ $well^{-1}$, and assayed 24 hours post transfection. Treatment groups: **1**= cells were treated with 1.0μg DNA + 5.0μl SuperFect™; **2**= 1.0μg DNA + 7.5μl SuperFect™; **3**= 1.0μg DNA + 10μM Lipofectin™; **4**= 2.0μg DNA + 5.0μl SuperFect™; **5**= 2.0μg DNA + 7.5μl SuperFect™; **6**= 2.0μg DNA + 10μM Lipofectin™. A control group, DNA with no transfection reagent produced no detectable light emission.

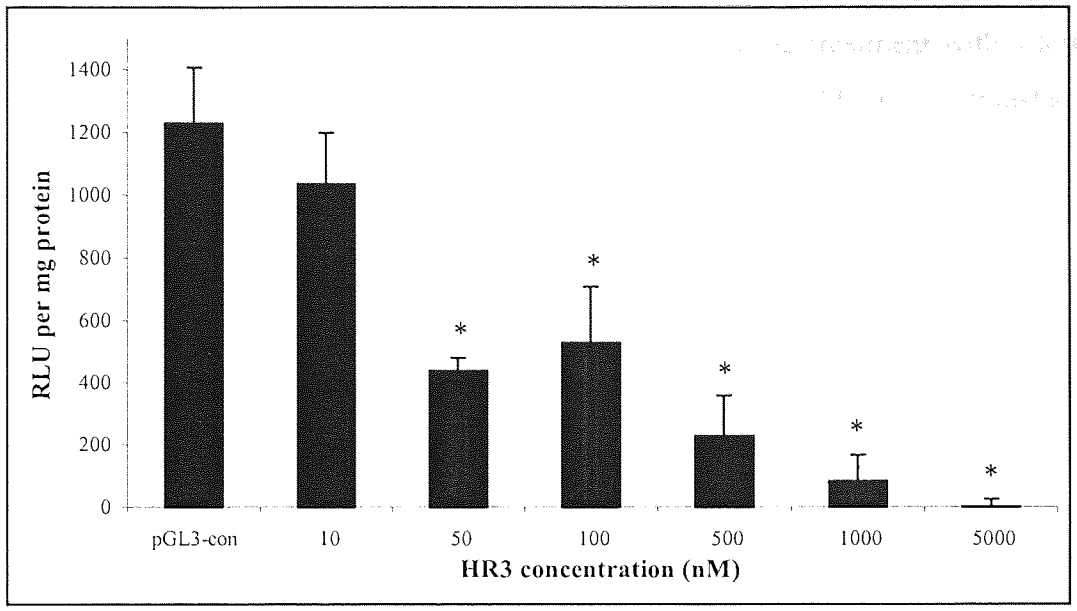
The results of the transfection optimisation experiments can be summarised as follows: HeLa cells to be seeded at an initial density of $1x10^5$ cells $well^{-1}$ in a 24 well plate and incubated at 37°C for 24 hours post seeding. Subsequently, cells treated with DNA-SuperFect™ complexes, consisting of 1.0μg DNA with 5.0μl SuperFect™ in a volume of 200μl, for four hours at 37°C. 24 hours post-transfection, cells were assayed for luciferase activity by lysing in 500μl lysis buffer and reacting 400μl lysate with 100μl reagent for 60 seconds. This reaction was read in luminometer as described (section 2.12.2). After reading, protein estimates were made using the BioRad assay to enable results to be expressed per mg of protein. These parameters were found to be optimum and gave maximal light output. These factors were adhered to when looking at the effect of DNA enzymes on luciferase activity.

6.5 DNA Enzyme Inhibition of Luciferase Activity

The first experiment to evaluate the effect of the 10-23 based DNA enzyme (HR3), using the optimised transfection conditions, was to treat plasmid-transfected cells with HR3. Plasmid and DNA enzyme were co-transfected using SuperFect™. Increasing concentrations of HR3 were co-transfected with both pGL3-con and the modified pGL10-23. The results of this are illustrated in Figure 6.12. Similar luciferase expression levels were attained with the modified plasmid as the unmodified control vector in the absence of DNA enzyme.

The interesting result is obtained when using 10nM of HR3. When HR3 at 10nM is co-transfected with pGL3-con, no significant decrease in light emission was observed. In contrast, when the same concentration *i.e.* 10nM of HR3 was co-transfected with the modified pGL10-23 plasmid, a sharp, significant fall in relative light units was recorded. Above 10nM, a progressive decrease in light emission was seen with both plasmids. This decrease could possibly be ascribed to decreased transfection efficiency when large concentrations of DNA enzyme are present and/or toxicity to cells. The latter explanation is less likely as protein levels were comparable at all concentrations of HR3 in both experimental setups (data not shown).

a)



b)

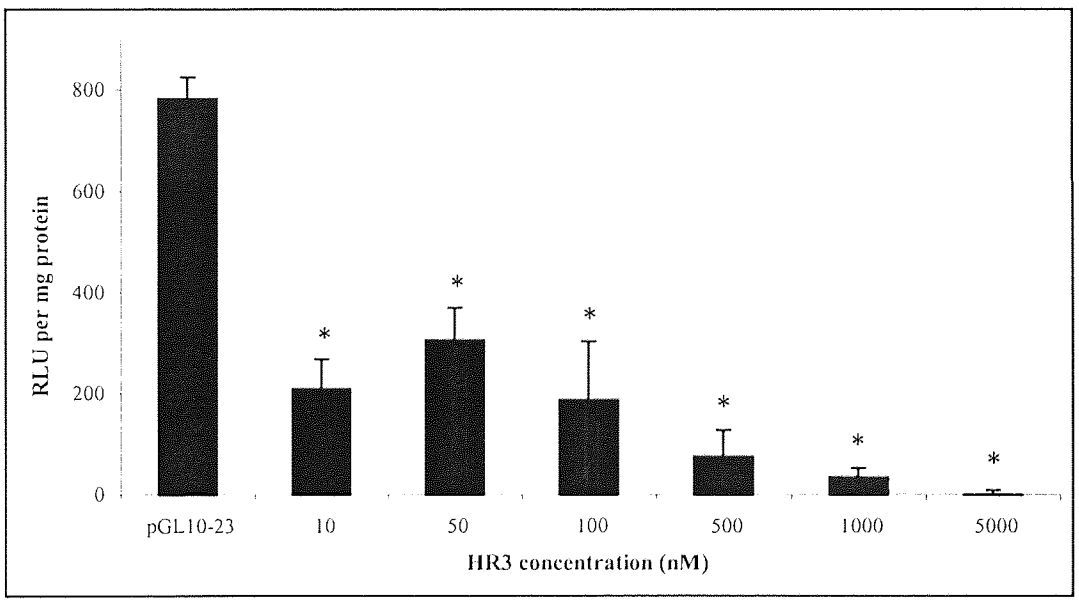


Figure 6.12 Effect of HR3 (10nM-5μM) on luciferase expression from pGL3-con and pGL10-23.

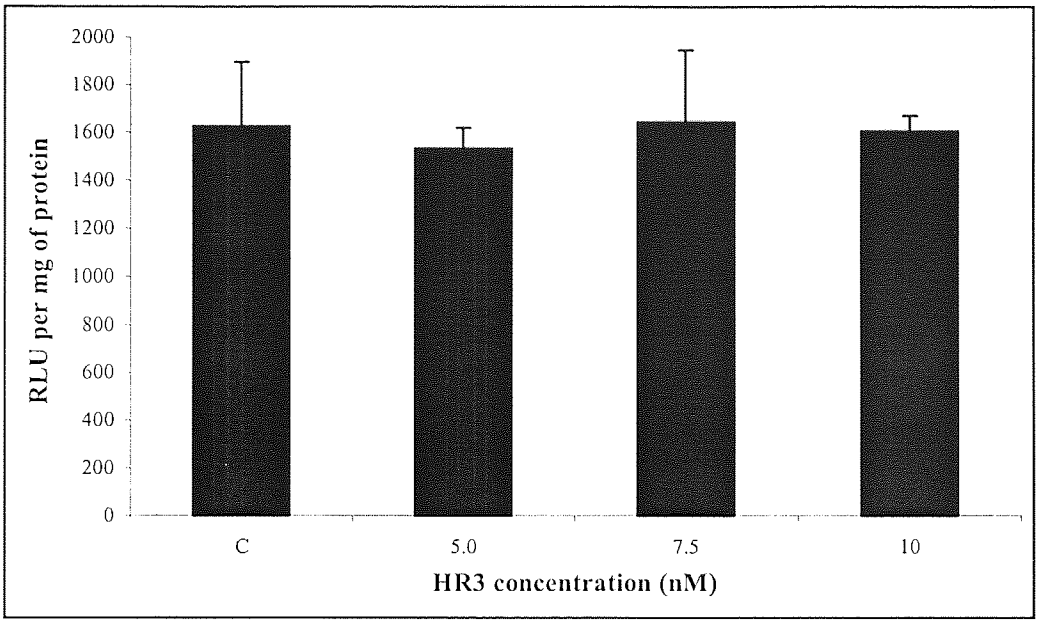
Luciferase light emission from HeLa cells transfected with **a)** pGL3-con and **b)** pGL10-23, and treated with increasing concentrations of HR3 as indicated. Cells were seeded at $1x10^5$ $well^{-1}$ and incubated at 37°C for 24 hours prior to treatment. HR3 was co-transfected with 1μg plasmid DNA using 5μl SuperFect™. Luciferase activity was assessed 24 hours post–transfection. Results (n=3±S.D.) are expressed as relative light units per mg of protein. A control group, treated with DNA without transfection reagent produced no detectable light emission. * denotes a significant reduction ($p<0.05$) from the control.

In an effort to validate the inhibitory activity of HR3, the luciferase expression from the pGL3-con and pGL10-23 plasmids was re-evaluated following treatment with a lower range of HR3 concentrations. HR3 concentrations from 5 to 10nM were co-transfected with 0.75μg plasmid. In a number of experiments (data not shown), it was observed that using 1μg plasmid gave inconsistent results of inhibition via DNA enzymes. It was envisaged that perhaps 1μg DNA was saturating 5μl SuperFect™ and the addition of any extra DNA, with the introduction of the DNA enzyme, was simply affecting the amount of plasmid transfection. To discount this possibility, the transfection experiments were repeated using only 0.75μg of each plasmid instead of 1μg, and this was a successful approach.

Figure 6.13 shows the comparative effect of HR3 on the luciferase expression from HeLa cells transfected with 0.75μg of pGL3-con or pGL10-23. No inhibition of pGL3-con was observed at 5-10nM HR3, whilst the same concentrations produced significant, dose-dependent inhibition of pGL10-23. This effect was reproduced in at least three independent experiments.

The selective inhibition of the target plasmid by HR3 was further corroborated by an additional experiment. Cells were plated as usual and co-transfected with 0.75μg pGL10-23 and a range of HR3 concentrations. A further sample of cells was co-transfected with plasmid and HR15 (8-17 DNA enzyme) at a concentration of 7.5nM. As illustrated in Figure 6.14, there is inhibition of luciferase activity with HR3 from 5 to 10nM. HR15 however, at 7.5nM produces similar light emission to the non-treated control. This result provides further evidence of the specific targeting by HR3 of its target sequence within the modified pGL10-23 plasmid.

a)



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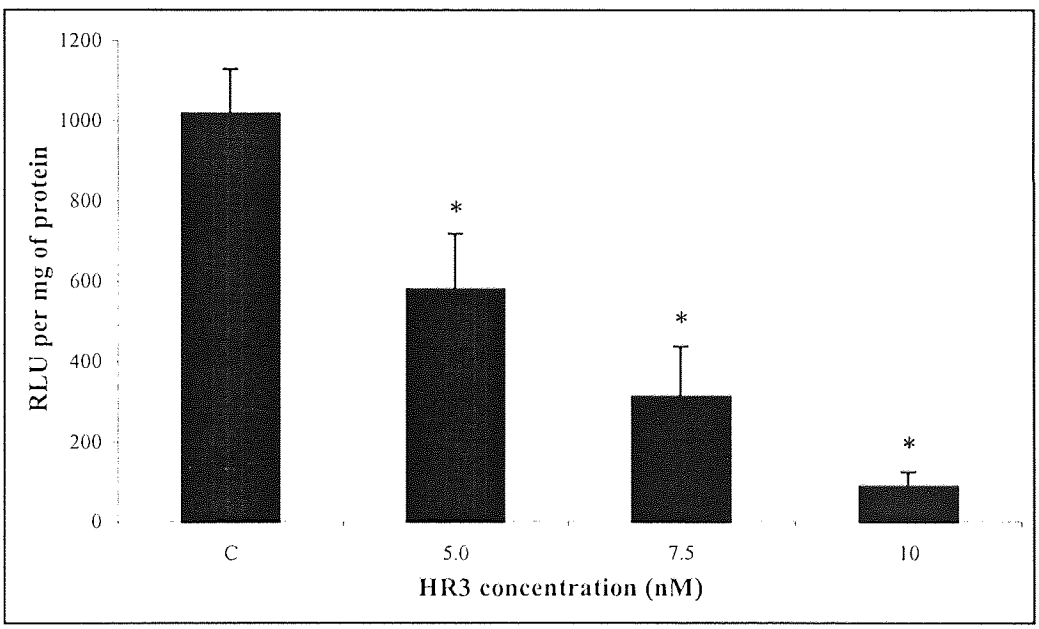

Figure 6.13 Comparative effect of HR3 on luciferase expression from HeLa cells transfected with pGL3-con and pGL10-23.

Luciferase light emission from HeLa cells transfected with **a)** pGL3-con and **b)** pGL10-23, and treated with increasing concentrations of HR3 as indicated. Cells were seeded at $1x10^5$ $well^{-1}$ and incubated at 37°C for 24 hours prior to treatment. HR3 was co-transfected with 0.75µg plasmid DNA using 5µl SuperFect™. Luciferase activity was assessed 24 hours post–transfection. Results (n=3±S.D.) are expressed as relative light units per mg of protein. A control group, treated with DNA without transfection reagent produced no detectable light emission. * denotes a significant reduction ($p<0.05$) from the control.

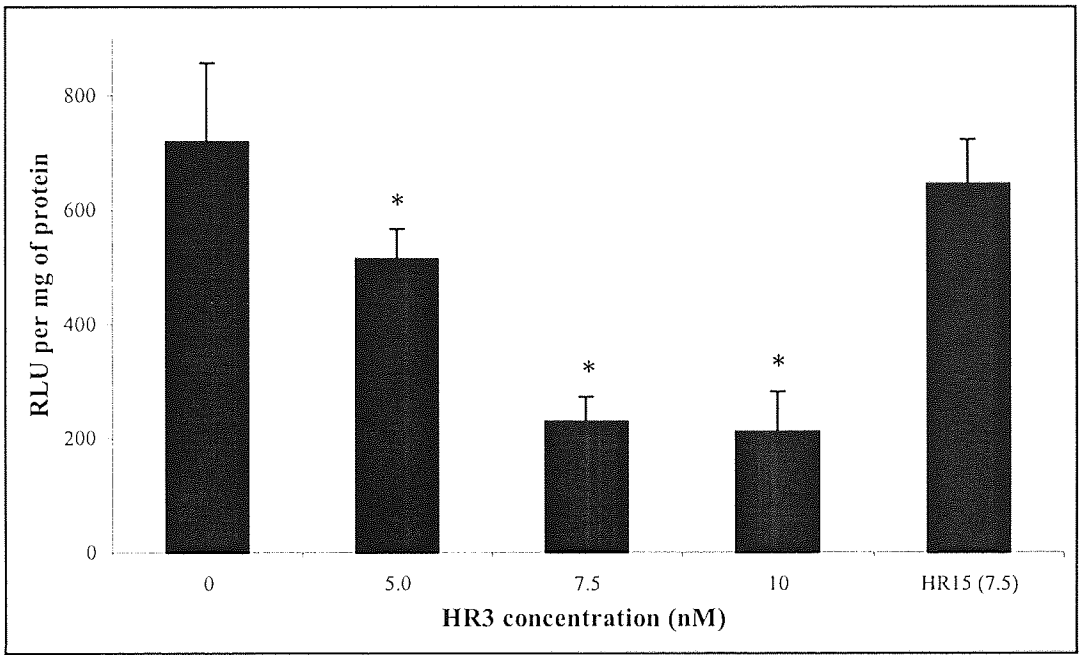

Figure 6.14 Comparative effect of HR3 and HR15 on luciferase expression from HeLa cells transfected with pGL10-23.

Luciferase light emission from HeLa cells transfected with pGL10-23, and treated with increasing concentrations of HR3 as indicated and also with 7.5nM HR15 as a control. Cells were seeded at $1x10^5$ $well^{-1}$ and incubated at 37°C for 24 hours prior to treatment. HR3 was co-transfected with 0.75μg plasmid DNA using 5μl SuperFect™. Luciferase activity was assessed 24 hours post–transfection. Results (n=3±S.D.) are expressed as relative light units per mg of protein. A control group, treated with DNA without transfection reagent produced no detectable light emission. * denotes a significant reduction ($p<0.05$) from the control.

6.6 Concluding Remarks

The type of luciferase assay used here is useful for estimating the efficacy and specificity of functional nucleic acid based therapies in mammalian cells. The results presented in this chapter suggest that DNA enzymes may be active in a cellular environment. HR3, at concentrations up to 10nM, produced inhibition of luciferase expression in cells transfected with pGL10-23 but not in cells transfected with pGL3-con, which lacked the target sequence for HR3. Furthermore, HR15 at a concentration of 7.5nM produced no inhibition of luciferase expression in HeLa cells transfected with pGL10-23. These results clearly demonstrate the sequence specificity of inhibition by DNA enzyme. An antisense mechanism cannot be ruled out but inhibition at concentrations below what would normally be required for antisense action suggests the presence of a catalytic mechanism.

The luciferase reporter system is an artificial system and as such it may not reflect interactions that would occur in a natural setting. Nevertheless, in the absence of a natural model, the system does serve to illustrate the obvious potential of DNA enzymes as novel therapies. This approach has been used in many studies (Warashina *et al.*, 1999; Oketanani *et al.*, 1999). DNA enzymes may prove useful against endogenous cellular targets, the inhibition of which have more immediate effects than the effect of telomerase inhibition on telomere length and consequently cell health.

CHAPTER SEVEN

DISCUSSION

Malignant gliomas account for the largest number of human malignant brain tumours, and are generally considered incurable (Kondo *et al.*, 1998). Of these, glioblastoma multiforme (GBM) is the most malignant and frequently occurring subtype, and it accounts for one-third of all primary brain tumours (Wong *et al.*, 1994). Traditional therapies are of limited value in GBM; hence the search is on for novel treatments to improve the prognosis of this invariably fatal disease. In recent years, DNA enzymes, deoxyribonucleic acid molecules with enzymatic activity, have emerged as novel therapeutic tools for the treatment of diseases at the molecular level. In a similar manner to ribozymes, DNA enzymes are able to catalyse cleavage of RNA in a sequence-specific manner. The major advantage of DNA enzymes over ribozymes is their superior stability. Also, the versatility of DNA chemistry means they are more amenable to rational design. DNA enzymes are a relatively new discovery but they have already demonstrated therapeutic potential. Sioud and Leirdal (2000) demonstrated the ability of DNA enzymes to induce apoptosis in malignant cells. This has significant therapeutic potential as a resistance to apoptosis has an important role in tumour growth and resistance to current chemotherapeutic agents. In this study, two DNA enzymes were designed to target the template region of human telomerase RNA (hTR), utilising the 10-23 and 8-17 catalytic motifs elucidated by Santoro and Joyce (1997). The design, activity and delivery of these two DNA enzymes were evaluated here.

The ends of eukaryotic chromosomes are capped with telomeres, specialised structures that prevent terminal fusions, recombination and degradation. Telomerase (discussed in detail in section 1.2) is an RNA-dependent DNA polymerase, which stabilises telomere lengths by adding hexameric repeats (TTAGGG in humans) to chromosome termini, thus preventing the telomere shortening that usually occurs during mitotic cell division. Telomerase activity, whilst absent in normal somatic tissues, is present in almost 90% of all tumours. The presence of telomerase activity is also correlated with poor clinical outcome of cancer patients. These facts have raised speculation that telomerase may be the much sought universal target for therapeutic intervention in cancer.

Chapter 3 outlines the work done in the design and subsequent *in vitro* activity testing of the two DNA enzymes. Based on the 10-23 and 8-17 catalytic motifs, the DNA

enzymes were designed to target the template region of hTR. The 11-base template within hTR is intrinsically accessible (Pitts and Corey, 1999) to binding by telomere ends and this recognition is essential for maintaining telomere length, suggesting it is an ideal target for antisense-based therapies. This accessibility also overcomes the problem of trying to predict the complex secondary and tertiary structures of RNA.

The potential cleavage sites were chosen within or very close to the template region of hTR. The 10-23 and 8-17 motifs cleave their target RNA between purine and pyrimidine, and adenosine and guanosine residues respectively. Flanking the catalytic cores, the substrate binding arms were kept to 7 or 8 nucleotides in length with sequences complementary to the target RNA. It is estimated that approximately 14 nucleotides are necessary to define a unique RNA sequence within human cells (Santoro and Joyce, 1998). It is agreed that the length of the binding arms is of importance to substrate affinity and specificity, but there is no consensus opinion on the exact optimum length. Warashina *et al.* (1999) tested DNA enzymes with longer (15 deoxynucleotides each) binding arms and found them to have lower substrate specificity than enzymes with the standard (8 nucleotides) arms. Toyoda *et al.* (2000) report that decreasing the length of the binding arms by just one nucleotide, from 8-mer each to 7-mer each, reduced the efficiency of their DNA enzymes. Conversely, Oketani *et al.* (1999) after testing DNA enzymes with equal arm lengths of either 11 or 15 nucleotides, found higher catalytic activity associated with longer length arms under cell-free conditions. However, when the DNA enzymes were transfected into cells, the DNA enzyme with longer arms (15 nucleotides each) showed lower suppressive activity, perhaps due to limited access to the target site (Oketani *et al.*, 1999).

Unmodified phosphodiester (PO) backbone DNA is liable to rapid degradation in the biological milieu. To overcome this problem a common approach is to introduce phosphorothioate (PS) linkages, where an oxygen atom in the phosphodiester backbone is replaced with a sulphur atom. However, extensive modifications may be detrimental to catalytic activity. Hence the *in vitro* cleavage activity and stability of the DNA enzymes were assessed after the introduction of various numbers of PS linkages. Complete PS modification increased the serum stability of the unmodified DNA enzymes from a $t_{1/2}$ of 10 minutes to 5 hours. However, complete modification also

caused the catalytic activity to diminish below detection. Conversely, the introduction of only 3 PS linkages at the 3-'end had no substantial effect on either the activity or stability of the DNA enzymes. DNA enzymes with a PO core and PS arms were chosen as the best compromise between activity and stability and used in future cell work. These enzymes were HR3 and HR15, containing the 10-23 and 8-17 motifs respectively. It is important to study activity in cellular as well as cell-free conditions as a DNA enzyme with lower cleavage activity under cell-free conditions has been shown to have more suppressive effect on target gene expression (Oketani *et al.*, 1999).

An interesting possibility would be to investigate the effect of 2'-*O*-methyl substitutions (see section 1.3.2) on the activity and stability of the DNA enzymes. Warashina *et al.*, (1999) tested the stability of various modified DNA enzymes in 90% human serum. They report that the introduction of two PS linkages or two 2'-*O*-methyl-substituted residues at both the 5' and 3' ends (to a total of four modifications) of a DNA enzyme conferred equal resistance to nucleases. The authors also report that neither modification had adverse effects on the activity or specificity of a 10-23 based DNA enzyme, probably because the modifications were sufficiently far from the catalytic domain. Another alternative would be the use of an inverted thymidine residue at the 3'-end. Sioud and Leirdal (2000) investigated the introduction of a 3'-3' inverted thymidine nucleotide to a 10-23 based DNA enzyme targeting protein kinase Cα. They found a DNA enzyme thus modified showed no loss of cleavage activity and was approximately 6 fold more stable in serum than its unmodified counterpart.

Control DNA enzymes; an equivalent antisense, sense and scrambled sequences were synthesised and tested. Lack of detectable cleavage with these controls demonstrated the cleavage activity specificity of HR3 and HR15. The cleavage of HR3 and HR15 was further tested under the influence of various factors *i.e.* temperature, $MgCl_2$ concentration, enzyme concentration and pH. All these variables affected the cleavage of HR3 and HR15 in a similar fashion. Maximum cleavage occurred at 30°C, 200mM $MgCl_2$, 2μM enzyme and pH 9. Some cleavage did occur in physiological conditions (quoted as 37°C, 2mM $MgCl_2$, pH 7.5) albeit it was not optimal. However, as DNA enzymes are synthetically engineered, once their limitations have been recognised steps can be taken to improve their design and subsequent activity.

The effect of metal ions on DNA enzyme cleavage is the factor most commonly studied in the literature. Santoro and Joyce (1997) found that the cleavage activity of a 10-23 based DNA enzyme increased with increasing $MgCl_2$ concentrations from 0 to 300mM. Elsewhere they report that the catalytic rate of a 10-23 DNA enzyme increased approximately linearly with increasing concentrations of various divalent metal cations. They explain that this correlation is consistent with a chemical mechanism involving metal-assisted deprotonation of the 2'-hydroxyl adjacent to the cleavage site (Santoro and Joyce, 1998). Surprisingly, Santoro and Joyce (1998) found that the catalytic rate of the 10-23 was higher in the presence of Mn^{2+} than Mg^{2+}, despite the fact that only Mg^{2+} was used in the selection procedure. This fact suggests that the *in vitro* activity of selected DNA enzymes should be tested in the presence of many common ions, in addition to the metal ion used in selection, in order to maximise activity potential.

The 10-23 DNA enzyme, in the presence of a divalent metal cation, stabilises the transition state leading to the forward cleavage reaction. Kinetic analysis of the 10-23 motif revealed that the enzyme has a strong preference for substrate cleavage over product ligation under simulated physiological conditions (Santoro and Joyce, 1998). The catalytic efficiency of the DNA enzyme is determined by the rate of enzyme-substrate association, which in turn is limited by the rate of nucleation of the corresponding DNA-RNA hetroduplex. The authors conclude that, unlike the hammerhead and group I ribozymes, the 10-23 DNA enzyme takes full advantage of the inherent rate of duplex formation, presumably by avoiding alternative structures that would limit the rate of substrate binding.

The ability of the DNA enzymes to inhibit telomerase activity was assessed using the telomerase repeat amplification protocol (TRAP), as outlined in Chapter 4. The TRAP reactions were performed in lysates of U87-MG cells, which have been shown to express high levels of telomerase (Kiaris and Schally, 1999). DNA enzymes of PO chemistry were used as these were shown to remain stable in a mock TRAP reaction mix (Figure 4.4). Treatment of 2μg cell lysates with various concentrations of DNA enzymes resulted in a dose-dependent inhibition of telomerase activity and an IC_{50} of 100nM was determined. This is much lower than previous work in which a hammerhead ribozyme was used to inhibit telomerase activity with an IC_{50} of 0.4μM.

In an effort to elucidate the mechanism of telomerase inhibition, various control DNA enzymes were tested in the TRAP reactions at 100nM. Results from the control DNA enzymes of both motifs indicated a mainly antisense mechanism of action. There was no significant difference between the active DNA enzymes, both unmodified and those with PS arms; the antisense and scrambled controls inhibited telomerase activity with similar efficiency to the active enzymes; the sense control did not inhibit telomerase activity to any significant effect. Thus base complementarity is very important but the catalytic competence, as demonstrated *in vitro*, does not appear to have any substantial benefit in cell lysates. In contrast to the results obtained here, it has been suggested that PS ODNs may exhibit higher activity with respect to telomerase inhibition than unmodified PO ODNs, but this activity is not sequence specific (Pitts and Corey, 1999). Matthes and Lehmann (1999) suggest that the reason for this is that PS ODNs bind to the protein motif of telomerase, at the primer binding site, rather than hTR. The authors suggest that a way to harness this effect would be to design a chimaeric ODN, with a PS modified portion targeting the primer binding site and another unmodified portion targeting hTR.

There are many reports of successful inhibition of telomerase activity using antisense-based therapies. Phosphodiester-linked 2'-*O*-methyl-RNA has proved to be a highly selective and potent inhibitor of telomerase (Pitts and Corey, 1998). Introduction of phosphorothioate linkages at the 3' and 5' termini of the 2'-*O*-methyl-RNA increased nuclease resistance without negative effects on potency or specificity. Also, this modified 2'-*O*-methyl-RNA inhibited telomerase sequence-selectively within human-tumour-derived (DU145) cells when delivered with cationic lipids. Kondo *et al.* (1998) demonstrated effectiveness of antisense against telomerase RNA *in vitro* and *in vivo*. An extra 2-5A moiety (2',5'oligoadenylate) was added to the antisense ODNs, which activates RNase L (a single strand specific endoribonuclease) and degrades the target RNA. The treatment of malignant glioma cells with antisense caused the death of the vast majority of cells within 14 days. Furthermore, following injection of antisense molecules directly into tumours in nude mice, tumour mass was reduced by 50% over a 14 day period. Shortly afterwards, Kondo *et al.* (1998) reported that antisense telomerase treatment inhibits telomerase activity subsequent to the induction of one of

two distinct pathways, either apoptosis or differentiation. Regulation of these two pathways may be dependent on expression of different cellular proteins.

Kiaris and Schally (1999) adopted a slightly different approach to target telomerase using an antagonist of growth hormone-releasing hormone (GH-RH). GH-RH antagonists were found to inhibit tumours in U87-MG glioblastomas, associated with the down-regulation of the hTERT gene, resulting in a decrease in telomerase activity.

Recently, arsenic has found an unexpected role as a powerful and dramatic therapy for acute promyelocytic leukaemia. Analysis of the mRNA levels in different cell types exposed to arsenic showed suppression of telomerase activity, which correlated with a dramatic decrease in the mRNA for the hTERT gene (Chou *et al.*, 2001). The authors propose that arsenic acts to inhibit telomerase by reducing the transcription of c-myc, a transcription factor necessary for the production of mRNA from the gene encoding hTERT.

3'-Azido-2',3'-dideoxythymidine (AZT) has also been shown to be an effective telomerase inhibitor. Gomez *et al.* (1995) demonstrated the preferential incorporation of AZT, over thymidine, into telomeric sequences of cell. The authors later studied the effect on telomere length and telomerase activity following long term (15 passages) exposure to AZT at 800μM (Gomez *et al.*, 1998). Here they found that AZT was able to shorten the size of telomeric repeats of tumour cells in culture, linear with the time in culture. This telomere shortening was irreversible on removal of AZT from the cell growth medium. Telomerase activity was also lost after AZT treatment. Thus AZT is an effective telomerase inhibitor and a combination with DNA enzymes targeting telomerase would be an interesting avenue to explore.

Much work in our research group has centred on using the epidermal growth factor receptor (EGFR), which is overexpressed in many gliomas, as a target in novel therapies for GBM (Hussain, 2002; Petch, 2002; Fell, 1999). Interestingly, recent findings indicate that telomerase and EGFR may be linked. Maida *et al.* (2002) examined the effect of epidermal growth factor (EGF) on telomerase activity. The authors conclude that EGF activates telomerase through the direct activation of hTERT transcription via a

specific transduction pathway. Tian *et al.* (2002) adopted an antisense approach to inhibit EGFR expression of U87-MG cells. Compared to controls, antisense treated cells showed a 54-fold reduction in telomerase activity, had shortened telomeres and displayed lower tumorigenecity. These results suggest EGFR plays an important role in the regulation of telomerase activity of glioma cells. Moreover, the inhibition of telomerase may reflect an additional mechanism through which the antisense mediated inhibition of EGFR leads to tumour suppression. Hence a combination approach of inhibiting telomerase and EGFR could be an interesting future development of research in our group.

It was endeavoured to develop an internal control for the TRAP assays to account for the possible presence of PCR inhibitors in the reactions. The resulting construct could not be optimised to work within the TRAP assay; however, its successful amplification in the presence of DNA enzymes discounted the possibility that the observed telomerase inhibition was due to inhibition of *Taq* activity only. An alternative option would have been to use the TRAP-eze™ kit from Oncor. The main advantage of this would be the inclusion of a 36bp internal standard, which helps quantitation and takes account of any PCR inhibitors. However, this internal standard could lead to false negatives as the standard appears to be amplified in samples that clearly contain PCR inhibitors (Holt *et al.*, 1996).

The ultimate aim of our research group is towards the elucidation of novel treatments and delivery systems to be of use in the treatment of GBM. Accordingly, in Chapter 5, a glioma cell line was used to evaluate the activity of the DNA enzymes in a cellular environment. Activity in cell culture models is very pertinent with regards to the inhibition of telomerase. Using animal models is very difficult as mouse, rat, monkey and even pig appear to have telomerase activity in vital organs which is only marginally different to levels in tumour tissues (Burger, 1997).

DNA enzymes modified with PS arms were used in the cellular studies. DNA enzymes are polyanionic molecules, which do not readily cross biological membranes. The cellular association of naked DNA enzymes, similar to naked ODNs, is inefficient with less than 2% of DNA enzyme associating with cells at 37°C. A significant decrease in

uptake was observed at 4°C compared to 37°C. This dependence is indicative of an active, energy-dependent process suggesting uptake is mediated via endocytosis as has previously been cited for ODNs (Akhtar and Juliano, 1992). Two commercial cationic lipid formulations, Lipofectin™ and LipofectAMINE™, were used to enhance the transfection of DNA enzymes to U87-MG cells. Parallel studies on the toxicity and enhancement of delivery were performed on both lipids. The importance of this was evident because concentrations of lipid that were found to be toxic to the cells could still display an apparent increase in uptake of DNA enzyme. Considering both toxicity and enhancement of cellular association, 10μM Lipofectin™ and 5μM LipofectAMINE™ were found to be optimum, enhancing the delivery of HR3 and HR15 to approximately 10%. Cationic lipids have been very successful for the delivery of oligonucleotides. The fact that the enhancement of oligonucleotides cellular uptake is not sufficient to explain the increase of oligonucleotides activity suggests that cationic lipids perform other roles such as improving the oligonucleotides stability (Mönkkönen and Urti, 1998) and modifying intracellular localization of ODNs (Zelphati and Szoka, 1996). For example, DOTAP has been reported to markedly prevent degradation of ODN by nucleases, which was explained by a collapse or a 'coating' of the ODN after aggregation of the complexes leading to structures where ODN is completely covered by lipid bilayers (Zelphati and Szoka, 1996).

Having optimised the lipid-mediated delivery of DNA enzymes, their anti-proliferative effects were assessed. However, Lipofectin™-aided delivery of HR3 and HR15 to U87-MG cells over a 4 hour period did not significantly inhibit cell proliferation compared to controls. This lack of observable effect can probably be ascribed to the expected lag period between inhibition of telomere maintenance and cell death. Studies have shown that it is necessary to down-regulate telomerase for at least 40 generations in order to induce growth arrest and apoptosis (Herbert *et al.*, 1999).

Recently, it has emerged that there may be a way of overcoming the lag in treatment when using telomerase inhibitors. Komata *et al.* (2002) describe two ways of telomerase-specific cancer therapy targeting telomerase-positive cells to directly kill them. Firstly immunotherapy directed against telomerase-positive cells. Secondly, the

use of the hTERT promoter to restrict the expression of suicide gene or toxin to telomerase-positive tumour cells.

Conversely, there are studies illustrating that even when there is inhibition of telomerase, telomeres may not shorten. Bisoffi *et al.* (1998) showed that telomerase-negative cell clones remained viable and grew similarly to control cells. Another group (Blasco *et al.*, 1997) documented telomerase-negative murine finroblasts with critically shortened telomeres, derived from mice lacking the gene encoding mTR (mouse analogue of hTR), had undiminished proliferative capacities. Gomez *et al.* (1998) found no senescence after 15 cell passages despite a loss of telomerase activity and shortening of telomeres. Of course these studies go some way to supporting the presence of telomerase-independent telomere maintenance (Bryan and Reddel, 1997).

If inhibitors of telomerase are to be of use in the clinic, it may be that a method for prolonged and sustained delivery is required. Thus PLGA microspheres were investigated as a possible device for providing sustained delivery of the DNA enzymes. HR3 and HR15 were encapsulated into polymer microspheres using a double emulsion method. Small (mean diameter=2.5μm), spherical particles of a narrow size range and possessing a smooth morphology were achieved. The microspheres demonstrated a biphasic release profile *i.e.* the initial burst effect was followed by a much slower, sustained release over the 35 day study period. These findings indicate that PLGA microspheres may be a promising method for the delivery of DNA enzymes. Formulations of biodegradable microspheres have been extensively reported in the literature for the delivery of a wide range of drugs including cytotoxics such as 5-fluorouracil (Menci *et al.*, 1999). ODNs have been widely delivered using biodegradable polymer microspheres *in vitro* (Lewis *et al.*, 1998; 1995) with reports of successful use *in vivo* (Khan *et al.*, 2000). The sustained delivery of hammerhead ribozymes has been achieved using biodegradable polymer film matrices (Hudson *et al.*, 1996). Direct single dose administration (i.c.v. or intracerebral injection) of nucleic acids appears to be effective for some CNS applications targeting short-lived proteins such as the immediate-early gene family (Grzanna *et al.*, 1998). However, for long-lived proteins and/or for mRNA targets exhibiting a slow turnover, repeated

administration is usually required but this has associated problems such as toxicity (Ho *et al.*, 1998).

The final stage of these studies involved the development of a luciferase-based assay for the assessment of DNA enzyme activity in cells, as described in Chapter 6. The aim was to use a reporter assay to allow the evaluation of DNA enzymes in a cellular environment. The commercially available pGL3-Control (pGL3-con) vector was modified by the insertion of the target (complementary) sequences of the DNA enzymes. This modification was achieved with the design of a PCR reaction utilising the proof reading DNA polymerase *Pwo*. Sequencing of the resultant modified plasmids showed that the 8-17 DNA enzyme target sequence did not insert correctly. Due to time constraints, work was done only on the plasmid with an inserted 10-23 target sequence, named pGL10-23. HeLa cells were used in preference to U87-MG cells due to lower levels of intrinsic telomerase RNA. Delivery of the modified and unmodified plasmids to HeLa cells was optimised using SuperFect™, an activated dendrimer reagent. The effect of HR3 on luciferase expression from cells transfected with pGL3-con and pGL10-23 was studied. HR3, at concentrations of up to 10nM, was found to produce significant inhibition of luciferase expression in cells transfected with pGL10-23 but not in cells transfected with pGL3-con, which lacked the target sequence for HR3. Furthermore, HR15 at 7.5nM produced no inhibition of luciferase expression in HeLa cells transfected with pGL10-23. These results clearly demonstrate the sequence-specificity of luciferase inhibition by a DNA enzyme. The contribution of an antisense mechanism cannot be ruled out; however, inhibition at concentrations below what is usually required for antisense action suggests the presence of a catalytic cleavage mechanism. Moreover, these results reveal DNA enzyme potential against endogenous cellular targets.

Using reporter assays is a popular way of assessing cellular activity of molecules. Warashina *et al.* (1999) investigated the specificity and cleavage activity of DNA enzymes in mammalian cells using reporter constructs that included a luciferase gene. Similar to our work, they tested various DNA enzymes against two luciferase-expressing plasmids using Lipofectin™-aided co-transfection. One of the plasmids encoded the target sequence (*BCR-ABL* fusion mRNA) whilst the other encoded for

normal *ABL* mRNA. The authors report that 0.3μM of the active 10-23 based DNA enzyme suppressed the expression of the *BCR-ABL*-luciferase gene in HeLa cells by approximately 99%. Moreover, the same concentration had no inhibitory effect on the expression of the normal *ABL*-luciferase gene. A DNA enzyme with an inactivated catalytic core was found to have no inhibitory effect on the expression of either gene. Another significant finding by Warashina *et al.* (1999) was the superiority of DNA enzyme over an equivalent antisense sequence. When antisense sequences were tested with the luciferase reporter assay, there was non-specific inhibition of both constructs. The authors propose that although DNA enzymes include antisense sequences that allow them to recognise their target, these sequences are interrupted by the catalytic loop resulting in lower affinity and higher specificity for the substrate. The non-specificity of the antisense DNA supports that the inhibitory action of the DNA enzymes was by a mechanism of cleavage.

Akin to the assay developed in Chapter 6, Oketani *et al.* (1999) used a luciferase reporter assay to assess the inhibitory effects of DNA enzymes on hepatitis C virus (HCV) gene expression in a human hepatoma cell line. The target sequence of the 10-23 based DNA enzymes was placed immediately upstream from a luciferase marker gene by cloning strategy. A construct lacking the HCV target sequence was used as a control. Luciferase activity was determined 24 hours after co-transfection of 0.1μg plasmid and 1μM DNA enzyme. The findings reported by the authors are similar to the results outlined in this chapter. The phosphorothioate modified DNA enzymes suppressed HCV-luciferase fusion gene expression by approximately 60% of control. Conversely, the same concentration of DNA enzyme had no significant effect on luciferase gene expression from the control plasmid lacking the target sequence. Furthermore, co-transfection of an unrelated DNA enzyme failed to have any significant effect on luciferase gene expression from the target plasmid.

One interesting possibility would have been to use a dual reporter assay. Promega have recently developed a dual-luciferase assay system. This involves the co-transfection of firefly luciferase and *Renilla reniformis* luciferase, which acts as a control reporter. Both of these are immediately active upon mRNA translation, neither are present naturally in mammalian cells, and as they have independent biochemistries, their

luminescent reactions can be distinguished. The activities of both are measured sequentially from a single sample. Hawkins *et al.* (2002) outline the benefits of such a system. A dual reporter assay can potentially decrease the variability of measurements as well as acting as an internal control for cell number, cell health, transfection efficiency and non-specific cellular responses. That is it enables the distinction of a promoter-specific (down-regulation) and a non-specific (cytotoxic) response (Hawkins *et al.*, 2002).

There has been a huge surge in interest about telomerase in recent years with an explosion in the number of papers published with regards to the structure, function, activity and inhibition of telomerase. The discovery of new agents for cancer is urgent. Telomerase inhibitors may limit the growth of human tumours directly but may also emerge as adjuncts to existing therapies *e.g.* after surgery, telomerase inhibitors may limit the recovery of residual cancer cells, making them more susceptible to attack by the immune system or killing by existing chemotherapeutic agents. The arrival of DNA enzymes has been an exciting development in the field of nucleic acid based therapies. These 'deoxyribozymes' essentially combine the benefits of highly sequence-specific, ribonuclease-independent RNA destruction, with the stability of ODNs. As prospective therapeutic agents, DNA enzymes face many of the same challenges that confront other nucleic acid based gene-inactivation agents. DNA enzymes are a relatively new discovery but increasing research into issues such as effective cellular delivery and target co-localisation should help DNA enzymes realise their therapeutic potential.

To conclude, the work in this thesis has met the aims outlined in Section 1.6. Two active DNA enzymes, based on the 10-23 and 8-17 motifs, were designed against the template region of hTR. Activity of these two DNA enzymes was demonstrated *in vitro*, in glioma cell extracts and within a cellular environment. If this work was carried further, a more detailed kinetic analysis of the DNA enzymes *e.g.* in multiple turnover conditions would be pertinent. Also, further experiments to ascertain the mechanism of action within the luciferase assay would be performed. In the long term, it would be interesting to divide this research into two arms; one to further investigate DNA enzymes and their properties, the second to focus on telomerase inhibition with the exploration of possible combination therapies.

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APPENDIX I

Papers:

Hughes M.D., Hussain M., Nawaz Q., **Sayyed P.** and Akhtar S. (2001) The Cellular Delivery of Antisense Oligonucleotides and Ribozymes. *Drug Discovery Today.* **6**, 303-315.

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