Smart screens for thyroid disrupting substances in the environment

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DECLARATION

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Last, but by no means least - y teulu. Diolch i chi gyd, Mam a Dad yn enwedig.
Words are pitifully inadequate – suffice it to say that it makes me immeasurably proud to dedicate this Thesis to Daff and Rhen. I know you'll both feel the same.

ABSTRACT.

Thyroid hormones are essential for the normal development and the maturation of all vertebrates. They exert their effects via thyroid receptors; ligand – activated transcription factors that regulate gene expression by binding to specific DNA sequences (TREs - thyroid response elements) in the promoter-region of target genes and through interactions with a number of regulatory proteins, termed coactivators and corepressors. The balance of this finely tuned system can be disrupted by exposure to environmental contaminants. The development of high – throughput, *in vitro* screens for such contaminants, will allow more informed risk assessments to be made concerning the potential human health impact of the chemicals which are already in use, as well as facilitating the screening of newly - developed compounds.

The murine thyroid receptor (α and β) cDNAs were PCR-amplified and cloned into a pET expression vector. Recombinant His-tagged fusion proteins were expressed in *E.coli* and purified by affinity chromatography.

A duel approach was used in order to monitor the effects of putative disruptors on transcription. Firstly, a cell culture reporter system was developed. CV1 cells were stably transfected with thyroid-responsive luciferase reporter construct and a number of clonal populations were isolated. Although an up-regulation of luciferase protein levels was observed in repsonse to treatment with thyroid hormone (T3), it was not as large as expected and the amount of thyroid receptor in cells was subsequently found to be a limiting factor.

Secondly, *in vitro* transcription assays comprising a nuclear extract and the thyroid-responsive luciferase reporter construct were employed. Transcription efficiency was assessed using radioactive primer extension assays, QPCR and the Hybridistion Protection Assay.

The interaction of recombinant TR with TRE-containing double-stranded DNA duplexes was monitored using the electrophoretic mobility shift assay (EMSA). A protein titration allowed the calculation of the K_d of TR for DNA. This quantitation of the affinity of TR for DNA was subsequently measured in the presence of known T3 analogues, thus providing the basis of a TR-DNA binding assay.

CONTENTS

ABBREVIATIONS AND CONVENTIONS

AE	Acridinium Ester
AF	Activation Function
AP	Alkaline Phosphatase
BCIP	5 Bromo-4-chloro-3-iodinylphosphate
BSA	Bovine Serum Albumin
bp	Nucleotide Base Pair
cAMP	Cyclic Adenosine Monophosphate
CBP	CREB Binding Protein
cDNA	Complementary DNA
cpm	Counts per Minute
CoRNR	Corepressor of Nuclear Receptor
CREB	cAMP-Response Element Binding Protein
C-terminal	Carboxy Terminal
CTE	Carboxy-Terminal Extension
CV	Coefficient of Variation
DBD(s)	DNA Binding Domain(s)
ddH_20	Double Distilled Water
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
dNTP(s)	Deoxynucleotide Triphosphate(s)
DR	Direct Repeat
DTT	Dithioerythreitol
ds	Double Stranded
EBDC	Ethylenebis(dithiocarbamate)
EDC	Endocrine Disrupting Chemical
EDTA	Ethylenediaminetetracetic acid
E-DTA	Endocrine Disruptor Testing and Assessment Task Force
EED(s)	Environmental Endocrine Disruptor(s)

EMSA	Electrophoretic Mobility Shift Assay
EPA	Environmental Protection Agency
ER	Everted Repeat
Et-Br	Ethidium Bromide
ETU	Ethylene Thiourea
FAM	6-carboxyfluroescein
FBS	Foetal Bovine Serum
GTF	General Transcription Factor
h	Hour(s)
HBS	HEPES Buffered Saline
hCMV	Human Cytomegalovirus
HDAC(s)	Histone Deacetylases
HPA	Hybridisation Protection Assay
HPLC	High Performance Liquid Chromatography
ID	Interaction Domain
IE	Immediate Early
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Inverted Repeat
IVT	*In vitro* Transcription
kb	Nucleotide Kilo Base Pairs
kDa	Kilo Daltons
LB	Luria Bertani
LBD(s)	Ligand Binding Domain(s)
mA	Milliamps
ME	Malic Enzyme
min	Minute(s)
mRNA	Messenger RNA
NBT	Nitro Blue Tetrazolium
NCo-A2	Nuclear Coactivator 2
NCoR	Nuclear Corepressor
NE	Nuclear Extract
NR(s)	Nuclear Receptor(s)
N-terminal	Amino Terminal

O/N	Overnight
OD_λ	Optical Density (measured at specified wavelength)
PBS	Phosphate Buffered Saline
PSB	Probe Storage Buffer
pCAF	P300/CBP Associated Factor
PCB(s)	Polychlorinated Biphenyls
PCDD(s)	Polychlorinated Dibenzodioxins
PCDF(s)	Polychlorinated Dibenzofurans
pCIP	CBP Interacting Protein
PCR	Polymerase Chain Reaction
PIC	Pre-initiation Complex
Poly(dI-dC)	Poly-(deoxyinosinic-deoxycytidilic) Acid Sodium Salt
QPCR	Quantitative PCR
RLB	Reporter Lysis Buffer
RNA	Ribonucleic Acid
ROX	Carboxy-X-rhodamine, Succinimidyl Ester
RT	Room Temperature
RXR	9-*cis* Retinoic Acid Receptor
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
sec	Second(s)
SMRT	Silencing Mediator for RXR and TR
SRC-1	Steroid Receptor Coactivator 1
SSC	Saline Sodium Citrate
ssDNA	Single Stranded DNA
SSPE	Sodium Chloride, Sodium Phosphate and EDTA
T25	$25cm^2$ cell culture flask
T75	$75cm^2$ cell culture flask
T175	$175cm^2$ cell culture flask

TAE	Tris Acetate EDTA Buffer
TAF	TBP-associated factor
TAMRA	6-carboxyl-tetramethyl-rhodamine
TBP	TATA Box-binding Protein
TE	Tris EDTA Buffer
TH(s)	Thyroid Hormone(s)
Tm	Melting Temperature
TK	Thymidine Kinase
TR(s)	Thyroid Receptor(s)
TRAP(s)	TR-associated Proteins
TRE(s)	Thyroid Response Elements
TRF	Thyrotrophin Releasing Factor
TSH	Thyroid-stimulating Hormone (thyrotrophin)
U	Units
UTR	Untranslated Region
UV	Ultraviolet
V	Volts
VDR	Vitamin D Receptor
v/v	Volume by Volume
WWF	World Wildlife Fund
w/v	Weight by Volume

The one and three letter abbreviations used for nucleotides and amino acids are those recommended for use by the Biochemical Journal. Policy of the Journal and Instructions to Authors. Biochem. J., 209: 1-27.

All laboratory materials are detailed according to their commercial name, as given in the appropriate manufacturer's catalogue.

CHAPTER ONE

GENERAL INTRODUCTION.

1.1. Environmental Endocrine Disruption.

All living organisms depend upon a large and intricate array of chemical signalling systems to guide biological development and regulate cell and organ activity (McLachlan, 2001). Over the past two decades, scientific interest and public concern regarding the ability of many environmental contaminants to interfere with these sensitive systems has grown dramatically. These molecules are believed to have the potential to alter the normal functioning of the various endocrine systems in wildlife and humans. Initially, concern was centered upon environmental chemicals-both synthetic and natural-that have the potential to mimic the action of the female hormone estrogen. Such pollutants were termed "environmental estrogens" or "xenoestrogens" and they have been linked to a variety of effects in a number of species. Examples include: imposex (masculinisation) of female gastropods of various species exposed to the anti-fouling agent, tributyltin (TBT; Mathiessen and Gibbs, 1998); infertility and reproductive tract tumours in mice exposed to diethylstilbesterol (Newbold *et al.*, 2000); and reproduction-related changes in many fish species upon exposure to sewage and industrial effluent, including intersex - the production in male fish of the female egg yoke lipoprotein, vitellogenin and testis abnormalities, (Copeland, *et al.*, 1986).

Following the initial concerns regarding environmental estrogens, and their effects on the reproductive system (particularly in females), the issue subsequently developed to encompass the concern that environmental chemicals could affect other elements of the endocrine system and impact on developmental as well as reproductive processes (Phillips and Harrison, 1999). Myers *et al.*, (2003) defined environmental endocrine disruptors (EEDs) as:

"*chemicals that can disrupt thyroid hormones, androgens, estrogens and other endocrine processes... by interfering with the hormonal signals that control normal development of the brain and other organ systems*".

1.2. Thyroid Hormones.

1.2.1. Physiological Importance of Thyroid Hormones.

Thyroid hormones (THs; 3,5,3'-$_L$-triiodothyronine, T3 and $_L$-thyroxine, T4, structures shown in Figure 1.1) play critical roles in differentiation, growth, and metabolism. The effects of THs are mediated predominantly at the level of gene expression via thyroid hormone receptors (TRs).

TRs are ligand-activated transcription factors whose main function is to regulate target gene expression directly through binding to specific DNA sequences, called thyroid response elements (see Section 1.4.1). The TRs are encoded by two separate genes, designated TRα and TRβ, located in different chromosomes (17 and 3, respectively, in humans). Alternative splicing of transcripts from each gene generates multiple isoforms, including TRα1, TRα2 and TRα3 from the TRα gene, and TRβ1 and TRβ2 from the TRβ gene (Zhang and Lazar, 2000). Overall, the proteins encoded by TRα and TRβ have similar biochemical and functional properties, although they do have distinct tissue distributions. In the adult rat, the α1 and β1 receptor mRNAs are found in all T3-sensitive tissues, although with differences in their relative abundancies. During brain development, TRα expression predominates at early stages and remains steady, whereas TRβ expression is intially very low but exhibits a dramatic transient increase during the critical period of the central nervous system maturation (Jeannin *et al.*, 1998).

TH levels are self-regulated by a negative feedback mechanism, and are required for the normal function of nearly all tissues, and is involved in the regulation of numerous processes, including: cardiovascular function – heart rate, blood pressure and breathing; postnatal development - bone lengthening, gut maturation, inner ear, retina and muscle development; liver metabolism, the digestive system and body temperature (Flamant and Samarut, 2003 and Yen 2001). Basic foetal development begins within the first few weeks of gestation, and the foetus is entirely depedent on maternal thyroid hormones until the beginning of the second trimester. Although the foetal thyroid begins to grow towards the end of the first trimester, the production of thyroid hormones does not begin until the second trimester, and the hypothalamic-pituitary-thyroid axis is not mature until the third trimester. Thus, maternal thyroid

hormone must be continuously available until birth, as crucial developmental processes take place before the foetal thyroid system is up and running. These include; spinal cord and hindbrain development, neuron formation, synapse development and formation of myelin (Brown, 2003).

The importance of TH-mediated gene expression, via the TRs has been demonstrated by the production of knockout mice. Impaired TH function, due to TR knockouts, leads to increased cell death and a delay in cerebellar development (O'Shea and Williams, 2002; Koibuchi and Chin, 2000). Transgenic mice lacking a functional TRα gene exhibit progressive hypothyroidism from birth. Animals display multiple disorders, which eventually lead to death, shortly after the weaning period. These include; a body temperature 0.5°C lower than normal, an average heart rate 20% lower than control animals, and delayed maturation of bone and small intestine (Gauthier *et al.*, 1999; Wikstrom *et al.*, 1998). A lack of the TRβ isoform leads to impaired auditory development whilst mice lacking both a functional TRα and TRβ display hyperthyroidism, consistent with the negative regulation of TSH by liganded TRs (described in Section 1.2.3; Zhang and Lazar, 2000; Gauthier *et al.*, 1999). TH action, mediated by a specific TR isoform, TRβ2, has been shown to be necessary for the normal development of green cone photoreceptors, in rats (Ng *et al.*, 2001).

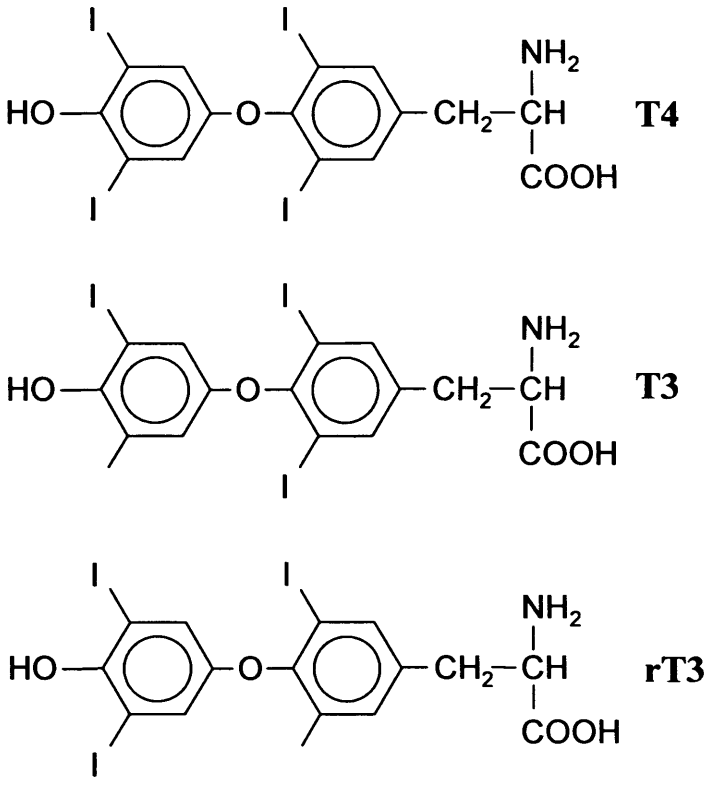


Figure 1.1. The naturally-occurring thyroid hormones. T3 (3,5,3'-$_L$-triiodothyronine) and reverse T3 (rT3; 3,5',3'-$_L$-triiodothyronine) are generated by outer-ring and inner-ring deiodination of T4 ($_L$-thyroxine), respectively.

1.2.2. Thyroid Hormone Synthesis.

The functional unit of the thyroid gland is the thyroid follicle. The first step in TH synthesis involves the active uptake of iodide (I^-), from the blood, by the thyroid follicle cells. This requirement for iodide in TH synthesis necessitates a sufficient intake of dietary iodine (I_2). Dietary iodine (I_2) is reduced to the oxidative level of iodide (I^-) before virtually complete absorption in the small intestine. The iodide is then transported across the follicular epithelium and concentrated in the follicular lumen. Iodide molecules are then oxidised by the enzyme thyroid peroxidase (with hydrogen peroxidase accepting the electrons) and bound in a matter of seconds to the 3 position of tyrosine molecules which are attached to the large glycoprotein, thyroglobulin, to form Monoiodotyrosine (MIT; Connors, 1997; and Ganong, 1995). MIT is then iodinated in the 5 position to form diiodotyrosine (DIT). Two iodotyrosine molecules are then coupled to yield the iodothyronine hormones, T4 and T3 (Figure 1.1.), a process that is also thought to be catalysed by thyroid peroxidase (Connors, 1997).

All of these processes are contained within thyroglobulin, whose three dimensional structure promotes interaction between the MIT and DIT molecules. T4 and T3 are then stored, bound to thyroglobulin, in the follicular lumen. THs are released into the blood by proteolytic cleavage of thyroglobulin, when required (Brent, 1994).

1.2.3. Regulation of Thyroid Hormone Secretion.

The mechanisms regulating TH secretion are summarised in Figure 1.2. Release of thyrotrophin-releasing factor (TRF) from the hypothalamus induces the anterior pituitary to produce thyroid stimulating hormone (TSH or thyrotrophin). TSH stimulates both the synthesis and secretion of THs. TSH acts via G-linked receptors in the cell surface membrane of thyroid follicle cells. Binding of TSH leads to the activation of adenylate cyclase, a subsequent rise in cAMP levels, leading to the synthesis and release of THs (Connors, 1997; Ganong, 1995).

The majority of hormone released from the thyroid gland is in the form of T4, as total serum T4 is 40-fold higher than serum T3. Only 0.03% of the total serum T4 is free (unbound), with the remainder bound to carrier proteins such as thyroxine binding globulin (TBG), albumin, and thyroxine-binding prealbumin (transthyretin). Approximately 0.3% of the total serum T3 is free, with the remainder

bound to the thyroid-binding proteins. Protein-bound THs and free hormone are in dynamic equilibrium and it is the free TH that enters target cells and generates a biological response (Yen, 2001). At the target tissues, approximately 85% of T4 may be converted to T3 by outer-ring deiodination at the 5 position, by thyroxine 5' – deiodinase (Brent, 1994). TH levels are balanced by negative feedback mechanisms. High levels of THs inhibit secretion of TSH from the pituitary, and TRF from the hypothalamus, thus limiting the amount of TH produced (Connors, 1997).

1.2.4. Thyroid Hormone Metabolism.

Deiodination is the most important metabolic pathway of thyroid hormones, in quantitative terms and because of its dual role in the activation and inactivation of T4.

Outer ring deiodination (ORD) of T4 by type I or type II 5'-deiodinases generates the biologically active T3, whilst inner ring deiodination (IRD) of T4 by tyrosyl-ring deiodinase generates the inactive metabolite reverse T3 (rT3; 3,5',3'-$_L$-triiodothyronine). T3 and rT3 undergo further deiodination, predominantly to the common metabolite 3,3'-diiodothyronine (3,3'-T2), which is generated by IRD of T3 and by ORD of rT3. Thus, ORD is an activating pathway by which the prohormone T4 is converted to active T3, whereas IRD is an inactivating pathway by which T4 and T3 are converted to the metabolites rT3 and 3,3'-T2, respectively (Yen, 2001). See Figure 1.1 for the structures of T4, T3 and rT3.

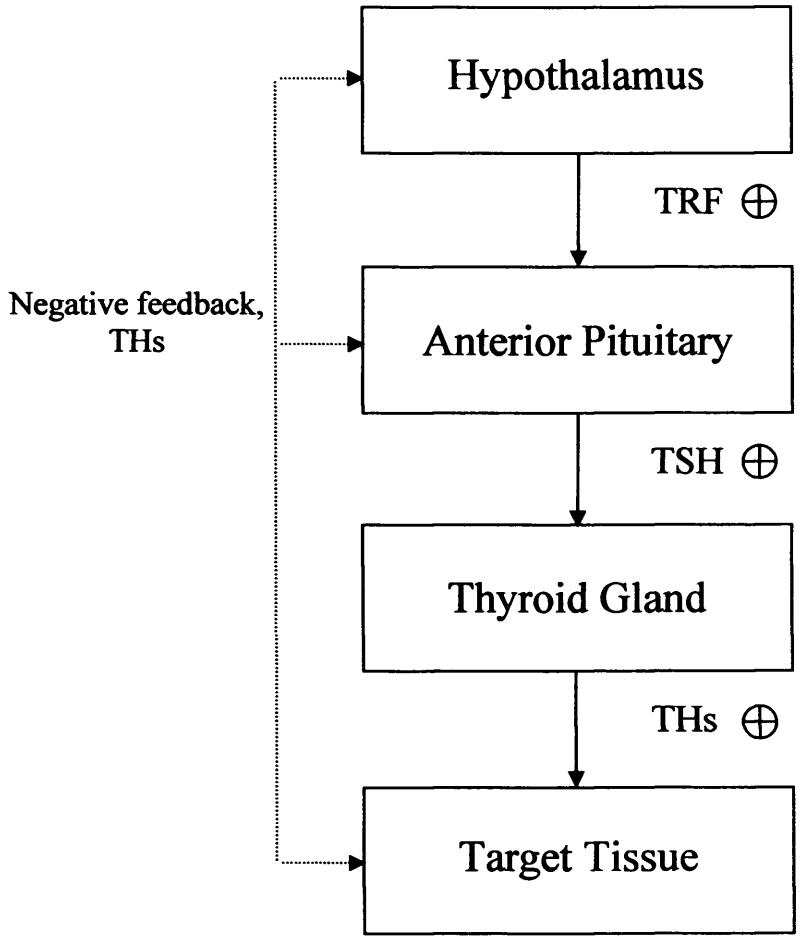


Figure 1.2. The self-regulatory control of thyroid hormone (TH) synthesis. Thyrotrophin releasing factor (TRF) induces the anterior pituitary to produce thyroid stimulating hormone (TSH, or thyrotrophin), which in turn stimulates the synthesis and secretion of THs from the thyroid gland. High levels of of THs inhibit secretion of TRH and TSH.

1.3. Thyroid Disease.

Thyroid disease is manifested as a decrease in the endocrine activity of the thyroid gland (hypothyroidism), as an excessive activity of the gland (hyperthyroidism), or as structural and morphologic changes that do not necessarily result in a change in thyroid hormone status (euthyroidism).

1.3.1. Hypothyroidism.

Hypothyroidism refers to the exposure of body tissues to a subnormal amount of thyroid hormones, due to under secretion of THs from the thyroid gland. Hypothyroidism may arise from a number of causes, including, insufficient dietary intake of iodine, a failure by the thyroid gland to efficiently utilise accumulated iodide, and the ingestion of substances (foodstuffs, drugs, pollutants) that inhibit thyroid function. The symptoms and signs of hypothyroidism in adults are myriad, and can include one or more of the following: fatigue, weight gain, dry skin, yellow skin, cold intolerance, constipation, mental impairment, infertility, coarseness of hair and goiter (Connors, 1997). Hypothyroidism in adults can also lead to myxedema, which is characterised by infiltration of liquid into the subcutaneous tissue, leading to swelling (Connors, 1997; Pitt-Rivers and Tata, 1960). Although the term myxedema refers specifically to the associated skin changes, the syndrome of adult hypothyroidism is also sometimes called myxedema.

In infants, the most serious consequences of TH deficiency involve mental and physical development. Without treatment, TH deficiency causes severe developmental impairment, and neurological disturbances. The result is cretinism (see Section 1.3.1.b.).

a) Goiter.

A goiter is an enlargement of the thyroid gland. It has previously been defined as a thyroid gland that is twice larger than normal, or about 40g in weight (Burrow, 1989). The classic explanation for goiter formation is that, due to iodide deficiency, the thyroid gland must grow as a homeostatic compensatory mechanism. Iodide deficiency results in inadequate TH production, giving rise to a compensatory release of TSH (see Figure 1.2.), leading eventually to goiter formation. This allows retention of TH synthesis and secretion. Specific problems may arise from the development of a goiter; the goiter may become large enough to constrict the neck and

interfere with eating and breathing. In addition, the cosmetic effect can also be undesirable (Connors, 1997).

Historically, the connection has often been made between the environment and its ability to interfere with the thyroid system. McCarrison (1917) recognised contamination of the water supply as being goitrogenic in low-iodine areas. He noted that goiter prevalence increased downstream from the water supply in a group of villages in the Himalayan foothills. The incidence of goiter increased from 12% at the source to 45% at the terminus of the river, which served as both a source of drinking water, and as an open sewer for the villages along the banks of the river. Subsequently, the residents of this area were found to be markedly iodine deficient. McCarrison was able to produce goiter in himself and his colleagues by the ingestion of suspended matter from the river water. More recent studies showed that *E.coli* in drinking water could produce anti-thyroid compounds (Gaitan *et al.*, 1980).

Cassava (also known as tapioca and manioc) is a major world-source of carbohydrate, especially in developing countries. The root contains compounds that release free cyanide on hydrolysis. Following ingestion of improperly prepared cassava, the cyanide is converted to thiocyanate, which blocks thyroid function. A significant difference in the prevalence of goiter has been shown to occur among individuals living in different parts of an isolated island on Kivu Lake, in Zaire (Burrow, 1989). The differences in goiter prevalence were due to the different methods of preparing cassava.

b) Cretinism.

In areas of severe iodine deficiency, goiter may become endemic. Endemic goiter is common in many areas in which the soil is deficient in iodine. The resultant low dietary iodine intake can result in the development of a euthyroid goiter, or, if the deficiency is severe enough, of a hypothyroid goiter (Connors, 1997). A consequence of the hypothyroidism that may develop in endemic-goiter regions is the occurrence of cretinism.

Cretinism has been defined as a "permanent neurologic and skeletal retardation resulting from an inadequate supply of thyroid hormone during gestation" (Burrow, 1989). The intrauterine period is crucial in the development of cretinism – most of the neural effects and mental retardation, which accompany the disease, are determined prenatally. Cretins may display a variety of neurologic defects, including

mental retardation, deafness, mutism, and spasmic diplegia. The hypothyroidism that may also be present, leads to retarded linear growth and maturation of body parts. Cretinism, can, however be prevented. Iodine prophylaxis has been shown to be effective in preventing the disease (Burrow, 1989).

1.3.2. Hyperthyroidism.

Hyperthyroidism may be defined as a group of disorders resulting from supraphysiologic tissue and circulating concentrations of thyroid hormones. Hyperthyroidism may result from a number of causes including, thyroid carcinoma, exposure to excess dietary iodine, over administration of thyroid hormone, and the most common cause, Graves' disease (see next Section).

The symptoms and signs of hyperthyroidism include the following: nervousness, weight loss, heat intolerance, sweating, tremor, alterations in appetite, fatigue, muscle weakness and sleep disturbances (Connors, 1997; and Ganong, 1995).

a) Graves' Disease.

In Graves' disease the thyroid gland is diffusely enlarged and hyperplastic, and there is a protrusion of the eyeballs, called exopthalmos. These symptoms give rise to some of the many synonyms that exist for Graves' disease, including exopthalmic goiter, toxic diffuse goiter, primary hyperthyroidism, and autoimmune thyroidism.

It is now generally accepted that Graves' disease is an autoimmune disorder. Antibodies (called TSAb, for thyroid stimulating antibody) are produced against components of the TSH receptor on the thyroid cell membrane, and the receptor is stimulated. This leads to an increase in adenylate cyclase, and consequently, cAMP (see Section 1.2.3.). The net result is a stimulation of the thyroid cell, resulting in increased TH secretion (see Figure 1.2; Ganong, 1995; and Volpe, 1989). Increasing levels of THs lead to decreased levels of circulating TSH, but tend to cause increased formation of TSAbs, leading to yet further TH secretion (Ganong, 1995). TSAbs are capable of stimulating the thyroid cell for a much longer interval than TSH (Volpe, 1989). Graves' disease can be treated, by lowering levels of circulating THs. This tends to lower, but not completely prevent, formation of the antibodies.

1.4. Thyroid Hormone Receptors.

1.4.1. Mode of Action.

The effects of THs are mediated predominantly at the level of gene expression via thyroid hormone receptors (TRs). TRs are ligand activated transcription factors whose main function is to regulate target gene expression directly, through binding to specific DNA sequences, known as thyroid response elements (TREs), in the regulatory regions of target genes (Zhang and Lazar, 2000; Wu and Koenig, 2000). The ability to bind specific sequences of DNA in target genes is critical to TR function. The minimal core hexad consensus sequence recognised by nuclear receptors (NRs) often contains the sequence AGGTCA known as the half site (Zhang and Lazar, 2000; Mangelsdorf and Evans, 1995). This half site can be configured in a variety of structured motifs (Figure 1.3.A). The most potent of TREs is the direct repeat, although TRs also bind to everted repeats (ER) and inverted repeats (IR), also known as palindrome TRE. Although TRs are capable of binding to TREs as monomers or homodimers, they preferentially bind to TREs as heterodimers with the 9-*cis* retinoic acid receptor (RXR), another member of the NR Superfamily. RXR/TR heterodimer formation is required for high affinity binding to the TREs (Mangelsdorf and Evans, 1995). TR will only form a heterodimer with ligand-free RXR, and dimerisation actually precludes binding of ligand to RXR – in this instance, RXR is referred to as the "silent partner" (Aranda and Pascual, 2001). TRs can enhance or inhibit gene expression depending on the nature of the TRE. Most of the natural, characterised TREs are "positive" TREs, in which gene transcription is repressed by unliganded TRs and activated by T3-occupied TRs (e.g. growth hormone, malic enzyme, myosin heavy chain-α). In contrast, on negatively regulated genes (e.g. TSHα and β subunits, myosin heavy chain-β), gene expression is activated in the absence of ligand and repressed in the presence of ligand (Wu and Koenig, 2000).

Heterodimer formation is thought to enhance DNA-binding affinity, as well as provide target gene specificity, determined by the spacing between the two half sites (Mangelsdorf *et al.*, 1995). Accordingly, TR/RXR binds DR4, that is two half sites in one orientation separated by 4bp (Zhang and Lazar, 2000). In addition to spacing, subtle differences in the sequence of the hexad half site and the 5' extension

of the TREs can have dramatic effects on the activity of a receptor (Mangelsdorf *et al.*, 1995).

TRs are members of the nuclear receptor (NR) superfamily. Other members of this family include receptors for the retinoids: retinoid X receptors (RXRs) and retinoic acid receptors (RARs), vitamin D receptors (VDRs) and steroid receptors (SRs). To date, there are over 150 different members of the family, spanning a large diversity of species, from worm to human (Mangelsdorf and Evans, 1995). Also contained within the family, are a growing number of receptors for which the cognate ligands have yet to be identified, the so-called orphan receptors. The family is often divided into the steroid receptor family and the TR/VDR/retinoid receptor, (or non-steroid) family. Members of the NR superfamily exhibit a modular structure with distinct functional domains. They are characterised by the highly conserved DNA- (DBD) and ligand-binding domains (LBD) (Wu and Koenig, 2000; Mangelsdorf *et al.*, 1995). The general modular organisation of a typical member of the NR superfamily is shown in Figure 1.3B.

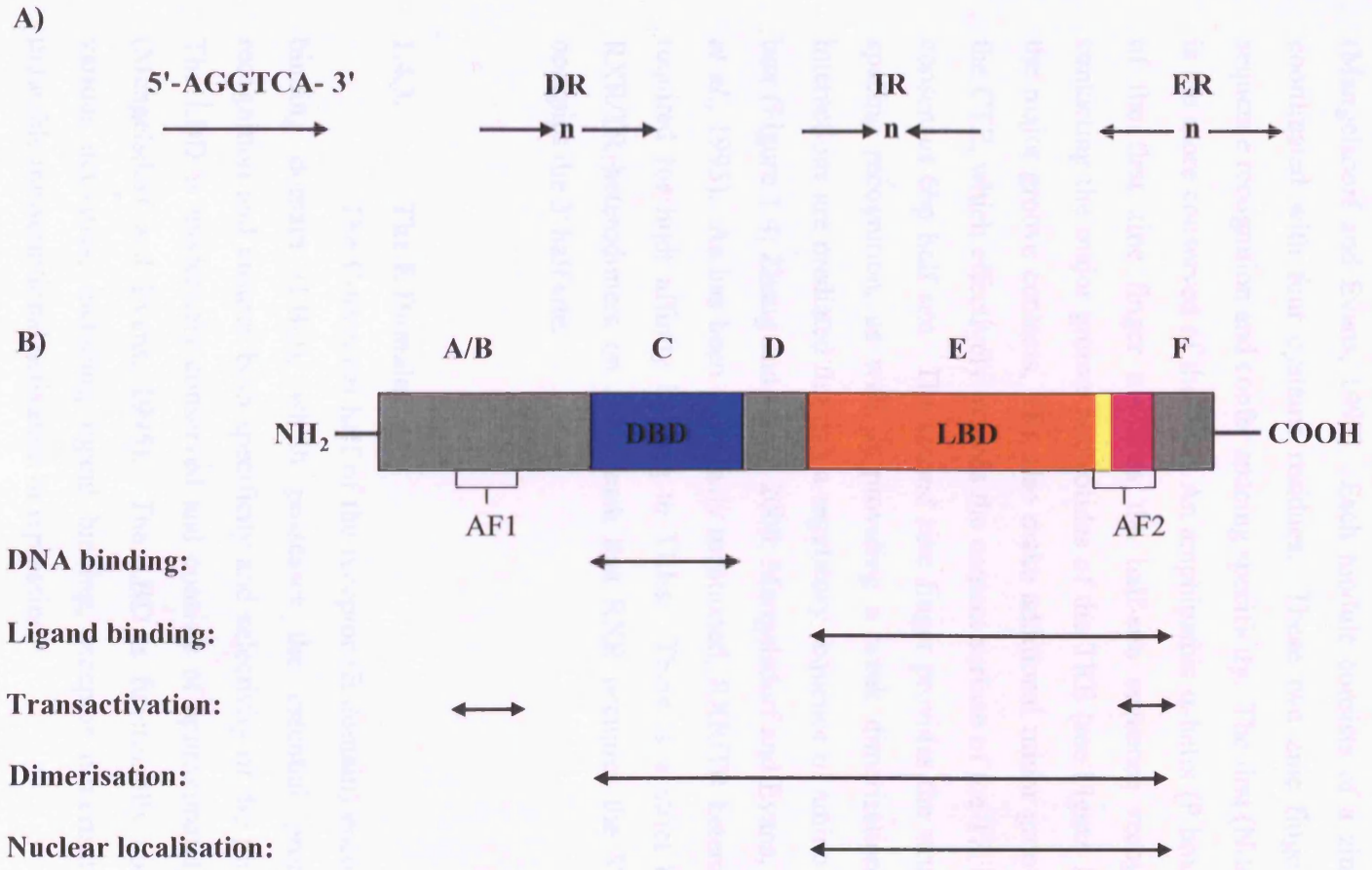


Figure 1.3. Panel A: Nuclear Receptor response elements. The core sequence AGGTCA can be arranged in a number of ways, separated by any number of nucleotides, "n".
Panel B: A schematic representing the modular structure of a typical nuclear receptor. The ninth heptad is coloured yellow.
Abbreviations: AF1 activation domain 1; AF-2, activation domain 2; DBD DNA-binding domain; LBD ligand-binding domain; DR direct repeat; IR inverted repeat. (adapted from Wu and Koenig, 2000).

1.4.2. The C Domain.

The C domain encompasses the DBD of all NRs. The DBD is the most conserved region of the NR superfamily. It spans a region of 66 amino acids, and is composed of two globular modules, followed by a carboxy-terminal extension (CTE) (Mangelsdorf and Evans, 1995). Each module consists of a zinc ion, tetrahedrally coordinated with four cysteine residues. These two zinc fingers mediate specific sequence recognition and confer spacing specificity. The first (N-terminal) zinc finger is the more conserved of the two. An amphipathic α-helix (P box) in the C-terminus of the first zinc finger mediates the half-site sequence recognition by directly contacting the major groove nucleotides of the TRE (see Figure 1.4). In addition to the major groove contacts, TRs also make additional minor groove contact, through the CTE, which effectively extends the contact surface of the TR DBD to beyond the consensus 6bp half site. The second zinc finger provides the structural basis for the spacing recognition, as well as providing a weak dimerisation interface. These interactions are mediated through a regulatory sequence of amino acids, called the D box (Figure 1.4; Zhang and Lazar, 2000; Mangelsdorf and Evans, 1995; Mangelsdorf *et al.*, 1995). As has been previously mentioned, RXR/TR heterodimer formation is required for high affinity binding to TREs. There is a strict binding polarity of RXR/TR heterodimers on DR4, such that RXR occupies the 5' half site, and TR occupies the 3' half site.

1.4.3. The E Domain.

The C-terminal half of the receptor (E domain) encompasses the ligand binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response. The LBD is moderately conserved and consists of approximately 225 amino acids (Mangelsdorf and Evans, 1995). The LBD is functionally complex, performing various activities, including ligand binding, receptor dimerisation and hormone-inducible transcriptional activation or repression.

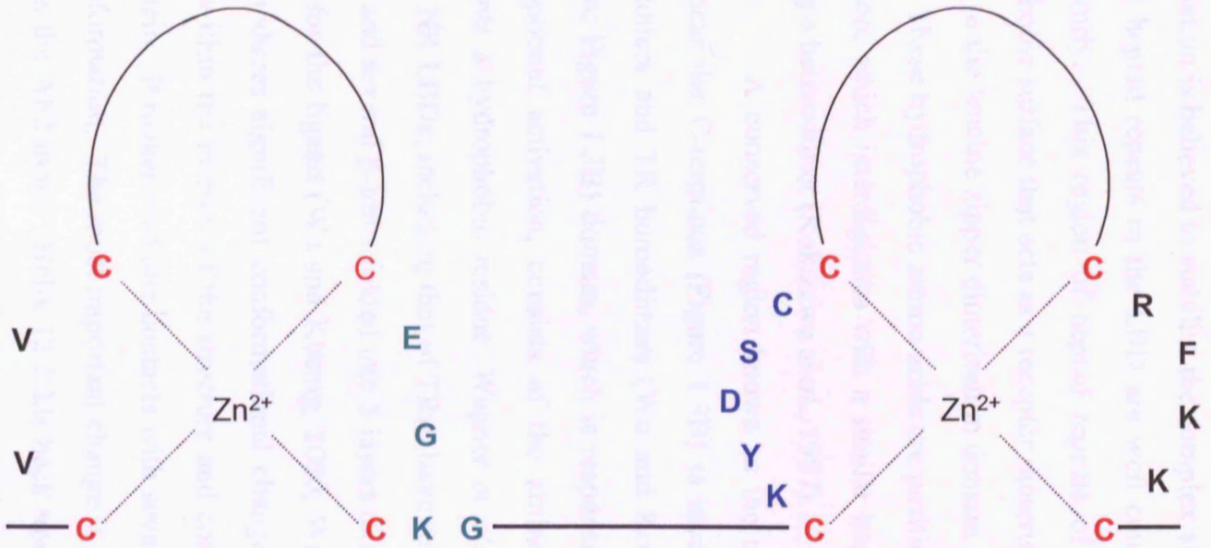


Figure 1.4. Schematic diagram detailing the modular arrangement of the DBD from Human TRα1. Each finger contains a central zinc ion, tetrahedrally coordinated to four cysteine residues. The first zinc finger mediates TRE recognition by direct contact with the nucleotides of the major groove, via the P-box (green residues). The second zinc finger is involved in receptor dimerisation, and spacing specificity, effects mediated via the D-box (blue residues).

In contrast to the dimerisation interface in the DBD, dimerisation motifs in the LBD permit the heterodimeric complex to interact with response elements. The LBD is responsible for DNA-independent dimerisation that *in vitro* allows formation of certain dimers in solution before DNA targeting (Kakizawa *et al.*, 1997). This dimerisation is believed to stabilise the complex and promote the recognition of DNA. Several heptad repeats in the LBD are well conserved among members of the NR superfamily. This region of heptad repeats of Ile, Leu or Val residues forms a hydrophobic surface that acts as a receptor dimerisation interface, which is structurally similar to the leucine zipper dimerisation domain found in Jun-Fos (Landschulz *et al.*, 1988). These hydrophobic amino acids are positioned on the same side of a α-helical formation, which interdigitates with a similar heptad from the second receptor, thus forming a heterodimer (Kakizawa *et al.*, 1997).

A conserved region known as the ninth heptad, located in the 11^{th} α-helix, near the C-terminus (Figure 1.3B) is involved in the formation of TR-RXR heterodimers and TR homodimers (Wu and Koenig, 2000). The AF2 (activation function; Figure 1.3B) domain, which is responsible for mediating ligand-dependent transcriptional activation, consists of the amino acid motif XXLEXX (where X represents a hydrophobic residue, Wagner *et al.*, 1995). The crystal structures of several NR LBDs, including that of TRα, have revealed a common structure of 12 α-helices and several β-turns folded into 3 layers to create a central hydrophobic binding pocket for the ligand (Wu and Koenig, 2000; Wagner *et al.*, 1995). The binding of ligand induces significant conformational changes. The ligand itself is completely buried within the interior of the structure and contributes to the hydrophobic core of the protein. It makes multiple contacts with several helices and β-strands to stabilise the conformation. The most important change is the repositioning of helix 12, which contains the AF2 motif. Helix 12 folds back toward the LBD, realigns with helix 3 and 4, and contacts the ligand, thereby closing the ligand cavity and generating a surface for coactivator recruitment (mousetrap model, Wu and Koenig, 2000; Zhang and Lazar, 2000; Mangelsdorf and Evans, 1995; Wagner *et al.*, 1995). The repositioning of helix 12 plays an important role in NR function: studies identified a hydrophobic cleft, composed of helices 3-5 and 12 that is crucial for coactivator binding (Wagner *et al.*, 1995).

1.4.4. The A/B Domain.

The N-terminal region (A/B domain) is the least conserved among the NR superfamily. It is a hypervariable region of between 25 (VDR) and 603 (mineralocorticoid receptor) amino acid residues in length. The A/B domain is highly divergent between the TRs. It varies not only between TRα and TRβ, but also between the two TRβ isoforms, TRβ1 and TRβ2. The A/B domain of TR contains a ligand-independent transactivation factor (AF1), which appears to modulate A/B domain function through interaction with basal transcription factors, especially TFIIB (Hadzic *et al.*, 1995). It is likely that this N-terminal domain is a modulator of TR function as whose presence is required to allow full transcriptional activation as opposed to being an essential factor, as illustrated by the fact that the A/B domain is not required for ligand-dependent transcriptional activation by rat TRβ1 (Zhang and Lazar, 2000).

In addition to modulating ligand-independent activation function, the A/B domain also modulates ligand-dependent interaction with corepressors. Hollenberg *et al.*, (1996) showed that NCoR functions as a ligand-dependent transcriptional repressor in the presence of TRβ1 and TRα1. However, this function was isoform-specific in that the ligand-independent and -dependent activity of the TRβ2 isoform was unaffected by the presence of NCoR. They showed that TRβ2 was able to interact physically with NCoR, but that a functional interaction was prevented. Oberste-Berghaus *et al.*, (2000) went on to show that TRβ2 exhibited greater T3-independent activation on TREs compared to TRα and TRβ1 due to its ability to interact with certain coactivators (CBP, SRC-1, and pCIP) in the absence of T3 through a domain which mapped to the amino-terminal half of its A/B domain. Since TRβ2 is found almost exclusively in the pituitary and hypothalamus it is likely, that it plays a unique role in negative regulation by thyroid hormone of centrally located genes, for example thyrotropin releasing factor (TRF).

1.4.5. The D Domain.

The region between the DBD and LBD consists of a short hinge region, comprised of hydrophilic residues. This hinge region allows retention of spatial flexibility. This flexibility gives the DBDs rotational capabilities, which allow them to swivel according to the orientations of the half sites to which they bind. Each

DNA-binding head must be able to rotate at least 180° relative to the LBD, in order to accommodate rotational differences associated with receptor-TRE binding (Mangelsdorf and Evans, 1995).

1.4.6. The F Domain.

The F domain is hypervariable in length and is the least understood of all the NR superfamily domains. It is absent in some receptors, including TRs, and has been removed from others, with no loss in function (Tsai and O'Malley, 1994).

1.5. Transcriptional Regulation.

1.5.1. The Basal Transcriptional Machinery.

Promoters transcribed by RNA polymerase II (RNAP II) are recognised by two types of transcription factor: the basal or general transcription factors (GTFs) that interact with the core promoter elements, and the sequence-specific transcription factors, including the nuclear receptors (NRs), which generally interact with sequences located further upstream. The core promoter may contain the TATA box close to an initiator sequence that spans the transcriptional start site where RNAP II binds (Aranda and Pascual, 2001). Transcription requires the assembly of the pre-initiation complex (PIC), composed of template DNA, RNAP II and five GTFs.

TFIID (general transcription factor IID) is composed of TBP (TATA binding protein) and TBP-associated factors (TAFs). The TBP of TFIID binds to the TATA box, sitting astride it like a molecular "saddle", inducing a sharp bend in the DNA and contacting the largest subunit of RNAP II (Woychik and Hampsey, 2002). TFIID binding is followed by the concerted recruitment of TFIIB, which binds the promoter through base-specific contacts in the major groove upstream and in the minor groove downstream of the TATA box. This asymmetrical binding is likely to account for the unidirectional assembly of the PIC and the direction of transcription. The subsequent binding of RNAP II (unphosphorylated) and TFIIF stabilizes the DNA-TBP-TFIIB ternary complex, and is a prerequisite for entry of TFIIE and TFIIH into the PIC (Woychik and Hampsey, 2002).

TFIIE affects late events in PIC assembly, including recruitment of TFIIH and subsequent regulation of TFIIH activities. TFIIE and TFIIH are required

for ATP-dependent formation of the open promoter complex prior to formation of the first phosphodiester bond. TFIIH consists of nine subunits and has a molecular mass similar to that of RNAP II. It can be resolved into two subcomplexes: core-TFIIH, which includes two ATP-dependent DNA helicases of opposite polarity (XPB and XPD), and a cyclin-dependent protein kinase complex. Following TFIIH binding, promoter melting is facilitated by XPB and XPD and the C-terminal domain of the largest RNAP II subunit is phosphorylated by TFIIH, facilitating transcription initiation, promoter clearance and progression into the elongation phase of transcription. Following termination, a phosphatase recycles RNAP II, allowing the GTFs and RNAP II to initiate another cycle of transcription (Woychik and Hampsey, 2002).

1.5.2. Chromatin Structure and the Histone Code.

Mammals package 2-3 billion base pairs of chromatinised DNA into the nucleus of a cell. Chromatin is a compact and ordered structure composed of a basic repetitive structural element known as the nuclesome. Nucleosomes consist of 146bp of DNA wrapped almost twice around a protein core containing two copies each of four histone proteins: H2A, H2B, H3 and H4 (Orphanides and Reinberg, 2002). An array of nucleosomes folds into a 30nm chromatin fibre, which must be compacted at least 200 to 300-fold in order achieve the final compacted size - a total of 2metres of human DNA ultimately fits within the nucleus of a cell (Khorasanizadeh, 2004). This condensation of chromatin provides a major impediment to transcription by restricting the access of transcription factors to DNA.

In addition to the structured histone fold octameric core, each histone forms extensions consisting of disordered N-terminal tails that protrude from the nucleosome. The histone tails are only partially visible in all these structures and show no secondary structures. By extending beyond the disk-shaped nucleosome surface, the tails form ideal surfaces for covalent modifications (Khorasanizadeh, 2004). The N-terminal tails of all four histones contain multiple targets for covalent modifications and various combinations of these modifications have been observed (Orphanides and Reinberg, 2002).

Histones are subject to a range of posttranslational modifications including: the acetylation of lysines, the methylation of lysines and arginines, the phosphorylation of serines and threonines, the ubiquitination of tyrosines, the

sumolation of lysines and ADP-ribosylation of glutamic acids (Dutnall, 2003). This led to the proposal that the modification of the histone N-termini make up a "histone code" and that specific histone modification patterns control the association of proteins with chromatin, through their effect on the strength and specificity of direct physical interaction with histones (Orphanides and Reinberg, 2002). The association of histone binding proteins with chromatin leads directly or indirectly to changes in the functional state of the underlying DNA (e.g. transcriptionally active, or inactive), through changes either in the higher order structure of chromatin or in the ability to subsequently recruit transcriptional machinery, or a combination of both. Histone acetylation by histone acetylases (HAT) and the reverse effect carried out by histone deacetylases (HDACs) has been correlated with activation or repression of transcription, respectively. The deacetylation of histone tails leads to a more compact chromatin structure that is much less accessible to the transcriptional machinery and is therefore transcriptionally silent.

In addition to the covalent modification of histones, the physical structure of chromatin can be perturbed by the ATP-dependent chromatin remodelling complexes. For example, the SWI/SNF complexes facilitate access of DNA-binding proteins to DNA by using the energy of ATP-hydrolysis to alter nucleosome structure and/or facilitate nucleosome mobility (Li *et al.*, 2003).

1.5.3. Role of TR.

As a transcription factor, a key function of the TR is to regulate target gene expression in response to multiple signalling pathways. As has been previously stated, TR homodimers and TR/RXR heterodimers constitutively bind TREs in the absence and presence of ligand. TRs are one of a limited number of transcription factors that retain the capacity to bind to their recognition elements even when they are wrapped into nucleosomes (another example being the glucocorticoid receptor; Collingwood *et al.*, 1999). Unliganded TR represses basal transcription. Ligand binding causes derepression and enhances transcriptional activation. A group of regulatory proteins, called coactivators and corepressors mediate activation and repression, respectively, by acting as bridging factors between the heterodimer and the basal transcription machinery (Kim *et al.*, 1999; Beato *et al.*, 1995). These coregulators cannot bind DNA, but interact directly with DNA-bound TRs, as a result of which they are recruited to the proximity of the target gene promoter region (Zhang

and Lazar, 2000). The coregulatory proteins (coactivators and corepressors) elicit their effects in a number of ways, including:

i) ATP-dependent remodelling of chromatin,

ii) Covalent modification of chromatin,

iii) Contacting the general transcription factors.

1.5.4. Gene Activation - the Role of Coactivators.

a) Chromatin Remodelling.

One of the primary functions of activated receptors can be viewed essentially as facilitating access of the basal transcription machinery to the promoter to initiate transcription. Chromatin plays an important role in regulating the basal activity of many promoters and molecules designed to overcome the thermodynamic restraints it imposes are thought to be recruited at an early stage in the model of receptor action. One of the best-characterised chromatin remodelling complexes is the ATPase-containing SWI/SNF multiprotein complex (McKenna and O'Malley, 2002). The role of chromatin remodelling in TR-mediated transcription has been well established, with strong evidence provided that transactivation by liganded TR/RXR heterodimers is dependent on chromatin remodelling complexes (Wong *et al.*, 1998). However, further studies have shown that in addition to chromatin remodelling, histone acetyltransferase (HAT) activity is also requried in order to fully activate the TSHα promoter (Li *et al.*, 2003).

b) Acetyltransferases.

A number of the coactivator proteins that interact with TR have been shown to possess intrinsic histone acetyltransferase activity (Oberste-Berghaus, 2000; Spencer *et al.*, 1997). Acetylation of histone lysines disrupts the electrostatic interactions between DNA and the core histone proteins, thus facilitating the access of the basal transcriptional machinery to the promoter (Zhang and Lazar, 2000).

The steroid receptor coactivator (SRC) family of proteins interact directly with liganded TRs and also serve as adaptor molecules to recruit other coactivators, such as p300/CBP (CREB-binding protein; Leo and Chen, 2000). Although some studies have found the SRCs to possess a weak intrinsic acetyltransferase (HAT) activity, p300/CBP and pCAF (p300/CBP-associated factor) possess potent acetyltransferase activity and appear to be the predominant acetyltransferases for histones (McKenna and O'Malley, 2002). p300/CBP is recruited indirectly to liganded TR via the SRCs, whilst pCAF is part of a multipolypeptide coactivator complex that can interact directly with liganded TR and p300/CBP (Li *et al.*, 2003).

Li *et al.*, (2003) showed that the SRC proteins and p300/CBP function together to form an activation pathway: TRβ/RXRα heterodimers induce promoter-

proximal disruption of nucleosomal arrays upon binding to chromatin, and acetylation of nucleosomal histones enhances T3-dependent activation. They showed that the primary role of SRC coactivators is to recruit p300, consistent with the observation that p300 does not exhibit strong direct binding to TR and that deletion of the SRC interaction domain in p300 greatly diminishes the coactivator activity of p300 with TRβ (Li *et al.*, 2003).

The SRC family of coactivators mediate ligand-dependent direct interactions with nuclear receptors via a centrally located receptor-interacting domain (RID) which contains the conserved motif LXXLL called the NR box (where "L" is leucine and "X" is any amino acid). The RID of SRCs contains three NR boxes, which interact with the AF2 domain of liganded TRs (Heery *et al.*, 1997; Section 1.4.3) by formation of an amphipathic α-helix, with the leucine residues comprising a hydrophobic surface on one side of the helix. The helix interacts with the AF2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3,4,5 and 12 that is the result of the conformational change induced by hormone binding (Leo and Chen, 2000). Nuclear receptors can differentially interact with different combinations of NR boxes to form distinct coactivator complexes, and the sequences adjacent to the motif determine the preference for receptor binding (Wu and Koenig, 2000). Mutation of a single NR box however, does not completely abolish coactivator binding, suggesting that multiple NR boxes contribute to the overall high-affinity binding of the receptor (Leo and Chen, 2000).

c) The TRAP/Mediator Complex.

The most universal cofactor that serves to transduce regulatory information between gene-specific transcription factors (TRs) and the basal transcriptional machinery is a large, modular complex, known as Mediator. Mediator itself is unable to bind specific DNA sequences, but interacts physically with RNA Polymerase II (RNAP II), although RNAP II itself is not a component of mediator (Woychik and Hampsey, 2002; Cosma, 2002).

The human TRAP/Mediator is a 1.5-2.0MDa transcriptional regulatory complex that is composed of more than 25 polypeptides. It was originally isolated as a TR-associated protein (TRAP) complex that mediates TR-activated transcription from DNA templates in conjunction with the general transcription machinery. A

small subset of the TRAP complex subunits are homologous to the multifunctional cofactor complex in yeast, termed Mediator (Ito and Roeder, 2001).

The TRAP complex is targeted to the LBD of liganded TR through a single protein, TRAP220 (Yuan *et al.*, 1998). This protein contains two LXXLL motifs within its RID (LXM1 and LXM2), and interactions with TR are again mediated via hydrophobic interactions with the AF2 domain (Coulthard *et al.*, 2003; Yuan *et al.*, 1998). As described in the previous section for the SRC family of proteins, the LXXLL motifs of TRAP220 also exhibit preferential binding to certain nuclear receptors, with LXM1 and LXM2 binding preferentially to RXR and TR, respectively (Coulthard *et al.*, 2003). In addition to the binding preferences of the LXXLL motifs, the sequences immediately flanking the motifs as well as the spacing between motifs further determines binding specificity (Coulthard *et al.*, 2003).

Whilst TRAP220 is also able to interact directly with the basal transcription factor TATA box-binding protein (TBP), and has been shown to enhance TRα function in a cell free environment (Yuan *et al.*, 1998), the entire TRAP/Mediator complex is required for efficient transactivation of TR-mediated transcription (Coulthard *et al.*, 2003).

d) An Ordered Recruitment.

A study carried out by Sharma and Fondell (2002) demonstrated that following the binding of ligand *in vivo*, TRβ first recruits SRC proteins and p300, resulting in the acetylation of histones with subsequent generation of a less compact chromatin structure. This, in turn, results in the recruitment of the TRAP complex to the promoter, which in turn, directly interfaces with RNAP II and potentiates transcription initiation. This step-wise recruitment of SRC, p300 and finally the TRAP complex to the TR-bound promoter is consistent with the fact that the binding of SRCs and TRAP220 to TR is mutually exclusive (See Figure 1.5: Ito and Roeder, 2001).

It has been shown that coregulatory proteins (corepressors and coactivators) utilise a similar binding surface in TRs, composed of helices 3 and 5. Competition assays with SMRT (see next Section) and SRC1 demonstrate that SMRT is able to inhibit the interaction between SRC1 and the receptor. Furthermore, the same study suggests a competition between SMRT and SRC1 for receptor binding,

even in the presence of ligand (Sohn *et al.*, 2003). This is consistent with a recent study, which implies a dominant role of corepressor release to allow coactivator binding (primarily with RAR) (Kao *et al.*, 2003). Taken together it is possible to envisage the transcriptional switch between activation and repression not as a static on-off switch, but rather as a dynamic equilibrium which is shifted to one or the other conformation dependent on ligand, type of response element and cofactor ratios (Eckey *et al.*, 2003).

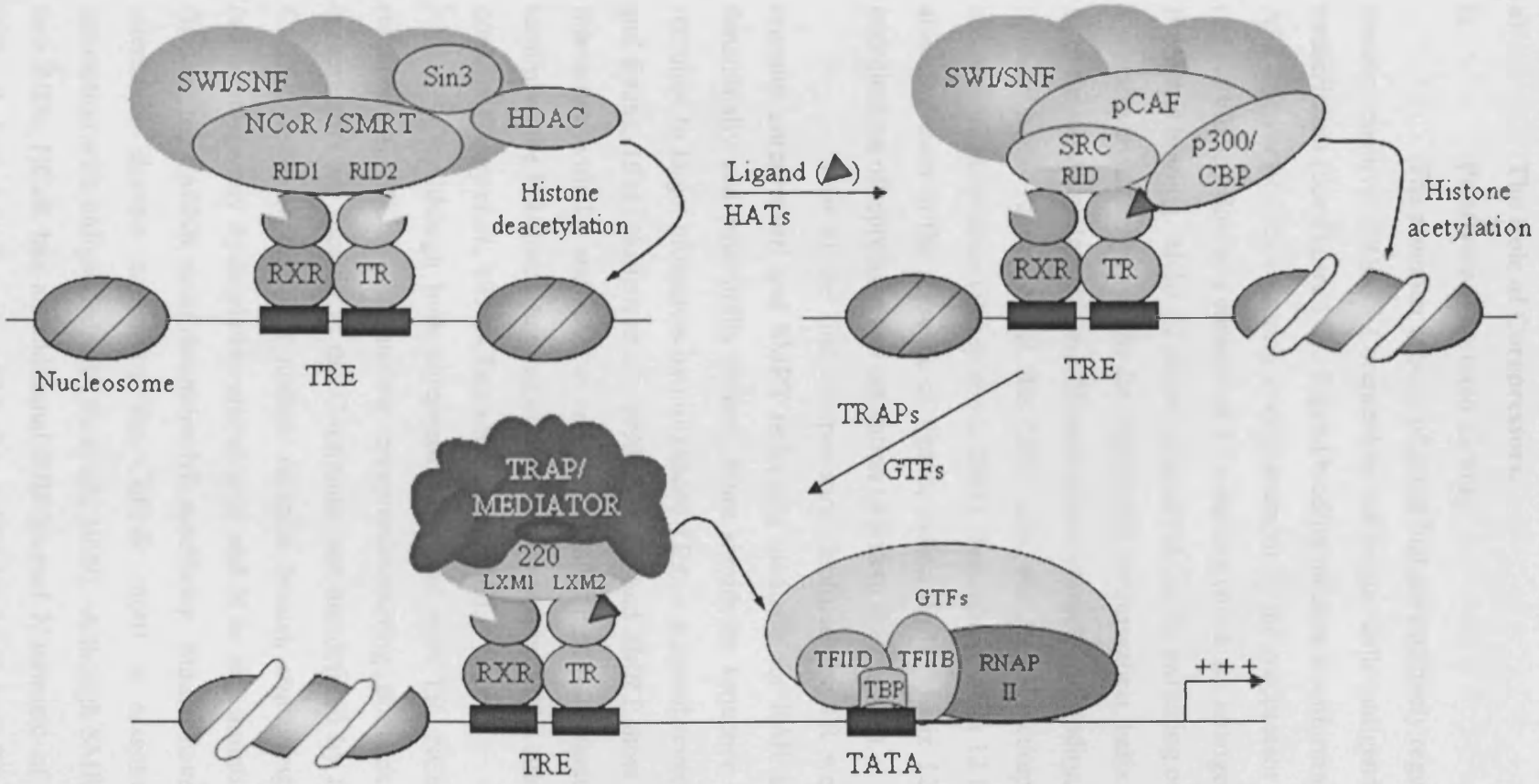


Figure 1.5. A multistep model for thyroid receptor (TR) function. Transcription from a positive TRE-containing target gene in a chromatin template (DNA plus associated histones and nucleosomal structures) is repressed in the absence of T3 (ligand) and activated in the presence of ligand. Binding of ligand leads to a conformational change in TR and subsequent release of corepressor proteins. ATP-dependent chromatin remodeling complexes are recruited, along with the SRC proteins, which subsequently recruit p300/CBP to ligand-bound TR. The acetyltransferase activity of p300/CBP and pCAF results in the loosening of DNA-histone interactions, thus facilitating the formation of the PIC (pre-initiation complex). PIC formation is mediated by the TRAP/Mediator complex, which replaces the SRC/p300/CBP coactivator proteins and interacts with ligand-bound TR via TRAP220. Abbreviations: NCoR, nuclear receptor corepressor; CBP, CREB-binding protein; GTF, general transcription factor(s); HAT, histone acetyltransferase; HDAC, histone deacetylase; pCAF, p300/CBP-associated factor; RID, receptor-interacting domain; RNAP II, RNA polymerase II; RXR, 9-*cis* retinoic acid receptor; SMRT, silencing mediator for retinoid and thyroid receptor; SRC, steroid receptor coactivator; TBP, TATA-binding protein; TR, thyroid receptor; TRAP, thyroid receptor-associated protein(s); TRE, thyroid response element (adapted from Ito and Roeder, 2001).

1.5.5. Gene Silencing.

a) The Role of Corepressors.

i) Positively-Regulated Genes.

The promoter regions of genes that are positively regulated by TRs contain positive TREs. Corepressors associate with unliganded TR to repress transcription (See Figure 1.5). Ligand binding induces a conformational change in the AF2 domain of TRs, resulting in displacement of the corepressor complex; the LBD (ligand binding domain) consists of 12 α-helices which are arranged to create a pocket for ligand binding. Helix 12 plays a crucial role in the switching of the repressor from a silencer to an activator. In the unliganded conformation, helix 12 protrudes away from the core structure of the LBD and allows corepressor binding. Hormone binding induces helix 12 to contact the LBD core, resulting in corepressor release and coactivator association (Eckey *et al.*, 2003). Deletion of helix 12 leads to constitutive silencing even in the presence of ligand, indicating that helix 12 is involved in the coordination of repression and activation (Apriletti *et al.*, 1998).

Two of the first corepressors identified for TR were NCoR (Nuclear receptor corepressor) and SMRT (silencing mediator for RAR and TR). They are functionally and structurally related, share significant sequence homology, and are recruited to target promoters by unliganded TRs, in a ligand-reversible manner (Chen and Evans, 1995; Horlein *et al.*, 1995). NCoR and SMRT show ligand-independent interaction with TR and RAR *in vitro*, and exhibit modular structures, with carboxyl-termini as the NR-interacting regions and amino termini as the autonomous repression domains (Nagy *et al.*, 1997; Chen and Evans, 1995).

Although both corepressors interact with TR, NCoR is preferentially recruited to TR. The nuclear receptor-interacting domains (RIDs) of these corepressors are located in the C-terminus and are defined by the presence of the CoRNR boxes (corepressor nuclear receptor boxes), containing a ΦXXΦΦ motif (where Φ is any hydrophobic amino acid and X is any amino acid). Sequences flanking the CoRNR motif determine NR specificity. Studies have shown that a short interaction domain containing the CoRNR motif is essential for corepressor interaction with unliganded TR (Hu *et al.*, 1999). Although SMRT and NCoR share two RIDs, NCoR has an additional RID located N-terminal of the two conserved NIDs, that seems to be responsible for preferential binding to TR. NCoR appears to

stabilize the TR homodimer by preventing its dissociation from TREs, whilst it is postulated that the C-terminal NID interacts with the RXR of a TR/RXR heterodimer (Eckey *et al.*, 2003).

Both NCoR and SMRT have been shown to interact with the protein Sin3, which itself associates with histone deacetylases (HDACs) to form a corepressor complex (Nagy *et al.*, 1997). Antibody blocking of each component of the SMRT-Sin3-HDAC complex causes relief of repression by TR and RAR, suggesting that each component of the complex is important for repression (Wu and Koenig, 2000; Nagy *et al.*, 1997). The finding of HDACs in the SMRT-NCoR-Sin3 repressor, coupled with the fact that histone deacetylase contributes to a compact chromatin structure, suggests that gene silencing is effected by excluding the recruitment of, or by restricting the accessibility of, basal transcription factors to targeted promoters.

NCoR and SMRT have also been shown to interact with TBP (TATA-binding protein) and TAFs (TBP-associated factors), in line with the observation that overexpression of NCoR prevents the functional interaction of TFIIB with TAFII32, which is a critical step in the assembly of the transcription initiation-complex (Wong and Privalsky, 1998).

ii) Negatively-Regulated Genes.

The promoter regions of genes that are negatively regulated by TRs contain negative TREs (nTREs). In contrast to the TR-mediated transcriptional regulation of positive genes, the mechanisms underlying the TR-mediated control of negatively regulated genes remain relatively poorly understood.

For all nTREs, transcription is constitutively activated in the presence of ligand-free TR, whilst the addition of ligand reverses this stimulation. Tagami *et al.*, (1997) showed that the overexpression of NCoR and SMRT enhanced basal transcription from negative TREs, in a ligand-reversible fashion. A more recent study by Berghagen *et al.*, (2002) demonstrated that TRα-SMRT complexes bound to a positive TRE had a different conformation to those bound to a nTRE, suggesting that allosteric changes resulting from binding of TR to different types of TREs can dictate whether a cofactor acts as a coactivator or corepressor. A study by Sanchez-Pacheco and Aranda (2003) subsequently showed that binding of TR to a nTRE was associated with histone acetylation, and that this acetylation was reversed in the presence of ligand.

b) The Role of TRβ.

TR can also mediate gene silencing by directly targeting the basal transcriptional machinery. TRβ has been shown to interact with the basal transcriptional factor TFIIB, whilst the addition of hormone (T3) leads to a significant decrease of this interaction (Baniahmad *et al.*, 1993). Tong *et al* (1995) showed that ligand-free TRβ or a TRβ-RXR heterodimer was able to inhibit the formation of a pre-initiation complex (PIC) by interacting with the TFIIB-TBP protein complex

1.6. Environmental Endocrine Disruptors.

Potential sources of exposure to EEDs are myriad, and include contaminated food and water, combustion sources and contaminated consumer products. EEDs are often pervasive and tend to bio-accumulate in the environment. Some are very persistent, are transported over large areas and are found even in the last remaining pristine habitats. Others are rapidly degraded in the environment or animal body, or may be present for short periods of time, but at critical periods of development of the organism (McLachlan, 2001). The effects of EED's on developing organisms are of greatest concern, since developmental stages are typically more vulnerable to signal disruption than adult stages. This is thought to occur for several reasons, including the absence of fully developed protective enzyme systems and higher metabolic rates. Altered gene expression can induce dramatic changes in developmental outcomes, and the disruptive effects of developmental exposure are permanent and irreversible ("organisational" effects). In adults, however, adverse effects can often be reversed by removing the EED, thus returning gene expression levels and organ functioning to normal ("activational" effects; Myers *et al.*, 2003).

There are now over 600 registered pesticides and 80,000 industrial chemicals in commercial use today (Keith, 1997). Whilst the potential action on the thyroid system of many of these chemicals has yet to be characterised, there are numerous chemicals which are recognised as having potential thyroid-disrupting properties. A group of twelve chemical compounds have been identified by the United Nations Environment Programme (UNEP) as powerful threats to human and wildlife health on a global basis (the "dirty dozen"). These chemicals belong to a class known as persistent organic pollutants (POPs). POPs are organic compounds that resist photolytic, biological, and chemical degradation. Most are semivolatile, which allows them to move long distances across the globe in the atmosphere before deposition occurs by a process called the "grasshopper effect." This process, which is often seasonal, involves a repeated pattern of release of a chemical into the atmosphere, such as through evaporation, and its subsequent deposit elsewhere, such as through rainfall. Due to the persistent nature of POPs, the chemicals are spread widely throughout the world through numerous iterations of this cycle (Fisher, 1999).

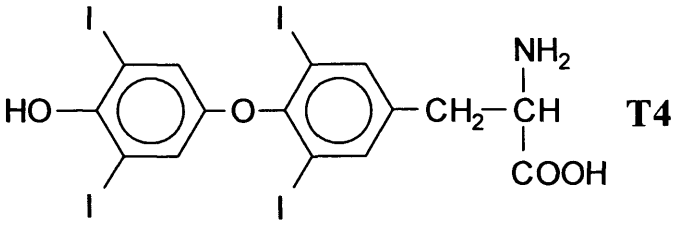
Some of the synthetic chemical which have been linked to adverse thyroid-mediated effects in animals are included on UNEP's list of 12 POPs which require urgent attention. These include the pesticides DDE, DDT, dieldrin, and their metabolites, polychlorinated biphenyls (PCBs) and dioxins. In addition, bisphenol A (used in epoxy resins), phthalates (widely found in plasticizers, used in many industrial and manufacturing contexts including the production of PVC), polybrominated diphenyl ethers (PBDEs – used as flame retardants in plastics, paints, furniture and upholstery) and the heavy metals cadmium, lead and mercury have also been implicated (Myers *et al.*, 2003; Brucker-Davies, 1998).

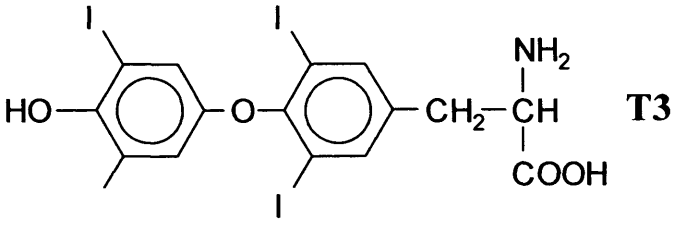
1.6.1. Agonists and Antagonsists of the Thyroid System.

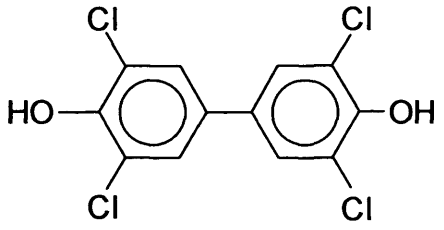
There are a number of ways in which, in principle, a chemical might exert an influence on the thyroid system. These include:

- Inhibiting iodine uptake by the thyroid gland and/or inhibiting hormone synthesis in the gland.
- Binding of exogenous chemicals to the serum proteins normally intended to transport T4 to target cells.
- Altering the breakdown and elimination of THs in the liver.
- Antagonising the effect of T3 by residing on the thyroid receptor and thereby denying access to T3.
- Mimicking the ways in which the natural hormone may operate in its binding to the hormone-receptor molecule.
- Modulating the interactions of co-regulator proteins with TRs.

Shown below are the molecular structures of some of the chemicals which have been identified as being putative EEDs (see text for specific references.)

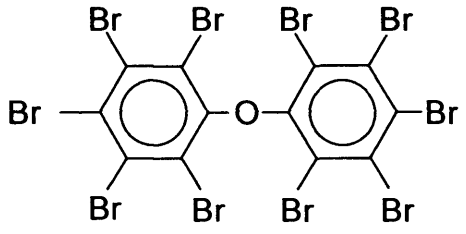




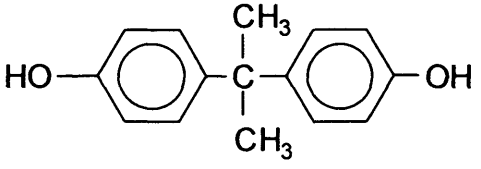


4,4′-diOH-

3,3′,5,5′-TCB



PBDE



BPA

Figure 1.6. Structures of thyroid hormones and chemicals which have been identified as possessing putative thyroid-modulating activity.
Abbreviations: TCB tetrachlorinated biphenyl; PDBE polybrominated diphenyl ether; BPA bisphenol A (4,4′ isopropylidenediphenol).

a) Polychlorinated Biphenyls.

Polychlorinated biphenyls (PCBs) are a strongly suspected EED that are listed by the EPA, the Centres for Disease Control and Prevention, and the WWF as a potential endocrine modifying chemical (Keith, 1997). These compounds were used as coolant fluid in transformers and fire retardants in capacitors and were produced in vast quantities from the 1930s to the late 1970s. Although their production was banned in 1979, they are still leaching from industrial and waste sites and leaking from old capacitors or transformers still in use today. As mentioned previously, PCBs are included on UNEP's list of 12 POPs which require urgent attention. Consistent with many POPs, PCBs exhibit low solubility in water and high solubility in lipids. Combined with the chemicals' persistence, these properties allow PCBs to bioconcentrate and bioaccumulate in the fatty tissues of organisms. They are present as a mixture of lipophilic congeners that can cross the placenta. Some of the congeners are very persistent, with a half-life of elimination from adipose tissue of several decades (Brucker-Davies, 1998). There are 209 congeners in total, with a variable number (1-10) of chlorine atoms attached to the benzene rings. Bioconcentration is the uptake of a chemical directly from water into an aquatic organism and is measured as a ratio of the amount of a chemical concentrated in an organism compared to the amount of the chemical in the surrounding environment. Bioaccumulation refers to the process of uptake from both water and dietary sources. These processes lead to biomagnification, in which tissue concentrations of a contaminant increase as it passes through two or more trophic levels in the food chain (Fisher, 1999).

PCBs in the environment may associate with organic components of soils, sediments, and biological tissues, or with dissolved organic carbon in aquatic systems. Environmental PCBs have been show to induce thyroid-related changes in rats, mice, mink, seals, birds and primates. The main effects in mammals are the occurrene of goiter, lowered T4 levels, elevated thyroid stimulating hormone (TSH) levels and normal or decreased T3 levels (Goldey and Crofton, 1998; Brucker-Davies, 1998). The main source of human PCB exposure is through food, especially fish. In Norway, the consumption of crabs from a contaminated fjord was found to be associated with the presence of PCBs in serum. Humans consuming large amounts of contaminated seafood show elevated concentrations of PCBs in their tissues, due to

the low biodegradation and excretion of these compounds (Johansen *et al.*, 1997). PCBs have been detected in human milk, and studies suggest that mothers accidentally exposed to PCBs may give birth to children who display neurological disturbances (Johansen *et al.*, 1997; Huisman *et al.*, 1995). PCBs decrease circulating levels of T4 in animals (Hallgren and Darnerud, 2002; Zoeller *et al.*, 2000; Brucker-Davies, 1998; Goldey and Crofton, 1998) and it has been speculated that PCBs exert neurotoxic effects on the developing brain by causing a state of relative hypothyroidism (Crofton et al., 2000). This concept it supported by the observations of Goldey and Crofton, (1998) who used T4 replacement therapy in rats to show that the toxic effects of PCB exposure (hearing loss) could be partially ameliorated by T4 replacement. A study by Stewart *et al.*, (2003) used magnetic resonance imaging to show that the association between PCB body burden and behavioural measures of response-inhibition is stronger in young children that have a smaller corpus callosum, an area of the brain affected by thyroid hormone.

Although the effects of PCBs on brain development may be attributable to their ability to reduce circulating levels of thyroid hormone, the developmental effects of PCB exposure are not fully consistent with hypothyroidism. Zoeller *et al.*, (2000) demonstrated that a mixture of PCBs, (Aroclor 1254, A1254) caused a dose-dependent increase in expression of the thyroid responsive gene RC3/Neurogranin, despite observing a severe reduction in circulating concentrations of T4. This is consistent with the observations that PCBs (or their metabolites) are able to bind TR and act as agonists *in vivo*. Cheek *et al.*, (1999) examined the ability of PCBs to bind to human thyroid hormone receptors and thyroid hormone transport proteins. Out of all the compounds, only hydroxylated PCBs bound to the human thyroid receptor, but had a greater binding affinity to the thyroid transport protein, transthyretin. This was followed up by a later study which showed that hydroxylated PCBs exhibited thyroid hormonal activity through interaction with TR; Kitamura *et al.*, (2005) reported that nine separate hydroxylated PCB congeners could bind to the rat TR with an IC_{50} as low as 50μM. These results suggest that hydroxylated PCBs have the potential to disrupt thyroid hormonal activity *in vivo* by interaction with TR; hydroxylated PCBs were shown to have agonistic activity for thyroid hormone on GH3 cells, a rat pituitary cell line where proliferation and growth hormone secretion is dependent on thyroid hormones. The structure of 4,4′-diOH-3,3′,5,5′-TCB (tetrachlorinated biphenyl), which was shown to bind most potently to TR by Cheek *et al.*, and was also

shown to bind TR by Kitamura *et al.*, (2005) as well as exhibiting agonistic activity for thyroid hormone on GH3 cells, is shown in Figure 1.6.

However, not all studies indicate that PCBs act as agonists on TRs. Bogazzi *et al.*, (2003) showed that A1254 (Aroclor 1254) was able to bind specifically to the rat TRβ at approximately 10 μM and that this concentration inhibited CAT activity on the ME-TRE in cultured cells by approximately 50%. Although A1254 reduced the binding of T3 to TR by 30%, it did not affect the interaction of TR with the ME-TRE in a gel shift (EMSA) assay. It was postulated that A1254 produced a conformational change in TR, leading to its increased proteolysis by trypsin. A later study by Miyazaki *et al.*, (2004) showed that PCBs could inhibit TH-mediated transcription by binding to thyroid receptors and causing partial dissocciation of TR/RXR heterodimers from the TRE.

It is clear that PCBs are neurotoxic in humans and animals, and that they can interact directly with the TR. However, the consequences of PCB exposure on TR action appear to be quite complex. This complexity includes acting as an agonist or antagonist and may include TR isoform selectivity in as much as most studies have been performed using TRβ, leaving TRα relatively unstudied in this context. In addition, considering that there are 209 different chlorine substitution patterns on the biphenyl backbone and that these can be metabolized (hydroxyl- and methylsulfonyl-metabolites), it is possible that different chemical species exert different effects. Finally, PCBs may exert different actions on TRs depending on associated heterodimer partners, promoter structure, or different co-factors (Zoeller, 2005).

b) Pesticides.

Ethylenebis(dithiocarbamate) (EBDC) fungicides are among the most common used in the USA. They are absorbed dermally and are metabolised to ethylene thiourea. The EPA classifies ETU as a carcinogen, as it has been shown to cause thyroid cancer in animal models, and it has also been shown to be genotoxic in bacterial and animal systems. In humans, the use of dithiocarbamate fungicides has been identified as causing an increase in circulating TSH amongst agricultural workers (Steenland *et al.*, 1997), consistent with previous studies in rats, whereby exposure to ETU caused a decrease in T4 and an increase in TSH levels. The observed disruption in thyroid homeostasis was correlated with morphological

changes in the thyroid gland and was postulated to be linked with the prevalence of thyroid tumours in the rats (Houeto *et al.*, 1995).

Crain *et al.*, (1998) examined the plasma levels of thyroxine and triiodothyronine in juvenile alligators of three lakes in Florida, as well as the relationship of the thyroid hormones to alligator size. The concentrations of thyroid hormones were found to be elevated in alligators from a lake contaminated by a pesticide spill in 1980, which consisted of as much as 15% DDE and DDT. In the reference lake thyroid hormone levels had a strong negative correlation to alligator size. Although it is recognized that pristine conditions no longer exist, and that in practice, the characteristics of an acceptable reference site may be influenced by socioeconomic demands the reference lake in this study was situated in a National Wildlife Refuge and considered to be a relatively pristine, natural environment with minimal influences from agricultural and municipal activities. Thyroxine levels in the males of the contaminated lakes were negatively correlated to size, but there was no clear relationship in the females. Triiodothyronine levels of the females in the contaminated lake had a negative relationship to size, but there was no correlation in the males of either contaminated lake to triiodothyronine. These results clearly indicate the alteration of the thyroid hormone system in alligators living in contaminated lakes. There are two significant questions that emerge from this study; first, if thyroid hormone levels are abnormal during a juvenile state is this an indication of embryonic exposure and is, therefore, a permanent alteration; and second, will the observed alterations have a significant impact of the reproductive potential of this cohort of alligators? (Crain *et al.*, 1998).

c) Brominated Flame Retardants.

Polybrominated diphenyl ethers (PBDEs) are a group of aromatic compounds in which one to ten hydrogens in the diphenyl oxide structure are replaced by bromine (See Figure 1.6 for structure). PBDEs with three to ten bromine atoms are used in commercial flame retardants. These are commonly used in a wide variety of products and processes, including plastics, paints, electronic parts and fabrics. The emission of flame retardants to the indoor environment from products in service has been a focal point in the debate about the use of brominated flame retardants. The

most obvious sources of emissions to the air would be from products where the flame retardants are used as additives, for example in printed circuit boards in consumer electronics or thermoplastic components that heat up during operation, e.g. computer monitors and TV screens. The emission of brominated flame retardants from office machines has been demonstrated by the detection of these compounds in the indoor atmosphere of office rooms and computer halls and PBDEs have been detected in human breast milk (Darnerud *et al.*, 1998). Fowles *et al.*, (1994) exposed mice (orally) to a commercial PBDE mix, DE-71, and looked for endocrine effects. Mice that were treated with a range of concentrations of DE-71 in a single dose exhibited significantly lower T4 serum concentrations at all doses, bar one. Total and free T4 concentrations were dose-dependently decreased in mice following subchronic exposure over a 14 day period. Ilonka *et al.*, (2000) conducted competitive binding experiments to measure the potency of brominated flame retardants to intefere with thyroid hormone function. Their results showed that brominated flame retardants, especially the brominated phenols and tetrabromobisphenol A, were very potent competitors for T4 binding to human transthyretin (a TR-binding transporter protein) *in vitro*. A study by Hallgren and Darnerud (2002) observed similar effects upon exposure of rats to the PBDE, DE-47, whereby T4 serum concentrations were lowered. They also postulated that this was due to the binding of *in-vivo* formed DE-47 metabolites to transthyretin.

d) Bisphenol A.

Bisphenol-A (BPA, 4,4′ isopropylidenediphenol, see Figure 1.6 for structure) is produced at a rate of over 800million kg/annum in the U.S. alone (Keith, 1997). It is used primarily in the manufacture of plastics including polycarbonates, epoxy resins that coat food cans, and in dental sealants. It has been reported at concentrations of between 1–10ng/ml in serum of pregnant women, in the amniotic fluid of their fetus, and in cord serum taken at birth. In addition, BPA concentrations of up to 100ng/g have been reported in placenta (Schonfelder *et al.*, 2002).

Moriyama *et al.*, used transient gene expression studies to show that Bisphenol A acts as a T3 antagonist. T3 induced transcriptional activity on a positive TRE was suppressed by BPA in a dose-dependent manner and these effects were

observed in the presence of physiological concentrations of T3. In contrast, BPA activated gene transcription on the TSHα negative TRE, which is suppressed by T3. Using a mammalian two-hybrid system, they demonstrated that BPA was able to recruit the corepressor N-CoR to TRβ and so postulated that BPA's antagonistic effects were due to it's replacement of T3 on the TR and subsequent recruitment of N-CoR.

A study by Zoeller *et al.*, (2005) showed that developmental exposure of rats to BPA gave rise to elevated T4 levels in the pups of BPA-exposed animals, whilst TSH levels remained unaltered. This is consistent with the inhibition of TRβ-mediated negative feedback. However, expression of the thyroid-responsive RC3 gene in BPA-treated animals was increased in the dentate gyrus. Since TRα is expressed in the dentate gyrus, Zoeller postulated that BPA may act as a selective TRβ antagonist *in vivo*.

1.7. International Chemical Assessment Activities.

In the U.S. in 1996, Congress passed a Food Quality Protection Act and amendments to the Safe Drinking Water Act, directing the Environmental Protection Agency (EPA) to determine whether, and to what extent, industrial chemicals disrupt reproductive and thyroid hormones. In reponse to its congressional mandate, the EPA formed the Endocrine Disruptor and Testing Advisory Committee (EDSTAC). In its 1998 final report, EDSTAC recommended that the EPA address the effects of pesticides, commercial chemicals and other environmental chemicals on the endocrine system – which comprises the pituitary and adrenal glands, the ovaries and testes, the pancreas, the hypothalamus, the parathyroid and the thyroid gland (Brown, 2003).

Efforts to test chemicals for endocrine modulating effects are being coordinated at the international level by the Organisation for Economic Cooperation and Development (OECD). In 1996, the OECD established a Task Force on Endocrine Disruptor Testing and Assessment (E-DTA), with representation from both governmental and non-governmental entities. From the E-DTA emerged two "validation management groups" (VMG), each with a mandate to oversee the development and validation of various *in vivo* test methods, for mammalian and ecotoxicological effects, respectively. However, in June 2002, a special session of the E-DTA recommended the addition of a number of non-animal approaches (including the *in vitro* thyroid screening assays) to the OECD's proposed "toolbox" of assay systems. This significant move culminated in a unanimous recommendation to establish a third VMG specific to non-animal screening and testing methods. The E-DTA's recommendations were subsequently ratified at the OECD Joint Meeting in November 2002.

1.7.1. EDSTAC – Tiered Testing.

The EDSTAC proposal from its final report (1998) included: (1) a process to prioritise chemicals for evaluation, along with tiered; (2) screening (Tier 1); and (3) testing (Tier 2) batteries. Since there are currently in excess of 80,000 chemicals in commercial use today (Keith, 1997), only a subset of high priority chemicals would initially enter the screening program. Prioritization would include an estimation of the chemical's ability to interact with steroid hormone receptors using either quantitative structure activity modelling (QSAR) for chemicals that bind steroid

receptors, or high-throughput pre-screening (HTPS) using hormone-dependent gene expression assays, among several other factors (Gray Jr *et al.,* 2002).

The screening battery recommended by EDSTAC was designed to detect alterations of hypothalamic-pituitary-gonadal (HPG) function, estrogen, androgen and thyroid hormone synthesis and AR-, ER- and TR- (androgen, estrogen and thyroid receptor, respectively) mediated effects in mammals and other taxa. Chemicals positive in Tier 1, based upon a "weight-of-evidence" analysis, would be considered as potential EEDs and subjected to testing (Tier 2). Tier 1 screening assays (T1S) should be sensitive enough to detect EEDs, whilst issues of dose-response, relevance of the route of exposure, sensitive life stages and adversity are resolved in Tier 2 Testing (T2T; Gray Jr, L.E. 1998). In this tiered approach, use of animals is minimised, as only chemicals displaying positive responses in T1S would be evaluated further in T2T. In addition, testing statistical "false positives" can be almost entirely eliminated by assuring that T1S assay results are replicated before moving to T2T.

T1S *in vitro* assessments of EEDs would compliment the results of the T1S *in vivo* assays. Short-term *in vivo* mammalian assays were proposed for the Tier 1 Screening Battery because of major limitations of *in vitro* assays. *In vitro* assays cannot account for metabolic activation of xenobiotics, resulting in "false negative" responses (positive *in vivo,* but negative *in vitro*). These assays can also yield false positive responses when chemicals are examined at *in vitro* concentrations which are orders of magnitude above those seen *in vivo* (Gray Jr *et al.,* 2002).

1.7.2. Extant Tier 1 Screening Assays.

1.7.2.1. Estrogen Disruption.

a) *In Vitro* Assays.

A number of assays have been developed to screen substances for estrogenicity. These assays include competitive ligand-binding assays, recombinant receptor-reporter assays, and yeast-based screens. Murk *et al.*, (2002) describe a sequential testing strategy for the identification of xenoestrogens found in wastewater. The binding of a compound to the estrogen receptor (ER) is measured in the ER competitive ligand binding assay (radioactive). Binding to the ER of both agonists

and antagonists gives a positive response, and all compounds can reach the ER without having to pass a cell membrane. The next assay to be used is the Yeast Estrogen Screen (YES) assay. This assay uses a yeast cell transformed with a human ER and a plasmid containing the estrogen-responsive element (ERE) upstream of the *LacZ* reporter gene, encoding β-galactosidase. Activation of the receptors results in increased red colouring of the assay medium. This assay is a measure of agonistic action. In the ER-CALUX (chemically activated luciferase gene expression) assay, reporter gene expression is also a measure of the ER-mediated cascade of events resulting in gene expression. In this assay, the T47D human breast adenocarcinoma cells were stably-transfected with an estrogen-responsive luciferase reporter construct. Both hydrophilic and lipophilic compounds can pass the cell membrane and antiestrogenic potency can be detected as well. In all assays, ERα is the important isoform (Murk *et al.*, 2002).

b) ***In vivo* Assays.**

Estrogen agonists and antagonists are detected using the "pubertal female rat assay", described by Goldman *et al.*, (2000). In this assay, weanling female rats are dosed daily by gavage for 21days and the age at vaginal opening (puberty) is monitored and the females are necropsied at approximately 42 days of age. Measurements taken include uterine and ovarian weight and histology, as well as serum thyroid hormones. This assay detects alterations in estrogens, HPG function, inhibition of steroidogenesis and thyroid hormone status.

1.7.2.2. Androgen Disruption.

a) ***In Vitro* Assays.**

Wong *et al.*, (1995) used recombinant human androgen receptor (AR) in a whole-cell binding assay (radioactive) to compare the binding affinities of known androgen disruptors. They also utilised a transient transfection approach in order to assess the effects of disruptors on AR-mediated transcription. Cell lines which have been stably-transfected with androgen responsive luciferase reporter constructs have since been developed (Wilson *et al.*, 2002).

b) *In Vivo* Assays.

Androgen agonists and antagonists can be detected using the Hershberger assay (Gray, 1998). In this assay, (anti)androgenic activity is assessed simply by weighing androgen dependent tissues in the castrated male rat. The weights of the ventral prostrate, Cowper's glands, seminal vesicle, glans, penis and levator ani/bulbocavernous muscles are measured in castrated male rats (with or without exogenous testosterone) after 10 days of oral treatment with the test compound.

A second T1S *in vivo* assay which can be used as an alternative to the Hershberger assay is the "pubertal male rat assay". Intact weanling males are exposed to the test substance for about 30days, the age at puberty is determined and reproductive tissues are evaluated and serum taken for hormonal analyses.

1.7.2.3. Estrogen and Androgen Disruption.

A second non-mammalian vertebrate T1S assay is a short-term reproduction test with the fathead minnow (*Pimephales promelas*) that can be used to detect chemicals that would adversely affect processes controlled by the estrogens and androgens. This test, also known as the "fish gonadal recrudescence assay", is initiated with sexually mature animals. Reproductive performance of the fish is monitored for two to three weeks prior to chemical exposure in order to provide baseline data for pre- versus post-exposure statistical analyses. The duration of chemical exposure is about 21days, during which survival, behaviour, appearance (e.g. secondary sex characteristics), fecundity and fertility are monitored. At conclusion of the test, the adults are sampled to determine gonadal condition (relative gonad weight, histopathology) and plasma concentrations of sex steroids (17 β-estradiol, testosterone, 11-ketotestosterone) and vitellogenin (Gray Jr *et al.*, 2002).

1.7.2.4. Thyroid Disruption.

a) ***In Vitro* Assays.**

There exist, as yet, no recognised *in vitro* assays for the T1S screening of chemicals for potential thyroid-disrupting properties.

b) ***In Vivo* Assays.**

There exist a number of T1S *in vivo* assays for the detection of thyroid-disrupting chemicals. The pubertal male and pubertal female rat assays have been described above. In addition to these two assays, EDSTAC recommended a further two assays with non-mammalian vertebrates for T1S screening of EEDs, one of which is specific to thyroid disruption; a metamorphosis assay with the amphibian *Xenopus Laevis* was designed to detect EEDs that could affect the vertebrate thyroid system. *X.Laevis* is a particularly sensitive environmental species, and it was suggested that the assay be initiated with stage 60 *X.laevis*, and rate of tail resorption be monitored over the course of a 14 day chemical exposure; an agonist would enhance resorption, while an antagonist would inhibit the process (Gray Jr *et al.*, 2002).

1.8. Aims of this Project.

The ultimate aim of this project is the development of simple *in vitro* tests, which will act in accordance with the EDTA's recommendations and augment the OECD's proposed "toolbox" of assays, providing evidence of thyroid disruption by chemicals which are, or which might be released into the environment. High-throughput screening assays will be developed, which may utilise non-radioactive detection technologies, such as the chemiluminescent acridinium ester (AE).

1.8.1. Transcriptional Activation.

Thyroid hormones act predominantly at a pre-translational level, selectively affecting gene expression through thyroid receptors (TRs), whose mode of action is modulated by regulatory proteins. Although TR-DNA binding is a pre-requisite for the modulation of gene expression, it does not reflect the complexity of other cellular factors that influence T3-moderated gene transcription.

In order to address the problems of both the complexity of the modulation of gene expression and the effects of biological processing, two parallel approaches will be used. Cell-free (*in vitro*) systems, using a nuclear extract and purified recombinant TR will be developed. A cell culture approach will also be developed, in order to address two important factors, cell entry and metabolism.

Constructs containing known TREs upstream from a reporter sequence (luciferase) will be introduced into both cell-free and cell culture systems, together with standard control constructs (where required). Reporter gene expression levels (luciferase mRNA and luciferase protein for cell-free and cell culture systems, respectively) will initially be quantified using standard techniques. Following optimisation of assays, including extraction and purification procedures, quantification of the reporter gene mRNA synthesis and transcription of the internal control will be detected using the HPA technology.

The development of *in vitro* transcription assays will require pure, recombinant TR; murine TRs (TRα and TRβ) will be inserted into an expression vector and transformed into *E.coli*. Authentic recombinant receptors will be liberated from the purification fusion protein by proteolytic cleavage. Recombinant TR will subsequently be utilised in the *in vitro* transcription assays and will also allow the development of TR-DNA binding assays.

1.8.2. Binding Studies.

A pre-requisite of TR-mediated control of gene expression is the binding of TR to DNA. In order to assess the impact of any chemical moderator on this protein-DNA interaction it is essential to have a sensitive and quantitative assay technique. Therefore, assay technology will be developed to probe the interaction of TR with DNA.

The binding of recombinant TR to DNA will be monitored using the electrophoretic mobility shift assay (EMSA). Modulation of binding in the presence of T3 and known T3 analogues is a complexity that will be studied after rapid binding assay systems have been developed. Utilisation of the acridinium ester (AE) technology will allow the development of non-radioactive screens for compounds that disrupt the interactions of recombinant TR with AE-labelled oligos.

The development of these screens will allow more informed risk assessments to be made concerning the potential human health impact from the release of novel chemicals into the environment, or the risk to communities/workforces exposed to chemicals that are already in use.

CHAPTER TWO

MATERIALS AND METHODS.

2.1 Materials.

Routine Laboratory materials were purchased from Fisher Scientific Ltd (Loughborough, Leicestershire, UK) and Sigma Chemicals Ltd (Poole, Dorset, UK). The suppliers of more specific chemicals, reagents and equipment are listed in Table 2.1.

Reagent	Supplier
Hybond™ -C extra nitrocellulose membrane Hybond™ -N neutral nylon membrane Megaprime™ DNA Labelling System	Amersham Biosciences UK Ltd (Buckinghamshire, UK)
Splittix™ Foetal bovine serum Foetal bovine serum (stripped)	Autogen Bioclear (Calne, Wiltshire, UK)
BCIP/NBT premixed colour development reagent	BioRad Laboratories Ltd (Watford, Hertfordshire, UK)
Dynabeads® mRNA DIRECT™ Kit	Dynal Biotech ASA (Oslo, Norway)
Culture flasks/dishes and disposable plastics	Greiner (Stonehouse, UK)
Chicken TRα	Insight Biotechnology Ltd (Wembley, Middlesex, UK)
BenchMark™ prestained protein ladder pcDNA3.1(-) vector TOPO® XL PCR Cloning Kit	Invitrogen (Paisley, UK)
0.22μm Nucleopore™ filters	Millipore UK Ltd (Watford, Hertfordshire, UK)
Autodetect 1 Autodetect 2 Hybridisation buffer Probe storage buffer Selection reagent	MLT Research Ltd (Cardiff, Wales, UK)
Oligonucleotide primers	MWG Biotech UK (Milton Keynes, UK)

Protogel Acrylamide Solution	National Diagnostics, Hessle, Yorkshire, UK
Restriction endonucleases	New England Biolabs (Beverly, MA, USA)
pET15b vector ProteoJuice™ Protein Transfection Reagent	Novagen (Oxfordshire, UK)
ABI Prism™ Dye Terminator Cycle Sequence Reaction Ready Kit	Perkin Elmer Applied Biosystems (Warrington, UK)
Sephadex-G50 Nick columns	Pharmacia Ltd (Tadworth, Surrey, UK)
DNA ligase DNA molecular weight markers(λ and ϕ) dNTPs G418 (neomycin) – sulphate HeLaScribe Nuclear Extract *In Vitro* Transcription System, Luciferase Assay Reagent MMLV reverse transcriptase pGEM® -T vector Reporter Lysis Buffer (RLB) Taq DNA polymerase TransFast™ Transfection Reagent Wizard SV Plus™ Mini and Maxi prep DNA Purification Systems, Wizard Genomic DNA Purification Kit	Promega Ltd (Southampton, UK)
QIAquick™ Gel Extraction Kit Ni-NTA AP Conjugate NiNTA agarose	QIAGEN (Crawley, UK)
Expand™ High Fidelity PCR System	Roche Diagnostics Ltd (Sussex, UK)
75 x 12mm Polystyrene Assay Tubes	Sarstedt (Leicester, UK)
10x TAE Ficoll (Type 400) poly(dI-dC) Salmon testes DNA Triiodothyronine (T3) TRI reagent™	Sigma Chemicals Ltd (Poole, Dorset, UK)
pRG224 Luciferase reporter vector	A gift from Dr. R.C. Caswell, Cardiff University, Cardiff, Wales

Table 2.1. Materials and Reagent supply.

All standard buffers and reagents required for molecular biological procedures were prepared according to the protocols of Sambrook *et al.*, (1989). Sterilisation was achieved by autoclaving at 121°C and 15p.s.i. for 20min. A list of buffers and solutions along with their compositions is shown in Table 2.2.

Solution	Components
Preparation of Plasmid DNA Section 2.2.1.3 a) Small-scale b) Large-scale	
Cell Resuspension Solution	50 mM Tris (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A
Cell Lysis Solution	0.2 M NaOH, 1% SDS
Neutralisation Solution	4.09 M Guanidine Hydrochloride, 0.759 M Potassium Acetate, 2.12 M Glacial Acetic Acid, (adjusted to pH 4.2)
Column Wash	60 mM Potassium Acetate, 10 mM Tris-HCl (pH 7.5), 60 % Ethanol
Reverse Transcriptase (Section 2.2.1.11.)	
5x MMLV-RT buffer	50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT
Isolation of Genomic DNA (Section 2.2.1.16.)	
Nuclei Lysis Solution	
Protein Precipitation Solution	
Southern Blotting (Section 2.2.1.17.)	
Denaturing solution	0.5M NaOH, 1.5M NaCl
Transfer solution	0.25M NaOH, 1.5M NaCl
Hybridisation buffer	50% (v/v) formamide, 5x SSPE, 5x Denhardt's solution (see below), 0.1% (w/v) SDS and 200µg/ml salmon testes DNA
20x SSC	3M NaCl, 0.3M sodium citrate, pH 7.0
20x SSPE	3M NaCl, 200mM $NaH_2PO_4.H_2O$, 20mM EDTA,

	pH 7.7
50x Denhardt's reagent	1%(w/v) BSA, 1% (w/v) ficoll type 400 and 1% (w/v) polyvinylpyrrolidone
Wash Solution I (low stringency)	2x SSC, 0.1% (w/v) SDS
Wash Solution II (medium stringency)	0.5x SSC, 0.1% (w/v) SDS
Wash Solution III (high stringency)	0.1x SSC, 0.1% (w/v) SDS
***In vitro* transcription reactions** (Section 2.2.1.18.)	
HeLa Nuclear Extract 1x Transcription Buffer	20mM HEPES (pH 7.9 at 25°C), 100mM KCl, 0.2mM EDTA, 0.5mM DTT, 20% Glycerol
HeLa Extract Stop Solution	0.3M Tris-HCl (pH 7.4 at 25°C), 0.3M sodium acetate, 0.5% SDS, 2mM EDTA, 3μg/ml tRNA
Formamide Loading Dye	98% formamide, 10mM EDTA, 0.1% bromophenol blue
Quantitative PCR (Section 6.3.1.2)	
10x QPCR buffer	200 mM Tris-HCl (pH 8.3), 500 mM KCl, 40 mM $MgCl_2$, 10 μM ROX
Transient transfection using calcium-phosphate precipitation (Section 2.2.2.3.a.)	
2x HBS	250mM NaCl, 50mM HEPES, 1.5mM Na_2PO_4, pH 7.15.
0.1x TE	1mM Tris, 0.1mM EDTA
Acridinium Ester Assays (Section 3.)	
Hybridisation buffer (2x)	250mM lithium hydroxide, 190mM succinic acid, pH 5.2, 3mM EDTA, 3mM EGTA, 17% (w/v) lithium lauryl sulphate
Selection reagent	190mM sodium tetraborate, 6.4% Triton X-102, pH 7.5
Autodetect 1	0.032M hydrogen peroxide, 0.001M nitric acid
Autodetect 2	1.575M sodium hydroxide
Wash Buffer	10mM Tris-HCl, pH 7.5, 0.15M LiCl, 1mM EDTA, 0.1% 0.1% (w/v) lithium lauryl sulphate
Protein SDS-PAGE (Section 2.2.3.3.)	
	4% (w/v) SDS, 125mM Tris-HCl (pH 6.8), 20% (v/v) Glycerol, 0.02% (w/v) Bromophenol Blue,

2x SDS-PAGE sample loading buffer	2% (v/v) β-mercaptoethanol.
10x SDS-PAGE running buffer	1.92M Glycine, 250mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS
Coomassie blue solution	0.025% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid
Destain solution	10% (v/v) methanol, 10% (v/v) acetic acid
Western Blotting (Section 2.2.3.4.)	
Anode I solution	300mM Tris, 20% (v/v) methanol
Anode II solution	25mM Tris, 20% (v/v) methanol
Cathode solution	25mM Tris, 20% (v/v) methanol, 40mM caproic acid
Detection of recombinant proteins using the His conjugate (Section 2.2.3.5.)	
TBS buffer	10mM Tris-HCl pH 7.5, 150mM NaCl
TBS-Tween buffer	20mM Tris-HCl pH 7.5, 500mM NaCl, 0.05% Tween 20
Purification of recombinant proteins using Ni-NTA agarose (Section 2.2.3.6.)	
Lysis buffer	50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0
Wash buffer	50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0
Elution buffer	50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 8.0
Storage buffer	50% glycerol in PBS, 5mM DTT
EMSA (Section 2.2.3.7.)	
Binding buffer (10x)	340mM KCl, 50mM $MgCl_2$, 1mM DTT
Dilution buffer	40mM KCl, 0.1mM EDTA
Miscellaneous	
1x TE buffer	10mM tris, 1mM EDTA
PBS	137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4

Table 2.2. Buffer, Solution and Reagent composition.

2.1.1. Water.

Where the grade of water is not specified, sterile ddH2O was used. For all applications involving DNA manipulations, sterile HPLC-grade water was used.

2.1.2. Antibiotics.

Stock solutions of antibiotics were dissolved in the appropriate solvent (sterile ddH_2O for ampicillin and kanamycin and 100mM HEPES pH 7.3 for G418) to the required concentration, passed through a 0.22μm Nucleopore™ filter and stored at -20°C in 250μl volumes (thus avoiding multiple freeze-thaw cycles). The stock concentrations of antibiotics prepared were 100 mg/ml ampicillin, 30 mg/ml kanamycin and 50mg/ml G418.

2.1.3. Sterilisation Conditions.

Where necessary, all equipment and reagents were sterilised prior to employing any technique involving nucleic acid manipulations. Sterilisation was achieved by autoclaving at 121°C and 15psi for 20 min. Any heat-sensitive components were sterilised by filtration through a 0.22μm Nucleopore™ filter.

2.1.4. Media.

LB nutrient broth and LB agar were prepared as described by the Manufacturer (Sigma), that is, by the addition of the appropriate number of capsules to water. Antibiotics, where required, were added at an appropriate concentration – usually a 1:1000 dilution of the stock solution.

2.1.5. Oligonucleotide Primers.

In order to minimise mispriming, primers were designed using the OLIGO© [1991] Primer Analysis Software (Version 4.0, Wojcieck Rychiick, National Biosciences, Plymouth, MN, USA). Primers selected for optimal Tm at the given length were assessed for self-complementarity as well as complementarity to the second primer, in order to reduce the risk of the formation of primer-dimers.

Lyophilised oligonucleotides were resuspended in water (100pmol/μl) and stored at -20°C. For specific oligonucleotide sequences, see Appendix 1.

2.1.6. DNA vectors and *E.coli* Host Strains.

DNA fragments generated by PCR and restriction endonuclease digestion were ligated into pGEM® -T. Recombinant protein expression was performed in pET15b. A list of the *E.coli* host cell strains used for manipulations of recombinant DNA is shown in Table 2.3.

Bacterial host strain	Selectable marker	Supplier
E.coli DH5α	N/A	Invitrogen
E.coli BL21 (DE3)	N/A	Novagen
E.coli BL21 (DE3) Rosetta pLysS	Chloramphenicol	Novagen

Table 2.3. List of all *E.coli* host strains employed for manipulations of recombinant DNA. The respective selectable markers and suppliers are also shown.

2.1.7. DNA Markers.

The DNA markers used were λ/*Hin*dIII and ϕ174/*Hae*III. The fragment sizes of these markers are given in Table 2.4.

DNA Marker	Fragment Sizes (bp)
λ/*Hin*dIII	23130, 9416, 6557, 4361, 2322, 2027, 564, 125
ϕ174/*Hae*III	1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72

Table 2.4. DNA marker fragment sizes.

2.1.8. Standard Cell Culture Materials.

Routine media and solutions for mammalian cell culture were obtained from Autogen Bioclear, Calne, Wiltshire, UK. The suppliers of more specific materials are listed in Table 2.1. The compositions of buffers and solutions used in cell culture techniques are detailed in Table 2.2.

2.1.9. Cell Lines.

Human Cell Lines were obtained from the European Collection of Cell Cultures (ECACC), Porton Down, Dorset, UK. Details of cell lines used are shown in Table 2.5.

Cell Line	Description	ECACC Number
U373 MG	Human glioblastoma astrocytoma	89081403
CV-1	African green monkey kidney fibroblast	87032605

Table 2.5. Cell line, with description and ECACC Number.

2.1.10. Protein Markers.

The protein markers used were BenchMark™ Prestained Protein Ladder. Although it was possible for specific fragment sizes to vary between different batches of marker, they were, on the whole, consistent. An example of the fragment sizes of a typical batch of markers is given in Table 2.6.

Protein Marker	Molecular weights (kDa)
BenchMark™ Prestained Protein Ladder	176.5, 113.7, 80.9, 63.8, 49.5, 37.4, 26.0, 19.6, 14.9, 8.4.

Table 2.6. Protein marker molecular weights (kDa).

2.2. Methods.

2.2.1. Molecular Biology Methods.

2.2.1.1. Agarose Gel Electrophoresis.

DNA samples were routinely resolved by agarose gel electrophoresis (0.5% - 2.0% w/v agarose gels, containing 500μg/l ethidium bromide). Samples were run at 120V for approximately 30min in a Pharmacia GNA-100 submarine tank, containing 1x TAE buffer. Nucleic acid bands were visualised under UV light. The size of each separated fragment was estimated relative to the migration of DNA markers of known size, as detailed in Section 2.1.7.

2.2.1.2. PCR Amplification.

PCR was performed using a reaction mixture containing target DNA, 5 units of Taq polymerase, forward and reverse primers each at 1μM, dNTPs each at 0.02mM, 25mM $MgCl_2$, and 1x PCR reaction buffer (containing 50mM Tris-HCl buffer, pH 8.0, 100mM NaCl, 0.1mM EDTA, 5mM DTT). Preparative mixtures (20μl) were contained within 0.2ml thin-walled PCR tubes. The thermocycling reactions were carried out in a Primus 25/96 Thermocycler (MWG Biotech).

2.2.1.3. Preparation of Plasmid DNA.

a) Small-scale Preparation of Plasmid DNA.

Plasmid DNA was purified from overnight cultures using the Wizard Plus™ SV Miniprep DNA purification system as described in the manufacturer's protocol (Promega).

b) Large-scale Preparation of Plasmid DNA.

Plasmid DNA was purified from overnight cultures using the Wizard Plus™ SV Maxiprep DNA purification system as described in the manufacturer's protocol (Promega).

2.2.1.4. Purification of DNA.

a) Phenol-chloroform Extraction and Ethanol Precipitation.

i) Phenol-chloroform Extraction.

DNA was purified by the addition of an equal volume of phenol (pH 8.0), followed by thorough vortexing and centrifugation at 13,000 x *g* for 3min. The aqueous layer was removed and placed in a fresh tube. An equal volume of phenol was again added and the sample was mixed, centrifuged and separated as before. An equal volume of chloroform was added and the sample mixed and centrifuged for a third time. The aqueous phase, containing purified DNA was removed to a clean tube.

ii) Ethanol Precipitation.

Purified DNA was precipitated overnight at -80°C by the addition of 2 volumes (v/v) of 100% ethanol and 0.1 volume (v/v) of 3M sodium acetate (pH 4.0). The DNA was recovered by centrifugation at 13,000 x *g* for 15min at 4°C. The supernatant was removed and the pellet air-dried at room temperature. The DNA was resuspended in an appropriate volume of water.

b) Extraction of DNA from Agarose Gel.

DNA was fractionated by agarose electrophoresis as described in Section 2.2.1.1. The desired band was excised and purified using the QIAquick™ Gel Extraction Kit, as described in the manufacturer's protocol (Qiagen).

2.2.1.5. Assessment of DNA Concentration and Purity.

Estimated DNA concentrations were obtained by spectroscopic analysis at 260nm, using a diode array spectrophotometer (Hewlett Packard model 825A , Meridien, CN, USA). Absorbance readings at 230nm and 280nm also allowed an estimation of DNA purity to be made.

2.2.1.6. Restriction Digest.

DNA samples were digested with restriction enzymes according to the manufacturer's recommendations. Typically, 5-10 U of enzyme was added per 1-2μg of DNA and reaction incubated at 37°C for 2h.

2.2.1.7. Dephosphorylation of Digested DNA.

Digested vector was dephosphorylated in order to prevent re-ligation. Where necessary, calf intestinal phosphatase (1U/μg DNA) was added to a restriction endonuclease reaction after 1h. The reaction was then incubated for a further hour (37°C). In order to terminate the dephosphorylation reaction, samples were resolved by agarose gel electrophoresis, and the DNA purified as detailed in Sections2.2.1.1. and 2.2.1.4.b. respectively.

2.2.1.8. Ligation of DNA into Plasmid Vectors.

Ligation reactions typically consisted of a vector:insert ratio of 1:3 (on the basis of molarity) and were carried out in a final volume of 10μl. DNA ligase was added to 1μl of 10x DNA ligase buffer (300mM Tris-HCl, pH 7.8, 100mM $MgCl_2$, 100mM DTT and 10mM ATP) and the reaction mixture was incubated at 16°C overnight.

2.2.1.9. Transformation of Competent *E.coli* Cells by Plasmid DNA.

Aliquots (50μl) of competent DH5α cells were thawed on ice, prior to the careful introduction of the ligated sample (3μl). Cells were incubated on ice for 30min, then heat shocked at 37°C for 20sec. Following a further 2min on ice, 0.95ml of 37°C LB-broth was added to the sample, prior to a 1h incubation at 37°C. The cells were then pelleted by spinning at 2000 x *g* for 8min, resuspended in 150μl LB-broth and plated out on LB-agar plates containing the ampicillin selection marker. The plates were allowed to stand for 2min to dry, before inversion and incubation at 37°C for 16h.

2.2.1.10. Preparation of total RNA.

Total RNA was prepared using the TRI reagent™ method. A whole mouse heart was homogenised in 1ml of TRI reagent™ using an homogeniser, and the mixture allowed to stand at RT for 5min. Chloroform (200μl) was added and the sample vortexed (5sec), before a further incubation of 15min at RT. The phases were separated by centrifugation (10,000 x *g* for 15min at 4°C) and the upper, aqueous phase removed to a clean microcentrifuge tube. Iso-propanol (500μl) was added, and the mixture was incubated at RT for 10min. Precipitated RNA was recovered by

centrifugation (10,000 x *g* for 15min at 4°C). The RNA pellet was washed in 70% (v/v) ethanol, vortexed and centrifuged (10,000 x *g* for 5min at 4°C). The RNA pellet was air-dried at RT and resuspended in 100µl H_2O. RNA samples were quantified and evaluated for purity by spectrophotometry (GeneQuant, Amersham Pharmacia Biotech, UK). RNA integrity was analysed by agarose gel electrophoresis (Section 2.2.1.1.). Samples were stored at -70°C until required.

2.2.1.11. Reverse Transcriptase Reaction.

A reverse transcriptase reaction containing the following components was made up in a sterile 1.5ml microcentrifuge tube: 50pmol oligo-dT primer, 50pmol random hexamers, 1mM dNTPs, 400 U MMLV reverse transcriptase, reverse transcriptase reaction buffer, 10mM DTT and 20U RNasin® made up to a final volume of 15µl with H_2O.

The RNA to be reverse transcribed was made up to up to 15µl with H_2O in a separate tube and denatured by heating at 65°C for 5min. The template (15µl) was then added to the reverse transcriptase mix, bringing the final volume to 30µl. A negative control mix was also produced, where all reaction components were combined in a sterile microcentrifuge tube, apart from the reverse transcriptase enzyme. Reaction mixes were briefly centrifuged (5sec pulse, 13,000 x *g*) and incubated at 37°C for 2h. The reactions were terminated by denaturing the enzyme (80°C for 10min), and the cDNA stored at –20°C until required.

2.2.1.12. PCR Screening of Bacterial Colonies.

PCR was used to amplify cDNA inserts from bacterial colonies harbouring plasmid DNA. LB medium (2ml) was inoculated with a single, random, recombinant bacterial colony. Following a 3h incubation (37°C with constant agitation) 1ml of bacterial culture was removed and pelleted by centrifugation (13,000 x *g* for 1min at RT). The supernatant was removed and the pellet resuspended in 100µl H_2O. Cells were lysed (95°C for 5min) and cell debris pelleted (13,000 x *g* for 1min at RT). An aliquot (1µl) of supernatant was used as template in the PCR mix. Amplification reactions were subsequently carried out as detailed in Section 2.2.1.2.

2.2.1.13. Sequencing of Plasmid DNA – ABI Prism™ Dye Terminator Cycle Sequencing.

Sequencing reactions were carried out according to the manufacturer's recommendations (ABI, 1995), with minor alterations. Terminator Ready Reaction mix was mixed with approximately 290ng of purified plasmid DNA (Section 2.2.1.3.) 1.6pmol of the appropriate primer, and made up to a final volume of 15µl with water. Thermal cycling was carried out in a Primus 25/96 Thermocycler (MWG Biotech), using the following cycling parameters:

Chain denaturation	95°C	10sec	25 cycles
Primer annealing	50°C	5sec	
Chain extension	60°C	4min	
Soak	4°C		

Isopropanol (90µl) 70% (v/v) was added, the samples were vortexed and the DNA was allowed to precipitate in the dark (20min at RT). Samples were centrifuged at RT for 30min at 13,000 x *g*. The isopropanol was removed, and the pellet rinsed with a further 150µl of 70% (v/v) isopropanol. Following vortexing, samples were again spun at RT for 5min at 13,000 x *g* and the supernatant removed. Pellets were allowed to air-dry in the dark and the tubes wrapped in foil before storage at -20°C, prior to sequencing.

The resulting amplified DNA fragments, now fluorescently labelled, were separated (using an acrylamide/urea gel matrix), detected and the sequence determined using an ABI ™ 377 DNA sequencer.

2.2.1.14. Sequence Analysis.

a) Bioedit Sequence Alignment.

The Bioedit software – sequence alignment editor version 4.8.10, was used for various DNA sequence analysis functions, including open reading frame analysis, translation and multiple alignments using the CLUSTAL W method (Thompson *et al.*, 1994).

b) DNAsis.

The computer software, DNAsis, was used for sequence analysis, including procedures as mentioned in Section 2.2.1.14.a. as well as the generation of consensus sequences.

2.2.1.15. Sequence Identification – Database Searches.

DNA sequences were compared to all database entries, searching for protein similarities (in all six reading frames), (BlastX searches), and nucleotide similarities (BlastN searches), using the Blast search analysis programme (Altschul *et al.*, 1997) which is part of the EBI online software suite (www.ncbi.nlm.nih.gov/).

2.2.1.16. Isolation of genomic DNA from Cultured Cells.

Genomic DNA was isolated from cultured cells using the Wizard Genomic DNA Purification Kit according to the manufacturer's instructions (Promega) with some minor modifications. CV1 cells from a confluent T175 were detached by scraping with a rubber policeman and pelleted by spinning at 16,000 x *g* for 1min. The supernatant was removed and the cells resuspended by pipetting in 500µl cold (4°C) PBS before a further 1min spin at 16,000 x *g*. The supernatant was decanted and the cell pellet was subjected to four cycles of freeze-thaw as follows; the pellet was frozen in liquid nitrogen and then thawed by heating to 95°C in a water bath.

Nuclei lysis solution (600µl) was added and the cells resuspended by pipetting. DNase-free RNase was added (12µg) and the sample was mixed by inversion before a 30min incubation at 37°C. Following a further 5min incubation to allow the sample to cool to RT, Protein Precipitation Solution was added (200µl) and the sample was vortexed vigorously for 20sec before a 4min spin at 16,000 x *g*. The DNA-containing supernatant was then carefully removed and added to 600µl isopropanol in a clean microcentrifuge tube. Gentle mixing by inversion caused the gradual appearance of white thread-like strands of DNA. The sample was then spun for 1min at 16,000 x *g* and the supernatant decanted. Pelleted DNA was washed by the addition of 600µl 70% (v/v) ethanol and gentle inversion of the tube. Following a 1min spin at 16,000 x *g*, the ethanol was decanted and the pellet allowed to air-dry at

RT for 20mins. TE buffer (100µl) was added to the pellet, which was subsequently rehydrated at 65°C for 1h. Rehydrated samples were stored at 4°C.

2.2.1.17. Southern Blotting

a) Preparation of [α32P]-dCTP Labelled Probes.

DNA fragments were radiolabelled by the random priming method using the Megaprime DNA Labelling System, according to the manufacturer's instructions (Amersham). In this way, 25-50ng of DNA was combined with random hexamers and boiled for 5min, prior to chilling on ice. Following the annealing of primers, labelling buffer (containing unlabelled dATP, dGTP and dTTP) was added along with Klenow polymerase (2µl) and 3µl of [α-^{32}P]-dCTP (Amersham). Following an incubation of 15min at 37°C the reaction was terminated by the addition of TE buffer (50µl).

b) Purification of Radiolabelled Probes.

A Sephadex-G50 Nick column (Pharmacia) was equilibrated with 9ml of TE buffer. The terminated labelling reaction (100µl) was carefully layered onto the column, followed by a further 300µl of TE buffer and the subsequent eluate (400µl) was collected as fraction 1. This step was repeated in order to obtain a second fraction, which contained the radiolabelled probe.

c) Downward Alkaline Transfer.

Genomic DNA (50µg) extracted from CV1 cells as described in Section 2.2.1.16, was digested with *Bam*HI and *Sma*I, as described in Section 2.2.1.6.. Following separation by electrophoresis on a 1% agarose gel, DNA fragments were denatured by soaking the gel in denaturing solution for 30min and then transferred to a neutrally-charged nylon membrane (Hybond™-N). The assembly used in this method is shown in Figure 2.7; a layer of tissue paper 3-4cm thick was overlaid with 5 sheets of Whatmann 3mm paper with the top piece having been soaked in transfer buffer. A piece of the nylon membrane cut to the same size as the gel was also soaked in transfer buffer and placed on top of the stack. The agarose gel containing the denatured DNA was then placed on top of the nylon membrane, with care taken to ensure that the gel and nylon were correctly lined up and that no air bubbles had been

trapped between the two. Three sheets of Whatmann paper (pre-soaked in transfer buffer) were then placed on top of the nylon, followed by two further sheets of Whatmann paper, cut larger than the previous pieces and also soaked in transfer buffer, which acted as a bridge with the tank containing transfer buffer. A plastic lid was placed on top of the stack in order to prevent evaporation, prior to a 5h incubation at RT to allow transfer of DNA to the nylon membrane.

Following transfer of DNA, the membrane was washed in 3x SSC in order to remove residual agarose and then wrapped in Saran wrap. Transferred DNA was irreversibly fixed to the membrane by U.V. cross-linking, using 12 x 10^4μJ in a U.V. Strata Linker 2400 bench top transilluminator (Stratagene).

d) Prehybridisation and Hybridisation of Nylon Membranes.

The membrane was placed between two sheets of nylon gauze, which were rolled up and placed in a hybridisation bottle. Hybridisation buffer (10ml) was added and the bottle placed in a rotary hybridisation oven at 42°C for 6h. Following pre-hybridisation, radiolabelled probe (previously denatured by boiling for 5min) was added at approximately 10pmol/ml and the membrane incubated O/N at 42°C. The hybridisation solution was decanted, and the membrane subjected to two 30min washes at 65°C in wash solution I (30ml). An increased washing stringency was then achieved by washing for 30min at 65°C in wash solution II. If the observed radioactivity was still above 50 cpm on a GMG Geiger Muller counter, washing stringency was increased still further by washing in wash solution III. Membranes were subsequently wrapped in Saran wrap and autoradiographed.

e) Autoradiography.

Hybridised and washed membranes were exposed to a Kodak phosphoimager screen in an autoradiography cassette (with two intensifying screens) at RT, initially O/N. Bands were visualised using a BioRad Phosphoimager and subsequent exposure times were determined depending on the intensity of the signals observed. The radioactivity associated with bands was quantified as described in Section 2.2.4.9.e.

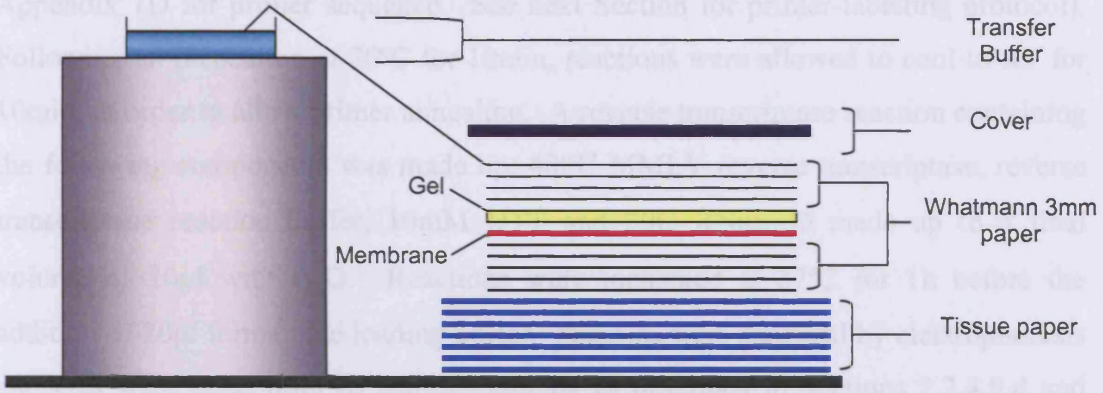


Figure 2.7. A schematic diagram showing the apparatus used in the downward alkaline transfer method (Southern Blotting). See text for details.

2.2.1.18. *In vitro* Transcription.

a) *In vitro* Transcription Reactions.

The HeLaScribe Nuclear Extract *in vitro* Transcription System (Promega) was used to allow the *in vitro* transcription of supercoiled DNA templates. HeLa nuclear extract was used as a source of transcriptional machinery, and reactions were performed essentially as per the manufacturer's protocol (Promega), except that no radionucleotide was incorporated into the transcript. Reactions consisted of: 4U HeLaScribe HeLa Nuclear Extract (3.6μl), HeLa Extract 1x Transcription Buffer (7.4μl), 8mM $MgCl_2$, recombinant TR and H_2O to a final volume of 25μl. Reaction mixes contained T3 (100nM) where indicated. Following a 30min incubation at 30°C to allow formation of the PIC, transcription was initiated by the addition of the rNTP mix (1μl, at 10mM each), before a further incubation at 30°C for 1h. Transcription was terminated by the addition of HeLa Stop buffer. RNA was phenol:choloroform extracted and ethanol precipitated as described in Section 2.2.1.4.a. Precipitated RNA was resuspended in 9.5μl nuclease-free H_2O.

b) Analysis of RNA by Primer Extension.

RNA samples were combined with dNTPs (1µl; 10mM) and the primer pGL2 - specific to the luciferase ATG-codon, 5'- labelled with [γ-^{32}P] (100fmol; see Appendix 1D for primer sequence. See next Section for primer-labelling protocol). Following an incubation at 70°C for 10min, reactions were allowed to cool to RT for 10min, in order to allow primer annealing. A reverse transcriptase reaction containing the following components was made up: 400U MMLV reverse transcriptase, reverse transcriptase reaction buffer, 10mM DTT and 20U RNasin® made up to a final volume of 20µl with H_2O. Reactions were incubated at 37°C for 1h before the addition of 20µl formamide loading buffer. Samples were analysed by electrophoresis and gels were dried prior to autoradiography as described in Sections 2.2.4.9.d and 2.2.4.9.e, with modifications; samples were run on 10% polyacrylamide gels containing 7M urea, alongside end-labelled ϕ174/*Hae*III DNA markers.

c) End-labelling of pGL2 Primer and ϕ174/*Hae*III Markers.

DNA (100fmol primer, 250ng ϕ174/*Hae*III Marker) was combined with T4-polynucleotide kinase (PNK; 10U) and 3µl of [γ-^{32}P]-dATP (Amersham) in 1x PNK Buffer. Following an incubation at 37°C for 10min the enzyme was deactivated by a 10min incubation at 90°C. H_2O (90µl) was added and radiolabelled primer was purified, as described in Section 2.2.1.17.b.

2.2.2. Cell Culture Methods.

2.2.2.1. Maintenance of Cell Lines in Culture.

Routine cell culture work was carried out under the laminar flow of a Class II hood (MDH, Andover, Hampshire, UK). Cells were maintained at 37°C in a hydrated atmosphere of 5% CO_2 and air in DMEM supplemented with 10% FBS, 2mM glutamine and 5000 units per ml (each) of penicillin and streptomycin. Cells which had been stably transfected with a plasmid containing the G418 selection marker (aminoglycoside phosphotransferase) were maintained as above, except that G418 was added to the growing medium to a final concentration of 800μg/ml.

2.2.2.2. Sub-culturing of Cells.

Confluent cells were washed twice with PBS, then sufficient Splittix was added to cover the cell layer (6ml for a 175cm^2 flask). The cells were then incubated at 37°C for 10-15min, during which time the cells became detached. Pre-warmed (37°C) medium was then added to the flask, to give an appropriate final volume. The cell suspension was then plated out into new tissue culture flasks, at a ratio of approximately 1:7. The medium was changed after 24h, and subsequently every 3-4 days.

2.2.2.3. Transient Transfection of Adherent Cells.

Plasmid DNA constructs, containing the firefly luciferase gene, under the control of the human cytomegalovirus (hCMV) major immediate-early promoter, were prepared according to Section 2.2.1.3.b. DNA was ethanol-precipitated as described in Section 2.2.1.4.a. to a final concentration of 1mg/ml. Assessment of purity and determination of concentration was performed as described in Section 2.2.1.5.

Each subsequent step of the Transfection procedure was carried out under the laminar flow of a Class II hood. Cells were seeded consistently at approximately 1 x 10^{6}cells/10cm^2 of surface area in a cell-culture vessel (usually a 6-well plate; surface area ~10cm^2, or a T75; 75cm^2 tissue culture flask). Cells were grown at 37°C for 16h. This incubation period allowed the attainment of between 70

– 80% confluency, which then permitted effective transfection of DNA into the adherent cells.

a) Calcium-phosphate Precipitation.

Stock solutions required for the transient transfection of cells using calcium phosphate precipitation (2x HBS, 0.1x TE and 2.5M $CaCl_2$, Table 2.2.) were prepared in advance and stored at 4°C. Cells were seeded and allowed to grow to 70-80% confluence as described above.

Four hours prior to transfection, the growth medium was removed and replaced with an equivalent volume of pre-warmed medium. All volumes are appropriate to a 6-well plate; multiply x8 for equivalent T75 volumes. Transfection solution A (DNA in 0.1x TE, total volume 225µl) and transfection solution B (2x HBS, 250µl) were prepared in sterile universal tubes. 2.5M $CaCl_2$, (25µl), was added to tube A and immediately mixed by pipetting with a plastic, sterile, Pasteur pipette. The resulting solution was then added drop-wise to tube B, with continuous agitation of the tube. A DNA-calcium phosphate precipitate was then allowed to form during a 30min RT incubation period. This precipitate was then added drop-wise to the cells, prior to a 16h incubation at 37°C. Following this incubation period, the cells were washed twice with PBS, fresh media introduced and culture vessels returned to 37°C for a further 24h incubation.

b) TransFast™ Reagent.

TransFast™ reagent was resuspended, as recommended in the manufacturer's protocol. (Promega). Cells were seeded and allowed to grow to 70-80% confluence as described in Section 2.2.2.3. The required amount of DNA was added to 1/3 the final flask volume of media, in a sterile tube (1ml or 5ml, for 6-well plate and T75 respectively), and vortexed for 10-15sec. TransFast™ (3µl/µg DNA) was added to the DNA/medium mix, and once again, the solution was vortexed thoroughly. Each DNA/medium/TransFast™ mixture was incubated at room temperature for 15min, to allow the formation of the plasmid DNA-TransFast™ complex. The growth medium was then removed from a culture vessel, and the transfection mixture (1ml or 5ml) was layered carefully over the monolayer of adherent cells and incubated at 37°C for 1h. The DNA/medium/TransFast™ mixture

was then removed from the culture vessel, and the cells were overlaid with fresh medium, prior to a further 24h incubation at 37°C.

2.2.2.4. Isolation of Cell Clones.

Cloning rings were made by cutting a 1cm length from the large end of a 1ml pipette tip. Cloning rings and Vaseline were sterilised by autoclaving. Following the removal of growing medium from the cells, individual cell clones were located by holding the plate up to the light, and the location of the clones marked on the underside of the plate using a permanent marker. Once the growing medium had been removed, all work was carried out rapidly so as to minimise the drying out of cells. Using a sterile forceps, the cloning rings were lightly dipped in Vaseline before being placed on the surface of the plate, so as to surround a single clone. Splittix (2-3 drops, from a sterile Pasteur pipette) was added, and following an incubation of 30s, Splittix and detached cells from a single clonal population were transferred to one well of a 24-well plate which already contained pre-warmed (37°C) medium (1ml, non-selective i.e. containing no G418). Following a 48h incubation at 37°C, clonal populations of cells were sub-cultured in growth medium containing G418 (800μg/ml) until sufficient quantities of cells were available for freezing of stocks.

2.2.2.5. Harvesting of Adherent Cells.

Following transfection, cells were incubated at 37°C for 24 hours. A protocol was developed which allowed preparation of a cell lysate and isolation of mRNA from the same sample of transfected, adherent cells.

a) Preparation of Cell Lysate.

Cells were harvested from the growth surface, into 1/3 the original volume of medium in which they had grown, using a rubber policeman. Detached cells were resuspended, by pipetting, and centrifuged at 2,000 x *g* for 5min, at 4°C. The supernatant was removed and the pelleted cells resuspended by vortexing in 1ml PBS at 4°C prior to a further spin at 4,000 x *g* for 5min. The supernatant was again discarded, and the cells resuspended by pipetting, in 250μl of 1x RLB (Reporter Lysis Buffer). Following a 10min incubation at RT, and a single round of freeze-thaw (30sec on ice), the cells were vortexed thoroughly for 15sec, and spun at 13,000 x *g*

for 2min. The supernatant (crude cell lysate) was removed to a fresh tube, and snap-frozen (liquid nitrogen). Each resulting lysate was assayed for firefly luciferase activity and protein concentration. The remaining pellets were also snap-frozen, subsequent to mRNA extraction.

b) Extraction of mRNA.

mRNA was isolated from cell pellets using the Dynabeads mRNA DIRECT™ Kit, as directed by the manufacturer's protocol (Dynal Biotech). mRNA was eluted from the beads in an appropriate volume of water by heating at 75°C for 2min. The tube was placed on the Magnetic Particle Concentrator (MPC) and allowed to stand for 2min. The supernatant, containing the mRNA, was removed to a fresh tube and stored at -80°C.

2.2.2.6. Measurement of Firefly Luciferase Activity in Cell Lysates.

Cell lysates prepared, as described in Section 2.2.2.5.a. were allowed to thaw to room temperature. Extracts were vortexed for 10-15sec and centrifuged for 30sec at 13,000 x *g*. A volume (20µl, unless otherwise stated) of cell extract was added to 100µl of Luciferase Assay Reagent in a reaction tube, and placed in a luminometer. Following an initial 2 second delay to allow sample stabilisation, the emitted luminescence was monitored over a period of 20sec. The relative light units (RLUs) generated by each sample were recorded directly, thus permitting further manipulation. If the RLU counts generated were above the upper limit of sensitivity of the luminometer (9999 RLUs), a smaller volume of cell extract was assayed and this dilution factor was taken into account prior to further manipulations of results.

2.2.2.7. Measurement of Total Protein of Cell Lysates.

The total protein concentration of cell lysates was determined by the method of Bradford (1976), using a known concentration range of BSA as a standard.

2.2.3. Protein Methods.

2.2.3.1. Analytical Scale Expression of Recombinant Proteins.

LB medium (10ml) containing the appropriate antibiotic selection was inoculated with an *E.coli* colony harbouring the required pET plasmid. The culture was grown at 37°C with constant agitation (225 rpm), overnight. A volume of this culture (200µl) was used to inoculate 10ml of fresh LB medium (1:50 dilution). The newly inoculated culture was incubated at 37°C until an OD_{600} of between 0.4 and 0.6 was reached. Expression of recombinant protein was induced by the addition of IPTG. Final IPTG concentrations are detailed in the relevant sections of text. Induced cultures were subsequently grown at 20°C, 30°C or 37°C, with agitation.

Samples (1ml) were removed at appropriate time points following induction, and pelleted by centrifugation (13,000 x *g* for 1min at RT). Cell pellets were stored at -20°C, until required.

2.2.3.2. Production of Soluble and Insoluble Protein Fractions.

The frozen cell pellets were thawed and resuspended in 50µl of PBS. The cell suspensions were subjected to 3 cycles of freeze-thaw (alternating between an ice/water bath a 37°C water bath) and the lysates centrifuged (13,000 x *g* for 1min at RT). The pellet (insoluble fraction) was resuspended in 50µl of PBS and 25µl 2x SDS-PAGE sample loading buffer. To the supernatant (soluble fraction) 25µl of 2x SDS-PAGE sample loading buffer was added. Fractions were analysed by SDS-PAGE. All samples were stored at -20°C.

2.2.3.3. SDS-PAGE.

Recombinant protein samples were resolved by SDS-PAGE. The running gel contained 12.5% (v/v) Protogel acrylamide solution (Protogel is a 30% (w/v) acrylamide/methylene bisacrylamide solution, in a 37.5:1 ratio), 375mM Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.225% (w/v) APS, 13.2mM TEMED and H_2O in a final volume of 10ml. The stacking gel contained 3.5% (v/v) Protogel acrylamide solution, 190mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.45% (w/v) APS, 13.2mM TEMED and H_2O in a final volume of 5ml.

Prior to loading, samples (with 2x loading buffer already added) were heated at 100°C for 5mins. Samples were spun briefly (13,000 x *g* for 15sec at RT) and 10µl of each sample was loaded onto the stacking gel. Gels were run at a constant 70mA current to allow sufficient separation of the protein samples in a Biorad-miniprotean II™ protein gel apparatus containing 1 x SDS-PAGE running buffer. BenchMark™ Prestained Protein Ladder (Invitrogen) was also loaded and run simultaneously on the gel, in order to allow estimation of molecular weight (kDa) of recombinant protein samples. Proteins were visualised by staining in Coomassie blue solution for 45min followed by immersion in de-stain solution for 1h. Gels were viewed on a white light source and a photographic record taken.

2.2.3.4. Western Blot Analysis.

Following separation by SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Hybond™-C extra nitrocellulose membrane) by electroblotting. Five pieces of Whatmann 3MM paper, cut to the same size as the protein gel, were soaked in the appropriate blotting buffers – two pieces each in anode I and in cathode solution, and one piece in anode II solution. In order to assemble the electroblotting stack, the anode I filter papers were placed on the bottom half of a Sartorius Semi-Dry Blotter (Sartorius Ltd, Epsom, Surrey, UK) followed by the anode II filter paper. The nitrocellulose was then put down on the anode filter papers, followed by the gel. Finally, the two cathode filter papers were placed on top of the gel, completing the stack. The blotter lid was placed on the stack and the proteins were transferred onto the membrane at a constant 150mA current for 45min.

2.2.3.5. Detection of Recombinant Proteins using the Ni-NTA AP Conjugate.

Following the transfer of proteins, the nitrocellulose membrane was washed three times for 10min each, in TBS buffer. Blocking was achieved by incubating the membrane in 3% (w/v) BSA in TBS buffer. The membrane was then washed a further three times for 10min each, in TBS-Tween buffer followed by an hour's incubation with a 1:1000 (v/v) dilution of the Ni-NTA AP conjugate, in TBS-Tween buffer. The Ni-NTA AP conjugate binds directly to His-tagged proteins and can be used for their detection without the need for antibodies. Following the incubation period, the membrane was washed three times (10min each) in TBS-Tween

buffer, prior to colour development in premixed colour development buffer containing BCIP and NBT solutions. The developed membrane was rinsed three times in distilled water to stop the colour reaction, and allowed to air-dry. A photographic record was taken before storage, wrapped in aluminium foil.

2.2.3.6. Purification of Recombinant Proteins using Ni-NTA Agarose.

To allow purification using Ni-NTA agarose, the target recombinant protein must contain a 6 His-Tag (six consecutive histidine residues). Large-scale expression of recombinant protein was performed in 1l of LB medium. Cultures were induced as described previously (Section 2.2.4.1.), and incubated under optimal conditions, as detailed in the relevant sections of text. Cells were harvested by centrifugation (10,000 x *g* for 10min at 4°C) and stored overnight at -20°C. Cells from 1l of culture were resuspended in 20ml lysis buffer and then subjected to four cycles of French pressing.

Following cell lysis by French pressing, cell debris was pelleted by centrifugation (10,000 x *g* for 20min at 4°C). The cell lysate, containing the soluble His-tagged recombinant protein was removed. Ni-NTA 50% slurry (5ml) was added to the cell lysate (20ml) and the resulting solution was mixed for 1h at 4°C, with constant agitation.

The slurry-lysate mix was poured onto a column with the bottom outlet capped. The bottom cap was removed and the flow-through collected. The column was washed with wash buffer, until the OD_{280} of the flow-through dropped to zero. Recombinant protein was eluted in four fractions of elution buffer, of 1ml each. All samples were analysed by SDS-PAGE and stored at -20°C until required further.

2.2.3.7. Protein Quantification.

a) Bradford Assay.

Proteins were quantified, where specified, using a Bradford assay reagent. BSA standards from 2mg/ml to 0.06125mg/ml, were made by the serial dilution of a stock solution (10mg/ml) with protein buffer (i.e. the buffer in which the protein was dissolved). Protein samples or BSA standards (20 µl) were then added to Bradford reagent (180µl) in a well of a 96 well plate. Absorbance at 590nm was

measured, and unknown protein concentrations estimated from the standard curve produced.

b) The Bicinchoninic Acid (BCA) Protein Assay.

The BCA assay was used as an alternative method for protein quantification as it is less sensitive to detergents than the Bradford assay.

BSA standards from 1000µg/ml to 200µg/ml, were made by the serial dilution of a stock solution (1mg/ml) with protein buffer (i.e. the buffer in which the protein was dissolved). Protein sample or standard (25µl) was added to BCA assay working solution (200µl, Sigma) in a well of a 96 well plate (25 µl). The plate was covered and incubated at 37°C for 30min. Absorbance at 562nm was measured, and unknown protein concentrations estimated from the standard curve produced.

2.2.3.8. Protein Buffer Exchange – Dialysis.

Pre-treated cellulose dialysis tubing (Fisher, UK) was prepared by boiling in distilled water for 10 minutes, until the tubing was softened. The protein-containing solution was placed in the tubing, and sealed using dialysis clips. The sealed tubing was then placed in a beaker containing a 200-fold volume of dialysis buffer, prior to overnight dialysis - carried out at 4°C on a magnetic stirrer.

2.2.3.9. Electrophoretic Mobility Shift Assay (EMSA).

a) Generation of Double-Stranded Oligonucleotides.

EMSA analysis was carried out using a double-stranded DNA probe, which was made by annealing complementary oligonucleotide pairs; forward and reverse oligos (400pmol of each) were incubated together at 100°C for 10min in a medium salt buffer (Promega Buffer E to a final volume of 200µl) prior to chilling on ice. Each oligo was designed in such a way so that upon annealing, 5'- overhangs were created, which contained G residues (For oligo sequences see Appendix 1E.). [α-^{32}P]-dCTPs were then base-paired with the overhanging G residues as part of the "filling in" reaction, described in the next Section.

b) Radiolabelling of Double-Stranded Oligonucleotides.

Double stranded oligonucleotide was end-labelled with [α-^{32}P]-dCTP using the Megaprime DNA Labelling System, according to the manufacturer's instructions (Amersham). In this way, double stranded oligonucleotide (40pmol in a final volume of 35µl) was combined with labelling buffer (containing unlabelled dATP, dGTP and dTTP), Klenow polymerase (2µl) and finally, 3µl [α-^{32}P]-dCTP. Reaction mixtures were incubated at 37°C for 30min and the reaction terminated by the addition of 50µl TE. Labelled probe was separated from unincorporated nucleotide as described in Section 2.2.1.17.b. The radioactivity of the probe was assessed by spotting 2µl on a 1cm^2 piece of blotting paper, placing the paper in a scintillation vial and measuring the activity on a Wallace 1209 Rackbeta Liquid Scintillation Counter. If the radioactivity of the probe was greater than or equal to approximately 50,000 c.p.m./µl, the probe was considered suitable for use in EMSA analysis.

c) Binding-Reaction Conditions.

Various combinations of the indicated amounts of specific reaction components (recombinant protein, T3, antibody, competing oligonucleotides) were combined with dilution buffer to a final volume of 30µl. Binding buffer (10x, 4µl) and poly[dI-dC] (1µg/ml, 3µl) were added, followed by radiolabelled oligonucleotide (3µl), bringing the final volume of all binding reactions to 40µl. Samples were incubated at RT for 20min, then 5µl of 20% (w/v) Ficoll was added prior to electrophoresis (Section 2.2.4.7.d) in order to facilitate gel-loading.

d) Electrophoresis of DNA-Protein Complexes.

DNA-protein complexes were resolved by electrophoresis using 5% (v/v) polyacrylamide gels (29:1 acrylamide:bisacrylamide) containing 0.5x TBE. Electrophoresis was carried out for 3-4h at 150V at 4°C using a vertical gel apparatus (Scotlab) with 0.5x TBE running buffer. In order to monitor the progress of electrophoresis, one lane of the gel was loaded with 10µl of 10x DNA-loading dye. Following electrophoresis, the gel was transferred to a piece of Whatmann 3MM paper, covered in clingfilm and dried under vacuum at 80°C for 1h. DNA-protein complexes were visualised by autoradiography, as per Section 2.2.1.17.e.

e) Quantification of Radioactivity Associated with Bands.

Autoradiograms of the dried gels were analysed densitometrically. Following visualisation of bands by autoradiography, as described in Section 2.2.1.17.e, the radioactivity associated with individual bands was estimated using the Quantity One® quantitation software. Following the subtraction of background noise, the area under the intensity profile curve for each band was measured.

2.2.3.10. Competition EMSA.

In order to determine the specificity of DNA-protein interactions, an excess quantity of unlabelled-specific and non-specific oligonucleotides were added to the binding reactions. The specific oligonucleotide was of the exact same sequence as the radiolabelled probe, whilst the non-specific contained an unrelated sequence. Electrophoresis of DNA-protein complexes was then carried out as described in Section 2.2.4.7.d.

CHAPTER THREE

DEVELOPMENT OF A CHEMILUMINESCENT ASSAY TO MEASURE LUCIFERASE MRNA.

3.1. Introduction.

The effects of thyroid hormones are mediated predominantly at the level of gene expression via the binding of the thyroid hormone receptors (TRs) to specific DNA sequences in the promoter region of target genes, known as thyroid response elements (TREs). In order to analyse the functional significance of such sequences, DNA from recognised TREs can be fused to a reporter gene. A commonly used reporter gene is the luciferase gene from the common firefly (*Photinus pyralis*). The reporter construct can be transfected into eukaryotic cells and the activity of the reporter gene can be estimated by measuring the luciferase protein activity levels. Alternatively, the construct could be used in cell-free *in vitro* systems which utilise an eukaryotic nuclear extract to produce mRNA, but which do not produce luciferase protein. Such systems would require a method of quantifying luciferase transcript levels.

The aim of this project, as outlined in Section 1.8, is "the development of simple *in vitro* tests, which will provide evidence of thyroid disruption by chemicals". In order to develop systems which monitor the effects of putative disruptors on the formation of a transcriptionally active complex, (Section 1.8.2), a prerequisite is the ability to quantify the end point of such diagnostic tests. Although it is possible to measure the levels of protein expressed from a reporter gene in cellular systems, it is also possible to measure the levels of luciferase mRNA transcribed, both from a cell culture or a cell-free *in vitro* system. The quantification of luciferase mRNA and luciferase protein levels from a cell culture system would allow a comparison to be made between the temporal profiles for luciferase mRNA and luciferase protein expression. This would allow a distinction to be made between chemicals which have an adverse effect on mRNA transcription or on the translation of protein. Many of the current techniques commonly used for quantification of mRNA have disadvantages

associated with them; transcript run-off assays rely on the use of a radioactive isotope and quantitative PCR relies on amplification and is both labour- and time-intensive.

The Hybridisation Protection Assay (HPA) is a technique which employs chemiluminescent technology and can be used for the detection of virtually any target nucleic acid, provided the sequence is known (Nelson *et al.*, 1995). It does not employ an amplification step, and so is rapid and easy to perform. The HPA is quantitative and relatively sensitive. It follows a homogeneous format, which lends itself to a high-throughput format which would be advantageous in the development of systems that allow for the screening of large numbers of compounds. It is proposed to use the HPA to quantify luciferase mRNA from cellular and *in vitro* systems. The objective, therefore, was to design and optimise an HPA to allow the quantification of luciferase mRNA.

3.2. Approach.

3.2.1. The Acridinium Ester.

Chemiluminescence is the process by which certain chemical reactions produce energy in the form of photons rather than as heat. The reactions are generally oxidative and involve the formation of excited intermediate molecules, which revert to their ground state by photonic emission. The concept of using non-isotopic labels to provide alternatives to radioimmunoassay is not new. Legislative restrictions on radioisotope usage as well as the increasing problem of radioactive waste disposal have spurred the development of alternatives (Woodhead, 1995).

The acridinium ester (AE) depicted in Figure 3.1 was developed for use as a chemiluminescent label in bioassays by Weeks *et al.*, (1983). Advantages of using acridinium esters in a detection system include high sensitivity; ease of use, handling and disposal; precise control of detection (chemiluminescence can be measured against a background of virtually zero); and long shelf lives. The AE reacts rapidly with hydrogen peroxide under alkaline conditions (typically 1-5 seconds, see Figure 3.2), to produce light at 430nm. These rapid reaction kinetics permit detection over a very short time frame, thereby minimising background noise and improving overall sensitivity. Detection in a standard luminometer exhibits a linear response over an AE range of more than four orders of magnitude, with a detection limit of less than 1fmol/reaction (Nelson *et al.*, 1995). An oligonucleotide labelled with AE can be used in a variety of simple assay formats to directly target nucleic acids. One such of these assays is the Hybridisation Protection Assay (HPA), which is described in detail in the next section.

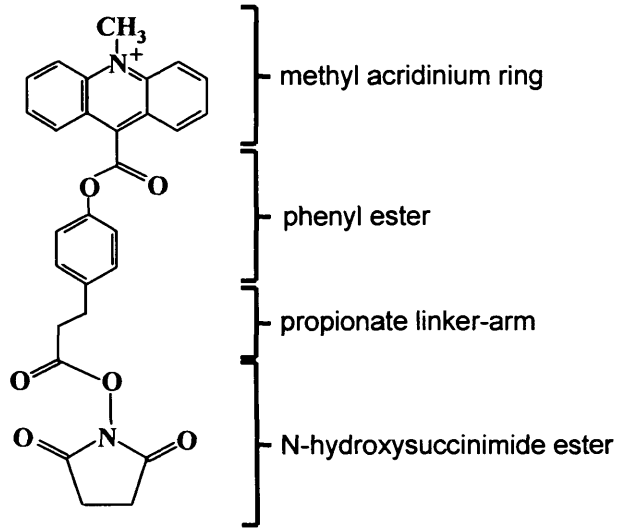


Figure 3.1. The structure of the *N*-hydroxysuccinimide ester of the *N*-methyl acridinium ester.

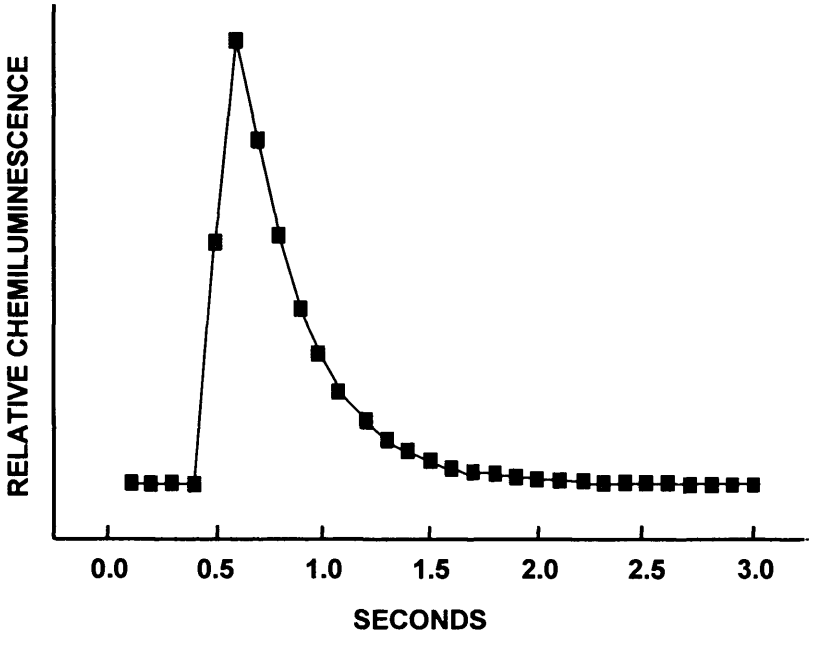


Figure 3.2. The acridinium ester reacts with hydroperoxy anions (HOO^-) to produce light. Shown is a typical kinetic profile of this light output, measured at 430nm (adapted from Nelson, *et al.*, 1996).

3.2.2. The Hybridisation Protection Assay (HPA).

An overview of the steps involved in the HPA is shown in Figure 3.3. The HPA is a three-step assay which utilizes an acridinium ester-labelled oligonucleotide (AE-probe) to quantify specific target nucleic acid sequences. It is based on the selective chemical degradation of the AE label such that chemiluminescence associated with unhybridised probe is rapidly lost, whereas chemiluminescence associated with hybridised probe is minimally affected. Underlying this selective degradation (or differential hydrolysis) process is a highly specific chemical hydrolysis reaction, controlled by the local environment of the acridinium ester.

In the first step of the HPA, the AE-probe is hybridised to the nucleic acid to be quantified. The second step involves the hydrolysis of unhybridised AE-probe and the assay is completed by the detection of chemiluminescence associated with hybridised AE-probe; the third step.

3.2.2.1. Hybridisation.

An AE-probe is hybridised with the nucleic acid to be quantified, during which time the AE-probe will form a double-stranded complex with any complementary target present. The AE-labelled probe is designed in such a way that the AE is positioned in an interior portion of the probe to protect it. Upon hybridisation of probe to the target nucleic acid, this interior placement stabilizes the AE to hydrolysis by hydroxide ions, by providing it with an intercalation site within the double-stranded duplex. Hybridisation of probe to the target sequence is facilitated by the presence of helper oligos. These are unlabelled oligonucleotides (30mers) which are complementary to the 5' and 3' sequences which immediately flank the region of mRNA to which the probe is designed. They facilitate access to the probe-binding site by opening out the adjacent RNA sequence, allowing probe binding which might otherwise be prevented by the presence of any secondary structure present (Hogan and Milliman, 1991).

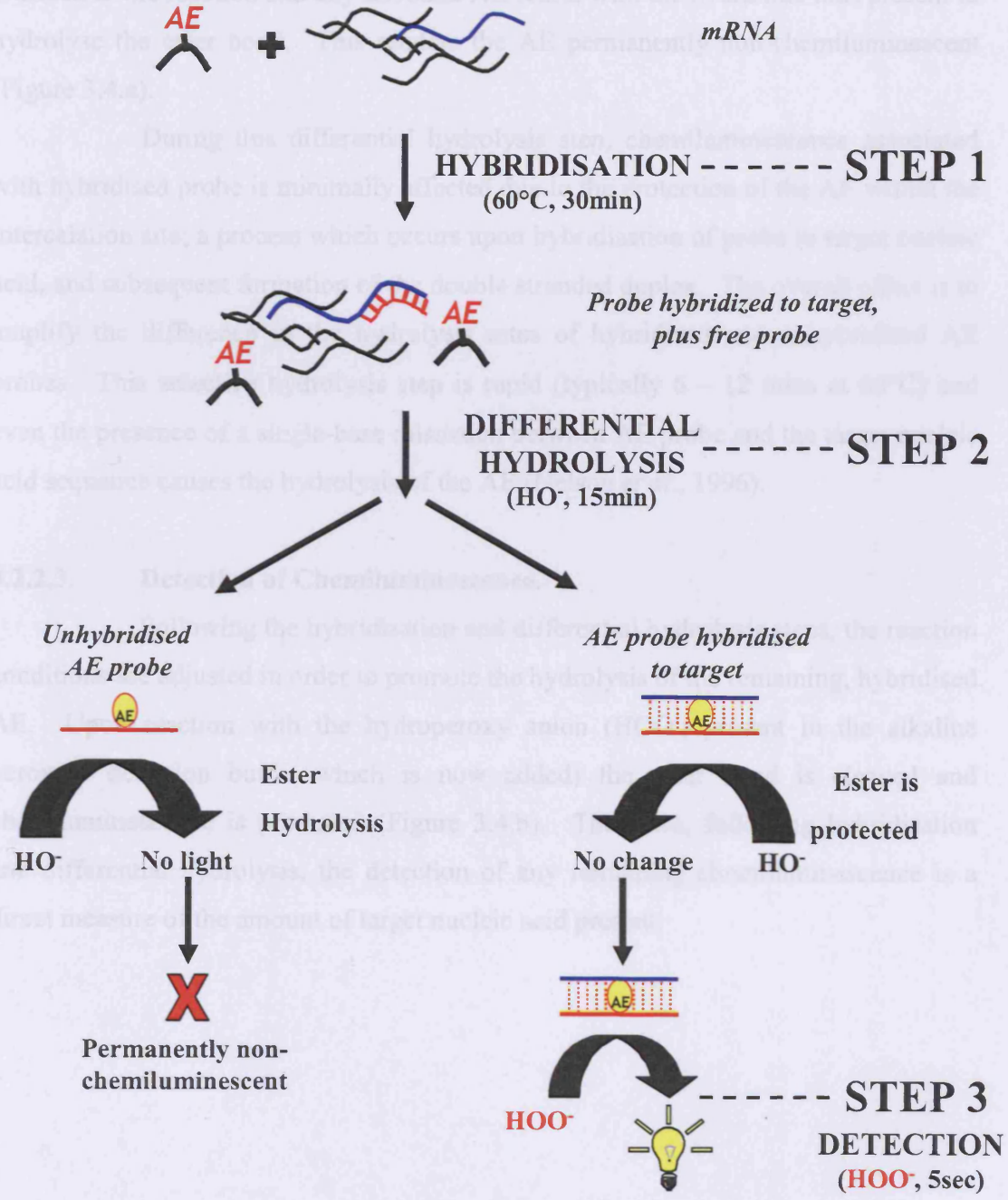


Figure 3.3. A schematic representation showing the principle steps of the HPA (Hybridisation Protection Assay). AE: Acridinium Ester.

3.2.2.2. Differential Hydrolysis.

Following the hybridisation step an alkaline differential hydrolysis buffer is added to the reaction and any unbound AE reacts with the hydroxide ions present to hydrolyse the ester bond. This renders the AE permanently non-chemiluminescent (Figure 3.4.a).

During this differential hydrolysis step, chemiluminescence associated with hybridised probe is minimally affected due to the protection of the AE within the intercalation site; a process which occurs upon hybridisation of probe to target nucleic acid, and subsequent formation of the double stranded duplex. The overall effect is to amplify the difference in the hydrolysis rates of hybridised and unhybridised AE probes. This selective hydrolysis step is rapid (typically 6 – 12 mins at 60°C) and even the presence of a single-base mismatch between AE probe and the target nucleic acid sequence causes the hydrolysis of the AE (Nelson *et al.*, 1996).

3.2.2.3. Detection of Chemiluminescence.

Following the hybridisation and differential hydrolysis steps, the reaction conditions are adjusted in order to promote the hydrolysis of the remaining, hybridised AE. Upon reaction with the hydroperoxy anion (HOO^-; present in the alkaline peroxide detection buffer which is now added) the ester bond is cleaved and chemiluminescence is produced (Figure 3.4.b). Therefore, following hybridisation and differential hydrolysis, the detection of any remaining chemiluminescence is a direct measure of the amount of target nucleic acid present.

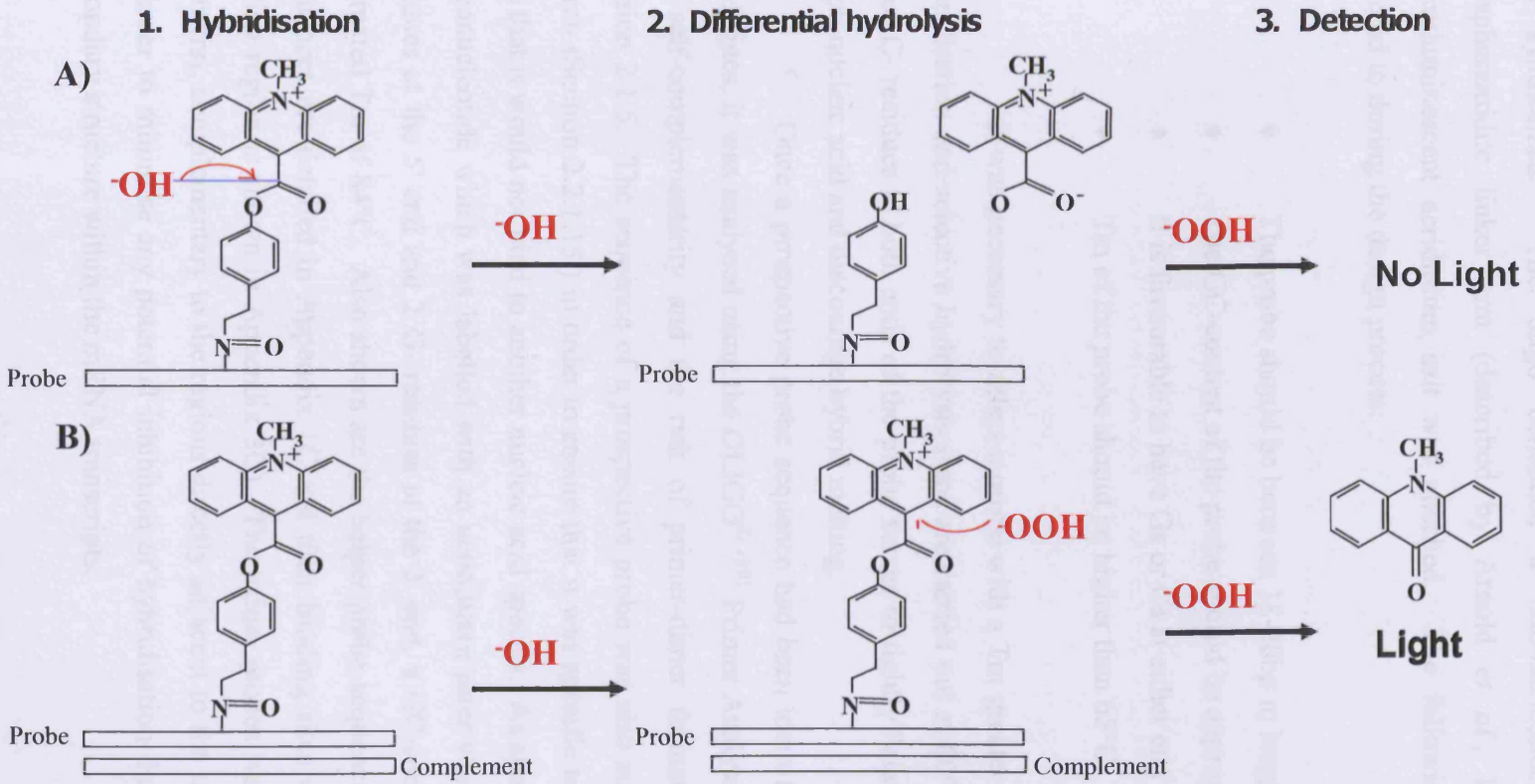


Figure 3.4. **Panel A)** Non-hybridised (single-stranded) AE-probe reacts with hydroxide to hydrolyse the ester bond, which yields the non-chemiluminescent acridinium carboxylic acid.

Panel B) Hybridised (double-stranded) AE-probe is protected from hydrolysis by hydroxide ions during the differential hydrolysis step. Hydrolysis by the hydroperoxy anion (HOO^-) leads to production of light.

3.2.3. Design and Synthesis of Chemiluminescent Oligonucleotide Probes.

A region of the firefly (*P.pyralis*) luciferase gene was selected and a 28bp oligomer complementary to the corresponding section of the mRNA transcript was synthesized. The oligo contained a non-nucleotide amine–terminated phospharamidite linker arm (described by Arnold *et al.*, 1989), to which a chemiluminescent acridinium salt was attached. The following guidelines were adhered to during the design process:

- The probe should be between 18-28bp in length.
- The GC-content of the probe should be approximately 50%.
- It is favourable to have Gs or Cs at either end of the probe.
- Tm of the probe should be higher than 65°C.

It was necessary to select a probe with a Tm greater than 65°C since the hybridisation and selective hydrolysis steps are carried out at 60°C. The presence of G- or C- residues at both ends of the probe serves to tightly "clamp" the probe to its target nucleic acid and discourage hybrid melting.

Once a prospective probe sequence had been identified using the above guidelines, it was analysed using the OLIGO© [1991] Primer Analysis Software to check for self-complementarity and the risk of primer-dimer formation, as detailed in Section 2.1.5. The sequence of a prospective probe was also subjected to a BlastN search (Section 2.2.1.15.) in order to ensure that it was specific to the luciferase gene and that it would not bind to another nucleic acid species. As shown in Table 3.5, the oligonucleotide which was labelled with an acridinium ester was a 28mer with 3 C-residues at the 5' end and 2 G- residues at the 3' end, a GC-content of 50% and an estimated Tm of 84°C. Also shown are the helper probe sequences (Probe and helper sequences are detailed in Appendix 1C and their binding sites within the luciferase coding region is shown in Appendix 3C). The helper probes were unlabelled oligos (30mers), complementary to the regions directly adjacent to the target sequence, used in order to minimise any potential inhibition of hybridisation due to the presence of secondary structure within the mRNA transcripts.

Oligo	Sequence (5'→3')
Luciferase AE-probe	CCC TTA GGT AAC CTA GT#A GAT CCA GAG G
5' - helper	AAT TCA TTA TCA GTG CAA TTG TTT TGT CAC
3' - helper	ACG CAG GCA GTT CTA TGC GGA AGG GCC ACA

Table 3.5. DNA sequences of the AE-probe and helper oligonucleotides which were used in the luciferase HPA (see text for probe design criteria). The position of the linker arm to which the AE was attached is indicated by #.

An AE-labelled oligonucleotide probe was prepared by GenProbe (San Diego, CA, USA), essentially as described by Nelson *et al* (1995). The AE reagent shown in Figure 3.1 can be covalently attached to primary amine-containing compounds via a specific reaction of the N-hydroxysuccinimide (NHS) ester of the AE with the primary amine of the compound. The oligonucleotide was therefore directly labelled with AE, by first incorporating a primary amine into the DNA, then reacting this amine with the NHS-AE. An oligonucleotide labelled in this way displays exactly the same chemiluminescence characteristics (reaction kinetics, specific activity and sensitivity of detection) as the free label. The detection properties of the label are not compromised by attachment to oligonucleotides (Nelson, *et al.*, 1995).

3.2.4. Preparation and Storage of Probes, Helper and Target Oligos.

Stock AE (Acridinium Ester) probes were stored in 10µl aliquots at 20°C. A working dilution (1:10, v/v; ~ 100fmol/µl) of probe was prepared and stored for one week. Subsequent probe dilutions, specific to each procedure, were made from the working dilution. AE probes, helper probes and target oligos (short oligonucleotides complementary to and of the exact same size as the AE-probe; see Appendix 1C) were diluted in HPLC–grade water and stored at –20°C. Prior to use, stock solutions of target and helper were heated at 60°C for 2min and vortexed thoroughly. All mRNA was stored at -80°C. Prior to use, mRNA was heated at 60°C for 5min, and vortexed for 20-30sec in order to destroy any intrinsic secondary structure. All AE-probes were stored as a 1:10 (5fmol/µl) dilution at -20°C. The 1:10 dilutions of AE-probe were heated at 60°C for 2min, vortexed and the tube pulsed in a bench top microfuge prior to use.

3.2.5. Detection of Chemiluminescence.

Reaction tubes (Sarstedt 75 x 12mm polystyrene assay tubes) which had been incubated in a water bath were blotted with a moist tissue in order to remove any droplets of water, and the chemiluminescence associated with each tube was measured in a single-tube portable luminometer (Stratec Electronic, Lumino, Birkenfeld, Germany), by automatic sequential injection of 200µl Autodetect 1 and 200µl Autodetect 2, followed by the measurement of the light emission over a period of 5sec.

3.3. Results.

3.3.1. Calculation of Differential Hydrolysis Ratio.

The HPA technique is based on selective chemical degradation of the AE label such that chemiluminescence associated with unhybridised probe is rapidly lost, whereas chemiluminescence associated with hybridised probe is minimally affected. In order to maximise the signal to noise ratio, it is necessary to select a differential hydrolysis time which allows the hydrolysis of as much unbound probe as possible, whilst minimally affecting the hydrolysis of bound probe.

Reaction components were combined in a 1.5ml microfuge tube, as detailed in Table 3.6. "Hybrid" reactions consisted of AE-probe which would hybridise with the complementary target oligo present, "Control" reactions contained AE-probe, but no target with which to hybridise and "Blank" reactions contained target but no AE-probe. All reactions were incubated at 60°C for 1h, and 1x hybridisation buffer (300µl) was added to each microfuge tube. Eleven sets of duplicate aliquots (10µl) from each reaction (Hybrid, Control and Blank) were pipetted into 75mm Sarstedt reaction tubes. In order to determine the initial levels of chemiluminescence present in each reaction, a "time-zero" measurement was taken; that is the chemiluminescence was measured when the samples had been subjected to (virtually) zero time under the selective hydrolysis conditions. Selection reagent (100µl) was added to a duplicate set of tubes for each preparation (Hybrid, Control and Blank), the contents mixed briefly by swirling, and the chemiluminescence associated with each tube was measured, as detailed in Section 3.2.5.

Selection reagent (100µl) was then added to all the other tubes, which were vortexed and returned to 60°C. At the desired time points, duplicate tubes were removed from the waterbath and placed in an ice/water slurry for 30sec, prior to detection of chemiluminescence. Results are shown in Figure 3.7.

	HYBRID	CONTROL	BLANK
2x hybridisation buffer	15µl	15µl	15µl
AE probe (0.01pmol/µl)	5µl	5µl	-
Target (0.1pmol/µl)	10µl	-	10µl
H_2O	-	10µl	5µl
Final Volume	30µl	30µl	30µl

Table 3.6. Assay components as used during the optimisation of differential hydrolysis conditions. A probe concentration of 0.01pmol/µl and a target concentration of 0.1pmol/µl was used. The above reactions were combined in 1.5ml microfuge tubes and incubated at 60°C for 1h, prior to the addition of 1x hybridisation buffer (300µl). Duplicate aliquots (10µl) from each reaction tube were then pipetted into 75mm Sarstedt reaction tubes and Selection reagent (100µl) was added. The Sarstedt tubes were vortexed and returned to 60°C. At the desired time points, duplicate tubes were removed from the waterbath and placed in an ice/water slurry for 30sec, prior to detection of chemiluminescence

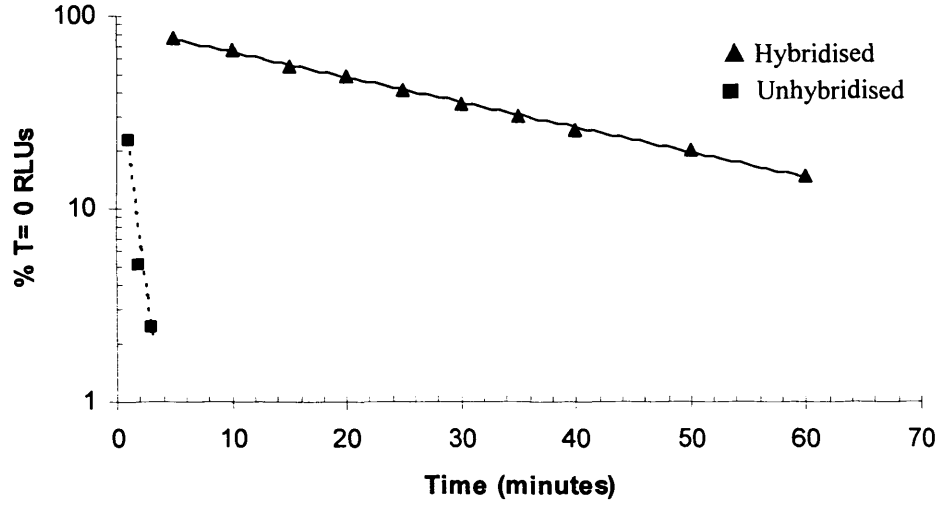


Figure 3.7 Acridinium ester hydrolysis of hybridised (▲) and unhybridised (■) Probe. Reaction components were combined in a 1.5ml microfuge tube, as detailed in Table 3.6; "Hybrid" reactions consisted of AE-probe and complementary target oligo in a ratio of 1:20 (0.05pmol:1pmol), "Control" reactions contained AE-probe but no target oligo. All reactions were incubated at 60°C for 1h, and 1x hybridisation buffer (300µl) was added to each microfuge tube. Selection reagent (100µl) was then added to duplicate aliquots (10µl) from each reaction, prior to incubation at 60°C. Samples were removed at the desired timepoints and the associated chemiluminescence detected. Chemiluminescence is expressed as a percentage of the activity of unhydrolysed label and is plotted relative to the time of exposure to hydrolysing conditions. The chemiluminescence of unhydrolysed probe was measured immediately upon the addition of selection reagent to samples, i.e. when the time of exposure to hydrolysing conditions was nil (T=0). Although the trend observed whilst calculating the differential hydrolysis ratio was reproducible, it was possible that the actual figures would vary due to the following factors; the number of freeze-thaw cycles to which the probe had been subjected, batch-to-batch variation between probes, batch-to-batch variation between reagents (buffers manufactured in-house).

Best line fit of data was obtained by regression analysis. The equations generated from regression analyses for the hybridised and unhybridised probe are shown below, along with the R^2 values for each fit:

Hybrid	y = -0.0131x + 1.9438	$R^2 = 0.998$
Control	y = -0.4858x + 1.7899	$R^2 = 0.964$

Using the data from the equations generated by regression analysis, the differential hydrolysis (DH) ratio was calculated as follows:

$$\text{Hybrid half-life} = \frac{\log 50\% \text{ hydrolysis}}{\text{Gradient (log/min)}} = \frac{0.301}{0.0131} = 22.97(\text{min})$$

$$\text{Control half-life} = \frac{0.301}{0.4854} = 0.62(\text{min})$$

$$\text{DH ratio} = \frac{\text{hybrid } t_{0.5}}{\text{control } t_{0.5}} = \frac{22.97}{0.62} = 37.04$$

Since the DH ratio is calculated by dividing the hybrid half-life by the half-life for control samples, then the larger this figure, the greater the signal to noise ratio. The impact of these hydrolysis half-lives (22.97min and 0.62min for hybridisied and unhybridised probe, respectively) becomes clear if the amount of chemiluminescence that remains for the hybridised and unhybridised forms of the probe with time is calculated. The theoretical percentage of remaining chemiluminescent label after a given hydrolysis time can be calculated using the equation:

$$(0.5)^n \times 100 = \text{percentage remaining chemiluminescence}$$

where n is the ratio of elapsed time to the half-life of chemiluminescence loss (i.e. the number of half-lives transpired during a given incubation time, $T/t^{1/2}$). Using the half-

lives given above, the calculated values for percentage remaining chemiluminescence after the 15min differential hydrolysis step would be 64% for hybridised probe and 0.0000052% for unhybridised probe. This represents greater than a 12million-fold discrimination between hybridised and unhybridised probe.

3.3.2. Residual Fraction.

In order to determine conditions which allow maximal hydrolysis of unhybridised probe during the differential hydrolysis step, it was necessary to generate a Residual Fraction profile for the AE-probe. This involved hydrolysing free (unhybridised) AE-probe in selection reagent, across a range of time points. Thus, it was possible to estimate the hydrolysis time which would give minimal levels of background chemiluminescence. A series of 14 time points were investigated; the following components were combined in triplicate sets of 75mm Sarstedt reaction tubes:

25µl 2x hybridisation buffer

10µl AE probe (5fmol/µl)

15µl H_2O

All tubes were incubated at 60°C for 30min. Selection reagent (300µl) was added to the duplicate tubes for the time-zero measurement, the contents mixed by swirling and the associated chemiluminescence measured as detailed in Section 3.2.5. Selection reagent (300µl) was added to all other tubes, which were vortexed briefly and returned to 60°C. Duplicate tubes were removed at the desired time points, placed in an ice/water slurry for 30sec and the associated chemiluminescence was measured. Results are shown in Figure 3.8.

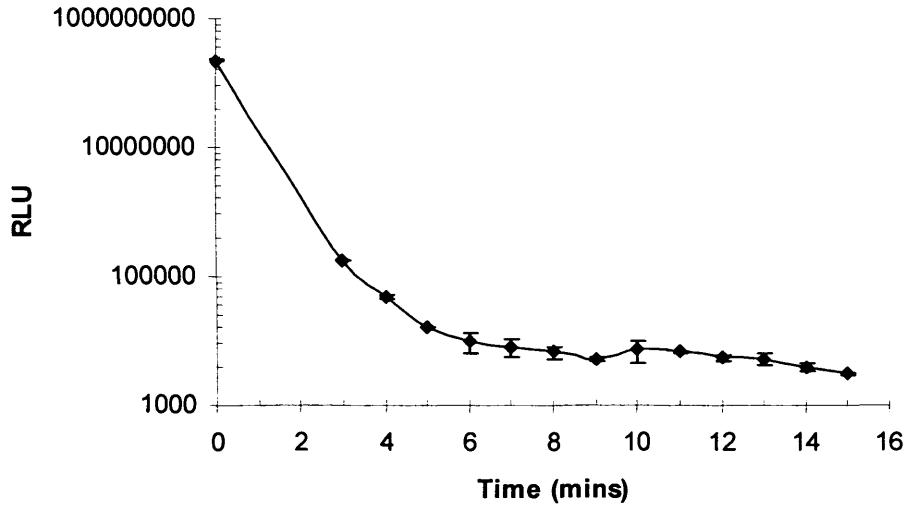


Figure 3.8. The residual fraction profile of the unhybridised luciferase AE-probe. A probe concentration of 50fmol/reaction was used. Triplicate reactions consisting of 2x hybridisation buffer (25µl), AE probe (10µl) and H_2O (15µl) were incubated at 60°C for 30min. Selection reagent (300µl) was added, reactions vortexed and returned to 60°C. Tubes were removed at the desired time points, placed in an ice/water slurry for 30sec and the associated chemiluminescence measured. Error bars represent standard deviations for each set of triplicate reactions. As with differential hydrolysis experiments, the trend observed was consistently reproducible, although it was possible that the actual figures would vary due to the factors mentioned previously; the number of freeze-thaw cycles to which the probe had been subjected, batch-to-batch variation between probes, batch-to-batch variation between reagents (buffers manufactured in-house).

The residual fraction profile for unhybridised luciferase AE-probe (Figure 3.8) shows a rapid decrease in chemiluminescence following the addition of selection reagent. This is due to the hydrolysis of the AE by the hydroxide ions, present in the differential hydrolysis buffer. Although the number of RLUs appears to have reached a plateau at a time of approximately 12 minutes, and the decrease in the level of RLUs between the times of 12 and 15 minutes is negligible, a hydrolysis time of 15min was found to produce consistently lower RLUs for control samples (i.e. unhybridised AE-probe, data not shown). It can be seen, also, from the differential hydrolysis data (Figure 3.7) that an additional 3min hydrolysis time causes a relatively small decrease in RLUs for AE-probe hybridised samples. It was decided, therefore, using both the differential hydrolysis data (Figure 3.7), and the residual fraction profile (Figure 3.8) to use a selective hydrolysis time of 15 minutes in all assays utilising the luciferase AE probe.

3.3.3. Hybridisation Kinetics.

In order to determine the incubation time which allowed maximal hybridisation of AE-probe to target, the following components were combined in 13 duplicate sets of 75mm Sarstedt reaction tubes:

25µl 2x hybridisation buffer

5µl H_2O

10µl AE probe (2.5fmol/µl)

10µl target (0.1fmol/µl)

For every timepoint, a duplicate set of control (probe, no target) and blank (no probe, target and reagents only) tubes were also set up. All reactions were incubated at 60°C, and a duplicate set removed to an ice/water slurry at every minute, between 3 and 15mins. Selection reagent (300µl) was then added and the tubes incubated for a further 15min at 60°C. Chemiluminescence associated with each sample was detected as before (Section 3.2.5).

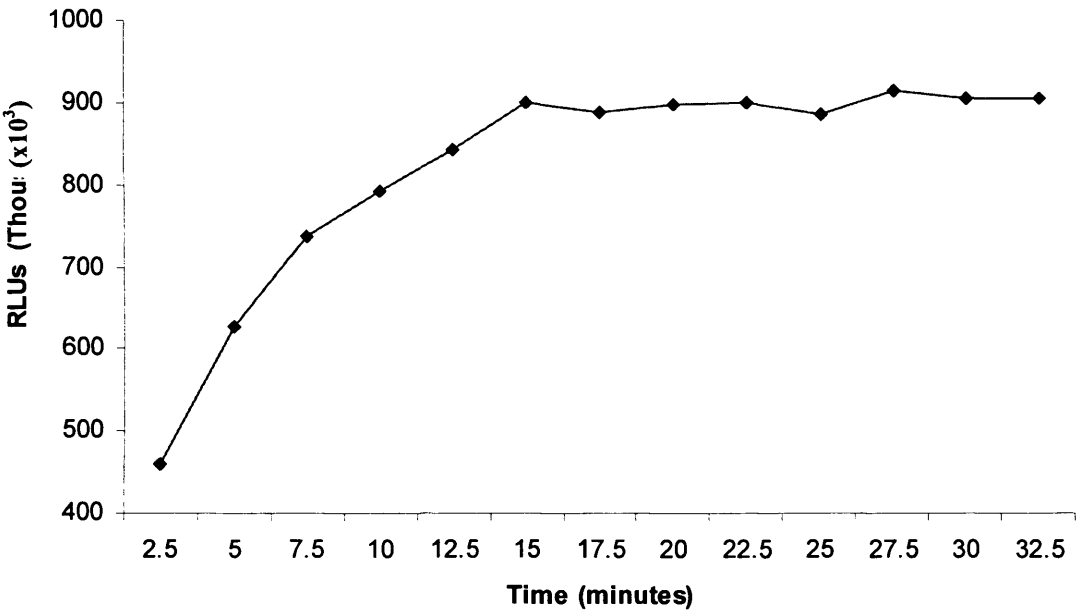


Figure 3.9. Hybridisation kinetics of the luciferase AE-probe. 2x hybridisation buffer (25µl), H_2O (5µl), AE probe (10µl at 2.5fmol/µl) and target (10µl at 0.1fmol/µl) were combined in duplicate sets of 75mm Sarstedt reaction tubes. For every timepoint, a duplicate set of control (probe, no target) and blank (no probe, target and reagents only) reactions were also set up. All reactions were incubated at 60°C, and at each time point a duplicate set was removed to an ice/water slurry. Selection reagent (300µl) was added prior to a further incubation (15min) at 60°C. Chemiluminescence associated with each sample was then detected as before (Section 3.2.5).

The data for the hybridisation kinetics of the luciferase AE-probe (Figure 3.9) indicate that maximal hybridisation levels appear to be achieved after approximately 15 to 18 minutes. However, in order to ensure consistently optimal hybridisation of probe to target, and since it is the hybridisation incubation period used in a typical HPA format (Nelson *et al.*, 1995), a hybridisation time of 30min was used in all subsequent assays utilising the luciferase AE probe.

3.3.4. Thermal Stability.

In order to determine the melting temperature of the AE-probe hybridised to target, the hybrid was subjected to an incubation at a range of temperatures prior to selective hydrolysis and detection of chemiluminescence. The following components were combined in a microfuge tube:

15µl 2x hybridisation buffer

5µl AE probe (0.01pmol/µl)

10µl target (0.1pmol/µl)

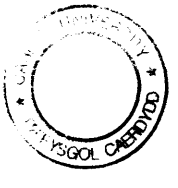
A control mix was also set up, with H_2O (10µl) replacing the target. The contents were mixed and incubated at 60°C for 30min. 1x hybridisation buffer (1000µl) was added to each tube. Ten sets of duplicate aliquots (50µl) for each mix were pipetted into 75mm luminometer tubes. A duplicate set of hybrid and control reactions were incubated at each temperature for 7min, before being placed on ice. Following the completion of all hybridisations, tubes were removed from the ice, and selection reagent (150µl) added. Tube contents were mixed by vortexing, incubated at 60°C for 15min and the chemiluminescence associated with each tube was detected as before (Section 3.2.5).

Hybridised luciferase AE-probe bound to target, is stable at temperatures up to and including 58°-62°C (Figure 3.10). Using the AE-probe data, as described in Table 3.5 the Tm of the AE-probe was estimated, using the method described by Sambrook *et al.*, (1989):

$$\text{AE-Probe Tm} = (\%GC \times 0.41) + 64.9 - (600/\text{oligo length})$$

$$= (50 \times 0.41) + 64.9 - (600/28) = 64°C$$

This estimation of AE-probe Tm correlates with the profile shown in Figure 3.10, where temperatures of 64°C and above lead to melting of the AE-probe-target hybrid. The melting of the hybrid, caused by incubation at 64°C and above, leads to hydrolysis of the AE molecule during the differential hydrolysis step, and the subsequent loss of RLUs, as observed in Figure 3.10.



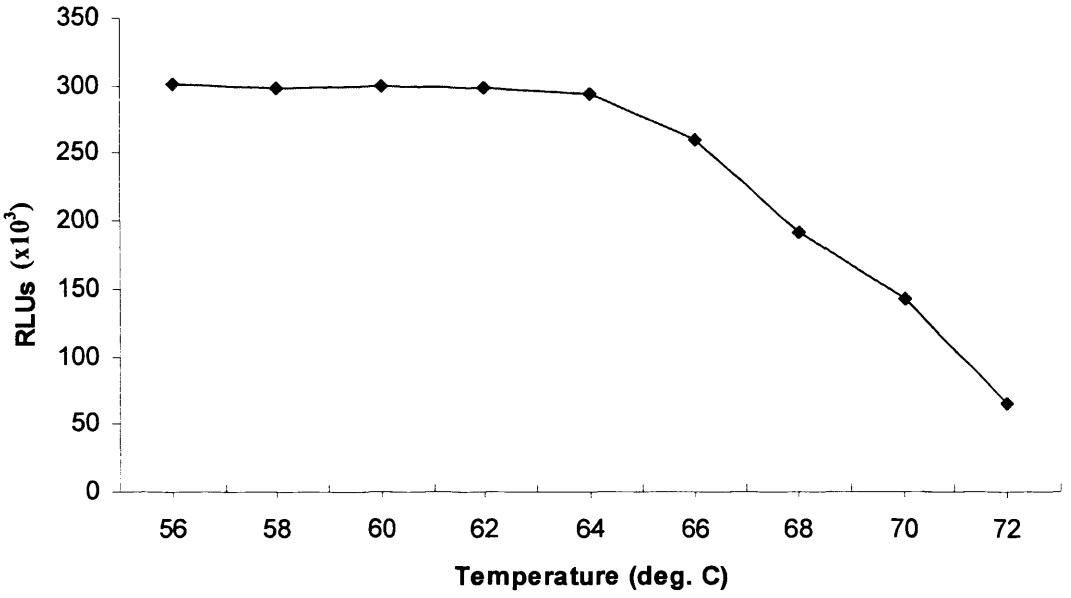


Figure 3.10. The thermal stability profile for the luciferase AE-probe hybridised to its complementary target oligonucleotide. 2x hybridisation buffer (15µl), AE probe (5µl at 0.01pmol/µl) and target (10µl at 0.1pmol/µl) were combined in a microfuge tube. Control reactions were also set up, with H_2O (10µl) replacing the target. Reactions were mixed and incubated at 60°C for 30min. 1x hybridisation buffer (1000µl) was then added to each reaction and ten sets of duplicate aliquots (50µl) for each mix were pipetted into 75mm luminometer tubes. A duplicate set of hybrid and control reactions were incubated at each temperature for 7min, before being placed on ice. Following the completion of all hybridisations, tubes were removed from the ice, and selection reagent (150µl) added. Tube contents were mixed by vortexing, incubated at 60°C for 15min and the chemiluminescence associated with each tube was detected as before (Section 3.2.5).

3.3.5. Helper Probe Assessment.

A helper probe assessment was carried out in order to determine whether or not the helper probes affected the efficiency of AE-probe binding. Since the purpose of the helper probes is to facilitate AE-probe binding in the presence of mRNA secondary structure (Section 3.2.2.1), it was necessary to use luciferase mRNA in this assessment. Luciferase mRNA was produced using the control plasmid from the RiboMax™ *In Vitro* Transcription Kit as per the manufacturer's instructions (Promega), and was kindly donated by Dr Ceri Morris (MLT Research Ltd, Cardiff, UK).

Reaction components were combined in duplicate 75mm luminometer tubes as indicated in Table 3.11 Where only one helper was used, two reactions were set up – one for the 5' and one for the 3' primer. Following an incubation at 60°C for 30min, selection reagent (300µl) was added, tubes were mixed by vortexing and returned to the 60°C waterbath for 15min prior to the detection of chemiluminescence associated with each tube, as described previously (Section 3.2.5).

		No helpers		One helper		Two helpers	
	Blank	**Control**	**Hybrid**	**Control**	**Hybrid**	**Control**	**Hybrid**
2x hyb.	50	50	50	50	50	50	50
Probe	-	10	10	10	10	10	10
5' helper	-	-	-	5	5	5	5
3' helper	-	-	-	-	-	5	5
mRNA	-	-	10	-	10	-	10
H_2O	50	40	30	35	25	30	20
Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Table 3.11. Assay components, as used for the assessment of helper probes. All figures shown are volumes, in microlitres. The probe concentration used was 2.5fmol/µl, the helper oligos were 5pmol/µl and the luciferase mRNA concentration was 0.24ng/µl.

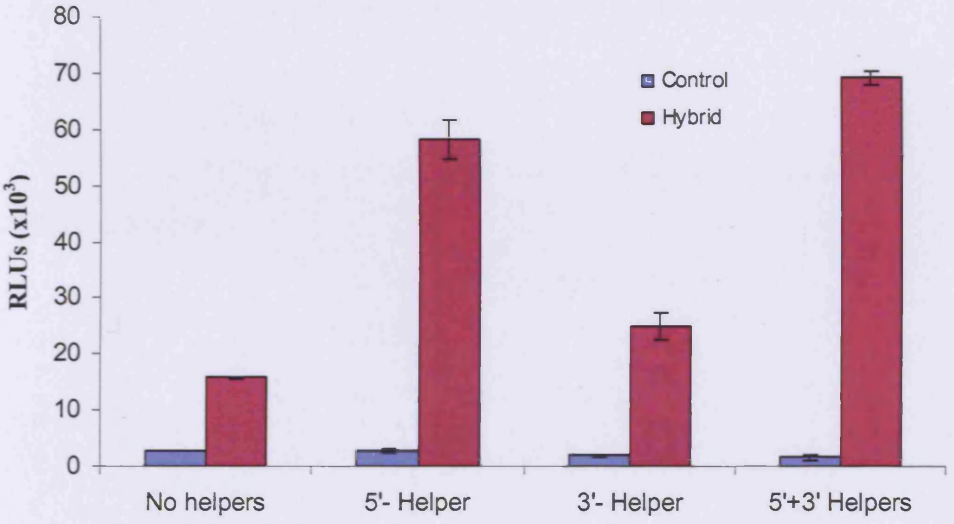


Figure 3.12. The facilitation of AE-probe binding to synthetic luciferase mRNA by the inclusion of helper oligos. AE-probe (25fmol) was incubated in the presence ("Hybrid") or absence ("Control") of luciferase mRNA for 30min at 60°C, as detailed in Table 3.11. Selection reagent (300µl) was added, tubes were mixed by vortexing and returned to the 60°C waterbath for 15min prior to the detection of chemiluminescence associated with each tube, as described previously. Reactions were carried out in duplicate – mean values have been plotted and error bars represent standard deviation.

The hybridisation of the luciferase AE probe to luciferase mRNA is greatly facilitated by the presence of helper probes (Figure 3.12). Binding of AE probe is improved in the presence of either helper individually, but maximal binding of AE probe to mRNA is achieved in the presence of both 5' and 3' helper probes. It is likely that this is due to the helper probes binding the mRNA and opening out any

secondary structure present; helper probes are 30bp oligos that are complementary to the regions of mRNA directly 5' and 3' to the site of binding of the AE probe thus making the binding site for the AE probe more accessible. Figure 3.12 represents data from a single experiment, however the experiment was run three times to ensure reproducibility.

3.3.6. Sensitivity.

In order to determine the limit of sensitivity of the luciferase HPA, a fixed concentration of AE-probe was firstly titrated against serial dilutions of the target oligo, and secondly against serial dilutions of luciferase mRNA in the presence of non-luciferase mRNA - in order to more closely mimic the actual experimental conditions under which the HPA technique might be applied; namely, for the quantification of luciferase mRNA extracted from cultured, adherent cells.

a) Target Oligo Titration.

The following components were combined in triplicate sets of 75mm luminometer tubes:

50µl 2x hybridisation buffer

30µl H_2O

10µl AE probe (5fmol/µl)

10µl target (serial dilutions)

A set of 5 control reactions (probe, no target) and triplicate blank (target, no probe) tubes were also set up. Two-fold dilutions of target were used, starting at the highest concentration of 100fmol/µl. Tubes were incubated at 60°C for 30min. Selection reagent (300µl) was added and the tubes vortexed, before being returned to 60°C. Samples were subjected to selective hydrolysis for 15min - as determined by the differential hydrolysis ratio (Section 3.3.1). Following hydrolysis, tubes were removed from the waterbath, and the associated chemiluminescence was measured, as detailed in Section 3.2.5.

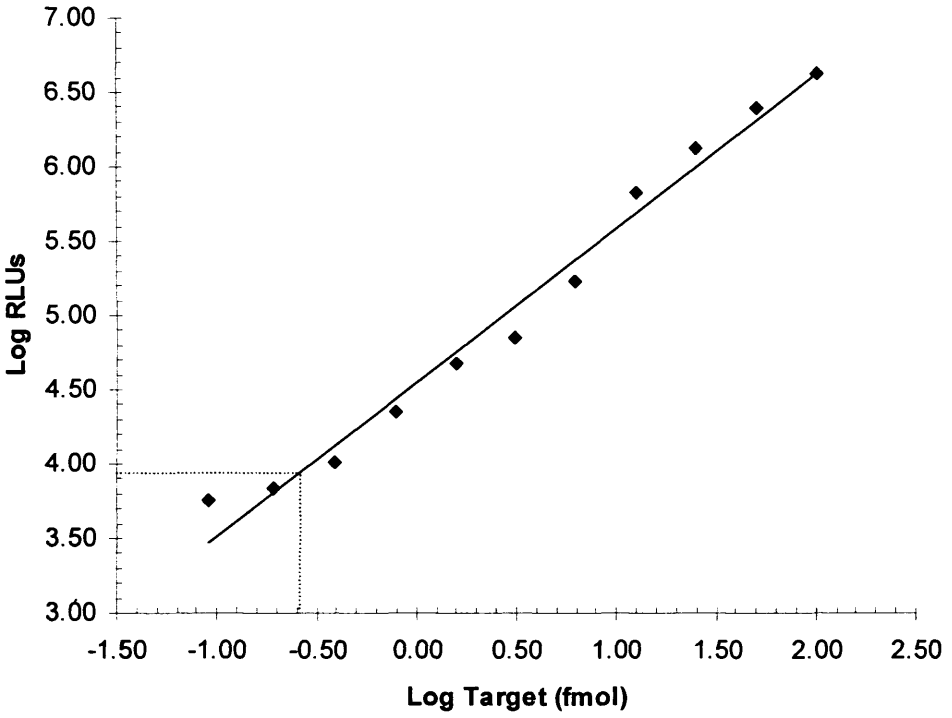


Figure 3.13.A. The sensitivity profile for the luciferase AE-probe. AE-probe (50fmol/reaction) was hybridised to serial dilutions of target oligo. A set of control (probe, no target) and blank (target, no probe) reactions were also set up. Tubes were incubated at 60°C for 30min. Selection reagent (300µl) was added, tubes vortexed and returned to 60°C for 15min selective hydrolysis - as determined by the differential hydrolysis ratio (Section 3.3.1). Following hydrolysis, chemiluminescence associated with tubes was measured as detailed in Section 3.2.5. Best line fit of data was obtained by regression analysis. The R^2 value for the fit was $R^2 = 0.989$. Log of the average RLUs for control samples (5 replicates containing probe, no target) was 3.60 and the standard deviation for these 5 values was 0.15.

b) Luciferase mRNA Titration.

The luciferase mRNA used in this experiment was produced using the control plasmid from the RiboMax™ *In Vitro* Transcription Kit as per the manufacturer's instructions (Promega), and again, was kindly donated by Dr Ceri Morris (MLT Ltd). The non-luciferase mRNA was simply mRNA extracted from non-transfected, cultured cells, as described in Section 2.2.2.5.b. The following components were combined in triplicate sets of 75mm luminometer tubes:

50µl 2x hybridisation buffer
10µl H_2O
10µl non-luciferase mRNA (from T75)
10µl AE probe (5fmol/µl)
10µl luciferase mRNA (serial dilutions)
5µl 5'- helper (5pmol/µl)
5µl 3'- helper (5pmol/µl)

A set of 5 control (all components, except luciferase mRNA) and triplicate of blank (all components, except probe) tubes were also set up. Two-fold dilutions of luciferase mRNA were used, starting at the highest concentration of 10fmol/µl.

Tubes were incubated at 60°C for 30min. Selection reagent (300µl) was added and the tubes vortexed, before being returned to 60°C. Samples were subjected to selective hydrolysis for 15min - as determined by the differential hydrolysis ratio (Section 3.3.1). Following hydrolysis, tubes were removed from the waterbath, and the associated chemiluminescence was measured, as detailed in Section 3.2.5. The results are shown in Figure 3.13.B.

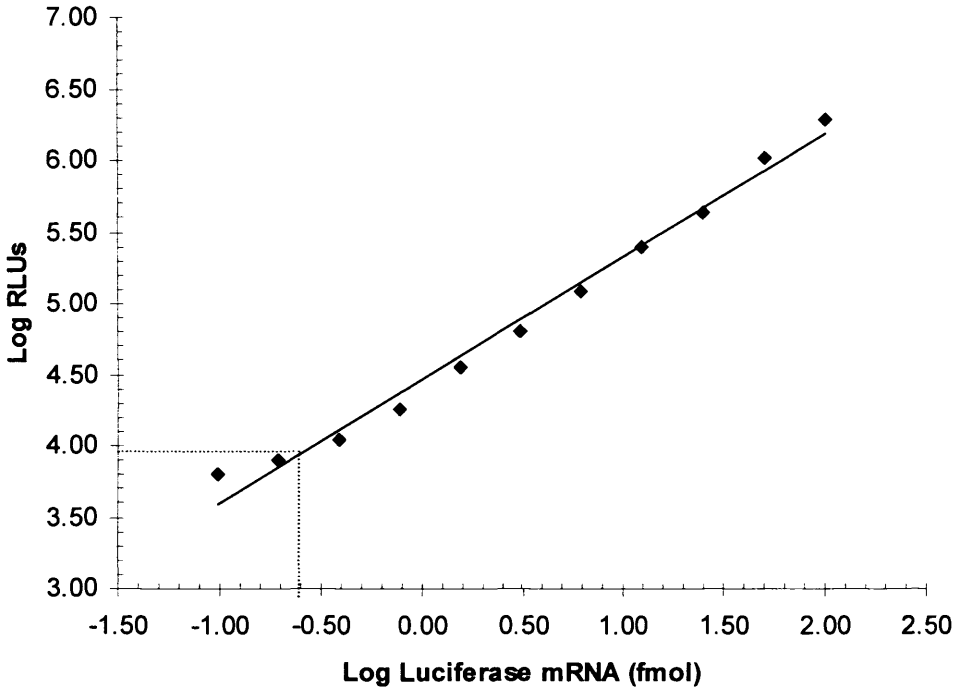


Figure 3.13.B. The sensitivity profile for the luciferase AE-probe. AE-probe (50fmol) was hybridised with serial dilutions of luciferase mRNA for 30min at 60°C. A set of control (probe, no target) and blank (target, no probe) reactions were also set up. Selection reagent (300µl) was added, tubes vortexed and returned to 60°C for 15min selective hydrolysis - as determined by the differential hydrolysis ratio (Section 3.3.1). Following hydrolysis, chemiluminescence associated with tubes was measured as detailed in Section 3.2.5. Best line fit of data was obtained by regression analysis. The R^2 value for the fit was $R^2 = 0.997$. Log of the average RLUs for control samples (5replicates containing probe, no luciferase mRNA) was 3.63 and the standard deviation for these 5 values was 0.16.

c) Determination of the Sensitivity Limit.

In order to determine the limit of sensitivity of the luciferase AE-probe from the plots shown above, the standard deviation of the log RLUs for the five control values ("controls" contained AE probe but no target) was calculated at 0.15 and 0.16 for target and mRNA titration experiments respectively. This value was doubled and subsequently added to the mean of the log RLUs for the control readings. This gave values of 3.92 and 3.95 for the target and mRNA titrations, respectively. Extrapolation to the x-axis estimates a limit of sensitivity of 0.251fmoles when detecting both complementary target oligonucleotide, and luciferase mRNA.

3.3.7. HPA for Luciferase mRNA.

Following optimisation of the various parameters described above, it was now possible to quantify the concentration of unknown luciferase mRNA samples by comparing chemiluminescence of the unknown samples against the chemiluminescence of a standard curve generated using serial dilutions of target oligo – that is, a Hybridisation Protection Assay for luciferase mRNA had now been optimised. Assay components were combined in duplicate, as shown in Table 3.14. The final volume of helper added equates to half a volume of 5' helper and half a volume of 3' helper. Tubes were agitated gently by hand, covered with standard laboratory label tape, and incubated at 60°C for 30min (hybridisation). Selection reagent (300µl) was added and the tubes incubated for a further 15min at 60°C (selective hydrolysis). Chemiluminescence was detected as described in Section 3.2.5. The RLUs generated by each tube were recorded directly, and a standard curve was generated, in order to allow the calculation of luciferase mRNA concentration in fmol/ml.

	BLANK	CONTROL	STANDARD	TEST
Hybridisation Buffer	50	50	50	50
H_2O	50	30	20	20
Helper (5pmol/µl)	-	10	10	10
Target	-	-	10	-
mRNA sample	-	-	-	10
Diluted Probe (5fmol/ µl)	-	10	10	10
Final Volume	100	100	100	100

Table 3.14. Luciferase mRNA assay components. All figures given represent volumes, in microlitres.

3.3.8. Detection of Luciferase mRNA Using a Two-Step Hybridisation and Direct Capture of AE-Labelled Target Nucleic Acids.

a) Introduction.

Although the AE-probe can be detected with a relatively high degree of sensitivity as part of the HPA, there is interplay involved between the amount of AE-probe present and the background readings, which must be kept relatively low to fully exploit AE sensitivity. The effects of background on sensitivity are especially a problem in the development of a homogeneous assay format such as the HPA, because of the difficulty in discriminating between hybrid and unhybridised AE-probe molecules in solution. Although the differential hydrolysis step (Section 3.2.2.2) effectively reduces levels of chemiluminescence associated with unhybridised AE-probe to relatively low levels, a background level of chemiluminescent AE-probe remains. This inherent background, the Residual Fraction, has been described previously and is calculated as detailed in Section 3.3.2.

Whilst the chemiluminescence levels associated with the Residual Fraction do not present a problem when the HPA is used for the detection of a relatively abundant target nucleic acid, it can be desirable to further lower levels of background chemiluminescence (effectively increasing the signal:noise ratio) if the target nucleic acid is present at a relatively low concentration. The lowering of background levels of chemiluminescence can be achieved by separating hybridised and unhybridised AE-probe. This can be done by hybridising the AE-probe to the target nucleic acid (luciferase mRNA), followed by the subsequent pulling-down or "direct capture" of the labelled nucleic acid by a "bridging" oligonucleotide, which binds in turn, to a solid support (magnetic particles). AE-probe not hybridised to the target nucleic acid is readily washed away from the solid support and thus, the detection of any remaining chemiluminescence is a direct measure of the amount of target nucleic acid present. A schematic representation of the two-step, direct-capture technique is shown in Figure 3.16.

b) Probe Design.

Wherever possible, reaction conditions and oligonucleotide sequences that had been previously optimised for the HPA were utilised. The sequences of the AE-probe and the 5' and 3'- helper oligos have been detailed previously (Table 3.5),

and since the binding of helper oligos had already been shown to facilitate the hybridisation of AE-probe to luciferase mRNA (Section 3.3.5), it was decided to utilise the sequence of the 5'-helper in the design of the bridging probe. A string of 14 A- residues were added to the 3'- end of the 5'- helper to make the "PolyA bridge" (Table 3.15). Consequently, following the hybridisation of the AE-probe, the 3'-helper and the PolyA bridge to the luciferase mRNA, it was possible to immobilise or "capture" the AE-labelled mRNA on magnetic oligo dT_{14} beads. Appendix 3C shows where the 5' – helper (and therefore, the PolyA bridge) binds within the luciferase coding sequence.

Oligo	**Sequence (5'→3')**
PolyA bridge	AAT TCA TTA TCA GTG CAA TTG TTT TGT CAC AAA AAA AAA AAA AAA

Table 3.15. The sequence of the PolyA bridge, which was utilised in direct-capture assays, along with the luciferase AE-probe (see text for details).

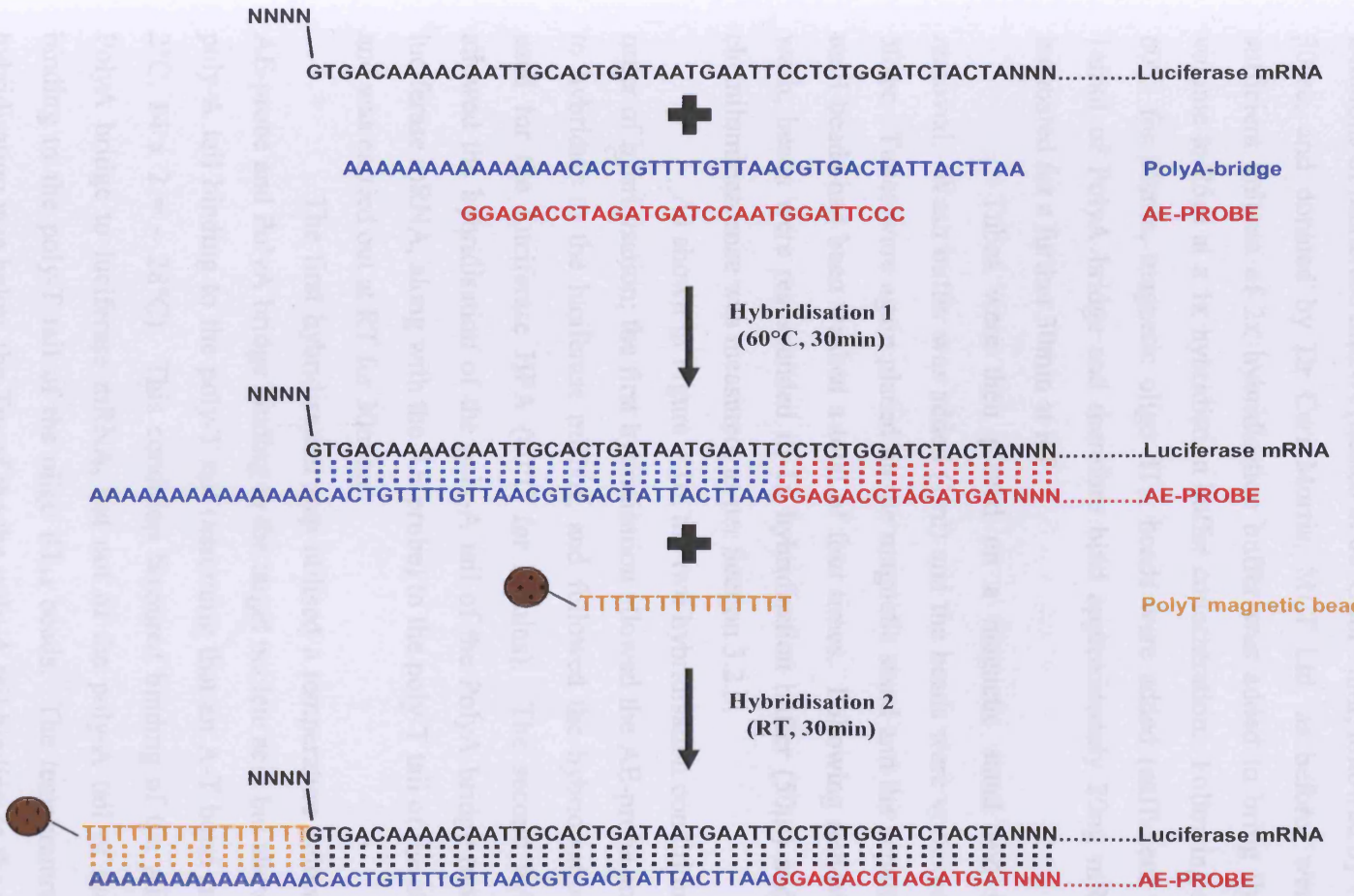


Figure 3.16 The two-step hybridisation protocol used in the direct-capture method for quantification of luciferase mRNA using the AE-probe. The full sequence of the PolyA-bridge is shown, whilst only part of the AE-probe sequence is displayed, for orientation purposes. The relevant, complementary regions of the 1,653bp luciferase mRNA are also shown. The remainder of the sequence is designated "-NNNN-".

c) Assay Format.

Luciferase AE-probe (50fmol) was combined with the luciferase 3'-helper probe (10fmol) and the PolyA bridge (1nmol) in a 75mm luminometer tube. Dilutions of luciferase mRNA (heated at 60°C for 5min, followed by vortexing for 20-30sec, and donated by Dr Ceri Morris, MLT Ltd, as before) were added and a sufficient volume of 2x hybridisation buffer was added to bring the final reaction volume to 25µl at a 1x hybridistion buffer concentration. Following an incubation at 60°C for 30min, magnetic oligo dT_{14} beads were added (sufficient volume to bind 1nmol of PolyA bridge and therefore bind approximately 20ng mRNA) and tubes incubated for a further 30min at RT.

Tubes were then placed on a magnetic stand and the supernatant removed. Wash buffer was added (1ml) and the beads were vortexed vigorously for 30sec. Tubes were again placed on the magnetic stand and the supernatant removed, until beads had been washed a total of four times. Following removal of the fourth wash, beads were resuspended in 1x hybridisation buffer (50µl) and the associated chemiluminescence was measured, as per Section 3.2.5.

As shown in Figure 3.16, the two hybridisation conditions controlled the order of hybridisation; the first hybridisation allowed the AE-probe and PolyA bridge to hybridise to the luciferase mRNA, and followed the hybridisation conditions as used for the Luciferase HPA (60°C for 30mins). The second hybridisation step allowed the hybridisation of the poly-A tail of the PolyA bridge (now bound to the luciferase mRNA, along with the AE-probe) to the poly-T tail of the oligo dT_{14} beads, and was carried out at RT for 30mins.

The first hybridisation step utilised a temperature below the Tm of the AE-probe and PolyA bridge binding to the target nucleic acid but above the Tm of the poly-A tail binding to the poly-T tail (assuming that an A-T bond raises the Tm by 2°C, 14 x 2 = ~ 28°C). This condition favoured binding of the AE-probe and the PolyA bridge to luciferase mRNA, but not of the poly-A tail of the PolyA bridge binding to the poly-T tail of the oligo dT_{14} beads. The temperature of the second hybridisation was below the Tm of the the poly-A tail binding to the poly-T tail, and thus was compatible with the immobilisation of AE-labelled luciferase mRNA to the magnetic oligo dT_{14} beads. ("Tm" refers to the melting temperature at which 50% of hybridisation complexes formed between two nucleic acid sequences are denatured.

At a temperature below the Tm, hybridisation is favoured, whereas at a temperature above the Tm, hybrid formation is not favoured).

d) Sensitivity.

In order to determine the limit of sensitivity of the direct-capture assay, a series of dilutions of luciferase mRNA (produced using the RiboMax™ *In Vitro* Transcription Kit (Promega), and donated by Dr Ceri Morris, MLT Ltd) were made and reactions were carried out as above. The limit of sensitivity was calculated as before for the luciferase HPA (Section 3.3.6.c); the standard deviation of the log RLUs for the five negative control values (containing 1x hybridisation buffer and oligo dT_{14} beads only – no AE probe) was doubled and added to the mean of the log RLUs for these five values (Figure 3.17). This gave a value of 2.65. Intrapolation to the x-axis estimates a limit of sensitivity of 26amoles for the detection of luciferase mRNA. This compares with a value of 251amoles for the HPA (Section 3.3.6.c).

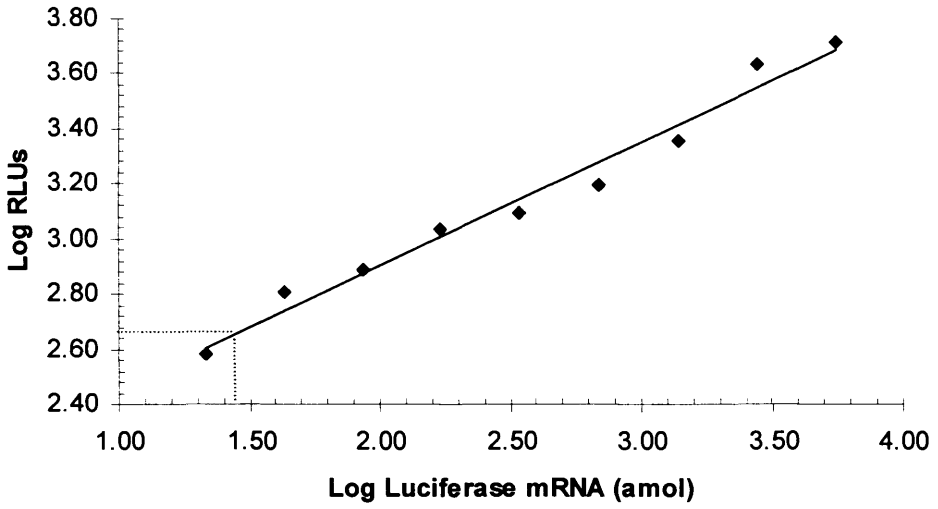


Figure 3.17. The sensitivity profile for the luciferase AE-probe utilised in a direct-capture assay at a final probe concentration of 50fmol/reaction. AE-probe, 3'- helper and the PolyA bridge were hybridised with serial dilutions of luciferase mRNA for 30min at 60°C, prior to the addition of magnetic oligo dT_{14} beads and a further 30min incubation at RT. Beads were washed four times, resuspended in 1x hybridisation buffer (50µl), and the associated chemiluminescence detected.

Best line fit of data was obtained by regression analysis. The R^2 value for the fit was $R^2 = 0.980$. Log of the average RLUs for negative control samples (1x hybridisation buffer and oligo dT_{14} beads only) was 2.49 and the standard deviation for these 5 values was 0.08.

3.4. Discussion.

The most common approach used to determine the sensitivity of an analytical technique is to assay the zero standard repeatedly and define the limit of sensitivity as being the concentration corresponding to two or three standard deviations above the mean. This equates to the probability that a single replicate of sample does not form part of the distribution of zero analyte values. In this way, the limit of sensitivity of the luciferase HPA was calculated by adding two standard deviations to the mean value for five replicates of the negative control sample (the negative controls contained AE probe but no target sequence) to give estimations for the limit of sensitivity of 0.25fmoles per reaction when quantifying both complementary oligonucleotide and luciferase mRNA. These estimations compare favourably with the value of 0.60fmoles, calculated by Arnold *et al.*, (1989).

It was also possible to further increase the limit of sensitivity of AE detection, with an adaptation of the HPA. In this Direct Capture assay, hybridised AE-probe was physically separated from unhybridised AE-probe, effectively lowering the background levels of chemiluminescence. The AE-probe (from the luciferase HPA) and a bridging oligo (consisting of the sequence of the 5'- helper from the HPA, followed by a further 14 A- residues) were hybridised to the luciferase mRNA. The mRNA could then be pulled down onto oligo dT_{14} magnetic beads, prior to detection of associated chemiluminescence. A simple luciferase mRNA titration demonstrated that the direct-capture assay was more sensitive than the HPA; 26amoles compared to 251amoles.

As well as increased sensitivity, a second advantage of the direct capture assay is that luciferase mRNA (hybridised to AE probe and bridging oligo) can be separated from any other nucleic acids present (by pulling down of mRNA to oligo dT_{14} magnetic beads). This could be an advantage when quantifying luciferase transcript levels from an *in vitro* transcription reaction (Chapter 6) which would include template (plasmid) DNA; non-specific binding of AE probe to the template DNA could lead to an increase in background levels of chemiluminescence and subsequent loss of sensitivity.

In order to develop assays which examine the effects of putative disruptors on transcription, it is essential that gene transcript levels can be quantified. Although it has been shown that the HPA can be successfully used for the

quantification of luciferase mRNA, there are also a number of other techniques which can be employed in the detection of gene transcripts.

The RNAse Protection Assay (RPA) typically requires approximately 10µg of total RNA (Sambrook *et al.*, 1989). Assuming that the mRNA fraction of a typical cell makes up about 2% of total RNA, that the average length of a mRNA molecule is 1.5kb and that the average relative molecular mass of a nucleotide is 350, this equates to approximately 0.38pmoles of mRNA in a single sample. An mRNA is usually considered to be abundant if it constitutes >1% of the mRNA fraction (Sambrook *et al.*, 1989). Therefore, if the RPA were to be used for the detection of an abundant mRNA from 10µg of total RNA, it can be estimated that it would be possible to detect at least 3.8fmoles of mRNA, using the RPA. It is possible that the limit of sensitivity of the RPA lies below this level and is comparable with the limit of sensitivity of the HPA (0.25fmol). However, the RPA relies on the use of radioactive isotopes and is more likely to take days, rather than hours to perform.

In comparison, a band can be detected from a Northern blot carried out under ideal conditions from as little as 100ng of total RNA, according to the estimates of Sambrook *et al.*, (1989). Following the same logic as above for an abundant mRNA, this equates to an approximate limit of sensitivity of 0.038fmoles. This is more sensitive than the HPA and is approximately comparable to the limit of sensitivity for the direct capture assay (0.026fmol). However, although a Northern blot is potentially as sensitive as the direct capture method for the analysis of gene transcripts, it provides only qualitative data, whereas the direct capture is quantitative. In addition to this, Northern blots again rely on the use of radioactive isotopes, as well as taking days, rather than hours to perform.

An alternative method for the quantification of transcript levels is TaqMan® probe real-time quantitative PCR (QPCR; described in Chapter 5). The limit of sensitivity for a luciferase-specific primer and probe set was estimated at ~ 1fg/µl of plasmid DNA (Section 5.4), equating to approximately 2amoles. This is significantly more sensitive than the HPA and the direct capture method. However, the QPCR technique is dependent on an amplification step and therefore exhibits relatively high CVs (10-20%), as well as being considerably more labour-intensive and expensive than the HPA.

It is hoped, therefore, that the successful optimisation of the HPA will provide a level of sensitivity within the measurement range required to quantify

luciferase mRNA transcribed from a luciferase reporter construct, both from cellular and from *in vitro* transcription systems. Furthermore, this approach may provide additional advantages, including ease of use and the generation of fully quantitative results, with CVs of less than 7.5% (Thomas-Jones *et al.*, 2003), thereby allowing more exacting measurements to be performed.

Since the *in vitro* transcription of the luciferase gene from a thyroid-responsive reporter construct is mediated via the binding of the thyroid receptor to a thyroid-reponse element in the promoter region of the gene, this necessitates the availability of purified, recombinant thyroid receptor.

CHAPTER FOUR

THE CLONING, EXPRESSION AND CHARACTERISATION OF THE MURINE THYROID HORMONE RECEPTORS.

4.1 Introduction.

DNA-binding and transcriptional-activation assays will be developed in order to assess the impact of potential chemical modulators on the molecular machinery of the thyroid hormone system. Since a necessary component for each of these assays is pure thyroid receptor(s), a powerful expression system was required for the production of sufficient quantities of homogeneous recombinant protein.

The pET family of expression vectors (Novagen) offers a powerful, versatile and rapid system, which allows the expression of heterologous genes in *E.coli*. This family of vectors was developed for the cloning and expression of DNA sequences under the control of the T7 promoter, hence their name: **p**lasmid for **e**xpression by **T**7 RNA polymerase. This system harnesses the transcription potential of the bacteriophage T7 promoter, as the host carries a fragment of DNA encoding the T7 RNA polymerase gene downstream from the the *lac*I gene and the *lac*UV5 promoter. Thus, the addition of IPTG (a stable lactose analogue) induces expression of T7 polymerase, which in turn expresses the cloned DNA sequence from the pET vector.

It was decided to amplify, clone and express the murine forms of the thyroid receptors, as they share significant homology with the human forms of the protein. On an amino acid level, the TRα1 isoforms share 79% identity, whilst the TRβ1 isoforms are 94% identical. Functional analysis of recombinant protein can be assessed using the electrophoretic mobility shift assay (EMSA).

The EMSA is a powerful technique which allows both qualitative and quantitative analysis of protein-DNA interactions. It is based on the observation that the electrophoretic mobility of a nucleic acid through a polyacrylamide gel can be

altered by the binding of protein. A radiolabelled oligonucleotide (probe) containing the sequence of interest is incubated with the recombinant protein and DNA-protein complexes and free probe are then separated by elecrophoresis on a non-denaturing polyacrylamide gel and visualised by autoradiography.

The malic enzyme (ME) gene is expressed ubiquitously, but is only up-regulated by T3 in the rat kidney, heart, and most prominently – the liver (Jeannin *et al.*, 1998). The presence of a positive TRE in the promoter region of the ME gene has been confirmed (Desvergne *et al.*, 1991; Petty *et al.*, 1990) and subsequent studies (Miyamoto *et al.*, 1993) have employed the electrophoretic mobility shift assay (EMSA) to demonstrate that both TRα and TRβ are able to bind to an oligo containing the ME-TRE.

4.1.1. The pET Expression System.

Target genes are cloned into the multiple cloning site of a pET plasmid, under the control of the bacteriophage T7 promoter. Expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 polymerase is so selective and active that, when fully induced, almost all the cells's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein just a few hours after induction (Studier and Moffatt, 1986).

Target genes are intially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, target protein expression is initiated by transferring the plasmid into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under *lac*UV5 control. These hosts are lysogens of bacteriophage DE3 (itself a λ derivative), and carry a DNA fragment containing the *lac*I gene, the *lac*UV5 promoter, and the gene for T7 RNA polymerase (Studier and Moffatt, 1986). This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter.

The *lac*UV5 promoter is a mutant of the P_{lac} promoter, which is part of the lac operon, found in *E.coli*; *E.coli* are able to utilise lactose as a source of carbon. The enzymes required for the use of lactose as a carbon source, are only synthesized

when lactose is available as the sole carbon source. As part of the lactose (*lac*) operon, the three structural genes *lacZ*, *lacY* and *lacA* (encoding β-galactosidase, galactoside permease and thiogalactoside transacetylase, respectively) are transcribed from a single transcription unit, *lacZYA,* under the control of a single promoter P_{lac} (Turner *et al.*, 1997). The *lacZYA* transcription unit contains an operator site O_{lac} which is positioned at the 5'-end of the P_{lac} promoter region. This site is bound with very high affinity by a protein called the *lac* repressor, which is a potent inhibitor of transcription when bound to the operator. The *lac* repressor is encoded by a separate regulatory gene *lacI*, which is also part of the *lac* operon; *lacI* is situated immediately upstream from P_{lac}.

In the absence of lactose, the *lac* repressor occupies the operator-binding site, whilst the RNA polymerase simultaneously occupies the *lac* promoter binding site. All but very low levels of transcription are blocked. When lactose is added, the low basal level of galactoside permease expressed allow it's uptake, and β-galactosidase catalyses the conversion of some of this lactose to allolactose. Allolactose acts as an inducer and binds to the *lac* repressor, it causes a change in protein conformation which leads to dissociation of the repressor from the operator binding site (Turner *et al.*, 1997). RNA polymerase is now able to rapidly transcribe the *lacZYA* genes. Thus, the addition of lactose, or a synthetic inducer such as isopropyl-β-D-thiogalactopyranoside (IPTG), very rapidly stimulates transcription of the *lac* operon structural genes, or whatever gene(s) may be under the control of the inducible promoter – in the case of a DE3 lysogen host-strain, the T7 polymerase gene under the control of the *lac*UV5 promoter (Figure 4.1).

The P_{lac} promoter is not a particularly strong promoter. For high level transcription, it depends on the activity of a specific activator protein called cAMP receptor protein (CRP). Binding of CRP to the P_{lac} promoter immediately upstream of the RNA polymerase binding site induces a 90° bend in DNA, increasing the affinity of RNA polymerase binding to the promoter and thus increasing transcription by up to 50-fold.

The *lac*UV5 promoter is a P_{lac} promoter mutant, which is strongly and constitutively active independent of CRP. Thus, the addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase expression, which in turn transcribes the target DNA from the pET plasmid.

Even in the absence of IPTG, there is some expression of T7 RNA polymerase from the *lac*UV5 promoter in the λDE3 lysogens. If target proteins are sufficiently toxic to affect cell growth and viability this basal level can be sufficient to prevent the establishment of plasmids in the λDE3 lysogens. One way of providing stability to target genes is to express them in host strains containing a compatible plasmid that encodes T7 lysozyme, a natural inhibitor of T7 RNA polymerase which works by binding to and inhibiting the polymerase enzyme. Upon induction of expression, however (achieved by the addition of IPTG), the T7 polymerase gene is up regulated and the low levels of lysozyme present are no longer able to inhibit the resultant high levels of T7 polymerase, and so the target gene is transcribed.

A further advantage of employing a strain which expresses T7 lysozyme, is that T7 lysozyme is a bifunctional protein; it cuts a specific bond in the peptidoglycan layer of the *E.coli* cell wall. Treatments that disrupt the inner membrane (e.g. freeze-thaw or mild detergents) thus induce rapid lysis of cells.

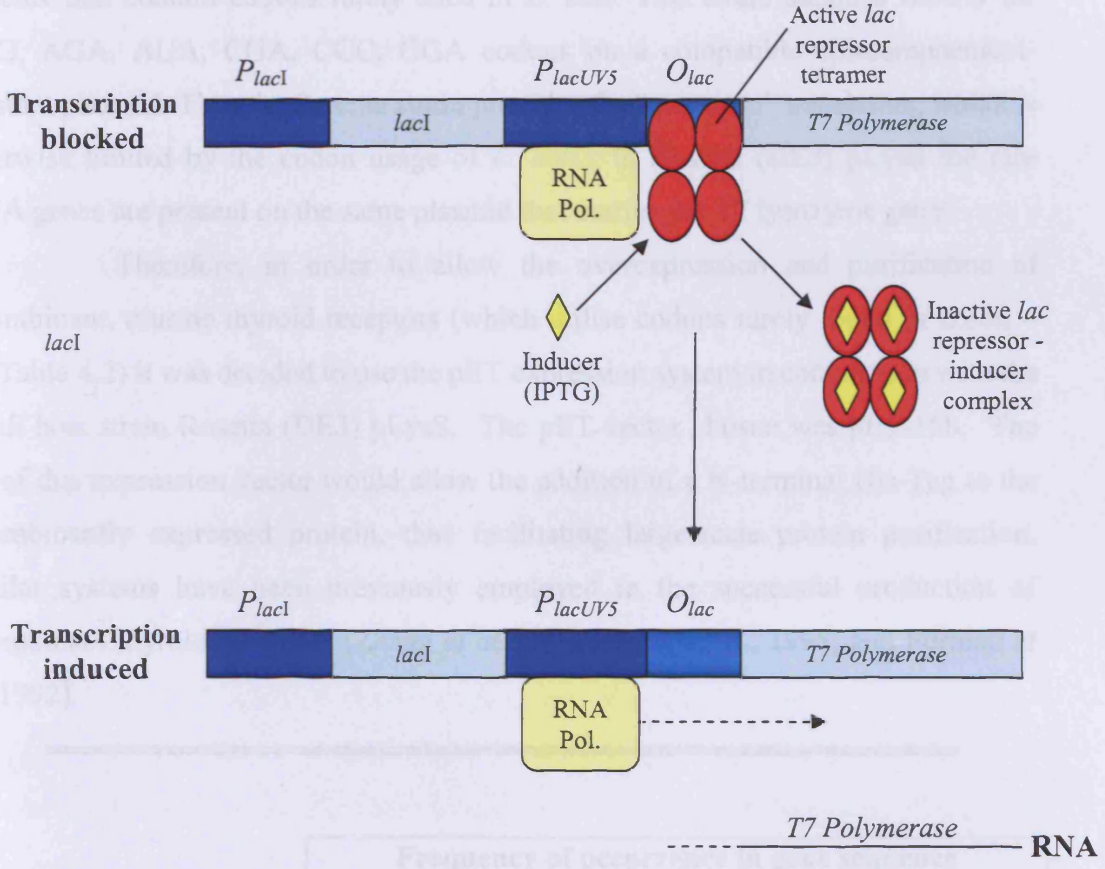


Figure 4.1. Inducible transcription of the T7 polymerase gene in λDE3-lysogenic *E.coli*. In the absence of lactose, the *lac* repressor occupies the operator-binding site (O_{lac}), and blocks all but very low levels of transcription. When the inducer is added (IPTG), it binds to the *lac* repressor and causes a change in protein conformation which leads to dissociation of the repressor from the operator binding site. The promoter-bound RNA polymerase is no longer blocked, and transcription is induced.

The *E.coli* host strain, Rosetta™ BL21 (DE3) pLysS is a λDE3 lysogen, carrying the T7 polymerase gene under the control of the *lac*UV5 promoter. It also carries a pLysS plasmid, which encodes T7 lysozyme. The Rosetta host strain offers an added advantage, in that it is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. This strain supplies tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons on a compatible chloramphenicol-resistant plasmid. Thus the Rosetta strain provides for "universal" translation, which is otherwise limited by the codon usage of *E. coli*. In Rosetta (DE3) pLysS the rare tRNA genes are present on the same plasmid that carries the T7 lysozyme gene.

Therefore, in order to allow the overexpression and purification of recombinant, murine thyroid receptors (which utilise codons rarely found in *E.coli* – see Table 4.2) it was decided to use the pET expression system in conjunction with the *E.coli* host strain Rosetta (DE3) pLysS. The pET vector chosen was pET-15b. The use of this expression vector would allow the addition of a N-terminal His-Tag to the recombinantly expressed protein, thus facilitating large-scale protein purification. Similar systems have been previously employed in the successful production of recombinant thyroid receptors (Zhang *et al.*, 1996; Tong *et al.*, 1995; and Forman *et al.*, 1992).

	Frequency of occurrence in gene sequence	
CODON	***mTRα***	***mTRβ***
AGG	4	4
AGA	1	4
AUA	1	4
CUA	3	5
CCC	7	7
GGA	2	4

Table 4.2. The codons encoded extra-chromosomally in the *E.coli* host strain Rosetta™ BL21 (DE3) pLysS and their frequency of occurrence in the gene sequences of the murine thyroid receptors α and β.

4.2. Experimental Approach and Results.

4.2.1. Amplification and Cloning of the DNAs Encoding the Murine Thyroid Receptors, Alpha and Beta.

Total RNA was prepared from a whole mouse heart as described in Section 2.2.1.10. Although there is a tissue-specific distribution of TR isoforms (Section 1.2.1 from the Intro), nearly equal levels of TRα and TRβ are seen in both the kidney and the heart (Hsu and Brent, 1998). The mRNA (~200ng) was reverse transcribed (Section 2.2.1.11) and an aliquot (1μl) of the resulting cDNA was used as a template for PCR. A PCR product encompassing the full-length cDNA sequence was amplified for both TRα and TRβ.

Specific forward and reverse primers were designed for each gene. The forward primer was designed against the 5' region encoding the start codon, whilst the reverse primer was designed against the 3' region and encoded the stop codon. Forward and reverse primers were designed in such a way so as to engineer *Bgl*II sites on to each end of the amplified PCR product, so as to facilitate cloning of the fragment into pET-15b. For primer sequences see Appendix 1B.

Amplification of the PCR products was achieved using the Expand™ High Fidelity PCR System. This consists of an enzyme mixture containing *Taq* DNA polymerase and a proofreading polymerase – both thermostable enzymes. Reactions were carried out following the conditions described in Section 2.2.1.2 and the thermocycling parameters were as follows:

Chain denaturation	95°C	3min	
	95°C	1min	30 cycles
Primer annealing	60°C	1min	
Chain extension	72°C	1.5min	
Final extension	72°C	10min	
	↓		
Soak	4°C		

A fragment which corresponded approximately to the predicted size was amplified for TRα (1233bp) and TRβ (1383bp), see Figure 4.3. PCR products were purified by agarose gel electrophoresis, and ligated into the pGEM®-T vector, (Section 2.2.1.4.b and Section 2.2.1.8, for vector map see Appendix 2A). The resultant plasmids were subsequently transformed, separately, into competent DH5α cells, as described in Section 2.2.1.9.

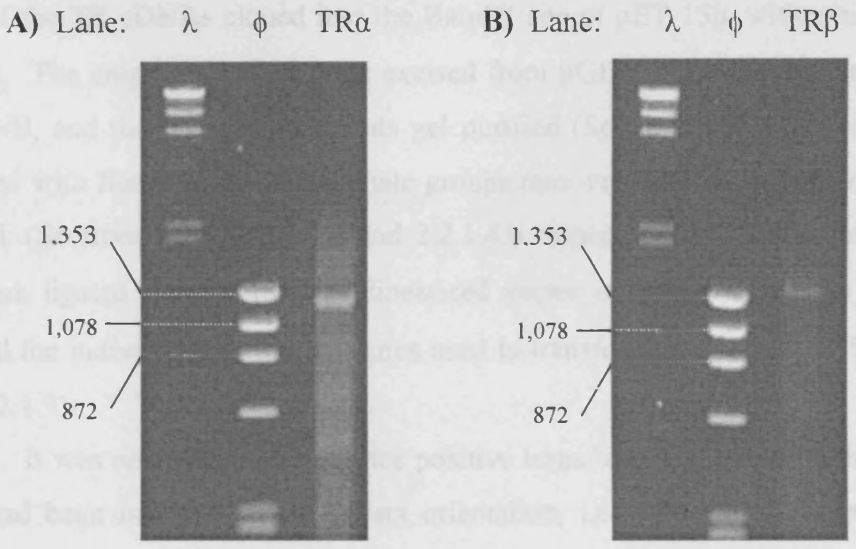


Figure 4.3. Analysis of the TRα and TRβ cDNA amplification reactions, performed by PCR. Mouse-heart cDNA was the template used. PCR products were analysed on a 1% agarose gel containing 0.5μg/ml ethidium bromide.
Panel A): Amplification of TRα
Panel B): Amplification of TRβ
In both panels, the markers are as follows: λ = λ/*Hin*dIII and ϕ = ϕ174/*Hae*III (See Table 2.4 for full list of DNA marker fragment sizes).

4.2.2. Subcloning of the DNAs Encoding the Murine Thyroid Receptors Into the Expression Vector pET-15b.

The pET vector chosen was pET-15b (for vector map, see Appendix 2B). This vector was chosen in order to allow the introduction of a N-terminal His-tag to the recombinantly expressed protein (Section 4.1.1). The His-tag sequence encodes a six-histidine peptide, whose presence can be exploited for both detection and purification of the recombinant protein. The cDNAs encoding TRα and TRβ were cloned into the vector downstream from, and in-frame with, the His-tag. The *Bam*H1 site of pET-15b was chosen, in order to minimise the number of extra amino acid residues that would be added to the recombinant protein (Appendix 3B shows the sequence of the TR cDNAs cloned into the BamHI site of pET-15b, with amino acid translation). The amplified DNAs were excised from pGEM®-T with the restriction enzyme *Bgl*II, and the released fragments gel purified (Section 2.2.1.4.b). pET15-b was digested with *Bam*H1, the 5' phosphate groups removed and the linearised vector gel purified. (Sections 2.2.1.6, 2.2.1.7 and 2.2.1.4.b respectively). Purified, digested cDNAs were ligated directly into the linearised vector overnight at 16°C (Section 2.2.1.8) and the individual ligation mixtures used to transform competent DH5α cells (Section 2.2.1.9).

It was necessary to screen for positive transformants, in which the DNA fragment had been inserted in the correct orientation, i.e. with the TR start codon downstream from and in frame with the N-terminal His-tag. Transformants were screened, using PCR, as described in Section 2.2.1.12. The primers used were gene-specific forward and T7 reverse (for primer sequences, see Appendices 1B and 1A, respectively). Positive clones for TRα and TRβ (which will subsequently be called pETTRα and pETTRβ) were identified, and an overnight culture inoculated from the respective colony. Plasmid DNA was extracted (Section 2.2.1.3.a) and subsequently used to transform competent *E.coli* expression host cells Rosetta™ BL21 (DE3) pLysS. The induction of expression from pETTRα and pETTRβ resulted in the production of a recombinant, N-terminal His-tagged fusion protein which will henceforth be referred to as TRα and TRβ respectively, unless otherwise specified.

4.2.3. Analytical Scale Expression of pETTRβ and Analysis of Cell Extracts.

An overnight culture of Rosetta™ BL21 (DE3) pLysS cells transformed with pETTRβ was used to inoculate 10ml of LB medium, containing the antibiotics ampicillin (100μg/ml) and chloramphenicol (34μg/ml), as described in Section 2.2.4.1. The culture was induced by the addition of IPTG to a final concentration of 1mM, and samples (1ml) were taken through a timecourse of 0 – 240 minutes post-induction. Soluble and insoluble protein fractions were prepared (Section 2.2.4.2) and analysed by SDS PAGE (Section 2.2.4.3) and western blotting, followed by detection with the Ni-NTA AP conjugate (Sections 2.2.4.4 and 2.2.4.5).

Expression of the cDNA encoding TRβ resulted in the appearance of a single band of approximately 55kDa, which is consistent with the anticipated size for the recombinant His-tagged fusion protein. The Coomassie Blue stained gel and the western blot both indicate the accumulation of a band of the predicted size, but restricted to the insoluble fraction (Figure 4.4). The band was visible on the SDS gel from 30min post induction. The intensity of this band increasing progressively up to 240min post induction and it was possible to confirm the presence of a His-tag using the western blot and Ni-NTA AP conjugate system (Sections 2.2.4.4. and 2.2.4.5.).

4.2.4. Optimisation of Induction Conditions for Expression of Recombinant TRβ.

Due to the lack of solubility initially observed for recombinant TRβ (Section 4.2.3.), it was decided to manipulate the induction conditions in order to investigate whether it was possible to obtain protein in a soluble form.

Analytical scale induction cultures were grown at 37°C to an OD_{600} of between 0.4 and 0.6, as described in Section 2.2.4.1. Cultures were induced by the addition of IPTG to final concentrations of 0.25mM, 0.5mM and 1mM. Cultures induced with each concentration of IPTG were then incubated at 20°C, 30°C and 37°C. Samples were taken at times 0, 0.5, 1, 2, 3 and 4h. Soluble and insoluble samples were prepared (Section 2.2.4.2), analysed by SDS PAGE (Section 2.2.4.3) and western blots performed with the Ni-NTA AP conjugate (Sections 2.2.4.4 and 2.2.4.5). The Coomassie Blue stained gel and the western blot showed that a band of predicted size could only be detected in the soluble fraction, which had been induced

with 0.5mM IPTG, and grown, subsequently at 20°C (temporal profile is shown in Figure 4.5). However, the amount of soluble protein was a relatively small proportion of the total quantity of total recombinant TRβ as exemplified when comparing the amount of soluble and insoluble recombinant TRβ expressed at 4 hours (Figure 4.5 Lanes 240 and I).

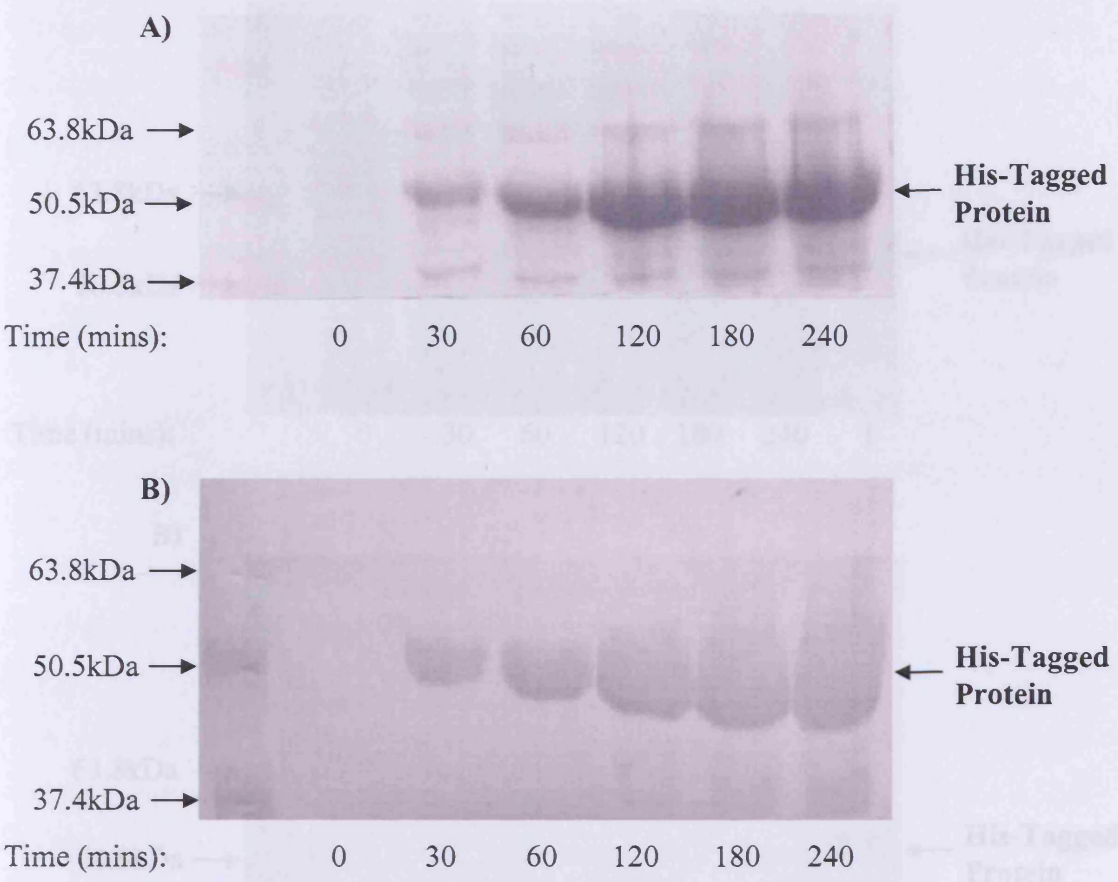


Figure 4.4. Analytical-scale expression of recombinant His-tagged TRβ. Analytical scale cultures were induced with 1mM IPTG and subsequently grown at 37°C. SDS-PAGE and western blot analysis of the insoluble fraction (10µl sample volume) along a temporal profile is shown in:
Panel A: Coomassie Brilliant Blue stained gel and,
Panel B: Western Blot probed with Ni-NTA AP conjugate.

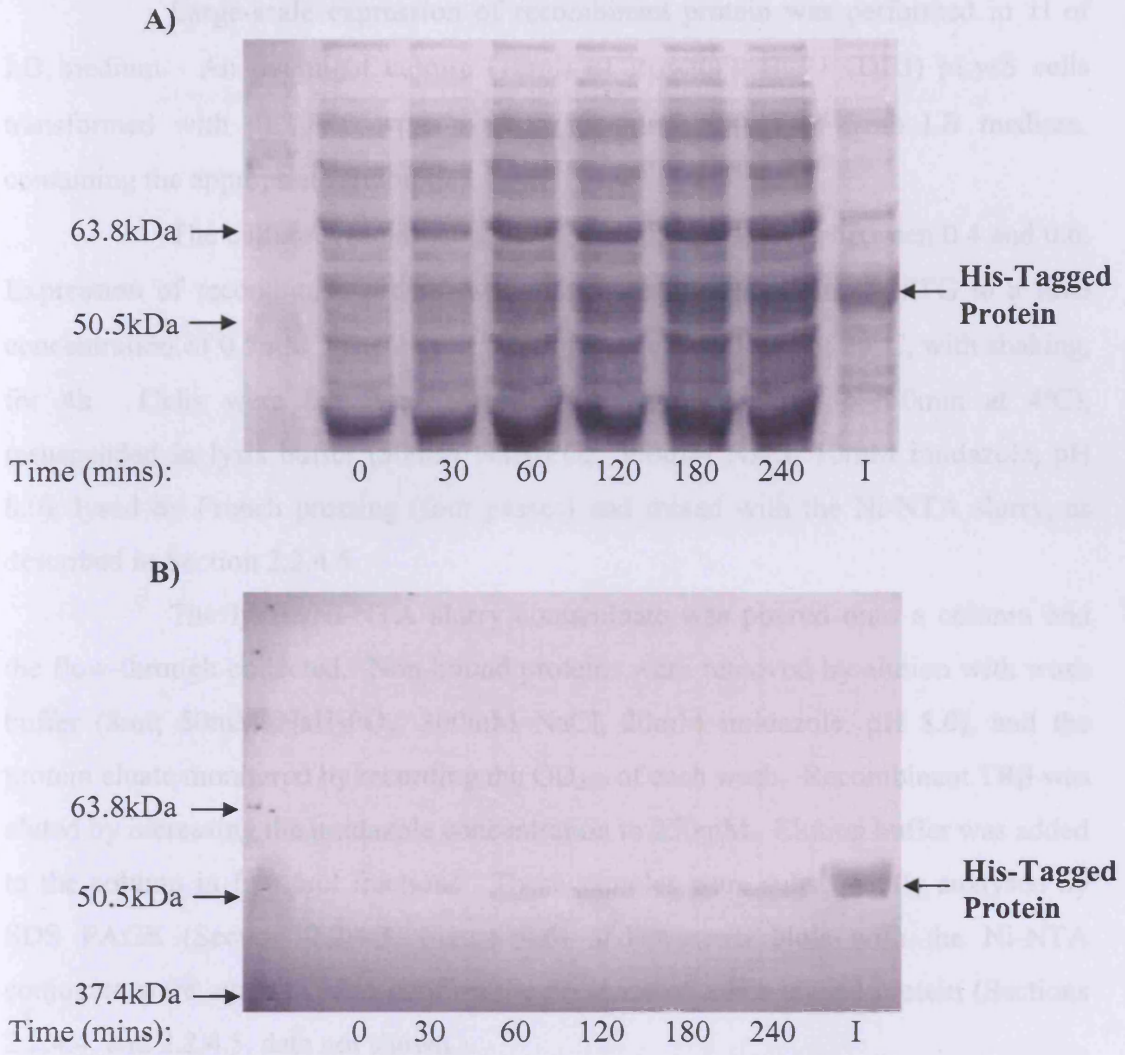


Figure 4.5. Production of soluble recombinant His-tagged TRβ. Analytical scale cultures were induced with 0.5mM IPTG and subsequently grown at 20°C. A Coomassie Brilliant Blue stained SDS-PAGE gel (**Panel A**) and a western blot probed with Ni-NTA AP conjugate (**Panel B**) was performed along a temporal profile of protein induction. Soluble extracts (10µl sample volume) from samples harvested at 0, 30, 60, 120, 180, and 240 minutes are shown in the respective lanes whilst Lane I shows 10µl of the insoluble fraction harvested after 240 minutes.

4.2.5. Preparative Scale Expression of TRβ, and Purification of Recombinant Protein using Ni-NTA Agarose.

Large-scale expression of recombinant protein was performed in 1l of LB medium. An overnight culture (10ml) of Rosetta™ BL21 (DE3) pLysS cells transformed with pETTRβ, was used to inoculate 500ml of fresh LB medium, containing the appropriate antibiotics.

The culture was grown at 37°C until the OD_{600} was between 0.4 and 0.6. Expression of recombinant protein was induced by the addition of IPTG to a final concentration of 0.5mM. The culture was subsequently grown at 20°C, with shaking, for 4h. Cells were harvested by centrifugation (10,000 x *g*, 10min at 4°C), resuspended in lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0), lysed by French pressing (four passes) and mixed with the Ni-NTA slurry, as described in Section 2.2.4.6.

The lysate/Ni-NTA slurry contaminate was poured onto a column and the flow-through collected. Non-bound proteins were removed by elution with wash buffer (8ml; 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0), and the protein eluate monitored by recording the OD_{280} of each wash. Recombinant TRβ was eluted by increasing the imidazole concentration to 250mM. Elution buffer was added to the column in five 1ml fractions. These samples were subsequently analysed by SDS PAGE (Section 2.2.4.3, Figure 4.6) and western blots with the Ni-NTA conjugate were again used to confirm the presence of a His-tagged protein (Sections 2.2.4.4. and 2.2.4.5, data not shown.).

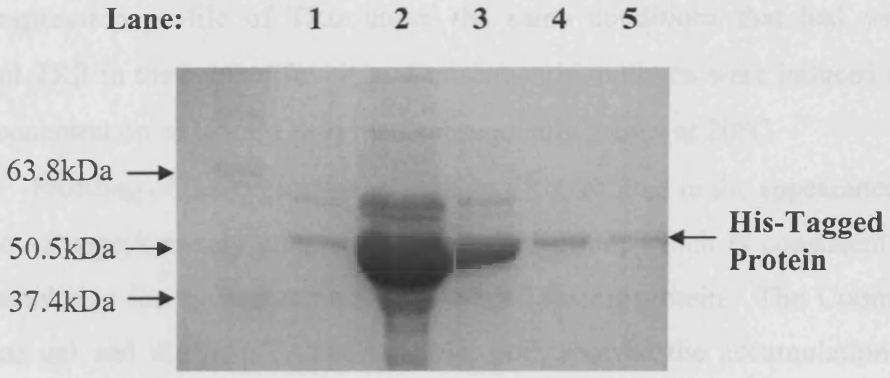


Figure 4.6. Large-scale purification of recombinant TRβ, using Ni-NTA resin. 1ml of an overnight culture (10ml) of Rosetta™ BL21 (DE3) pLysS cells transformed with pETTRβ, was used to inoculate 500ml of fresh LB medium. The culture was grown at 37°C until the OD_{600} was between 0.4 and 0.6 and recombinant protein expression was induced by the addition of 0.5mM IPTG followed by growth at 20°C. Cells were harvested after 4hours, lysed (French Press) and mixed with the Ni-NTA slurry. The lysate/Ni-NTA slurry contaminate was poured onto a column and non-bound proteins removed by elution with wash buffer (containing 20mM Imidazole). Recombinant TRβ was eluted from the column by increasing the imidazole concentration to 250mM. A Coomassie Brilliant Blue stained SDS-PAGE gel shows five 1ml elutions (5µl sample volume).

4.2.6. Optimisation of Induction Conditions for Expression of Recombinant TRα.

The TRβ cDNA was cloned and recombinant protein was successfully expressed before completion of the TRα cDNA subcloning. Therefore, upon completion of the cloning and subcloning of TRα, it was decided to study the temporal expression profile of TRα under the same conditions that had yielded recombinant TRβ in the soluble fraction. Consequently, cultures were induced using an IPTG concentration of 0.5mM, and then subsequently grown at 20°C.

Profiling of the expression of soluble TRα resulted in the appearance of a single band of approximately 46kDa 1hour post-induction, which is consistent with the anticipated size for the recombinant His-tagged fusion protein. The Coomassie Blue stained gel and the Ni-NTA western blot both showed the accumulation of a band of the expected size (Figure 4.7). Thus, it was possible to successfully express and purify TRα, on a preparative scale, following identical conditions used in the expression and purification of recombinant TRβ, as described in Section 4.2.5, (Figure 4.8).

4.2.7. Long-term Storage of Purified, Recombinant TRα and TRβ.

In order to allow long-term storage of purified recombinant proteins at -20°C, it was necessary to dialyse the protein into the appropriate storage buffer (50% glycerol in PBS, 5mM DTT). In each instance, fractions 2, 3 and 4 (from Figures 4.6 and 4.8, for TRβ and TRα respectively) were pooled and dialysed into storage buffer, as described in Section 2.2.4.8. The storage of recombinant protein in any buffer other than the above storage buffer was found to cause precipitation of protein. Protein concentration was measured using the Bradford Asssay, as described in Section 2.2.4.7.a.

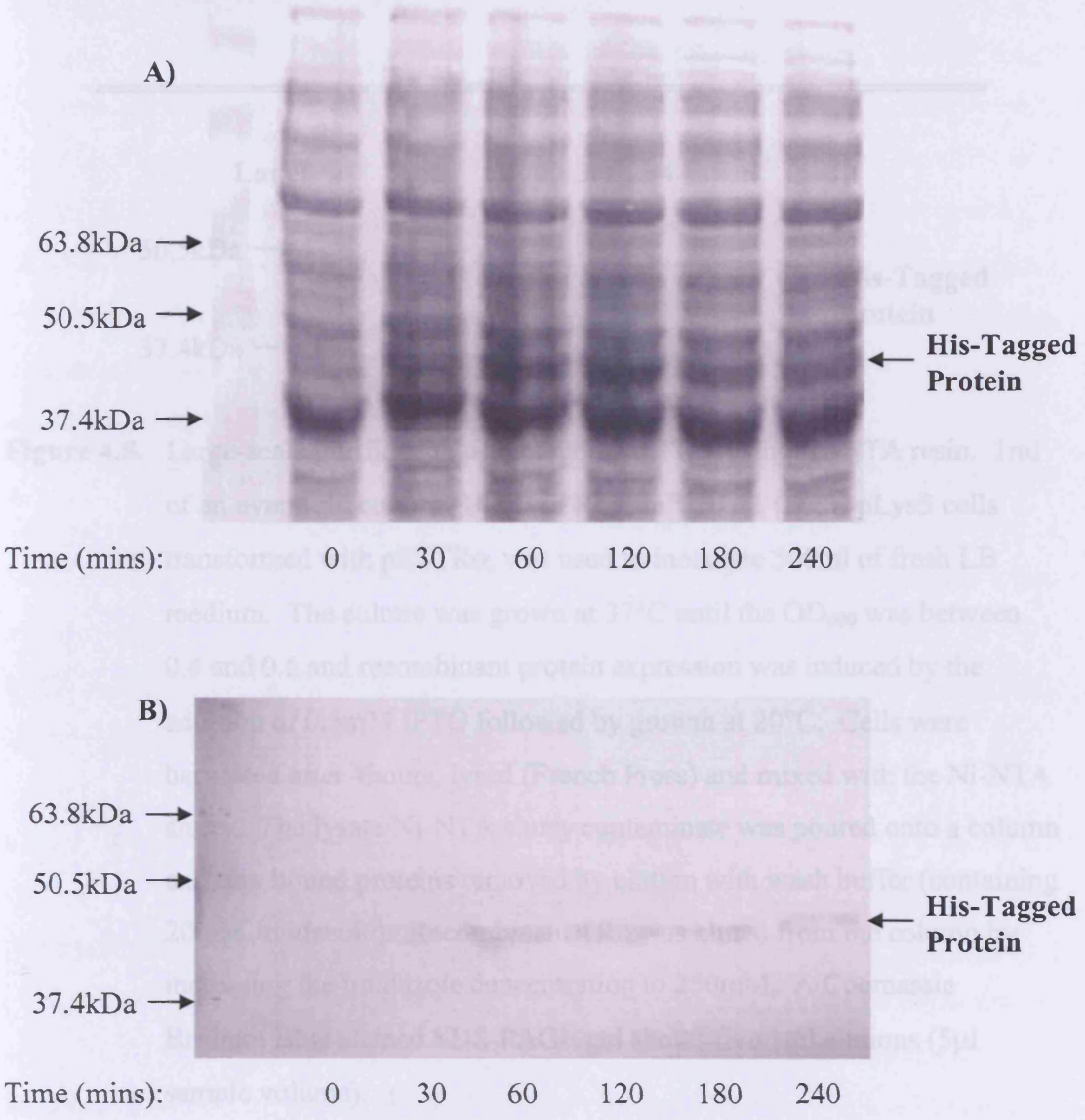


Figure 4.7. Production of soluble recombinant His-tagged TRα Analytical scale cultures were induced with 0.5mM IPTG and subsequently grown at 20°C. A Coomassie Brilliant Blue stained SDS-PAGE gel (**Panel A**) and a western blot probed with Ni-NTA AP conjugate (**Panel B**) was performed along a temporal profile of protein induction. Soluble extracts (10µl sample volume) from samples harvested at 0, 30, 60, 120, 180, and 240 minutes are shown in the respective lanes whilst Lane I.

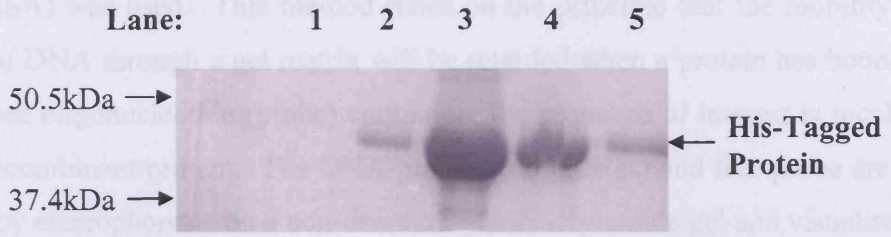


Figure 4.8. Large-scale purification of recombinant TRα, using Ni-NTA resin. 1ml of an overnight culture (10ml) of Rosetta™ BL21 (DE3) pLysS cells transformed with pETTRα, was used to inoculate 500ml of fresh LB medium. The culture was grown at 37°C until the OD_{600} was between 0.4 and 0.6 and recombinant protein expression was induced by the addition of 0.5mM IPTG followed by growth at 20°C. Cells were harvested after 4hours, lysed (French Press) and mixed with the Ni-NTA slurry. The lysate/Ni-NTA slurry contaminate was poured onto a column and non-bound proteins removed by elution with wash buffer (containing 20mM Imidazole). Recombinant TRα was eluted from the column by increasing the imidazole concentration to 250mM. A Coomassie Brilliant Blue stained SDS-PAGE gel shows five 1ml elutions (5µl sample volume).

4.2.8. EMSA Analysis.

4.2.8.1. Introduction.

In order to assess whether or not the recombinant protein was functional (i.e. was correctly folded and able to bind DNA) the electrophoretic mobility shift assay (EMSA) was used. This method relies on the principle that the mobility of a fragment of DNA through a gel matrix will be retarded when a protein has bound. A radiolabelled oligonucleotide (probe) containing the sequence of interest is incubated with the recombinant protein. The DNA-protein complex(es) and free probe are then separated by elecrophoresis on a non-denaturing polyacrylamide gel and visualised by autoradiography.

A double-stranded DNA duplex containing the ME-TRE (malic enzyme thyroid response element, see next Section, below) was made by annealing a complementary oligonucleotide pair as described in Section 2.2.4.9.a (for oligo sequences, see Appendix 1E). The annealed duplex was radiolabelled with [α-^{32}P]-dCTP as described in Section 2.2.4.9.b. The annealed, radiolabelled duplex will henceforth be referred to as the "probe".

4.2.8.2. Probe Design.

The ME gene is involved in lipid metabolism, in particular, lipid synthesis. Its expression is ubiquitous, but is only up-regulated by T3 in the rat kidney, heart, and most prominently – the liver, which is a major target organ for the action of T3 (Jeannin *et al.*, 1998; Petty *et al.*, 1990). Liver malic enzyme (ME) is a tetrameric protein that catalyzes the reversible oxidative decarboxylation of L-malate to give carbon dioxide and pyruvate with the concomitant reduction of $NAD(P)^+$ to NAD(P)H:

$$NAD(P)^+ + \text{L-malate} \rightleftharpoons CO_2 + \text{pyruvate} + NAD(P)H + H^+$$

The NAD(P)H produced is utilised in the biosynthesis of long-chain fatty acids or steroids, hence the classification of ME as a lipogenic enzyme (Chang and Tong, 2003). As has been previously mentioned (Section 1.5.1) TRs regulate target gene expression through binding to specific DNA sequences called TREs (thyroid

response elements), in the regulatory regions of target genes. TRs can enhance or inhibit gene expression depending on the nature of the TRE. Heterodimer formation with RXR enhances DNA-binding affinity, and provides target gene specificity, as determined by the spacing between the two half sites. Accordingly, TR/RXR binds DR4, that is, a direct repeat - two half sites in one orientation, separated by 4bp. The half-site consensus sequence recognised by TRs often contains the sequence AGGTCA, and the direct repeat is the most potent, although other arrangements are possible (detailed in Section 1.5.1).

The ME-TRE (malic enzyme thyroid-responsive element), is a direct repeat motif with a 4-bp gap between the two hexamers, a DR4:

5' -----aggacgttgGGGTTAggggAGGACAgtg----- 3'

Initial studies confirmed that the ME-TRE is a positive TRE, i.e. the transcription of genes under its control is activated by ligand-bound TRs, whilst transcription is repressed by unliganded TRs (Desvergne *et al.*, 1991; Petty *et al.*, 1990). Subsequent studies (Miyamoto *et al.*, 1993) employed the electrophoretic mobility shift assay (EMSA) to demonstrate that both TRα and TRβ are able to bind the ME-TRE as monomers and homodimers. It was for these reasons that it was decided to use the ME-TRE to assess whether or not the purified TRs were able to bind DNA.

4.2.8.3. Analysis of Protein-DNA Interactions.

Each EMSA experiment was run on a minimum of two but more typically on three or more separate occasions in order to ensure reproducibility. In each instance, data shown is representative of a single experiment. Unless otherwise specified, "TR" refers to the purified, recombinant, N-terminal His-tagged murine forms of the fusion protein.

EMSA experiments were carrried out following the conditions outlined in Section 2.2.3.9.c. Figure 4.9 shows the binding of purified recombinant TRα and TRβ (40pmol of each) to radiolabelled probe (3pmol). Shown also, as a positive control, is the binding of chicken TRα to probe (purified, recombinant C-terminal GST-tagged chicken TRα fusion protein, cTRα, 40pmol; Insight Biotech Ltd).

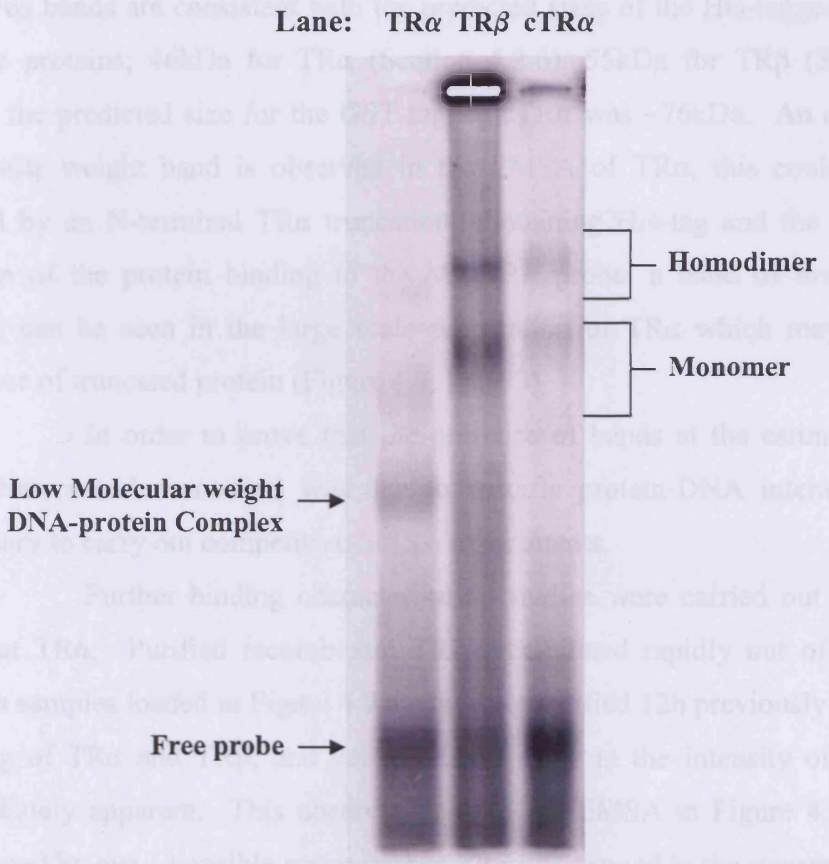


Figure 4.9. The binding of recombinant TRs to the ME-TRE probe, analysed by the electrophoretic mobility shift assay. Recombinant TR (~40pmol) was combined with radiolabelled probe (3pmol) and subjected to electrophoresis through a non-denaturing acrylamide gel matrix. Following electrophoresis, the gel was dried and bands were visualised by autoradiography. Recombinant TRα, TRβ and cTRα was loaded in Lane 1, 2 and 3 respectively.

Figure 4.9 shows that purified, recombinant TRα and TRβ were able to bind the ME-TRE probe. This result is confirmed by a previous study (Miyamoto *et al.*, 1993) which also employed the EMSA to demonstrate that TRα and TRβ are able to bind the ME-TRE as monomers and homodimers. The approximate sizes of the observed bands are consistent with the predicted sizes of the His-tagged recombinant murine proteins; 46kDa for TRα (Section 4.2.6), 55kDa for TRβ (Section 4.2.3), whilst the predicted size for the GST-tagged cTRα was ~76kDa. An additional low molecular weight band is observed in the EMSA of TRα, this could possibly be caused by an N-terminal TRα truncation, containing His-tag and the DNA-binding domain of the protein binding to the ME-TRE probe; a band of lower molecular weight can be seen in the large scale preparation of TRα which may indicate the presence of truncated protein (Figure 4.8, Lane 3).

In order to prove that the presence of bands at the estimated sizes for homodimers and monomers was due to specific protein-DNA interactions it was necessary to carry out competitive EMSA experiments.

Further binding characterisation studies were carried out only on TRβ and not TRα. Purified recombinant TRα precipitated rapidly out of solution; the protein samples loaded in Figure 4.9 had been quantified 12h previously to allow even loading of TRα and TRβ, and yet the discrepancy in the intensity of the bands is immediately apparent. This observation from the EMSA in Figure 4.9 was further confirmed by eye – a visible precipitate of TRα had formed in the storage tube.

4.2.8.4. Examination of the Specificity of DNA-Protein Complex Formation; Competition EMSA Analysis.

In order to confirm whether the DNA-protein complexes shown in Figure 4.9 represented specific interactions of recombinant TRβ with the radiolabelled probe, competition EMSA was carried out on TRβ (40pmol) as described in Section 2.2.4.10.

As shown in Figure 4.10, the intensities of the bands in complexes C1 and C2 decreased as the fold excess of the unlabelled ME-TRE oligo was increased. However, when the maximum (500-fold) excess of unrelated, non-specific, unlabelled duplex containing the NFκB binding site was used, no such competition was observed. This indicated that both complexes represented specific interactions of TRβ with the radiolabelled ME-TRE probe.

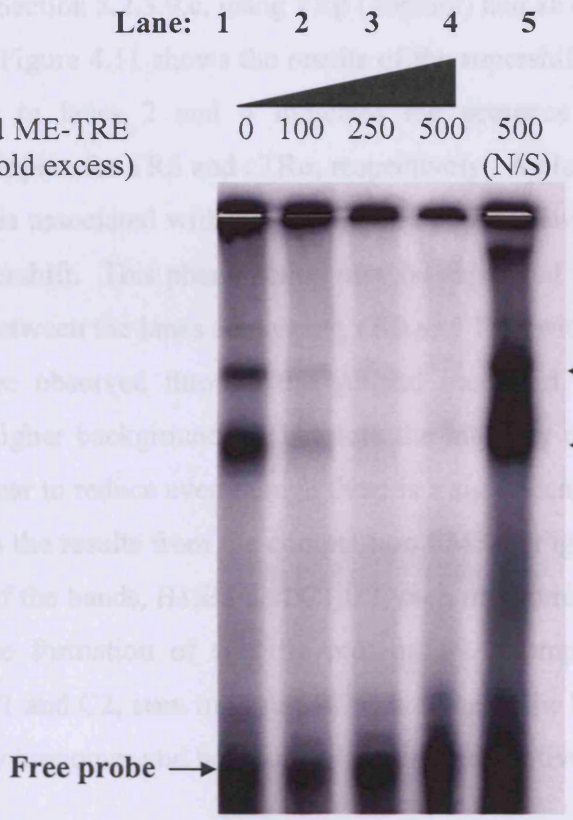


Figure 4.10. Competition studies of binding of TRβ to the ME-TRE probe, analysed by the electrophoretic mobility shift assay (EMSA). Recombinant TRβ (40pmol), combined with radiolabelled probe (3pmol) and the indicated fold (Molar) excess of either non-labelled ME-TRE (lanes 1-4) or non-specific (N/S) probe (lane 5) were subjected to electrophoresis through a non-denaturing acrylamide gel matrix. Following electrophoresis, the gel was dried and bands were visualised by autoradiography. C1 and C2 refer to the DNA-protein complexes observed.

4.2.8.5. Antibody Supershift Assays.

In order to confirm the identity of the DNA-protein complexes (C1 and C2) seen in Figure 4.10, antibody supershift experiments were performed using an antibody which recognises both the TRα and TRβ isoforms (a gift from Prof. Jiemin Wong, Baylor College of Medicine, Houston, Texas, USA). Reactions were set up as described in Section 2.2.3.9.c, using TRβ (40pmol) and an excess of anti-TR antibody.

Figure 4.11 shows the results of the supershift assay. The appearance of complex C3 in lanes 2 and 4 indicates the presence of an antibody-TR-DNA supershift complex for TRβ and cTRα, respectively. A clear reduction in the intensity of cTRα C1 is associated with the appearance of C3, however this is not observed in the TRβ supershift. This phenomenon may be explained through differential loading of material between the lanes containing TRβ and TRβ with antibody, an indication of which can be observed through the general increased intensity of the supershift separation (higher background). Therefore the intensity of C1 in the TRβ supershift does not appear to reduce even though there is a significant C3 band. This data, taken together with the results from the competition EMSA (Figure 4.10), confirms that the appearance of the bands, B1;B2 and C1;C2, seen in Figures 4.9 and 4.10 respectively, is due to the formation of specific protein-DNA complexes. In the same way, complexes C1 and C2, seen in Figure 4.11, are due to the binding of TR to DNA, and are caused by monomer and homodimer binding, respectively.

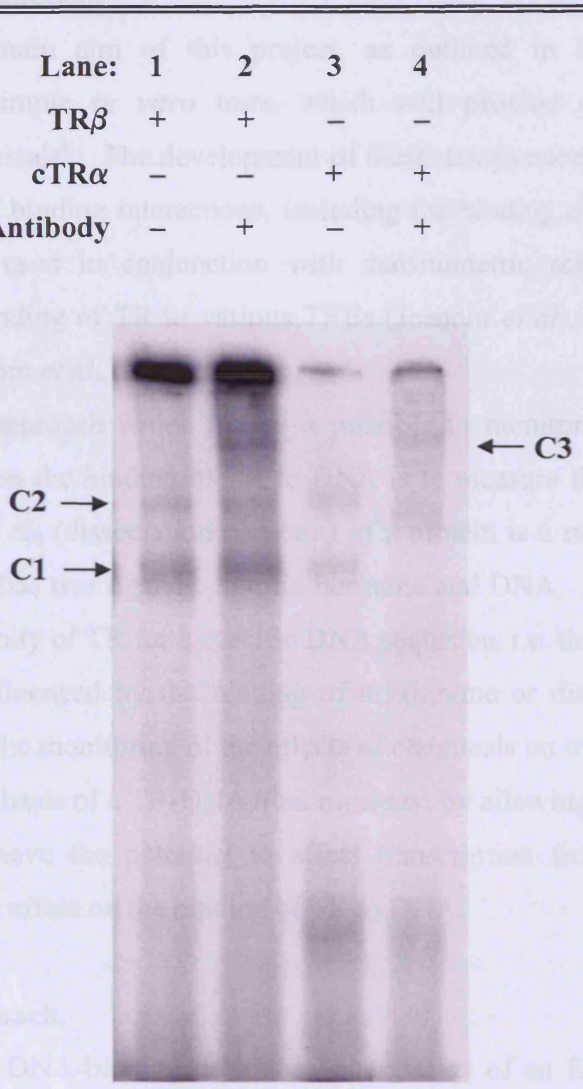


Figure 4.11. EMSA analysis of antibody supershift assays, showing binding of an anti-TR antibody to TRβ and cTRα. Recombinant TR (40pmol) and radiolabelled probe (3pmol) were combined with an excess of the anti-TR antibody (which recognises TRα and TRβ) and subjected to electrophoresis through a non-denaturing acrylamide gel matrix. Following electrophoresis, the gel was dried and bands were visualised by autoradiography. Supershifts with TRβ and cTRα were performed with independent batches of probe and a composite autoradiogram generated.

4.2.9. EMSA Analysis as a way of Monitoring the Disruption of DNA-Protein Binding.

4.2.9.1. Introduction.

The main aim of this project, as outlined in Section 1.9, is "the development of simple *in vitro* tests, which will provide evidence of thyroid disruption by chemicals". The development of these assays necessitates being able to probe a number of binding interactions, including the binding of TRs to DNA. The EMSA has been used in conjunction with densitometric scanning of results to characterise the binding of TR to various TREs (Jeannin *et al.*, 1998; Darling *et al.*, 1993; and Wahlström *et al.*, 1992).

One approach which makes it possible to monitor whether a potential disruptor impacts on the binding of TR to DNA is to measure the affinity of the TR for the DNA. The K_d (dissociation constant) of a protein is a measure of its affinity for its ligand. TR has two ligands; thyroid hormone and DNA. It should be possible to quantify the affinity of TR for a specific DNA sequence, i.e. the K_d of TR for DNA. This affinity is influenced by the binding of a hormone or disruptor to the ligand binding domain. The monitoring of the effects of chemicals on the K_d of TR for DNA should provide the basis of a TR-DNA binding assay, by allowing the identification of chemicals which have the potential to affect transcription from thyroid-regulated genes by having an effect on the binding of TR to DNA.

4.2.9.2. Approach.

For a DNA-binding protein in the context of an EMSA, the K_d of the protein is approximately equal to the protein concentration at which half the available DNA (probe) has become bound (Carey, J., 1991). The first step in attempting to develop a system to monitor the modulation of DNA-binding therefore, was to estimate the K_d of TR for the ME-TRE probe. This was done using the EMSA; TR was titrated against a constant amount of probe, the amount of free probe was quantified by densitometric scanning of gels and the K_d was estimated from a plot of TR concentration vs. percentage of free probe.

In order to determine whether or not a potential DNA-binding modulator had an effect on TR binding to DNA the potential modulator was titrated against a constant amount of TR and probe and free probe was quantified as before

(densitometric scanning of gels). The TR concentration used was the K_d as determined for TR in the absence of T3. An assay in this format allowed the effects of a large range of concentrations of potential binding modulators to be assessed. Therefore, it was possible to assess T3 and its analogues for potential effects on the binding of TR to DNA.

It was necessary to dissolve THs and their analogues in the required solvent, as per the manufacturer's instructions (Sigma). In order to ensure that any observed effects were not caused by the presence of these solvents a negative control reaction was performed alongside each TH / TH-analogue titration. Negative reactions consisted of an equivalent final concentration of the solvent, in the absence of any TH / TH-analogue. All negative control reactions showed that the solvent present had no effect on the binding of TR to DNA. Each experiment was run on a minimum of two but more typically on three or more separate occasions in order to ensure reproducibility. In each case, data shown is from a single experiment but is representative of multiple experiments.

4.2.9.3. Estimating the K_d of Recombinant TRβ.

a) Ligand-free TRβ.

A wide range of TRβ concentrations (0-400pmol/reaction) were titrated against a constant probe concentration (0.3pmol/reaction). The midpoint concentration was estimated by eye from the radiogram in order to give an indication of the approximate magnitude of the dissociation constant, K_d (data not shown). This allowed a second protein titration, of a narrower concentration range, to be carried out. TRβ (0-120pmol/reaction) was combined with ME-TRE probe (0.3pmol/reaction), as described in Section 2.2.3.9.c and complexes were separated by gel electrophoresis (Section 2.2.3.9.d). The results are shown in Figure 4.12. Following electrophoresis and gel-drying, the radioactivity associated with bands was quantified as described in Section 2.2.3.9.e. The quantitative data was plotted as percentage of free probe vs TRβ concentration, Figure 4.13. An estimation of the TRβ concentration which binds approximately half the probe from Figure 4.13 gives a K_d of approximately 1.0μM (final volume of an EMSA reaction is 40μl, Section 2.2.4.9.c). This estimate of K_d is only valid if the DNA concentration is negligible compared to the protein concentration at the midpoint (at least 10-, and preferably 100-fold lower; Carey,

1991). A constant amount of probe (0.3pmol) was added to each titration reaction (final volume 40μl) – this equates to a probe concentration of 0.0075μM.

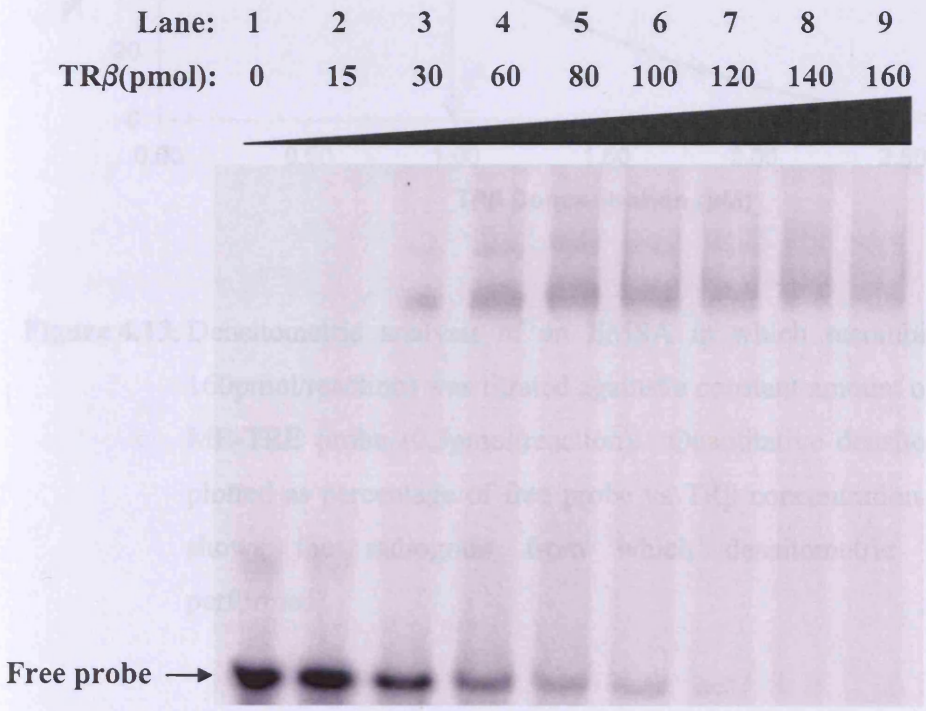


Figure 4.12. EMSA analysis showing a titration of TRβ binding to the ME-TRE probe. Recombinant TRβ (0-160pmol) was titrated with radiolabelled probe (0.3pmol) and subjected to electrophoresis through a non-denaturing acrylamide gel matrix. Following electrophoresis, the gel was dried and bands were visualised by autoradiography.

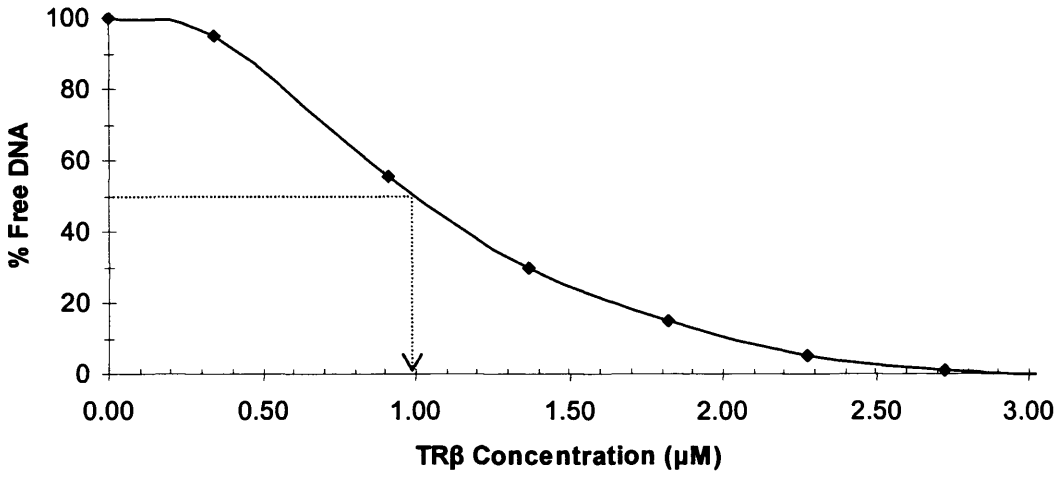


Figure 4.13. Densitometric analysis of an EMSA in which recombinant TRβ (0-160pmol/reaction) was titrated against a constant amount of radiolabelled ME-TRE probe (0.3pmol/reaction). Quantitative densitometric data is plotted as percentage of free probe vs TRβ concentration. (Figure 4.12 shows the radiogram from which densitometric analysis was performed).

b) Ligand-bound TRβ.

In order to assess whether the above method for estimating the K_d of TR is valid, it was decided to carry out a second protein titration, this time in the presence of T3 (100nM). Since T3 has been shown to lower the affinity of TR for DNA (Piedrafita *et al.*, 1995; Sugawara *et al.*, 1994 and Yen *et al.*, 1992) it should be possible to measure an increase in the K_d of TR for the ME-TRE probe. A TRβ titration was carried out following the conditions detailed above, except with the addition of T3 to a final concentration of 100nM. Complexes were separated by electrophoresis and gels dried. Results were analysed as before, and the radiogram and quantitative data are shown in Figures 4.14 and 4.15, respectively. An estimation of the TRβ concentration which binds approximately half the probe from Figure 4.15 gives a K_d of approximately 1.8μM.

This increase in K_d is reflective of a decrease in the affinity of TRβ dimers for the ME-TRE DNA and is consistent with previous studies. Miyamoto *et al.*, (1993) compared a non-T3-binding mutant with a wild-type receptor (TRβ) and demonstrated that the addition of T3 induced dimer-specific dissociation from the ME-TRE whilst monomer binding remained virtually unaffected. The observed ligand-dependant disruption of dimer-DNA complex occurs due to a conformational change in TR which is caused by the binding of ligand to TR (Miyamoto *et al.*, 1993).

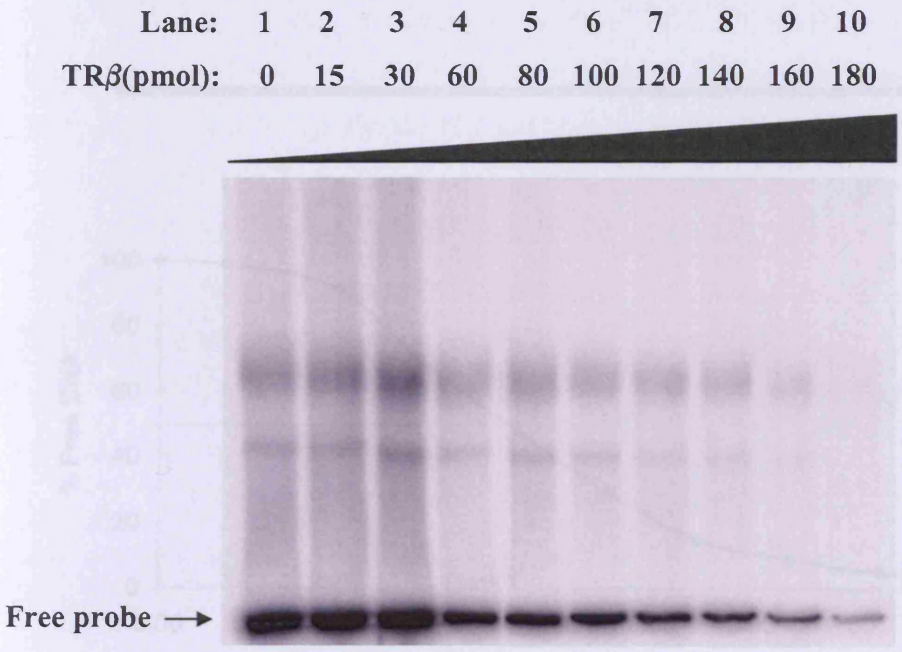


Figure 4.14. EMSA analysis showing a titration of ligand-bound TRβ binding to the ME-TRE probe. Recombinant TRβ (0-180pmol) was titrated with radiolabelled probe (0.3pmol) in the presence of T3 (100nM) and subjected to electrophoresis through a non-denaturing acrylamide gel matrix. Following electrophoresis, the gel was dried and bands were visualised by autoradiography.

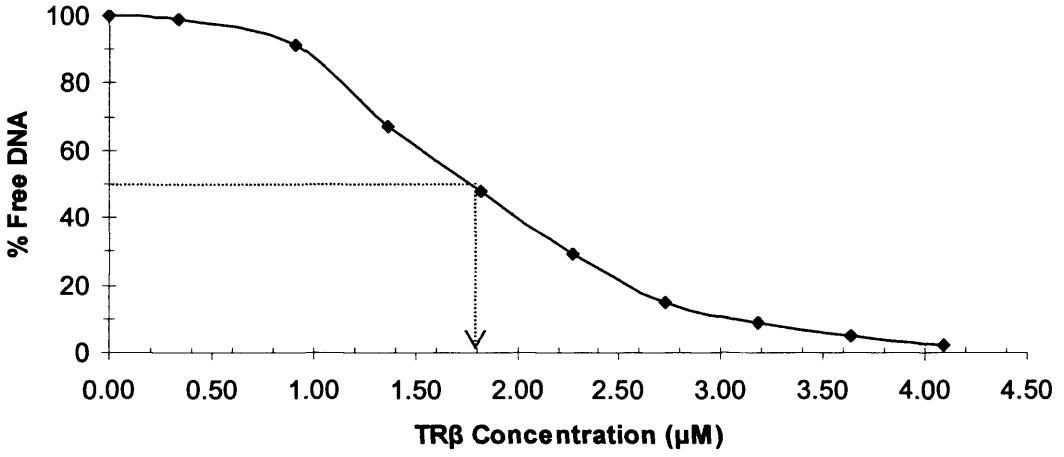


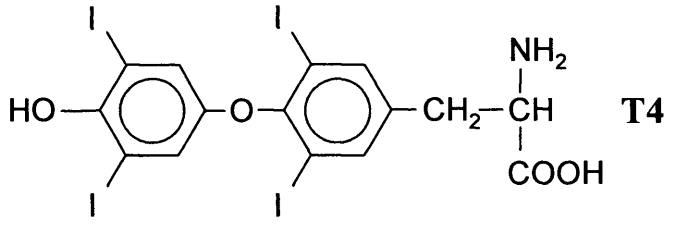
Figure 4.15. Densitometric analysis of an EMSA in which ligand-bound recombinant TRβ (100nM T3, 0-180pmol TRβ/reaction) was titrated against a constant amount of radiolabelled ME-TRE probe (0.3pmol/reaction). Quantitative densitometric data is plotted as percentage of free probe vs TRβ concentration. (Figure 4.14 shows the radiogram from which densitometric analysis was performed).

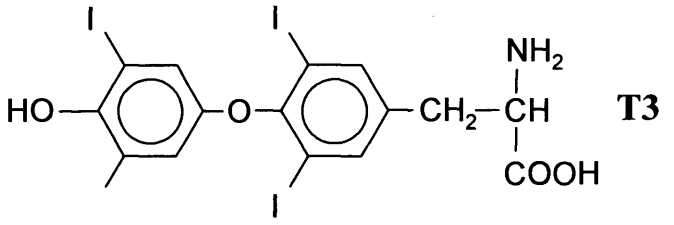
4.2.9.4. The Effect of Thyroid Hormones and their Analogues on the Binding of Recombinant TRβ to DNA.

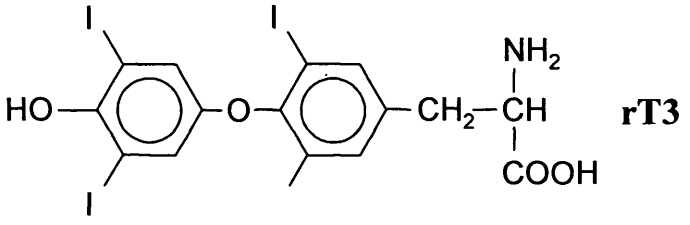
The relative affinity of TR for THs and their analogues have been described previously (Cheek *et al.*, 1999 and Ribeiro *et al.*, 1992) and are as follows: T3 > T4 > Tetrac (tetraiodothyroaceticacid) > rT3". Outer-ring deiodination of T4 gives the biologically active T3, whilst inner-ring deiodination converts T4 to the biologically inactive metabolite rT3 – which accounts for about 40% of T4 turnover and is the key step in the inactivaton of TH. The structures of the THs and the TH analogues which were analysed are shown in Figure 4.16.

Following previous studies, which showed that the higher the affinity of TR for the ligand, the greater the disruption of TR binding to DNA (Miyamoto *et al.*, 1993 and Yen *et al.*, 1992) it was decided to compare these results with the results gained using the technique described above.

Recombinant TRβ to a final concentration of 1.0μM (the K_d of ligand-free TRβ as determined in Section 4.2.9.3.a) was combined in an EMSA reaction with a constant amount of radiolabelled ME-TRE probe (0.3pmol/reaction) and varying concentrations of either THs or their analogues, as described in Section 2.2.3.9.c. Complexes were separated by gel electrophoresis, the gels dried and the radioactivity associated with bands was quantified as described in Sections 2.2.3.9.d. and 2.2.3.9.e, respectively. The quantitative data is plotted relative to the amount of free probe observed in the absence of the relevant compound (arbitarily designated as 1).







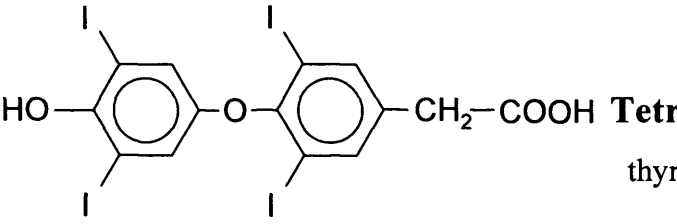


Figure 4.16. Thyroid hormones and their analogues.

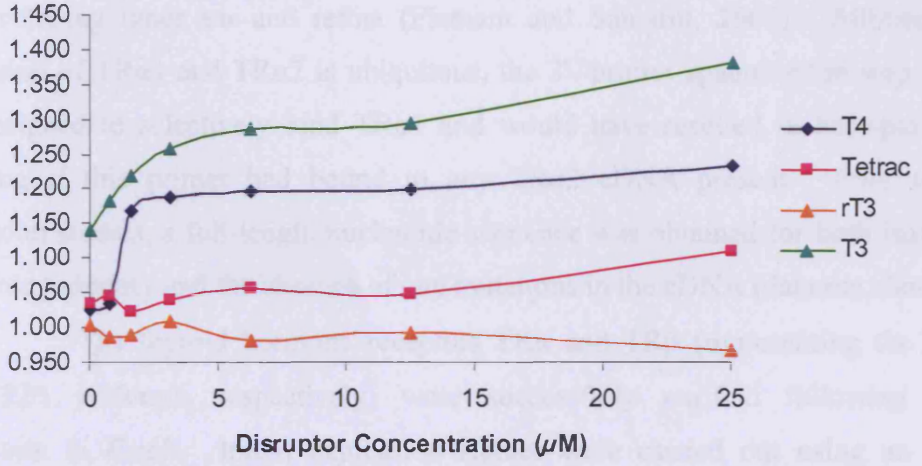


Figure 4.17. Densitometric analyses of EMSAs in which the effect of THs and their analogues on binding of TRβ to the ME-TRE probe was analysed. A constant concentration of recombinant TRβ (1μM) and radiolabelled ME-TRE probe (0.3pmol/reaction) was incubated with different concentrations of T3 (▲), T4 (♦), rT3 (▲) and Tetrac (■). Quantitative densitometric data is plotted relative to the amount of free probe observed in the absence of the relevant compound. Each data set shown is representative of a single experiment. Abbreviations: T3, triiodothyronine; T4, tetraiodothyronine; rT3, reverse triiodothyronine and Tetrac, Tetraiodothyroaceticacid (see Figure 4.16 for chemical structures; see Appendix 4 for individual densitometric plots.

4.3 Discussion.

The amplification of the cDNAs for TRα1 and TRβ1 from mouse heart cDNA, using gene-specific primers, resulted in the appearance of a single major product in both cases (Figure 4.3), even though there is more than one isoform of TRα and TRβ (discussed previously, Section 1.2.1). This was as expected for TRβ, as TRβ2 expression is limited mainly to the pituitary, the hypothalamic TRH neurons, the developing inner ear and retina (Flamant and Samarut, 2003). Although the expression of TRα1 and TRα2 is ubiquitous, the 3'- primer spanning the stop codon was designed to selectively bind TRα1 and would have resulted in base-pair mis-matching if this primer had bound to any TRα2 cDNA present. Prior to any expression studies, a full-length nucleotide sequence was obtained for both isoforms, confirming identity and the absence of any mutations in the cDNA (data not shown).

The thyroid hormone receptors TRα and TRβ (representing the TRα1 and TRβ1 isoforms, respectively) were successfully purified following over-production in *E.coli.* Initial expression studies were carried out using an IPTG concentration of 1mM and an induction growth temperature of 37°C. This resulted in the production of insoluble recombinant TR (TRβ: Figure 4.4, TRα: data not shown). Subsequent optimisation of the expression conditions allowed the expression of soluble protein; culture conditions can have a dramatic effect on solubility and localization of expressed protein - conditions that decrease the rate of protein synthesis, such as low induction temperatures, tend to increase the percentage of target protein found in soluble form by decreasing the rate of protein self-assocation and the formation of inclusion bodies (insoluble protein). This would explain the observation of increased expression levels of soluble TRα and TRβ when induced at 0.5mM IPTG with subsequent growth at 20°C (Figures 4.5 and 4.7 respectively).

The expression of recombinant TRs as His-tagged fusion proteins allowed confirmation of protein identity following small-scale optimisation experiments. An Ni-NTA AP conjugate, which binds to His-tagged proteins, was used to probe Western blots of recombinant protein samples (Figures 4.5.b and 4.7.b for TRβ and TRα respectively). A second advantage of expressing TRs as His-fusions was exploited to allow a one-step purification following a large-scale induction, using Ni-NTA agarose as described in Section 4.2.5. Production of recombinant TR in a

soluble form did not necessarily indicate that the protein was folded properly. It was essential to establish that the recombinant TRs exhibited DNA binding activity.

EMSA analysis was used to demonstrate that both recombinant TRα and TRβ were able to bind the ME-TRE probe as monomers and dimers (Figure 4.9). These results were in agreement with previous studies (Miyamoto *et al.*, 1993), which also showed that recombinant TRα and TRβ were able to bind to an ME-TRE containing oligo. The specificity of protein-DNA interactions was verified by carrying out a competition EMSA (Figure 4.10) and the identity of protein-DNA complexes was confirmed using an antibody supershift EMSA, which employed a TR-specific antibody (Figure 4.11).

Previous studies have used EMSA to characterise the DNA binding properties of TR monomers, TR-TR homodimers and TR-RXR heterodimer in the absence and presence of T3. The overall number of TR homodimers binding to inverted palindromic TREs was reduced by T3 but the affinity of the bound TR homodimers for the TREs was unaltered (Ribeiro *et al.*, 1992). This occurs due to a conformational change caused by ligand binding, as confirmed by a later study (Miyamoto *et al.*, 1993). This study also demonstrated that the binding of TR homodimers to the ME-TRE is decreased in the presence of T3, whilst the binding of a TR mutant (with no ligand-binding ability) to the ME-TRE was not altered in the presence of T3. Yen *et al.*, (1992) showed that the addition of TH analogues led to a decrease in binding of TR homodimers to an inverted palindromic TRE, but that the binding of TR monomers was unaffected. Miyamoto *et al.*, (1993) showed that addition of T3 (and its analogues), whilst decreasing homodimer binding, did not lead to a decrease in the binding of TR monomers to the ME-TRE. Both studies also showed that the higher the affinity of TR for a particular ligand, the greater the disruption of TR binding to DNA (Miyamoto *et al.*, 1993 and Yen *et al.*, 1992).

Hence, the results shown in Figure 4.17 are consistent with those published previously. This indicates that the EMSA can be used to monitor suspected chemicals for potential effects on the binding of recombinant TR to a TRE-containing oligo and so is a convenient starting point for the development of non-radioactive TR-DNA binding assays. There are a number of technical limitations associated with the EMSA technique which also make it desirable to develop non-radioactive assays. There is inherent variability stemming from differences in efficiency of probe-labelling, differential loading of samples onto the gel and radioactive decay of the

probe. These issues may be addressed cosmetically by optimising the exposure time of the gel to film but to establish true reproducibility the specific activity of each probe must be constant and the quantity loaded on the gel identical. EMSA assays were exploited in the characterisation of the recombinant TR in a qualitative mode to establish whether the recombinant molecule bound the target the DNA sequence. Deployment of EMSA assays to determine binding coefficients (in the presence or absence of a potential disruptor) required only quantitative comparison within a gel, where the specific activity of the probe was identical.

Therefore, an assay system was successfully developed, which allowed the monitoring of TR binding to DNA in the presence of a potential disruptor. The potential now exists to further optimise this system to allow the development of a non-radioactive, high-throughput assay. Recombinant His-tagged TR could be immobilised to a solid phase (Ni-NTA beads) and bound to oligonucleotide probes labelled with the previously described chemiluminescent molecule, the acridinium ester. Any free AE-labelled probe could then be washed away and thus, detection of the remaining chemiluminescence associated with the beads would allow the quantification of DNA bound to the immobilised TR. If such an assay were optimised it would then be possible to monitor the effects of potential disruptors on the binding of recombinant TR to its cognate DNA.

CHAPTER FIVE

CELL CULTURE SYSTEMS.

5.1. Introduction

The effects of THs are mediated predominantly at the level of gene expression via thyroid hormone receptors (TRs: Section 1.5.1), which are ligand activated transcription factors that regulate target gene expression directly through binding to specific DNA sequences, known as TREs, in the promoter regions of target genes (Zhang and Lazar, 2000; Wu and Koenig, 2000).

The deployment of a cell culture approach to monitor the effects of putative disruptors on T3-moderated gene transcription will act in accordance with the EDTA's recommendations to develop non-animal screening assays, whilst still maintaining a degree of biological relevance. Whilst revealing the effects of putative disruptors on the formation of a transcriptionally active complex a cell culture approach will also allow the addressing of two important factors which act to modulate gene expression; cell entry and the metabolism of potential disruptors.

The thyroid-responsive luciferase reporter construct pcTRELuc consists of the thyroid response element from the Malic Enzyme gene (ME-TRE) upstream of the coding sequence for the firefly luciferase gene (see Appendix 2D for vector map). In order to analyse the impact of potential disruptors on the transcriptional response mediated by TRs via the ME-TRE in a cellular environment, the reporter construct can be transfected into eukaryotic cells and the activity of the reporter gene can be measured by quantifying the levels of translated protein produced. In addition to measuring the levels of protein expressed from the reporter gene, it is also possible to measure levels of luciferase mRNA transcribed, and this can be done using the HPA (Section 3.3.7). The quantification of luciferase mRNA synthesis by HPA, would allow a comparison to be made between the temporal profiles for luciferase mRNA and protein expression. It is essential, therefore, that the quantities of mRNA harvested from transfected cells fall well within the detection range of the HPA, in order that the HPA can be used as a detection method.

5.2. Approach.

Although the final aim was to be able to assess thyroid-responsiveness by measuring luciferase levels expressed from a thyroid-responsive reporter construct (pcTRELuc), initial transfection studies were carried out in order to determine the number of cells required to allow detection of luciferase mRNA by HPA under optimal conditions, i.e. in the presence of high levels of luciferase mRNA expression in a robust, well-characterised cell line. Transient transfections were carried out using the calcium phosphate precipitation method or TransFast™ Reagent (a liposome based transfection reagent; Sections 2.2.2.3.a and b, respectively) using U373s (a human glioblastoma astrocytoma, adherent cell-line). The pRG224 vector was used, which contains the luciferase gene under the control of the hCMV immediate-early (I.E.) promoter, which is strong and constitutively active.

A large-scale preparation of pRG224 was carried out (Section 2.2.1.3.b.) and the purity and concentration of DNA assessed (Section 2.2.1.5). pRG224 DNA was then transiently transfected into cells using either the calcium phosphate precipitation method or TransFast™ transfection reagent. Following a 24h incubation at 37°C in an atmosphere of 5% CO_2 in air, cells were harvested, lysates were prepared and mRNA was purified (Sections 2.2.2.5.a and b, respectively). Lysates were prepared from transfected cells (Section 2.2.2.5.a) and then analysed, in duplicate, for luciferase activity and total cellular protein concentration, as described in Sections 2.2.2.6. and 2.2.2.7, respectively. mRNA was extracted according to Section 2.2.2.5.b and HPAs performed in duplicate for luciferase (Sections 3.3.7) and β-actin mRNA. In order to allow the comparison of data sets from different experiments, all experimental data was normalised. In the case of cell lysates, this standardisation involved the calculation of a ratio of luciferase activity to total protein content of the lysate, that is, the specific luciferase activity. In the instance of HPA, the concentration of luciferase mRNA was normalised against the concentration of β-actin mRNA; a housekeeping gene whose expression is relatively constant within the experimental conditions used. Each experiment was carried out on two separate occasions, data is shown as mean ± standard deviation.

Following the optimisation of transient transfection, it was decided to produce a stably-transfected cell line, expressing the pcTRELuc construct in CV1 cells. CV1s have been widely used in the characterisation of TREs and express both TRα and the 9-*cis* retinoic acid receptor RXR. In order to generate a number of clonal populations of stably-transfected cells, pcTRELuc DNA was transfected into cells using TransFast™ Reagent (Section 2.2.2.3.b). Clonal populations of CV1 cells were isolated and cultured as described in Section 2.2.2.4 and lysates were assayed for luciferase protein activity, as before.

5.3. Results.

5.3.1. Transient Transfection using the Calcium-Phosphate Precipitation Method.

Initial optimisation experiments involved the transfection of the pRG224 luciferase reporter construct into the adherent cell-line, U373s. Cells were seeded at 1.5 – 2.0 x 10^6cells/ml and were allowed to achieve approximately 80% confluence prior to transfection. Varying amounts of DNA were transfected into cells as described in Section 2.2.2.3.a. Following a 24h incubation, cell lysates were prepared, and mRNA extracted from samples. Although it was possible to measure luciferase protein activity in the cell lysate of cells harvested from the well of a 6-well plate, it was not possible to detect either the luciferase or β-actin mRNA using the HPA technology (data not shown); the HPA for luciferase mRNA was carried out as described previously (Section 3.3.7) and the β-actin mRNA assay was performed as described in the manufacturer's Fathead Minnow β-Actin mRNA Assay Kit insert (MLT Research, Cardiff, UK). This kit allows detection of β-actin mRNA from a number of species, as the probe is designed to a highly conserved region of the β-actin gene.

It was decided, therefore, to "scale up" and subsequent transfection experiments were carried out as above, and involved the transfection of the pRG224 luciferase construct into U373s, seeded in T75s (75cm^2 tissue culture flasks). Although it was now possible to quantify the concentration of β-actin mRNA using the HPA, it was still not possible to detect a signal using the luciferase HPA (data not shown). It was therefore decided to conduct further transfection optimisations using a liposome-based transfection method, TransFast™, in the hope that this method would give a higher degree of transfection efficiency.

5.3.2. Optimisation of Transient transfection, using TransFast™ Reagent.

The transient transfection of U373s with pRG224 was optimised for i) the amound of plasmid DNA, ii) the transfection reagent (TransFast™) to plasmid DNA ratio, and iii) the exposure period of cells to the DNA-transfection reagent complex.

a) Amount of DNA.

Cells were seeded in a T75 flask and allowed to achieve approximately 80% confluence prior to transfection (approximately 24h). Increasing amounts of DNA (8 to 64μg), complexed with a constant amount of transfection reagent (1:1) were carefully layered over cells before a 1h incubation at 37°C in an atmosphere of 5% CO_2 in air. The complex was then removed from cells and replaced with an appropriate volume of pre-warmed growth medium, prior to a further 24h incubation. Cells were then harvested and the lysates assayed for luciferase protein activity. The results are shown in Figure 5.1.A. HPA analysis on mRNA extracted from the same samples indicated that the minimum amount of pRG224 DNA that had to be transfected into cells before luciferase mRNA levels could be measured was 16μg (Figure 5.1.B). For economic reasons, further optimisation experiments were therefore conducted using this amount of DNA, hence minimising the amount of TransFast™ required.

b) TransFast™:DNA Ratio, and Incubation Period.

Cells were transfected, as above, with a constant amount of pRG224 DNA (16μg) and different ratios of Transfection reagent:DNA (0.5:1 to 8:1). Each transfection was carried out separately and cells were exposed to the transfection reagent:DNA complex for a range of times. Complexes were then removed from the cells and replaced with an appropriate volume of growth medium, prior to a further 24h incubation. Cells were harvested and the lysates assayed for luciferase protein activity. The results from these optimisation experiments (carried out in duplicate) are shown in Figure 5.2.

A)

B)

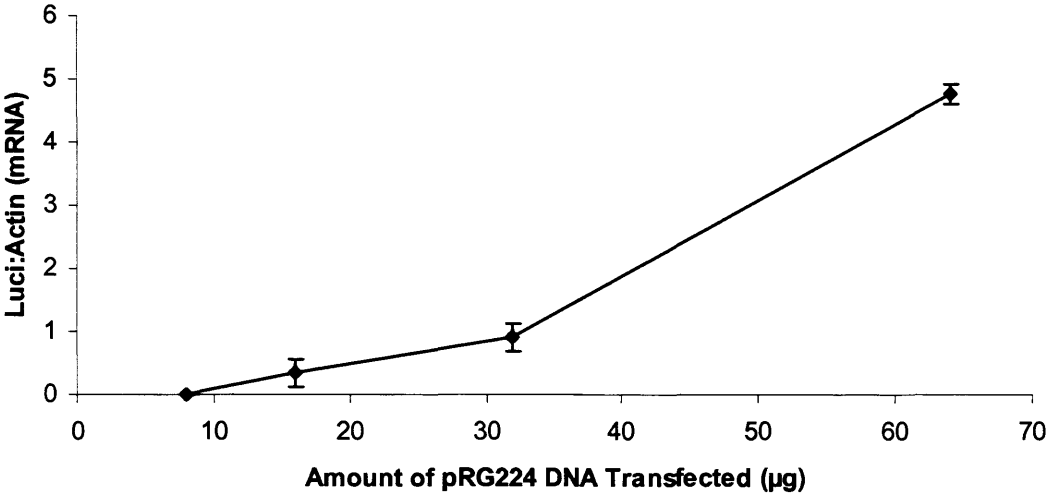
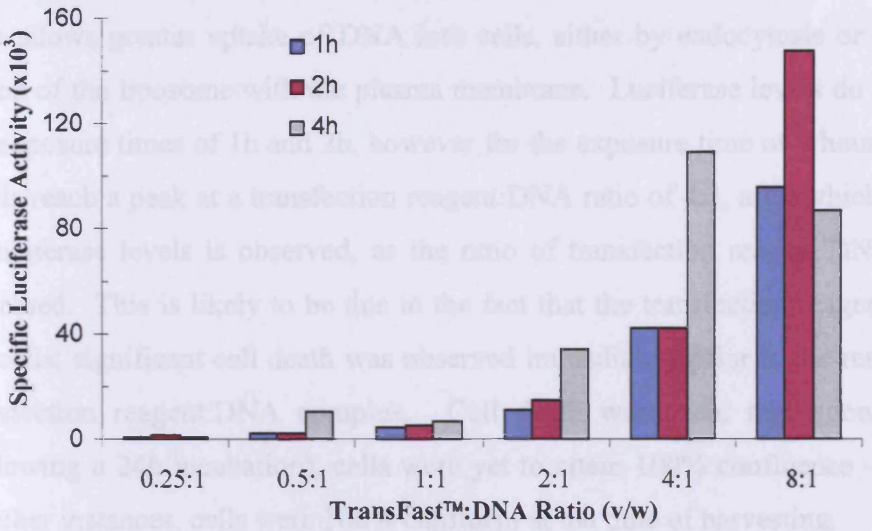


Figure 5.1. The effect of increasing amounts of DNA transfected on levels of luciferase mRNA and protein expression in adherent U373 cells. Cells were transfected with the indicated amount of pRG224 DNA, complexed with TransFast™ in a 1:1 ratio. Error bars indicate standard deviations from two separate experiments performed in duplicate.

Panel A: Specific luciferase protein activity. (Concentration of total protein of samples remained constant).

Panel B: Expression of luciferase mRNA, measured using the HPA. (levels of actin mRNA of samples remained constant).

A)



B)

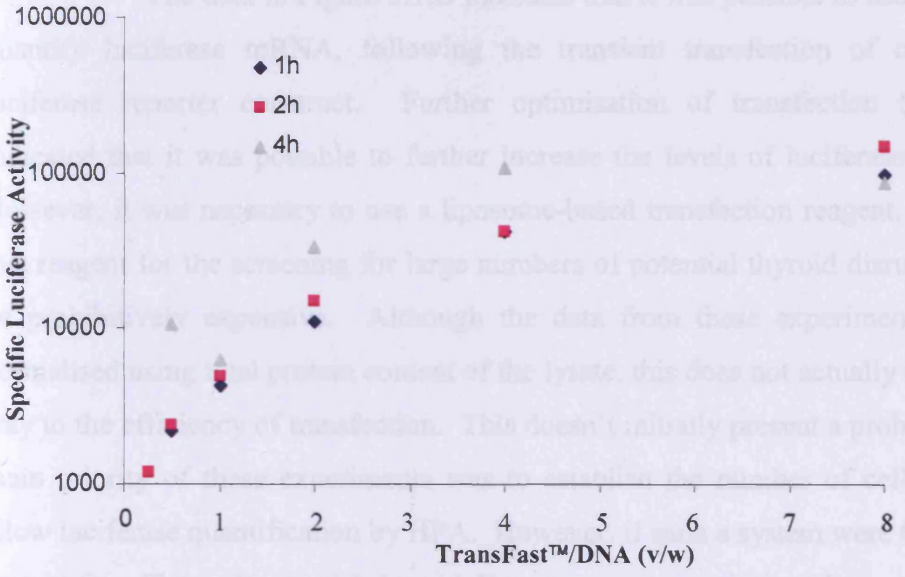


Figure 5.2. The effect of the ratio of TransFast™:DNA on luciferase protein production in U373 cells. Cells were exposed to pRG224 DNA (16µg), complexed with the indicated ratios of TransFast™ for 1, 2 and 4 hours. Cell lysates were prepared and luciferase activity measured and normalised to concentration of protein present in the cell lysates.

Panel A: A bar chart representation of the specific Luciferase activity, shown using linear scale. **Panel B**: A scatter plot representation of the quantity of specific Luciferase activity, shown using a logarithmic scale.

Extending the time of cellular exposure to the transfection reagent:DNA complex increased levels of luciferase activity. It is likely that a greater exposure time allows greater uptake of DNA into cells, either by endocytosis or possibly the fusion of the liposome with the plasma membrane. Luciferase levels do not peak for the exposure times of 1h and 2h, however for the exposure time of 4 hours, luciferase levels reach a peak at a transfection reagent:DNA ratio of 4:1, after which, a decrease in luciferase levels is observed, as the ratio of transfection reagent:DNA is further increased. This is likely to be due to the fact that the transfection reagent is toxic to the cells; significant cell death was observed immediately prior to the removal of the transfection reagent:DNA complex. Cell death was such, that upon harvesting, (following a 24h incubation), cells were yet to attain 100% confluence – whereas in all other instances, cells were 100% confluent at the time of harvesting.

The data in Figure 5.1.B indicates that it was possible to use the HPA to quantify luciferase mRNA, following the transient transfection of cells with a luciferase reporter construct. Further optimisation of transfection (Figure 5.2) indicated that it was possible to further increase the levels of luciferase expression. However, it was necessary to use a liposome-based transfection reagent. The use of this reagent for the screening for large numbers of potential thyroid disruptors would be prohibitively expensive. Although the data from these experiments has been normalised using total protein content of the lysate, this does not actually relate in any way to the efficiency of transfection. This doesn't initially present a problem – as the main priority of these experiments was to establish the number of cells needed to allow luciferase quantification by HPA. However, if such a system were to be used to look at the effects of potential thyroid disruptors on transcription, data would have to be normalised, with regard to efficiency of transfection. In order to allow quantification of transfection efficiency, it is usual to co-transfect a reporter construct that acts as a positive control (commonly a vector expressing β-galactosidase). Since it is only possible to transfect a limited concentration of DNA into a population of cells, having to co-transfect a control vector as well as an experimental reporter construct(s) into cells decreases the concentration of experimental construct it is possible to transfect. Coupled with the fact that there is a large degree of variability inherent to transient transfection systems, and that future studies would use a thyroid-

responsive luciferase reporter construct (even the most potent of TREs would not match the strength of the hCMV immediate-early promoter; personal communciation, Dr Peter Kille), it was decided to produce a cell-line which was stably-transfected with a thyroid-responsive luciferase reporter construct.

5.3.3. Production of a Stably-Transfected Cell Line.

The goal of stable, long-term transfection is to isolate and propagate individual clones which have incorporated the transfected DNA into their genome. It is therefore necessary to distinguish nontransfected cells from those that have taken up the exogenous DNA. This screening can be achieved by drug selection, when an appropriate drug resistance marker is included in the transfected DNA; the thyroid-responsive luciferase reporter construct pcTRELuc contains the neomycin phosphotransferase gene (see Appendix 2D for a vector map). The adherent African Green Monkey kidney cell line, CV1, has been widely used to study the characteristics of various TREs. Previous studies (Ribeiro *et al.*, 2001; Bhat *et al.*, 1997 and Zhang *et al.*, 1996) have co-transfected TR-expressing constructs or recombinant TR protein, along with the thyroid-responsive constructs. However, a PCR reaction using gene-specific primers and cDNA from CV1 cells showed that these cells express TRα but not TRβ (data not shown; mRNA was extracted from adherent, non-transfected cells and cDNA generated by reverse transcription, as described in Sections 2.2.2.5 and 2.2.1.11 respectively. Primers and thermocycling conditions were as described in Section 4.2.1). CV1 cells also express the 9-*cis* retinoic acid receptor (RXR; Safer et al., 1997), another member of the NR superfamily, which is the preferential heterodimer partner for TR when binding to a positive TRE (Zhang and Lazar, 2000). Thus, it was deemed that it would not be necessary to transfect a source of TR or RXR into cells, and so it was decided to stably transfect CV1 cells with the pcTRELuc reporter construct.

Cells were seeded at 1.5 – 2.0 x 10^6cells/ml in a 6-well plate and were allowed to achieve approximately 80% confluence prior to transfection. pcTRELuc DNA (10μg) complexed with TransFast™ was transfected into each well, as described in Section 2.2.2.3.b. Following a 24h incubation, non-selective growth medium was replaced with medium containing the neomycin analogue, G418. Cells were incubated at 37°C until individual cell clones were visible (approximately 5-6 days.) Individual clones (five in total) were isolated as described in Section 2.2.2.4. Cells were subsequently maintained in growth medium supplemented with hormone-stripped foetal bovine serum.

a) Construction of the Thyroid-Responsive Luciferase Reporter Construct, pcTRELuc.

The MAL-TKLuc vector has been described previously by Collingwood *et al.*, (1994) and was kindly donated by Richard Caswell (Cardiff University). It contains a natural direct repeat TRE from the malic enzyme gene (Desvergne *et al.*, 1991) upstream of the viral TK promoter and the luciferase gene. Components of MAL-TKLuc were used in the construction of a vector which would allow for the development of a stably-transfected cell line; the luciferase gene under the control of the ME-TRE from MAL-TKLuc was combined with the coding sequence for the neomycin phosphotransferase gene – allowing for selection of stably-transfected cells.

A schematic representation showing the important features of MAL-TKLuc is shown in Figure 5.3. A number of restriction digests (Section 2.2.1.6) were carried out to confirm the integrity of the vector. Restriction endonuclease digestion with various combinations of enzymes were analysed by agarose gel electrophoresis (Section 2.2.1.1, Figure 5.4).

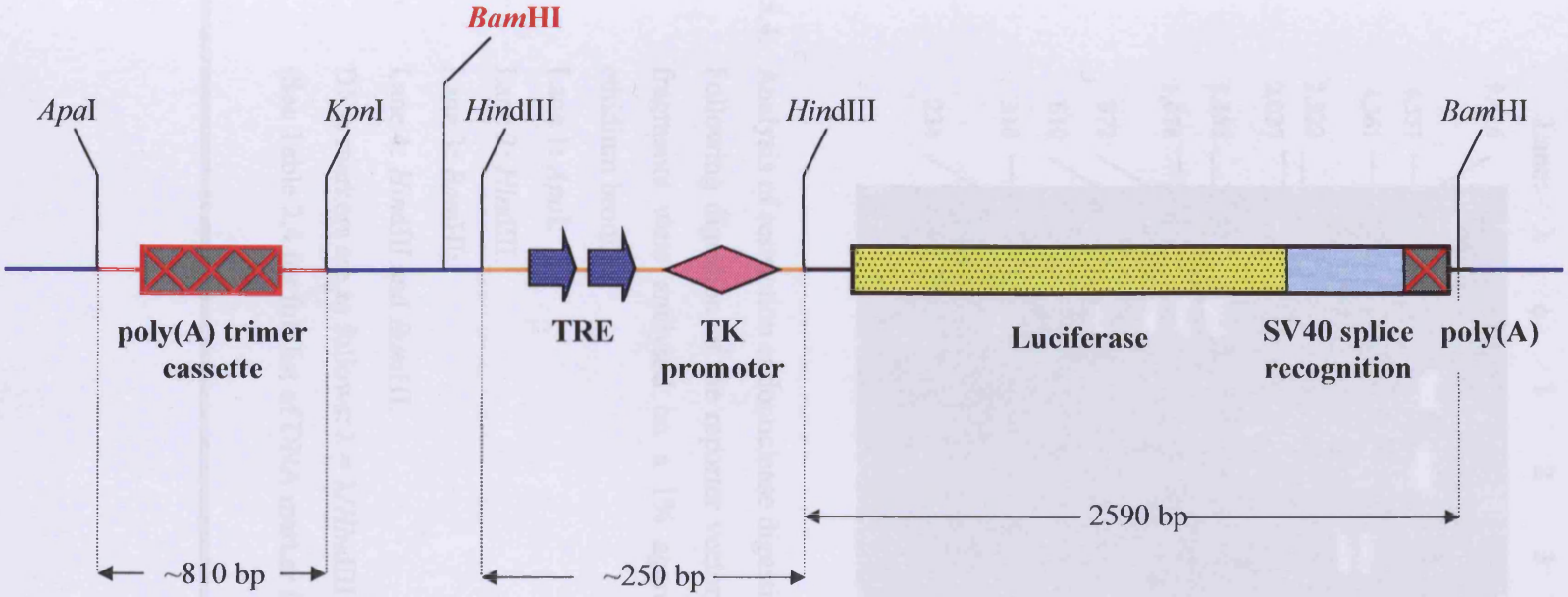


Figure 5.3. A schematic representation (not drawn to scale) showing the various components, along with approximate sizes, of the MAL-TKLUC reporter vector. Known restriction sites are also shown, with the approximate location of the anomalous *Bam*HI site shown in bold, red text. The construction of this vector has been described previously (Collingwood *et al.*, 1994). The blue lines represent pGEM7zf (+), which is the original vector, into which the various components were cloned. For a detailed description of how the vector was constructed, see text, Section 5.3.1.1.a.

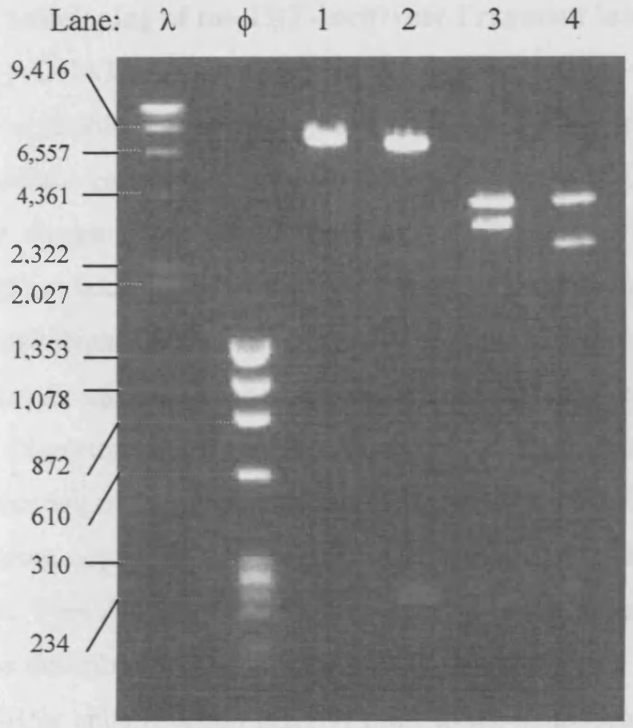


Figure 5.4. Analysis of restriction endonuclease digestion of MAL-TKLuc.
Following digestion of the reporter vector with the indicated enzymes, fragments were analysed on a 1% agarose gel containing 0.5µg/ml ethidium bromide.
Lane 1: *Apa*I;
Lane 2: *Hin*dIII;
Lane 3: *Bam*HI;
Lane 4: *Hin*dIII and *Bam*HI.
DNA markers are as follows: λ = λ/*Hin*dIII and ϕ = ϕ174/*Hae*III.
(See Table 2.4 for full list of DNA marker fragment sizes).

b) Construction and Verification of the Vector pcTRELuc.

i) Subcloning of the TRE-luciferase Fragment into pcDNA3.1.

pcDNA3.1 (Invitrogen) is a mammalian expression vector, which encodes the selectable marker neomycin (for plasmid map, see Appendix 2C). The hCMV immediate early promoter was removed from pcDNA3.1(-), by restriction endonuclease digestion with *Bgl*II and *Bam*H1 (Section 2.2.1.6). Sticky 5'- ends were dephosphorylated in order to prevent re-ligation of the vector (Section 2.2.1.7) and the digested dephosphorylated fragment, of approximately 4.4kb, was purified by extraction from an agarose gel as described in Section 2.2.1.4.b, (Figure 5.5).

Digestion of the reporter vector MAL-TKLuc with *Bam*HI liberated a fragment consisting of the malic enzyme TRE, the thymidine kinase promoter and the luciferase coding sequence. The fragment was purified by extraction from an agarose gel, as above. This *Bam*HI fragment was then ligated into the *Bam*HI/*Bgl*II digested pcDNA3.1 as described in Section 2.2.1.8. The ligation mix was used to transform competent DH5α cells (Section 2.2.1.9) prior to the small-scale isolation of plasmid DNA from a number of colonies (Section 2.2.1.3.a). Constructs were screened in order to determine the orientation of the TRE-containing insert, in relation to the pcDNA3.1 vector. pcTRELuc was constructed in this way in preference to an amplification-based method in order to eliminate the possibility of the introduction of mutations caused by the PCR process. Since the final construct contained the TK-TRE-Luciferase components and the neomycin resistance gene, it was therefore compatible with the propagation of a stably-transfected cell line, as well as with *in vitro* transcription systems.

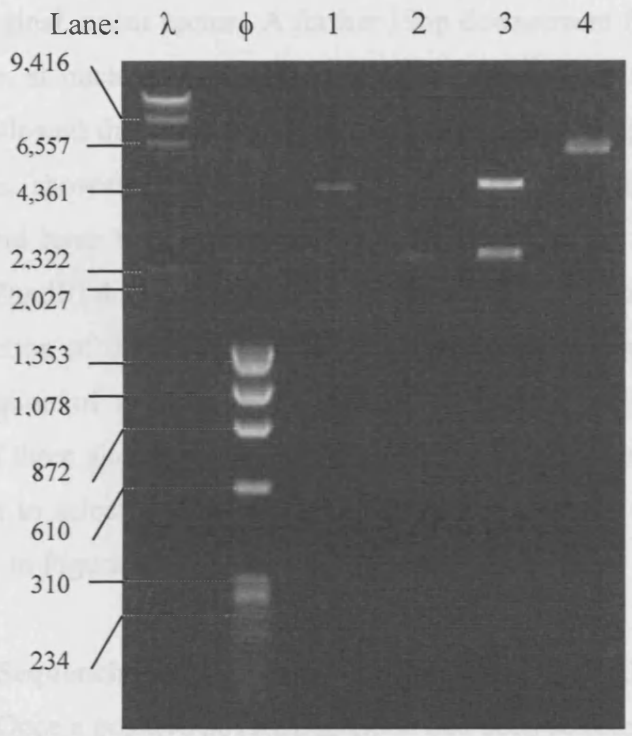


Figure 5.5. Analysis of the gel-purified constituent parts of the pcTRELuc vector and of restriction endonuclease digestion, confirming the integrity of the final construct. Fragments were analysed on a 1% agarose gel containing 0.5μg/ml ethidium bromide.

Lane 1: Gel purified *Bgl*II/*Bam*HI digested pcDNA3.1.;

Lane 2: Gel purified TRE-luciferase fragment, excised from MAL - TK Luc by *Bam*HI digestion.;

Lane 3: pcTRELuc digested with *Hin*dIII;

Lane 4: pcTRELuc digested with *Apa*I.

DNA markers are as follows: λ = λ/*Hin*dIII and ϕ = ϕ174/*Hae*III.

(See Table 2.4 for full list of DNA marker fragment sizes).

ii) Screening of Recombinant Clones.

The TRE-luciferase insert was cloned into *Bgl*II/*Bam*HI sites of pcDNA3.1. As is shown in Appendix 2C, the *Bam*HI site was at nucleotide number 977 in the original, uncut vector. A further 19bp downstream from this *Bam*HI site is a *Hin*dIII site, at nucleotide number 996. The proximity of the *Hin*dIII site to the *Bam*HI site allowed the screening of recombinant clones. Figure 5.6 is a schematic representation, showing alternative ways that the *Bam*HI-digested TRE-luciferase fragment could have been orientated, upon ligation into the BglII/*Bam*HI-digested pcDNA3.1. *Hin*dIII digestion of a clone with the fragment in one orientation resulted in the production of three bands, of approximate sizes 4500bp, 2609bp and 250bp. *Hin*dIII digestion of a clone in the second orientation, however, resulted in the production of three alternative bands: 6991bp, 250bp and 100bp. It was, therefore, a simple matter to select a clone with the fragment inserted in the correct orientation (orientation 1 in Figure 5.6).

iii) Sequencing of the Promoter Region from pcTRELuc.

Once a positive pcTRELuc clone had been selected, the promoter region (5' to the luciferase start codon) of the construct was sequenced. The primer pGL2 (see Appendix 1D for primer sequence) was used, and the sequencing reaction performed as described in Section 2.2.1.13. To confirm the exact sequence four independent sequencing reactions were performed and electrophoretograms compared to allow removal of any sequencing artefacts. The resulting sequence was reversed and complemented (Appendix 3A), using DNAsis (Section 2.2.1.14.b.) and BlastN search was performed (Section 2.2.1.15) and the malic enzyme TRE was identified, as was the herpes simplex virus thymidine kinase (TK) promoter.

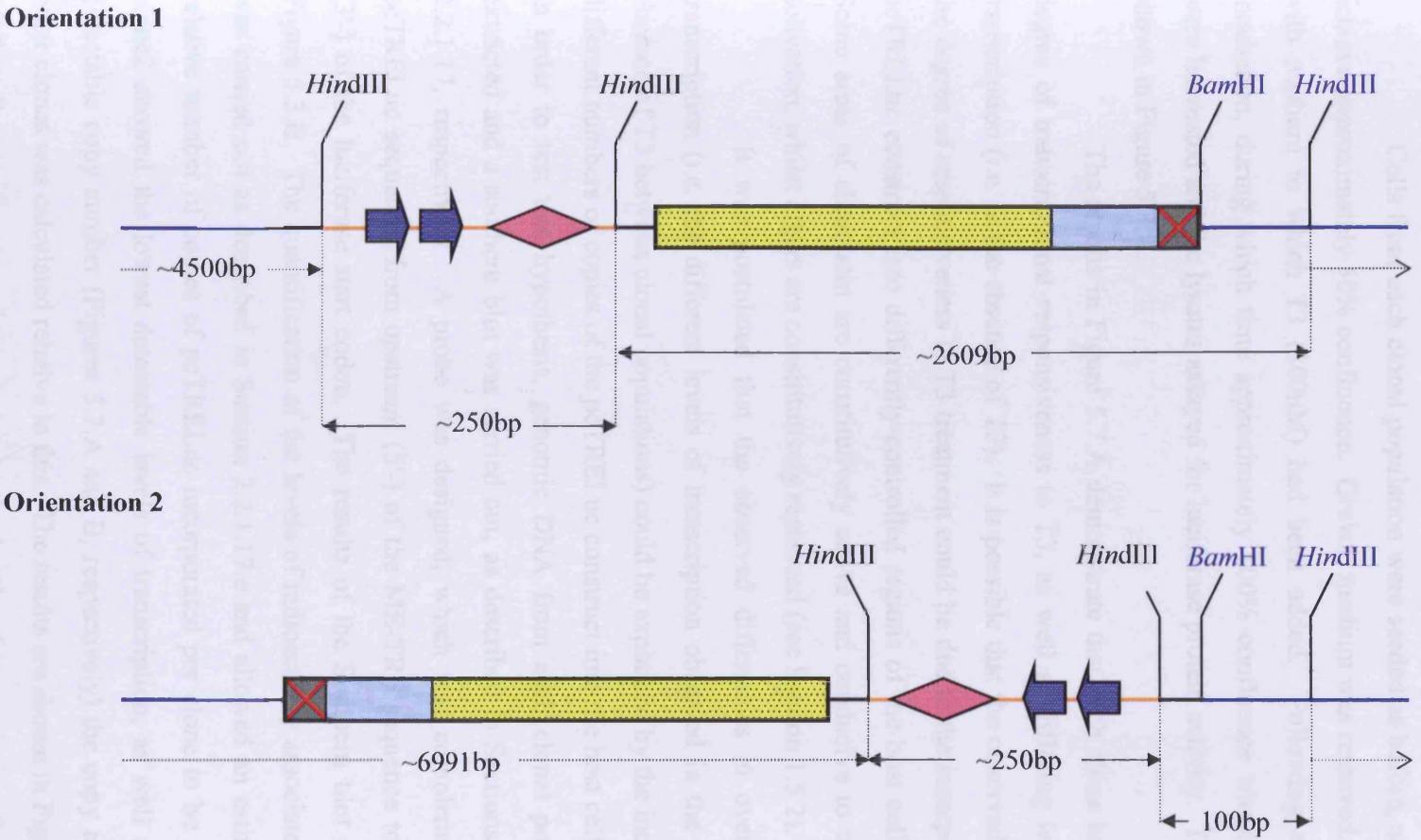


Figure 5.6. A schematic representation showing the different ways in which the TRE-luciferase fragment could have become orientated upon ligation into the *Bgl*II/*Bam*HI digested pcDNA3.1. Blue lines and blue text (restriction sites) represent sequence from pcDNA3.1.

5.3.3.1. Transcriptional Response of Clones to T3.

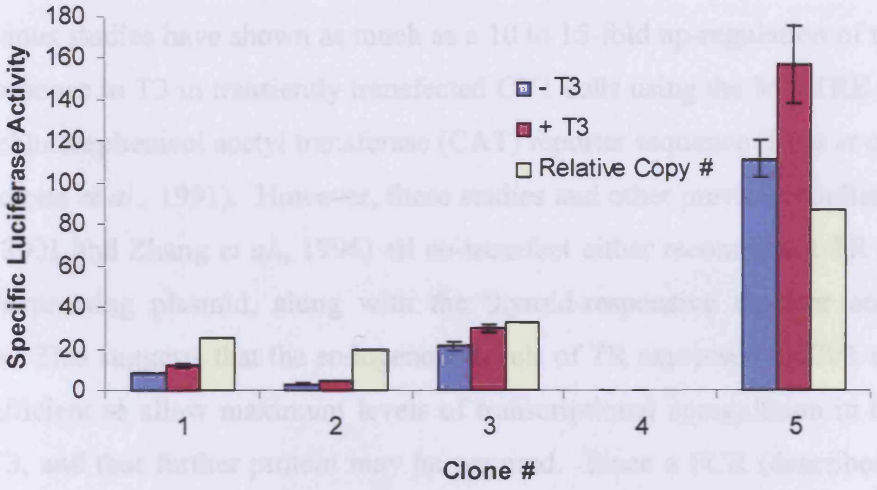
a) In the Absence of Exogenous TR.

Cells from each clonal population were seeded as before, and allowed to achieve approximately 50% confluence. Growth medium was removed, and replaced with medium to which T3 (100nM) had been added. Following a further 48h incubation, during which time approximately 100% confluence was attained, cells were harvested and the lysates assayed for luciferase protein activity. The results are shown in Figure 5.7.A.

The results in Figure 5.7.A demonstrate that each clone had a different degree of transcriptional responsiveness to T3, as well as differing levels of basal transcription (i.e. in the absence of T3). It is possible that the observed variations in the degree of responsiveness to T3 treatment could be due to the incorporation of the pcTRELuc construct into differently-controlled regions of the host cell's chromatin. Some areas of chromatin are constitutively active and conducive to transcriptional activation, whilst others are consititutively repressed (see Section 1.5.2).

It was postulated that the observed differences in overall levels of transcription (i.e. the different levels of transcription observed in the presence and absence of T3 between clonal populations) could be explained by the incorporation of different numbers of copies of the pcTRELuc construct into the host cell's chromatin. In order to test this hypothesis, genomic DNA from each clonal population was extracted and a southern blot was carried out, as described in Sections 2.2.1.16 and 2.2.1.17, respectively. A probe was designed, which was complementary to the pcTRELuc sequence, from upstream (5'-) of the ME-TRE sequence to downstream (3'-) of the luciferase start codon. The results of the Southern blot are shown in Figure 5.3.B. The quantification of the levels of radioactivity associated with bands was carried out as described in Section 2.2.1.17.e and allowed an estimation of the relative number of copies of pcTRELuc incorporated per clone to be made. Since clone2 showed the lowest detectable levels of transcription, as well as the lowest detectable copy number (Figures 5.7.A and B, respectively) the copy number of the other clones was calculated relative to this. The results are shown in Figure 5.7.A and confirm the positive correlation between the levels of transcription (both basal and induced) and the number of copies of the pcTRELuc construct incorporated into the host cell's genome.

A)



B) **Clone** 1 2 3 4 5

Figure 5.7. Panel A: The transcriptional response of the stably-transfected clonal populations. Cells were transfected with pcTRELuc and grown under selective ($G418^{+}$) conditions. Individual clones were isolated and cultured in growth medium supplemented with hormone-stripped serum. Cells were incubated for 48h in the presence or absence of T3 (100nM). Following harvesting, cell lysates were assayed for luciferase protein activity. Error bars represent standard deviations from two separate experiments, carried out in duplicate.

Also shown is the relative copy number of pcTRELuc, calculated by quantitative densitometric analysis of data from the southern blot in Panel B.

Panel B: A southern blot was carried out on gDNA extracted from adherent cells from each clonal population. A radiolabelled probe was designed to incorporate the ME-TRE and luciferase start codon.

b) In the Presence of Co-transfected TR.

The observed up-regulation in luciferase transcription seen in the presence of T3 (100nM) for all clones was of a lesser magnitude than expected. Previous studies have shown as much as a 10 to 15-fold up-regulation of transcription in response to T3 in transiently transfected CV1 cells using the ME-TRE upstream of the chloramphenicol acetyl transferase (CAT) reporter sequence (Bhat *et al.*, 1997 and Umesono *et al.*, 1991). However, these studies and other previous studies (Ribeiro *et al.*, 2001 and Zhang *et al.*, 1996) all co-transfect either recombinant TR protein or a TR-expressing plasmid, along with the thyroid-responsive reporter construct into cells. This suggests that the endogenous levels of TR expressed in CV1 cells may be insufficient to allow maximum levels of transcriptional upregulation in the presence of T3, and that further protein may be required. Since a PCR (described in Section 5.3.3) demonstrated that CV1 cells express TRα, it was decided to transfect cells with recombinant TRβ. Given that the cells from stably-transfected clone 5 gave the largest response to T3 (Figure 5.7.A), subsequent studies were carried out using cells from this clonal population. Cells were seeded as before, and allowed to achieve approximately 80% confluence. Recombinant TRβ, purified as described in Section 4.2.5, was transfected into cells (2μg/10mm dish) using ProteoJuice™ Protein Transfection Reagent, as recommended in the manufacturer's instructions (Novagen). After four hours, the transfection reagent was removed and replaced with growth medium, supplemented with stripped serum. Cells were maintained at 37°C until 100% confluence was achieved (approximately 48h) in the presence or absence of T3 (100nM). Following harvesting, cell lysates were assayed for luciferase protein activity. The results are shown in Figure 5.8.

The results shown in Figure 5.8 indicate that the absence of endogenous TRβ expression in CV1 cells may be limiting the T3-induced transcriptional response in the stably-transfected cells. In order to confirm that TRβ had been successfully transfected and that the observed effects were caused by the presence of exogenous TRβ, it would be necessary to wash the transfected cells and prepare a lysate which could be probed or the presence of the recombinant protein. This could be achieve by performing a western blot to confirm the presence of the His-tagged protein, using the Ni-NTA AP conjugate, as described previously (Section 2.2.4.5).

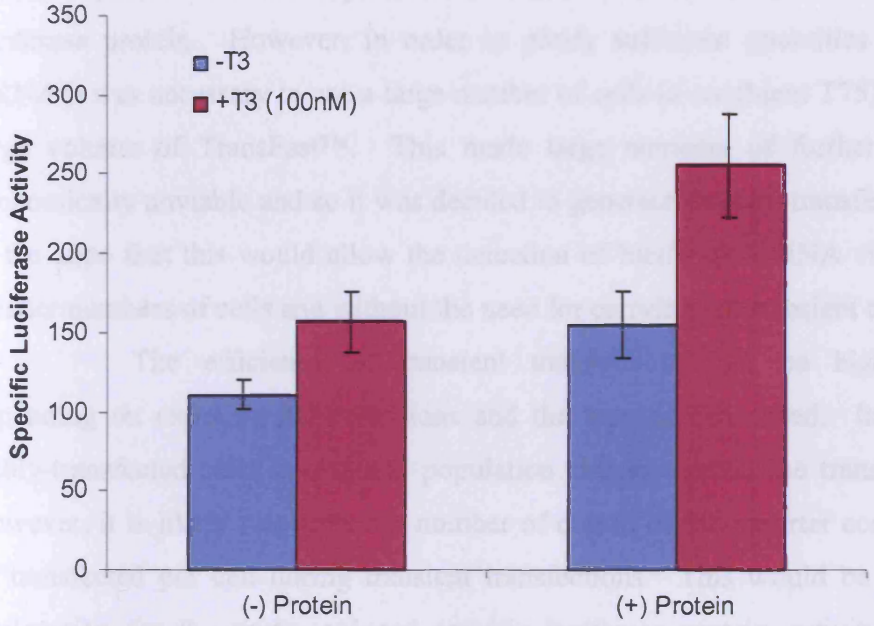


Figure 5.8. The transcriptional response of clone 5 cells to T3. Cells were subjected to ProteoJuice™ Protein Transfection Reagent for four hours, in the presence or absence of recombinant TRβ (2μg). The transfection reagent was replaced with growth medium supplemented with hormone-stripped serum in the presence or absence of T3 (100nM). Following attainment of 100% confluence, cells were harvested and lysates were assayed for luciferase protein activity. Error bars represent standard deviations from two separate experiments, carried out in duplicate.

5.4. Discussion.

The optimisation of transient transfections, using the liposome-based transfection reagent, Transfast™ allowed the detection of luciferase mRNA, using the HPA. It was hoped that it would be possible to carry out a comparison between the temporal profiles for the expression of luciferase mRNA and the translation of luciferase protein. However, in order to purify sufficient quantities of luciferase mRNA it was necessary to use a large number of cells (a confluent T75) and hence, a large volume of TransFast™. This made large numbers of further experiments economically unviable and so it was decided to generate a stably-transfected cell line, in the hope that this would allow the detection of luciferase mRNA via HPA, from smaller numbers of cells and without the need for carrying out transient transfections.

The efficiency of transient transfections can be highly variable, depending on experimental conditions and the type of cells used. In contrast, all stably-transfected cells in a clonal population should contain the transfected DNA. However, it is likely that a greater number of copies of the reporter construct would be transfected per cell during transient transfections. This would be one possible explanation for the vastly reduced specific luciferase protein activity seen in the stably-transfected clones, compared to the transient transfections (100s Vs 100,000s). A second reason for the observed decrease in specific luciferase activity would be the relative potencies of the promoters used. All transient transfection optimisations used the pRG224 vector, where the luciferase gene is under the control of the hCMV immediate-early promoter. The stably transfected CV1 cell-line was made using the pcTRELuc construct, where the luciferase gene is under the control of the much less potent malic enzyme thyroid responsive element (ME-TRE). These reasons, in turn, explain why it was not possible to use the HPA to measure luciferase mRNA purified from stably-transfected cells, either in the absence or presence of T3.

In addition to being unable to measure luciferase mRNA purified from stably-transfected cells, the observed up-regulation in T3-induced luciferase transcription for all clones was of a lesser magnitude than expected (Section 5.3.3.1.b). A possible explanation for the lower than expected observed up-regulation of luciferase transcription is provided by Tillman *et al.*, (1993), who used transient transfections to examine the effects of T3 and TRβ on luciferase expression in CV1 cells. Using the wild-type and a mutated form of TRβ (mTRβ, where cysteine-122 was changed to serine-122 in the first zinc finger of the DNA binding domain) they

demonstrated that the activity of the luciferase reporter was down-regulated in a T3 and TRβ dependent manner. They also showed that T3 and TRβ dependent inhibition of luciferase expression occurred consistently to a greater or lesser extent with various mutations of the herpes simplex virus thymidine kinase (TK) promoter sequence, i.e. was independent of a TRE sequence. A 1.5 to 2-fold inhibition of expression was observed when a construct was used which contained the non-mutated TK promoter sequence (i.e. the same TK sequence as is in the pcTRELuc vector). This inhibition was not reversed when a single copy of the rat growth hormone positive TRE was placed 5′ to the TK promoter (5′TRE-tkLUC). This TRE had previously been shown to stimulate CAT reporter expression approximately 2-fold (Brent *et al.*, 1989). The effects of a strong positive TRE were then investigated using the TREpX2-tks-LUC construct; two copies of TREp were inserted 5′ to the TK promoter (TREp is synthetic variant of the rat growth hormone TRE). T3 and TRβ dependent inhibition of luciferase expression was now reversed and was stimulated approximately 5-fold.

In order to confirm that this was a phenomenon specific to the luciferase sequence, Tillman *et al.*, (1993) conducted a series of experiments using the CAT reporter sequence. Expression from these constructs was not down-regulated in the presence of T3 and TRβ. These studies also demonstrated that the activity of a strong, positive TRE was unaffected by the use of a luciferase reporter construct; TREpX2-tks-LUC was stimulated 5.6 ± 1.6-fold in CV1 cells in the presence of T3 and TRβ. TREpX2-tks-CAT, a construct identical to TREpX2-tks-LUC except that CAT replaced the LUC reporter, was stimulated 6.3 ± 0.2-fold under identical conditions. These results were consistent with a previous study by Glass *et al.*, (1989) who also used a TREpx2-tk-LUC construct in CV1 cells to show an upregulation of luciferase expression of approximately 5-fold, in the presence of TRβ and T3. Although the exact mechanism of this unexpected response was unclear, Tillman postulated that regulation of luciferase activity could occur at the gene expression level, or at the level of luciferase mRNA or protein stability. The Tillman study was followed up by Maia *et al.*, (1996) who postulated that there may be a negative TRE in the luciferase cDNA. Consistent with this they transiently transfected a TK-LUC vector into JEG-3 cells to show that unliganded TR induced luciferase expression, whilst the addition of T3 reduced expression levels by up to as much as 80% (Maia *et al.*, 1997). In order to postulate the possible implications of these studies for the work performed in this thesis, it is necessary to estimate the relative strengths of the promoters used. Bhat *et*

al., (1997) reported the relative strengths of the promoters he employed to be as follows; ME-TRE ~10-fold upregulation compared to a 40-fold upregulation caused by TREp. The study by Umesono *et al.*, (1991) demonstrated that ME-TRE caused a 15-fold upregulation of expression, compared to a 20-fold upregulation caused by TREp. The combination of transcriptional suppression caused by the putative internal luciferase TRE and the unknown relative strength of the ME-TRE used in these studies limits the possible value of the data generated.

Although it was possible to detect the presence of TRα transcripts by PCR (Section 5.3.3) it is possible that protein levels were not sufficiently high to allow a maximal transcriptional response in the presence of T3. It may be possible that the small amounts of endogenous TRα present are sufficient to allow T3 induced upregulation under physiological conditions, however when overloaded with relatively large amounts of plasmid DNA containing the ME-TRE the amount of endogenous TR present becomes a limiting factor, hence the need to transfect exogenous TR. The results shown in Figure 5.8 illustrate that transfection of recombinant TRβ into cells lead to a greater transcriptional response in the presence of T3. This is consistent with previously published results (Jeannin *et al.*, 1998) where the increased expression levels of a CAT reporter where shown to be directly proportional to the amount of TR expression plasmid transfected into cells.

Although previous studies (Ribeiro *et al.*, 2001, Bhat *et al.*, 1997, Zhang *et al.*, 1996 and Umesono *et al.*, 1991) all co-transfect a source of recombinant TR into CV1 cells, they do not transfect a source of 9-*cis* retinoic acid receptor (RXR, the preferential heterodimer partner for TR when binding to a positive TRE) suggesting that endogenous levels of this protein are sufficient. The studies mentioned above co-transfect TR expression vectors encoding the human forms of TR, suggesting that they are able to heterodimerise with the endogenous RXR from the CV1 cells (green monkey kidney cell line). A number of studies have also utilised TR expression vectors encoding the rat forms of TR, suggesting that the rat protein is able to heterodimerise with the endogenous RXR found in human cell lines. Izumo and Mahdavi, (1988) showed that rat TRα (rTRα) was a T3 dependent transcriptional factor, which bound to αMHC-TRE (myosin heavy chain) and activated expression of a CAT reporter by approximately 10-fold in HeLa cells. Studies carried out to confirm the identity and location of the ME-TRE by Desvergne *et al.*, (1991) used a series of CAT reporter constructs under the control of various ME-TRE deletions in

NIH 3T3 cells. They co-transfected rTRα and T3 to demonstrate a 11-fold upregulation of CAT expression. Petty *et al.*, (1990) carried out studies similar studies except they utilised COS-7 cells. Co-transfection of rTRα and T3, led to a 5-fold upregulation of CAT expression.

Since a stably-transfected cell line was generated to minimise both expense and the inter-assay variability observed with transient transfections, a possible future objective would be to generate a stably-transfected cell line which expressed the luciferase gene under the control of the ME-TRE promoter as well as the TRβ protein-coding sequence, under the control of a separate promoter.

As has been previously stated, the effects of thyroid hormones (THs) are mediated at the level of gene expression via the thyroid hormone receptors (TRs), which bind to specific DNA sequences. TRs in turn, recruit coregulatory proteins which act as bridging molecules and contact the basal transcriptional machinery (Section 1.5.3). Although the effects of a putative disruptor on T3-mediated transcription can be monitored using a cell culture system, this system is reliant upon the entry of the disruptor into the cell. In addition, the disruptor may also be metabolised and altered upon cell entry. Therefore, the development of an *in vitro* (cell free) transcription system, to be used in conjunction with a cell culture system, will provide further insights into how a cellular environment effects the properties of a potential disruptor.

CHAPTER SIX

IN VITRO TRANSCRIPTION.

6.1. Introduction.

The Malic Enzyme TRE (ME-TRE) described in Chapter 4, is a natural direct-repeat, positive TRE; the most potent of the TREs. Although TRs are capable of binding to the ME-TRE as monomers or homodimers (see Section 4.2.8.3), they bind preferentially to positive TREs as heterodimers with the retinoid X receptor (9-*cis* retinoic acid receptor or RXR), (Mangelsdorf and Evans, 1995). Heterodimer formation between TR and RXR enhances DNA-binding affinity and provides target gene specificity (Mangelsdorf *et al.*, 1995).

In order to analyse the impact of potential disruptors on the transcriptional response mediated by TRs via the ME-TRE, the DNA sequence can be fused to a reporter gene, combined with a nuclear extract containing the necessary transcriptional machinery, and the levels of mRNA produced can be quantified. A commonly used reporter is the luciferase gene from the common firefly (*Photinus pyralis*) and a nuclear extract from HeLa cells can be used; HeLa cells do not express TR but express RXR and the cofactors necessary for TR function (Kim *et al.*, 1999). Quantification of luciferase mRNA expression from such a system would allow an assessment of the effect on transcription of a potential disruptor.

Traditional methods for mRNA detection from *in vitro* transcription systems, including the "run-off" assay and primer extension, involve the use of radioactive nucleotides. However, it has been shown previously (Section 3.3.7) that it is possible to quantify luciferase mRNA using the HPA, and it is proposed to use this quantification method as an alternative to the radionuclide-dependent primer extension assay.

6.2. Approach.

In order to develop a system which would allow the monitoring of the effects of putative disruptors on thyroid receptor-mediated transcription, an *in vitro* transcription system was developed which utilised a nuclear extract, a thyroid-reponsive reporter construct and recombinant TR. The ME-TRE (malic enzyme thyroid-reponsive element) was cloned upstream of the firefly luciferase gene sequence to give pcTRELuc (the reporter vector also contained the neomycin phosphotransferase gene which allowed the generation of stably–transfected cell lines; described in Chapter Five). Luciferase gene transcripts from *in vitro* reactions were initially quantified using the traditional radioactive isotope-dependent primer extension method. The possibility of quantifying luciferase mRNA with non-radioactive methods was then investigated; using both TaqMan® Probe real-time quantitative PCR and the Hybridisation Protection Assay (HPA) technologies.

6.3. Results.

6.3.1. *In vitro* Transcription Reactions.

The main aim of the work described in this chapter was to develop non-radioactive assays for the detection of luciferase mRNA from *in vitro* transcription systems. However, before such assays could be developed, it was necessary to confirm the integrity of the *in vitro* transcription system, which used the pcTRELuc reporter construct and purified recombinant TR. This was done using the traditional primer extension method, for detection of luciferase mRNA.

In vitro transcription (IVT) reactions were carried out as described in Section 2.2.1.18.a. Each set of IVT experiments was run on a minimum of two separate occasions, but more typically on three or more separate occasions in order to ensure reproducibility. Quantitative data represents the mean value from two separate experiments, carried out in triplicate. Error bars indicate the standard deviations for these data.

6.3.1.1. Detection of Luciferase mRNA by Primer Extension.

pcTRELuc template DNA and HeLa nuclear extract were combined with recombinant TRβ (either ligand-free or ligand-bound) as indicated (Figure 6.1). The pGL2 primer (see Appendix 1D for primer sequence) and ϕ174/*Hae*III Markers were end-labelled with γ-^{32}P-ATP and primer extension analysis of transcripts was performed as described in Sections 2.2.1.18.c. and 2.2.1.18.b, respectively. The results shown correspond with the previously published data of Lee *et al.*, (1994), who used an *in vitro* transcription system to show that ligand-free TR and RXR combined caused an increase in transcription from the ME-TRE, compared to levels observed in the presence of RXR alone (shown in Figure 6.1.A; compare lanes 4-6 with lanes 1-3). The same study by Lee *et al.*, also showed a further increase in the transcriptional response upon the addition of 100nM T3. In order to confirm these trends, quantitative data from two separate primer extension experiments was plotted (Figure 6.1.B. Although the transcriptional responses observed in Figure 6.1.B are of a lesser magnitude than those described by Lee *et al.*, (approximately 2.5 and 3.5 compared to 4 and 7), there are a number of differences between the two systems. Lee *et al.* used a nuclear extract solely as a source of basal transcriptional machinery, then added both

recombinant TR and RXR. The system described here uses the HeLa nuclear extract both as a source of basal transcriptional machinery and of RXR. Lee *et al.* investigated a range of concentrations of recombinant TRα whereas this study used a single concentration of TRβ.

The results in Figure 6.1.A show two primer extension products. Using the sequence information from Appendix 3A it was estimated that the desired product should be approximately 105bp long. For this reason it was only the primer extension product marked in Figure 6.1.A that was quantified by densitometry. The presence of a second product of higher molecular weight could possibly be due to the presence of an alternative transcriptional start site.

In its present format it would be difficult to exploit this technology to quantify disruptor activity - it may be possible that further optimisation of this system could have resulted in the observation of a larger transcriptional response in the presence of T3. However, the aim of the subsequent work in this chapter was the development of non-radioactive assays for the detection of luciferase mRNA from *in vitro* transcription systems. It was decided to try and reproduce the results shown in Figure 6.1 using an alternative method, before carrying out any further optimisations of the system.

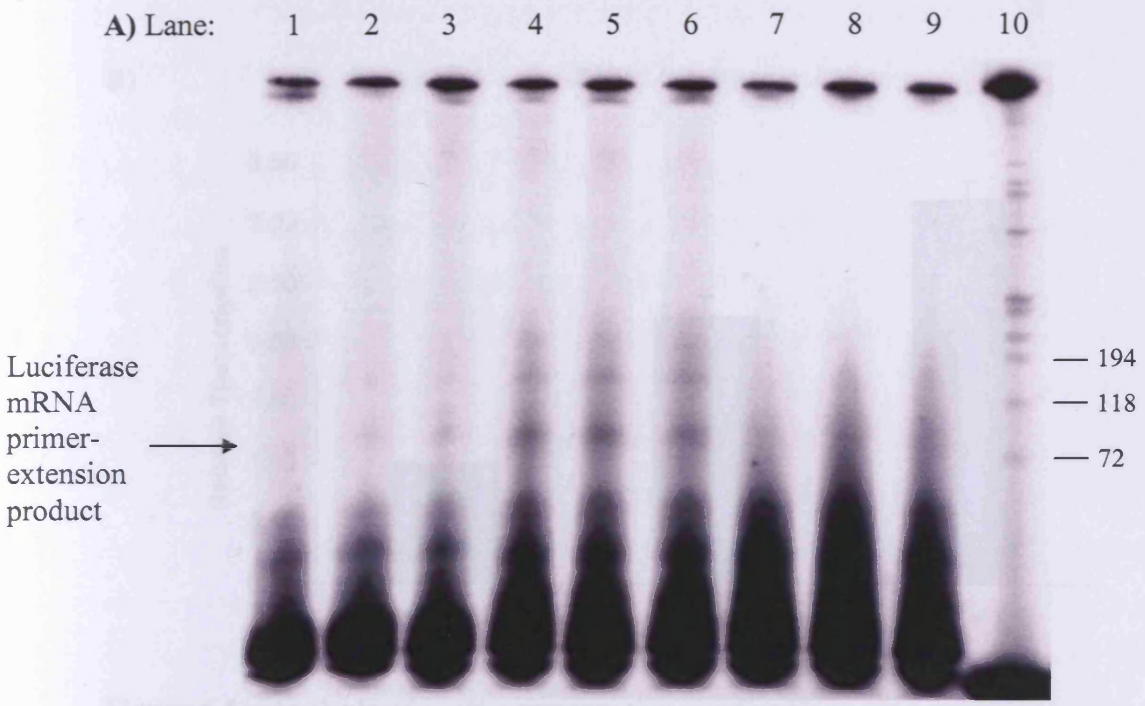


Figure 6.1. Analysis of *in vitro* transcription reactions by Primer Extension.

Panel A. An autoradiogram showing analysis of transcripts by primer extension. Lanes 1-3: pcTRELuc (4nM) was combined with HeLa nuclear extract. Lanes 4-6: pcTRELuc (4nM) was combined with HeLa nuclear extract and TRβ (80nM). Lanes 7-9: negative control, pcTRELuc (4nM) only (no nuclear extract or TRβ). Lane 10: ϕ174/*Hae*III size markers. (See Table 2.4 for full list of DNA marker fragment sizes).

B)

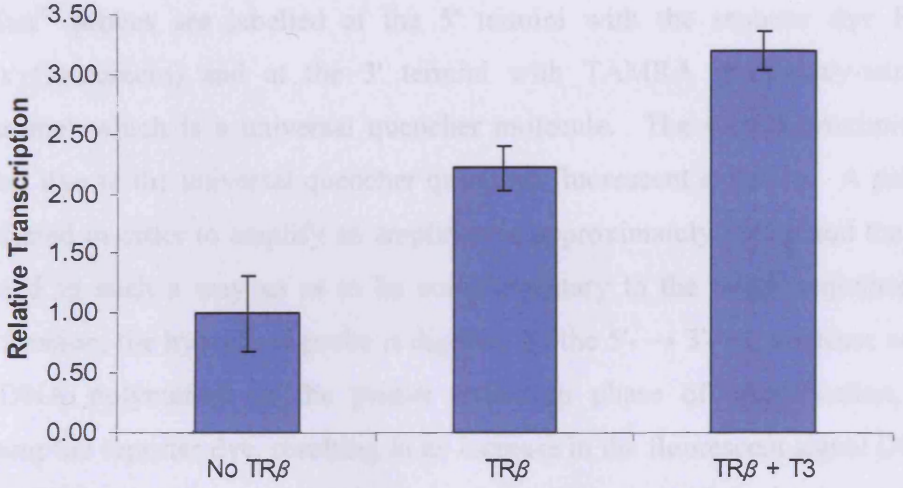


Figure 6.1. Analysis of *in vitro* transcription reactions.

Panel B: Quantitative densitometric analysis of the primer extension product indicated in Panel A was carried out. HeLa nuclear extract and pcTRELuc (4nM) were combined with: no TRβ or T3 ("No TRβ"), TRβ only (80nM, "TRβ") or TRβ (80nM) and T3 (100nM, "TRβ + T3"). The transcriptional response is plotted relative to basal levels observed (i.e. the level of transcription observed in the absence of TRβ; designated 1). No primer extension product was observed in the absence of HeLa nuclear extract or TRβ (see Panel A, lanes 7-9). Quantitative data is representative of the mean of six independent primer extensions. Error bars indicate the standard deviations for these data.

6.3.1.2 Detection of Luciferase mRNA using TaqMan® 5'-Exonuclease Probe Quantitative PCR.

TaqMan Probe® real-time quantitative PCR has been shown to be a sensitive method for measuring gene transript levels (Eun *et al.*, 2000). The detection methodology of the TaqMan® Probe real-time quantitative PCR uses specific fluorescent probes to hybridise template DNA within the specified amplicon. The TaqMan® probes are labelled at the 5' termini with the reporter dye FAM (6-carboxyfluorescein) and at the 3' termini with TAMRA (6-carboxy-tetramethyl-rhodamine) which is a universal quencher molecule. The spatial proximity of the reporter dye to the universal quencher quenches fluorescent emission. A primer pair is designed in order to amplify an amplicon of approximately 100bp and the probe is designed in such a way so as to be complementary to the target sequence. Upon amplification, the hybridised probe is digested by the 5'- → 3'- exonuclease activity of *Taq* DNA polymerase in the primer extension phase of amplification, thereby releasing the reporter dye, resulting in an increase in the fluorescent signal (Nasabardi *et al.*, 1999). A diagrammatic representation of this technology is shown in Figure 6.2. The sequences of the probe and primers designed for quantification of luciferase mRNA are given in Appendix 1F.

a) TaqMan® Probe and Primer Design.

Primers and probes were designed using the Primer Express ™ software package (PE Applied Biosystems; Section). TaqMan® probes were synthesized by QIAgen (Sussex, UK) with the reporter dye FAM and universal quencher TAMRA covalently attached to the 5' and 3' ends, respectively. The 3' ends were also phosphorylated in order to prevent probe extension. Primers were designed to amplify a region of approximately 100bp, whilst the probe was complementary to a region within this target area. The following guidelines were adhered to during the design process:

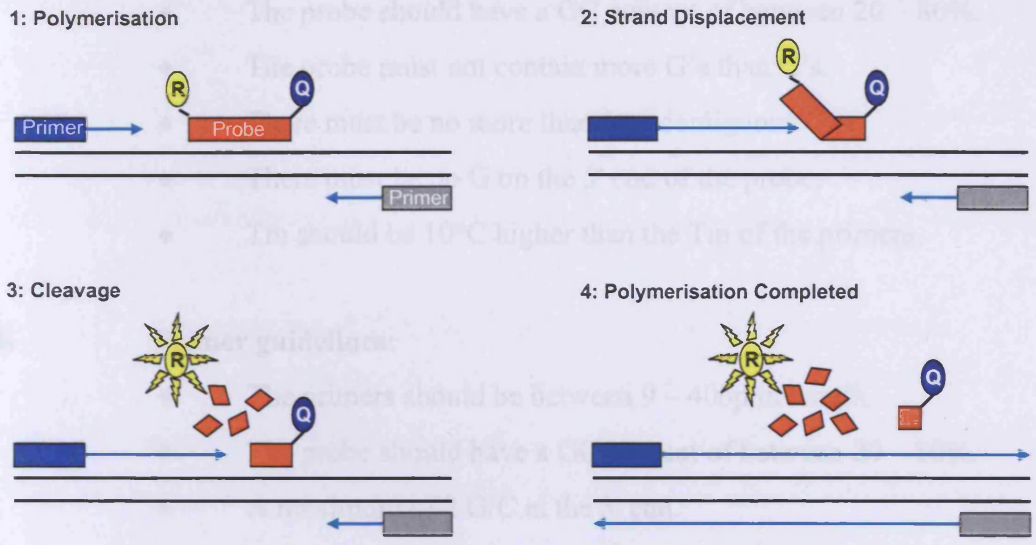


Figure 6.2. Illustration of TaqMan® probe technology quantitative PCR amplification reactions. The symbol "R" represents the reporter fluorescent molecule; "Q" represents the quencher molecule.

i) Probe guidelines:

- The probe should be between 9 - 40bp in length.
- The probe should have a GC content of between 20 – 80%.
- The probe must not contain more G's than C's.
- There must be no more than four contiguous G's.
- There must be no G on the 5' end of the probe.
- Tm should be 10°C higher than the Tm of the primers.

ii) Primer guidelines:

- The primers should be between 9 – 40bp in length.
- The probe should have a GC content of between 20 – 80%.
- A maximum of 2 G/C at the 3' end.
- Tm of the primers must be between 58 – 60°C.
- A maximum of 2°C difference allowed between the Tm of primer pairs.

iii) Amplicon guidelines:

- The amplicon should be between 50 – 150bp in length.
- The 3' end of the primer should be as close as possible to the probe, without overlapping.

b) Template Preparation.

i) Standard Dilution Series for Calibration.

Stock plasmid preparations containing an insert corresponding to the target sequence to be amplified for quantification purposes were purified, quantified and ethanol-precipitated to a known concentration as described in Sections 2.2.1.3.b., 2.2.1.5. and 2.2.1.4.a.ii., respectively.

A standard dilution series of calibration standards were prepared by diluting purified plasmid to a range of concentrations in H_2O, from 500pg/µl to 1fg/µl, at 10-fold intervals.

ii) Sample cDNA.

In vitro transcription reactions were carried out and mRNA purified as described in Section 2.2.1.18.a. Samples were treated with RQ1 RNAse-free DNAse (Promega) at 37°C for 1h to remove pcTRELuc template DNA, prior to enzyme inactivation (70°C for 10min). cDNA was generated from the purified mRNA by reverse transcription (Section 2.2.1.11.) and samples were diluted 1:10 in HPLC-grade water prior to quantitative analysis.

c) Optimisation of TaqMan® Quantitative PCR Amplifications.

Prior to optimisation, the amplification reactions consisted of the following : 2.5µl of template DNA, 5′ and 3′ primers , both at 0.9µM (synthesised by MWG Biotech, Germany), 200nM probe, 0.2mM dATP, dCTP and dGTP, 0.4mM dUTP, 1 Unit of in-house *Taq* DNA polymerase buffered in 20mM Tris-HCl (pH 8.3), 50mM KCl, 4mM $MgCl_2$, 1µM ROX (5-carbosy-X-Rhodamine, a machine calibration dye). The PCR reactions were performed in 25µl volumes in PCR 96-well plates, using optical lids, and subjected to the following thermal cycling parameters using the ABI Prism® 7700 Sequence Detection System:

Chain denaturation	95°C	10sec	
Primer annealing	50°C	5sec	25 cycles
Chain extension	60°C	4min	
	↓		
Soak	4°C		

As with any amplification technique, optimisation was required. Optimisation of two variables was necessary for the use of TaqMan® probe real-time PCR quantification in the ABI Prism® 7700 sequence detection system:

i) Oligonucleotide Primer concentrations, and
ii) Probe concentration.

i) Primer Concentration.

Forward and reverse primers were tested at three different concentrations, resulting in nine different primer-concentration combinations (Table 6.3). A final probe concentration of 200nM and 1ng of pcTRELuc DNA were used, and thermocycling reactions carried out under the conditions described in Section 6.3.2.2.c. The primer combination which gave the lowest Ct value and therefore the best amplification rate was taken as having the optimal reaction conditions. As can be seen from Figure 6.4, a concentration of 900nM for both forward and reverse oligonucleotide primers gave the largest fluorescent response. Therefore, all further optimisation and quantification reactions were carried out using this combination of concentrations.

Final Primer Conc. (nM)	**Primer Type**	**Final Primer Conc. (nM)**	**Primer Type**	**Final Primer Conc. (nM)**	**Primer Type**
1. 50	Forward	2. 50	Forward	3. 50	Forward
50	Reverse	300	Reverse	900	Reverse
4. 300	Forward	5. 300	Forward	6. 300	Forward
50	Reverse	300	Reverse	900	Reverse
7. 900	Forward	8. 900	Forward	9. 900	Forward
50	Reverse	300	Reverse	900	Reverse

Table 6.3. The nine combinations of final primer concentrations analysed in the optimisation of TaqMan® probe quantitative PCR reactions.

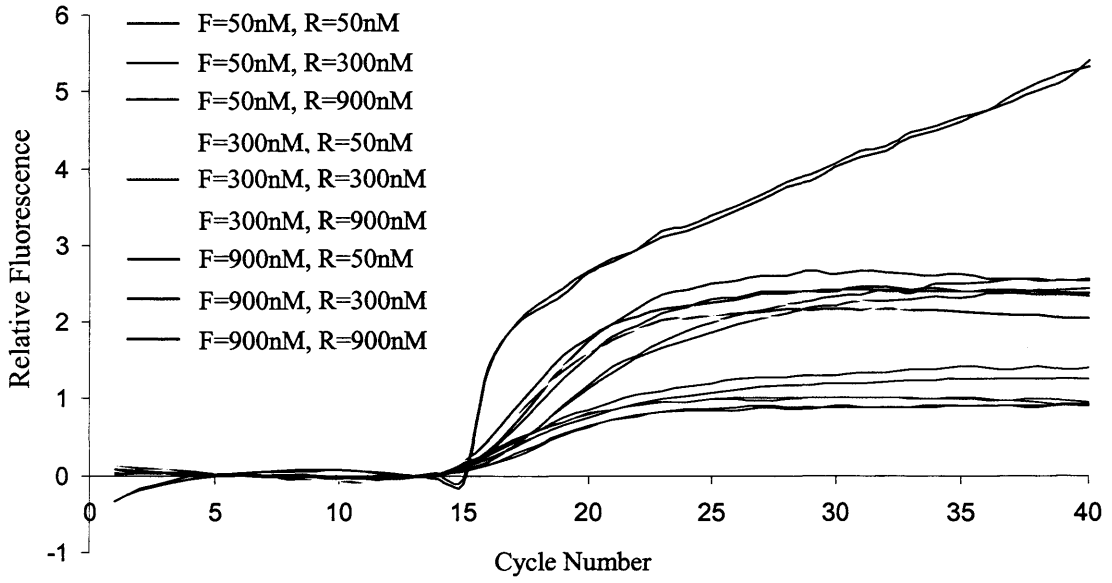


Figure 6.4. TaqMan® Probe real-time QPCR oligonucleotide primer concentration optimisation.

Oligonucleotide primer concentrations were tested in duplicate, using combinations of 3 primer concentrations; 50nM, 300nM and 900nM, as indicated. "F" refers to the concentrations of Forward primer and "R" to the concentration of Reverse primer. Cycling reactions were carried out as described, using the nine different oligonucleotide combinations in the presence of fluorescent probe (200nM) and pcTRELuc plasmid DNA (1ng).

ii) Probe Concentration.

Eight different probe concentrations were tested (Table 6.5) and reactions were performed as described in Section 6.3.2.2.c, using the forward and reverse primers at 900nM (as determined in Section 6.3.2.2.c.i, above). As can be seen from Figure 6.8, the optimal probe concentration (i.e. the concentration which gave the lowest Ct value and therefore the best amplification rate) was 200nM. (An additional set of negative reactions were also prepared with H_2O replacing the pcTRELuc template DNA, in order to ensure that any fluorescence observed could be attributed to the amplification of template DNA).

	Final Probe Concentration (nM)
i	25
ii	50
iii	75
iv	100
v	125
vi	150
vii	175
viii	200

Table 6.5. The eight final-reaction probe concentrations analysed in the optimisation of TaqMan® probe quantitative PCR reactions.

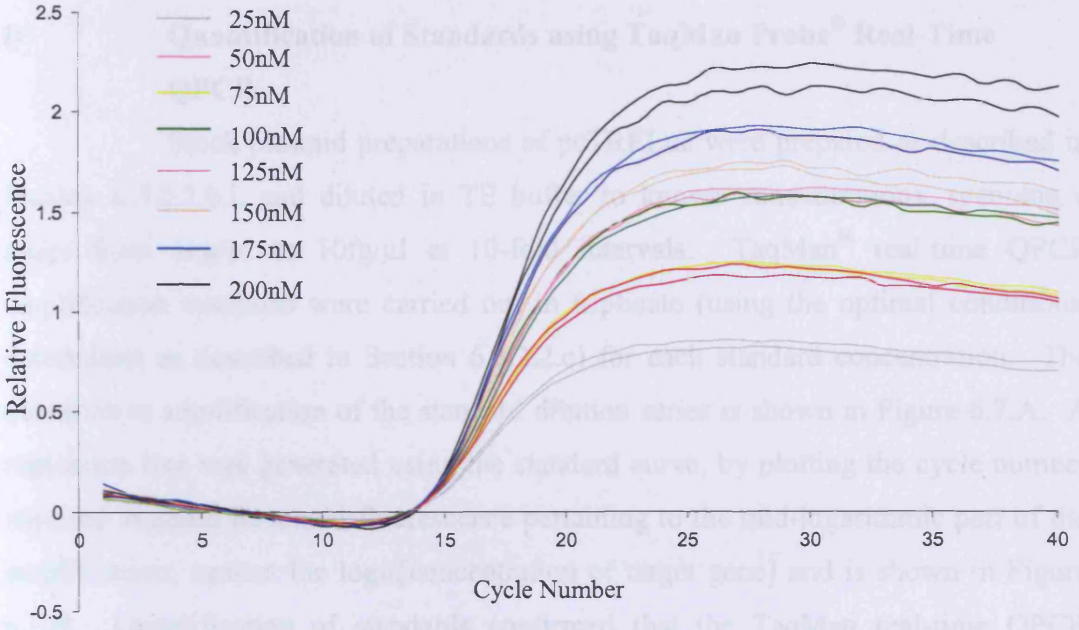


Figure 6.6. TaqMan® Probe real-time QPCR Probe concentration optimisation. Fluorescent probe concentrations were tested in duplicate using the concentrations 200, 175, 150, 125, 100, 75, 50 and 25nM. Cycling reactions were carried out as described, using 900nM each primer and pcTRELuc plasmid DNA (1ng).

d)

i) Quantification of Standards using TaqMan Probe® Real-Time QPCR.

Stock plasmid preparations of pcTRELuc were prepared as described in Section 6.3.2.2.b.i. and diluted in TE buffer to known concentrations, spanning a range from 1ng/µl to 10fg/µl at 10-fold intervals. TaqMan® real-time QPCR amplification reactions were carried out in triplicate (using the optimal conditions, determined as described in Section 6.3.2.2.c) for each standard concentration. The quantitative amplification of the standard dilution series is shown in Figure 6.7.A. A regression line was generated using the standard curve, by plotting the cycle number required to attain threshold fluorescence pertaining to the mid-logarithmic part of the amplification, against the $\log_{10}$[concentration of target gene] and is shown in Figure 6.7.B. Quantification of standards confirmed that the TaqMan real-time QPCR method had been correctly optimised and could now be used for the quantification of luciferase mRNA from *in vitro* transcription systems.

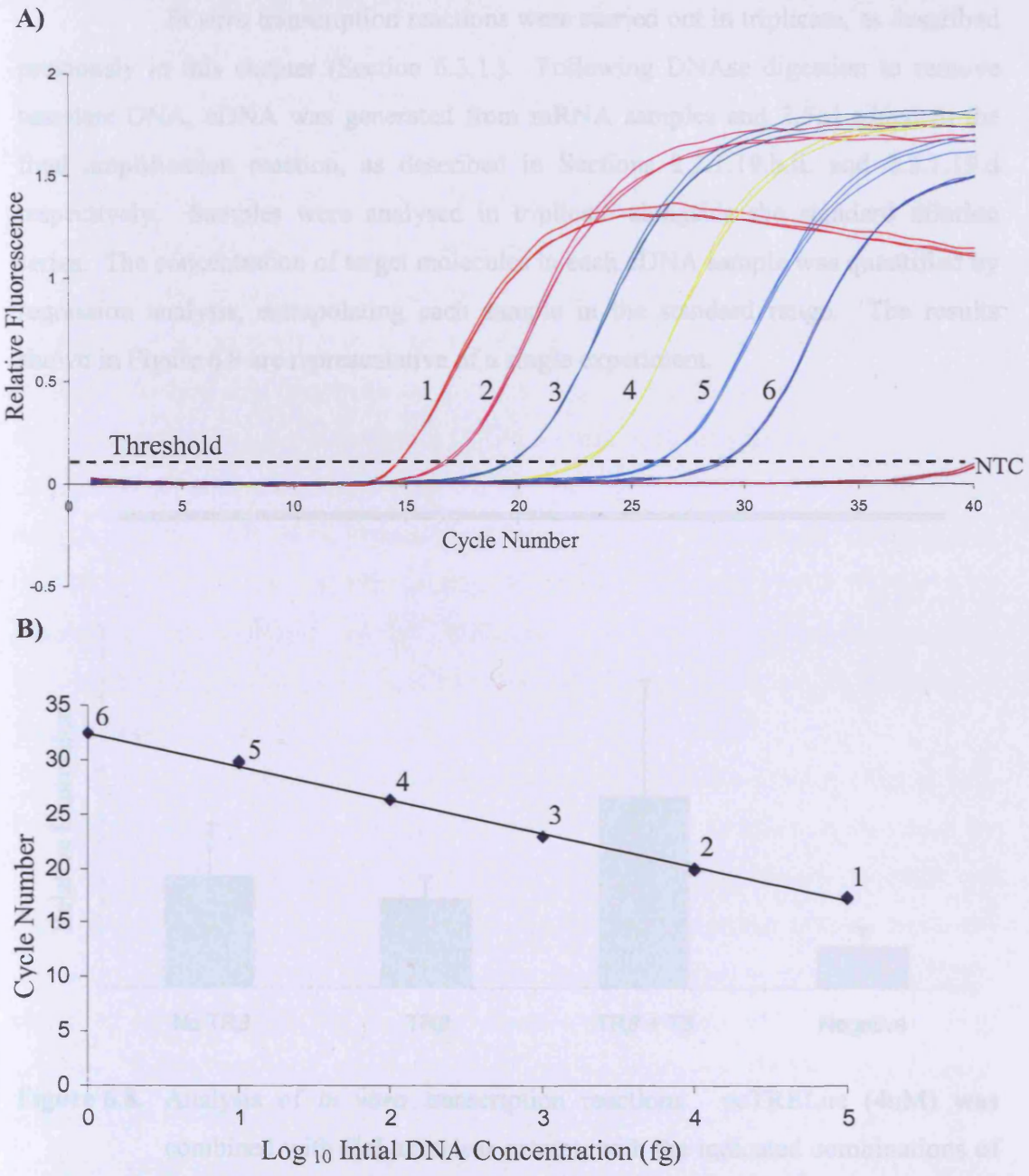


Figure 6.7. Quantitative amplification of a standard dilution series (raw data from pcTRELuc standards), analysed in triplicate. The concentration range 1-6 represents 1ng, 100pg, 10pg, 1pg, 100fg and 10fg respectively.

Panel A: Amplification curves for each standard. An example of threshold value of fluorescence is marked with a dashed line. A no template control (NTC) reaction was also performed.

Panel B: Linear regression analysis of the standard dilution series.

ii) Quantification of Samples using TaqMan Probe® Real-Time QPCR.

In vitro transcription reactions were carried out in triplicate, as described previously in this chapter (Section 6.3.1.). Following DNAse digestion to remove template DNA, cDNA was generated from mRNA samples and 2.5µl added to the final amplification reaction, as described in Sections 2.2.1.19.b.ii. and 2.2.1.19.d respectively. Samples were analysed in triplicate alongside the standard dilution series. The concentration of target molecules in each cDNA sample was quantified by regression analysis, extrapolating each sample in the standard range. The results shown in Figure 6.8 are representative of a single experiment.

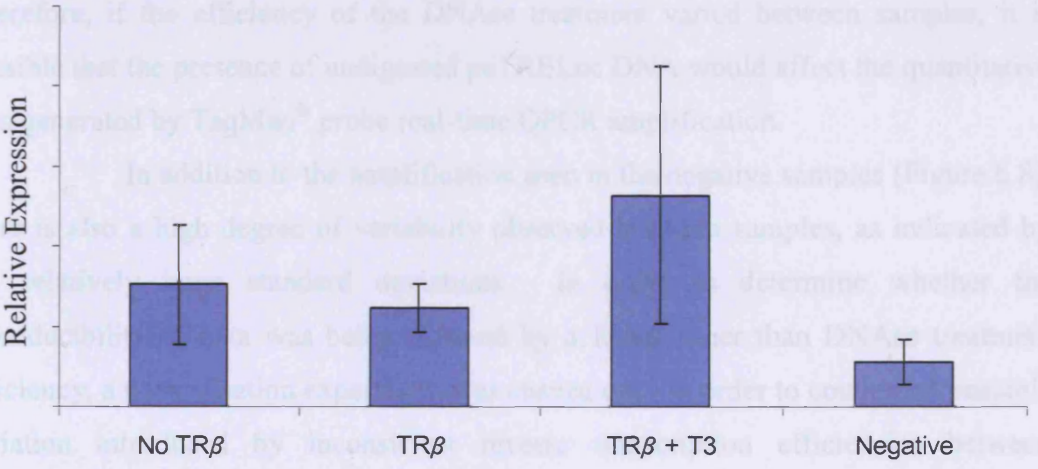


Figure 6.8. Analysis of *in vitro* transcription reactions. pcTRELuc (4nM) was combined with HeLa nuclear extract, with the indicated combinations of TRβ (80nM) and T3 (100nM). Negative samples contained only pcTRELuc (4nM), and no nuclear extract or TRβ. mRNA samples were DNAse-treated and reverse transcribed. Luciferase expression levels were quantified using TaqMan® probe real-time QPCR. Mean values from triplicate samples are shown, and error bars indicate the standard deviations of these triplicates.

A detectable amplicon was associated with the negative samples, containing plasmid, but no nuclear extract or TRβ (Figure 6.8). This amplification cannot be due to the presence of reverse-transcribed cDNA, as there is no transcriptional machinery present in the negative samples to allow for mRNA production. It is unlikely that this is due to contamination of the QPCR reaction since there is no amplification associated with the no template controls (Figure 6.7). It was therefore concluded that the amplification seen in the negative samples in Figure 6.8 were due to the presence of undigested pcTRELuc. Under optimal conditions, the DNAse used to treat samples prior to reverse transcription should digest double-stranded plasmid into fragments as small as 4bp in length (technical information provided by Promega). However, pcTRELuc was initially present at a very high concentration, when the sensitivity of the TaqMan® probe real-time QPCR method is considered; the final initial concentration of pcTRELuc was 20ng/μl (500ng/25μl reaction), whilst it was actually possible to detect as little as 1fg/μl (Figure 6.7). Therefore, if the efficiency of the DNAse treatment varied between samples, it is possible that the presence of undigested pcTRELuc DNA would affect the quantitative data generated by TaqMan® probe real-time QPCR amplification.

In addition to the amplification seen in the negative samples (Figure 6.8), there is also a high degree of variability observed between samples, as indicated by the relatively large standard deviations. In order to determine whether the reproducibility of data was being affected by a factor other than DNAse treatment efficiency, a normalisation experiment was carried out. In order to counteract possible variation introduced by inconsistent reverse transcription efficiencies between samples, values obtained from TaqMan® probe real-time QPCR amplification reactions are usually normalised. This is done by quantifying the expression levels of a control gene in the sample (e.g. actin), however, since the luciferase mRNA being quantified is from an *in vitro* transcription reaction, there is no control gene transcript. This issue was overcome by spiking each purified luciferase mRNA sample with a fixed amount of worm mRNA (2ng per sample; kindly donated by Huw Ricketts, Cardiff University), prior to the generation of cDNA. Consequently, it was possible to carry out TaqMan® probe real-time QPCR amplification reactions using the luciferase probe and primer set, as well as a probe and primer set (optimised previously) designed for the quantification of actin mRNA expression levels. The normalised data is shown in Figure 6.9. From the relatively large standard deviations shown in Figure

6.9, it can be seen that the normalisation of quantitative luciferase expression data against actin levels made virtually no difference to the reproducibility of experimental data, suggesting that the reverse transcription efficiencies are relatively constant and that the variation is introduced by the DNAse treatment step.

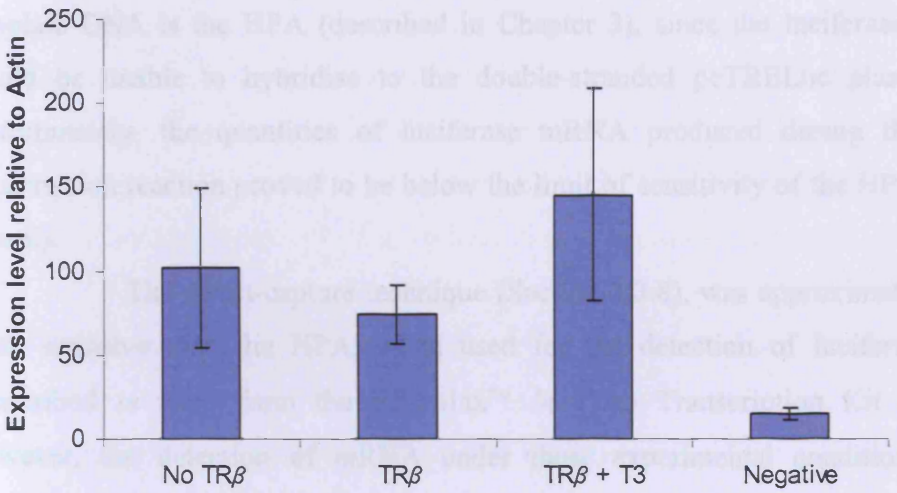


Figure 6.9. Analysis of *in vitro* transcription reactions, spiked with worm Actin mRNA. pcTRELuc (4nM) was combined with HeLa nuclear extract, with the indicated combinations of TRβ (80nM) and T3 (100nM). Negative samples contained only pcTRELuc (4nM), and no nuclear extract or TRβ. All samples were spiked with worm mRNA (2ng), prior to DNAse-treatment and reverse transcription. Luciferase and actin expression levels were quantified using TaqMan® probe real-time QPCR. The mean (+/- S.D.) expression levels of the luciferase gene normalised against actin levels, are shown.

iii) Quantification of Samples using Direct Capture of AE-Labelled Target Nucleic Acids.

An alternative, non-radioactive assay which would enable the specific quantification of luciferase mRNA and would not be affected by the presence of template DNA is the HPA (described in Chapter 3), since the luciferase AE-probe would be unable to hybridise to the double-stranded pcTRELuc plasmid DNA. Unfortunately, the quantities of luciferase mRNA produced during the *in vitro* transcription reaction proved to be below the limit of sensitivity of the HPA (data not shown).

The direct-capture technique (Section 3.3.8), was approximately 10-fold more sensitive than the HPA, when used for the detection of luciferase mRNA transcribed *in vitro* from the RiboMax™ *In Vitro* Transcription Kit (Promega). However, the detection of mRNA under these experimental conditions did not accurately reflect the experimental conditions which would be encountered upon quantifying an mRNA sample from the *in vitro* transcription system described previously in this chapter. The presence of template pcTRELuc DNA caused an increase in background levels of chemiluminescence. Although DNAse treatment of samples countered this increase, background levels of chemiluminescence were still higher than observed in the absence of template DNA. The use of DNAse caused further complications, as with any enzyme, it is most efficient under certain buffer conditions. These buffer conditions differed from the IVT optimal buffer conditions, which, in turn, differed from the optimal buffer conditions which allowed hybridisation of the AE-probe to luciferase mRNA. Although it would have been possible to purify nucleic acids between steps (using a phenol-chloroform extraction followed by ethanol precipitation), the relatively low abundance of luciferase mRNA present, meant that further purification steps would reduce yield and increase variability, therefore this approach was not taken.

6.4. Discussion.

The *in vitro* transcription system used a HeLa nuclear extract as a source of basal transcription factors. Reactions consisted of the pcTRELuc reporter construct, recombinant TRβ (ligand free and ligand-bound) and the nuclear extract. The radionuclide-dependent primer-extension method coupled with densitometric scanning of bands, was used as a means of estimating expression levels of luciferase mRNA. Once it had been established that the *in vitro* transcription system was successfully producing mRNA, it was decided to try and quantify mRNA using non-radioactive methods prior to further optimisation of the *in vitro* transcription system.

Following the optimisation of a probe and primer set specific for the luciferase gene-sequence, Taqman® probe real-time quantitative PCR was used as a detection method for mRNA. Although it was possible to measure mRNA, the reproducibility of experiments was poor, as indicated by the large standard deviations seen for triplicates in Figure 6.8. At first, it was postulated that this may have been due to the varying efficiency of the reverse-transcriptase reaction. However, the spiking of luciferase mRNA samples with worm mRNA allowed the quantification of actin mRNA, also using Taqman® probe QPCR. Results demonstrated that the efficiency of the RT reaction was having little or no effect on the reproducibility of results. The second variable to be investigated was the possible presence of pcTRELuc template DNA in amplification reactions. Although DNAse treatment was carried out pcTRELuc was initially present at a very high concentration; 20ng/μl (500ng/25μl reaction). The sensitivity of TaqMan® probe real-time QPCR amplification meant that it was actually possible to detect as little as 1fg/μl pcTRELuc, as shown by the standard curve in Figure 6.7. Therefore, it was concluded that the efficiency of DNAse treatment was inconsistent, and that the presence of undigested pcTRELuc DNA was affecting the quantitative data generated by TaqMan® probe real-time QPCR amplification. Template DNA removal was attempted using RNeasy columns (which bind DNA but not RNA), but was unsuccessful.

Hybridisation Protection Assay (HPA), an alternative non-radioactive technique, was used in an attempt to quantify luciferase mRNA. However, HPA was not sensitive enough to measure the relatively small amounts of mRNA produced from the *in vitro* transcription reaction. Luciferase mRNA quantification was also attempted using a second AE-based assay, the direct-capture technique, where the

sensitivity of AE detection is increased by separating hybridised AE-probe from unhybridised AE-probe, effectively lowering levels of background chemiluminescence. Although the direct-capture method has been shown to be approximately 10-fold more sensitive than the HPA, it still was not possible to quantify luciferase mRNA using this technique, possibly due to technical difficulties; buffer compatibility between different parts of the assay and the increase in background levels of chemiluminescence associated with the presence of the pcTRELuc template DNA.

The direct-capture technique still has potential as a non-radioactive technique which could be used to quantify luciferase mRNA from IVT systems. Further optimisation of buffer conditions could allow the development of a homogeneous assay, whilst it is possible that a selective hydrolysis step (described previously; Section 3.2.2.2.) could further lower background levels, prior to detection of the chemiluminescent signal.

Chapter Seven

General Discussion.

7.1. Introduction.

Scientific interest in possible thyroid modulation by chemicals present in the environment has intensified recently due to the central importance of this endocrine system to growth, development, reproductive viability and health (McLachlan, 2001).

Initial concern focussed on environmental chemicals that could impact on thyroid-mediated developmental, as well as reproductive, processes within wildlife species. However, in addition to the reports of abnormalities within wildlife populations (Brucker-Davies, 1998; Crain *et al.*, 1998), there are also a number of publications in which human reproductive health appears to be compromised, and a possible link to environmental chemical contamination suggested (Johansen *et al.*, 1997; Steenland *et al.*, 1997; see Section 1.6 for more detail).

The aim of this research was the development of *in vitro* assays, which would support a sequential testing strategy for potential thyroid disrupting chemicals. In this context, sequential testing refers to a gradual, step-wise strategy for the assessment of chemicals, beginning with the evaluation of the ability of a chemical to disrupt the interaction between the thyroid receptor and its cognate piece of DNA. Chemicals found to have an effect on TR-DNA binding could then be screened for transcriptional effects, using the cell-free transcriptional assay. Finally, prior to any animal testing, a cell culture system could be employed to provide additional information regarding the entry of chemicals into cells, as well as the effect of metabolism on the transcriptional modulation by chemicals.

7.2. Potential Tier 1 Screening (T1S) *In vitro* Assays.

Although a number of limitations are associated with the simple *in vitro* assays developed as part of this research (for example, they do not take into consideration the interaction of thyroid hormones with serum thyroid-binding proteins) they represent a convenient starting point for the identification of potential thyroid-disrupting chemicals - in view of the large numbers of chemicals that could be subject to testing for potential thyroid effects (a possible 80,000+, see Section 1.6), the development of rapid high-throughput assay systems would be a major advantage.

The proposed *in vitro* assays would fit within EDSTAC's tiered screening system (Section 1.7.1), and allow the identification of chemicals with potential thyroid-disrupting properties. This would allow a reduction in the number of compounds to be tested using *in vivo* studies, and/or would compliment the results of T1S *in vivo* studies such as the Amphibian Metamorphosis Assay and the Male and Female Rat Pubertal Assays.

7.2.1. Cell Culture Systems.

The cell culture system described in Chapter 5 consists of a luciferase reporter construct, where the luciferase gene is under the control of the Malic Enzyme TRE. The binding of TR to this TRE regulates luciferase gene expression in a ligand-dependent manner and thus it should be possible to measure the effect of a potential thyroid disruptor on the transcriptional response from this construct. The use of a cellular system in conjunction with the cell-free *in vitro* transcriptional system to study the impact of a potential disruptor on transcription, would allow an insight to be gained into the entry of potential disruptors into cells, as well as the effect of metabolism on a chemical's disruptor properties.

Initial experiments focussed on the transient transfection of the pRG224 vector (where the luciferase gene is under the control of the hCMV immediate-early promoter) into U373 cells. The two endpoints measured for the quantification of luciferase expression levels were luciferase protein (measured using the Promega Luciferase Assay) and luciferase mRNA (measured using the HPA). The eventual objective was to use a thyroid-responsive promoter upstream of the luciferase gene, in order to quantify the transcriptional and translated protein response to thyroid-mediated stimulus in the presence of potential disruptors. However, even using the pRG224 vector (where luciferase expression is under the control of a relatively potent

promoter), such large numbers of cells were required in order to purify sufficient quantities of mRNA for detection by HPA, that such experiments were deemed unviable.

There are a number of disadvantages associated with transient transfection systems, including an inherent variability of efficiency and the potential toxic effect of transfection reagents. In order to negate these variables and in an attempt to increase overall luciferase expression levels, a stably-transfected cell line was generated. pcTRELuc was transfected into CV1 cells (which express both TRα and the 9-*cis* retinoic acid receptor) which were subsequently grown under G418 (Neomycin) selective pressure. Following the isolation and propagation of a number of clonal populations of stably-transfected cells, the response of cells to T3 was quantified. Although a T3-dependent up-regulation of luciferase transcription was detected, this was of a lesser magnitude than expected. Further experiments indicated that this could possibly be due, in part, to a lack of endogenous TRβ in the cells. The discussion in Chapter Five details the work of Tillman *et al.*, (1993) and Maia *et al.*, (1996) who postulated that there may be a negative TRE present in the luciferase coding sequence. They concluded that any results drawn from experiments involving the use of thyroid-responsive luciferase reporter constructs in CV1 cells should be interpreted with caution. Many previous studies cited in the literature (Ribeiro *et al.*, 2001; Bhat *et al.*, 1997; Zhang *et al.*, 1996; Umesono *et al.*, 1991) utilise thyroid-responsive CAT reporter contructs in CV1 cells. A possible future directive might be the generation of a stably-transfected cell line using a different construct; the CAT gene under the control of a TRE one direction, and the coding sequence for TRα and/or TRβ under the control of a different promoter. In this way, the uncertainties surrounding the use of thyroid-responsive luciferase constructs would be avoided, whilst the assay would still benefit from the advantages offered by a stably transfected cell line. During the course of this thesis, Bogazzi *et al.*, (2005) utilised a ME-TRE CAT reporter construct and transient transfection studies to demonstrate the antagonistic effects of Aroclor 1254 (a commercial mix of PCBs) on the thyroid receptor. This latter study illustrates the potential benefits of a reporter based approach, as proposed in the aims of this thesis, unfortunately the specific genetic construct exploited did not facilitate comparable success within our studies.

7.2.2. *In Vitro* Transcription Systems.

The *in vitro* transcription (IVT) system described in Chapter 6 utilises pcTRELuc; the same ME-TRE luciferase reporter construct as the cell culture system described above. However, the IVT reactions do not utilise the host cell's transcriptional machinery, instead, they employ a HeLa nuclear extract. HeLa cells do not express TR, but express RXR and the cofactors necessary for TR function.

Used in conjunction with the cell culture system, the IVT system would allow for the screening of chemicals for any effects on TR-mediated transcription. The IVT system has the advantage that it does not require the chemical to enter the cell, and the metabolic processing of any chemical would be minimised. Also, if a chemical were to somehow affect translation of the luciferase protein (which is the endpoint measured from the cell culture system) it would be possible to differentiate this effect from an effect on gene transcription, by using these two systems together.

Following the intial optimisation of the IVT system and the quantification of luciferase mRNA transcripts using the radionuclide-dependent primer extension technique, the possibility of quantifying luciferase mRNA using non-radioactive techniques was investigated. A luciferase-specific primer and probe set was optimised, in order to allow transcript quantification using TaqMan® probe real-time QPCR. However, since TaqMan® QPCR is a highly sensitive technique that quantifies the reverse-transcribed products of the luciferase mRNA transcripts, it was necessary to remove the pcTRELuc template DNA, prior to the reverse-transcription reaction. A number of techniques were used, singly, or in combination, in an attempt to remove all template DNA. However it was not possible to consistently achieve reproducibly low background readings, and so TaqMan® probe QPCR was deemed unsuitable as a detection technique to quantify luciferase transcript levels from IVT systems.

Although the HPA had been successfully used previously in the quantification of luciferase mRNA, the small amounts of mRNA produced via the IVT system proved to lie below the limit of sensitivity of the HPA. Components of the HPA were used, however, in the development of an alternative AE-based assay. The direct-capture technique is described in Section 5.3.2.3 and was successfully used to detect as little as 26amoles of luciferase mRNA (compared to 251amoles of mRNA, detected using the HPA). Due to the limitations of sensitivity of the HPA, the absolute aim of the thesis was not achieved for the *in vitro* transcription systems.

However, although the direct-capture technique described still requires further refinement and optimisation, it has the potential to be developed into a novel, straightforward, high-throughput assay, which could be used to screen chemicals for effects on TR-mediated transcription. If an alternative Cell Culture system were developed which utilised a CAT reporter construct (Section 7.2.1, above), then it would be necessary to design and optimise an AE probe, helper and polyA-bridge to a region of the CAT reporter sequence.

7.2.3. TR-DNA Binding Assays.

The regulation of transcription on thyroid-responsive genes is mediated by the thyroid receptor, and a pre-requisite of this control is the binding of TR to DNA. TR-DNA binding was monitored using the electrophoretic mobility shift assay (EMSA). Since this technique relies upon the binding of protein to a radiolabelled probe (double-stranded oligonucleotide), it was possible to quantify the amount of free probe in a given reaction by densitometric scanning of dried gels. The K_d of a protein refers to the protein concentration at which approximately half the ligand (radiolabelled probe, in this instance) has become bound, and so is a measure of a protein's affinity for its ligand. Thus, the K_d of TR for DNA was estimated, and the effect of T3 and its analogues (whose relative affinities for TR were known) on the TR-DNA binding was monitored. Consistent with previously published data, the results from the EMSA studies revealed that the higher the affinity of TR for a particular ligand, the greater the disruption of TR-DNA binding.

Therefore, an assay system was successfully developed, which allowed the monitoring of TR binding to DNA and the quantification of binding disruption in the presence of a potential EED. The potential exists to further optimise this system, to allow the development of a non-radioactive, high-throughput assay. Recombinant His-tagged TR could be immobilised to a solid phase (Ni-NTA beads) and oligonucleotide probes labelled with the previously described acridinium ester could subsequently bind. Any free AE-labelled probe could be washed away and thus, the detection of any remaining chemiluminescence associated with the beads would allow the quantification of DNA binding to the immobilised TR.

7.2.4. Alternative Assays.

During the course of this thesis, a number of studies have described the development of assays which could potentially fit into EDSTAC's Tier 1 Screening and Testing batteries. Bogazzi *et al.*, (2005) used a thyroid-responsive CAT reporter construct to examine the effects of Aroclor 1254 (A1254, a commercial mix of PCBs) on gene expression. A1254 inhibited TR action on the ME-TRE CAT reporter construct, however A1254 did not alter the ability of TR to bind to the ME-TRE in a gel shift assay. This study illustrates the importance of the use of complimentary systems to screen chemicals for potential thyroid effects. Gutleb *et al.*, (2005) have developed the "T-test" which is an *in vitro* bioassay based on thyroid hormone dependent cell proliferation of a rat pituitary tumour cell line (GH3) in serum-free medium. This same system was utilised by Kitamura *et al.*, (2005) to demonstrate the thyroid agonistic effects of a number of hydroxylated PCBs. Opitz *et al.*, (2005) developed the *Xenopus* Metamorphosis Assay (XEMA) to detect thyroid-disrupting activities of potential EEDs. They monitored developmental rates as a means of assessing the thyroidal status of the differentially treated tadpoles. The metamorphic development of tadpoles to juvenile froglets is dependent on TH and in accordance with this, they showed that T4 accelerated tadpole development whereas mammalian anti-thyroidal compounds such as propylthiouracil (PTU) and ethylenethiourea (ETU) caused developmental retardation of *X. laevis* tadpoles.

It is possible that a QSAR (Quantitative Structure Activity Relationships) approach could be developed for predicting the binding affinities of chemicals to the TR. Hong *et al.*, (2002) developed a decision tree-based model to evaluate 58,000 chemicals for binding to the estrogen receptor by using physiochemical data and SARs from a training set of molecules with a known binding affinity for the rat estrogen receptor. The model involves using a set of exclusion filters to narrow the window of structural criteria for testing. This computational approach had a low false-negative rate and predicted that 80% of the 58,000 chemicals would show negligible binding to the estrogen receptor.

7.3. Concluding Remarks.

The thyroid system is so complex that understanding its normal function is difficult enough, but deciphering environmental disruptions to it is staggeringly difficult. Many of the chemicals of concern were produced to improve human welfare and provide economic benefit (e.g. to increase crop production). The science of endocrine disruption, however, is now revealing many unexpected adverse consequences, resulting from the ability of very low levels of these compounds to interfere with gene expression. Most of the chemicals now implicated have been subjected to little if any rigorous testing (Myers *et al.*, 2003). Krimsky (2000) states what he terms the "environmental endocrine hypothesis" which asserts that "a diverse group of industrial and agricultural chemicals in contact with humans and wildlife have the capacity to mimic or obstruct hormone function – not simply disrupting the endocrine system like foreign matter in a watchworks, but fooling it into accepting new instructions that distort the normal development of the organism...". From the standpoint of human pathology, the environmental endocrine hypothesis could turn out to be the most significant environmental health hypothesis since the discovery of chemical mutagenesis" (McLachlan, J.A., 2001).

The development of the OECD's "toolbox" of assays for potential thyroid disruptors (described in Section 1.8) will allow assessments to be made regarding the potential human health impact from the release of novel chemicals into the environment, as well as the risk associated with chemicals that are already in use. The "toolbox" is organised into a number of compartments or levels each corresponding to a different level of biological complexity. Even though the toolbox may be full of testing tools, this does not imply that they will all be needed for assessment purposes. Scientists are attempting to minimise animal use by using as few animals as possible in the most precise and sensitive assays, and by incorporating sensitive *in vitro* assays wherever possible (Gray Jr *et al.*, 2002). The use of the TR-DNA binding assay developed here, as well as other high throughput pre-screening assays, (e.g. quantitative structure activity modelling, QSAR), allows for the prioritisation of chemicals for further screening (see Figure 7.1). The cell culture and *in vitro* transcription systems could subsequently be employed, meaning that chemicals testing positive *in vitro* could then be evaluated further using more extensive, higher tier animal tests.

Thus, the concepts for three potential *in vitro* assays have been developed over the course of this thesis. Potential limitations were identified within each approach which significantly limit the utility of the assays developed, however, potential technical modifications (detailed in the relevant Chapters) have been proposed which would provide the basis for future developments. The requirement for the development of assays which could potentially fit into EDSTAC's Tier 1 Screening and Testing batteries has been recognised by the Scientific community, as illustrated by the previously mentioned studies of Bogazzi *et al.*, (2005) Gutleb *et al.*, (2005) Kitamura *et al.*, (2005), detailed in Section 7.2.4. Therefore we are now seeing the development of assays which are relatively straightforward and inexpensive, and would allow for the screening and prioritisation of large numbers of chemicals for potential thyroid-disrupting properties, prior to further *in vivo* studies.

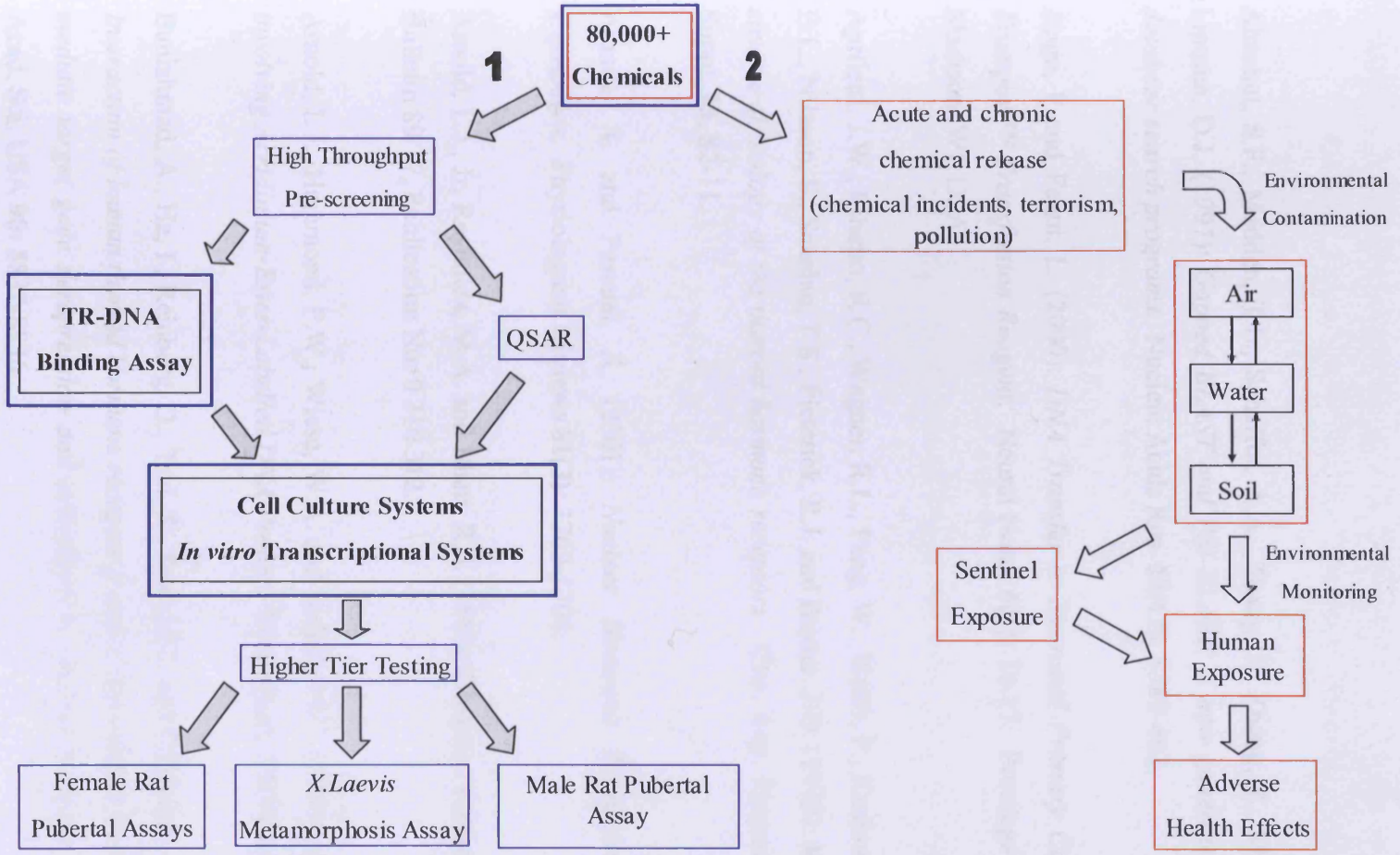


Figure 7.1. There are currently over 80,000 industrial chemicals in use today (Keith, L.H. 1997). The use of the *in vitro* assays developed as part of this thesis (double-boxed in blue and black) within a tiered screening strategy (EDSTAC Tier 1 Screening Assays; Route 1) allows the identification of chemicals with potential thyroid-dsirupting properties. This reduces the number of animal tests carried out, by limiting thenumber of chemicals subjected to higher Tier testing. The fate of chemicals released into the environment (with associated consequences) is illustrated in Route 2.

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APPENDIX 1

OLIGONUCLEOTIDE SEQUENCES.

A) Universal Primers and Their Sequences.

Primer	Sequence (5'→3')
M13 For	CAG GAA ACA GCT ATG AC
M13 Rev	GTA AAA CGA CGG CCA GT
T7 For	AAA TAC GAC TCA CTA TAG
T7 Rev	GCT AGT TAT TGC TCA GCG G
Random Hexamers	$(N)_6$
Oligo (dT)	TTT TTT TTT TTT TTT

B) Thyroid Receptor Primers, used for PCR Amplification.

(*Bgl*II restriction sites in bold).

Primer	Sequence (5'→3')
TRα For	G**AG ATC T**GA TGG AAC AGA AGC CAA GCA AG
TRα Rev	GAA **GAT CT**T TAG CGG CCG CTG ACT TCC TGA TCC TCA AAG AC
TRβ For	G**AGATC T**GA TGA CTC CTA ACA GTA TGA CAG
TRβ Rev	GAA **GAT CT**T TAG CGG CCG CTG TCC TCA AAT ACT TCT AAG AAC

C) Oligonucleotides used in the Luciferase HPA.

Primer	Sequence (5'→3')
AE-probe	CCC TTA GGT AAC CTA GTA GAT CCA GAG G
5' - helper	AAT TCA TTA TCA GTG CAA TTG TTT TGT CAC
3' - helper	ACG CAG GCA GTT CTA TGC GGA AGG GCC ACA
Target oligo	CCT CTG GAT CTA CTG GGT TAC CTA AGG G

D) The pGL2 used to Sequence Upstream of the Luciferase Start Codon.

Primer	Sequence (5'→3')
pGL2	CTT TAT GTT TTT GGC GTC TTC CA

E) The Malic Enzyme TRE-Containing Primer Pair used for Probe Generation in the EMSA Analysis of Recombinant Proteins.

Sequences in bold indicate non-complimentary regions which form 5'-overhangs upon annealing, allowing subsequent "filling in" by Klenow polymerase, and generation of a radiolabelled duplex.

Primer	Sequence (5'→3')
2G-ME-TRE-FOR	**GGC ATC** CAG GAC GTT GGG GTT AGG GGA GGA CA
2G-ME-TRE-BAK	**GGT CCA C**TG TCC TCC CCT AAC CCC AAC GTC CT

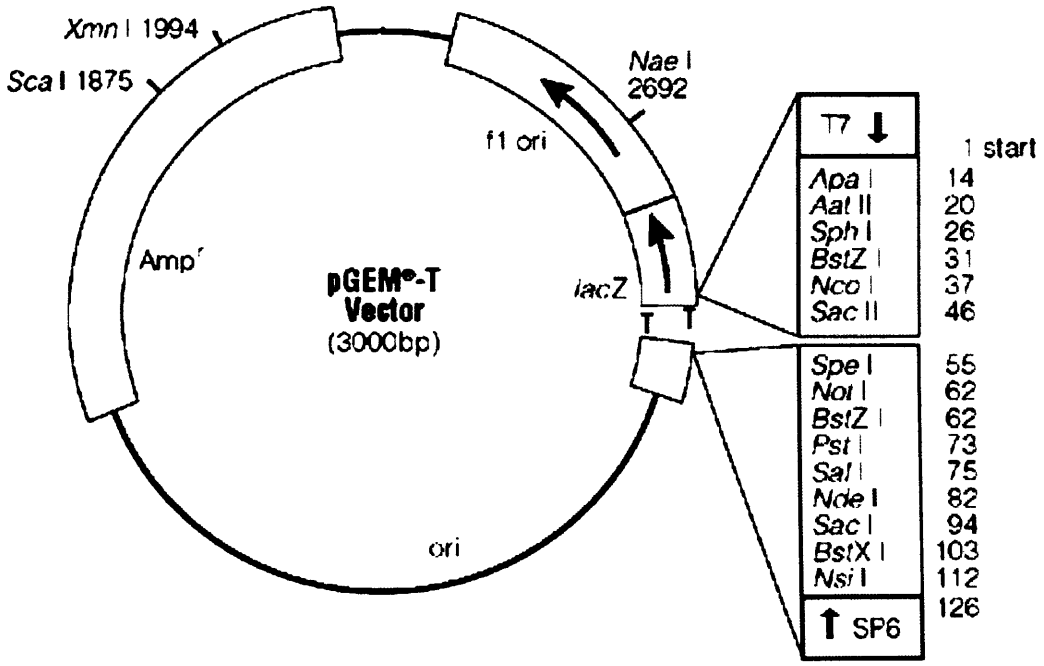
F) Probe and Primer Sequences, used in the Taqman Real-Time Quantitative PCR Analysis of Luciferase mRNA.

	Sequence (5'→3')
5' Primer	TGC TTT TAC AGA TGC ACA TAT CGA
3' Primer	TGC CAA CCG AAC GGA CAT
Probe	TGA ACA TCA CGT ACG CGG AAT ACT TCG A

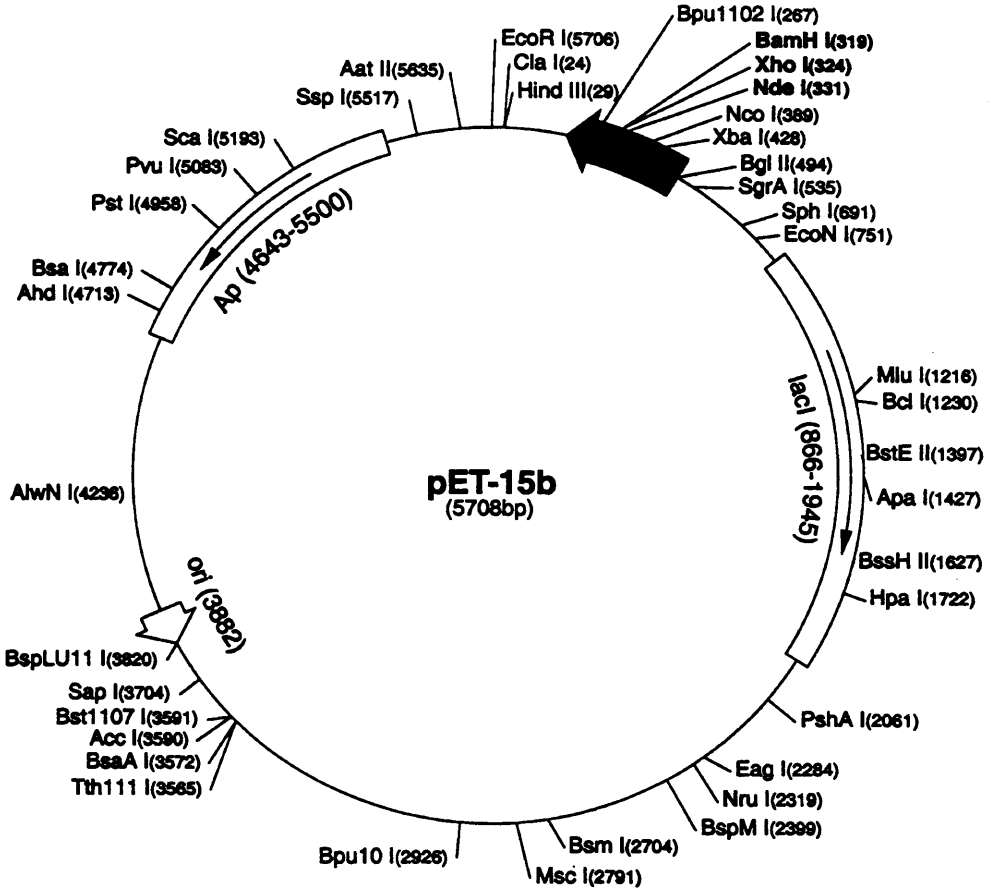
APPENDIX 2

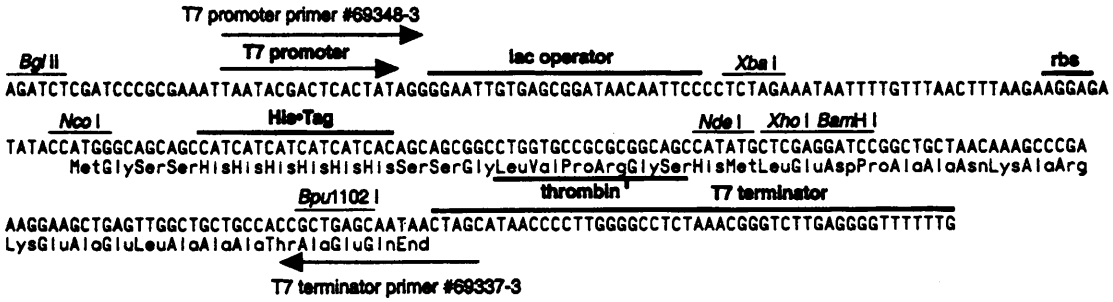
VECTOR MAPS.

A) pGEM-T (Promega).



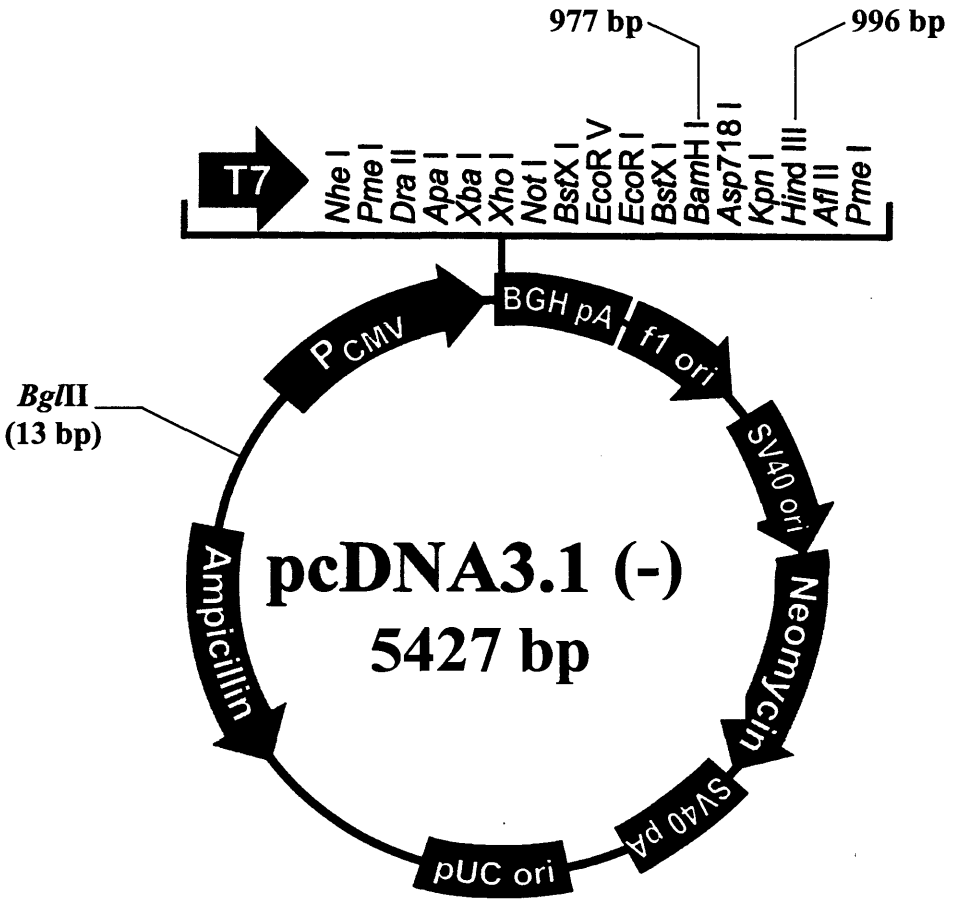
B) **pET-15b Vector map, showing multiple cloning sites, and position of His-Tag (Novagen).**



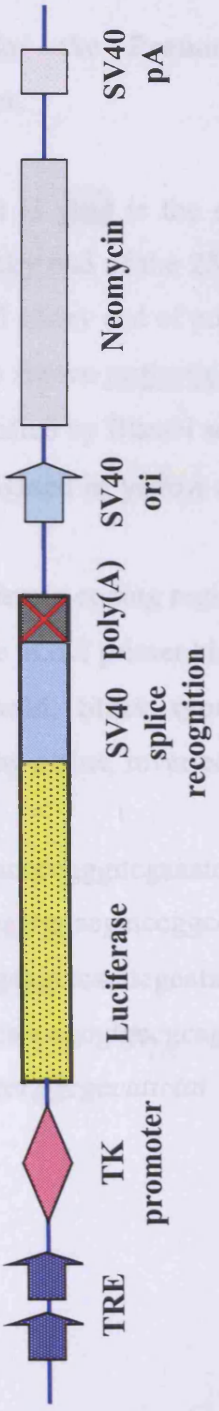


pET-15b cloning/expression region

C) pcDNA3.1(-) Expression Vector Map, with Multiple Cloning Site Shown (Invitrogen).



D) **The pcTRELuc Reporter Construct, with Important Features Shown.**



APPENDIX 3

DNA SEQUENCES.

A) DNA Sequence for the Promoter Region of the pcTRELuc Reporter Construct.

- The sequence highlighted in grey is the splice-sequence generated upon the ligation of the *Bam*HI sticky end of the 250bp TRE-containing fragment from MAL-TKLUC to the *Bgl*II sticky end of pcDNA3.1.
- The Malic enzyme TRE is shown **underlined, in red.**
- TheTK promoter, as identified by BlastN search, is in blue.
- The two sequences highlighted in yellow are the *Hin*dIII sites into which the ME-TRE was cloned.
- The beginning of the luciferase coding region is shown in *italics.*
- The sequence to which the pGL2 primer binds is highlighted in *green*.
- The TATA box is in **bold, black type**, and the estimated start site of transcription is indicated by a blue, inverted triangle.

tgacgtcgacggatcgggagatccccgggtaccccgggttcgaaatcgataagcttgcatgcctgcaggtcgactctaga
ggatcaggacgttggggttaggggaggacagtggacgatccggccccgcccagcgtcttgtcattggcgaattcgaac
acgcagatgcagtcggggcggcgcggtccgaggtccacttcgcatattaaggtgacgcgtgtggcctcgaacac▽cga
gcgaccctgcagcgacccgcttaacagcgtcaacagcgtgccgcagatctaagcttggcattccggtactgttggtaaaat
ggaagacgccaaaaacataaagaaaggcccggcgccattctat

B) Nucleotide Sequence and Amino Acid Translation for the N-terminal End of TRα and TRβ, Expressed from pET-15b.

The genes for the murine forms of TRα and TRβ were amplified by PCR and ligated into pGEM-T. cDNAs were excised by *Bgl*II digestion, gel-purified and ligated into the expression vector, pET-15b, which had been digested with *Bam*HI.

Nucleotide sequence from the pET-15b expression vector, beginning with the start codon from the vector, is in blue. The His-tag is highlighted in yellow. Sequence in red represents the ligated gene sequence for TRα or TRβ, with the start codon in italics, underlined and bold.

```
atg ggc agc agc cat cat cat cat cat cac agc agc ggc ctg gtg
Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val

ccg cgc ggc agc cat atg ctc gag gat ctg atg
Pro Arg Gly Ser His Met Leu Glu Asp Leu Met
```

C) Nucleotide Sequence for the *Photinus Pyralis* Luciferase Gene, Showing Probe and Helper Binding sites, as used in the Luciferase HPA.

The coding sequence for the firefly luciferase gene is shown in black. The highlighted sequences indicate the specific regions to which the components of the HPA assay bound. In red is the binding site for the Acridinium Ester Probe, in blue the 5' – helper (also the sequence used for the polyA bridge) and in purple the 3' – helper.

atggaagacgccaaaaacataaagaaaggcccggcgccattctatcctctagaggatggaaccgctggagagcaactgc
ataaggctatgaagagatacgccctggttcctggaacaattgcttttacagatgcacatatcgaggtgaacatcacgtacgcg
gaatacttcgaaatgtccgttcggttggcagaagctatgaaacgatatgggctgaatacaaatcacagaatcgtcgtatgcag
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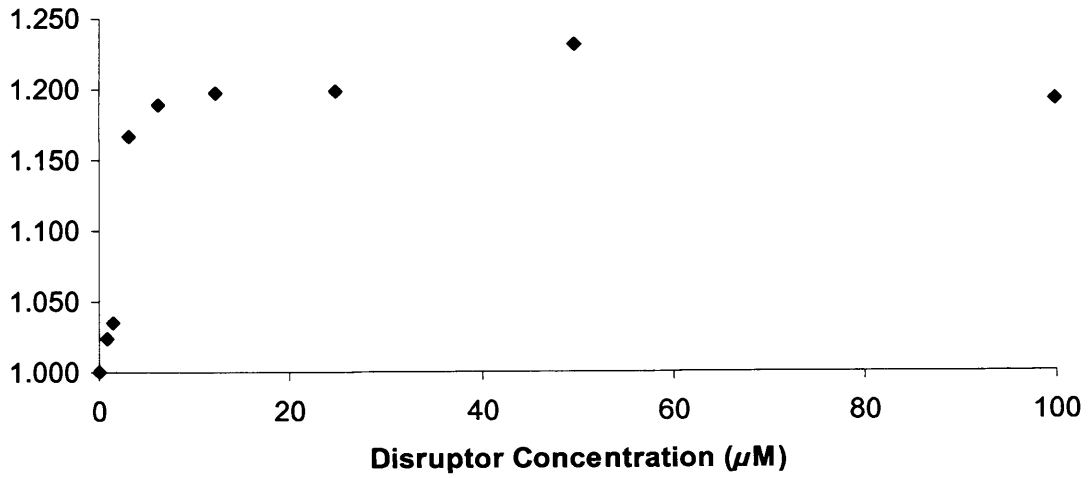
gcaaaaaaaattaccaataatccagaaaattattatcatggattctaaaacggattaccagggatttcagtcgatgtacacgttc
gtcacatctcatctacctcccggttttaatgaatacgattttgtaccagagtcctttgatc**gtgacaaaacaattgcactgata**
atgaattcctctggatctactgggttacctaagggtgtggcccttccgcatagaactgcctgcgtcagattctcgcatgc
cagagatcctatttttggcaatcaaatcattccggatactgcgattttaagtgttgttccattccatcacggttttggaatgtttact
acactcggatatttgatatgtggatttcgagtcgtcttaatgtatagatttgaagaagagctgtttttacgatcccttcaggattac
aaaattcaaagtgcgttgctagtaccaaccctattttcattcttcgccaaaagcactctgattgacaaatacgatttatctaattta
cacgaaattgcttctgggggcgcacctctttcgaaagaagtcggggaagcggttgcaaaacgcttccatcttccagggatac
gacaaggatatgggctcactgagactacatcagctattctgattacacccgagggggatgataaaccgggcggggtcggt
aaagttgttccatttttgaagcgaaggttgtggatctggataccgggaaaacgctgggcgttaatcagagaggcgaattatg
tgtcagaggacctatgattatgtccggttatgtaaacaatccggaagcgaccaacgccttgattgacaaggatggatggcta
cattctggagacatagcttactgggacgaagacgaacacttcttcatagttgaccgcttgaagtctttaattaaatacaaaggat
atcaggtggcccccgctgaattggaatcgatattgttacaacaccccaacatcttcgacgcgggcgtggcaggtcttcccga
cgatgacgccggtgaacttcccgccgccgttgttgttttggagcacggaaagacgatgacggaaaaagagatcgtggatta
cgtcgccagtcaagtaacaaccgcgaaaaagttgcgcggaggagttgtgtttgtggacgaagtaccgaaaggtcttaccg
gaaaactcgacgcaagaaaaatcagagagatcctcataaaggccaagaagggcggaaagtccaaattgtaa

APPENDIX 4

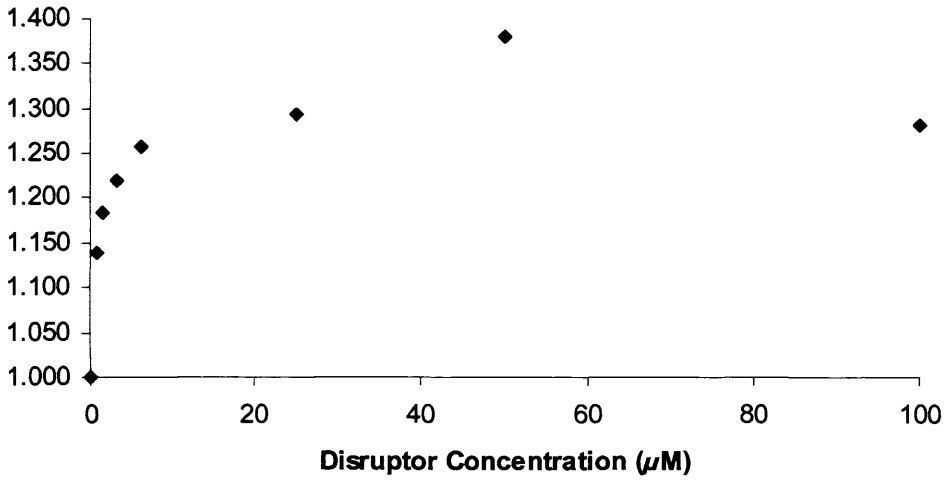
TH / T3-Analogue/ Binding Disruption Plots.

Recombinant TRβ to a final concentration of 1.0μM (the K_d of ligand-free TRβ as determined in Section 7.3.1.a) was combined in an EMSA reaction with a constant amount of radiolabelled ME-TRE probe (0.3pmol/reaction) and varying concentrations of either THs or their analogues, as described in Section 2.2.4.9.c. Complexes were separated by gel electrophoresis (Section 2.2.4.9.d), the gels dried and the radioactivity associated with bands was quantified as described in Section 2.2.4.9.e. The quantitative data is plotted relative to the amount of free probe observed in the absence of the relevant compound (arbitarily designated as 1).

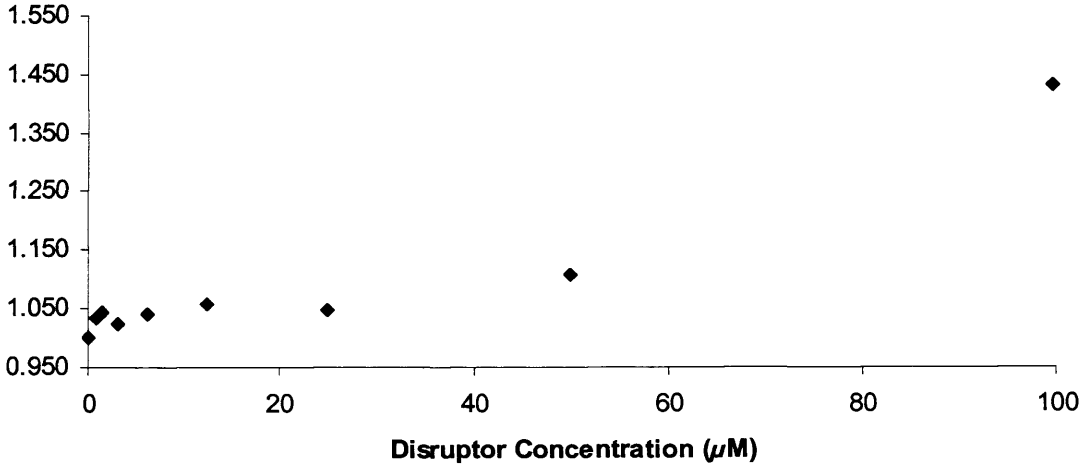
A) Triiodothyronine (T3).



B) Tetraiodothyronine (T4).



C) Tetraiodothyroaceticacid (TETRAC).



D) Reverse Triiodothyronine (rT3).