# Transcriptional Regulation of Human Topoisomerase II Beta

A thesis presented to Massey University in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry

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# Abstract

Topoisomerase II has an essential role in maintaining the DNA in the correct topological state required for various cellular processes. Its mechanism of action involves the introduction of a double-stranded break into the DNA, passage of a different piece of DNA through the break, followed by the religation of the DNA. Topoisomerase II, in humans, exists as two different isoforms: topoisomerase II alpha, which is cell cycle-regulated and highly expressed in rapidly proliferating cells, and topoisomerase II beta, which is ubiquitously expressed and it is not under the influence of the cell cycle.

Several chemotherapeutic drugs have been designed to interfere with the catalytic mechanism of the topoisomerase II enzyme. By either stabilising the DNA cleavage complex or interfering with another step of the mechanism, these topoisomerase II targeted drugs promote the entry of the cell into cell death pathways. An increasing problem in the treatment of cancer with these drugs is the rising number of patients with inherited or developed drug-resistance. It has been shown that drug-resistance, at least in part, results from the down-regulation of topoisomerase II expression.

The expression of a gene is a highly regulated process and the initiation of transcription represents a major point of regulation. Prior to this study little was known regarding the regulation of transcription of topoisomerase II beta. Understanding the processes surrounding the regulation of this enzyme would provide some insight as to how it is down regulated in drug-resistance.

The focus of this study was to examine the role of three elements in the topoisomerase II beta promoter, GC1, ICB1, and ICB2 and the transcription factors that bind to them. Electrophoretic mobility shifts assays revealed that Sp1, Sp3, NF-Y and two uncharacterised proteins are capable of binding to the promoter *in vitro*. Transient transfection assays showed *in vivo* that Sp1 was able to activate transcription and that Sp3 inhibited transcription driven by the topoisomerase II beta promoter. In addition the key activating elements appear to be ICB2 and GC1, while ICB1 is inhibitory.

# Abbreviations

In addition to the chemical symbols from the Periodic Table of Elements and the International System of Units (SI), the following abbreviations are used:

| | |
|---|---|
| Amp | Ampicillin |
| Ap-2 | Activator Protein 2 |
| ATF | Activating transcription factor |
| ATP | Adenosine triphosphate |
| ATPase | Adenosine triphosphotase |
| B-gal | β-galactosidase |
| bp | Base pairs of DNA |
| BSA | Bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| CAT | Chloramphenicol acetyltransferase |
| cDNA | Synthetic DNA, generated from mRNA |
| c/EBP | CCAAT/enhancer binding protein |
| ChIP | Chromatin immunoprecipitation assay |
| CMV | Cytomegalovirus |
| CTF/NF-1 | CCAAT transcription factor |
| DEAE | Diethylaminoethyl |
| DMSO | Dimethyl sulfoxide |
| dNTP | Deoxynucleoside triphosphate (dCTP, dGTP, dTTP, dATP) |
| *E.coli* | *Escherichia coli* |
| EDTA | Ethylene diamine tetra-acetic acid |
| EMSA | Electrophoretic mobility shift assay |
| FCS | Fetal calf serum |
| GCN5 | a histone acetyltransferase |
| GFP | Green fluorescent protein |

| | |
|---|---|
| G-segment | Gated segment of DNA |
| G(0)-phase | Resting phase of the cell cycle |
| G(1)-phase | Resting phase of the cell cycle |
| HAT | Histone acetyltransferase |
| HDAC | Histone deactyltransferase |
| HeLa | Human cervical carcinoma cells |
| ICB | Inverted CCAAT box |
| ICBP90 | Inverted CCAAT box binding protein of molecular weight 90 kDa |
| kb | kilobases of DNA |
| kDa | kiloDalton |
| LB | Luria Bertani bacteriological media |
| MCS | Multiple cloning site |
| MDR | Multidrug resistance |
| MDR1 | Multidrug resistance gene |
| MHC | Major histocompatibility complex |
| M-phase | Mitotic phase of the cell cycle |
| mt | mutant |
| NEB | New England Biolabs |
| NF-Y | Nuclear factor Y |
| NPR-A | Natriuretic peptide receptor A |
| ODF | Osteoclast differentiation factor |
| ONPG | o-Nitrophenol β-D-Galacto-pyranoside |
| PAGE | Polyacrylamide gel electrophoresis |
| pBS | plasmid BlueScript |
| PBS | Phosphate buffered saline |
| PBSE | Phosphate buffered saline with EDTA |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| pGL3Basic | pGL3Basic vector |
| PP1 | Protein phosphatase 1 |
| p/CAF | a histone acetyltransferase |
| rpm | revolutions per minute |
| $sH_2O$ | sterile water |
| siRNA | small interfering RNA |
| S-phase | DNA synthesis phase of the cell cycle |
| Sp1 | Specificity protein 1 |
| Sp3 | Specificity protein 3 |
| STET | Sucrose, Tris, EDTA, and triton-X buffer |
| SUMO | Small ubiquitin-like modification |
| T-segment | Transport segment of DNA |
| TAE | Tris acetate EDTA buffer |
| TAFs | TBP associated factors |
| TATA | TATA box |
| TBE | Tris borate EDTA |
| TBP | TATA binding protein |
| TE | Tris EDTA buffer |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| TEN | Tris EDTA buffer with sodium |
| TFIID | Transcripiton initiation factor complex containing TBP and TAFs |
| TF | Transcription factor |
| TIMP-2 | Tissue inhibitor of metalloproteinases-2 |
| TMTC | Too many to count |
| TsAP | Thermosensitive alkaline phosphatase |
| XK469 | Topoisomerase II beta poison |
| UV | Ultra-violet light |

| | |
|---|---|
| wt | wild type |
| 3C assay | Chromosome conformation capture assays |

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# 1. Introduction

## 1.1. Overview

Topoisomerases constitute a family of highly conserved nuclear enzymes, which have been found to exist in all investigated living prokaryotic and eukaryotic cells. By introducing transient single- or double-stranded breaks in DNA, topoisomerases can reduce the torsional strain placed on DNA, and thereby allow DNA to exist in the correct topological form required for many different cellular processes. There are three classes of topoisomerases (I, II, and III) of which topoisomerase II is the only one essential for viability in eukaryotes. Topoisomerase II is divided into two isoforms: alpha and beta. Mammalian topoisomerase II alpha expression is cell cycle-dependent and it is highly expressed in rapidly proliferating cells. Expression of topoisomerase II beta is not cell cycle-regulated, and it is expressed at similar levels in all cell types except in tumours where it may be up-regulated.

The two isoforms of topoisomerase II are important chemotherapy targets. Topoisomerase poisons stabilise the cleavage complex formed between the topoisomerase II and DNA, and this drug-induced DNA damage sends the cell into apoptotic pathways. Catalytic inhibitors however, work by inhibiting other steps of the topoisomerase II catalytic cycle. Increased levels of topoisomerase II alpha convey a greater sensitivity to chemotherapy; decreased levels lead to drug-resistance. Both the alpha and beta isoforms have been shown to be down-regulated in drug-resistant cell lines, however, the mechanisms involved remain largely unknown. It is possible that this down-regulation is caused by changes in mRNA stability (Goswami *et al.*, 1996), post-translational modifications of the enzyme (Burden and Sullivan, 1994), or transcriptional regulation (Isaacs *et al.*, 1998).

Marked differences in biochemical properties, nuclear localisation, and expression profiles suggest that the cellular role of topoisomerase II alpha and beta are very different. A large number of studies have been carried out to determine the mechanisms involved in the transcriptional regulation of topoisomerase II alpha, in order to explain its expression pattern and down-regulation associated with drug-resistance (Isaacs *et al.*, 1996; Magan

*et al.*, 2003; Allen *et al.*, 2004). Many of the transcription factors, which bind to the promoter, have been reported and functionally analysed with respect to their effects on expression of reporter genes driven by regions of the topoisomerase II alpha promoter (Magan, 2002; Magan *et al.*, 2003). Three previous studies have provided some insight into the mechanisms involved in the transcriptional regulation of topoisomerase II beta (Ng *et al.*, 1997; Lok *et al.*, 2002; Willingham, 2004), nevertheless these remain poorly characterised. The aim of this study was to identify transcription factors that bind to the topoisomerase II beta promoter, and to functionally characterise some of the observed interactions, as a means to understand the mechanism responsible for the down-regulation of topoisomerase II beta associated with drug-resistance.

## 1.2. Topoisomerases

Topoisomerases are a class of nuclear enzymes, which catalyse the interconversion of topological isomers of DNA. They relax supercoiled DNA, thereby relieving the torsional strain placed upon the DNA during common cellular processes such as replication, transcription, chromatin remodelling, and recombination. They also play a major role in the decatenation and unknotting of DNA (Shiozaki and Yanagida, 1991).

The mechanism of action for all topoisomerases involves the cleavage of the DNA phosphate ester backbone, followed by the formation of a covalent phosphotyrosyl link between the enzyme and the DNA. After the formation of the protein-DNA intermediate, a single- or double-stranded DNA segment passes through the break, and religation occurs once the correct DNA conformation is achieved (reviewed in Roca, 1995).

There are three different types of topoisomerases in higher eukaryotes: type I, II, and III. Topoisomerase I works to correct both overwound and underwound duplexes by introducing a single-stranded break in DNA. This enzyme does not require ATP, or a metal ion as a co-substrate. Topoisomerase I plays an important role in relaxing supercoils generated during transcription, and it is indispensable during development (Lee *et al.*, 1993; Morham *et al.*, 1996).

Topoisomerase III generally catalyses the relaxation of underwound DNA. It only relaxes overwound DNA if there is a pre-existing break in the DNA. The mechanism of action is fundamentally different to other classes of topoisomerases, and involves the unpairing of double-stranded DNA before a break is introduced into a single-strand (Wang, 1997). There are two isoforms of topoisomerase III, the alpha and beta isoforms. Topoisomerase III alpha is required during embryogenesis (Li and Wang, 1998), and topoisomerase III beta may play an important role in the resolution of double-Holliday junctions (Kwan *et al.*, 2003).

Topoisomerase II is an essential nuclear enzyme. These enzymes introduce a staggered double-stranded break into the DNA, which allows the passage of the intact double-stranded DNA through the break. This process requires ATP to catalyse the resetting of the enzyme before it can undertake a second round of catalysis. Topoisomerase II is

capable of relaxing both overwound and underwound DNA, decatenation, and unknotting of DNA.

Shiozaki and Yanagida (1991) determined the basic structure of yeast topoisomerase II. These authors found that the protein exists as a homodimer and that each subunit consists of three functional domains: an N-terminal ATPase domain, a central DNA breakage-rejoining domain containing the active site tyrosine residue, and a C-terminal domain required for nuclear localization (Figure 1.1). The C-terminal domain however, is not essential for catalytic activity.

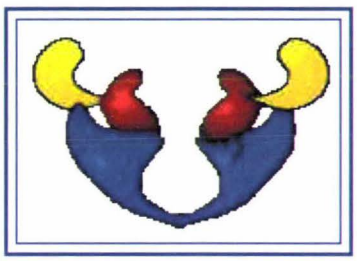


**Figure 1.1: Schematic Representation of Topoisomerase II.**

Topoisomerase II is a homodimeric enzyme and each half has an ATPase domain (Yellow), a DNA breakage-rejoining domain (Red) and a nuclear localization domain (Blue). Figure from Berger *et al.* (1996).

Berger *et al.* (1996) uncovered the general mechanism of action of topoisomerase II and since then it has been widely accepted as the model for topoisomerase II action (Figure 1.2). Topoisomerase II binds to a double-stranded segment of DNA (G-segment), and upon binding a conformational change in the enzyme occurs. A second segment of DNA (T-segment) is brought into the enzyme, and at the same time ATP binds to the ATPase domains. The enzyme then undergoes a series of conformational changes in which the enzyme closes up around the DNA strands. The cleavage process of the G-segment involves transesterification between two active site tyrosine residues (one in each half of this dimeric enzyme) and two DNA phosphate ester bonds four base pairs apart in the G-segment. The oxygens from the tyrosine residues covalently bind to the 5' phosphoryl groups of the broken DNA and the broken ends move away from each other. Concomitant with this process is the dimerisation of the ATPase domains. Next, the T-

segment is transported through the broken DNA and into the center of the enzyme. Once the T-segment has been passed through, a second transesterification occurs between the 3' hydroxyl group and the phosphotyrosyl bond. This transesterification step leads to religation of the DNA, and following this the T-segment is released through a second gate in the enzyme. ATP is then hydrolysed, and the enzyme undergoes a further conformational change, which returns it back into the starting state. The G-segment is either released or the process starts over again.

Topoisomerase II exists as two isoforms. Chung *et al.* (1989b) sequenced several partial topoisomerase II cDNA clones obtained from a human cDNA library, and found two classes of nucleotide sequences. One was identical to that of a previously sequenced cDNA, topoisomerase II alpha, and the other encoded a slightly larger protein, topoisomerase II beta.

Topoisomerase II alpha is encoded on human chromosome 17q21-22 (Tan *et al.*, 1992), and the protein is 170 kDa in size (Chung *et al.*, 1989b). Its expression levels vary throughout the different stages of the cell cycle, therefore it is speculated to have a role in cell cycle-regulation. It is expressed at low levels in quiescent cells, and at high levels in proliferating cells (Hsiang *et al.*, 1988) and therefore, topoisomerase II alpha is highly expressed in rapidly growing tissues, such as the spleen and the thymus (Tsutsui *et al.*, 1993).

The topoisomerase II beta gene resides on human chromosome 3p24 (Tan *et al.*, 1992), and it is slightly larger than the alpha isoform, 180 kDa in size (Chung *et al.*, 1989b). The cellular role of topoisomerase II beta is not fully understood, and its regulatory mechanisms are still under investigation. Topoisomerase II beta is expressed independently of cell cycle and growth status (Kimura *et al.*, 1994), localized mostly in the nucleolus (Woessner *et al.*, 1991), and it is expressed at similar levels in all tissues (Tsutsui *et al.*, 1993).

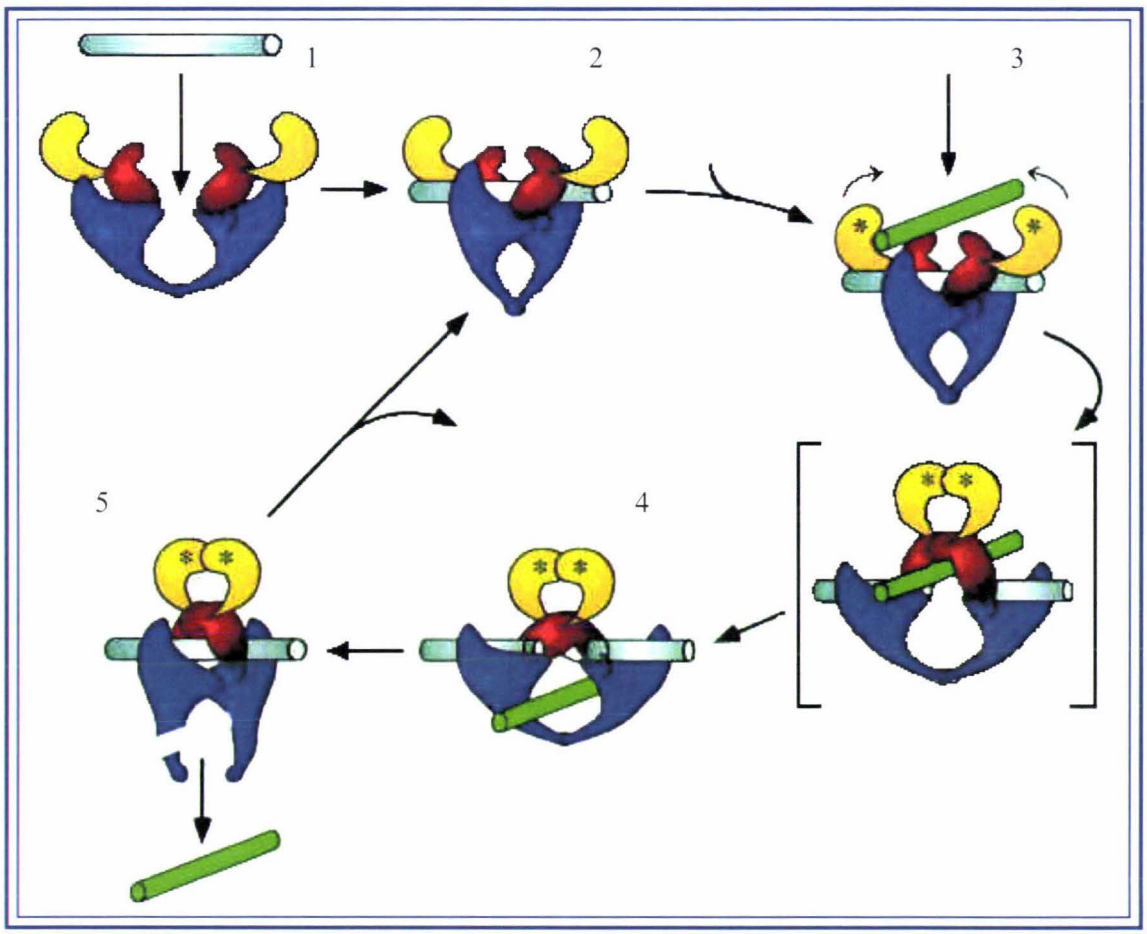



**Figure 1.2: Mechanism of action for Topoisomerase II.**

Topoisomerase II binds to double-stranded DNA, the Gate segment (G-segment), and a conformation change occurs in the enzyme (2). Simultaneously ATP binds, and the Transport DNA (T-segment) moves into the enzyme (3). The ATPase domains clamp shut, and the G-segment is cleaved (4). The T-segment moves through the cleaved G-segment, and is released from the enzyme. ATP is hydrolysed, and the G-segment is religated (5). Through a conformational change, topoisomerase II returns to its starting state, and the G-segment is either released or the process starts again (Berger *et al.*, 1996).

## 1.3. Topoisomerase II as a Chemotherapy Target

The first anti-cancer drugs that were designed and effective in treating cancer had no known cellular target. After approximately 20 years of use and a large amount of experimental work topoisomerases were shown to be a key intracellular target for these drugs (Ross *et al.*, 1978; Ross *et al.*, 1979). Topoisomerase II makes an ideal drug target, as it is an essential enzyme, which lacks functional redundancy within the cell.

The drugs that target topoisomerases can be divided into two classes: poisons and catalytic inhibitors. Topoisomerase poisons act by stabilising the DNA-topoisomerase cleavage complex, thus preventing the religation of the DNA. The double-stranded breaks in the DNA are recognised by the caspases, which then lead the cell into programmed cell death (Benjamin *et al.*, 1998). Topoisomerase inhibitors, interfere with the catalytic function of the enzyme without stabilising the DNA-topoisomerase cleavage complex. They may act either by preventing the topoisomerase enzyme from binding to the DNA, or by locking the DNA bound topoisomerase in the shape of a closed clamp.

Topoisomerase II alpha is the target of many anti-cancer drugs because of its increased expression in rapidly proliferating cells. Qiu *et al.* (1996) provided evidence that the anti-cancer drug etoposide (classified as an epipodophyllotoxin) was capable of stabilizing the topoisomerase II alpha-DNA cleavage complex. This DNA damage, which would cause a halt in transcription, is likely to be sensed by the cell, leading the cell into cell death pathways.

While topoisomerase II beta is expressed at lower levels in the cell (compared to topoisomerase II alpha) it does not mean it is less important in the treatment of cancer. Several studies have shown that some topoisomerase II poisons work by acting on topoisomerase II beta as well as the alpha form (Errington *et al.*, 1999) and that some selectively target this particular isoform (Gao *et al.*, 1999). Topoisomerase II beta may provide an ideal target in the treatment of solid tumours. Solid tumours are slow growing and therefore do not have an increased level of topoisomerase II alpha like the highly proliferating cells found in leukemias for example. Gao *et al.* (1999) reported the discovery of the topoisomerase II beta poison XK469 (classified as a quinoxaline phenoxypropionic acid derivative) that is effective in the treatment of solid tumours. They also reported that XK469 could be an alternative treatment for those cancers, which show multidrug resistance.

Drug-resistance is a major clinical problem when treating tumours with chemotherapeutic drugs. This resistance can either be inherited or acquired when repeatedly exposed to the drugs. Resistance occurs not only to the drug being used but also to a group of structurally unrelated compounds giving the multidrug resistance phenotype. An increase

in expression of the P170 glycoprotein (Grimaz *et al.*, 1998) or decreases in the sensitivity of topoisomerase II have both been implicated in this phenomenon.

Topoisomerase II alpha and beta expression is regulated in a number of ways. mRNA stability has been implicated to have a role in the regulation of topoisomerase II alpha (Goswami *et al.*, 1996). These authors showed that topoisomerase II alpha mRNA has the greatest stability during S-phase with a half life of 4 hours and the shortest half life during G(1)-phase of only 30 minutes. This correlates to the observed cell cycle-expression of topoisomerase II alpha. Topoisomerase II alpha and beta are also subject to phosphorylation, the levels of which are greatest at M-phase (Burden and Sullivan, 1994). Neither of these observations explains the down-regulation of topoisomerase II in many cell lines and tumours. Several studies have shown that this down-regulation has been due to a decrease in mRNA resulting from decreased transcriptional activity and not through mRNA stability (Kubo *et al.*, 1995; Asano *et al.*, 1996; Isaacs *et al.*, 1998).

Topoisomerase II alpha levels were measured in a human stomach-adenocarcinoma cell line either sensitive or resistant to adriamycin (Son *et al.*, 1998). Western blot analysis showed that in drug-resistant cells there was approximately a 20-fold decrease in topoisomerase II alpha in comparison to drug-sensitive cells. Son *et al.* (1998) also reported when the above cells were exposed to etoposide there was a 20-fold decrease in the number of topoisomerase II alpha-DNA covalent complexes formed in drug-resistant cells when compared to drug-sensitive cells. Another study showed that high levels of topoisomerase II alpha were observed in rapidly proliferating tumours (Turley *et al.*, 1997).

Several mutations in the topoisomerase II alpha gene have been shown to cause a drug-resistant phenotype. Wessel *et al.* (1999) examined a human small cell lung cancer cell line and found a substitution of an arginine to a glutamine (R162Q) in the ATP utilization domain, which conveyed resistance to the catalytic inhibitor ICRF-187 (classified as a bisdioxopiperazine). A 20-25% decrease in activity was observed for this mutation when compared to wild type topoisomerase II alpha. Previously, this same group had reported a tyrosine to phenylalanine (Y49F) substitution in a Chinese hamster ovary cell line, which is resistant to the topoisomerase II catalytic inhibitor ICRF-159 (classified as a

bisdioxopiperazine) (Sehested *et al.*, 1998). This mutation, located in the N-terminal clamp end of the enzyme, was introduced into topoisomerase II alpha in a ICRF-159 sensitive human cell line and resistance to ICRF-159 was subsequently observed. Furthermore, de Lucio *et al.* (2005) characterised a non-small cell lung cancer cell line in order to determine the cause of the observed resistance to the topoisomerase II poison etoposide (characterised as an epipodophyllotoxin). Northern blot analysis revealed two truncated mRNA fragments of which the 4.8 kb mRNA encoding the topoisomerase II alpha protein was used for subsequent analysis. Western blot analysis showed that it was the C-terminal end of topoisomerase II alpha that was absent, and this is the region required for the correct cellular localisation of the protein to the nucleus. Immunocytochemical detection showed that the truncated topoisomerase II alpha protein was located in the cytoplasm and therefore non-functional. From this the authors concluded that the observed resistance to etoposide was due to the incorrect cytoplasmic localisation of the protein and therefore it was unable to carry out its function in the nucleus.

Topoisomerase II beta expression in acute lymphoblastic leukemia cells showed a correlation with cytotoxicity to doxorubicin and etoposide (Brown *et al.*, 1995; Markovits *et al.*, 1995). Topoisomerase II beta knockout mouse cells showed that these cells were 3-times more resistant to XK469 than were wild type cells (Snapka *et al.*, 2001). Down-regulation of topoisomerase II beta is also a cause of resistance to the topoisomerase II inhibitor mitoxantrone (classified as an anthracycline) in leukemia (Harker *et al.*, 1991). Western blot analysis showed the topoisomerase II beta protein could no longer be detected in these cells, and therefore the catalytic activity was also reduced. This decrease in the drug target means that even though the drugs are being taken up by the cell there is no longer a sufficient level of target enzyme therefore they are no longer effective in treating the cancer.

Padget *et al.* (2000) used a quantitative western blot method to investigate the relative levels of topoisomerase II alpha and beta in drug-sensitive and drug-resistant cells. From this they suggested that the relative levels of topoisomerase II alpha and beta might be important in determining a tumours response to anti-cancer drugs.

All these studies directly implicate the level and integrity of topoisomerase II alpha and beta in the response of tumours to chemotherapy.

## 1.4. Transcription in Eukaryotes

One of the major points of regulation in the expression of a gene is at the transcriptional level, and this could have a very important role in the regulation of both topoisomerase II alpha and beta. Transcriptional regulation involves the formation of several different protein complexes binding to proximal and distal elements in the promoter of a gene, and the subsequent recruitment of RNA polymerase II. RNA polymerase II is responsible for transcribing the protein coding genes.

A large number of promoters contain a TATA box, which has a consensus sequence of TATAAAA. The TATA box is located on average 25 bases upstream of the transcription start site, and it provides a platform for the assembly and positioning of the pre-initiation complex close to the transcription start site. TFIID, made up of the TATA binding protein (TBP) and a number of TATA associated factors (TAFs), is the first to bind to the TATA box in association with TFIIA. TFIIB binds next, followed by TFIIF, which in turn recruits RNA polymerase II. TFIIE and TFIIH bind last in the complex assembly (Figure 1.3) (reviewed in Shidlovskii *et al.*, 2005). This precise and orchestrated assembly of proteins is important to correctly regulate the expression of a gene. Often, however, there are other upstream elements (other than a TATA box) both proximal and distal, which are utilized to direct transcription within a promoter.

Not all promoters contain a TATA element and this is common in housekeeping genes and developmentally-regulated genes. It therefore follows, that no canonical TATA box has been detected in either topoisomerase II alpha or beta. Genes that lack TATA boxes rely on other elements in the promoter region to recruit the proteins required to form the pre-initiation complex, for example a GC-rich element or a CCAAT box. Both the transcription factors Sp1 (which binds to GC-rich elements) and NF-Y (which binds to inverted CCAAT boxes) have been shown to interact with TAFs, and therefore, these

proteins may play a vital role in TATA-less promoters through interactions with their respective binding sites (reviewed in Suske, 1999; Frontini *et al.*, 2002).

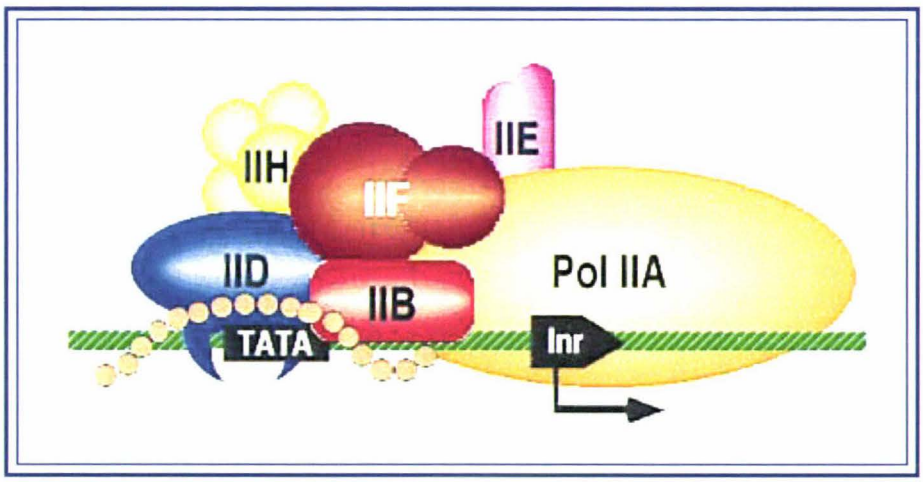



**Figure 1.3: Schematic Representation of the Pre-initiation Complex.**

Transcription factor IID (TFIID) is the protein complex which recognises the TATA element in a number of eukaryotic promoters. TFIID binding to a promoter starts a cascade of protein-protein interactions, which recruit RNA polymerase II to the promoter. Figure from Nikolov and Burley (1997).

One general mechanism involved in transcriptional regulation is via the acetylation state of the promoter. Histone acetyltransferases (HATs) bind to chromatin in the promoter region, and acetylate lysine residues at the N-terminal end of histones. This weakens the histone-DNA interactions, destabilises the nucleosomes, and results in the opening of the chromatin structure. Transcription factors can then gain access to the promoter region of the gene, and the pre-initiation complex can assemble. Histone deacetylases (HDACs), which are also capable of binding at promoter regions, remove the acetyl moiety. This leads to closing of the DNA structure. Through the opening and closing of the chromatin structure, the transcription of a gene can be regulated.

Basal transcription can either be enhanced or repressed by proteins binding to various elements, which can exist upstream or downstream of the transcription start site. Each promoter has its own unique set of transcription factors and elements, therefore the level of expression of a gene is dependent on which regulatory proteins are present and required to bind to the promoter.

## 1.5. Topoisomerase II Beta

A general role for topoisomerase II beta remains evasive. A possible role for this protein in neural development however, has been proposed (Yang *et al.*, 2000). These authors generated topoisomerase II beta knockout mice, and found that these mice failed to develop appropriate neural innervation of skeletal muscle. In these studies the mice died soon after birth due to a breathing impairment, which supports this speculation. Kondapi *et al.* (2004) further examined the topoisomerase II beta protein in rat brains at various developmental stages. These authors found a significant age dependent decline in topoisomerase II beta activity in the cerebellum. From this it was suggested that the low levels of topoisomerase II beta activity may contribute to genomic instability in the ageing cerebellum. The precise function of topoisomerase II beta, however, has yet to be elucidated.

### 1.5.1. The Topoisomerase II Beta Promoter

Ng *et al.* (1997) were the first group to clone and characterise the topoisomerase II beta promoter. Initially, two transcription start sites were identified; one was 193 nucleotides and the other was 89 nucleotides upstream of the translation start codon (ATG). Based on RNase protection assays the majority of transcription initiation occurred 193 nucleotides upstream of the translation start site, and therefore this position was designated +1 (Ng *et al.*, 1997).

The topoisomerase II beta promoter has a high GC content (77%) and a high frequency of CpG dinucleotides (Ng *et al.*, 1997). CpG dinucleotides are suspected sites for methylation and could be important for transcriptional repression. Like topoisomerase II alpha and other housekeeping genes, topoisomerase II beta lacks a TATA box.

Initial deletion assays suggested that one or more transcriptional activators may bind between –1000 and –500, and that one or more transcriptional inhibitors may bind between –500 and –14 (Ng *et al.*, 1997) relative to the transcription start site at +1. Later on, more extensive studies of the 5' upstream regulatory region were carried out by Lok *et al.* (2002) and Willingham (2004). Lok *et al.* (2002) cloned 1.3 kb of the 5' promoter and

carried out a deletion analysis to determine the boundaries of the minimal promoter. Deletion of a region from –555 to –456, within the 1.3 kb promoter, resulted in a 70% decrease in promoter activity, thus identifying this region as containing important transcription factor binding sites.

Initial computational analysis of the topoisomerase II beta promoter region between –456 and –555 identified two inverted CCAAT boxes (ICBs), which are possible binding sites for Nuclear Factor Y (NF-Y), and one GC rich element, which could bind Specificity factor 1 (Sp1) or Specificity factor 3 (Sp3) (Lok *et al.*, 2002). Magan (2002) also identified these putative transcription factor binding sites, as well as the following additional putative binding sites in the full length -1357 topoisomerase II beta promoter: two Activator Protein 2 sites (AP-2), an Activating Transcription Factor (ATF) site, a further GC rich element, and two additional ICB elements (Figure 1.4).

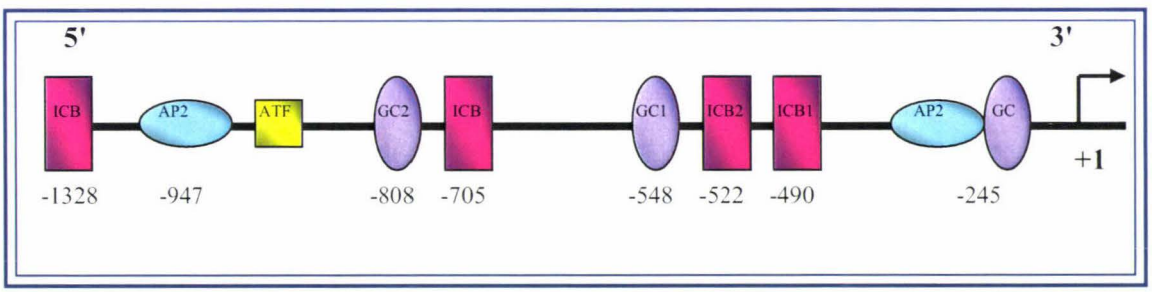



**Figure 1.4: Topoisomerase II Beta Promoter.**

Schematic representation of the putative regulatory elements in the topoisomerase II beta promoter relative to the major transcription start site, +1. Four inverted CCAAT boxes (ICB), two activator protein binding site (AP2), an activating transcription factor binding site (ATF) and three GC-rich elements (GC) have been identified (Adapted from Lok *et al.*, 2002; Magan, 2002).

## 1.5.2. CCAAT Boxes

The sequence CCAAT or ATTGG (reverse orientation) is defined as a CCAAT box. The CCAAT box is found in a large number of promoters, and is one of the most common elements in eukaryotic promoters. It can be in either the forward or reverse orientation, and in higher eukaryotes, it is found in the reverse orientation approximately 60% of the time (Mantovani, 1998). In promoters containing a TATA element the CCAAT box can

be found in either orientation, and on average it is located between –80 and –100 with respect to the transcription start site. In TATA-less promoters, however, the CCAAT box is more often in the reverse direction, and it is positioned closer to the transcription start site, between –41 and –80 with respect to the transcription start site (Mantovani, 1998). It is thought that both the orientation and location of the CCAAT box in TATA-less promoters may be of importance for the positioning of other transcription factors, and subsequently RNA polymerase II close to the transcription start site.

A number of proteins have been shown to bind to CCAAT boxes. CCAAT/enhancer binding protein, C/EBP, binds to this element, and has been shown to be an activator of transcription (Umek *et al.*, 1991). CCAAT transcription factor, CTF/NF-1, recognises the sequence $TGC(N)_6GCCAA$ (Zorbas *et al.*, 1992), where the T is not strictly required for binding. The CCAAT displacement protein was initially found to bind to CCAAT boxes in sea urchin and repress the expression of the sperm H2B gene (Barberis *et al.*, 1987). Inverted CCAAT box binding protein 90 kDa (ICBP90) was first identified as an ICB2 binding protein in the context of the topoisomerase II alpha promoter (Hopfner *et al.*, 2000). These authors showed ICBP90 is highly expressed in proliferating cells, as is topoisomerase II alpha, at both the mRNA and protein level. Over-expression of ICBP90 leads to an increase in topoisomerase II alpha expression, which suggests that ICBP90 may play a role in the regulation of topoisomerase II alpha. By far the most common CCAAT box binding protein is Nuclear Factor Y (NF-Y), and it has been shown to bind to this element in a large number of promoters (Mantovani, 1999). NF-Y has been shown to activate the transcription of a gene through interactions with the chromatin or through the recruitment of other proteins to the promoter (Motta *et al.*, 1999; Caretti *et al.*, 2003).

Many studies have been carried out to gain insight into the regulation of topoisomerase II alpha, and determine the regulatory elements of importance in its promoter. Hochhauser *et al.* (1992) first reported the presence of multiple CCAAT boxes in the topoisomerase II alpha promoter, and ICB1 (at position –68) was subsequently shown to be of particular importance for basal expression (Magan, 2002). Mutations introduced into ICB1 resulted in a 60–70% decrease in transcription (Magan *et al.*, 2003). ICB2 is essential for the down-regulation of topoisomerase II alpha at confluence-arrest (Isaacs *et al.*, 1996), and for cell cycle-regulated expression, specifically the down-regulation at G(0)/G(1) phase (Falck *et al.*, 1999). Numerous studies have shown that NF-Y is capable of binding to

this element, and that this interaction has a role in the regulation of topoisomerase II alpha.

The topoisomerase II beta promoter contains multiple ICBs. ICB1 and ICB2 are located 490 and 522 bases upstream of the transcription start site respectively (Willingham, 2004) and are considered to have functional significance. Mutations introduced into either of these two ICBs, in the context of the full-length promoter, results in a modest 20–25% decrease in transcription relative to the wild type full-length promoter. A double ICB1/ICB2 mutation in the promoter however resulted in a significant 70% decrease in the transcription of a reporter gene suggesting that there is a functional redundancy between these two elements (Lok *et al.*, 2002). Functionally, both elements are important for the expression of the topoisomerase II beta gene, possibly due to the transcription factors that they recruit to the promoter.

### 1.5.3. NF-Y

NF-Y, also known as CBF, CP1, or YEBP, has an absolute requirement for all five bases in the pentanucleotide sequence (CCAAT) and mutations introduced into any of these bases inhibits NF-Y binding and activity (Graves *et al.*, 1986; Myers *et al.*, 1986). In addition to this CCAAT sequence, the 3' flanking region is also considered to be of importance for NF-Y binding. Electrophoretic mobility shift competitor assays demonstrated that only when 13 bp or more of the 3' flanking sequence were present, in conjunction with the CCAAT box, full competition for NF-Y binding was observed (Sugiura and Takishima, 2003). These authors also found that the correct positioning of certain bases within the 3' flanking sequence were also important for stabilising NF-Y binding.

NF-Y is a protein that consists of three subunits: NF-YA, NF-YB and NF-YC, all of which are required for DNA binding. There is a high degree of evolutionary conservation of NF-Y, which suggests that NF-Y has a fundamentally important role in the cell. Sinha *et al.* (1995) showed that there was a precise way in which the NF-Y heterotrimer is assembled. Firstly, NF-YB and NF-YC form a tight interaction with each other, and this

provides the base for NF-YA association. NF-YA binding is weaker than the NF-YB/NF-YC interaction, and the whole complex is stabilized upon DNA binding.

NF-Y itself may not have the ability to directly activate the transcription of a gene, however it may play a role in the structural organization of the DNA. Ronchi *et al.*, (1995) showed that NF-Y forms interactions with the minor groove of the DNA double helix, and that upon DNA binding, introduces a distortion into the DNA double helix. DNA bending assays showed that NF-Y was capable of bending the DNA between 62° and 82°. It is possible that this bending may open up the DNA, thereby allowing the binding of other transcription factors. In addition, NF-Y has been shown to interact with the histone acetyltransferases, P/CAF and GCN5 (Currie, 1998; Jin and Scotto, 1998). This provides evidence for further opening of the chromatin structure upon NF-Y binding, making the DNA increasingly accessible to the proteins required for the formation of the pre-initiation complex.

NF-Y could also play an important role in the recruitment of other proteins to the promoter. It has been shown that NF-Y is responsible for recruiting TFIID to the major histocompatibility complex (MHC) class II Ea promoter (Frontini *et al.*, 2002). In fact, these authors demonstrated that NF-Y is able to interact with a number of different TAFs in the TFIID protein complex. Variations in the TAF that NF-Y binds to could be of importance for the assembly of the initiation complex in different core promoter architectures (Frontini *et al.*, 2002). To build on these findings Kabe *et al.* (2005) investigated the recruitment of RNA polymerase II to the osteoclast differentiation factor (ODF) gene promoter. A small interfering RNA (directed to NF-YA) knocked down NF-YA, thus preventing NF-Y associating with the CCAAT box in the ODF promoter. This decrease in NF-Y binding to the promoter was concomitant with a decrease in RNA polymerase II binding to the same promoter.

Experiments were undertaken to determine if there were any significant differences in transcription factor expression in a Chinese hamster cell line that has reduced levels of topoisomerase II alpha (Saxena *et al.*, 2004). Western blot analysis and quantitative real-time reverse-transcription PCR showed that NF-Y was decreased at both the protein and

mRNA level respectively, thus suggesting that NF-Y is activating topoisomerase II alpha expression.

In order to investigate the role of NF-Y in the context of the topoisomerase II beta promoter Lok *et al.* (2002) used a dominant negative NF-YA mutant in transient transfection experiments. These experiments resulted in decreased levels of reporter gene activity, relative to no addition of NF-YA mutant. This suggests that NF-Y may work to activate topoisomerase II beta transcription. Lok *et al.* (2002) also carried out transfection assays using a mutant ICB1/ICB2 promoter construct, and showed that the dominant negative NF-YA mutant had no effect on reporter gene expression. This confirmed that the dominant negative mutant of NF-YA was acting specifically though the two ICB elements. Over-expression of wild type NF-YA however, was insufficient to increase transcription, which is logical as all three NF-Y subunits are required for DNA binding. It is possible that NF-Y activates transcription of the topoisomerase II beta gene through the recruitment of other transcription factors to the promoter.

### 1.5.4. GC-rich Elements

The GC-rich element has the general consensus sequence of GGGCGG, and this element is found in a large number of eukaryotic promoters. GC-rich elements are of particular importance in many ubiquitously expressed genes, tissue-specific expression, and in the promoter regions of viral genes. They also occur frequently in promoter regions of genes involved in developmental patterning and hormonal activation.

It has been found that in the topoisomerase II alpha promoter a mutation in GC1 has no significant effect on the expression level. However, a mutation in GC2 results in a 50% increase in transcription, which suggests that there is a transcription factor that binds to the GC2 element that inhibits transcription (Magan *et al.*, 2003), or that a functional synergism may exist between both these GC-rich elements.

A mutation in GC1 in the topoisomerase II beta promoter shows that this element does not bind an inhibitory transcription factor, as there was no significant change in reporter gene activity (Lok *et al.*, 2002), however there may be some co-operation between GC1 and ICB1 or ICB2. When GC1 is mutated, in conjunction with either of these elements, at least a 50% decrease in transcription was observed. This suggests that the transcription factors binding at these sites may be able to recruit each other when a mutation is present in only one of their binding sites (Zhong *et al.*, 2000).

### 1.5.5. Sp Family of Transcription Factors

The Specificity protein (Sp) family of transcription factors have been shown to bind to DNA, and act through GC-rich or GC-rich like elements to modulate transcription. To date, nine members of the Sp family have been identified (namely Sp1 – Sp9). The Sp family of transcription factors is characterised by a conserved zinc finger DNA binding domain, near the C-terminal end of the protein, which facilitates binding to the GC-rich elements.

Sp1 and Sp3 are the most well characterised members of the family, and have been repeatedly shown to play a major role in the transcriptional regulation in a large number of genes (Suske, 1999). Sp2 is poorly characterised but may play a role in cell physiology (Moorefield *et al.*, 2004) and Sp4 expression is restricted to the brain (Black *et al.*, 1999). Sp5 has been proposed to have a role in patterning in the developing embryo (Harrison *et al.*, 2000), while Sp6 may play a role in the development of epithelial tissues (Nakamura *et al.*, 2004). Sp7, also known as Osterix, is only expressed in osteoblasts, and is a key regulator of bone cell differentiation (Gao *et al.*, 2004). The more recently discovered Sp8 and Sp9 have been shown to play a major role in limb outgrowth in embryos (Kawakami *et al.*, 2004).

Sp1 and Sp3 are ubiquitously expressed members of the Sp family, and it is therefore possible that these two transcription factors play a role in topoisomerase II beta regulation through the GC-rich elements located in the promoter.

### 1.5.6. Sp1

Sp1 is a ubiquitously expressed protein, and it is a bonafide transcriptional activator. The Sp1 gene is located on human chromosome 12q13 (Gaynor *et al.*, 1993) and there are several different isoforms of Sp1 (Thomas *et al.*, 2005).

Cloning and characterisation of the Sp1 promoter showed that it contained a number of GC-rich elements (Nicolas *et al.*, 2001). Transient transfection experiments showed that Sp1 activated its own expression, i.e. it is autoregulated, while another member of the Sp family, Sp3, is able to repress Sp1 activated transcription (Nicolas *et al.*, 2003). Transcriptional regulation of Sp1 also involves both NF-Y and E2F (Nicolas *et al.*, 2003). E2F plays a major role in the activation of expression of genes involved in the transition from G(1) to S phase in the cell cycle, and therefore provides a link to the cell cycle-regulated expression of topoisomerase II alpha.

Post-translational modification has an important role in the regulation of Sp1 at the protein level. Phosphorylation of serine and threonine residues in the DNA binding domain of Sp1 prevents Sp1 binding to the GC-rich element. The phosphorylation state of Sp1 is dynamic, Casein Kinase II has been shown to phosphorylate Sp1, and protein phosphatase 1 (PP1) is thought to dephosphorylate the protein (Armstrong *et al.*, 1997). Black *et al.* (1999) proposed an additional role for phosphorylation in the interaction with other transcription factors. Sp1 is also subject to O-glycosylation (Han and Kudlow, 1997). This modification may provide a link between nutrient availability and cell growth. In low nutrient states, Sp1 becomes hypoglycosylated, and this results in Sp1 being targeted to the proteasome for degradation (Han and Kudlow, 1997). From these results the authors suggested that the subsequent drop in Sp1 levels might result in a general reduction in transcription, thereby providing a possible mechanism for nutrient conservation, as Sp1 is involved in the transcriptional activation of many genes.

Sp1 has been found to be over-expressed in a large number of cancers such as breast cancer (Zannetti *et al.*, 2000), pancreatic cancer (Shi *et al.*, 2001) and fibrosarcoma (Lou *et al.*, 2005). Using U1snRNA/ribozyme (which is used to inhibit the expression of a specific gene, in this case Sp1) Lou *et al.* (2005) showed the levels of Sp1 within the cell

decreased and this resulted in a decrease in tumourigenicity, and a decrease in the levels of several other proteins involved in malignant transformation.

Magan *et al.* (2003) showed that over-expression of Sp1 caused a 6-fold increase in transcription of a reporter gene driven by the topoisomerase II alpha promoter, indicating that Sp1 is a strong activator of topoisomerase II alpha expression. Further evidence supporting the role of Sp1 as an activator of transcription came from Allen *et al.* (2004). These authors demonstrated that topoisomerase II alpha is down-regulated in breast cancer cells exposed to doxorubicin, and that the decrease in topoisomerase II alpha expression is concomitant with a decrease in Sp1 expression.

Only one study to date has investigated the role of Sp1 on topoisomerase II beta expression. Unlike topoisomerase II alpha, this preliminary study found that Sp1 had no effect on topoisomerase II beta expression (Willingham, 2004). This result is somewhat surprising, as Sp1 binds tightly to the GC1 element in the topoisomerase II beta promoter *in vitro* (Lok *et al.*, 2002; Willingham, 2004) and acts as a general transcriptional activator in a number of different promoters. The role of Sp1 in topoisomerase II beta regulation will require further investigation.

### 1.5.7. Sp-1 and NF-Y Interactions

NF-Y and Sp1 have been shown to co-operate in the regulation of a number of promoters. A mutation in the NF-Y binding site is partially compensated by a wild type Sp1 binding site and vice versa. This was demonstrated by Zhong *et al.* (2000) when these authors were investigating the regulation of the human tissue inhibitor of metalloproteinases-2 (TIMP-2) gene in response to cyclic AMP (cAMP). No response to cAMP was seen when both NF-Y and Sp1 binding sites were mutated; however, when only one was mutated, a partial response was observed.

Roder *et al.* (1999) wanted to determine the basis for the co-operation between Sp1 and NF-Y, which they had previously shown to occur in the fatty acid synthase insulin-responsive element 1 promoter (Roder *et al.*, 1997). Using yeast two-hybrid assays,

GST-pull-down assays, and co-immunoprecipitation experiments, these authors demonstrated conclusively that Sp1 and NF-Y physically interact both *in vivo* and *in vitro*. An independent study by Liang *et al.* (2001) also demonstrated that Sp1 and NF-Y could physically interact, and that this interaction may be of functional importance in the transcriptional regulation of the type A natriuretic peptide receptor (NPR-A) gene.

The promoter of the MHC Class II-associated invariant chain gene contains an imperfect ICB, and a GC-rich element within close proximity to each other. NF-Y and Sp1 were shown to bind to their respective elements; however binding of either transcription factor on its own had no effect on transcription (Wright *et al.*, 1995). Only when both transcription factors were present was there a significant increase in transcriptional activation by NF-Y and Sp1. It is possible that Sp1 binding stabilises NF-Y binding to the imperfect ICB, and that this interaction makes it possible for NF-Y to recruit the other transcription factors required for activating transcription.

### 1.5.8. Sp3

Sp3, like Sp1, is a ubiquitously expressed protein. Unlike Sp1, however, Sp3 exhibits two functions, as either an activator or a repressor of transcription. The Sp3 gene is located on human chromosome 2q31, and through differential translation initiation sites, three different size proteins can be produced; two small 58 – 60 kDa proteins, and one full-length 110 kDa protein (Kennett *et al.*, 1997). Kennett *et al.* (1997) also showed that the two smaller proteins had very little *trans*-activation activity, however they were shown to be potent inhibitors of Sp1- or Sp3-mediated *trans*-activation.

Unique to Sp3 is an inhibitor domain that enables it to repress transcription. By preparing a series of deletion constructs Dennig *et al.* (1996) narrowed the region responsible for the inhibitory function down to a short 13 amino acid sequence, consisting of highly charged residues. Further mutational analysis of these amino acids identified the triplet, lysine (K), glutamic acid (E), glutamic acid (E), (amino acids 423-425), as the essential factor for the inhibition of transcription (Dennig *et al.*, 1996).

Sp3 undergoes a range of post-translational modifications like Sp1. Phosphorylation of Sp3 has been shown to increase DNA binding affinity (Ge *et al.*, 2001). It is thought that the lysine (residue 423) located in the inhibitory domain may be important in the switch between Sp3 acting as a transcriptional repressor or activator. This residue is subject to acetylation (Braun *et al.*, 2001; Ammanamanchi *et al.*, 2003) and is also one of the essential amino acids in the SUMO (small ubiquitin-like modifier) modification motif (IKEE) (Ross *et al.*, 2002). When acetylated, Sp3 becomes a strong activator of transcription (Ammanamanchi *et al.*, 2003), however if this same lysine residue has a SUMO moiety added, it becomes a potent repressor of transcription (Ross *et al.*, 2002). Both acetylation and SUMO modification are reversible and therefore are interchangeable. This suggests that the post-translational modification present on Sp3 may be a key determinant in its function as either a transcriptional activator or repressor.

The relative levels of Sp1 and Sp3 are thought to be important in the transcriptional regulation of a gene. DeLuca *et al.* (1996) showed that Sp3 was able to repress Sp1 mediated activation of a gene by competing for DNA binding. More recently, Williams (submitted) used chromatin immunoprecipitation assays to show that Sp1 and Sp3 were able to compete for binding to the topoisomerase II alpha promoter, and that Sp3 was dominant over Sp1 and consequently repressed Sp1 mediated activation of transcription.

The role Sp3 plays in the regulation of topoisomerase II beta remains unknown. It has been shown to bind to the GC1 element *in vitro*, and when co-expression reporter gene experiments were carried out a modest 20% decrease in topoisomerase II beta promoter activity was observed (Willingham, 2004). Further work is required to establish the precise role of this transcription factor in topoisomerase II beta regulation, but preliminary studies suggest a role in repression.

## 1.6. Project Outline

To date, very few reports have been published regarding the transcriptional regulation of human topoisomerase II beta, while much is known about the mechanisms involved in the transcriptional regulation of topoisomerase II alpha.

It is clear that topoisomerase II alpha and topoisomerase II beta are regulated in different ways, even though they both have similar elements in their promoters. Topoisomerase II alpha is cell cycle-regulated, expressed at high levels in proliferating cells, and is the specific target of a number of chemotherapeutic drugs. Meanwhile, topoisomerase II beta is not cell cycle-regulated, it is ubiquitously expressed amongst all tissues, and it too is the target of chemotherapeutic drugs. The down-regulation of both of these isoforms of topoisomerase II is a major clinical problem as it leads to drug-resistance during chemotherapy for a number of cancers.

Further research into the transcriptional regulation of topoisomerase II beta would provide a better understanding of the underlying mechanisms involved in the development of resistance to drugs targeting this isoform. By investigating which proteins bind to the elements in the topoisomerase II beta promoter, and determining the functional effects of these protein-DNA interactions, an understanding of the down-regulation of this enzyme in drug-resistance may be achieved.

Research aims:

1. Determine the proteins which bind to the ICB1, ICB2, GC1 and GC2 element of the topoisomerase II beta promoter, using electrophoretic mobility shift assays.
2. Investigate the effect of over-expression of Sp1 and Sp3 on transcription from the topoisomerase II beta promoter in reporter gene assays.
3. Clone a series of topoisomerase II beta promoter constructs containing mutations in ICB1, ICB2 and GC1 and all combinations of these elements.
4. Establish the effect these mutations have on the level of transcription generated by the topoisomerase II beta promoter, using reporter gene assays.

# 2. Materials and Methods

## 2.1. Materials

Restriction endonucleases and associated buffers, *Taq* DNA Polymerase, Bovine Serum Albumin (BSA), 2-Nitrophenyl-β-D-galactopyranoside (ONPG), ampicillin, and agarose powder were purchased from the following sources: New England BioLabs, MA, USA, Roche, Mt Wellington, Auckland, and Boehringer Mannheim, Germany.

Thermosensitive Alkaline Phosphatase, T4 DNA Ligase, 1 kb Plus DNA Ladder, Trypsin, Penicillin/Streptomycin (5000 units/mL), foetal calf serum (FCS), and OptiMEM were all purchased from GIBCOBRL, Invitrogen Corporation, Invitrogen NZ Limited, Penrose, Auckland, New Zealand.

Topoisomerase II beta primers, oligonucleotides for electrophoretic mobility shift assays, RV3 primer, GL2 primer, ethidium bromide, lysozyme, ampicillin, DMSO, TEMED, and dNTPs were all purchased from Sigma Chemical Company, St Louis, MO, USA.

The Plasmid MiniPrep Kit was purchased from BioRad Laboratories, CA, USA.

The MaxiPrep, MidiPrep plasmid purification kits were purchased from QIAGEN, New Zealand distributors: Biolab Scientific Ltd, Auckland, New Zealand.

Plasticware used for tissue culture procedures was from Nunc Inc, Naperville, IL, USA, or Greiner One, New Zealand Distributor, RayLab, Mt Roskill, Auckland, New Zealand.

Fugene6™ transfection reagent was purchased from Roche Molecular Biochemicals, IN, USA.

The *Escherichia coli* XL-1 Blue strain was from Stratagene, La Jolla, CA, USA.

pGL3Basic and the Luciferase Assay system were purchased from Promega Corporation, WI, USA.

The FLUOstar Galaxy, used for luciferase assays, was obtained from BMG Labtechnologies Pty Ltd, Melbourne, Australia.

Poly(dIdC) was purchased from Amersham Parmacia Biotech AB, Uppsala, Sweden.

T4 polynucleotide kinase was from Roche Diagnostics, Germany.

$\gamma^{32}$P [ATP] was brought from Amersham BioSciences Corporation, NJ, USA.

The primary antibodies, Sp1, Sp3 and NF-YA, were all purchased from Santa Cruz Biotechnologies, CA, USA.

DE-81 and 3MM paper were purchased from Whatman, Maidstone, England.

X-ray film used for electrophoretic mobility shift assays came from KODAK, Christchurch, New Zealand.

Quantification Standards were kindly prepared by Carole Flyger, IMBS, Massey University, Palmerston North, New Zealand.

The original HeLa cells were a generous gift from Dr Rachel Page (Department of Biochemistry, University of Cardiff).

The pGL3Basic vector containing the –1357 bp topoisomerase II beta promoter construct and the –654 bp topoisomerase II beta promoter construct were kindly provided by Melanie Willingham, Massey University, Palmerston North, New Zealand.

All other reagents and chemicals used were of analytical grade or higher.

## 2.2. Methods

### 2.2.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis is commonly used to separate DNA. When subjected to an electrical current the negatively charged DNA migrates away from the negative electrode and towards the positive electrode. The rate of movement is directly proportional to the size of the fragment, with small fragments of DNA moving faster through the gel than larger fragments.

Ethidium bromide is a commonly used marker for identifying and visualising DNA after electrophoresis. Ethidium bromide is an intercalating agent, which inserts between the base pairs of the DNA. Upon exposure to UV light ethidium bromide fluoresces, a camera detects this fluorescence, and a photograph highlighting the DNA bands can be produced.

A 1% agarose gel was generally used and made by melting 50 mg of Agarose LE powder in 50 mL of 1X TAE (40 mM Tris.Acetate, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8) and left to cool to 55°C before 2 µL of ethidium bromide (10 mg/mL) was added. The gel was left to set before being immersed in 1X TAE. Samples were mixed with a DNA loading dye (0.25% bromophenol blue, 40% (w/v) sucrose in $H_2O$), which has a high density that ensures the DNA sinks to the bottom of the wells. A 1 kb ladder containing fragments of known sizes, was always loaded onto gels so estimates on the size of the linear DNA fragments could be made. Electrophoresis was carried out at 100 V for 1 hour after which the gel was visualised on a gel doc with the QuantityOne 4.4.0 software (BioRad).

### 2.2.2. Restriction Endonuclease Digests

Generally 30 µL digest reactions were prepared, however, when larger volumes of digested DNA was required 50 µL reactions were used and the volume of the various components adjusted accordingly.

To each restriction digest, 500-1000 ng of DNA was added along with 3 µL of the recommended 10X reaction buffer to give a final concentration of 1X reaction buffer. Some endonucleases required the addition of BSA to effectively and efficiently cut the DNA, therefore 0.5 µg of BSA was added accordingly. Five units of restriction enzyme were added and the digest reactions, which were made up to 30 µL with sterile $H_2O$ ($sH_2O$), were incubated at 37°C for 1 to 2 hours.

### 2.2.3. Removal of 5' Phosphate Groups

To prevent religation of the vector during the ligation procedure, 5' phosphate groups were removed using thermosensitive alkaline phosphatase (TsAP). TsAP was chosen because it is easily inactivated by heating at 65°C.

Four microliters of TsAP buffer (100 mM Tris.HCl) and 1 U of TsAP (1 U/µL, Roche) were added to the 30 µL vector restriction endonuclease digest and the reaction was made up to 40 µL with $sH_2O$. The reaction was incubated for 15 minutes at 37°C followed by the addition of 4.5 µL of STOP buffer (200 mM EDTA). A final incubation was carried out for 15 minutes at 65°C.

### 2.2.4. DNA Purification using the Freeze-Squeeze Method

The freeze-squeeze method of DNA purification (Thuring *et al.*, 1975) was used to purify DNA from an agarose gel. It is generally used to purify large amounts of DNA and the recovered DNA is suitable for cloning. One advantage of this method is that any size DNA fragment can be purified.

The entire PCR reaction was loaded onto a 1% agarose gel, and electrophoresis was carried out as described above in section 2.2.1. Long-wave UV was used to visualise the DNA in order to prevent the introduction of mutations which may have been created had short-wave UV been used. The band of corresponding size to that of the fragment of interest was excised and as much agarose was removed from around the band as possible. The excised gel slice was folded up in parafilm and frozen at –20°C for 1 hour. The gel

slice was then squeezed and the liquid collected. Fifty microliters of 1X TAE was added to the agarose, after which it was frozen again at -20°C, for 30 minutes. The gel was squeezed again and the liquid collected and pooled with the first collection. To remove all traces of agarose the collected liquid was centrifuged at 12,000 rpm for 30 seconds, and the supernatant transferred to a fresh microcentrifuge tube. The DNA was then concentrated by ethanol precipitation before use in cloning.

### 2.2.5. Ethanol Precipitation

Ethanol precipitation is a standard method used to recover DNA from aqueous solutions. It is simple, fast, efficient and removes salts at the same time as concentrating the DNA.

Two volumes of ice cold 95% ethanol and 1/10 the volume of 1 M sodium acetate (pH 4.8) were added to the DNA solution, after which it was left to stand at –20°C for 1 hour to allow the DNA to precipitate. Following this, the DNA was recovered by centrifugation at 4°C for 10 minutes at 12,000 rpm. The supernatant was removed and 500 µL of 70% ethanol was added. A final centrifugation step was carried out at 4°C for 2 minutes at 12,000 rpm and then the supernatant was removed. The tube, containing the DNA pellet, was left to dry at room temperature before the pellet was resuspended in an appropriate volume of TE (10 mM Tris-HCl pH 8, 0.1 mM EDTA) (Sambrook and Russell, 2001).

### 2.2.6. Oligonucleotides

Oligonucleotides were custom synthesised by Sigma or Invitrogen and provided as a dry stock. The DNA was resuspended in TE (10 mM Tris-HCl pH 8, 0.1 mM EDTA) to give 10 µg/µL stock solutions, which were diluted further as required. For PCR mutagenesis the primers were diluted to 50 ng/µL. For the labelling reactions required in Electrophoretic Mobility Shift Assays they were diluted to 100 ng/µL and competitor oligonucleotides were diluted to give 1 µg/µL stocks.

### 2.2.7. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was carried out using *Taq* polymerase. The PCR mixture contained 2 µL of DMSO, 5 µL dNTPs (3 mM), 5 µL 10X PCR buffer with $MgCl_2$ (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 5 µL of forward and reverse primer (50 ng/µL), 100 ng of plasmid DNA template, 0.5 µL *Taq* DNA polymerase (5 U/µL), and made up to 50 µL with $sH_2O$. The *Taq* DNA polymerase was added last and the samples mixed thoroughly and briefly centrifuged to ensure all components were together at the bottom of the tube. In addition, a negative control, which had no DNA added, was always included to ensure there was no contamination present.

PCR was carried out on the GeneAmp® PCR System 2700, Applied Biosystems (USA). The PCR program used was as follows: initially a 5 minute incubation at 95°C was carried out, followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes 30 seconds, and finally a 7 minute incubation at 72°C.

To determine if the PCR was successful 5 µL (10%) of each PCR reaction was loaded onto an agarose gel for analysis of size and amplification efficiencies.

### 2.2.8. Ligation Reactions

Restriction endonuclease digests were carried out on both the vector and insert to generate the sticky or blunt ends required to ligate the two together. For a ligation to occur between the insert and vector it is essential to have the insert in excess of the vector.

Ligations were carried out in a reaction containing 5X ligase buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 M DTT, 5% polyethylene glycol) and 1 U T4 DNA ligase (1 U/µL), with a vector to insert ratio of 1:3. Ligations were incubated at room temperature for three hours before being left overnight at 4°C.

## 2.2.9. Transformation of Escherichia coli XL-1 Cells

### 2.2.9.1. Grow on the Day Competent Cells

#### 2.2.9.1.1. *Preparation of Competent Cells*

A glycerol stock containing *Escherichia coli* (*E. coli*) XL-1 blue competent cells was used to prepare a single colony streak onto an LB agar plate containing the antibiotic tetracycline (10 mg/mL). The plate was incubated at 37°C overnight after which a single colony was picked and used to perform another single colony streak. A single colony was picked and used to inoculate a 5 mL LB broth containing 5 µL tetracycline (10 mg/mL). The 5 mL broth was incubated overnight at 37°C, with shaking at 220 rpm, before 200 µL of the broth was removed and used to inoculate another 5 mL broth. This broth was monitored and grown until the optical density, at 600 nm, was between 0.4 and 0.5. At this stage the *E.coli* XL-1 blue cells were growing exponentially, and were ready to be harvested and used for preparation of competent cells. A 1 mL aliquot of cells was taken and pelleted in a centrifuge for 1 minute at 12,000 rpm. The cells were resuspended in 100 µL of ice-cold TSS buffer (10 g/L Tryptone, 5 g/L yeast extract, 100 g/L PEG-4000, 0.085 M NaCl, 5% DMSO, 0.05 M $MgCl_2$, pH 6.5), stored on ice, and used within 3 hours (Chung *et al.*, 1989a).

#### 2.2.9.1.2. *Transformation Procedure*

The freshly prepared competent cells were split into 50 µL aliquots and 1 µL (50 ng) of plasmid DNA was added, followed by a 30 minute incubation on ice. The cells were then heat shocked for 2 minutes at 42°C and subsequently placed on ice for another 2 minutes to allow the cells to recover. Next, 200 µL of SOC (2% Bacterotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to the cells followed by incubation at 37°C for 1 hour with shaking. After this incubation 50 µL of the cell suspension was plated onto LB agar plates with or without the antibiotic ampicillin.

### 2.2.9.2. Calcium Chloride Competent Cells

#### 2.2.9.2.1. *Preparation of Competent Cells*

A 5 mL overnight culture of *E.coli* XL-1 blue cells was used to inoculate a 200 mL LB broth containing 200 µL of tetracycline (10 mg/mL). The broth was grown at 37°C, with shaking, until an optical density at 600 nm of 0.5 was reached. At this stage the cells were growing exponentially and were harvested at 4000 rpm, at 4°C, for 15 minutes. Next, 25 mL of freshly prepared, ice cold 100 mM $CaCl_2$ was used to resuspend the pellet containing the cells. A 20 minute incubation on ice followed, before being centrifuged again at 4000 rpm, at 4°C, and for 15 minutes. The cells were resuspended in 5 mL of ice cold 100 mM $CaCl_2$ and left to stand on ice for 1 hour. Glycerol was added to give a final glycerol concentration of 15%. The cells were then split into 300 µL aliquots and snap frozen using liquid nitrogen. The cells were stored at –70°C until required.

#### 2.2.9.2.2. *Transformation Procedure*

Typically, 1 µL of a ligation reaction, equivalent to 50 ng of plasmid DNA, was added to the cells, which were then incubated on ice for 30 minutes. The cells were then heat shocked for 5 minutes at 37°C after which they were placed on ice for 10 minutes to recover. One millilitre of LB broth was added and a 1 hour incubation at 37°C was carried out to allow the cells to begin growing again. After this incubation the cells were pelleted by centrifugation at 5000 rpm for 5 minutes and the supernatant decanted. Finally, 100 µL of LB broth was used to resuspend the cells and 20 µL of this was plated onto LB or LB-ampicillin plates.

## 2.2.10. Plasmid Preparations

### 2.2.10.1. Rapid Boil Plasmid Purification

Rapid boil plasmid preparations (Holmes and Quigley, 1981) generate impure DNA, which contains RNA and proteins, but this method is suitable for screening bacterial cultures for plasmids that contain the insert DNA required.

A single colony was picked from an agar plate and used to inoculate a 5 mL LB broth containing 5 µL of ampicillin (100 mg/mL). The cultures were then incubated overnight at 37°C with vigorous shaking. One millilitre of the overnight culture was placed in a microcentrifuge tube and centrifuged at 12,000 rpm for 1 minute to pellet the cells. The cell pellet was resuspended in 350 µL of STET (8% sucrose, 50mM EDTA, 50mM TrisBase, pH 8.0, 5%(v/v) TritonX-100), followed by the addition of 25 µL of freshly prepared lysozyme (10 mg/mL egg white lysozyme) and mixed using a vortex. The samples were boiled for exactly 40 seconds in a water bath and immediately centrifuged for 10 minutes at 12,000 rpm. The gelatinous pellet, which contained the proteins, was removed and 400 µL of isopropanol was added to the supernatant. The samples were then placed at – 70°C for 30 minutes, after which the DNA was pelleted by centrifugation at 12,000 rpm, for 15 minutes at 4°C. The pellet was washed with 500 µL ice cold 95% ethanol and centrifuged again at 12,000 rpm, 4 °C for 1 minute. The supernatant was decanted and the pellets left to dry so all traces of ethanol evaporated. The dried pellet was resuspended in 50 µL of TE (10 mM Tris.Cl, 1 mM EDTA, pH 8.0) and stored at 4°C or – 20°C.

### 2.2.10.2. MiniPrep Plasmid Purification

The Quantum Prep® Plasmid Miniprep Kit utilizes a silicon dioxide matrix to bind to the plasmid DNA. The high affinity for DNA, elution in deionised water, and the simplistic nature of the process made the kit ideal for producing the small amounts of high quality and high purity DNA required for automated DNA sequencing.

Overnight cultures were grown for *E. coli* containing the plasmid to be purified. The protocol recommended by the manufacturer was followed and the DNA was eluted from the matrix using 100 µL of deionised water. The eluted DNA was stored at 4°C or -20°C.

### 2.2.10.3. MaxiPrep Plasmid Purification

QIAGEN MaxiPrep kits were used for large-scale preparations of the plasmids that were used in transient transfections. The QIAGEN MaxiPrep kit makes use of an anion-exchange resin, which has a very high charge density. The positively charged diethylaminoethyl groups on the surface of the resin interacts with the negatively charged phosphate in the DNA backbone. All impurities were washed from the column using a salt concentration lower than that which was used to elute the DNA. A yield of up to 500 µg can be achieved using this kit.

The protocol was followed as recommended by the manufacturer. The plasmid DNA was resuspended in 200 µL of TE and stored at –20°C.

## 2.2.11. DNA Quantification

Two different methods of DNA quantification were carried out to ensure the most accurate measure of DNA concentration was obtained.

### 2.2.11.1. Quantification using Gel Electrophoresis

In order to quantify DNA using gel electrophoresis the plasmids had to be linear. To do this, a restriction endonuclease digest was performed using an enzyme which only cut once within the plasmid. The quantification standards were 10 ng/5µL, 20 ng/5µL, 50 ng/5µL, and 100 ng/5µL. The DNA to be quantified was diluted to give a range of concentrations and 5 µL of the diluted DNA was loaded onto an agarose gel along with the standards. Electrophoresis was carried out as described in section 2.2.1. By

comparing the different band intensities an estimate of DNA concentration could be made.

### 2.2.11.2. Quantification using UV Spectrophotometry

Plasmid DNA was quantified using UV spectrophotometry. At a wavelength of 260 nm double-stranded DNA, at a concentration of 50 µg/mL, generates an absorbance of 1.0. Plasmid DNA was diluted 1/50 and 1/100 in $sH_2O$ and 50 µL of the dilution was placed in a quartz cuvette. The cuvette was placed in the Ultraspec 300 UV visible spectrophotometer (Pharmacia Biotech, Cambridge, England) and the absorbances measured against a water blank. To determine DNA concentration the following formula was used:

$$\text{DNA concentration} = A_{260} \times 50\ \mu g/mL \times \text{Dilution Factor}$$

To determine the purity of the DNA sample the absorbance at 280 nm was also recorded and the following formula used:

$$\text{Purity} = \frac{A_{260}}{A_{280}}$$

A ratio of 1.8 indicated pure DNA, a ratio of less than 1.8 indicated protein contamination, and a ratio of more than 1.8 indicated RNA contamination.

## 2.2.12. Sequencing of DNA

DNA sequencing was carried out by Lorraine Berry at the Genome Service, Allan Wilson Centre, Massey University, Palmerston North, New Zealand. The DNA was sequenced using the 3730 DNA Analyser, ABI, (Applied BioSystems). The BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biochemistry) was used and the manufacturer's protocol was followed.

## 2.2.13. Electrophoretic Mobility Shift Assays

### 2.2.13.1. Oligonucleotide Labelling

To label the oligonucleotide, 100 ng of forward oligonucleotide, 1X $T_4$ polynucleotide kinase buffer (0.5 M Tris.HCl pH 7.5, 0.1 M $MgCl_2$, 50 mM DTT, 0.5 mg/mL BSA), 50 μCi of $\gamma^{32}P$ [ATP], and 1 μL of $T_4$ polynucleotide kinase (10 Units/μL, Roche) were added to a microfuge tube and the reaction mix was made up to 10 μL with $sH_2O$. The reactions were incubated at 37°C for 45 minutes. A 6X excess of complementary oligonucleotide was added along with 1 M KCl and made up to a total volume of 50 μL by adding $sH_2O$. The reactions were then incubated at 95°C, in a water bath, for 5 minutes. Following this the water bath was turned off, and left to cool with the reactions still in it to allow the two complementary oligonucleotides to anneal. An equal volume of gel shift buffer (40 mM Tris pH 7.6, 16% ficoll, 100 mM KCl, 0.4 mM EDTA, 1 mM DTT) was added to each of the reactions and purification of the double-stranded oligonucleotide was carried out immediately afterwards.

### 2.2.13.2. Purification of the labelled oligonucleotide

A 10% polyacrylamide gel was used to purify the double-stranded oligonucleotides from the single-stranded and shorter oligonucleotides (failed synthesis) as well as the unbound $\gamma^{32}P$ [ATP]. The gel was poured into sequencing plates (37 cm long with 0.4 mm spacers) and once set immersed in 1X TBE (0.09 M Tris, 0.09 M Boric Acid, 0.02 M EDTA, pH 8.0). The samples were loaded onto the gel and in one lane DNA loading dye was added to monitor progress of electrophoresis. Electrophoresis was carried out for 1 hour and 30 minutes, at 30 W and 1500 V. The gel apparatus was then dismantled, the gel wrapped in plastic film, and exposed to X-ray film for no longer than one minute before the film was developed. Following this the autoradiograph was matched up to the gel and the area corresponding to the double-stranded oligonucleotide was excised and eluted overnight, at 37°C, in 200 μL of 50 mM KCl. The following day the tubes were spun briefly and the supernatant transferred to another tube, which was stored at 4°C in a radioactive safe

Perspex box. The labelled double-stranded oligonucleotide was now ready to be used in electrophoretic mobility shift assays.

### 2.2.13.3. Preparing Double Stranded Competitor Oligonucleotides

Unlabelled double-stranded competitors were prepared by mixing 5 µL of the complementary oligonucleotides (1 µg/µL), and boiling in a water bath for 5 minutes. The water bath was turned off and the reactions were left in the water bath to cool slowly. The double-stranded competitor oligonucleotides were stored at –20°C until required.

### 2.2.13.4. Electrophoretic Mobility Shift Assays

A master mix, containing suitable volumes of all components, for all reactions used in one Electrophoretic Mobility Shift Assay (EMSA), was made. Each reaction required 10 µL of 2X gel shift buffer, 1 µL poly(dIdC), and between 4 and 16 µg of HeLa extract. The reaction mix was made up to 25 µL using $sH_2O$. If a competitor assay was being carried out 5 ng, 50 ng, or 100 ng (10, 100, and 200 fold excess, respectively) of double-stranded unlabelled competitor was also added to the reaction mix, and the volume of $sH_2O$ was adjusted to accommodate this. Likewise if an antibody supershift assay was being carried out 5 µL (1 µg) of the appropriate antibody was added to the reaction, and again the volume of $sH_2O$ added was adjusted accordingly. The reactions were incubated on ice for 10 minutes before 1 µL (0.5 ng) of labelled double-stranded oligonucleotide was added. Each reaction was mixed using a vortex, briefly centrifuged, and subsequently incubated at room temperature for 15 minutes. The reactions were then loaded onto a 4% polyacrylamide gel in 0.25X TBE. To an outer lane 2 µL of DNA loading dye was added to track the progress of electrophoresis, which was carried out at 200 V for 1 hour and 10 minutes. The gel was transferred to DE-81 paper, which was placed on top of 3MM paper, and dried for 15 minutes using a gel dryer. DE-81 paper is a weakly basic anion exchanger with diethylaminoethyl functional groups, and this was used to prevent the DNA being pulled through the gel during the gel drying process. The dried gel was then placed into a cassette with two intensifying screens, and X-ray film (Kodak) was placed over top. The cassette, containing the gel, was left at –70°C for

between 18 and 30 hours depending on the strength of the radioactively labelled oligonucleotides. The X-ray film was developed using a 100Plus Automatic X-Ray Film Processor.

### 2.2.14. Tissue Culture

Human cervical cancer cells, HeLa cells, were the cell line of choice for this study. All procedures involving the cells prior to harvesting were carried out in a laminar flow hood, using aseptic technique to prevent contamination. Cells were incubated at 37°C, 5% $CO_2$, and in humid conditions at all times.

#### 2.2.14.1. Growing HeLa Cells

HeLa cells were grown from stocks stored under liquid nitrogen. The cells were thawed, pelleted and resuspended in OptiMEM media (GIBCOBRL, Invitrogen) containing 2% (4 mL) foetal calf serum and 1% (2 mL) penicillin/streptomycin (5000 U/mL penicillin G sodium and 5 mg/mL streptomycin sulfate in 0.85% saline). The resuspended cells were then transferred to T80 flasks containing 14 mL of complete media and incubated as above. Cells were checked daily to monitor growth.

#### 2.2.14.2. Maintenance of HeLa Cells

At 80% confluence (as judged by eye) cells were passaged into new flasks. The media was removed and the cells were rinsed twice with trypsin (1 mL 10X Trypsin (GIBCOBRL) and 9 mL PBSE (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4.2H_2O$, pH 7.2, 0.5 mM EDTA)). The trypsin was removed and the cells were left to round up for no longer than 2 minutes. The flask was tapped sharply to dislodge the cells and 5 mL of media was added to resuspend them. A portion of these cells were transferred to a new flask containing 14 mL of media and incubated as above in section 2.2.14.

### 2.2.14.3. Freezing HeLa Cells

Cells were passaged as normal but resuspended in fetal calf serum containing 10% DMSO rather than in OptiMEM. The cells were frozen slowly, by wrapping in tissue paper and placing at –70°C overnight, before being transferred to liquid nitrogen for long-term storage.

## 2.2.15. Preparing HeLa Whole Cell Extracts

HeLa cells were grown in round plates (15 cm diameter) until confluent. The cells were washed twice with PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4.2H_2O$, pH 7.2) and then scraped off the bottom in 2 mL of TEN buffer (40 mM Tris.HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl). Aliquots of 1 mL were centrifuged for 1 minute at room temperature and the supernatant was discarded. Each pellet was resuspended in 200 – 500 µL (depending on cell density) of extraction buffer (40 mM Hepes pH 7.9, 0.4 M KCl, 1 mM DTT, 10% glycerol, containing Complete mini™ protease inhibitor) and put through three freeze-thaw cycles, using liquid nitrogen to snap freeze them. The samples were then centrifuged for 5 minutes, at 12,000 rpm, and the supernatant dispersed into 50 µL aliquots and snap frozen a final time. The whole cell HeLa extracts were stored at –70°C until required.

## 2.2.16. Bradford Protein Assays

Bradford protein assays using BioRad reagent were used to determine the protein concentrations of the whole cell extracts.

Bovine Serum Albumin (BSA) was used to construct a protein standard curve. A 1 mg/mL stock of BSA was diluted 1/5 with $sH_2O$. The range used was 0, 0.2, 0.4, 0.6, 1.0, 1.6, 2, and 2.4 mg of BSA. A 1/10, 1/20, 1/50 and 1/100 dilution of HeLa extract was carried out as well and 10 µL of the diluted protein was also used in the assay. The Bradford solution was diluted 1/5 and 100 µL of this was added to all the wells containing either the BSA standards or HeLa extract. The reactions were left to develop for 5 minutes after which the absorbances were read at 595 nm.

Using the absorbances of the standards, a standard curve was plotted, and the dilution of HeLa extract, which fell in the linear range of this graph, was used to determine the protein concentration of the HeLa extract.

### 2.2.17. Transient Transfections

Transient transfections were used to study transcriptional activity of the promoter-reporter gene constructs. Cells were passaged as per normal, but seeded into 12 well plates to be between 60% and 80% confluent the next day. The amounts of the various plasmid DNA constructs used were as follows: 0.5 μg of pGL3Basic reporter plasmid, 0.25 μg of pCMVSPORTβ-galactosidase reporter plasmid and 0 μg, 0.25 μg, 0.5 μg or 1 μg of either Sp1 or Sp3 co-expression plasmid. pBlueScript was used to ensure the amount of DNA added to all wells was the same. A Fugene6™ to DNA ratio of 3:2 was used and prior to being added to the DNA, the Fugene6™ was diluted in serum free media. Cells were then transfected with the DNA and incubated for 24 hours before being harvested.

### 2.2.18. Harvesting Cells

To harvest, the cells were rinsed twice with PBS and then lysed with 80 μL of cell lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid, 10% glycerol, 1% TritonX-100) for 15 minutes at room temperature. Each well was scraped, the liquid was collected, and then spun at 12,000 rpm for 5 minutes. The cell lysates (supernatants) were used for β-galactosidase and luciferase assays.

#### 2.2.18.1. β-galactosidase assays

β–galactosidase (β-gal assays) were carried out as a control for transfection efficiency. Five microliters of cell lysate was added to a single well of a flat-bottomed 96 well plates along with 100 μL of β-gal buffer (60 mM $NaH_2PO_4$, 40 mM $Na_2HPO_4$, 10 mM KCl, 1 mM MgCl) and 50 μL of ONPG (2 mg/mL ONPG, 60 mM $NaH_2PO_4$, 40 mM $Na_2HPO_4$).

Plates were incubated at 37°C, for up to 4 hours, until they had visibly turned yellow. To stop the reaction 50 µL of 1 M $Na_2CO_3$ was added, and their absorbance at 405 nm was measured using a plate reader.

### 2.2.18.2. Luciferase Assays

Luciferase assays were carried out to measure topoisomerase II beta promoter activity in the presence of a co-expression vector or when the promoter was mutated. Five microliters of each cell lysate was added to a different well of an opaque flat-bottomed 96 well plate. The Fluostar Galaxy Plate reader (BMG Labtechnologies Pty. Ltd) was programmed to add 20 µL of luciferase reagent (Promega Corporation) to each of the wells, and subsequently measure the maximum level of light emitted over a 3-minute time period. The maximum readings were then converted into an Excel Spreadsheet (Microsoft 97) and the maximum readings were used for further analysis of the data. Five microliters of cell lysis buffer was also added to three wells to serve as a control for background activity.

## 2.2.19. GMO Approval Codes

The work carried out in this thesis was done so with the following ERMA regulatory authority approvals:

- Topoisomerase II beta promoter plasmids – GMO/99/MU/25
- Co-expression Plasmids (Sp1, Sp3, pGL3Basic and pCMVSPORTβ-galactosidase) – GMO/00/MU/40

# 3. Electrophoretic Mobility Shift Assays

## 3.1. Introduction

Electrophoretic Mobility Shift Assays (EMSAs) are commonly used to investigate the interactions between DNA binding proteins and their specific binding sequences. This is an *in vitro* approach used to study protein/DNA interactions within promoter regions of a gene. EMSAs utilize the characteristic movement of protein-bound DNA and free DNA in a non-denaturing acrylamide gel. The mobility of a molecule in a gel is dependent on its size, shape, and charge. Therefore, free oligonucleotide will migrate faster through the gel than oligonucleotide-protein complexes. Furthermore, different oligonucleotide-protein complexes will have characteristic mobilities based on the associated proteins' size and charge. Due to the properties of the oligonucleotide-protein complexes and the high resolving power of acrylamide gels it is possible to differentiate various DNA/protein complexes.

The EMSA method involves labelling a single-stranded oligonucleotide with $\gamma^{32}$P[ATP] and annealing it to the complementary sequence. Protein is added to the labelled double-stranded oligonucleotide probe (oligonucleotide probe) and polyacrylamide gel electrophoresis is used to resolve complexes. The complexes are then visualised by autoradiography (Figure 3.1). Poly dIdC is also added to minimise non-specific oligonucleotide-protein interactions.

Competitor assays are carried out to determine the specificity of any oligonucleotide-protein interaction observed. An excess of unlabelled competitor is added to the binding reactions before the addition of oligonucleotide probe. The unlabelled oligonucleotide competes for binding of the proteins with the oligonucleotide probe, and if the proteins bind specifically to the unlabelled oligonucleotide a reduction in the corresponding oligonucleotide probe-protein complex is seen.

Antibody supershift assays can be used to identify the specific proteins that are present in the protein-oligonucleotide complexes. Antibodies against known proteins are added to the reactions before the addition of oligonucleotide probe. If the antibody interacts with a

protein, which in turn binds to the oligonucleotide probe, the resultant complex will be larger and have a lower mobility than a control without antibody. Alternatively, the antibody could bind to the protein in such a way that it prevents the protein forming a complex or interacting with the oligonucleotide probe, therefore the corresponding band will diminish in intensity.

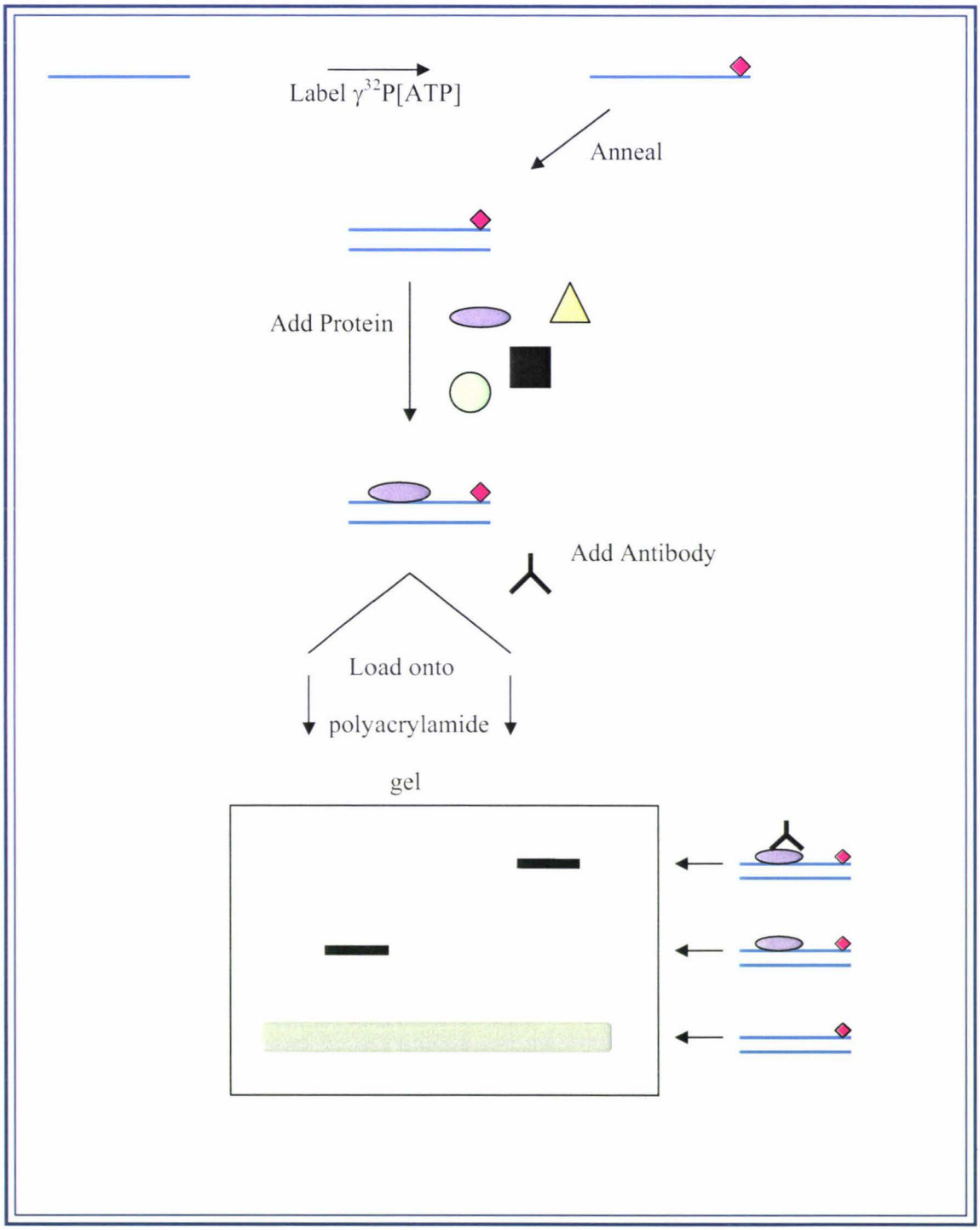



**Figure 3.1: Schematic Representation of an Electrophoretic Mobility Shift Assay.**

This figure shows the basic theory that is used to carry out an EMSA. The single-stranded oligonucleotide is labelled with $^{32}\gamma$P[ATP] and annealed to its complementary sequence. Protein extract is added to the labelled double-stranded oligonucleotide probe, and the reaction is loaded onto a polyacrylamide gel and the resulting bands are visualisedby exposure to X-Ray film.

## 3.2. Binding of Proteins to the Topoisomerase II Beta Promoter

Previous EMSAs, using oligonucleotides derived from the topoisomerase II beta promoter sequence, have shown that the GC1 element binds the transcription factor Sp1 and that the ICB1 and ICB2 elements bind to the transcription factor NF-Y (Lok *et al.*, 2002). There are however, other bands in these EMSAs that were not positively identified.

Magan *et al.* (2003) carried out a series of EMSAs using topoisomerase II alpha promoter sequences as the oligonucleotide probes, and found that both Sp1 and Sp3 bound to two GC elements. These authors suggested that NF-Y may recruit Sp1 to the ICB1 element of the topoisomerase II alpha promoter. The researchers then investigated a composite ICB1/GC1 oligonucleotide probe, which is similar to the ICB2/GC1 composite element of the topoisomerase II beta promoter. Interestingly they found that Sp1, Sp3, and NF-Y (as well as some unknown protein) bound to the ICB1/GC1 oligonucleotide probe in a GC1 dependent manner.

Based on these previous studies oligonucleotides were designed for the topoisomerase II beta promoter. GC1, GC2, ICB1, and ICB2 oligonucleotides were designed to incorporate the protein binding element as well as some of the flanking sequence (see Appendix 1 for oligonucleotide sequences). A slightly larger double element oligonucleotide was also designed containing both the ICB2 and GC1 elements as well as flanking sequence, which could be necessary to facilitate protein binding.

### 3.2.1. HeLa Protein Extract Preparations

Whole cell protein extracts, for use in EMSAs, were prepared from HeLa cells as described in section 2.2.15. HeLa cells were subsequently used in transient transfections, therefore the same complement of proteins were present in both *in vitro* binding assays and the *in vivo* functional assays.

### 3.2.2. Oligonucleotide Preparation

It has been previously been shown that Sp1 binds to the GC1 element, and that NF-Y binds to both the ICB1 and ICB2 elements within the topoisomerase II beta promoter (Lok *et al.*, 2002). To further investigate these interactions and to determine whether Sp3 can also bind to the promoter EMSAs were performed.

Commercially synthesised oligonucleotides were labelled using $\gamma^{32}$P[ATP] and annealed to the complementary oligonucleotide as outlined in section 2.2.13.1. The double-stranded labelled oligonucleotide was purified by polyacrylamide gel electrophoresis as described in section 2.2.13.2. Since the labelling of the single-stranded oligonucleotide and annealing the complementary sequence are unlikely to be 100% successful, it is necessary to remove the unlabelled and single-stranded oligonucleotide as well as shorter oligonucleotide. These smaller/contaminating oligonucleotides will migrate at a different rate through the polyacrylamide gel to the double-stranded oligonucleotides, and can thus be removed. The majority of the oligonucleotide was double-stranded and labelled and a corresponding band was clearly visible as shown on the autoradiograph (Figure 3.2). The band representing the labelled oligonucleotide was excised from the gel and the oligonucleotide eluted as described in section 2.2.13.2. The GC2 and GC1 oligonucleotides show higher mobility bands, which are most likely to be shorter oligonucleotides due to failed steps in the original synthesis by the manufacturer.

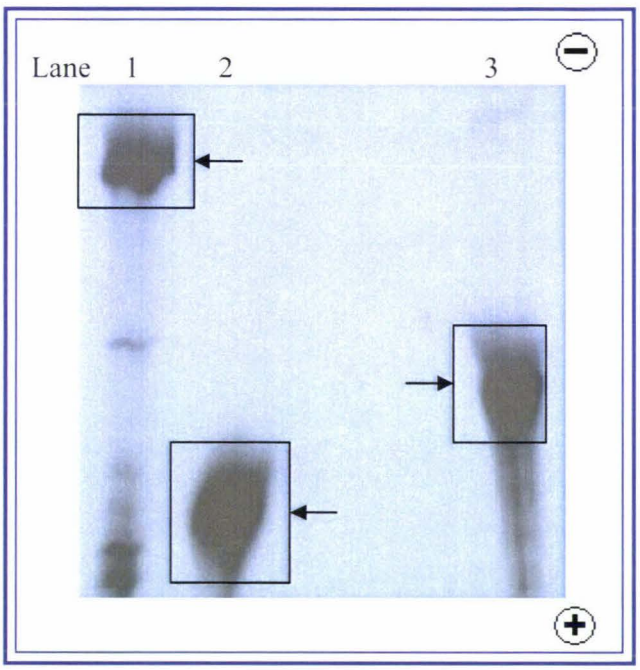



**Figure 3.2: Oligonucleotide Purification.**

100 ng of forward oligonucleotide was end-labelled using $\gamma^{32}$P[ATP]. 600 ng of the complementary oligonucleotide was annealed and the total reaction was loaded onto a 10% polyacrylamide gel, which was run at 30 W (1500 V) for 1 hour in 1X TBE. The gel was exposed to X-Ray film for less than 1 minute; the labelled double-stranded oligonucleotide bands excised and resuspended in 50 mM KCl to elute the oligonucleotide from the gel.

Lane 1: GC1 oligonucleotide

Lane 2: ICB2 oligonucleotide

Lane 3: GC2 oligonucleotide

### 3.2.3. Extract Titrations

As the recovery of the purified oligonucleotide probe could only be estimated, HeLa extract titrations were performed to determine the optimal ratio of oligonucleotide probe to protein. An extract titration was carried out to optimise the ratio of oligonucleotide probe to protein extract for each batch of whole cell extracts, and for each newly labelled double-stranded oligonucleotide. One microliter of oligonucleotide probe was used in each reaction along with increasing amounts of HeLa extract (0 μg, 4 μg, 8 μg and 16 μg).

#### 3.2.3.1. Single Element Extract Titrations

Figure 3.3 shows the protein extract titration for the three single topoisomerase II beta elements that are within the proximal promoter, GC1, ICB1, and ICB2, as well as the GC2 element, located further upstream of the transcription start site. As the amount of HeLa extract was increased the intensity of the bands also increased. Table 3.1 outlines the number of bands observed for each element and the optimal amount of protein.

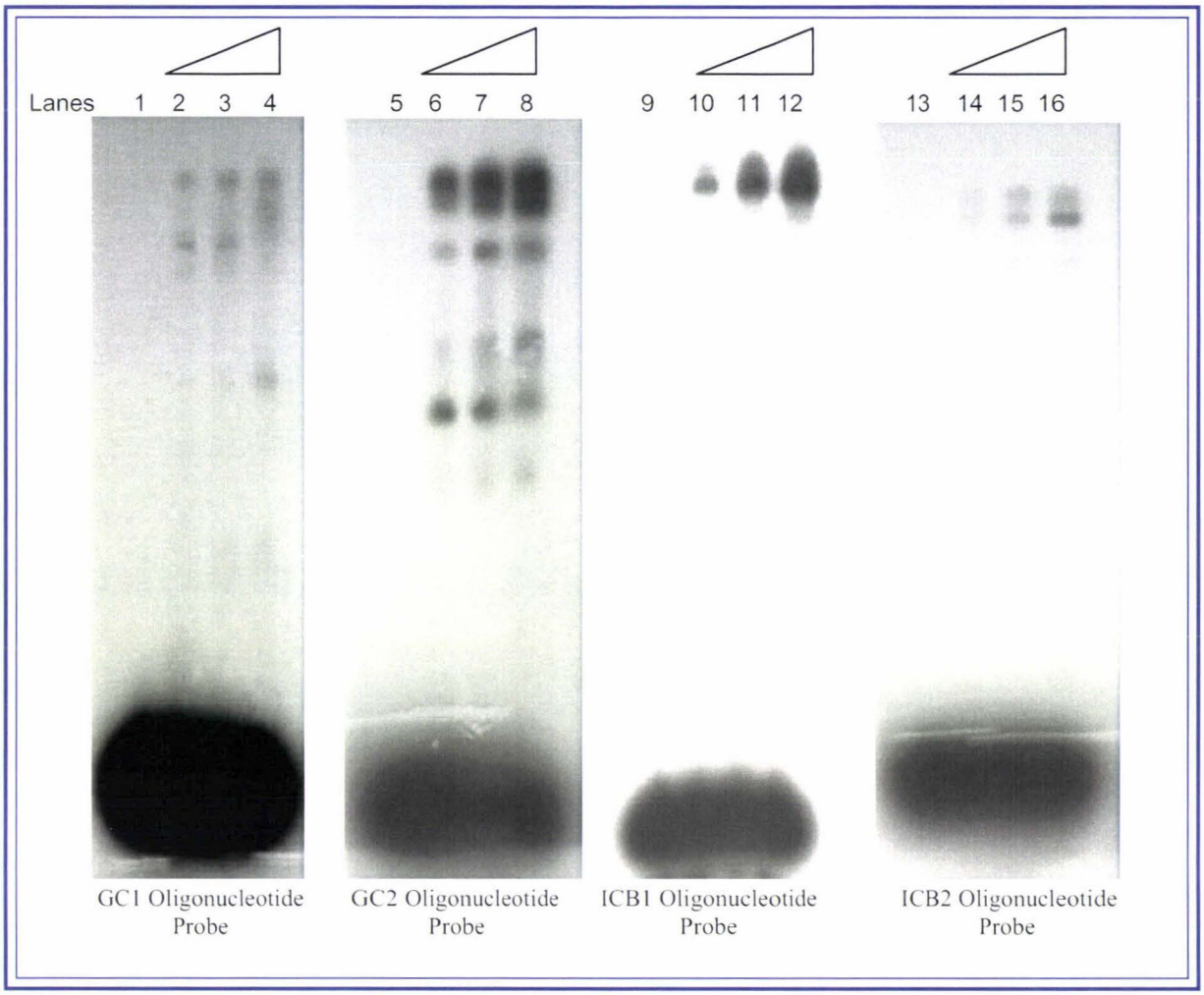



**Figure 3.3: GC1, GC2, ICB1 and ICB2 Extract Titrations.**

1 μL of oligonucleotide probe and increasing amounts of HeLa protein extract (0 μg, 4 μg., 8 μg and 16 μg) were used in the binding reactions. 10 μL (50%) of each reaction was loaded onto a 4% acrylamide gel in 0.25X TBE buffer and electrophoresis was carried out for 75 minutes at 200 V. The gel was dried and exposed to X-Ray film for approximately 15 hours then developed.

Lanes 1-4, GC1 oligonucleotide probe; lanes 5-8, GC2 oligonucleotide probe; lanes 9-12, ICB1 oligonucleotide probe; and lanes 13-16, ICB2 oligonucleotide probe. Lanes 1, 5, 9 and 13 have oligonucleotide probe only and are controls for the movement of free oligonucleotide probe. Lanes 2, 6, 10 and 14 contain 4 μg of HeLa protein extract, lanes 3, 7, 11 and 15 contain 8 μg and lanes 4, 8, 12 and 16 contain 16 μg of HeLa extract.

| Oligonucleotide Probe | Number of Bands | Optimal Amount of HeLa extract |
|---|---|---|
| GC1 | 4 | 16 μg |
| GC2 | 3 | 4 μg |
| ICB1 | 1 | 4 μg |
| ICB2 | 2 | 8 μg |
| ICB2/GC1 | 5 | 4 μg or 16 μg |

**Table 3.1: Extract Titration Results.**

Extract titrations were carried out using increasing amount to determine the optimal amount of HeLa extract to use in subsequent electrophoretic mobility shift assays.

### 3.2.3.2. Double Element Extract Titrations

The double element GC1/ICB2 oligonucleotide probe has five different bands in the extract titration as shown in Figure 3.4. When 4 μL (16 μg) of HeLa extract was added the bands are not as well defined as they were with only 1 μL (4 μg) of extract however, an extra band of lower mobility appeared, perhaps due to a larger protein complex forming along with the usual bands. Therefore, both amounts, 1 μL and 4 μL, have been used for further assays.

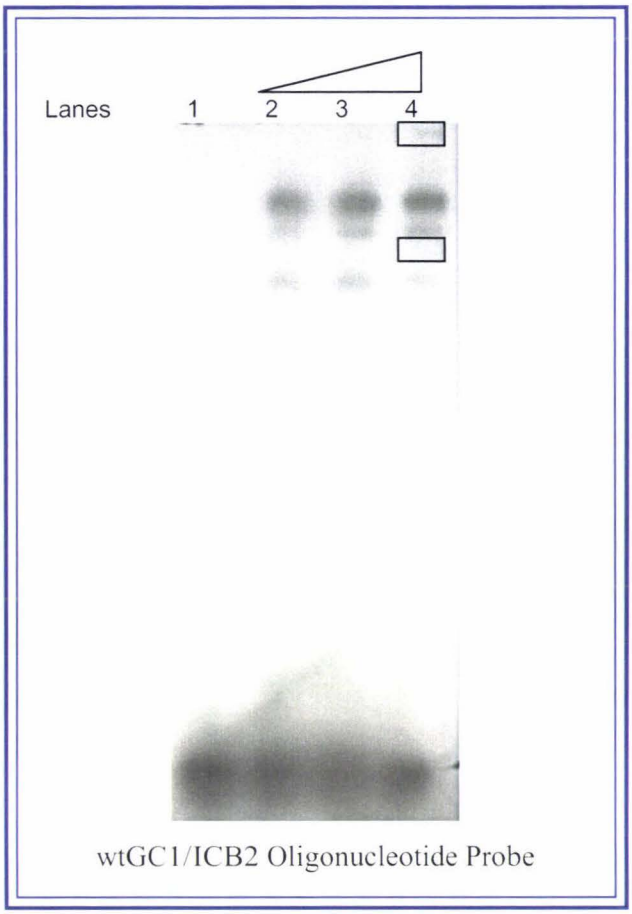



**Figure 3.4: GC1/ICB2 Extract Titration.**

1 μL of oligonucleotide probe and increasing amounts of HeLa protein extract (0 μg, 4 μg, 8 μg, and 16 μg) were used in the binding reactions. 10 μL (50%) of each reaction was loaded onto a 4% acrylamide gel in 0.25X TBE buffer and electrophoresis was carried out for 75 minutes at 200 V. The gel was dried and exposed to X-Ray film for approximately 15 hours then developed.

Lanes 1-4 contain the GC1/ICB2 oligonucleotide probe. Lane 1 has oligonucleotide probe only and is a control for the movement of free oligonucleotide probe. Lane 2 contains 4 μg of HeLa protein extract, lane 3 contains 8 μg and lane 4 contains 16 μg of HeLa extract.

### 3.2.4. Antibody Supershift Assays

Antibody supershift assays were carried out in order to identify the proteins that bound to the oligonucleotide probes derived from the topoisomerase II beta promoter. Sp1/Sp3 and NF-Y are transcription factors, which are known to bind to GC-rich elements and inverted CCAAT boxes, respectively. Sp1 and NF-Y have already been shown to bind to the topoisomerase II beta promoter (Lok *et al.*, 2002) and, therefore, these were used as the controls for the single element oligonucleotide probes to investigate the possibility of Sp3 binding to the promoter. Antibodies against each of these three proteins were added to separate reaction mixes, along with poly dIdC, and the previously optimised amount of HeLa protein extract.

Figure 3.5 shows an antibody supershift assay for the GC1 and GC2 oligonucleotide probes. For each oligonucleotide probe the first lane, (lanes 1 and 6) contains oligonucleotide probe alone, and the second lane has HeLa extract added. These lanes serve as controls to show the movement of free oligonucleotide probe, and to allow comparisons to be made between protein-oligonucleotide probe complexes and antibody shifted protein-oligonucleotide probe complexes. The third lane contains Sp1-specific antibody, the fourth lane contains Sp3-specific antibody and the fifth lane contains an antibody specific for the NF-YA subunit.

The GC1 oligonucleotide probe shown in lanes 1-5 binds four proteins. In lane 3 Sp1 antibody was added to the reaction and there is a shift in band 1, likewise when NF-YA antibody was added band 2 was shifted. This identifies these two bands as Sp1 and NF-Y respectively. Band 3 does not appear to shift upon addition of any of the antibodies and this protein remains uncharacterised. The faint fourth band is lost when Sp3 antibody is added, thereby identifying band 4 as an Sp3/oligonucleotide probe complex.

The GC2 oligonucleotide probe (lanes 6-10) binds three distinct proteins. Band 1 is shifted upon the addition of Sp1 antibody and band 3 is shifted when Sp3 antibody is added, thus identifying the two proteins as Sp1 and Sp3 respectively. There is also a faint band in the middle, band 2, which does not appear to shift with any of the 3 antibodies added, therefore, no conclusions could be made as to the proteins identity.

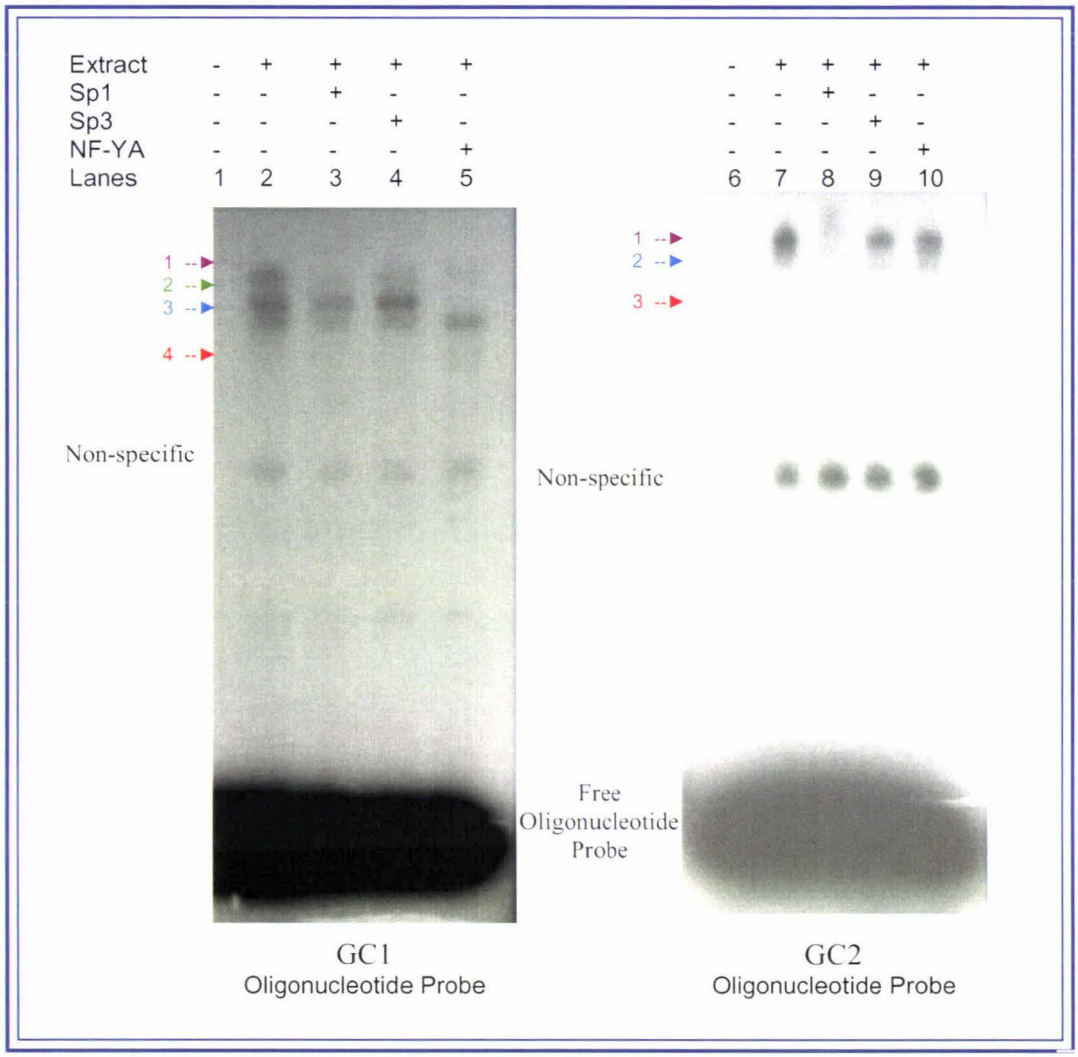



**Figure 3.5: GC1 and GC2 Antibody Supershift Assay.**

1 µL of oligonucleotide probe and 1 µg of antibody were added to each reaction along with 16 µg of HeLa extract for the GC1 oligonucleotide probe and 4 µg GC2 oligonucleotide probe. 12.5 µL (50%) of each reaction was loaded onto a 4% acrylamide gel in 0.25X TBE buffer. Electrophoresis was carried out at 200 V for 75 minutes after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The gel was exposed to X-Ray film for 18 hours at –70°C, after which the film was developed.

Lane 1 and 6 contain oligonucleotide probe. Lanes 2 and 7 contain HeLa extract and act as controls for normal binding. Lanes 3 and 8 contain Sp1 antibody; lanes 4 and 9 contain Sp3 antibody; and lanes 5 and 10 contain NF-YA antibody. Arrows indicate the bands of interest.

The bands with highest mobility in both the GC1 and GC2 antibody supershift assays (Figure 3.5) do not shift upon addition of any of the three antibodies used. The intensity of the band indicates that the oligonucleotide probe/protein interaction is strong in both the GC1 and GC2 antibody supershift assay. The specificity of these interactions will be determined in further EMSAs.

Figure 3.6 shows an antibody supershift assay for the ICB1 oligonucleotide probe, where one band is observed. Lanes 3 and 4 contain Sp1 and Sp3 antibody, respectively, and there is no mobility shift observed. Lane 5 contains NF-YA antibody and shows a shift, this indicates that only NF-Y is able to interact directly with ICB1.

The ICB2 oligonucleotide probe (lanes 6-10) binds two proteins, band 1 being identified as Sp1 and band 2 NF-Y, as there is a loss of these bands when the corresponding antibodies are added in lanes 8 and 10 respectively. Sp1 is not known to bind to ICBs and this could suggest that Sp1 is being recruited to the ICB by the NF-Y protein, through protein to protein interactions. Band 1, which contains Sp1, is more diffuse than band 2 and it is also less intense in lane 10, which contains the antibody against NF-Y. This suggests band 1 could be a complex of DNA with both NF-Y and Sp1.

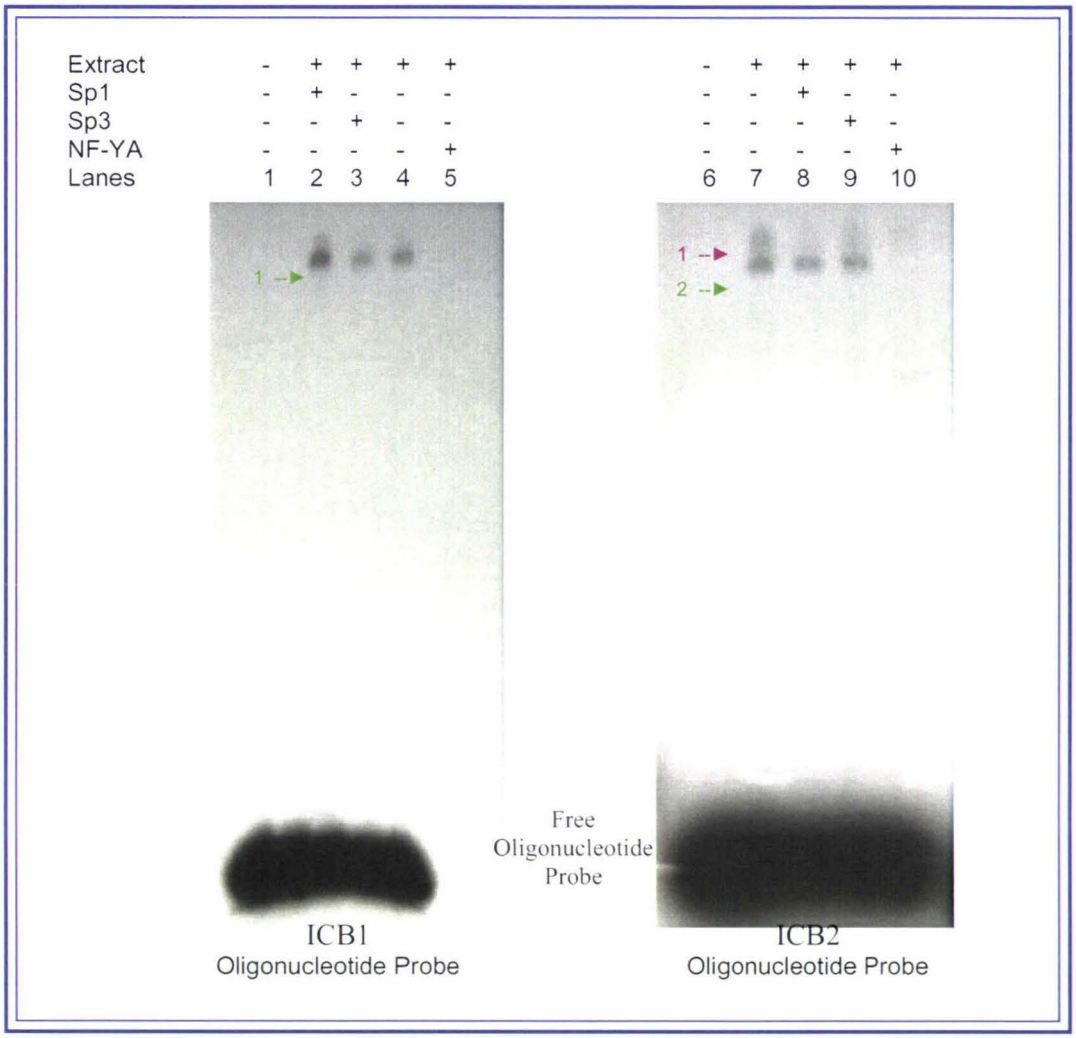



**Figure 3.6: ICB1 and ICB2 Antibody Supershift Assay.**

1 µL of oligonucleotide probe and 1 µg of antibody were added to each reaction along with 4 µg of HeLa extract for the ICB1 oligonucleotide probe and 8 µg ICB2 oligonucleotide probe. 12.5 µL (50%) of each reaction was loaded onto a 4% acrylamide gel in 0.25X TBE buffer. Electrophoresis was carried out at 200 V for 75 minutes after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The gel was exposed to X-Ray film for 18 hours at –70°C, after which the film was developed.

Lane 1 and 6 contain oligonucleotide probe. Lanes 2 and 7 contain HeLa extract and act as controls for normal binding. Lanes 3 and 8 contain Sp1 antibody; lanes 4 and 9 contain Sp3 antibody; and lanes 5 and 10 contain NF-YA antibody. Arrows indicate the bands of interest.

The antibody supershift assays confirmed that NF-Y, Sp1, and Sp3 bind to their respective elements in the promoter. Szremska (2000) suggested that Sp1 was capable of binding to ICB1 in the topoisomerase II alpha promoter and Magan *et al.* (2003) investigated this interaction further using a composite ICB1/GC1 oligonucleotide probe in EMSAs in order to determine the sequence specific for the binding of Sp1 and NF-Y. These authors unexpectedly uncovered an additional protein, which was found to bind to this composite ICB1/GC1 oligonucleotide probe only, and it was not recognised by antibodies to Sp1, Sp3 or NF-YA. The topoisomerase II beta promoter contains two elements, namely GC1 and ICB2, which resemble in sequence and spacing the ICB1 and GC1 elements of the topoisomerase II alpha promoter. It was therefore of interest to determine if the topoisomerase II beta ICB2/GC1 composite oligonucleotide probe was able to bind additional proteins, which may be the same uncharacterised protein observed in EMSAs with the topoisomerase II alpha oligonucleotide probes.

The antibody supershift EMSA for the double element, GC1/ICB2, oligonucleotide probe shows 5 distinct bands represented in lane 2 of Figure 3.7. Band 1 is shifted to some extent with each of the three antibodies, lanes 3-5, suggesting that a complex consisting of at least these three proteins is forming. When Sp1 antibody is added band 2 is partially shifted to form a new band of lower mobility, lane 3. Due to the large amount of Sp1 binding to the GC1/ICB2 oligonucleotide probe not all the Sp1-oligonucleotide probe complex is shifted upon addition of antibody. It may be possible to show a complete shift of this band with more antibody. When Sp3 antibody is added a shift in band 5 is observed (lane 4), and band 3 shifts with antibody to NF-YA (lane 5). There is also another weak band present, band 4, which does not appear to shift upon addition of any of the antibodies. This appears to be the same protein/oligonucleotide probe complex that is observed with the GC1 and GC2 single element oligonucleotide probes and further analysis will be required to uncover its identity. Notably, antibodies against NF-YA completely shift band 1 and band 3, confirming the presence of NF-YA in the complex represented by band 1. Sp3 antibodies partially shift band 1 and completely shift band 5. These differences may be due to different stoichiometries between proteins in the larger, less mobile complex, and proteins in the more mobile complexes.

All other bands seen in the antibody supershift assays are likely to be non-specific interactions. Specificity of oligonucleotide probe/protein interactions was investigated further using competitor assays.

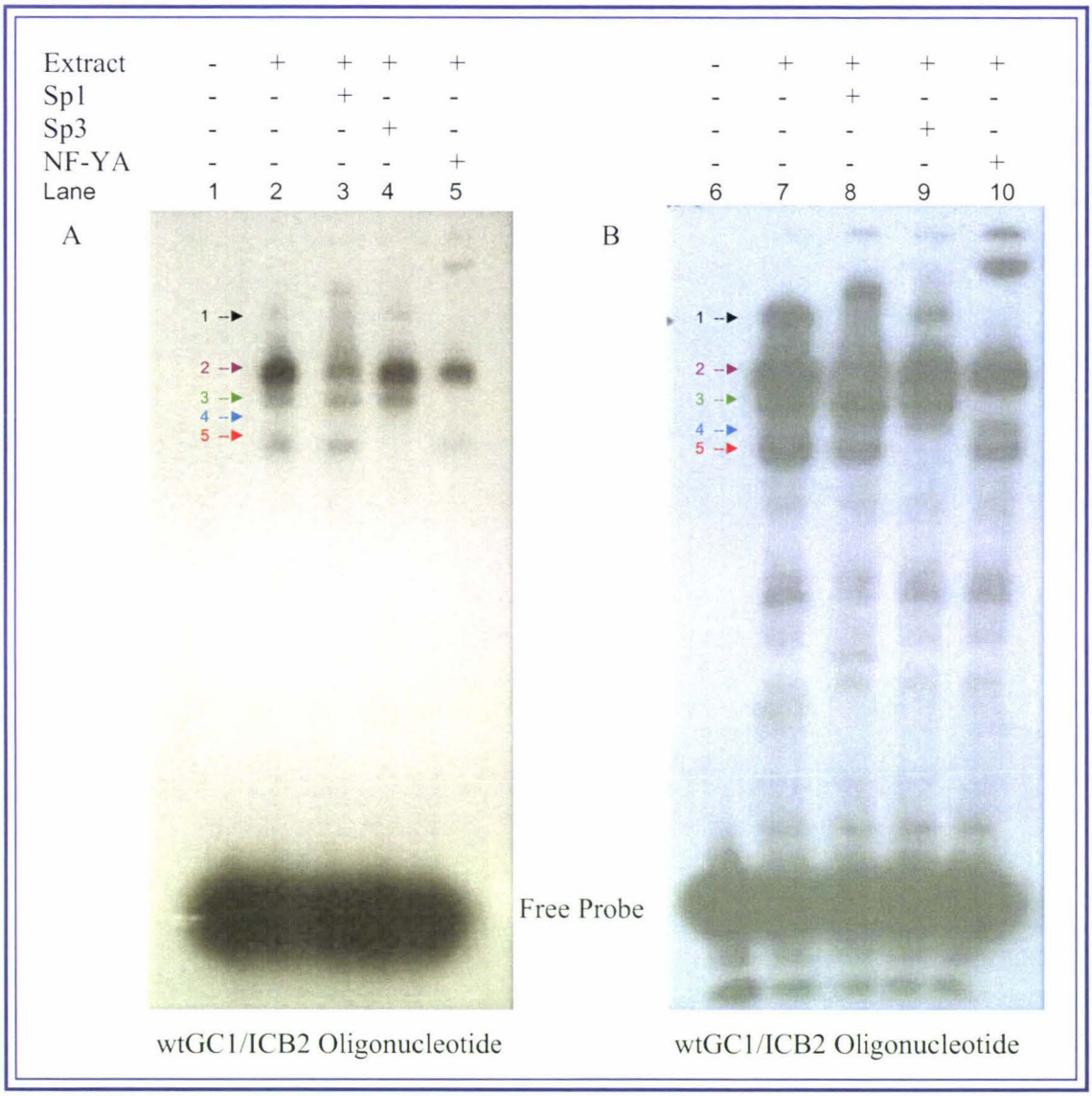



**Figure 3.7: GC1/ICB2 Antibody Supershift Assay.**

1 μL of oligonucleotide probe and 1 μg of antibody were added to each reaction along with 4 μg of HeLa extract (A) or 16 μg of HeLa extract (B). 12.5 μL (50%) of each reaction was loaded onto a 4% acrylamide gel in 0.25X TBE buffer. Electrophoresis was carried out at 200 V for 75 minutes after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The gel was exposed to X-Ray film for 18 hours (A) or 30 hours (B) after which the film was developed. Lanes 1 and 6 contain oligonucleotide probe. Lanes 2 and 7 contain HeLa extract and act as controls for normal binding. Lanes 3 and 8 contain Sp1 antibody; lanes 4 and 9 contain Sp3 antibody; and lanes 5 and 10 contain NF-YA antibody. Arrows indicate the bands of interest.

### 3.2.5. Competitor Assays

Competitor assays, using excess unlabelled oligonucleotide probe, were carried out for each of the different labelled oligonucleotide probes to determine the specificity of the interactions. A reduction in a labelled oligonucleotide probe /protein complex with a homologous competitor oligonucleotide and not with the mutated competitor oligonucleotide would suggest that the interaction seen is specific.

Four different competitor oligonucleotides were used in each competitor assay. The first competitor oligonucleotide was identical to the oligonucleotide probe, the second had the binding sequence mutated, the third competitor oligonucleotide was a similar element found elsewhere in the promoter sequence, and the fourth was a mutated form of the third. The first lane of each competitor assay contained oligonucleotide probe alone and lanes 2, 6, 10, and 14 contained HeLa extract with no competitor oligonucleotide to show normal binding. The following three lanes in each set contained 5 ng, 50 ng, or 100 ng of competitor oligonucleotide. The approximate amount of oligonucleotide probe used in each reaction was 0.5 ng therefore approximately 10X, 100X, and 200X excess competitor oligonucleotide was used.

The competitor assay using the GC1 oligonucleotide probe is shown in Figure 3.8. Lane 2 shows the four proteins that bind to the GC1 oligonucleotide probe, then as the amount of wtGC1 competitor oligonucleotide increases the amount each of the bands attributed to Sp1, Sp3, NF-Y, and unknown protein binding to the labelled GC1 oligonucleotide probe decreases (lanes 3-5). When the GC1 element was mutated, and excess of this competitor oligonucleotide was added to the assay, there was no reduction in any of the observed bands, indicating that the proteins binding to the GC1 oligonucleotide probe do bind specifically (lanes 7-9). The GC2 competitor oligonucleotide competes strongly for the Sp1 band as illustrated by strong competition with only 5 ng of this competitor oligonucleotide (lane 11). This result indicates that Sp1 preferentially binds to the GC2 element *in vivo*. Sp3 is also weakly competed for by the GC2 competitor oligonucleotide, but NF-Y and the unknown protein were not competed for even when 100 ng of competitor oligonucleotide was added (lanes 11-13). The mutant GC2 competitor oligonucleotide has a very weak effect on the protein-GC1 oligonucleotide probe interactions across all the concentrations of competitor oligonucleotides, which further confirms the specificity of the observed interactions (lanes 15-17). The band located half

way down the EMSA was identified as a non-specific interaction, because the mtGC2 competitor oligonucleotide competed as effectively for the binding of the protein as the wtGC1 competitor oligonucleotide (lanes 15-17 and lanes 3-5 respectively). The wtGC2 competitor oligonucleotide and mtGC1 competitor oligonucleotide did not compete for proteins in this band.

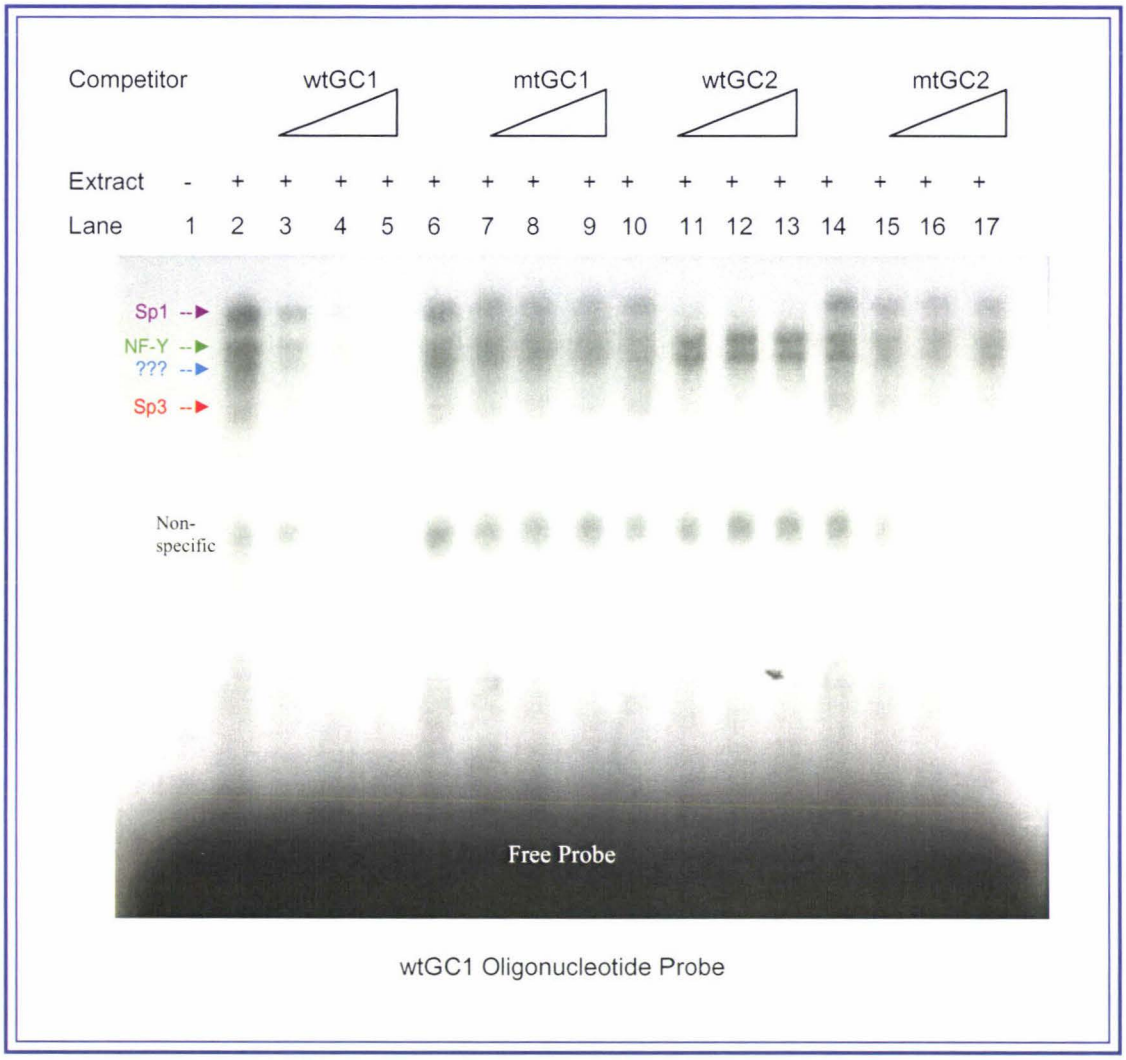



**Figure 3.8: GC1 Competitor Assay.**

16 µg of HeLa protein extract and 1 µL of oligonucleotide probe were added to each reaction along with increasing amounts of competitor oligonucleotide (0 ng, 5 ng, 50 ng, and 100 ng). 12.5 µL (50%) of each reaction was loaded into a 4% acrylamide gel. Electrophoresis was carried out for 75 minutes at 200 V in 0.25X TBE, after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The EMSA was then exposed to X-Ray film for 17 hours before the film was developed.

Lane 1 contains oligonucleotide probe alone and lanes 2, 6, 10, and 14 contain no competitor oligonucleotide. Arrows indicate specific interactions and all other bands observed are non-specific interactions.

The results shown in Figure 3.9 are representative of a competitor assay with the GC2 oligonucleotide probe, and are similar to the GC1 competitor assay results (Figure 3.8). With no competitor oligonucleotide, the standard three bands are observed using the GC2 oligonucleotide probe. However, when only 5 ng of GC2 competitor oligonucleotide is added nearly all Sp1, Sp3, and the unknown protein are competed for with equal affinity (lane 3). The GC1 competitor oligonucleotide appears to be less effective in competing for the binding of the proteins, as faint bands representing all three proteins remain even when 200X excess competitor oligonucleotide is added (lanes 11-13). Both the mutant competitor oligonucleotides, mtGC2 (lanes 7-9) and mtGC1 (lanes 15-17), had no effect on the binding patterns observed, thus indicating the three identified proteins are participating in specific interactions with the oligonucleotide probe. There also appears to be another specific interaction occurring further down the gel (labelled unidentified protein), but the identity of this protein was not established in the antibody supershift assays. This interaction is much weaker than that observed for either Sp1 or Sp3. The oligonucleotide probe/protein band with highest mobility appears to be non-specific as competition with any of the competitor oligonucleotides was not consistent.

Only one band is observed when the ICB1 oligonucleotide probe is used, indicating that ICB1 binds only one protein *in vitro*, which was identified as NF-Y in the antibody supershift assay. Figure 3.10 shows NF-Y is competed for with 10X excess wtICB1 competitor oligonucleotide (lane 3) and 10X excess wtICB2 competitor oligonucleotide (lane 12). This suggests that NF-Y binds to the ICB1 element and ICB2 element with similar affinity. No competition is observed when mutated ICB1 competitor oligonucleotide (lanes 7-9) or mutated ICB2 competitor oligonucleotide (lanes 15-17) is added, thus confirming the specificity of the interaction.

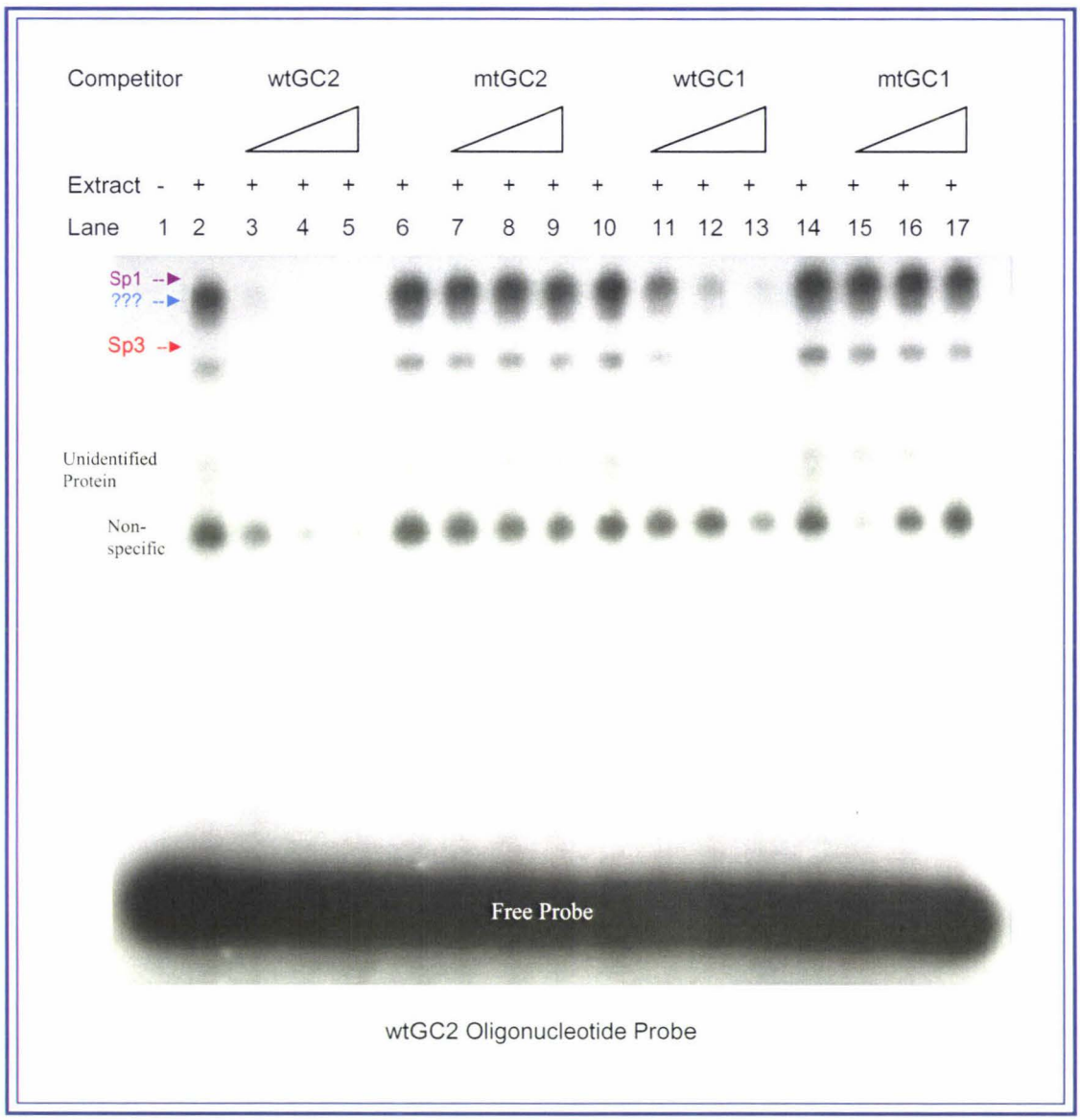



**Figure 3.9: GC2 Competitor Assay.**

4 µg of HeLa protein extract and 1 µL of oligonucleotide probe were added to each reaction along with increasing amounts of competitor oligonucleotide (0 ng, 5 ng, 50 ng, and 100 ng). 12.5 µL (50%) of each reaction was loaded into a 4% acrylamide gel. Electrophoresis was carried out for 75 minutes at 200 V in 0.25X TBE, after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The EMSA was then exposed to X-Ray film for 17 hours before the film was developed.

Lane 1 contains oligonucleotide probe alone and lanes 2, 6, 10, and 14 contain no competitor oligonucleotide. Arrows indicate specific interactions and all other bands observed are non-specific interactions.

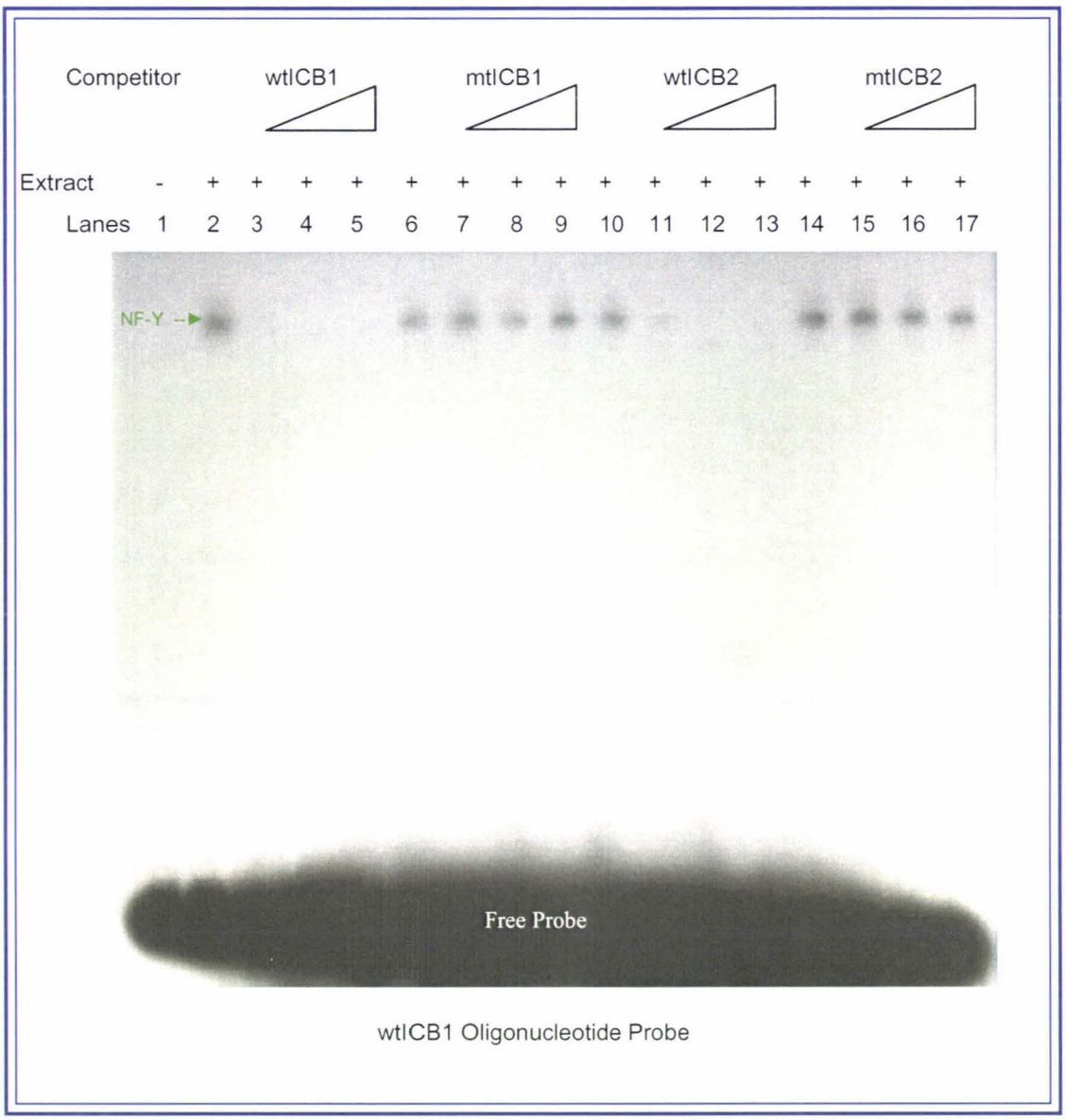



**Figure 3.10: ICB1 Competitor Assay.**

4 µg of HeLa protein extract and 1 µL of oligonucleotide probe were added to each reaction along with increasing amounts of competitor oligonucleotide (0 ng, 5 ng, 50 ng, and 100 ng). 12.5 µL (50%) of each reaction was loaded into a 4% acrylamide gel. Electrophoresis was carried out for 75 minutes at 200 V in 0.25X TBE, after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The EMSA was then exposed to X-Ray film for 17 hours before the film was developed.

Lane 1 contains oligonucleotide probe alone and lanes 2, 6, 10, and 14 contain no competitor oligonucleotide. Arrows indicate specific interactions and all other bands observed are non-specific interactions.

The ICB2 oligonucleotide probe binds two proteins, NF-Y and Sp1. Both of these were competed for when 5 ng of ICB2 competitor oligonucleotide was added (Figure 3.11, lane 3). However, the wtICB1 competitor oligonucleotide only competed for the binding of NF-Y, and Sp1 remained bound to the ICB2 oligonucleotide probe (lanes 11-13). This was expected because the ICB1 element appears to only bind NF-Y (Figure 3.6). Neither the ICB2 mutant competitor oligonucleotide (lanes 7-9), nor the ICB1 mutant competitor oligonucleotide (lanes 15-17) had any effect on the binding of the two proteins to the ICB2 oligonucleotide probe, again confirming the specificity of these interactions.

Figure 3.12 shows the double element competitor assay using the GC1/ICB2 oligonucleotide probe. The extract titration for this oligonucleotide probe showed that there were five oligonucleotide probe/protein complexes forming (Figure 3.4, lanes 2-4). The wtGC1/wtICB2 competitor oligonucleotide competes for all proteins but the multi-protein complex disappeared when only 10X excess competitor oligonucleotide was added (lane 3). This suggests the interactions in this complex are weak under the conditions used. All the other bands were competed for when 100X excess competitor oligonucleotide was added (lane 4). These results suggest that these interactions are specific. The mtGC1/wtICB2 competitor oligonucleotide would be expected to compete for NF-Y and Sp1, but not Sp3 since ICB2 is wild type and has been shown to bind both Sp1 and NF-Y. Sp3 is not bound to ICB2 but can bind to GC1; therefore no competition would be expected. When this mtGC1/wtICB2 competitor oligonucleotide was added there is weak competition for both Sp1 and Sp3 and strong competition for NF-Y (lanes 6-9). This suggests that NF-Y, which can still bind to the ICB2 element of the competitor oligonucleotide, may be interacting with both Sp1 and Sp3 independently of GC1. Using the wtGC1/mtICB2 element as a competitor oligonucleotide, NF-Y should still bind to the oligonucleotide probe but the binding of both Sp1 and Sp3 would be expected to reduce, as the wtGC1 sequence (of the competitor oligonucleotide) should be able to sequester both these proteins. Lanes 10-13 show that there is no reduction in intensity of the NF-Y band, as expected, however, there is only a slight reduction in the intensity of the Sp1/ oligonucleotide probe complex. The Sp3 band is competed with a 10X excess of competitor oligonucleotide (lane 11). This suggests that Sp1 is able to interact more strongly with the oligonucleotide probe when both ICB2 and GC1 binding elements are present. When the mtGC1/mtICB2 competitor oligonucleotide is added to the binding reactions there is no competition for the binding of NF-Y and Sp1, but there is weak

competition for the complex and Sp3. Taken together these results confirm the specificity of binding for NF-Y, Sp1, and Sp3 to the composite promoter element, but the interaction of NF-Y and Sp1 with the oligonucleotide probe is stronger than that of both the complex and Sp3.

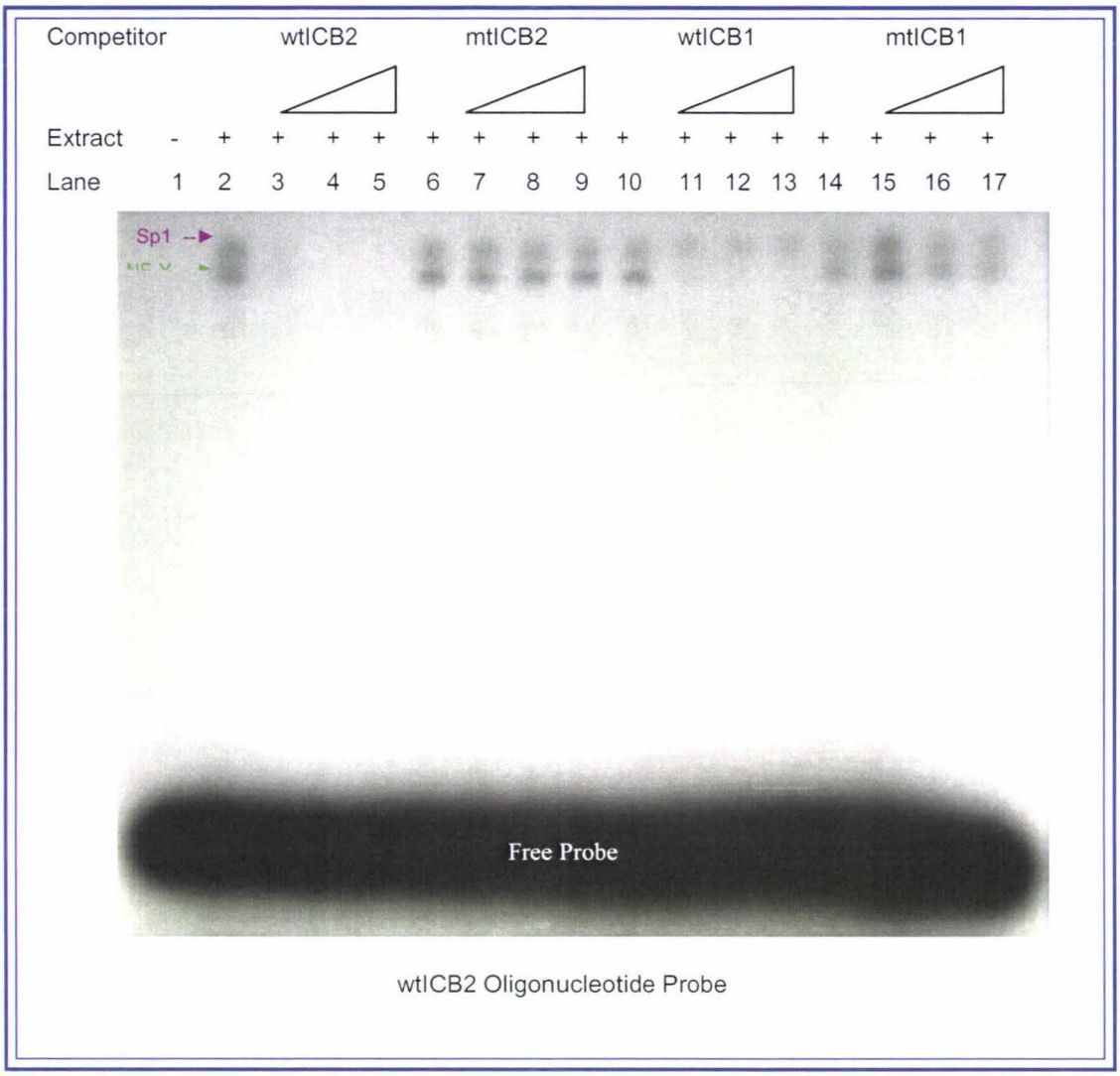



**Figure 3.11: ICB2 Competitor Assay.**

8 µg of HeLa protein extract and 1 µL of oligonucleotide probe were added to each reaction along with increasing amounts of competitor oligonucleotide (0 ng, 5 ng, 50 ng, and 100 ng). 12.5 µL (50%) of each reaction was loaded into a 4% acrylamide gel. Electrophoresis was carried out for 75 minutes at 200 V in 0.25X TBE, after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The EMSA was then exposed to X-Ray film for 17 hours before the film was developed.

Lane 1 contains oligonucleotide probe alone and lanes 2, 6, 10, and 14 contain no competitor oligonucleotide. Arrows indicate specific interactions and all other bands observed are non-specific interactions.

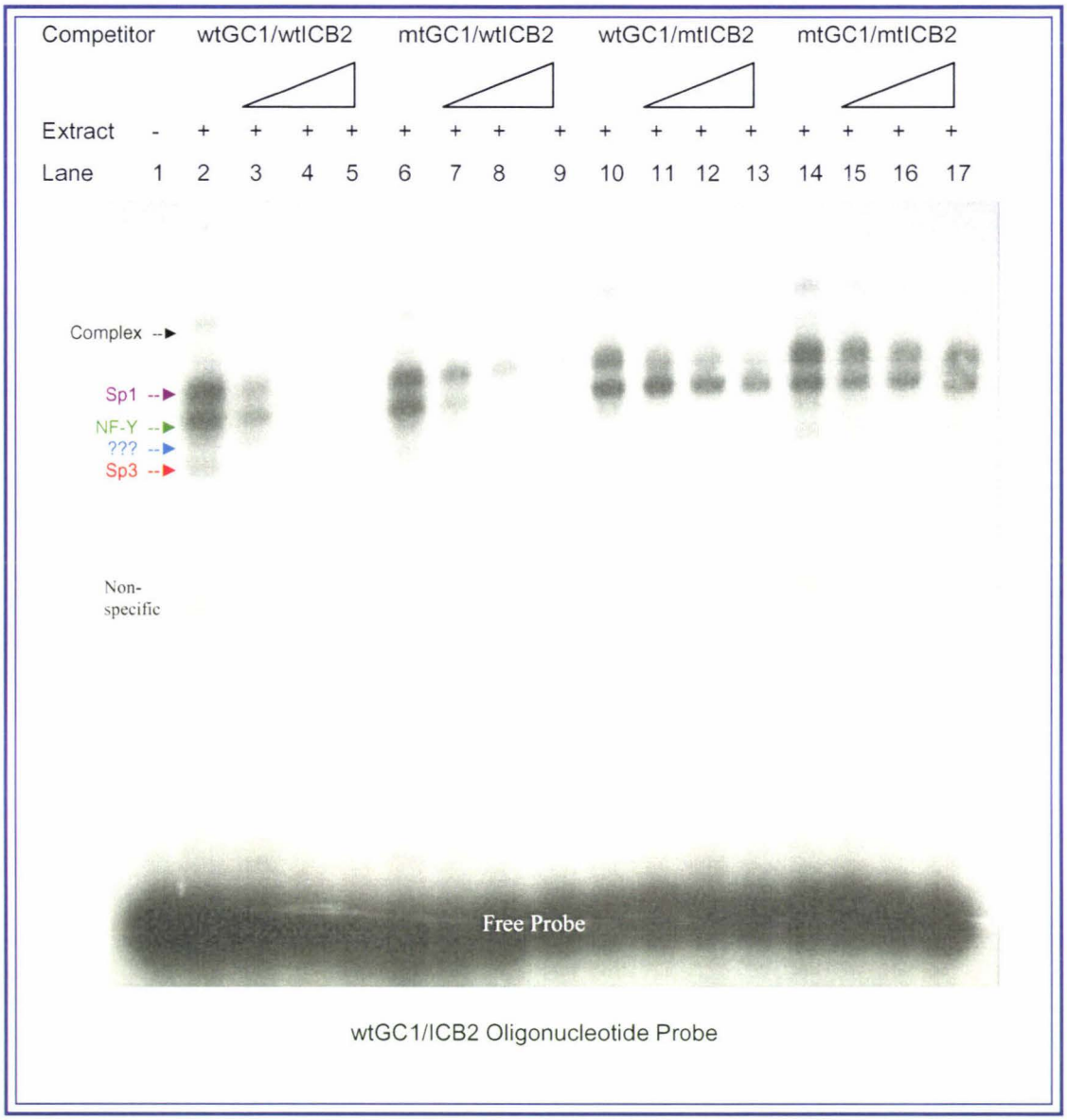



**Figure 3.12: GC1/ICB2 Double Element Competitor Assay.**

16 µg of HeLa protein extract and 1 µL of oligonucleotide probe were added to each reaction along with increasing amounts of competitor oligonucleotide (0 ng, 5 ng, 50 ng, and 100 ng). 12.5 µL (50%) of each reaction was loaded into a 4% acrylamide gel. Electrophoresis was carried out for 75 minutes at 200 V in 0.25X TBE, after which the gel was dried on DE-81 paper using a gel dryer at 80°C, for 15-20 minutes. The EMSA was then exposed to X-Ray film for 17 hours before the film was developed.

Lane 1 contains oligonucleotide probe alone and lanes 2, 6, 10, and 14 contain no competitor oligonucleotide. Arrows indicate specific interactions and all other bands observed are non-specific interactions.

## 3.3. Chapter Summary

Electrophoretic mobility shift assays showed that there are at least five different proteins that bind to the topoisomerase II beta promoter *in vitro*. Three of these proteins were identified, using antibody supershift assays, as the transcription factors Sp1, Sp3, and NF-Y. The identity of the other two proteins remains unknown. A complex comprising at least Sp1, Sp3, and NF-Y was also shown to form, suggesting some type of higher order structure between the DNA and multiple proteins at the proximal promoter.

Sp1 strongly interacted with the GC2 element, and also bound to the GC1 and ICB2 elements in isolation. NF-Y was found to bind to three elements in isolation: GC1, ICB1 and ICB2. Normally Sp1 does not interact with ICB elements and NF-Y does not bind GC elements. These results would suggest that these two transcription factors strongly interact with each other, and that they are capable of recruiting the other in the absence of its preferred binding element, as suggested previously (Zhong *et al.*, 2000). More evidence for this occurring is provided by the wtGC1/wtICB2 protein/oligonucleotide probe complexes being competed for with the wtGC1/mtICB2 competitor oligonucleotide, where the amount of NF-Y binding does not decrease, and competition for Sp1 is minimal. Sp3 bound to both GC elements but did not appear to be recruited to the ICB elements by NF-Y. It was also found to bind to the double GC1/ICB2 element, and as part of the complex that assembled.

It is well documented that both Sp1 and Sp3 can interact with NF-Y, and this has been recently shown for the topoisomerase II alpha promoter (Magan *et al.*, 2003). The interaction with the topoisomerase II alpha promoter also occurs at a composite element where a GC1 rich element is adjacent to an inverted CCAAT box. More importantly, the interaction at the topoisomerase II alpha promoter also involves a fourth uncharacterised protein. It is possible that the same protein also associates with the topoisomerase II beta promoter at the adjacent GC1/ICB2 elements. Sp1 is known to be an activator of topoisomerase II alpha expression (Magan *et al.*, 2003), and a similar role for Sp1 in topoisomerase II beta expression was investigated in a series of experiments described in Chapter 4.

The functional significance of these DNA/protein interactions was analysed with transient transfection assays using a series of topoisomerase II beta promoter-reporter gene constructs carrying mutations in each of the elements.

# 4. Cloning of the Topoisomerase II Beta Promoter

## 4.1. Introduction

The topoisomerase II beta upstream regulatory region was partially characterised by Ng *et al.* (1997). These authors cloned over 3 kb of the 5' flanking region of the human topoisomerase II beta gene and analysed the DNA sequence. It was reported that the region from –1000 to –500 was critical for the transcription of the topoisomerase II beta gene, as when it was deleted there was approximately a 60% decrease in promoter activity. This suggested that there are important regulatory elements in this region.

Further characterisation of the topoisomerase II beta promoter was carried out by Lok *et al.* (2002). These authors characterised 1.3 kb of upstream flanking sequence and the Ng *et al.* (1997) findings were confirmed. Sequence analysis identified one GC-rich element at –533 and two inverted CCAAT boxes (ICBs), ICB1 and ICB2, at –490 and –522 respectively. *In vitro* binding studies indicated Sp1 bound the GC-rich element and NF-Y bound the ICB elements. Functional studies were then carried out by introducing mutations into the different elements, in the context of the -1067 promoter construct, and using them in transient transfections. Mutations introduced into each of the individual elements had little effect on the relative promoter activity, however, when any combinations of mutations were made there was a large decrease in promoter activity. This suggests that there is some functional redundancy between the elements in the topoisomerase II beta promoter.

A study concurrent with that of Lok *et al.* (2002) also cloned and characterised the topoisomerase II beta promoter (Willingham, 2004). Deletion studies indicated that each of the four elements (GC2 as well) could have a functional significance. Mutations were introduced independently into the two ICB elements, in the context of the –654 bp promoter construct, and used for functional analysis in transient transfection assays. A mutation in the ICB1 element resulted in an increase in the topoisomerase II beta promoter activity, suggesting ICB1 is a negative element, and a mutation in ICB2 caused a decrease in activity, which suggests it is a positive element.

In the current study combinations of mutations were introduced collectively into the ICB1, ICB2, and GC1 elements to both confirm the previous findings by Willingham (2004), and to further investigate possible functional interactions between these elements.

## 4.2. PCR Mutagenesis

PCR mutagenesis is often used to introduce either specific or random mutations into DNA. Specific point mutations can be introduced by designing primers incorporating the required mutation.

PCR mutagenesis (Ho *et al.*, 1989) was used to create mutations in each of the three binding elements in the -654 promoter construct, ICB1, ICB2 and GC1. The introduction of each mutation required two rounds of PCR. For each element the two first round products were generated in separate reactions using the pGL3Basic-654 vector, which has 654 bp of the topoisomerase II beta 5' regulatory region, as the template. The RV3 primer and the reverse mutant primer were used in one reaction, and the GL2 primer and the forward mutant primer were used in the other reaction (see appendix 1 for primer sequences). The second round of PCR utilized the two first round products as the template and the two flanking primers, RV3 and GL2, to produce the final product (Figure 4.1).

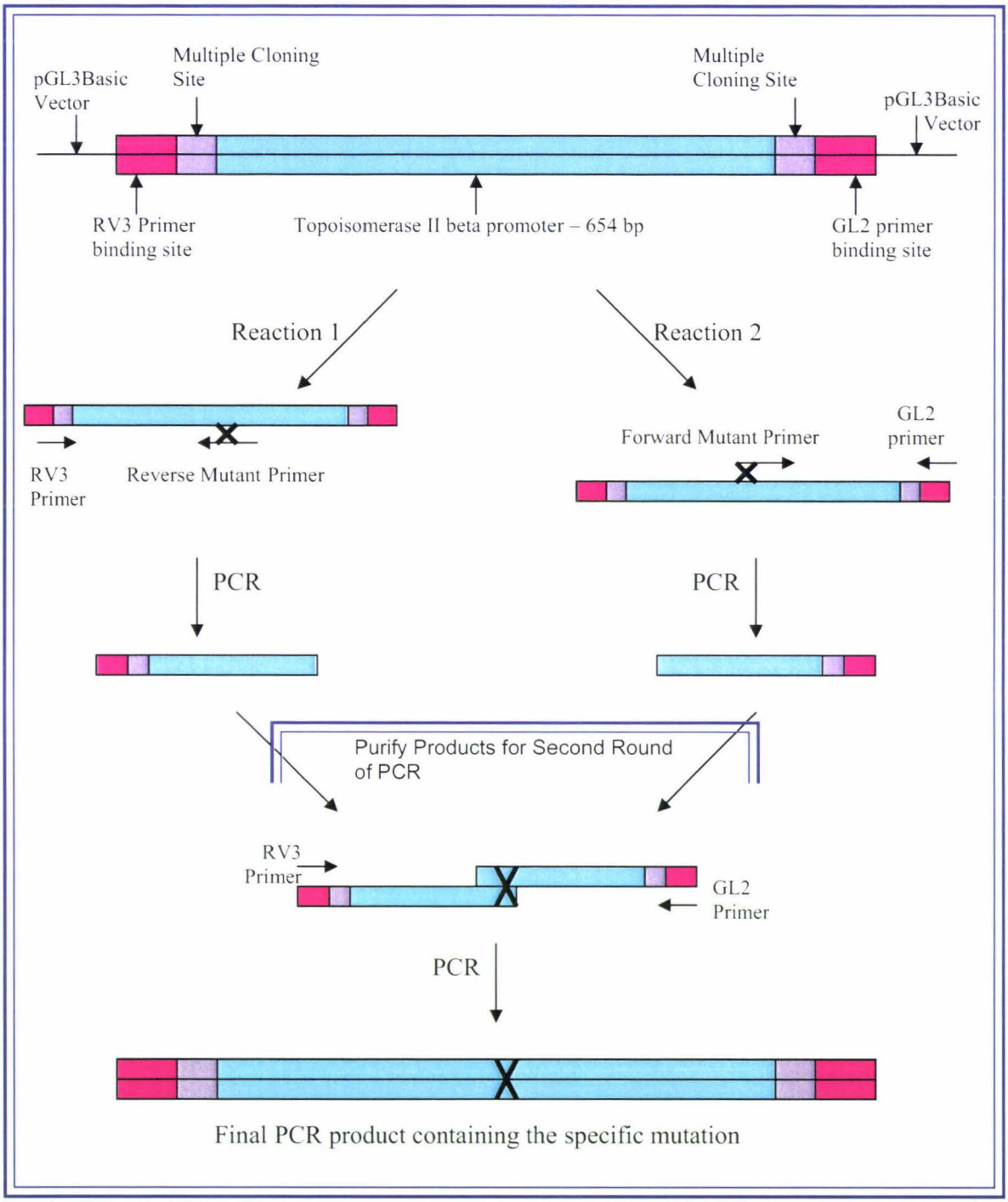



**Figure 4.1: Schematic Representation of PCR Mutagenesis Procedure.**

The pGL3Basic-654 topoisomerase II beta promoter construct was used as a template for the two first round reactions in conjunction with a flanking and mutant primer. The products generated from the first round were used as the template DNA for the second round of PCR, along with the flanking primers to give the final product.

### 4.2.1. First Round PCR Mutagenesis

The first round of PCR was carried out with the reaction mixes shown in Table 4.1. The 10X reaction buffer used in the PCR reaction contained $MgCl_2$; therefore the addition of $Mg^{2+}$ to the reaction was unnecessary. DMSO was added to each reaction to prevent the formation of secondary structures in the DNA, which would inhibit the amplification of the DNA and *Taq* polymerase was chosen to synthesise the new DNA.

The primers, which contained the mutated elements, were designed so new restriction sites would be formed (Willingham, 2004). The ICB1 mutant primers introduced a new *Xba* I site and the ICB2 mutant primers introduced a new *Bgl* II site. These two restriction sites appear once in the pGL3Basic vector, therefore introducing new restriction sites when the elements are mutated provides a simple diagnostic test in the form of a restriction endonuclease digest using these two enzymes.

A negative control was included to show that any products observed were not due to contamination of the reaction components.

| Reaction | | Negative Control | Reaction 1 | Reaction 2 |
|---|---|---|---|---|
| 10X PCR Buffer with $MgCl_2$ | | 5 µL | 5 µL | 5 µL |
| dNTPs (3 mM) | | 5 µL | 5 µL | 5 µL |
| DMSO | | 2 µL | 2 µL | 2 µL |
| Primers | RV3 (50 ng/µL) | 5 µL | 5 µL | - |
| | GL2 (50 ng/µL) | - | - | 5 µL |
| | Forward mutant (50 ng/µL) | - | - | 5µL |
| | Reverse mutant (50 ng/µL) | 5 µL | 5 µL | - |
| DNA (50 ng/µL) | | 0 µL | 2 µL | 2 µL |
| *Taq* Polymerase (5 U/µL) | | 0.5 µL | 0.5 µL | 0.5 µL |
| $sH_2O$ | | 26 µL | 24 µL | 24 µL |
| TOTAL | | 50 µL | 50 µL | 50 µL |

**Table 4.1: Standard First Round PCR Mutagenesis Reactions.**

Reaction 1 utilizes the RV3 primer, which has a binding site in the pGL3Basic vector, and the reverse mutant primer, which spans the element to be mutated. Reaction 2 uses the GL2 primer, which binds to the vector, and the forward mutant primer, which spans the element to be mutated. *Taq* polymerase was used to carry out the synthesis of the new DNA. The negative control was included to ensure the reagents used had no contaminating DNA that could be amplified.

PCR was carried out under the previously optimised conditions shown in Table 4.2 (Willingham, 2004). An initial denaturation step was required to ensure all the plasmid DNA was single-stranded and accessible to the primers. This was followed by 45 cycles of denaturation, primer annealing, and elongation, before a final elongation step was carried out to make certain all synthesis had been completed. At the end of the run the samples were cooled and incubated at 4°C. PCR was carried out on a GeneAmp® PCR System 2700 from Applied Biosystems (USA).

| | Temperature | Time | Cycles |
|---|---|---|---|
| | 95°C | 5 minutes | 1 cycle |
| Denaturation | 95°C | 30 seconds | 45 cycles |
| Annealing | 30°C | 30 seconds | |
| Elongation | 72°C | 2 minutes 30 seconds | |
| | 72°C | 7 minutes | 1 cycle |

**Table 4.2: PCR Conditions.**

PCR was performed using the GeneAmp® PCR System 2700, Applied Biosystems (USA). 45 cycles of denaturation, annealing and elongation were carried out and the final products were loaded onto an agarose gel to visualise the results.

Product sizes were dependent on the element being mutated and the expected sizes of the products are outlined in Table 4.3. The final products were loaded onto an agarose gel to visualise the results (Figure 4.2).

| Mutated Element | Expected Product Size | | |
|---|---|---|---|
| | Reaction 1 | Reaction 2 | Reaction 3 |
| ICB1 | 201 bp | 606 bp | 780 bp |
| ICB2 | 168 bp | 639 bp | 780 bp |
| GC1 | 137 bp | 370 bp | 780 bp |

**Table 4.3: Expected Products for First and Second Round of PCR Mutagenesis.**

Reaction 1 is the product generated using the reverse mutant primer and the flanking RV3 primer, reaction 2 the forward mutant primer and the flanking GL2 primer, and reaction 3 is the final product generated in the second round of PCR mutagenesis.

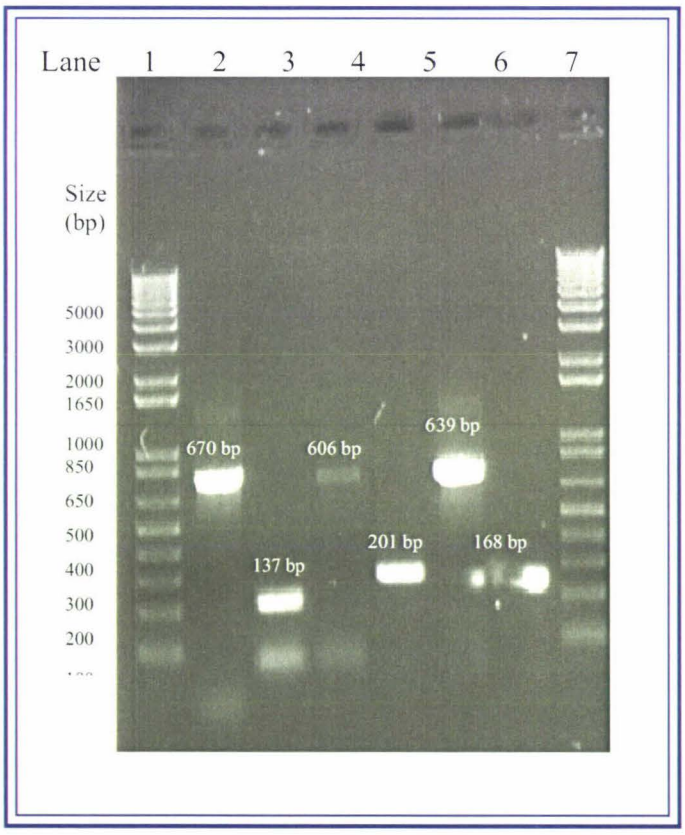



**Figure 4.2: Agarose Gel of the First Round PCR Mutagenesis Products.**

5 µL of each PCR reaction was loaded into a 1% agarose gel, containing 0.4 µg/mL ethidium bromide, in 1X TAE. The gel was electrophoresed at 100 V for 1 hour. Labelled products were gel purified. Lane 1 contains a 1 kb ladder, included to allow estimates of product size to be made.

Lane 2: GC1m - Reaction 2

Lane 3: GC1m - Reaction 1

Lane 4: ICB1m - Reaction 2

Lane 5: ICB1m - Reaction 1

Lane 6: ICB2m - Reaction 2

Lane 7: ICB2m - Reaction 1

The PCR products were gel purified using the Freeze-Squeeze method (Thuring *et al.*, 1975) as outlined in section 2.2.4. This process removed all primers, smaller and larger pieces of DNA and enzyme. The products were quantified (section 2.2.11) and then used in the second round of PCR.

### 4.2.2. Second Round PCR Mutagenesis

The products from the first rounds of PCR were used as templates for the second round of PCR. The end of the two products, containing the mutated binding element, are complementary and stick to each other during the annealing step of PCR (Figure 4.1). The flanking primers RV3 and GL2 were used and *Taq* polymerase synthesised the new DNA. DMSO was also added to this reaction and a negative control included. Table 4.3 shows the general second round PCR reaction.

| Reaction | Negative Control | Reaction 3 |
|---|---|---|
| 10X PCR Buffer with $MgCl_2$ | 5 µL | 5 µL |
| dNTPs (3 mM) | 5 µL | 5 µL |
| DMSO | 2 µL | 2 µL |
| RV3 Primer (50 ng/µL) | 5 µL | 5 µL |
| GL2 Primer (50 ng/µL) | 5 µL | 5 µL |
| DNA – Reaction 1 (100 ng/µL) | - | 1 µL |
| DNA – Reaction 2 (100 ng/µL) | - | 1 µL |
| *Taq* Polymerase (5 U/µL) | 0.5 µL | 0.5 µL |
| $sH_2O$ | 26 µL | 24 µL |
| TOTAL | 50 µL | 50 µL |

**Table 4.4: Standard Second Round PCR Mutagenesis Reactions.**

The second round of PCR mutagenesis used the two first round products (from reactions 1 and 2 in Table 4.1) as the template DNA. The two products anneal through the complementary sequence where either mutant primer bound to the original template DNA. The two flanking primers, RV3 and GL2, were used along with *Taq* polymerase. The negative control was included to ensure the reagents used had no contaminating DNA that could be amplified.

The PCR conditions used were the same as those used for the first round and are outlined in Table 4.2. Figure 4.3 shows the final product, which migrated at a rate consistent with a 780 bp product, as expected.

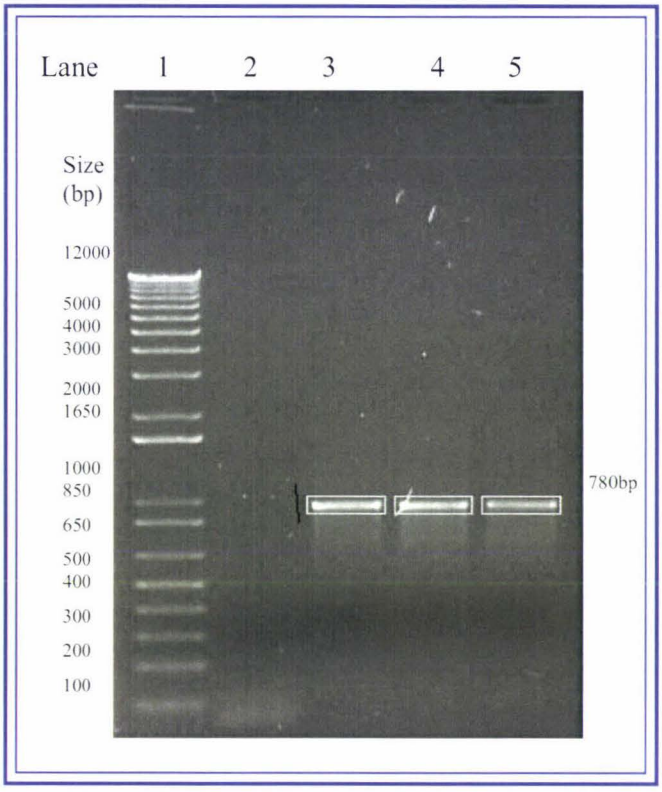



**Figure 4.3: Agarose Gel of the Second Round PCR Mutagenesis Products.**

5 µL of each PCR reaction was loaded into a 1% agarose gel, containing 0.4 µg/mL ethidium bromide, in 1X TAE. The gel was electrophoresed at 100 V for 1 hour. Products in the boxes were gel purified. Lane 1 contains a 1 kb ladder, included to allow estimates of product size to be made.

Lane 2: Negative Control

Lane 3: GC1 Mutant

Lane 4: ICB1 Mutant

Lane 5: ICB2 Mutant

### 4.2.3. Digestion and Removal of 5' Phosphate Group

The PCR product was then digested so that it could be ligated into the pGL3Basic vector for functional analysis. The flanking primers, RV3 and GL2, bind either side of the multiple cloning site (MCS) in the pGL3Basic vector. The restriction sites chosen to digest the PCR product, *Kpn* I and *Hin*d III, only cut once in the MCS. The same restriction sites were used to digest an empty pGL3Basic vector so that ligation of the PCR product into the vector was possible (Figure 4.4).

Phosphate groups were removed from the cut vector, to prevent religation, as described in section 2.2.3. Thermosensitive alkaline phosphatase was used to catalyse the reactions, as it could be inactivated by heating for a short period of time at 65°C.

The DNA was again run on a gel and purified using the freeze-squeeze method (Thuring *et al.*, 1975). This removed all enzymes and contaminating shorter pieces of DNA

generated from the digestion and phosphatase reactions. The purified vector and PCR product (insert) were then ready to be used in ligations.

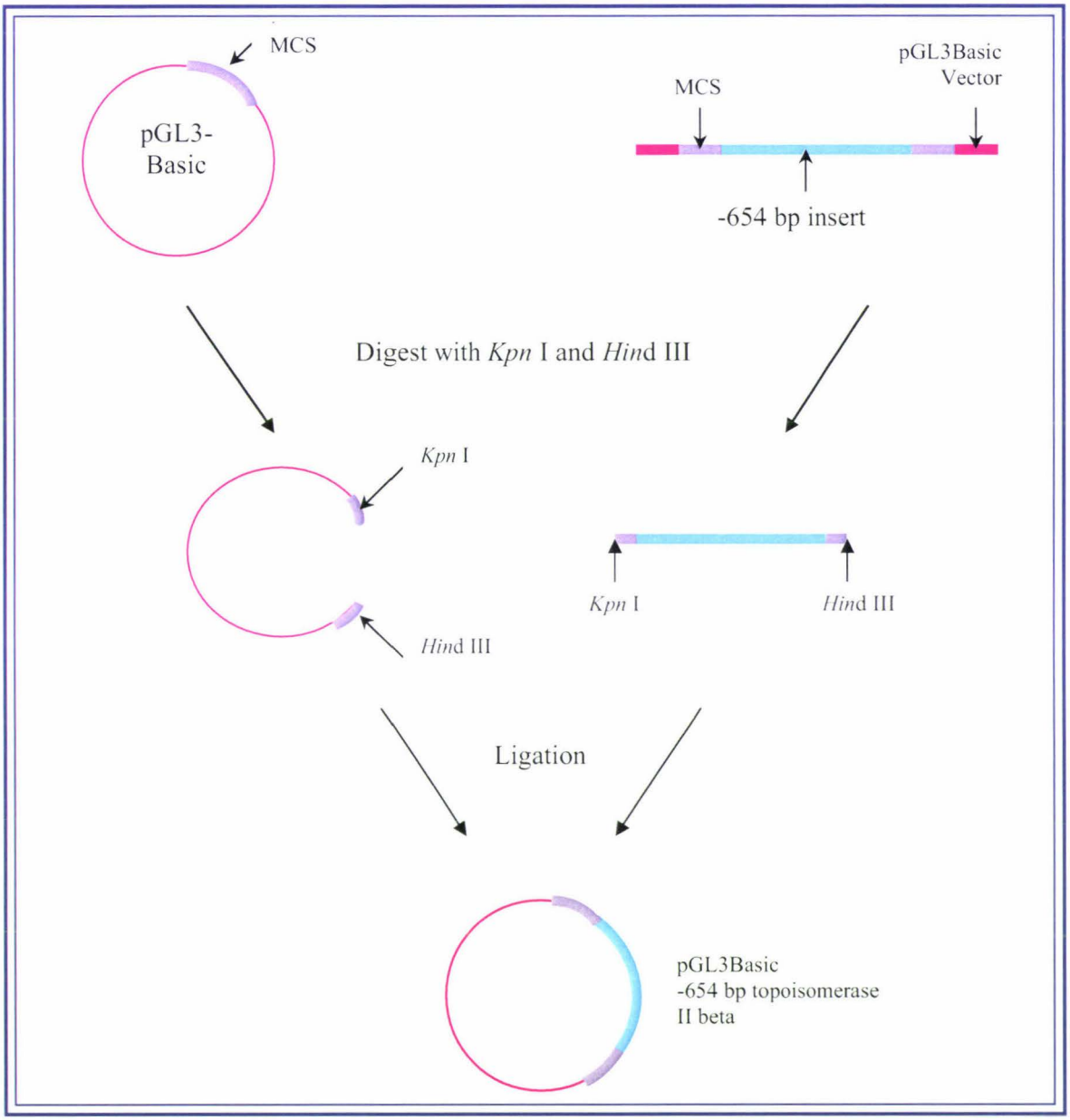



**Figure 4.4: Schematic Representation of Cloning Strategy.**

An empty pGL3Basic vector and the final PCR products were digested with *Kpn* I and *Hind* III, the vector was then phosphatased and both digested products were purified before being used in the ligation reaction.

## 4.2.4. Ligations

Ligations were set up as stated in section 2.2.8 and a standard ligation reaction is shown in Table 4.5. Ligations were incubated at room temperature for 3 hours and then overnight at 4°C.

| Reaction | Control 1 (no insert) | Control 2 (no ligase) | Control 3 (Vector cut | Insert + Vector |
|---|---|---|---|---|
| Vector (50 ng/μL) | 0.5 μL | 0.5 μL | 0.5 μL | 0.5 μL |
| Insert (50 ng/μL) | - | - | - | 2 μL |
| 5X Ligase Buffer | 4 μL | 4 μL | 4 μL | 4 μL |
| T4 DNA Ligase (1 U/μL) | 1 μL | - | 1 μL | 1 μL |
| $sH_2O$ | 14.5 μL | 15.5 μL | 14.5 μL | 12.5 μL |
| TOTAL | 20 μL | 20 μL | 20 μL | 20 μL |

**Table 4.5: Standard Ligation Reactions.**

The final PCR product and an empty pGL3Basic vector were digested with *Kpn* I and *Hind* III and purified for use in ligation reactions. A 3:1 ratio of insert DNA to vector DNA was used to optimise the ligation reactions. Three control reactions were included to: 1) ensure there was no vector religation, 2) determine if there is any uncut vector present and 3) to ensure the ligase enzyme is active. Reactions were incubated for 3 hours at room temperature and then overnight at 4°C before being used for transformations.

## 4.2.5. Transformations

The ligation reactions were used to transform *E.coli* XL-1 Blue Competent Cells, which had been prepared as outlined in section 2.2.9.1.1 or 2.2.9.2.1. A heat shock method was used to transform the cells as described in section 2.2.9.1.2 or 2.2.9.2.2. The various controls included are outlined in Table 4.6 along with results representative of a standard transformation reaction.

| Reaction | Competent Cells | Ligation Mix | SOC | Volume Plated | Number of Colonies |
|---|---|---|---|---|---|
| XL-1 Blue Positive Control | 50 µL | - | 200 µL | LB plates 50 µL | TMTC* |
| XL-1 Blue Negative Control | 50 µL | - | 200 µL | LB Amp 50 µL | 0 |
| Control 1 (no insert) | 50 µL | 2 µL | 200 µL | 50 µL | 0 |
| Control 2 (no ligase) | 50 µL | 2 µL | 200 µL | 50 µL | 0 |
| Control 3 (vector cut once) | 50 µL | 2 µL | 200 µL | 50 µL | TMTC* |
| Transformation Control (uncut vector) | 50 µL | 2 µL | 200 µL | 50 µL | TMTC* |
| pGL3 Basic + Insert | 50 µL | 2 µL | 200 µL | 50 µL | 135 |

**Table 4.6: Standard Transformation Reactions.**

After being incubated overnight, at 4°C, the ligation reactions were used to transform *E.coli* XL-1 Blue competent cells. A heat shock method was used to facilitate plasmid DNA uptake and SOC was added as a rich nutrient source to enable the cells to recover. Each transformation reaction was plated onto LB-Ampicillin plates, unless stated otherwise, and incubated overnight at 37°C to allow single colonies to form. The number of singles colonies on each plate were counted and the table above is representative of the results obtained. Colonies, from the pGL3Basic + insert plates, were then randomly selected and cultured. The two XL-1 Blue controls ensured viability and that the ampicillin was effective, and the transformation control was included to determine if the transformation procedure was successful. All ligation controls were also used to transform XL-1 Blue competent cells. *Too many to count.

The XL-1 Blue positive control was to test the cells viability after undergoing heat shock treatment. These cells were plated on LB agar and as expected countless bacterial colonies were formed. The negative control was performed to ensure that the cells had no intrinsic ability to grow on agar containing the antibiotic ampicillin (amp). When plated on LB-amp agar no cells grew, showing that the ampicillin was effective in killing any bacteria that had not been transformed. This means that any colonies formed were likely to contain the pGL3Basic vector, which conveys ampicillin resistance.

The ligation control, which did not contain insert DNA, was included to show the background rate of vector religation. No colonies were observed when the cells were transformed using this mixture, which means there was either no, or a very small level of vector religation. The other ligation control had no ligase included in the reaction. This control was included to show that there was no undigested vector present. Together these two reactions indicated that it was highly likely that any colonies formed contained the insert DNA. The final ligation control, containing the vector cut once, was included to determine if the ligase enzyme was active. The formation of hundreds of colonies indicated that it was.

To ensure that the cells were capable of plasmid DNA uptake a transformation control was included. This reaction contained uncut pGL3Basic, and as expected there were a large number of colonies observed on the LB-amp plate. This showed the cells were highly competent.

The ligation with vector plus insert worked well and many bacterial colonies formed upon the transformation of XL-1 Blue competent cells. Inserting DNA into the pGL3Basic vector does not change the phenotype of the bacterial colonies therefore, several colonies were selected at random to be screened for the presence of insert DNA. The colonies selected were cultured in 5 mL LB broths overnight and the plasmid DNA isolated to screen for vectors that not only contained the insert DNA but also the correct mutation.

### 4.2.6. Screening for Mutants

The rapid boil method of plasmid purification (Holmes and Quigley, 1981) was used to isolate the plasmid DNA from the 5 mL bacterial cultures (Section 2.2.10.1) The extracted DNA was quantified, then used in diagnostic digests to ascertain if the plasmids contained the insert DNA. *Kpn* I and *Hin*d III, the same enzymes used to prepare the vector and insert for ligations, were used to digest the vector. If the vector contained the inserted DNA this digest would release the 654 bp insert from the 4.8 kb vector. The digests were run on an agarose gel to visualise the results (data not shown).

A second diagnostic digest was also performed using the *Xba* I and *Bgl* II restriction enzymes to determine if either the ICB2 or ICB1 elements had been mutated (Figure 4.5). If PCR mutagenesis had successfully mutated the ICB1 element then two fragments of DNA would be generated from the *Xba* I digest, one being 2.262 kb and the other 3.210 kb. If the ICB1 element was not mutated then the enzyme would only cut once in the vector giving a linear piece of DNA 5.472 kb in size. The *Bgl* II enzyme was used to determine if the ICB2 element had been mutated. If it was mutated a 0.639 kb fragment and a 4.833 kb fragment of DNA would be generated. If the ICB2 element had not been mutated then a 5.472 kb fragment would be observed. The appropriate fragments were observed (Figure 4.5) therefore PCR mutagenesis was successful. The GC1 element could not be checked in this way so DNA sequencing of the plasmids was also performed.

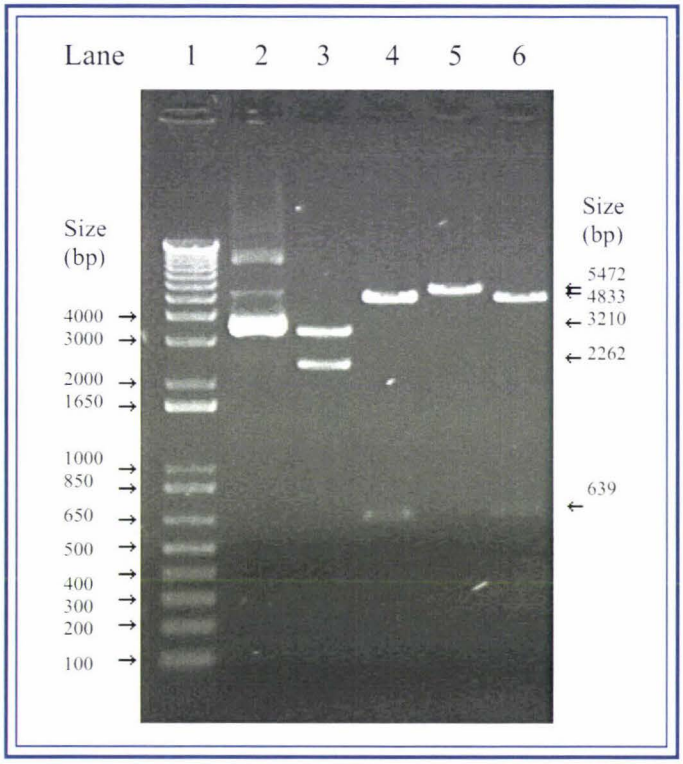



**Figure 4.5: Agarose Gel of a Diagnostic Digest.**

5 µL of each digest was loaded into a 1% agarose gel, containing 0.4 µg/mL ethidium bromide, in 1X TAE. The gel was electrophoresed at 100 V for 1 hour. Lane 1 contains the 1 kb ladder, included to allow estimates of product size to be made.

Lane 2: Uncut Plasmid

Lane 3: ICB1/ICB2 mutant *Xba* I digest

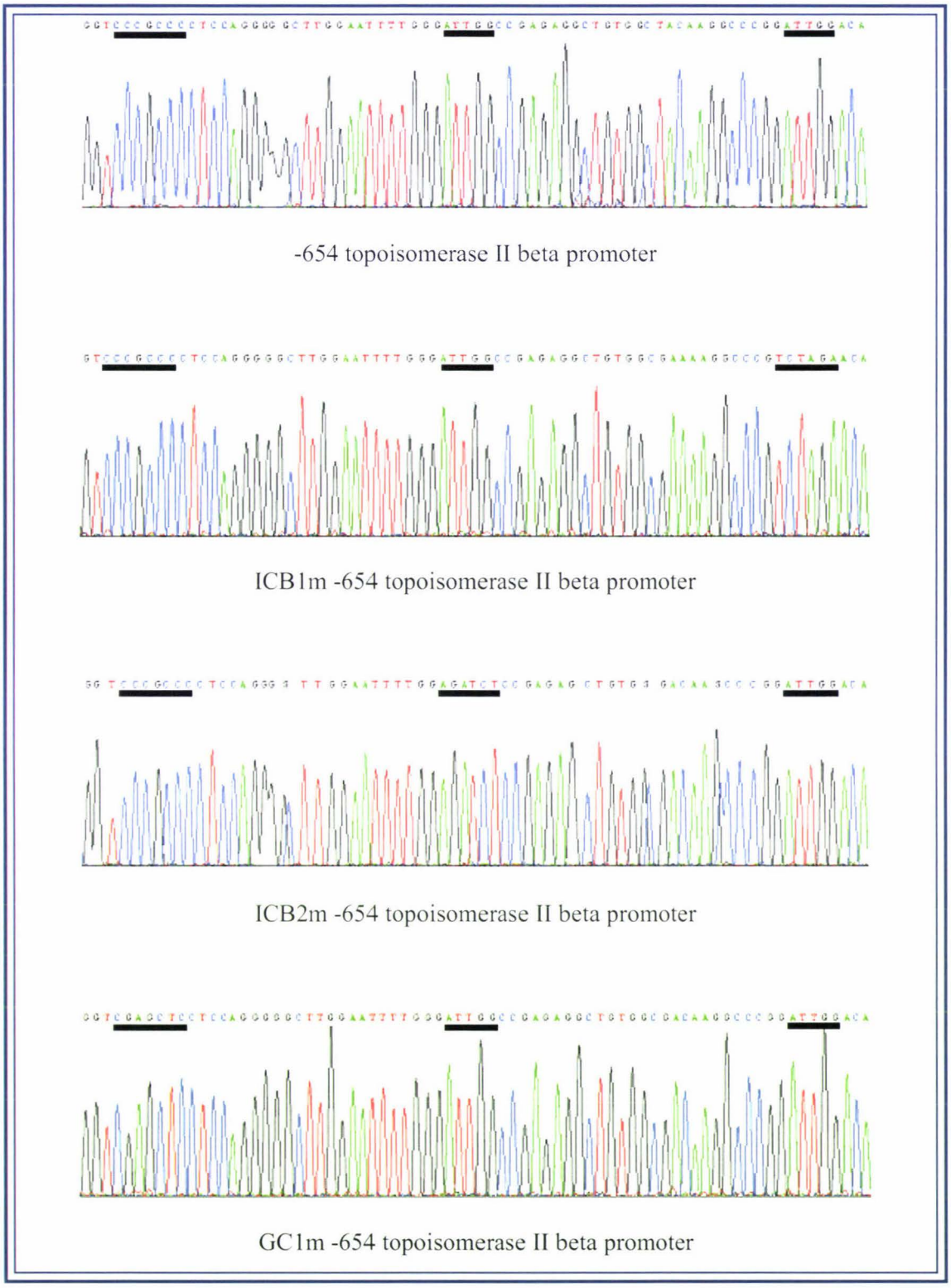
Lane 4: ICB1/ICB2 mutant *Bgl* II digest

Lane 5: ICB2 mutant *Xba* I digest

Lane 6: ICB2 mutant *Bgl* II digest

## 4.2.7. Sequencing

The cultures used to isolate the plasmid DNA were saved and used to inoculate another 5 mL broth. The broth was used for plasmid DNA extraction using a BioRad MiniPrep Kit. This method of plasmid purification produces the level of purity required for DNA sequencing. Sequencing of the insert was performed using the RV3 primer binding site located in the pGL3Basic vector. The Allan Wilson Centre Genome Service, Massey University, Palmerston North, carried out the sequencing reactions. Analysis of the results confirmed that the various constructs contained the correct mutations (Figure 4.6a, Figure 4.6b and Appendix 4), and the constructs were now able to be used for functional analysis.



-654 topoisomerase II beta promoter

ICB1m -654 topoisomerase II beta promoter

ICB2m -654 topoisomerase II beta promoter

GC1m -654 topoisomerase II beta promoter

**Figure 4.6a: Sequencing Results for Mutant Constructs.**

The sequencing reactions contained 400 ng of plasmid DNA, 5 pmol of primer, and 1 µL of DMSO (which reduces secondary structure formation). The Allan Wilson Centre Genome Service at Massey University, Palmerston North, performed the sequencing reactions.

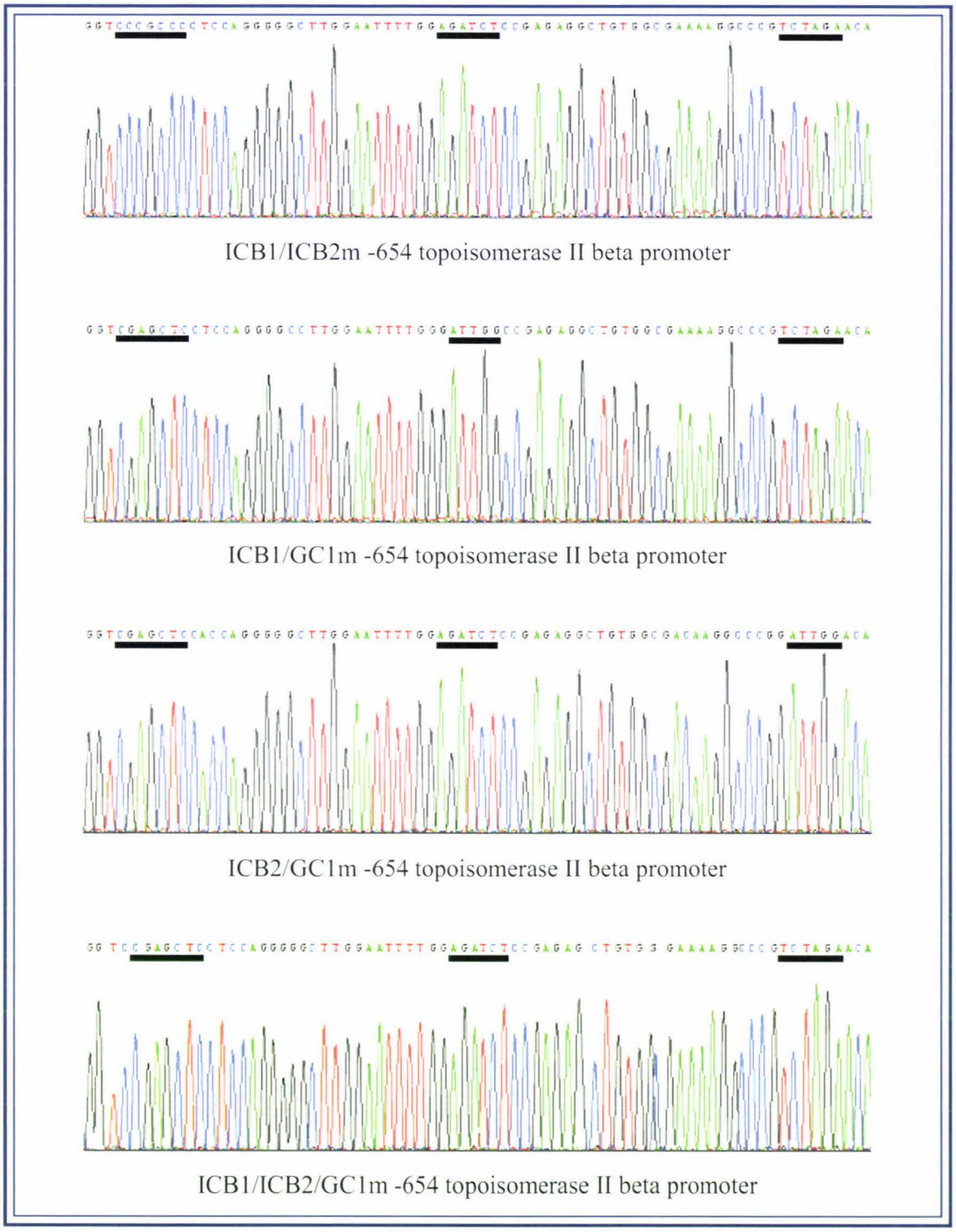



**Figure 4.6b: Sequencing Results for Mutant Constructs.**

The sequencing reactions contained 400 ng of plasmid DNA, 5 pmol of primer, and 1 µL of DMSO (which reduces secondary structure formation). The Allan Wilson Centre Genome Service at Massey University, Palmerston North, performed the sequencing reactions.

## 4.3. Summary

Mutations were successfully introduced into the pGL3Basic-654 promoter construct. Single element mutations were made using PCR mutagenesis giving pGL3-654 -mICB1, -mICB2 and –mGC1. Electrophoretic mobility shift assays showed that proteins bound to each of these individual elements *in vitro*. By mutating these elements and inserting them into the pGL3Basic vector it should be possible to determine if these interactions have any functional significance *in vivo*.

Electrophoretic mobility shift assays also showed that a complex of proteins formed when the double element, GC1/ICB2, probe was used. This suggested that there might be an interaction occurring between the two elements. To investigate this possibility the following mutations were made: pGL3-654 –mICB1/mICB2, -mICB1/mGC1, -mICB2/mGC1 and mICB1/mICB2/mGC1. To generate the double and triple element mutant constructs, the single element mutant constructs were used as the template for PCR mutagenesis with additional mutations then being introduced.

Transient transfection assays were then carried out to determine the effect of each element on the transcriptional activity of the topoisomerase II beta promoter.

# 5. Transient Transfections

## 5.1. Introduction

Transfection is a process that introduces nucleic acids into eukaryotic cells by non-viral methods. Transfection is a commonly used technique to study gene expression, gene function, and protein function.

The introduction of DNA into a cell can either be stable or transient. Stable transfection refers to cells which have not only taken up the DNA but that have incorporated this DNA into one or more of their chromosomes. The DNA is permanently integrated into the genome, and therefore the new DNA is reproduced and passed on to the daughter cells.

Transient transfection does not involve the integration of the DNA into the genome. Plasmid DNA is taken up by the cell where it is transiently expressed. This DNA can be lost at any stage depending on environmental factors and it is lost indefinitely at mitosis. The products of transiently expressed genes can be assayed to determine expression levels 24 – 72 hours after transfection.

There are four common techniques for introducing DNA into a mammalian cell. Diethylaminoethyl-dextran (DEAE-dextran) and calcium phosphate are two methods which both rely on chemical reagents for DNA uptake. DEAE-dextran is a positively charged polymer that forms complexes with the negatively charged DNA. These complexes have an overall positive charge, which allows them to be associated with the cell membrane, and it is thought that endocytosis is responsible for the uptake of the complexes. The calcium phosphate method involves the formation of a positively charged DNA precipitate, which is dispersed onto the cultured cells and uptake of the precipitate is through endocytosis or phagocytosis.

Electroporation is often used as a means to transfer DNA into cells resilient to other techniques, for example plant protoplasts. An electrical pulse is used to disrupt the cell

membrane. Pores are formed in the cell membrane and this allows the uptake of DNA into the cells. Pulse strength and duration is dependent on cell type and needs to be adjusted accordingly.

In 1980 artificial liposomes began to be used to facilitate the uptake of DNA. This new technique, liposome-mediated DNA delivery, offers many advantages over the previous two methods based on chemical reagents. It has a relatively high efficiency of gene transfer, it has the ability to transfect cell lines the previous chemical methods can not, and it can deliver a range of molecules, such as DNA of all sizes, RNA and proteins. Lipids with an overall positive charge are most commonly used, and the positively charged part interacts with the negatively charged DNA to form overall positively charged complexes capable of associating with the membrane of the cells to be transfected. The complex enters the cell through endocytosis and the DNA moves to the nucleus.

There are several different reporter genes available to study eukaryotic gene expression. An ideal reporter vector has no endogenous expression and reporter gene expression must be able to be assayed with rapidity, sensitivity, quantitation and simplicity. Chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) and luciferase represent a selection of reporter genes. In this study, firefly luciferase was used as a reporter to study transcription driven by the topoisomerase II beta promoter, and a β-galactosidase reporter vector was used as a transfection control.

### 5.1.1. Luciferase Assays

Firefly luciferase is a commonly used reporter gene to study gene expression because the assay has a high level of sensitivity, is rapid, and the protein product requires no post-translational modifications. The pGL3Basic vector contains the cDNA that encodes the *Photinus pyralis* (North American firefly) luciferase enzyme. The firefly luciferase enzyme catalyses the bioluminescent reaction of luciferin being oxidised to oxyluciferin (Figure 5.1). This reaction requires ATP as an energy source as well as $Mg^{2+}$ as a co-substrate.

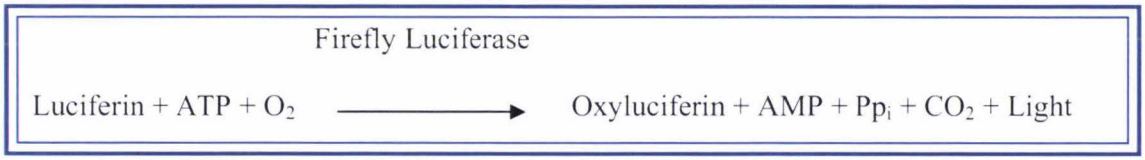



**Figure 5.1: Reaction Catalysed by Firefly Luciferase.**

The firefly luciferase enzyme catalyses the oxidation of beetle luciferin to oxyluciferin. During the reaction a flash of light is emitted, which is measured at 560 nm using a FLUOstar galaxy plate reader.

The FLUOstar galaxy was programmed to automatically inject luciferase reagent into a 96 well plate containing the cell extracts, and take a reading every second over a 3 minute time period commencing immediately after luciferase reagent was added. A flash of light (photons) is generated, followed by a rapid decay that gives a long period of low light intensity, and it is for this reason that the maximum reading is taken and used for all calculations. The photons are detected by the reading head and relayed to Microsoft Excel (Microsoft Office, 97), for use in further calculations.

The amount of light emitted and subsequently detected is directly proportional to the amount of luciferase enzyme present, which reflects the strength of the promoter used to drive the expression of the reporter gene. Therefore, the amount of light measured changes to reflect the effect of any mutations introduced into the promoter, or the use of a co-expression vector whose gene product may modulate transcription.

### 5.1.2. β-Galactosidase Assays

β-galactosidase is a useful internal control for normalizing variability in reporter gene activity due to transfection efficiency or cell extract preparation (Fowler and Zabin, 1983). The β-galactosidase enzyme, encoded by the *lacZ* gene of *E.coli*, can use o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. ONPG, which is colourless, is cleaved by β-galactosidase to produce galactose and o-nitrophenol, which is bright yellow in colour (Figure 5.2).

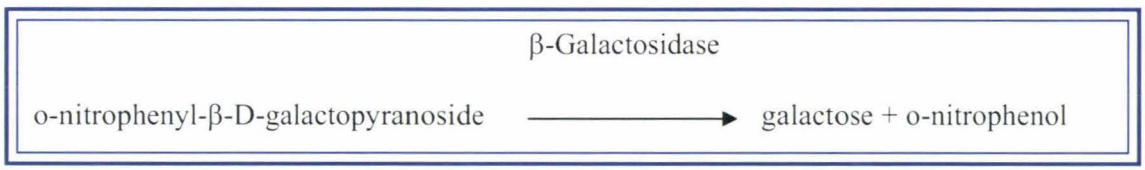



**Figure 5.2: Reaction Catalysed by β-Galactosidase.**

β-galactosidase catalyses the cleavage of ONPG to form galactose and o-nitrophenol. A colour change, from colourless to yellow, is observed which is assayed at 420 nm using a microplate reader.

If ONPG is in excess of the β-galactosidase enzyme then the rate of the reaction is dependent on the concentration of the enzyme. Therefore, the more cells that take up the β-galactosidase plasmid, the more enzyme will be produced which means the colour change will proceed faster. The same amount of β-galactosidase plasmid was added to each reaction therefore, any difference observed in colour intensity reflects the difference in plasmid DNA uptake by the cells in each reaction. The reaction can be stopped by adding 1 M $Na_2CO_3$, following which the amount of o-nitrophenol can be easily assayed at 420 nm using a spectrophotometer or a microplate reader.

A range of promoters can be used to drive the expression of the β-galactosidase enzyme, and in this case the cytomegalovirus (CMV) promoter was used. The CMV promoter is a strong constitutively expressed promoter and was used because its promoter has no binding sites for either Sp1 or Sp3, potential modulators of topoisomerase II beta transcription.

### 5.1.3. Analysis of Results

Due to the sensitive nature of the luciferase assay and the unavoidable small amount of error within each experiment, each reaction was carried out in triplicate, and to ensure the validity of the results each experiment was carried with at least three replicates.

To correct for any background luciferase or β-galactosidase activity a blank assay was carried out for each, containing cell lysis buffer only. The value recorded for each blank was subtracted from the respective value. The corrected luciferase values were then divided by their respective corrected β-galactosidase values to adjust for any difference in plasmid uptake, and the average values were calculated (Table 5.1).

To allow for comparisons to be made between data sets, all transient transfections were adjusted relative to the wild type –1357 topoisomerase II beta promoter construct, which was arbitrarily assigned a value of 100%. A sample with a higher value indicates a relative increase in transcription, and a sample with a lower percentage indicates a relative decrease in transcription. The average corrected luciferase value for the wild type –1357 topoisomerase II beta promoter construct was divided by the average corrected luciferase value for each sample, and multiplied by 100 to generate relative luciferase activity.

| Construct | Luciferase Maximum | Luciferase - Blank | B-gal Values | B-gal - Blank | Corrected Luciferase | Average | Relative to -1357 |
|---|---|---|---|---|---|---|---|
| Blank | 246 | - | 0.044 | - | - | - | - |
| | 316 | - | 0.047 | - | - | | |
| | 292 | - | 0.046 | - | - | | |
| -1357 0 μg Spl | 18017 | 17732 | 0.38 | 0.334 | 53037 | 51729 | 100% |
| | 18034 | 17749 | 0.391 | 0.345 | 51397 | | |
| | 18167 | 17882 | 0.398 | 0.352 | 50754 | | |
| -1357 0.25 μg Spl | 22519 | 22234 | 0.301 | 0.255 | 87079 | 77913 | 150% |
| | 25867 | 25582 | 0.37 | 0.324 | 78876 | | |
| | 24099 | 23814 | 0.397 | 0.351 | 67782 | | |

**Table 5.1: Transient Transfection Calculations.**

Representative data set outlining the calculations involved in analysing the luciferase assay and β-galactosidase assay data. Microsoft Excel (Microsoft Office, 97) was used to perform the calculations and graph the data obtained.

A small amount of error within the triplicates of one experiment and within a data set is unavoidable, and for this reason the standard deviation was calculated (Figure 5.3). The standard deviation is a statistical measurement that indicates how closely the samples are grouped around the mean in a set of data. A small standard deviation indicates low variation within a data set, and a large standard deviation indicates a high amount of variation within a data set.

$$\frac{1}{n} \sum X - \bar{x}$$

Where n = the number of observations
X = the individual observation
x = the average

**Figure 5.3: Standard Deviation Formula.**

The standard deviation gives an indication of how much variability there is in a data set. Microsoft Excel (Microsoft Office, 97) was used to perform the calculation to establish the amount of variation between the triplicates of one experiment, and between the three replicates of an experiment.

A sample standard deviation calculation is shown below:

Standard Deviation = ((53037 – 51729) + (51397 – 51729) + (50754 – 51729)) x $\frac{1}{3}$

= 871.67 Luciferase Units

Percentage Error = (Average Standard Deviation/Average) x 100

= 1.69% error within the triplicates.

The student t-test can be used to determine whether two groups of data are statistically different from each other (Figure 5.4). In this case it was used to determine whether the relative luciferase activity was significantly different after the addition of Sp1, Sp3, or the introduction of mutations into the topoisomerase II beta promoter in comparison to the wild type promoter construct with no co-expression vector. The student t-test assumes that there are two independent, simple, random samples from two distinct populations, and that both populations are normally distributed with unknown means and standard deviations.

Estimate of pooled variance ($S^2$):

$$S^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

Where $n$ = number of data points in a population

$S^2$ = variance of a population

$$t = \frac{\text{Average}_1 - \text{Average}_2}{\sqrt{S^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

**Figure 5.4: Two Sample t-test Formula.**

The t-test is used to determine if two samples are statistically different from each other. The t-test can be used to generate a p-value that determines the levels of significance for any difference observed.

The p-value represents the probability that the sample could have been drawn from the population being tested given the assumption that the null hypothesis is true. A p-value of 0.05 is the typical threshold and any value ≤ to this shows the difference observed is statistically significant (Table 5.2). This value represents a less than a 5% chance of both data sets sharing the same mean.

| p-value | Evidence for significant difference between two samples | Significant |
|---|---|---|
| $p > 0.10$ | No Evidence | No |
| $0.05 < p \leq 0.10$ | Slight Evidence | No |
| $0.01 < p \leq 0.05$ | Moderate Evidence | Yes * |
| $0.001 < p \leq 0.01$ | Strong Evidence | Yes ** |
| $p \leq 0.001$ | Very Strong Evidence | Yes *** |

**Table 5.2: p-values and Their Significance.**

The p-values obtained from the student t-test were analysed for their significance. A p-value of 0.001 provides very strong evidence that there is a statistically significant difference between the two samples being tested.

## 5.2. Plasmid Preparation

Large-scale preparations of each plasmid were prepared before use in transient transfections. pGL3Basic, pCMVSPORTβ-galactosidase, Specificity protein 1 (Sp1), Specificity protein 3 (Sp3), and the various topoisomerase II beta promoter constructs in pGL3Basic were all streaked, from glycerol stocks, onto LB-Amp plates and incubated overnight at 37°C. Colonies were then randomly selected and cultured overnight, at 37°C, in 5 mL LB broths containing ampicillin, in a shaking incubator. Rapid boil plasmid preparations (Holmes and Quigley, 1981) were carried out on the 5 mL broths, and to confirm the identity of the plasmids restriction endonuclease digests were performed as outlined in section 2.2.2. The enzymes used and the expected sizes of the products are shown in Table 5.3.

| | pGL3Basic | pCMVSPORT-β-galactosidase | Sp1 | Sp3 | Reporter Construct |
|---|---|---|---|---|---|
| Buffer | 3 µL | 3 µL | 3 µL | 3 µL | 3 µL |
| DNA (200 ng/µL) | 3 µL | 3 µL | 3 µL | 3 µL | 3 µL |
| $sH_2O$ | 23 µL | 23 µL | 23 µL | 23.5 µL | 23 µL |
| *Bam* HI | 0.5 µL | - | - | - | - |
| *Eco* RI | - | 0.5 µL | 0.5 µL | - | - |
| *Hind* III | 0.5 µL | 0.5 µL | - | - | 0.5 µL |
| *Kpn* I | - | - | - | - | 0.5 µL |
| *Not* I | - | - | - | 0.5 µL | - |
| Expected Product sizes | 1951 kb<br>2867 kb | 3536 kb<br>4381 kb | 4 kb<br>5 kb | 5.4 kb<br>2.3 kb | Insert size +<br>4.8 kb |

**Table 5.3: Diagnostic Digest Reactions and the Expected Product Sizes.**

3 µL of each rapid boil preparation (600 ng of DNA) was digested at 37°C for 2 hours. 5 µL of each digest was loaded onto an agarose gel to visualise the results.

The digests were loaded onto a gel and electrophoresis was carried out (Figure 5.5). This shows that each plasmid produced the expected restriction fragments, thus confirming their identity. Once the identity of the plasmids was confirmed, a small amount of the previous 5 mL broth was used to inoculate a 200 mL LB broth containing ampicillin. The 200 mL broths were grown at 37°C overnight in a shaking incubator and 50 mL of the culture was used in a QIAGEN MaxiPrep. The MaxiPrep kit was used to isolate the plasmid DNA from the bacteria because it purifies large amounts of highly pure plasmid DNA suitable for transfection. The manufacturers protocol was followed for this procedure. The pellet was resuspended in 100 μL of TE and the DNA was quantified by UV spectrophotometry and agarose gel electrophoresis. The resuspended pellets were then used in transient transfections.

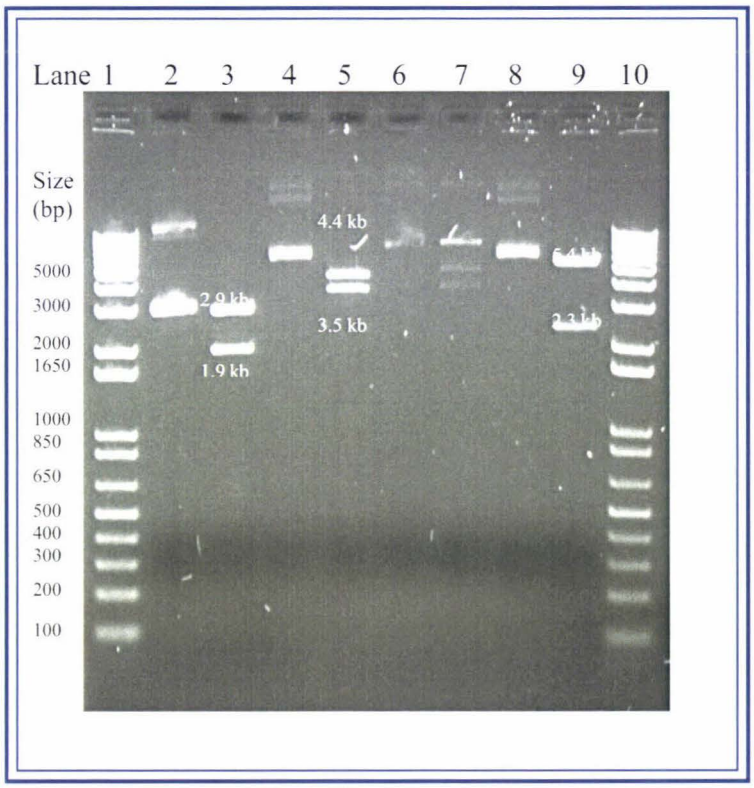



**Figure 5.5: Agarose Gel of the Diagnostic Digests.**

5 µL of each digest was loaded into a 1% agarose gel, containing 0.4 µg/mL ethidium bromide, in 1X TAE. The gel was electrophorsised at 100 V for 50 minutes. Lanes 1 and 10 contain the 1 kb ladder so that product size can be estimated.

Lane 2: pGL3Basic Undigested

Lane 3: pGL3Basic Digested with *Bam* HI and *Hin*d III

Lane 4: pCMVSPORTβ-galactosidase Undigested

Lane 5: pCMVSPORTβ-galactosidase Digested with *Eco* RI and *Hin*d III

Lane 6: Sp1 Undigested

Lane 7: Sp1 Digested with *Eco* RI

Lane 8: Sp3 Undigested

Lane 9: Sp3 Digested with *Not* I

## 5.3. Transient Transfection in HeLa Cells

HeLa cells were used for transient transfection assays. The cells were grown to 80% confluence and then passaged into 12 well plates to be 50–70% confluent the next day. This level of confluence is optimal for the transfection reagent, Fugene6™, to facilitate DNA uptake by the cells. Fugene6™ is a multi-component lipid based reagent, which forms complexes with the DNA and transports it across the cell membrane into the cell. Cells were transfected with 0.5 μg pGL3Basic reporter vector, 0.25 μg pCMVSPORTβ-galactosidase, varying amounts of co-expression vector and pBlueScript (pBS) to normalise plasmid quantity. Fugene6™, pre-mixed with serum free OptiMEM, was used at a Fugene6™ to DNA ratio of 3:2 and mixed with the DNA for 15 minutes before being added to the cells. The cells were then incubated for 24 hours, at 37°C, 5% $CO_2$, and in humid conditions, before they were harvested and cell extracts used in luciferase and β-galactosidase assays.

### 5.3.1. Sp1 Co-Expression Transfections

Transient co-expression transfections are often used to determine the effect of overexpressing a transcription factor on the level of transcription, and therefore provide evidence for its *in vivo* function in relation to a particular promoter (Figure 5.6).

Sp1 is a known transcriptional activator. Magan *et al.* (2003) investigated the effect of Sp1 on the topoisomerase II alpha promoter using transient transfection with the Sp1 co-expression plasmid, which overexpresses Sp1. A 6-fold increase in promoter activity was observed; this shows that Sp1 is a transcriptional activator of human topoisomerase II alpha. Because the topoisomerase II beta promoter has similar elements to those found in the topoisomerase II alpha promoter the effect of Sp1 on transcriptional regulation of topoisomerase II beta was studied. Sp1 has also been shown to bind to the topoisomerase II beta promoter *in vitro* (see Chapter 3).

In a preliminary study Willingham (2004) carried out transient transfection experiments with the Sp1 co-expression vector, and found that the addition of 0.5 μg of Sp1 had no significant effect on the –654 or the -1357 wild type topoisomerase II beta promoters.

Because both GC1 and GC2 were shown to bind Sp1 (Chapter 3), the effect of this transcription factor was studied further over a range of Sp1 levels. Both the –654 and the –1357 topoisomerase II beta promoter constructs were used for these experiments. This could determine if elements upstream of –654 were responsible for any effect observed upon addition of Sp1.

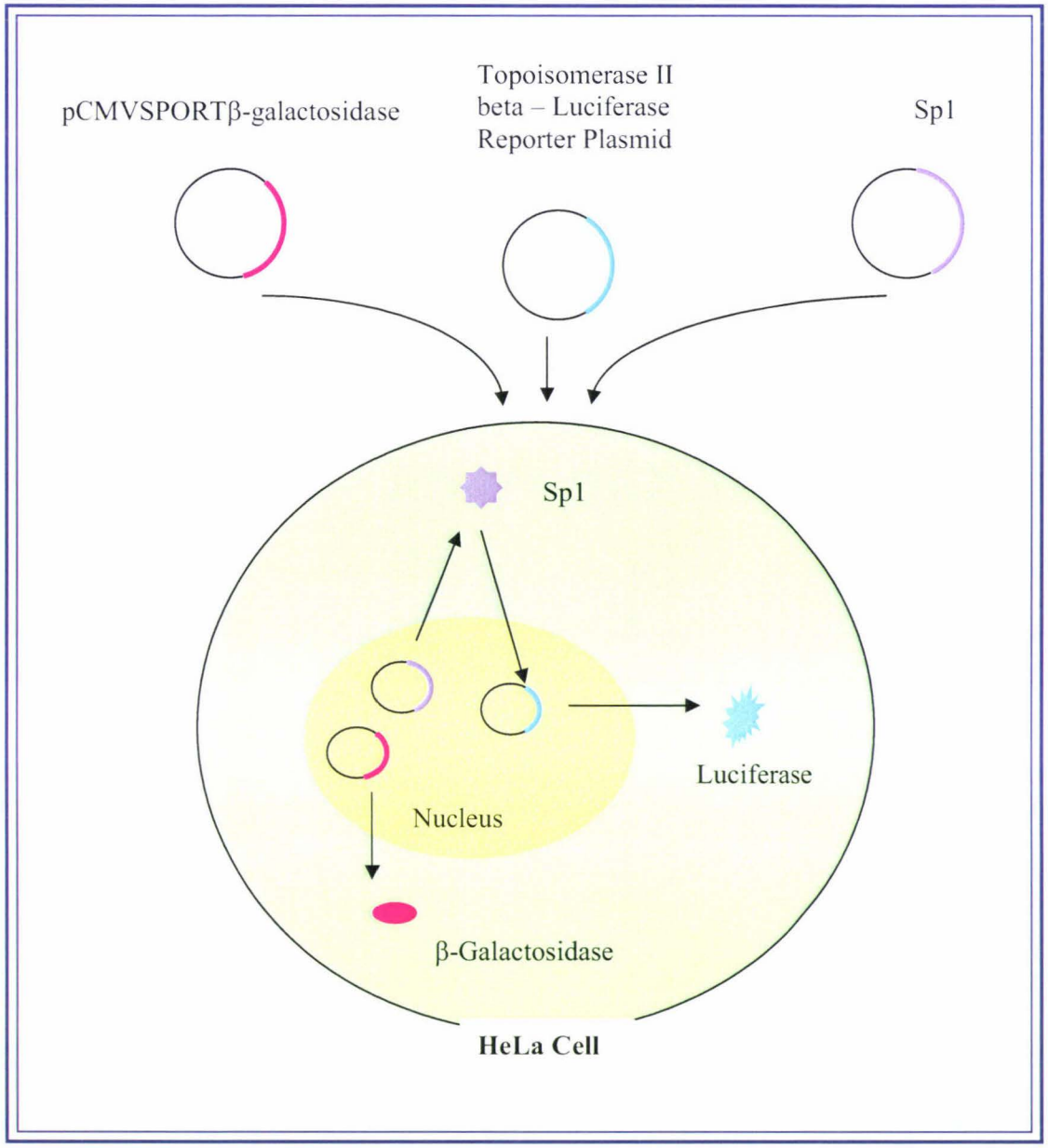



**Figure 5.6: Schematic Representation of a Transient Transfection using a Co-expression Vector.**

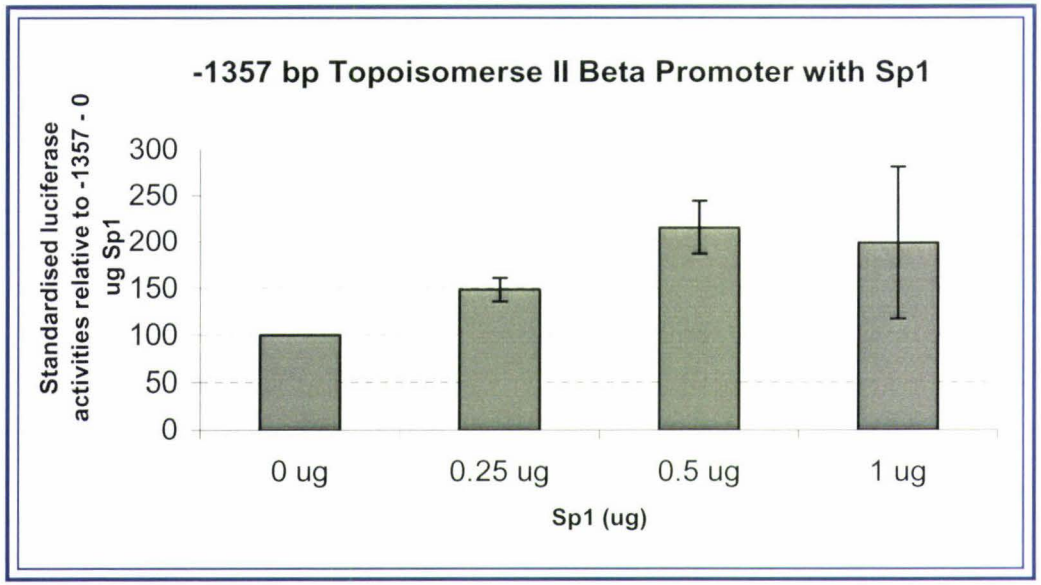
HeLa cells were grown in 12 well plates for 24 hours before being transfected. Cells were transfected with the topoisomerase II beta luciferase reporter plasmid and Sp1 as the co-expression vector, as well as the pCMVSPORTβ-galactosidase vector, which is an internal control for plasmid uptake. Cells were incubated for 24 hours at 37°C, 5% $CO_2$, and in humid conditions before being harvested. The cell extracts were used in β-galactosidase assays and luciferase assays.

Co-expression transfections were carried out using 0 μg, 0.25 μg, 0.5 μg and 1 μg of Sp1 expression vector, along with pCMVSPORTβ-galactosidase and the –1357 topoisomerase II beta luciferase reporter plasmid (Table 5.4). When 0.25 μg of Sp1 was added, luciferase activity increased to 150%, relative to 0 μg of Sp1 (Figure 5.7). A larger increase in the luciferase activity, around 200%, was seen when either 0.5 μg or 1 μg of Sp1 was added, relative to 0 μg of Sp1. These results confirm that Sp1 is an activator of topoisomerase II beta transcription, and under these experimental conditions Sp1 is saturating with the addition of 0.5 μg of expression vector. When 0.25 μg or 0.5 μg of Sp1 is added, the difference in relative luciferase activity observed (compared to 0 μg of Sp1 added) is statistically significant, with a p-value of 0.04 and 0.03 respectively, giving moderate evidence the difference observed is real. The variability of results with the transient transfection experiments using 1 μg Sp1 is too high to establish significance.

| | pGL3 Basic | pCMVSPORTβ-galactosidase | 1357 bp Construct | Sp1 | pBS |
|---|---|---|---|---|---|
| pGL3Basic Control | 0.5 μg | - | - | - | 1.25 μg |
| pCMV Control | - | 0.25 μg | - | - | 1.5 μg |
| Sample 1 | - | 0.25 μg | 0.5 μg | - | 1.0 μg |
| Sample 2 | - | 0.25 μg | 0.5 μg | 0.25 μg | 0.75 μg |
| Sample 3 | - | 0.25 μg | 0.5 μg | 0.5 μg | 0.5 μg |
| Sample 4 | - | 0.25 μg | 0.5 μg | 1.0 μg | - |

**Table 5.4: Quantities of Plasmid DNA used for Sp1 Co-expression Transient Transfections with the –1357 bp Topoisomerase II Beta Promoter Construct.**

Cells were transfected with a luciferase reporter plasmid, pCMVSPORTβ-galactosidase, Sp1, and pBS to give a total of 1.75 μg of plasmid DNA for each well. A Fugene6™ to DNA ratio of 3:2 was used and therefore 2.625 μL of Fugene6™ was used in each well. The transfected cells were incubated for 24 hours, harvested, and cell extracts were used in luciferase and β-galactosidase assays.




| Construct | Normalised Value | p-value | Significant Difference |
|---|---|---|---|
| -1357 topoisomerase II beta | 100% | - | - |
| -1357 topoisomerase II beta + 0.25 µg Sp1 | 148.2% | 0.04 | Yes* |
| -1357 topoisomerase II beta + 0.5 µg Sp1 | 215.2% | 0.03 | Yes* |
| -1357 topoisomerase II beta + 1.0 µg Sp1 | 198.9% | 0.2 | No |

**Figure 5.7: Effect of Sp1 on –1357 bp Topoisomerase II Beta Promoter Construct.**

HeLa cells were grown to 50-70% confluence before being transfected with 0.5 µg of luciferase reporter vector, 0.25 µg of pCMVSPORTβ-galactosidase and either 0 µg, 0.25 µg, 0.5 µg or 1.0 µg of the Sp1 co-expression vector. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed.

The normalised luciferase values were determined relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic with 0 µg of Sp1 added. To establish if there was any significant difference between 0 µg of Sp1 being added and the samples with Sp1, a t-test was performed and p-values were obtained. Yes or No indicates whether the samples are significantly different, and the * refer to Table 5.2.

In the context of the –1357 topoisomerase II beta promoter Sp1 acts as an activator of transcription. It was therefore of interest to see if this observed Sp1-mediated activation required the GC1 (position -548) or GC2 (position -808) binding element.

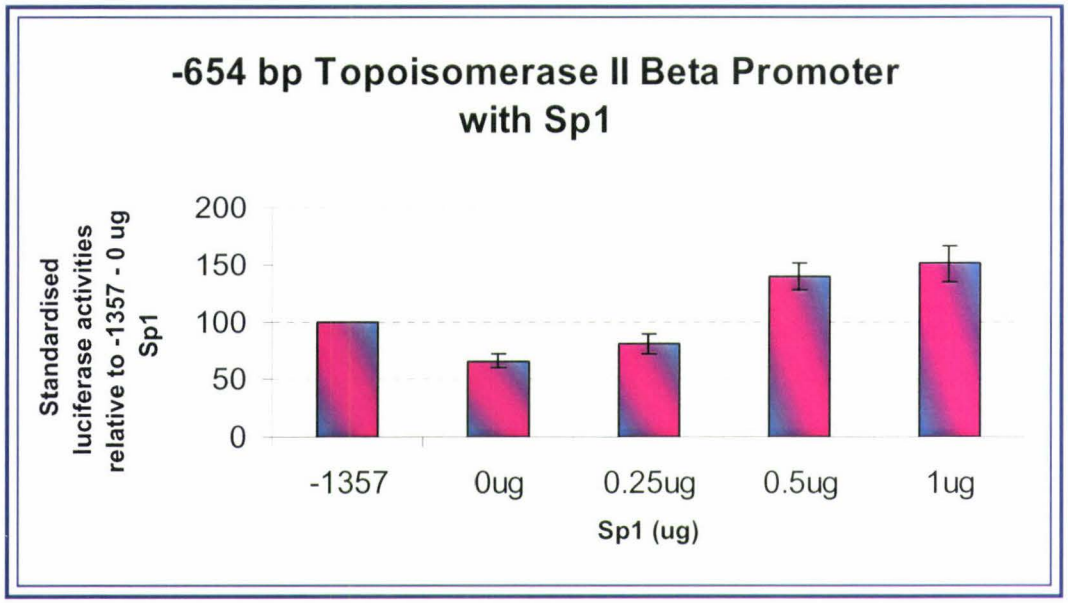
In this set of transfections the –654 topoisomerase II beta promoter construct was used in conjunction with 0 μg, 0.25 μg, 0.5 μg or 1 μg of Sp1 (Table 5.5). The –1357 topoisomerase II beta promoter construct with 0 μg of Sp1 was also included, and all results are expressed relative to this construct, so that comparisons could be made with the previous experiment (Figure 5.7). The –654 topoisomerase II beta promoter expresses at approximately two thirds of the level shown for the –1357 topoisomerase II beta promoter construct (Figure 5.8), which is in agreement with that reported by Lok *et al.* (2002) who demonstrated a small decrease when the shorter promoter was used in transfections. Upon addition of 0.25 μg of Sp1 a 20 % increase in luciferase activity, compared to the –654 topoisomerase II beta construct with 0 μg of Sp1, was observed and this was statistically significant with a p-value of 0.006. A statistically significant increase is observed when either 0.5 μg or 1 μg of Sp1 were added; the luciferase activity is approximately double compared to the –654 topoisomerase II beta with 0 μg of Sp1. This increase is equivalent to that observed for the –1357 topoisomerase II beta promoter construct with 0.5 μg or 1 μg of Sp1 added. These results also suggest the cells may be saturated with Sp1 and the addition of any more will have little effect. The data clearly demonstrate that Sp1 is capable of activating transcription from the shorter –654 topoisomerase II beta promoter construct, which lacks the GC2 element.

The same effect is observed for Sp1 when added to either the –654 or –1357 topoisomerase II beta promoter construct. The p-values for both sets of data show that the differences after Sp1 addition are statistically significant. A 2-fold increase in luciferase activity is detected when 0.5 μg of Sp1 is added to the –1357 and –654 topoisomerase II beta promoter constructs. As the increase is approximately the same with the –1357 construct (GC1 and GC2) as with the –654 construct, which lacks GC2, it is likely that GC1 is the element through which Sp1 activation is mediated. Transient transfections using a –654 topoisomerase II beta promoter construct, where GC1 is mutated, would help to clarify if GC1 is important in the observed activation of transcription by Sp1.

| | pGL3Basic | pCMVSPORTβ-galactosidase | -1357 bp Construct | -654 bp Construct | Sp1 | pBS |
|---|---|---|---|---|---|---|
| pGL3Basic Control | 0.5 μg | - | - | - | - | 1.25 μg |
| pCMV Control | - | 0.25 μg | - | - | - | 1.5 μg |
| -1357 Control | - | 0.25 μg | 0.5 μg | - | - | 1.0 μg |
| Sample 1 | - | 0.25 μg | - | 0.5 μg | - | 1.0 μg |
| Sample 2 | - | 0.25 μg | - | 0.5 μg | 0.25 μg | 0.75 μg |
| Sample 3 | - | 0.25 μg | - | 0.5 μg | 0.5 μg | 0.5 μg |
| Sample 4 | - | 0.25 μg | - | 0.5 μg | 1.0 μg | - |

**Table 5.5: Quantities of plasmid DNA used for Sp1 Co-expression Transient Transfections with the –654 bp Topoisomerase II beta Promoter Construct.**

Cells were transfected with a luciferase reporter plasmid, pCMVSPORTβ-galactosidase, Sp1, and pBS to give a total of 1.75 μg of plasmid DNA for each well. A Fugene6™ to DNA ratio of 3:2 was used and therefore 2.625 μL of Fugene6™ was used in each well. The transfected cells were incubated for 24 hours and then harvested for use in luciferase and β-galactosidase assays.




| Construct | Normalised Value | p-value | Significant Difference |
|---|---|---|---|
| -1357 topoisomerase II beta | 100% | - | - |
| -654 topoisomerase II beta | 65.9% | - | - |
| -654 topoisomerase II beta + 0.25 µg Sp1 | 80.57% | 0.006 | Yes** |
| -654 topoisomerase II beta + 0.5 µg Sp1 | 139.7% | 0.009 | Yes** |
| -654 topoisomerase II beta + 1.0 µg Sp1 | 150.9% | 0.017 | Yes* |

**Figure 5.8: Effect of Sp1 on –654 bp Topoisomerase II Beta Promoter Construct.**

HeLa cells were grown to 50-70% confluence before being transfected with 0.5 µg of luciferase reporter vector, 0.25 µg of pCMVSPORTβ-galactosidase and either 0 µg, 0.25 µg, 0.5 µg, or 1.0 µg of the Sp1 co-expression vector. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed.

The normalised luciferase values were determined relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic. To establish if there was any significant difference between 0 µg of Sp1 being added to the –654 topoisomerase II beta promoter construct and the samples with Sp1, a t-test was performed and p-values were obtained. Yes or No indicates whether the samples are significantly different and for the significance values and the * refer to Table 5.2.

### 5.3.2. Sp3 Co-Expression Transfections

A large number of studies have shown Sp3 to be an inhibitor of transcription via its inhibition domain. In a preliminary study no effect was seen when Sp3 was added to the –654 bp topoisomerase II beta promoter construct, however when added in the presence of the -1357 bp topoisomerase II beta promoter a small decrease in transcription was observed (Willingham, 2004). This finding suggests that Sp3 is a transcriptional inhibitor in the context of the full-length topoisomerase II beta promoter.

Transient co-expression transfection experiments were carried out to further examine what role, if any, Sp3 played in the transcriptional regulation of topoisomerase II beta. Transfections were carried out similar to those outlined in Table 5.5, except increasing amounts of Sp3 expression vector were used instead of Sp1, with and without the addition of pCMVSPORTβ-galactosidase control vector. Twenty-four hours after transfection the cells were harvested and assayed for β-galactosidase and luciferase activity.

The Sp3 co-expression transfections with the –1357 topoisomerase II beta promoter construct, were performed without the inclusion of the pCMVSPORTβ-galactosidase plasmid, which is a control for transfection efficiency. This is because when increasing amounts of Sp3 were added, the β-galactosidase values decreased (Figure 5.9). This suggested that Sp3 was having an inhibitory effect on the expression of β-galactosidase in this vector. The same trends were seen in the luciferase assay regardless of whether β-galactosidase was added, and the trends observed were reproducible without significant errors in the absence of β-galactosidase. Therefore, the pCMVSPORTβ-galactosidase vector was omitted from this set of transfections.

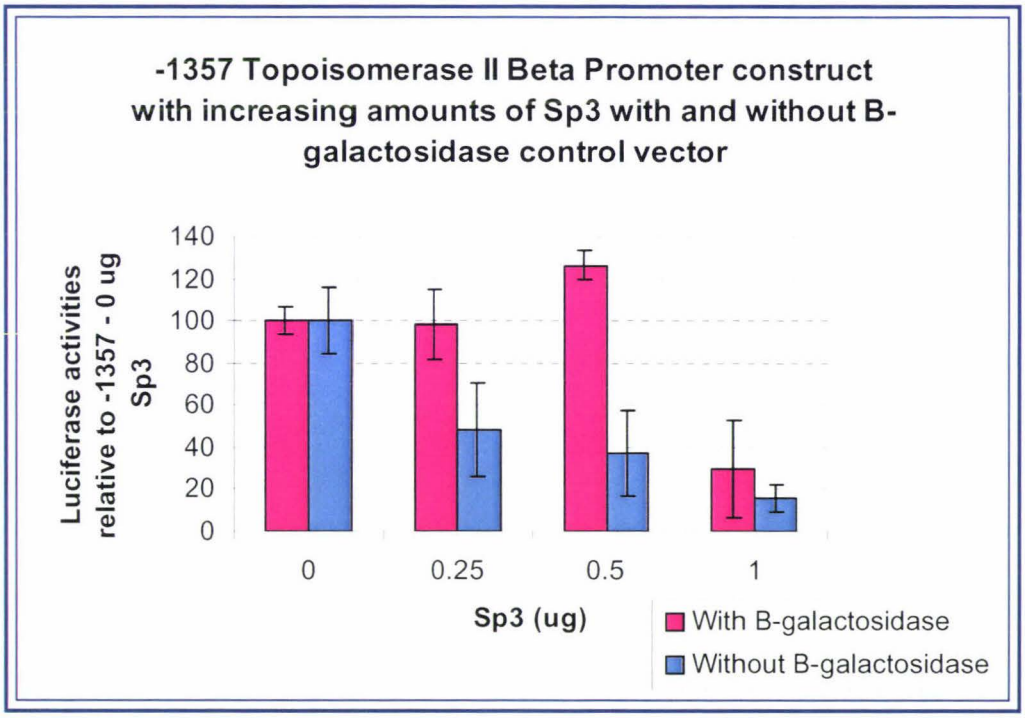
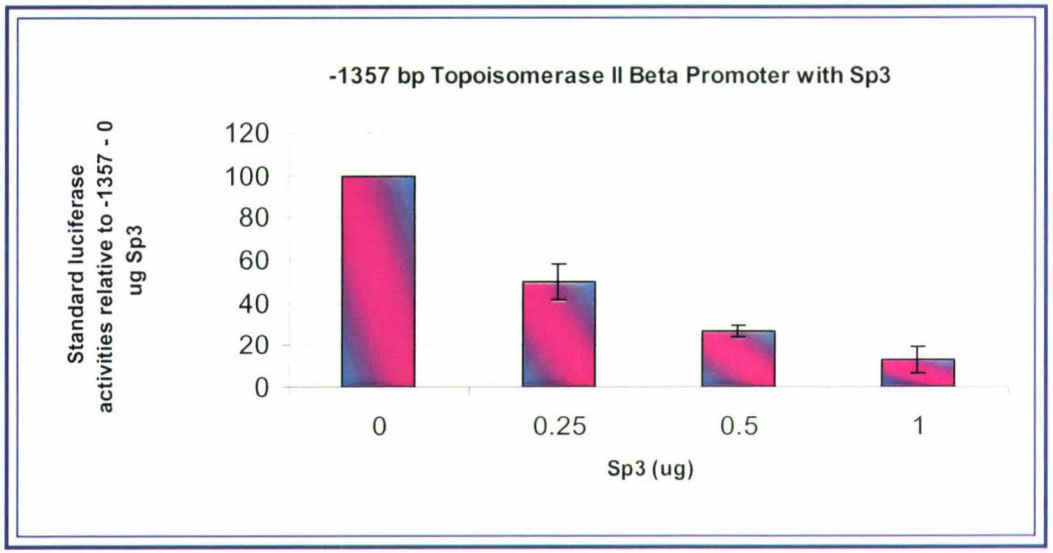



**Figure 5.9: Effect of β-galactosidase on Relative Luciferase Activities.**

HeLa cells were grown to 50-70% confluence before being transfected with 0.5 μg of luciferase reporter vector, with or without 0.25 μg of pCMVSPORTβ-galactosidase and with either 0 μg, 0.25 μg, 0.5 μg, or 1.0 μg of the Sp3 co-expression vector. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed. The normalised luciferase values were determine relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic with 0 μg of Sp3 added. This graph is representative of the results observed when β-galactosidase was included in the Sp3 transient transfections when using the –1357 topoisomerase II beta promoter construct.

Addition of only 0.25 μg of Sp3 vector resulted in a very significant 50% decrease in luciferase activity (Figure 5.10). Addition of 0.5 μg of Sp3 vector resulted in a further 25% decrease to give an overall 75% decrease in luciferase activity. Furthermore, addition of 1 μg of Sp3 vector resulted in a decrease to 13% of the luciferase activity compared to the activity in the absence of Sp3 vector. This decrease, when 1 μg of Sp3 was added, had a p-value of 0.003 providing strong evidence the observed decrease was significant. These data show that Sp3 is a potent inhibitor of topoisomerase II beta transcription.




| Construct | Normalised Value | p-value | Significant Difference |
|---|---|---|---|
| -1357 topoisomerase II beta | 100% | - | - |
| -1357 topoisomerase II beta + 0.25 µg Sp3 | 49.7% | 0.02 | Yes* |
| -1357 topoisomerase II beta + 0.5 µg Sp3 | 26.6% | 0.001 | Yes*** |
| -1357 topoisomerase II beta + 1.0 µg Sp3 | 13.2% | 0.003 | Yes** |

**Figure 5.10: Effect of Sp3 on –1357 bp Topoisomerase II Beta Promoter Construct.**

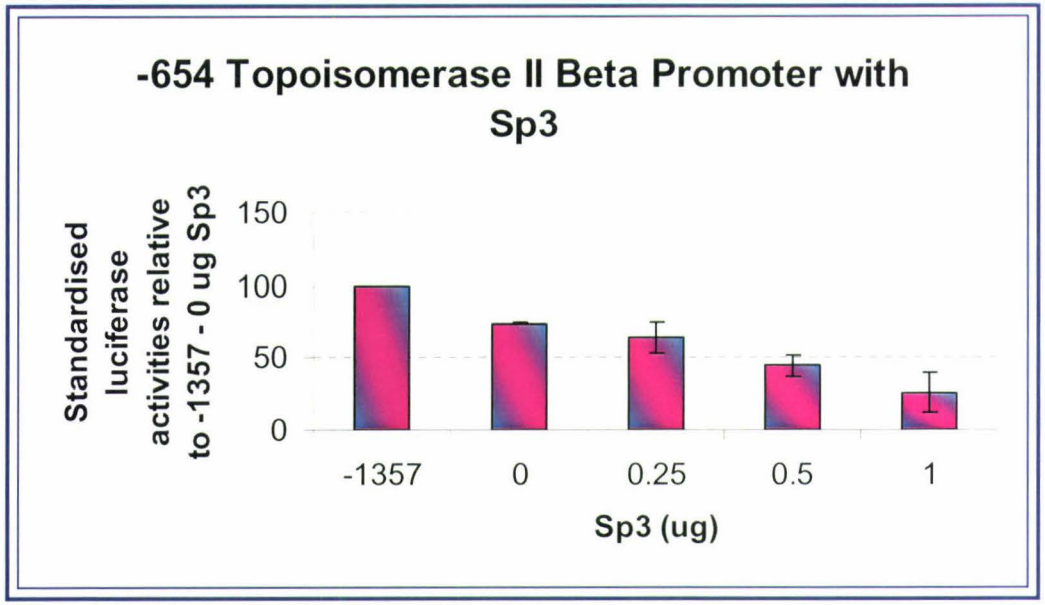
HeLa cells were grown to 50-70% confluence before being transfected with 0.5 µg of luciferase reporter vector, and either 0 µg, 0.25 µg, 0.5 µg, or 1.0 µg of the Sp3 co-expression vector. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed.

The normalised luciferase values were determined relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic with 0 µg of Sp3 added. To establish if there was any significant difference between 0 µg of Sp3 being added and the samples with Sp3, a t-test was performed and p-values were obtained. Yes or No indicates whether the samples are significantly different and for the significance values and the * refer to Table 5.2.

Willingham (2004) carried out a preliminary study to determine the role of Sp3 in topoisomerase II beta expression and found it may play a role as an inhibitor. The data shown in Figure 5.10 adds to that of Willingham (2004) and confirms Sp3 is a inhibitor of topoisomerase II beta expression with respect to the –1357 promoter construct.

Sp3 co-expression transfections were also carried out with the -654 topoisomerase II beta promoter reporter vector, this construct lacks the GC2 element. This set of experiments was designed to investigate the importance of GC1, in the absence of GC2, in the Sp3-mediated down-regulation of topoisomerase II beta transcription. A decrease in relative luciferase activity similar to that observed for -1357 topoisomerase II beta construct would suggest GC1 was more important, while any differences observed would suggest GC2 has a fundamental role. In a preliminary investigation Sp3 had no effect on the -654 topoisomerase II beta promoter construct (Willingham, 2004). Sp3 was shown to bind to both GC1 and GC2 *in vitro* (Chapter 3), therefore the functional significance of these interactions were further investigated.

The Sp3 co-expression transfection, with the -654 topoisomerase II beta promoter, was carried out similar to that outlined in Table 5.5, using Sp3 in place of Sp1. A similar difference in luciferase activity was observed for the -1357 topoisomerase II beta promoter construct and the -654 topoisomerase II beta promoter construct as that observed in Figure 5.8. Statistically significant decreases were observed after the addition of 0.5 μg, and 1 μg of Sp3 expression vector. After the addition of 1 μg of Sp3 the relative luciferase activity dropped to 26% of the -1357 topoisomerase II beta promoter activity. The decrease observed for the -654 topoisomerase II beta reporter vector was slightly less than that observed when Sp3 was added to the -1357 topoisomerase II beta reporter vector. This suggests that Sp3 may bind to both GC1 and GC2 *in vivo*, and act through both elements to repress the transcription of topoisomerase II beta, although GC1 appears to be dominant over GC2.




| Construct | Normalised Value | p-value | Significant Difference |
|---|---|---|---|
| -1357 topoisomerase II beta | 100% | - | - |
| -654 topoisomerase II beta | 74.2% | - | - |
| -654 topoisomerase II beta + 0.25 μg Sp3 | 63.5% | 0.146 | No |
| -654 topoisomerase II beta + 0.5 μg Sp3 | 44.4% | 0.022 | Yes* |
| -654 topoisomerase II beta + 1.0 μg Sp3 | 25.7% | 0.028 | Yes* |

**Figure 5.11: Effect of Sp3 on –654 bp Topoisomerase II Beta Promoter Construct.**

HeLa cells were grown to 50-70% confluence before being transfected with 0.5 μg of luciferase reporter vector, 0.25 μg of pCMVSPORTβ-galactosidase and either 0 μg, 0.25 μg, 0.5 μg, or 1.0 μg of the Sp3 co-expression vector. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed.

The normalised luciferase values were determined relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic. To establish if there was any significant difference between 0 μg of Sp3 being added to the –654 topoisomerase II beta promoter construct and the samples with Sp3, a t-test was performed and p-values were obtained. Yes or No indicates whether the samples are significantly different and for the significance values and the * refer to Table 5.2.

### 5.3.3. Transient Transfection using the Mutant -654 Topoisomerase II Beta Promoter Constructs

Electrophoretic mobility shift assays showed that Sp1 and NF-Y bind to both the GC1 element and the ICB2 element, and that Sp3 also binds to the GC1 element *in vitro* (refer to section 3.2.4). Furthermore, a complex of proteins was shown to bind to the composite ICB2/GC1 element, with the functional significance unknown. Electrophoretic mobility shift assays also showed that NF-Y was capable of binding to ICB1 *in vitro*, and that this was the only transcription factor shown to bind to this element. To further investigate these interactions, functionally characterise them, and to build on the results demonstrated by Willingham (2004), transient transfections were carried out with the –654 topoisomerase II beta construct containing mutations in GC1 and/or ICB1 and/or ICB2.

The mutant topoisomerase II beta promoter constructs (cloned in Chapter 4) were used in transient transfections to determine the effects of these mutations on the expression of topoisomerase II beta. The amounts of the various plasmids used in transient transfections are outlined in Table 5.6.

| | pGL3 Basic | pCMVSPORTβ -galactosidase | -1357 bp Construct | -654 bp Construct | Mutant Construct | pBS |
|---|---|---|---|---|---|---|
| pGL3Basic Control | 0.5 µg | - | - | - | - | 0.25 µg |
| pCMV Control | - | 0.25 µg | - | - | - | 0.5 µg |
| -1357 Control | - | 0.25 µg | 0.5 µg | - | - | - |
| -654 Control | - | 0.25 µg | - | 0.5 µg | - | - |
| ICB1 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| ICB2 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| GC1 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| ICB1/ICB2 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| ICB1/GC1 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| ICB2/GC1 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |
| ICB1/ICB2/GC1 Mutant | - | 0.25 µg | - | - | 0.5 µg | - |

**Table 5.6: Quantities of plasmid DNA used for Topoisomerase II Beta Mutant Promoter Construct Transient Transfections.**

Cells were transfected with a luciferase reporter plasmid, pCMVSPORTβ-galactosidase, and pBS to give a total of 0.75 µg of plasmid DNA for each well. A Fugene6™ to DNA ratio of 3:2 was used and therefore 1.125 µL of Fugene6™ was used in each well. The transfected cells were incubated for 24 hours and then harvested for use in luciferase and β-galactosidase assays.

Figure 5.12 is representative of the results obtained for the –654 topoisomerase II beta mutant promoter constructs. When the ICB1 element was mutated there was approximately a 20% increase in reporter gene expression. This result had a p-value of 0.002 therefore it is statistically significant and suggests that ICB1 is a positive element, which binds a transcriptional activator. A mutation introduced into either ICB2 or GC1 reduced reporter gene activity by 50% and 36%, respectively, when compared to the wild type –654 topoisomerase II beta promoter construct. With p-values of 0.0006 and 0.0008 respectively there is very strong evidence that the differences observed are statistically significant. This result suggests these two elements have an important role in basal transcription driven by the –654 topoisomerase II beta promoter. When ICB1 is mutated in combination with either GC1 or ICB2 its ability to repress transcription is lost as the ICB1/ICB2 mutant construct and the ICB1/GC1 mutant construct have a 50% and 44% reduction in reporter gene activity respectively (compared to the wild type –654 topoisomerase II beta promoter construct). Furthermore, no further reduction in reporter gene activity was observed for the ICB2/GC1 mutant construct or when all three elements were mutated, compared to that observed for the single ICB2 mutant and GC1 mutant constructs, with both results being significant. Taken together these results suggest that ICB2 and GC1 are the dominant elements in the –654 topoisomerase II beta promoter construct and that in this context they are the most important elements for basal transcription. This is however, only a preliminary study and the experiment will need to be repeated to confirm the findings. It is possible that the assembly of the protein complex, shown to form on the ICB2/GC1 composite element in electrophoretic mobility shift assays, is important for the correct expression of topoisomerase II beta, as if it cannot form, due to mutations in either ICB2 or GC1, reporter gene activity decreases.

Lok *et al.* (2002) demonstrated that mutations introduced into ICB1 and /or ICB2 and/or GC1 resulted in a decrease in reporter gene activity. In this study the mutations were introduced into a –1067 promoter construct. The results shown in Figure 5.12 confirm the role of ICB2 and GC1 as activator binding sites.

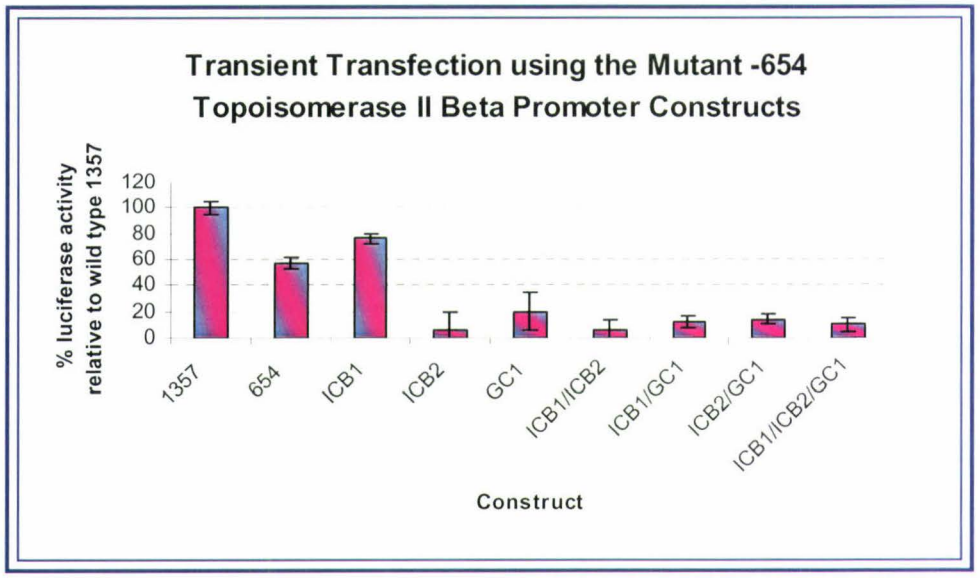



**Figure 5.12: Effect of Mutations on Topoisomerase II Beta Expression.**

HeLa cells were grown to 50-70% confluence before being transfected with 0.5 µg of luciferase reporter vector and 0.25 µg of pCMVSPORTβ-galactosidase. A Fugene6™ to DNA ratio of 3:2 was used to facilitate DNA uptake. The cells were left to grow for 24 hours after which they were harvested and β-galactosidase and luciferase assays were performed. These data are from one experiment and show arrays of triplicates. The normalised luciferase values were determined relative to the –1357 bp topoisomerase II beta promoter construct in pGL3Basic. To establish if there was any significant difference between wild type and mutant –654 topoisomerase II beta constructs, a t-test was performed and p-values were obtained. Yes or No indicates whether the samples are significantly different and for the significance values and the * refer to Table 5.2.

| Construct | Normalised Value | p-value | Significant Difference |
|---|---|---|---|
| -1357 topoisomerase II beta | 100% | - | - |
| -654 topoisomerase II beta | 56.9% | - | - |
| -654 ICB1 Mutant | 76.0% | 0.002 | Yes** |
| -654 ICB2 Mutant | 5.6% | 0.0006 | Yes*** |
| -654 GC1 Mutant | 20.1% | 0.0008 | Yes*** |
| -654 ICB1/ICB2 Mutant | 6.2% | 0.0008 | Yes*** |
| -654 ICB1/GC1 Mutant | 12.4% | 0.001 | Yes** |
| -654 ICB2/GC1 Mutant | 14.0% | 0.0009 | Yes*** |
| -654 ICB1/ICB2/GC1 Mutant | 9.8% | 0.002 | Yes** |

## 5.4. Summary

Transient co-expression transfections identified a functional role for the observed *in vitro* binding of Sp1 and Sp3 to the topoisomerase II beta promoter. Overexpression of Sp1, in the context of either the –654 or –1357 topoisomerase II beta promoter constructs resulted in a statistically significant increase in reporter gene activity. This confirmed that Sp1 is a transcriptional activator of the topoisomerase II beta promoter. Overexpression experiments using the Sp3 co-expression vector in conjunction with either the –654 or –1357 topoisomerase II beta promoter constructs resulted in a decrease in expression. Therefore, Sp3 is an inhibitor of topoisomerase II beta expression.

For both Sp1 and Sp3, the level of activation or inhibition was similar for both the –654 or –1357 topoisomerase II beta promoter constructs. The –1357 promoter construct contains an additional binding site for Sp1 and Sp3, the GC2 element. These results suggest that Sp1 and Sp3 exert their function primarily by binding to the GC1 element. This raises the possibility that Sp1 and Sp3 compete for the binding of GC1 and it would be of interest to determine under what conditions which of the two transcription factors is dominant.

The transfections using the mutant topoisomerase II beta promoter constructs showed that an inhibitor binds to the ICB1 element as when this element was mutated there was an increase in reporter gene expression. The ICB2 and GC1 elements appear to be both required to maintain the correct expression of topoisomerase II beta as a mutation in either considerably reduced reporter gene activity.

It will be important to carry out mutational analysis in the full-length (-1357) topoisomerase II beta promoter and combine the mutations with an additional mutation in the upstream GC2 element. This will further clarify the specific roles of each of the elements in the topoisomerase II beta promoter.

# 6. Summary and Future Directions

Electrophorectic mobility shift assays and transient transfection assays were used to investigate the regulation of expression of topoisomerase II beta. Previous work had identified the regions important for topoisomerase II beta promoter expression and several transcription factors have been shown to bind to the promoter *in vitro* (Lok *et al.*, 2002; Willingham, 2004). In this study the focus was to confirm the previous findings, and identify more transcription factors that bind to the topoisomerase II beta promoter. Transient transfection experiments were designed to investigate the functional significance of the DNA/protein interactions, and to determine the specific roles of ICB1, ICB2, and GC1 in topoisomerase II beta expression.

## 6.1. Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) showed that Sp1, Sp3, and NF-Y were all capable of binding to the topoisomerase II beta promoter *in vitro*. GC1 was found to bind all three proteins, while GC2 bound Sp1 and Sp3. NF-Y was the only protein capable of binding to ICB1, and ICB2 bound both NF-Y and Sp1. These experiments suggest that protein-protein interactions may be responsible for recruiting transcription factors to binding elements within the topoisomerase II beta promoter that they would not normally bind to (i.e. Sp1 would not normally be expected to interact with a CCAAT box, and NF-Y would not normally be expected to bind a GC-rich element). When a composite GC1/ICB2 oligonucleotide probe was used in EMSAs, all three proteins (NF-Y, Sp1, and Sp3) were shown to bind, as well as a complex containing all three proteins.

EMSAs also uncovered two proteins that specifically bind to the topoisomerase II beta promoter, which were not identified by the NF-Y, Sp1, or Sp3 antibodies used in the antibody supershift assays (Figures 3.5 and 3.7). One of the proteins bound specifically to the GC2 oligonucleotide probe, and the other bound to the GC1, GC2, and GC1/ICB2 oligonucleotide probe, suggesting that it binds to DNA in a GC-rich element-dependent manner.

## 6.2. Transient Transfections

Following the *in vitro* binding assays transient transfection assays were performed to determine if these observed interactions had any functional significance *in vivo*. Transient co-expression transfection assays showed that Sp1 was capable of increasing reporter gene expression, driven by either the –1357 or –654 wild type topoisomerase II beta promoter constructs (Figures 5.7 and 5.8) to a similar level. The –1357 topoisomerase II beta promoter construct contains the GC2 element, which is not present in the shorter –654 topoisomerase II beta promoter construct. Therefore, this result suggests that the observed Sp1-mediated activation of transcription acts primarily through the GC1 element, rather than the GC2 element. Similar studies using the Sp3 co-expression vector (in place of the Sp1 co-expression vector) showed that Sp3 is a strong inhibitor of topoisomerase II beta expression, and that this effect is also likely to be mediated through the GC1 element. The supporting evidence for this conclusion is that when either the –654 or –1357 wild type topoisomerase II beta promoter constructs were used a similar level of inhibition was observed, although the presence of modulatory elements between –1357 and –654 of the promoter cannot be ruled out.

To further examine the precise roles of the GC1, ICB1, and ICB2 elements, in the expression of topoisomerase II beta, a series of –654 promoter constructs were cloned containing mutations in GC1 and/or ICB1 and/or ICB2. These mutated promoter constructs were subsequently used in transient transfection assays, and preliminary data suggests that an inhibitor of transcription binds to ICB1. The data also suggested that activators of transcription bind to GC1 and ICB2, and more importantly that both of these elements are required for the expression of a reporter gene driven by the –654 topoisomerase II beta promoter.

## 6.3. Future Directions

The results thus far indicated that both the topoisomerase II alpha (Magan *et al.*, 2003) and topoisomerase II beta promoters appear to be regulated by the same set of transcription factors in essentially the same manner. The regulation by Sp1, Sp3, and NF-Y is therefore unlikely be responsible for the differential expression of these two isoforms as regards to the cell cycle (Woessner *et al.*, 1991; Kimura *et al.*, 1994). Since regulation of gene expression correlating with the cell cycle is important for both cancer cell progression, and targeted chemotherapy, it will be important to further investigate the transcriptional regulation of topoisomerase II beta to begin to understand its role in drug-resistance.

### 6.3.1. Transcription Factor Binding Assays

Additional electrophoretic mobility shift competitor assays could be performed using the GC1/ICB2 oligonucleotide probe with the single element (ICB1, ICB2, GC1, and GC2) competitor oligonucleotides. This could provide evidence as to which element forms the strongest interaction with the DNA binding proteins and possibly lead to differential expression due to differing amounts of these cognate factors.

It will be important to determine if the proteins shown to bind to the topoisomerase II beta promoter *in vitro* also bind *in vivo*. Chromatin immunoprecipitation assays (ChIP assays) (Kuo and Allis, 1999) could be used for this purpose. This assay involves crosslinking the proteins to the chromatin *in vivo*, sonification of the DNA to produce small fragments, immunoprecipitation using an antibody against the protein of interest, and final PCR-amplification of the target sequence. If the protein does bind to the topoisomerase II beta promoter *in vivo* a specific PCR product will be generated. Antibodies against Sp1, Sp3, and NF-Y could be used to determine if the observed *in vitro* binding of these proteins is significant *in vivo*. Real time PCR would be useful to quantify any interactions observed. Repeating these assays with cells exposed to topoisomerase II-targeting drugs, may highlight differences between protein/DNA interactions at the topoisomerase II alpha and topoisomerase II beta promoters. A cell line resistant to XK469 could be used, because this poison specifically targets topoisomerase II beta (Gao *et al.*, 1999). Any differences

observed could provide information on the mechanism involved in the observed down-regulation of topoisomerase II beta in drug-resistance.

### 6.3.2. Cloning and Transient Transfections

To confirm the results described in Chapter 5, that Sp1 is likely to act through the GC1 element, the topoisomerase II beta mutant promoter constructs (generated in section 4.2) could be used in conjunction with Sp1 in transient co-expression transfection assays. If no increase, or only a small increase, in reporter gene expression is observed upon the addition of Sp1 to the mutant GC1 topoisomerase II beta promoter construct, the conclusions from the previous experiment will be confirmed, i.e. Sp1 binds to the GC1 element and works through this element to activate the transcription of topoisomerase II beta. Conversely, a mutation could be introduced into the GC2 element in the context of the wild type –1357 topoisomerase II beta promoter. If Sp1 does act through GC1 alone then the same level of activation should be seen in both mutant and wild type GC2 –1357 promoter constructs, unless an alternative transcription factor interacts with the GC2 element.

A similar set of experiments using the Sp3 co-expression vector (in place of the Sp1 co-expression vector) could be carried out. This could clarify which of the two GC-rich elements interacts functionally with Sp3, thereby mediating the down-regulation of reporter gene expression driven by the topoisomerase II beta promoter (Figures 5.10 and 5.11).

Since topoisomerase II beta is ubiquitously expressed, the involvement of one or more of the other tissue-specific Sp-family members (Suske, 1999) in a tissue-specific manner cannot be ruled out.

If ChIP assays showed that both Sp1 and Sp3 bound to the topoisomerase II beta promoter *in vivo*, as suggested by *in vitro* EMSA assays (Chapter 3), it would be of interest to investigate the importance of the relative levels of these two transcription factors, which are both present in drug treated cells, and bound to the topoisomerase II

beta promoter. The relative levels of Sp1 and Sp3 have been reported to be of importance in the regulation of gene expression (*DeLuca et al.*, 1996); Williams *et al.*, submitted). Williams *et al.* (submitted) showed that Sp3 was dominant over Sp1, and had the ability to repress Sp1-mediated activation of transcription from the topoisomerase II alpha promoter. This has also been shown for other promoters (Majello *et al.*, 1995; Nicolas *et al.*, 2003). Transient co-expression assays using both Sp1 and Sp3 at varying levels would provide information on the functional significance of the two transcription factors relative to each other. Both have been shown to bind to the topoisomerase II beta promoter *in vitro* (Figure 3.5), and an *in vivo* functional experiment could determine whether either of the two transcription factors has a dominant effect when both are present.

To further investigate the physiological importance of Sp1, Sp3, and NF-Y, RNA interference could be used to knock down the expression of these specific transcription factors. siRNAs, with homology to NF-YA, Sp1, or Sp3, could be transfected into HeLa cells. Following the knockdown of the transcription factor, whole cell protein extracts could be prepared for analysis by immunoblotting. Antibodies to topoisomerase II beta, the specific transcription factor (to confirm knockdown), and an α-tubulin control, would highlight any changes in topoisomerase II beta expression at a protein level. Another subset of cells could be used for mRNA preparation for use in quantitative real time reverse transcription PCR. Primers designed to amplify the mRNA of topoisomerase II beta, the transcription factor knocked down, and a β-actin control could be used to assess any effect seen at the mRNA level. This series of experiments would give a clear indication of the specific role of NF-Y, Sp1, and Sp3 as either activators or inhibitors of topoisomerase II beta expression.

### 6.3.3. Characterisation of the Unidentified Proteins

The two proteins shown to bind specifically to the GC-rich elements in EMSAs to date remain uncharacterised, and their roles in topoisomerase II beta expression are unknown. To fully understand the mechanism underlying the expression of topoisomerase II beta, it will be essential to identify these two proteins and functionally characterise them.

In order to identify the two unknown proteins they first must be purified from whole cell extracts. One way to do this would be by exploiting the strong biotin/streptavidin interaction in combination with a biotinylated oligonucleotide (encompassing regions of the promoter), and streptavidin magnetic beads. One of the unknown proteins was found to bind to the GC2 oligonucleotide probe and the other to the GC1/ICB2 composite oligonucleotide probe used in EMSAs. These two probes could be synthesised with a biotin moiety attached to one end of the probe, and used to extract the unknown binding proteins from the whole cell extract. In principle, only the proteins specific to the elements in the probe being used would bind, and the rest of the proteins could be washed away. Western blot analysis could be used to identify the proteins known to bind to the probe, and through the process of elimination the remaining unidentified protein would be the protein of interest (Figure 6.1a). This could then be gel purified and subjected to N-terminal sequencing, or trypsin digestion followed by peptide mass fingerprinting to identify the proteins.

An alternative method that could be used is co-immunoprecipitation. Both proteins were bound to their respective elements in the presence of other transcription factors; therefore it is likely the unknown proteins interact with the other proteins. Indeed, protein/protein interactions may be stronger than DNA/protein interactions, as the bands representing these unknown proteins were relatively indistinct in gel shift experiments. Western blot analysis could eliminate the known proteins and indicate which one is the unknown protein (Figure 6.1b).

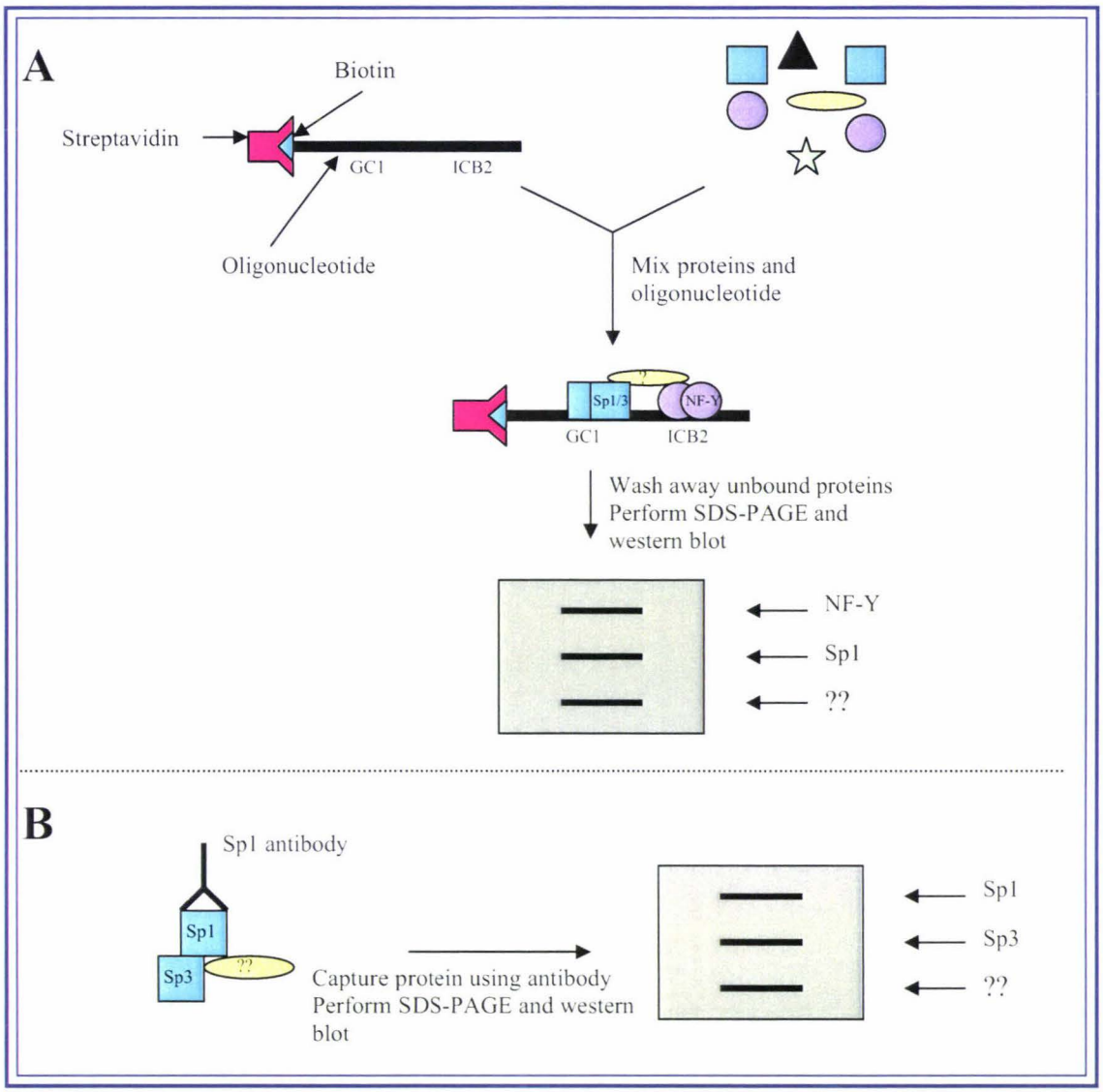



**Figure 6.1: Schematic Representation of Two Purification Procedures.**

A) Streptavidin/Biotin Purification. The oligonucleotide probe is modified to contain a biotin moiety on one end. Streptavidin beads are then used to capture the biotinylated oligonucleotide probe and whole cell extracts are passed over it. Unbound proteins are washed away, and the bound proteins run on a SDS-PAGE. Western blot analysis is used to identify the known proteins and the remaining band should represent the unknown protein. B) Immunoprecipitation. An antibody to a known protein is used to capture the protein complex and isolate it from all the other proteins. The purified complex is run on a SDS-PAGE and western blot analysis used to identify the known proteins, and the remaining band should represent the unknown protein.

In order to confirm the identity of the proteins, suggested from the amino acid sequencing or peptide analysis, a western blot could be carried out using antibodies that are specific to the protein, if available.

Once the proteins have been identified, the cDNAs encoding them could be cloned into mammalian expression vectors for use in transient co-expression transfection assays. The over-expression of the cDNA would allow a functional characterisation of the protein, with respect to transcription from the topoisomerase II beta promoter. An increase in reporter gene activity would suggest the protein is an activator; a decrease in reporter gene activity would suggest that the protein is an inhibitor. To reinforce the conclusions from the over-expression experiments, RNA interference could be used to determine the effect of knockdown of the mRNA and protein levels on topoisomerase II beta expression.

### 6.3.4. Chromosome Conformation Capture Assays

If GC2 is shown to be functionally significant, Chromosome Conformation Capture Assays (3C Assays) could be performed. This could determine if two elements (GC1 and GC2), which are separated in the promoter of the topoisomerase II beta gene, are capable of interacting through DNA looping and protein-protein interactions (Dekker *et al.*, 2002).

The general method is outlined in Figure 6.2. The proteins are crosslinked and following this the DNA is digested using a suitable restriction enzyme. Next, the DNA is religated. A large dilution preceding religation is required to promote intramolecular ligations over intermolecular ligations. Therefore, only if the two elements are connected though protein-protein interactions will the DNA religate. Crosslinks are reversed, the proteins removed, and PCR performed. Primers are designed to bind to either side of the elements thought to interact. If the two elements do interact then a product of a specific size will be formed, whereas if the two elements do not interact no product will be formed.

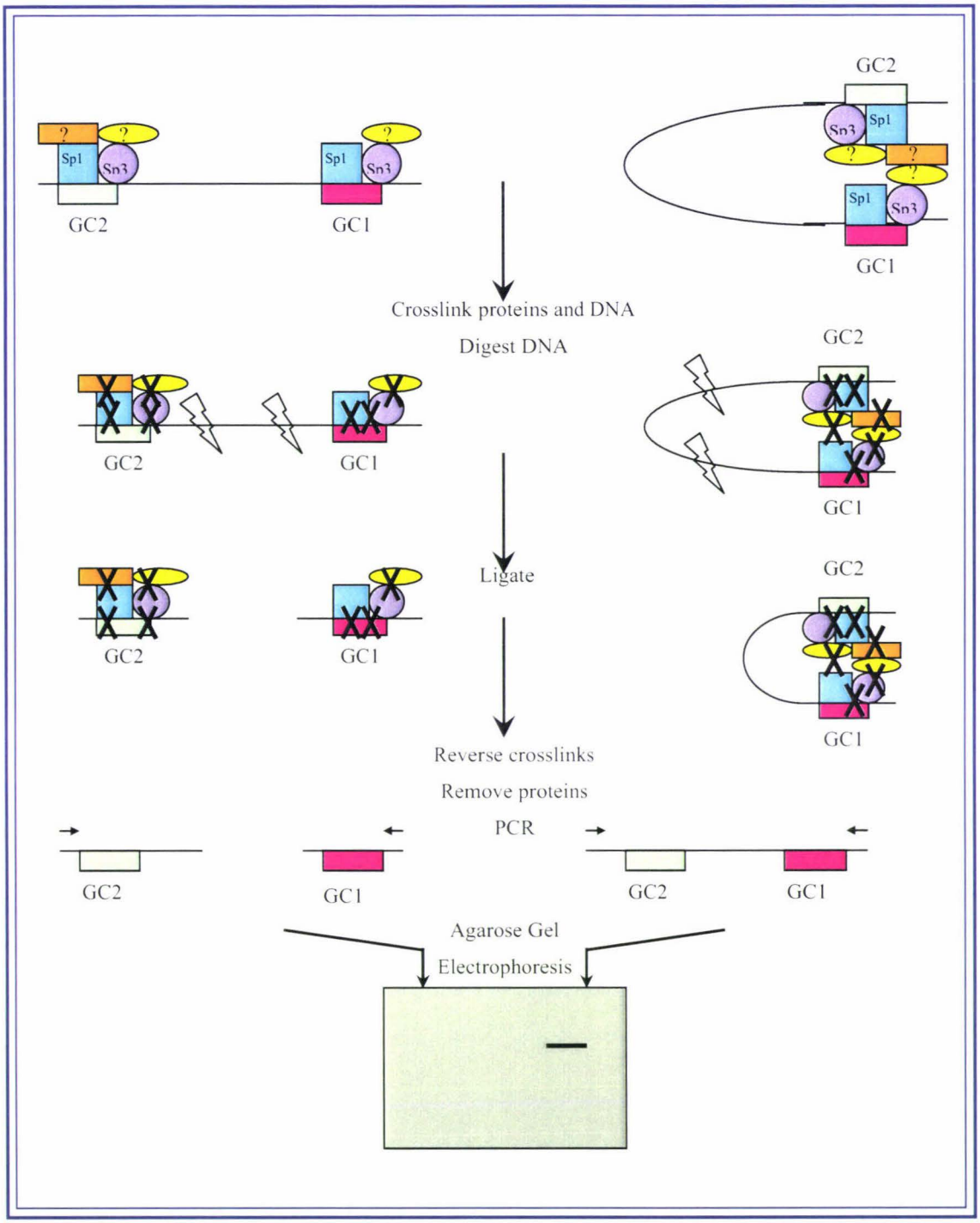



**Figure 6.2: Schematic Representation of a Chromosome Capture Conformation Assay.**

Whole cells are used and the proteins and DNA crosslinked. The protein/DNA complexes are then purified and the DNA digested. A large dilution is carried out, followed by a ligation. The crosslinks are then reversed and the DNA purified. PCR is carried out using primers directly upstream and downstream of the two elements thought to interact, and agarose gel electrophoresis is carried out to visualise the results.

Williams *et al.* (submitted) has performed 3C assays for the topoisomerase II alpha promoter, and shown that the GC1 and GC2 elements interact via protein-protein interactions. It is possible that in the topoisomerase II beta promoter the GC2 element interacts, through the bending of the DNA, with one of the downstream elements (GC1). A 3C assay would therefore determine if this did in fact occur. On the other hand, this DNA looping of the topoisomerase II alpha promoter may be the major differentiating factor in determining the cell cycle-specific transcriptional regulation, which is not observed for topoisomerase II beta.

3C assays could also be useful to perform for drug-sensitive and drug-resistant cell lines. Cells sensitive or resistant to XK469 (topoisomerase II beta specific poison) could be used for 3C assays. This would determine if there are any changes in DNA conformation brought about by protein-protein interactions, which may alter transcription initiation potential. Transcription factors (NF-Y and Sp1) have been shown to be differentially expressed in drug-resistant cell lines (Allen *et al.*, 2004), therefore it is possible that changes in promoter conformation could occur if DNA looping was a consequence of Sp1/Sp3/GC1 and Sp1/Sp3/GC2 interactions.

### 6.3.5. Investigation of Topoisomerase II alpha and Topoisomerase II beta

It has been suggested that it is the relative levels of topoisomerase II alpha and topoisomerase II beta in the cell that is important in the response to chemotherapeutic drugs (Padget *et al.*, 2000). However, little has been reported on the levels of the two isoforms in the cell during drug-resistance.

To investigate this possibility a number of drug-sensitive and drug-resistant cell lines would be required. Using several different cell lines would indicate if any variation of the relative levels is cell line specific, or if it is a universal trend.

Measurements of RNA and protein levels of topoisomerase II alpha and topoisomerase II beta could be determined. Western blot analysis could be used to measure protein levels

and reverse transcription real time PCR could be used to measure the relative mRNA levels of the two isoforms. This would establish if there are any differences in the ratio of topoisomerase II alpha to topoisomerase II beta in drug-sensitive and drug-resistant cells.

Phosphorylation of topoisomerase II alpha and topoisomerase II beta has been suggested to activate the enzyme (Saijo *et al.*, 1990), however, no conclusive data has been reported. Therefore it would also be interesting to study the relative levels of phosphorylation of topoisomerase II alpha and beta in drug-sensitive and drug-resistant cells. Western blot analysis, using an antibody to the phosphorylated residues would highlight any differences occurring. If any differences were observed, then further exploration into the role of the phosphorylation of the alpha and beta isoforms would be required to determine the *in vivo* significance of the observation. For example, if phosphorylation of topoisomerase II does activate the enzyme, it is possible that there is a decrease in the phosphorylation of (one or both of the topoisomerase II isoforms) in drug-treated or drug-resistant cells.

## 6.4. Conclusion

DNA binding studies showed that *in vitro* a number of proteins bind specifically to the elements located within the topoisomerase II beta promoter. Sp1, Sp3, and NF-Y bind to the GC and ICB elements respectively. In the absence of their binding sequences these transcription factors can still be recruited to the promoter through protein-protein interactions. Electrophoretic mobility shift assays also revealed two proteins that specifically bind to the GC1 and GC2 elements in the topoisomerase II beta promoter, and which were not identified in this study; to date they remain uncharacterised.

Functional studies carried out using over expression of Sp1 or Sp3 revealed the relative roles of these two transcription factors. Sp1 activated expression of the reporter gene driven by the topoisomerase II beta promoter, while Sp3 repressed transcription. It is likely that both of these transcription factors exert their function solely through the GC1 element in the topoisomerase II beta promoter. This means therefore, that Sp1 and Sp3 may compete for binding to GC1.

Mutational analysis of the topoisomerase II beta promoter elements revealed the specific roles of ICB1, ICB2, and GC1 in the context of the –654 promoter construct. ICB1 acts as an inhibitor of transcription through the binding of a transcription factor (possibly NF-Y). ICB2 and GC1 both bind transcription factors that activate transcription, and both these elements are required for normal transcription (driven by the –654 topoisomerase II beta promoter construct) as when either are deleted there is a large decrease in reporter gene activity. This suggests that the complex of proteins shown to form in Electrophoretic mobility shift assays has functional significance *in vivo*.

This study provides further information on the mechanisms underlying the regulation of expression of topoisomerase II beta, however, little is known about the transcriptional response to drug treatment or the mechanisms that result in drug-resistance. It will be essential to identify and characterise the unknown proteins, shown to bind to the topoisomerase II beta promoter *in vitro*, in order to fully understand the regulation of this gene. It will also be of importance to carry out a series of comparative experiments in drug-sensitive and drug-resistant cell lines and tumour samples to determine: whether topoisomerase II beta is down-regulated in cells or tumours resistant to chemotherapeutic

drugs, how this down-regulation occurs, and whether relative levels of topoisomerase II beta in tumours has any relevance for prognosis or targeted therapies.

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# Appendix

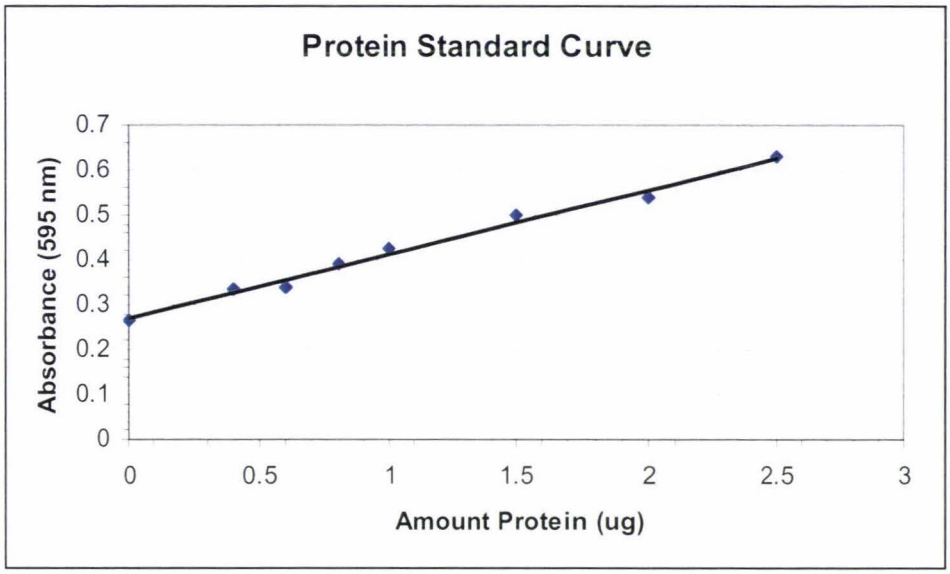
## Appendix 1: Primer and Oligonucleotide Sequences

Sequences of oligonucleotides used in this study. The oligonucleotides were used in two different experiments: electrophoretic mobility shift assays (EMSA) or PCR mutagenesis (PCR). The underlined sequences represent the binding elements and the bold sequences represent the mutations.

| Oligoucleotide | Experiment | Sequence (5' - 3') |
|---|---|---|
| GC1wtF | EMSA | CGGGTCCCGCCCCTCCAG |
| GC1wtR | EMSA | CTGGAGGGGCGGGACCCG |
| GC1mtF | PCR and EMSA | CTCGGGTC**GA**GC**T**CCTCCAGG |
| GC1mtR | PCR and EMSA | CCTGGAGG**A**GC**TC**GACCCGAG |
| GC2wtF | EMSA | TCCCCACCCCGCCCCCATCCT |
| GC2wtR | EMSA | AGGATGGGGGCGGGGTGGGGA |
| GC2mtF | EMSA | TCCCCACATCGCATCCATCCT |
| GC2mtR | EMSA | AGGATGGATGCGATGTGGGGA |
| ICB1wtF | EMSA | ACAAGGCCCGGATTGGACAGCATGGCG |
| ICB1wtR | EMSA | CGCCATGCTGTCCAATCCGGGCCTTGT |
| ICB1mtF | PCR and EMSA | ACAAGGCCCG**TCTAGA**ACAGCATGGCG |
| ICB1mtR | PCR and EMSA | CGCCATGCTGT**TCTAGA**CGGGCCTTGT |
| ICB2wtF | EMSA | TTGGGATTGGCCGAG |
| ICB2wtR | EMSA | CTCGGCCAATCCCAA |
| ICB2mtF | PCR and EMSA | GGAATTTTGG**AGATCT**CCGAGAGGCTG |
| ICB2mtR | PCR and EMSA | CAGCCTCTCGG**AGATCT**CCAAAATTCC |
| GC1wtICB2wtF | EMSA | CTCGGGTCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTG |
| GC1wtICB2wtR | EMSA | CAGCCTCTCGGCCAATCCCAAAATTCCAAGCCCCCTGGAGGGGCGGGACCCGAG |
| GC1mtICB2wtF | EMSA | CTCGGGTC**GA**GC**T**CCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTG |
| GC1mtICB2wtR | EMSA | CAGCCTCTCGGCCAATCCCAAAATTCCAAGCCCCCTGGAGG**A**GC**TC**GACCCGAG |

| Oligoucleotide | Experiment | Sequence (5' - 3') |
|---|---|---|
| GC1wtICB2mtF | EMSA | |
| | | CTCGGGTCCCGCCCCTCCAGGGGGCTTGGAATTTTGGAGATCTCCGAGAGGCTG |
| GC1wtICB2mRF | EMSA | |
| | | CAGCCTCTCGGAGATCTCCAAAATTCCAAGCCCCCTGGAGGGGCGGGACCCGAG |
| GC1mtICB2mtF | EMSA | |
| | | CTCGGGTCGAGCTCCTCCAGGGGGCTTGGAATTTTGGAGATCTCCGAGAGGCTG |
| GC1mtICB2mtR | EMSA | |
| | | CAGCCTCTCGGAGATCTCCAAAATTCCAAGCCCCCTGGAGGAGCTCGACCCGAG |

## Appendix 2: Protein Standard Curve



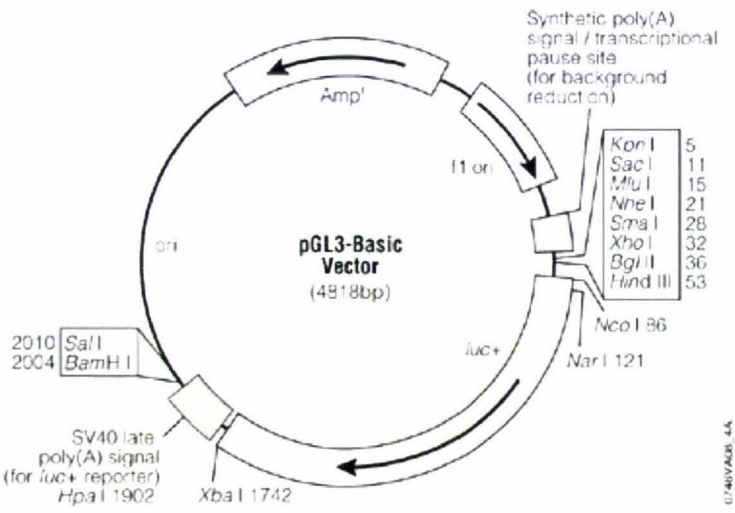

| BSA Standard ug | Average Absorbance at 595 nm |
|---|---|
| 0 | 0.266 |
| 0.4 | 0.333667 |
| 0.6 | 0.339667 |
| 0.8 | 0.390333 |
| 1 | 0.425333 |
| 1.5 | 0.498667 |
| 2 | 0.539333 |
| 2.5 | 0.629 |

| Absorbance at 595 nm HeLa Extract 1:40 dilution, 10 uL | Amount of Protein (ug) |
|---|---|
| 0.426 | 1.10 |
| 0.427 | 1.11 |
| 0.422 | 1.07 |
| | Average 1.09 |
| Concentration = 4.36 ug/ul | |

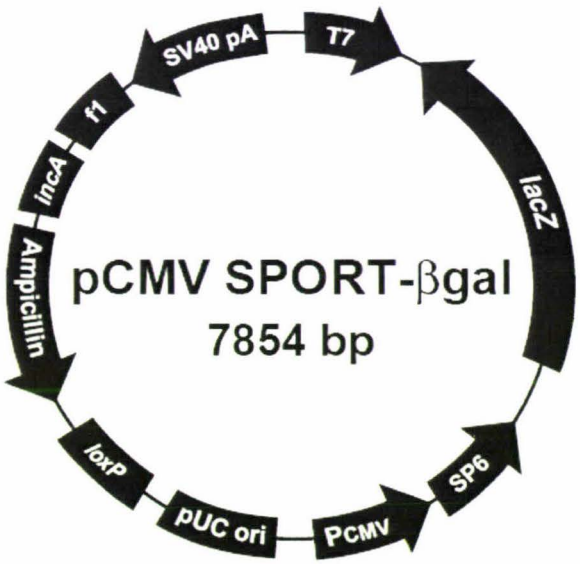
### Protein Standard Curve Results

The Bradford protein assay was carried out as described in section 2.2.16. To construct the standard curve, BSA was diluted to give several different concentrations, following which it was mixed with Bradford reagent and the absorbance at 595 nm read. HeLa extracts were also mixed with Bradford reagent, and the dilution that fell within the range of the standard curve and its absorbances at 595 nm are shown in the table on the left. Each dilution was carried out in triplicate; therefore the amount of protein in the HeLa extract was averaged and then used to calculate the concentration of protein in the extract.

## Appendix 3: Vector Maps




www.promega.com




www.invitrogen.com

The Sp1 expression vector was a gift from Dr Merlin Crossley, University of Sydney, Australia

The Sp3 expression vector was a gift from Dr Guntram Suske, Institute für Molekularbiologie and Tumorforschung, Marburg

## Appendix 4: Topoisomerase II Beta Sequences

### -654 Topoisomerase II Beta Sequence

```
WT      CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
654     CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
        ************************************************************

WT      CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
654     CCTTTCGGTTGTTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
        *********** ************************************************

WT      TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
654     TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCTACAAGGCCCGGATTGGACAGCATGGCG
        ********************************* ****************************

WT      CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
654     CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
        ************************************************************

WT      TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
654     TGTGCGGTGTTATGCCGGACAGGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
        ********************** ***************************************

WT      TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
654     TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
        ************************************************************

WT      CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
654     CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
        ************************************************************

WT      GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
654     GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
        ************************************************************

WT      CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
654     CCCCGACCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
        ***** ********************************************************

WT      GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
654     GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
        ************************************************************

WT      CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
654     CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACSCCGCSCCTCGAGTTTGAGGGCAGCCGG
        ********************************** **** ************************

WT      CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
654     CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
        ************************************************************

WT      CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
654     CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
        ************************************************************

WT      CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGGC
654     CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGGC
        ************************************************************

WT      ACTCGCCATG
654     ACTCGCCATG
        **********
```

## -654 ICB1 Mutant Topoisomerase II Beta Sequence

```
WT      CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
ICB1    CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
        ************************************************************

WT      CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
ICB1    CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
        ************************************************************

WT      TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
ICB1    TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGAAAAGGCCCGTCTAGAACAGCATGGCG
        ********************************** ********  * * ************

WT      CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
ICB1    CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
        ************************************************************

WT      TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
ICB1    TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
        ************************************************************

WT      TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
ICB1    TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
        ************************************************************

WT      CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
ICB1    CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
        ************************************************************

WT      GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
ICB1    GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
        ************************************************************

WT      CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
ICB1    CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
        ************************************************************

WT      GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
ICB1    GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
        ************************************************************

WT      CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
ICB1    CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
        ************************************************************

WT      CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
ICB1m   CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
        ************************************************************

WT      CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
ICB1m   CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
        ************************************************************

WT      CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
ICB1m   CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
        ************************************************************

WT      CACTCGCCATG
ICB1m   CACTCGCCATG
        ***********
```

## -654 ICB2 Mutant Topoisomerase II Beta Sequence

```
WT      CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
ICB2    CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
        ************************************************************

WT      CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
ICB2    CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
        ************************************************************

WT      TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
ICB2    TGGAATTTTGGAGATCTCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
        ***********     *  ******************************************

WT      CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
ICB2    CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
        ************************************************************

WT      TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
ICB2    TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
        ************************************************************

WT      TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
ICB2    TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
        ************************************************************

WT      CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
ICB2    CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
        ************************************************************

WT      GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
ICB2    GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
        ************************************************************

WT      CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
ICB2    CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
        ************************************************************

WT      GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
ICB2    GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
        ************************************************************

WT      CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
ICB2    CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
        ************************************************************

WT      CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
ICB2m   CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
        ************************************************************

WT      CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
ICB2m   CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
        ************************************************************

WT      CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
ICB2m   CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
        ************************************************************

WT      CACTCGCCATG
ICB2m   CACTCGCCATG
        ***********
```

## -654 GC1 Mutant Topoisomerase II Beta Sequence

```
WT      CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
GC1     CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
        ************************************************************

WT      CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
GC1     CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCGAGCTCCTCCAGGGGGCT
        ******************************************  ** **************

WT      TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
GC1     TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
        ************************************************************

WT      CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
GC1     CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
        ************************************************************

WT      TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
GC1     TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
        ************************************************************

WT      TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
GC1     TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
        ************************************************************

WT      CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
GC1     CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
        ************************************************************

WT      GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
GC1     GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
        ************************************************************

WT      CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
GC1     CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
        ************************************************************

WT      GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
GC1     GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
        ************************************************************

WT      CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
GC1     CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
        ************************************************************

WT      CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
GC1m    CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
        ************************************************************

WT      CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
GC1m    CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
        ************************************************************

WT      CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
GC1m    CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
        ************************************************************

WT      CACTCGCCATG
GC1m    CACTCGCCATG
        ***********
```

# -654 ICB1/ICB2 Mutant Topoisomerase II Beta Sequence

```
WT          CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
ICB1/ICB2m  CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
            ************************************************************

WT          CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
ICB1/ICB2m  CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
            ************************************************************

WT          TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
ICB1/ICB2m  TGGAATTTTGGAGATCTCCGAGAGGCTGTGGCGAAAAGGCCCGTCTAGAACAGCATGGCG
            ***********   *  ***************** ********  * * ************

WT          CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
ICB1/ICB2m  CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
            ************************************************************

WT          TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
ICB1/ICB2m  TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
            ************************************************************

WT          TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
ICB1/ICB2m  TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
            ************************************************************

WT          CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
ICB1/ICB2m  CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
            ************************************************************

WT          GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
ICB1/ICB2m  GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
            ************************************************************

WT          CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
ICB1/ICB2m  CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
            ************************************************************

WT          GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
ICB1/ICB2m  GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
            ************************************************************

WT          CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
ICB1/ICB2m  CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
            ************************************************************

WT          CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
ICB1/ICB2m  CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
            ************************************************************

WT          CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
ICB1/ICB2m  CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
            ************************************************************

WT          CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
ICB1/ICB2m  CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
            ************************************************************

WT          CACTCGCCATG
ICB1/ICB2   CACTCGCCATG
            ***********
```

## -654 ICB1/GC1 Mutant Topoisomerase II Beta Sequence

```
WT          CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
ICB1/GC1    CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
            ************************************************************

WT          CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
ICB1/GC1    CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCGAGCTCCTCCAGGGGGCT
            ******************************************  ** **************

WT          TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
ICB1/GC1    TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGAAAAGGCCCGTCTAGAACAGCATGGCG
            ********************************** ********  * * ************

WT          CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
ICB1/GC1    CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
            ************************************************************

WT          TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
ICB1/GC1    TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
            ************************************************************

WT          TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
ICB1/GC1    TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
            ************************************************************

WT          CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
ICB1/GC1    CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
            ************************************************************

WT          GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
ICB1/GC1    GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
            ************************************************************

WT          CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
ICB1/GC1    CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
            ************************************************************

WT          GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
ICB1/GC1    GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
            ************************************************************

WT          CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
ICB1/GC1    CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
            ************************************************************

WT          CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
ICB1/GC1    CGGCGCKGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCKCGATGGGGCG
            ****** ****************************************** ***********

WT          CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGC
ICB1/GC1    CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGC
            ************************************************************

WT          CCAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGA
ICB1/GC1    CCAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGA
            ************************************************************

WT          GGCACTCGCCATG
ICB1/GC1    GGCACTCGCCATG
            *************
```

## -654 ICB2/GC1 Mutant Topoisomerase II Beta Sequence

```
WT          CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
ICB2/GC1m   CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
            ************************************************************

WT          CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
ICB2/GC1m   CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCGAGCTCCACCAGGGGGCT
            ****************************************  ** ** **********

WT          TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
ICB2/GC1m   TGGAATTTTGGAGATCTCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
            ***********    *  ******************************************

WT          CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
ICB2/GC1m   CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
            ************************************************************

WT          TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
ICB2/GC1m   TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
            ************************************************************

WT          TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
ICB2/GC1m   TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
            ************************************************************

WT          CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
ICB2/GC1m   CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
            ************************************************************

WT          GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
ICB2/GC1m   GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
            ************************************************************

WT          CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
ICB2/GC1m   CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
            ************************************************************

WT          GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
ICB2/GC1m   GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
            ************************************************************

WT          CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
ICB2/GC1m   CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
            ************************************************************

WT          CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
ICB2/GC1m   CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGCG
            ************************************************************

WT          CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
ICB2/GC1m   CGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC
            ************************************************************

WT          CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
ICB2/GC1m   CAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
            ************************************************************

WT          CACTCGCCATG
ICB2/GC1m   CACTCGCCATG
            ***********
```

## -654 ICB1/ICB2/GC1 Mutant Topoisomerase II Beta Sequence

```
WT          CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
All_3       CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTT
            ************************************************************

WT          CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCT
All_3       CCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGTCCGAGCTCCTCCAGGGGGCT
            ****************************************  *  ** **************

WT          TGGAATTTTGGGATTGGCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCG
All_3       TGGAATTTTGGAGATCTCCGAGAG-CTGTGS-GAAAAGGCCCGTCTAGAACAGCATGGCG
            ***********   *  ******* *****  ** ********  * * ************

WT          CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
All_3       CTGACTGACAGCGGGGGCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTG
            ************************************************************

WT          TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
All_3       TGTGCGGTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGT
            ************************************************************

WT          TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
All_3       TTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTG
            ************************************************************

WT          CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
All_3       CCTTTGAAGCAGCGGCGGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTC
            ************************************************************

WT          GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
All_3       GCGGAGTGTGAGAAGGACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCT
            ************************************************************

WT          CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
All_3       CCCCGGCCGCCCTCGCGGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGC
            ************************************************************

WT          GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
All_3       GGGCGGGCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTCTCCT
            ************************************************************

WT          CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
All_3       CAGCCGCCGCGCTAGGCCCGGGCGACGCGGACGCCGCGCCTCGAGTTTGAGGGCAGCCGG
            ************************************************************

WT          CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGC
All_3       CGGCGCGGCCTCCTCAGCGGGCTCGGCTGGACGTCCGCTCCGGATCTTCGCGATGGGGC
            ************************************************************

WT          GCGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGC
All_3       GCGGGGGTCGGCGCGGCTAGGAGTGCGGCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGC
            ************************************************************

WT          CCAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
All_3       CCAGCGGCCCGCAGGGAGGCGGGAGCGGCGGCTGGGGCCTCAGGGCCTGTGAGCTGGAGG
            ************************************************************

WT          CACTCGCCATG
All_3       CACTCGCCATG
            ***********
```

## Appendix 5: Transient Transfection Data

### -1357 topoisomerase II beta promoter activity in the presence of Sp1

Experiment 1

**Luciferase Maxima**

| pcmvSPORT | pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|
| 68 | 93 | 572 | 1342 | 1646 | 2757 | 246 |
| 39 | 46 | 682 | 1320 | 1389 | 3086 | 316 |
| 43 | 39 | 635 | 1413 | 1497 | 2270 | 292 |
| | | | | | | 284.6667 |

**B-Gal Results**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.079 | 0.056 | 0.221 | 0.348 | 0.281 | 0.415 | 0.061 |
| 0.078 | 0.053 | 0.212 | 0.356 | 0.255 | 0.415 | 0.058 |
| 0.075 | 0.062 | 0.212 | 0.354 | 0.281 | 0.356 | 0.06 |
| | | | | | | 0.059667 |

**Luciferase minus blank**

| | | | | | |
|---|---|---|---|---|---|
| -216.667 | -191.667 | 287.3333 | 1057.333 | 1361.333 | 2472.333 |
| -245.667 | -238.667 | 397.3333 | 1035.333 | 1104.333 | 2801.333 |
| -241.667 | -245.667 | 350.3333 | 1128.333 | 1212.333 | 1985.333 |

**B-Gal minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 0.019333 | -0.00367 | 0.161333 | 0.288333 | 0.221333 | 0.355333 |
| 0.018333 | -0.00667 | 0.152333 | 0.296333 | 0.195333 | 0.355333 |
| 0.015333 | 0.002333 | 0.152333 | 0.294333 | 0.221333 | 0.296333 |

**Corrected: luciferase/B-gal**

| | | | | | |
|---|---|---|---|---|---|
| -11206.9 | 52272.73 | 1780.992 | 3667.052 | 6150.602 | 6957.786 |
| -13400 | 35800 | 2608.315 | 3493.813 | 5653.584 | 7883.677 |
| -15760.9 | -105286 | 2299.781 | 3833.522 | 5477.41 | 6699.663 |

**Averages**

| | | | | | |
|---|---|---|---|---|---|
| -13455.9 | -5737.66 | 2229.696 | 3664.796 | 5760.532 | 7180.375 |

**Corrected average relative to -1357**

| | | | | | |
|---|---|---|---|---|---|
| -603.487 | -257.329 | 100 | 164.363 | 258.355 | 322.0338 |

Average deviation from the mean

| | | | | | |
|---|---|---|---|---|---|
| 1536.632 | 66365.37 | 299.1362 | 113.9883 | 260.047 | 468.868 |

**Percentage error**

| | | | | | |
|---|---|---|---|---|---|
| -11.4197 | -1156.66 | 13.41601 | 3.11036 | 4.514288 | 6.529853 |

Experiment 2

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|
| 45 | 10 | 18017 | 22519 | 39211 | 23786 | 246 |
| 88 | 187 | 18034 | 25867 | 36704 | 25963 | 316 |
| 31 | 45 | 18167 | 24099 | 34005 | 17160 | 292 |
| | | | | | | 284.6667 |

**B-Gal results**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.291 | 0.069 | 0.38 | 0.301 | 0.379 | 0.397 | 0.044 |
| 0.232 | 0.065 | 0.391 | 0.37 | 0.382 | 0.378 | 0.047 |
| 0.281 | 0.07 | 0.398 | 0.397 | 0.402 | 0.403 | 0.046 |
| | | | | | | 0.045667 |

**Luciferase minus blank**

| | | | | | |
|---|---|---|---|---|---|
| -239.667 | -274.667 | 17732.33 | 22234.33 | 38926.33 | 23501.33 |
| -196.667 | -97.6667 | 17749.33 | 25582.33 | 36419.33 | 25678.33 |
| -253.667 | -239.667 | 17882.33 | 23814.33 | 33720.33 | 16875.33 |

**B-Gal minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 0.245333 | 0.023333 | 0.334333 | 0.255333 | 0.333333 | 0.351333 |
| 0.186333 | 0.019333 | 0.345333 | 0.324333 | 0.336333 | 0.332333 |
| 0.235333 | 0.024333 | 0.352333 | 0.351333 | 0.356333 | 0.357333 |

**Corrected: luciferase/B-gal**

| | | | | | |
|---|---|---|---|---|---|
| -976.902 | -11771.4 | 53037.89 | 87079.63 | 116779 | 66891.84 |
| -1055.46 | -5051.72 | 51397.68 | 78876.67 | 108283.4 | 77266.8 |
| -1077.9 | -9849.32 | 50754.02 | 67782.73 | 94631.43 | 47225.75 |
| | | Averages | | | |
| -1036.75 | -8890.82 | 51729.86 | 77913.01 | 106564.6 | 63794.8 |

**Corrected average relative to -1357**

| | | | | | |
|---|---|---|---|---|---|
| -2.00417 | -17.187 | 100 | 150.6152 | 206.0021 | 123.323 |

**Average deviation from the mean**

| | | | | | |
|---|---|---|---|---|---|
| 39.90122 | 2559.399 | 872.0152 | 6753.52 | 7955.464 | 11046.03 |

**Percentage error**

| | | | | | |
|---|---|---|---|---|---|
| -3.84867 | -28.787 | 1.685709 | 8.668026 | 7.465389 | 17.31494 |

Experiment 3

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|
| 165 | 48 | 6401 | 8870 | 12744 | 11178 | 246 |
| 61 | 86 | 8120 | 9072 | 15380 | 12498 | 316 |
| 131 | 78 | 7342 | 9924 | 15318 | 9130 | 292 |
| | | | | | | 284.6667 |

**B-Gal results**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.502 | 0.214 | 0.317 | 0.3 | 0.305 | 0.31 | 0.077 |
| 0.469 | 0.238 | 0.327 | 0.312 | 0.39 | 0.32 | 0.067 |
| 0.481 | 0.218 | 0.296 | 0.321 | 0.335 | 0.311 | 0.071 |
| | | | | | | 0.071667 |

**Luciferase minus blank**

| | | | | | |
|---|---|---|---|---|---|
| -119.667 | -236.667 | 6116.333 | 8585.333 | 12459.33 | 10893.33 |
| -223.667 | -198.667 | 7835.333 | 8787.333 | 15095.33 | 12213.33 |
| -153.667 | -206.667 | 7057.333 | 9639.333 | 15033.33 | 8845.333 |

**B-Gal minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 0.430333 | 0.142333 | 0.245333 | 0.228333 | 0.233333 | 0.238333 |
| 0.397333 | 0.166333 | 0.255333 | 0.240333 | 0.318333 | 0.248333 |
| 0.409333 | 0.146333 | 0.224333 | 0.249333 | 0.263333 | 0.239333 |

**Corrected: luciferase/B-gal**

| | | | | | |
|---|---|---|---|---|---|
| -278.079 | -1662.76 | 24930.71 | 37600 | 53397.14 | 45706.29 |
| -562.919 | -1194.39 | 30686.68 | 36563.11 | 47419.9 | 49181.21 |
| -375.407 | -1412.3 | 31459.14 | 38660.43 | 57088.61 | 36958.22 |
| | | | **Averages** | | |
| -405.469 | -1423.15 | 29025.51 | 37607.84 | 52635.22 | 43948.57 |

**Corrected average relative to -1357**

| | | | | | |
|---|---|---|---|---|---|
| -1.39694 | -4.9031 | 100 | 129.5683 | 181.3412 | 151.4136 |

**Average deviation from the mean**

| | | | | | |
|---|---|---|---|---|---|
| 104.9673 | 159.7417 | 2729.869 | 701.722 | 3476.88 | 4660.237 |

**Percentage error**

| | | | | | |
|---|---|---|---|---|---|
| -25.8879 | -11.2245 | 9.405067 | 1.865893 | 6.605616 | 10.60384 |

Collated Data

| pcmvSPORT | pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug |
|---|---|---|---|---|---|
| | | **Expt 1 Corrected: luciferase/B-gal** | | | |
| -11206.9 | 52272.73 | 1780.992 | 3667.052 | 6150.602 | 6957.786 |
| -13400 | 35800 | 2608.315 | 3493.813 | 5653.584 | 7883.677 |
| -15760.9 | -105286 | 2299.781 | 3833.522 | 5477.41 | 6699.663 |
| | | **Average** | | | |
| -13455.9 | -5737.66 | 2229.696 | 3664.796 | 5760.532 | 7180.375 |
| | | **Corrected average relative to -1357** | | | |
| **-603.487** | **-257.329** | **100** | **164.363** | **258.355** | **322.0338** |
| | | **Average deviation from the mean** | | | |
| 1536.632 | 66365.37 | 299.1362 | 113.9883 | 260.047 | 468.868 |
| | | **Percentage error** | | | |
| -11.4197 | -1156.66 | 13.41601 | 3.11036 | 4.514288 | 6.529853 |
| | | | | | |
| | | **Expt 2 Corrected: luciferase/B-gal** | | | |
| -976.902 | -11771.4 | 53037.89 | 87079.63 | 116779 | 66891.84 |
| -1055.46 | -5051.72 | 51397.68 | 78876.67 | 108283.4 | 77266.8 |
| -1077.9 | -9849.32 | 50754.02 | 67782.73 | 94631.43 | 47225.75 |
| | | **Average** | | | |
| -1036.75 | -8890.82 | 51729.86 | 77913.01 | 106564.6 | 63794.8 |
| | | **Corrected average relative to -1357** | | | |
| **-2.00417** | **-17.187** | **100** | **150.6152** | **206.0021** | **123.323** |
| | | **Average deviation from the mean** | | | |
| 39.90122 | 2559.399 | 872.0152 | 6753.52 | 7955.464 | 11046.03 |
| | | **Percentage error** | | | |
| -3.84867 | -28.787 | 1.685709 | 8.668026 | 7.465389 | 17.31494 |
| | | | | | |
| | | **Expt 3 Corrected: luciferase/B-gal** | | | |
| -278.079 | -1662.76 | 24930.71 | 37600 | 53397.14 | 45706.29 |
| -562.919 | -1194.39 | 30686.68 | 36563.11 | 47419.9 | 49181.21 |
| -375.407 | -1412.3 | 31459.14 | 38660.43 | 57088.61 | 36958.22 |
| | | **Average** | | | |
| -405.469 | -1423.15 | 29025.51 | 37607.84 | 52635.22 | 43948.57 |
| | | **Corrected average relative to -1357** | | | |
| **-1.39694** | **-4.9031** | **100** | **129.5683** | **181.3412** | **151.4136** |
| | | **Average deviation from the mean** | | | |
| 104.9673 | 159.7417 | 2729.869 | 701.722 | 3476.88 | 4660.237 |
| | | **Percentage error** | | | |
| -25.8879 | -11.2245 | 9.405067 | 1.865893 | 6.605616 | 10.60384 |
| | | | | | |
| | | **SUMMARY OF ALL EXPTS THIS SERIES** | | | |
| -603.487 | -257.329 | 100 | 164.363 | 258.355 | 322.0338 |
| -2.00417 | -17.187 | 100 | 150.6152 | 206.0021 | 123.323 |
| -1.39694 | -4.9031 | 100 | 129.5683 | 181.3412 | 151.4136 |
| | | **Average (graphed)** | | | |
| -202.296 | -93.1398 | 100 | 148.1821 | 215.2328 | 198.9235 |
| | | **Percentage error(graphed)** | | | |
| 267.4606 | 109.4597 | 0 | 12.40926 | 28.74816 | 82.07357 |

**-654 topoisomerase II beta promoter activity in the presence of Sp1**

Experiment 4

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 247 | 232 | 12989 | 4879 | 8418 | 24555 | 20503 | 522 |
| 236 | 246 | 13910 | 4663 | 9595 | 25537 | 21098 | 570 |
| 229 | 226 | 12852 | 4729 | 9011 | 22662 | 21652 | 490 |
| | | | | | | | 527.3333 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.138 | 0.093 | 0.404 | 0.294 | 0.416 | 0.599 | 0.556 | 0.082 |
| 0.151 | 0.098 | 0.438 | 0.277 | 0.395 | 0.639 | 0.489 | 0.088 |
| 0.162 | 0.1 | 0.434 | 0.284 | 0.432 | 0.571 | 0.517 | 0.088 |
| | | | | | | | 0.086 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| -280.333 | -295.333 | 12461.67 | 4351.667 | 7890.667 | 24027.67 | 19975.67 |
| -291.333 | -281.333 | 13382.67 | 4135.667 | 9067.667 | 25009.67 | 20570.67 |
| -298.333 | -301.333 | 12324.67 | 4201.667 | 8483.667 | 22134.67 | 21124.67 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.052 | 0.007 | 0.318 | 0.208 | 0.33 | 0.513 | 0.47 |
| 0.065 | 0.012 | 0.352 | 0.191 | 0.309 | 0.553 | 0.403 |
| 0.076 | 0.014 | 0.348 | 0.198 | 0.346 | 0.485 | 0.431 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| -5391.03 | -42190.5 | 39187.63 | 20921.47 | 23911.11 | 46837.56 | 42501.42 |
| -4482.05 | -23444.4 | 38018.94 | 21652.71 | 29345.2 | 45225.44 | 51043.84 |
| -3925.44 | -21523.8 | 35415.71 | 21220.54 | 24519.27 | 45638.49 | 49013.15 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| -4599.51 | -29052.9 | 37540.76 | 21264.91 | 25925.19 | 45900.49 | 47519.47 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| -12.252 | -77.3903 | 100 | 56.64485 | 69.05879 | 122.2684 | 126.581 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 527.6803 | 8758.377 | 1416.701 | 258.5327 | 2280.004 | 624.7086 | 3345.366 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| -11.4725 | -30.1463 | 3.773767 | 1.215772 | 8.794552 | 1.361006 | 7.039991 |

Experiment 5

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 140 | 135 | 7335 | 3453 | 7272 | 16749 | 13724 | 537 |
| 165 | 184 | 7596 | 3615 | 7314 | 16256 | 15459 | 434 |
| 187 | 175 | 7219 | 3200 | 6712 | 15322 | 14266 | 469 |
| | | | | | | | 480 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.159 | 0.082 | 0.475 | 0.29 | 0.452 | 0.75 | 0.601 | 0.074 |
| 0.17 | 0.083 | 0.472 | 0.302 | 0.515 | 0.67 | 0.619 | 0.077 |
| 0.175 | 0.093 | 0.48 | 0.325 | 0.536 | 0.717 | 0.599 | 0.079 |
| | | | | | | | 0.076667 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| -340 | -345 | 6855 | 2973 | 6792 | 16269 | 13244 |
| -315 | -296 | 7116 | 3135 | 6834 | 15776 | 14979 |
| -293 | -305 | 6739 | 2720 | 6232 | 14842 | 13786 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.082333 | 0.005333 | 0.398333 | 0.213333 | 0.375333 | 0.673333 | 0.524333 |
| 0.093333 | 0.006333 | 0.395333 | 0.225333 | 0.438333 | 0.593333 | 0.542333 |
| 0.098333 | 0.016333 | 0.403333 | 0.248333 | 0.459333 | 0.640333 | 0.522333 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| -4129.55 | -64687.5 | 17209.21 | 13935.94 | 18095.91 | 24161.88 | 25258.74 |
| -3375 | -46736.8 | 18000 | 13912.72 | 15590.87 | 26588.76 | 27619.55 |
| -2979.66 | -18673.5 | 16708.26 | 10953.02 | 13567.49 | 23178.55 | 26393.11 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| -3494.74 | -43365.9 | 17305.82 | 12933.89 | 15751.43 | 24643.07 | 26423.8 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| -20.194 | -250.586 | 100 | 74.73723 | 91.01807 | 142.3975 | 152.6873 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 423.2107 | 16461.65 | 462.7846 | 1320.582 | 1562.992 | 1297.132 | 797.1647 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| -12.1099 | -37.9599 | 2.674155 | 10.21024 | 9.922863 | 5.263679 | 3.016844 |

Experiment 6

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 54 | 66 | 15736 | 8006 | 13775 | 35620 | 39375 | 157 |
| 57 | 252 | 16657 | 8131 | 18814 | 40526 | 48300 | 231 |
| 0.6 | 40 | 13886 | 7487 | 16477 | 41691 | 40157 | 223 |
| | | | | | | | 203.6667 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.1 | 0.062 | 0.273 | 0.213 | 0.289 | 0.44 | 0.328 | 0.063 |
| 0.099 | 0.06 | 0.272 | 0.243 | 0.353 | 0.417 | 0.469 | 0.062 |
| 0.102 | 0.063 | 0.256 | 0.202 | 0.343 | 0.367 | 0.394 | 0.061 |
| | | | | | | | 0.062 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| -149.667 | -137.667 | 15532.33 | 7802.333 | 13571.33 | 35416.33 | 39171.33 |
| -146.667 | 48.33333 | 16453.33 | 7927.333 | 18610.33 | 40322.33 | 48096.33 |
| -203.067 | -163.667 | 13682.33 | 7283.333 | 16273.33 | 41487.33 | 39953.33 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.038 | 0 | 0.211 | 0.151 | 0.227 | 0.378 | 0.266 |
| 0.037 | -0.002 | 0.21 | 0.181 | 0.291 | 0.355 | 0.407 |
| 0.04 | 0.001 | 0.194 | 0.14 | 0.281 | 0.305 | 0.332 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| -3938.6 | 0 | 73612.95 | 51671.08 | 59785.61 | 93694 | 147260.7 |
| -3963.96 | -24166.7 | 78349.21 | 43797.42 | 63953.04 | 113584 | 118172.8 |
| -5076.67 | -163667 | 70527.49 | 52023.81 | 57912.22 | 136024 | 120341.4 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| -4326.41 | -62611.1 | 74163.22 | 49164.1 | 60550.29 | 114434 | 128591.6 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| -5.83363 | -84.4234 | 100 | 66.29176 | 81.64463 | 154.3002 | 173.39 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 500.1718 | 67370.37 | 2790.659 | 3577.788 | 2268.499 | 14393.34 | 12446.03 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| -11.5609 | -107.601 | 3.762862 | 7.277237 | 3.74647 | 12.57785 | 9.678725 |

Collated Data

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug |
|---|---|---|---|---|---|---|
| | | **Expt 1 Corrected: luciferase/B-gal** | | | | |
| -5391.03 | -42190.5 | 39187.63 | 20921.47 | 23911.11 | 46837.56 | 42501.42 |
| -4482.05 | -23444.4 | 38018.94 | 21652.71 | 29345.2 | 45225.44 | 51043.84 |
| -3925.44 | -21523.8 | 35415.71 | 21220.54 | 24519.27 | 45638.49 | 49013.15 |
| | | | **Average** | | | |
| -4599.51 | -29052.9 | 37540.76 | 21264.91 | 25925.19 | 45900.49 | 47519.47 |
| | | | **Corrected average relative to -1357** | | | |
| **-12.252** | **-77.3903** | **100** | **56.64485** | **69.05879** | **122.2684** | **126.581** |
| | | | **Average deviation from the mean** | | | |
| 527.6803 | 8758.377 | 1416.701 | 258.5327 | 2280.004 | 624.7086 | 3345.366 |
| | | | **Percentage error** | | | |
| -11.4725 | -30.1463 | 3.773767 | 1.215772 | 8.794552 | 1.361006 | 7.039991 |
| | | | | | | |
| | | **Expt 2 Corrected: luciferase/B-gal** | | | | |
| -4129.55 | -64687.5 | 17209.21 | 13935.94 | 18095.91 | 24161.88 | 25258.74 |
| -3375 | -46736.8 | 18000 | 13912.72 | 15590.87 | 26588.76 | 27619.55 |
| -2979.66 | -18673.5 | 16708.26 | 10953.02 | 13567.49 | 23178.55 | 26393.11 |
| | | | **Average** | | | |
| -3494.74 | -43365.9 | 17305.82 | 12933.89 | 15751.43 | 24643.07 | 26423.8 |
| | | | **Corrected average relative to -1357** | | | |
| **-20.194** | **-250.586** | **100** | **74.73723** | **91.01807** | **142.3975** | **152.6873** |
| | | | **Average deviation from the mean** | | | |
| 423.2107 | 16461.65 | 462.7846 | 1320.582 | 1562.992 | 1297.132 | 797.1647 |
| | | | **Percentage error** | | | |
| -12.1099 | -37.9599 | 2.674155 | 10.21024 | 9.922863 | 5.263679 | 3.016844 |
| | | | | | | |
| | | **Expt 3 Corrected: luciferase/B-gal** | | | | |
| -3938.6 | 0 | 73612.95 | 51671.08 | 59785.61 | 93694 | 147260.7 |
| -3963.96 | -24166.7 | 78349.21 | 43797.42 | 63953.04 | 113584 | 118172.8 |
| -5076.67 | -163667 | 70527.49 | 52023.81 | 57912.22 | 136024 | 120341.4 |
| | | | **Average** | | | |
| -4326.41 | -62611.1 | 74163.22 | 49164.1 | 60550.29 | 114434 | 128591.6 |
| | | | **Corrected average relative to -1357** | | | |
| **-5.83363** | **-84.4234** | **100** | **66.29176** | **81.64463** | **154.3002** | **173.39** |
| | | | **Average deviation from the mean** | | | |
| 500.1718 | 67370.37 | 2790.659 | 3577.788 | 2268.499 | 14393.34 | 12446.03 |
| | | | **Percentage error** | | | |
| -11.5609 | -107.601 | 3.762862 | 7.277237 | 3.74647 | 12.57785 | 9.678725 |
| | | | | | | |
| | | **SUMMARY OF ALL EXPTS THIS SERIES** | | | | |
| -12.252 | -77.3903 | 100 | 56.64485 | 69.05879 | 122.2684 | 126.581 |
| -20.194 | -250.586 | 100 | 74.73723 | 91.01807 | 142.3975 | 152.6873 |
| -5.83363 | -84.4234 | 100 | 66.29176 | 81.64463 | 154.3002 | 173.39 |
| | | | **Average (graphed)** | | | |
| -12.7599 | -137.467 | 100 | 65.89128 | 80.57383 | 139.6554 | 150.8861 |
| | | | **Percentage error(graphed)** | | | |
| 4.956077 | 75.41287 | 0 | 6.164289 | 7.676696 | 11.59132 | 16.20341 |

**-1357 topoisomerase II beta promoter activity in the presence of Sp3 – with and without β-galactosidase**

Experiment 7

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|
| 103 | 197 | 7894 | 7361 | 3045 | 530 | 119 |
| 30 | 81 | 12134 | 8810 | 2871 | 333 | 100 |
| 46 | 230 | 8068 | 5169 | 2748 | 347 | 159 |
| | | | | | | 126 |

**B-Gal results**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.202 | 0.108 | 0.244 | 0.21 | 0.118 | 0.092 | 0.064 |
| 0.157 | 0.062 | 0.348 | 0.3 | 0.122 | 0.086 | 0.069 |
| 0.186 | 0.072 | 0.28 | 0.217 | 0.121 | 0.092 | 0.069 |
| | | | | | | 0.067333 |

**Luciferase minus blank**

| | | | | | |
|---|---|---|---|---|---|
| -23 | 71 | 7768 | 7235 | 2919 | 404 |
| -96 | -45 | 12008 | 8684 | 2745 | 207 |
| -80 | 104 | 7942 | 5043 | 2622 | 221 |

**B-Gal minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 0.134667 | 0.040667 | 0.176667 | 0.142667 | 0.050667 | 0.024667 |
| 0.089667 | -0.00533 | 0.280667 | 0.232667 | 0.054667 | 0.018667 |
| 0.118667 | 0.004667 | 0.212667 | 0.149667 | 0.053667 | 0.024667 |

**Corrected: luciferase/B-gal**

| | | | | | |
|---|---|---|---|---|---|
| -170.792 | 1745.902 | 43969.81 | 50712.62 | 57611.84 | 16378.38 |
| -1070.63 | 8437.5 | 42783.85 | 37323.78 | 50213.41 | 11089.29 |
| -674.157 | 22285.71 | 37344.83 | 33694.88 | 48857.14 | 8959.459 |

**Averages**

| | | | | | |
|---|---|---|---|---|---|
| -638.527 | 10823.04 | 41366.16 | 40577.09 | 52227.47 | 12142.37 |

**Corrected average relative to -1357**

| | | | | | |
|---|---|---|---|---|---|
| -1.5436 | 26.16399 | 100 | 98.09247 | 126.2565 | 29.3534 |

**Average deviation from the mean**

| | | | | | |
|---|---|---|---|---|---|
| 311.8234 | 7641.784 | 2680.89 | 6757.016 | 3589.584 | 2824.003 |

**Percentage error**

| | | | | | |
|---|---|---|---|---|---|
| -48.8348 | 70.60664 | 6.480876 | 16.65229 | 6.872981 | 23.25742 |

Experiment 8

**Luciferase maxima**

| pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|
| 197 | 8639 | 5058 | 2834 | 1288 | 119 |
| 81 | 8787 | 3845 | 3842 | 1287 | 100 |
| 230 | 5971 | 2582 | 2208 | 1456 | 159 |
| | | | | | 126 |

**Luciferase minus blank**

| | | | | |
|---|---|---|---|---|
| 71 | 8513 | 4932 | 2708 | 1162 |
| -45 | 8661 | 3719 | 3716 | 1161 |
| 104 | 5845 | 2456 | 2082 | 1330 |

**Averages**

| | | | | |
|---|---|---|---|---|
| 43.33333 | 7673 | 3702.333 | 2835.333 | 1217.667 |

**Corrected average relative to -1357**

| | | | | |
|---|---|---|---|---|
| 0.564751 | 100 | 48.25144 | 36.95208 | 15.8695 |

**Average deviation from the mean**

| | | | | |
|---|---|---|---|---|
| 58.88889 | 1218.667 | 830.8889 | 587.1111 | 74.88889 |

**Percentage error**

| | | | | |
|---|---|---|---|---|
| 135.8974 | 15.88253 | 22.4423 | 20.70695 | 6.150196 |

**-1357 topoisomerase II beta promoter activity in the presence of Sp3**

Experiment 9

**Luciferase maxima**

| pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|
| 109 | 17533 | 8207 | 4869 | 1348 | 36 |
| 88 | 13928 | 7821 | 4811 | 1686 | 127 |
| 17 | 13636 | 6540 | 4319 | 1221 | 167 |
| | | | | | 110 |

**Luciferase minus blank**

| | | | | |
|---|---|---|---|---|
| -1 | 17423 | 8097 | 4759 | 1238 |
| -22 | 13818 | 7711 | 4701 | 1576 |
| -93 | 13526 | 6430 | 4209 | 1111 |

**Averages**

| | | | | |
|---|---|---|---|---|
| -38.6667 | 14922.33 | 7412.667 | 4556.333 | 1308.333 |

**Corrected average relative to -1357**

| | | | | |
|---|---|---|---|---|
| -0.25912 | 100 | 49.67498 | 30.53365 | 8.767619 |

**Average deviation from the mean**

| | | | | |
|---|---|---|---|---|
| 36.22222 | 1667.111 | 655.1111 | 231.5556 | 178.4444 |

**Percentage error**

| | | | | |
|---|---|---|---|---|
| -93.6782 | 11.17192 | 8.837725 | 5.082059 | 13.63907 |

Experiment 10

**Luciferase maxima**

| pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|
| 67 | 32156 | 10164 | 6372 | 2545 | 347 |
| 303 | 27411 | 10943 | 7052 | 2608 | 190 |
| 265 | 26322 | 11588 | 6374 | 2385 | 179 |
| | | | | | 238.6667 |

**Luciferase minus blank**

| | | | | |
|---|---|---|---|---|
| -171.667 | 31917.33 | 9925.333 | 6133.333 | 2306.333 |
| 64.33333 | 27172.33 | 10704.33 | 6813.333 | 2369.333 |
| 26.33333 | 26083.33 | 11349.33 | 6135.333 | 2146.333 |

**Averages**

| | | | | |
|---|---|---|---|---|
| -27 | 28391 | 10659.67 | 6360.667 | 2274 |

**Corrected average relative to -1357**

| | | | | |
|---|---|---|---|---|
| -0.0951 | 100 | 37.54594 | 22.40381 | 8.009581 |

**Average deviation from the mean**

| | | | | |
|---|---|---|---|---|
| 96.44444 | 2350.889 | 489.5556 | 301.7778 | 85.11111 |

**Percentage error**

| | | | | |
|---|---|---|---|---|
| -357.202 | 8.280402 | 4.592597 | 4.744436 | 3.742793 |

Experiment 11

**Luciferase maxima**

| pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|
| 122 | 22407 | 14452 | 6927 | 5590 | 401 |
| 121 | 28324 | 14016 | 6305 | 5595 | 214 |
| 120 | 20886 | 16118 | 6581 | 5811 | 234 |
| | | | | | 283 |

**Luciferase minus blank**

| | | | | |
|---|---|---|---|---|
| -161 | 22124 | 14169 | 6644 | 5307 |
| -162 | 28041 | 13733 | 6022 | 5312 |
| -163 | 20603 | 15835 | 6298 | 5528 |

**Averages**

| | | | | |
|---|---|---|---|---|
| -162 | 23589.33 | 14579 | 6321.333 | 5382.333 |

**Corrected average relative to -1357**

| | | | | |
|---|---|---|---|---|
| -0.68675 | 100 | 61.80336 | 26.79742 | 22.81681 |

**Average deviation from the mean**

| | | | | |
|---|---|---|---|---|
| 0.666667 | 2967.778 | 837.3333 | 215.1111 | 97.11111 |

**Percentage error**

| | | | | |
|---|---|---|---|---|
| -0.41152 | 12.58102 | 5.743421 | 3.402939 | 1.804257 |

Collated Data

| pGL3Basic | 0 ug | 0.25 ug | 0.5 ug | 1 ug |
|---|---|---|---|---|
| | | **Expt 1 Average** | | |
| -38.6667 | 14922.33 | 7412.667 | 4556.333 | 1308.333 |
| | | **Corrected average relative to -1357** | | |
| **-0.25912** | **100** | **49.67498** | **30.53365** | **8.767619** |
| | | **Average deviation from the mean** | | |
| 36.22222 | 1667.111 | 655.1111 | 231.5556 | 178.4444 |
| | | **Percentage error** | | |
| -93.6782 | 11.17192 | 8.837725 | 5.082059 | 13.63907 |
| | | | | |
| | | **Expt 2 Average** | | |
| -27 | 28391 | 10659.67 | 6360.667 | 2274 |
| | | **Corrected average relative to -1357** | | |
| **-0.0951** | **100** | **37.54594** | **22.40381** | **8.009581** |
| | | **Average deviation from the mean** | | |
| 96.44444 | 2350.889 | 489.5556 | 301.7778 | 85.11111 |
| | | **Percentage error** | | |
| -357.202 | 8.280402 | 4.592597 | 4.744436 | 3.742793 |
| | | | | |
| | | **Expt 3 Average** | | |
| -405.469 | -1423.15 | 29025.51 | 37607.84 | 52635.22 |
| | | **Corrected average relative to -1357** | | |
| **-0.68675** | **100** | **61.80336** | **26.79742** | **22.81681** |
| | | **Average deviation from the mean** | | |
| 0.666667 | 2967.778 | 837.3333 | 215.1111 | 97.11111 |
| | | **Percentage error** | | |
| -0.41152 | 12.58102 | 5.743421 | 3.402939 | 1.804257 |
| | | | | |
| | | **SUMMARY OF ALL EXPTS THIS SERIES** | | |
| -0.25912 | 100 | 49.67498 | 30.53365 | 8.767619 |
| -0.0951 | 100 | 37.54594 | 22.40381 | 8.009581 |
| -0.68675 | 100 | 61.80336 | 26.79742 | 22.81681 |
| | | **Average (graphed)** | | |
| -0.34699 | 100 | 49.67476 | 26.5783 | 13.198 |
| | | **Percentage error(graphed)** | | |
| 0.226507 | 0 | 8.085882 | 2.782988 | 6.412538 |

**-654 topoisomerase II beta promoter activity in the presence of Sp3**

Experiment 12

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 60 | 60 | 8132 | 3993 | 2308 | 1296 | 398 | 130 |
| 56 | 75 | 8766 | 3915 | 2674 | 1371 | 405 | 185 |
| 65 | 53 | 7604 | 3685 | 2345 | 1321 | 386 | 184 |
| | | | | | | | 166.3333 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.302 | 0.066 | 0.534 | 0.368 | 0.339 | 0.263 | 0.288 | 0.067 |
| 0.287 | 0.069 | 0.546 | 0.358 | 0.338 | 0.28 | 0.305 | 0.064 |
| 0.314 | 0.067 | 0.517 | 0.351 | 0.369 | 0.274 | 0.311 | 0.065 |
| | | | | | | | 0.065333 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| -106.333 | -106.333 | 7965.667 | 3826.667 | 2141.667 | 1129.667 | 231.6667 |
| -110.333 | -91.3333 | 8599.667 | 3748.667 | 2507.667 | 1204.667 | 238.6667 |
| -101.333 | -113.333 | 7437.667 | 3518.667 | 2178.667 | 1154.667 | 219.6667 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.236667 | 0.000667 | 0.468667 | 0.302667 | 0.273667 | 0.197667 | 0.222667 |
| 0.221667 | 0.003667 | 0.480667 | 0.292667 | 0.272667 | 0.214667 | 0.239667 |
| 0.248667 | 0.001667 | 0.451667 | 0.285667 | 0.303667 | 0.208667 | 0.245667 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| -449.296 | -159500 | 16996.44 | 12643.17 | 7825.822 | 5715.008 | 1040.419 |
| -497.744 | -24909.1 | 17891.12 | 12808.66 | 9196.822 | 5611.801 | 995.8275 |
| -407.507 | -68000 | 16467.16 | 12317.39 | 7174.533 | 5533.546 | 894.1655 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| -451.516 | -84136.4 | 17118.24 | 12589.74 | 8065.726 | 5620.119 | 976.8041 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| -2.63763 | -491.501 | 100 | 73.54574 | 47.11772 | 32.83117 | 5.706217 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 30.81917 | 50242.42 | 515.2543 | 181.5679 | 754.0639 | 63.25984 | 55.09236 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| -6.82571 | -59.7155 | 3.009972 | 1.442189 | 9.34899 | 1.125596 | 5.640063 |

Experiment 13

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 1826 | 1357 | 10236 | 8096 | 10417 | 4699 | 3017 | 937 |
| 1386 | 1504 | 10076 | 7430 | 10649 | 5328 | 3011 | 987 |
| 624 | 1470 | 8736 | 7887 | 9398 | 4301 | 2673 | 985 |
| | | | | | | | 969.6667 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.123 | 0.066 | 0.281 | 0.293 | 0.396 | 0.265 | 0.19 | 0.066 |
| 0.112 | 0.066 | 0.313 | 0.287 | 0.442 | 0.283 | 0.226 | 0.066 |
| 0.124 | 0.066 | 0.305 | 0.365 | 0.388 | 0.254 | 0.221 | 0.063 |
| | | | | | | | 0.065 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 856.3333 | 387.3333 | 9266.333 | 7126.333 | 9447.333 | 3729.333 | 2047.333 |
| 416.3333 | 534.3333 | 9106.333 | 6460.333 | 9679.333 | 4358.333 | 2041.333 |
| -345.667 | 500.3333 | 7766.333 | 6917.333 | 8428.333 | 3331.333 | 1703.333 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.058 | 0.001 | 0.216 | 0.228 | 0.331 | 0.2 | 0.125 |
| 0.047 | 0.001 | 0.248 | 0.222 | 0.377 | 0.218 | 0.161 |
| 0.059 | 0.001 | 0.24 | 0.3 | 0.323 | 0.189 | 0.156 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| 14764.37 | 387333.3 | 42899.69 | 31255.85 | 28541.79 | 18646.67 | 16378.67 |
| 8858.156 | 534333.3 | 36719.09 | 29100.6 | 25674.62 | 19992.35 | 12679.09 |
| -5858.76 | 500333.3 | 32359.72 | 23057.78 | 26093.91 | 17626.1 | 10918.8 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| 5921.256 | 474000 | 37326.17 | 27804.74 | 26770.11 | 18755.04 | 13325.52 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| 15.86355 | | 100 | 74.49129 | 71.71942 | 50.24636 | 35.70021 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 7853.342 | 57777.78 | 3715.683 | 3164.643 | 1181.122 | 824.8757 | 2035.431 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| 132.6297 | 12.1894 | 9.954634 | 11.38167 | 4.412093 | 4.398154 | 15.27469 |

Experiment 14

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug | Blank |
|---|---|---|---|---|---|---|---|
| 59 | 193 | 23870 | 38659 | 19512 | 11859 | 3457 | 79 |
| 111 | 194 | 20560 | 44888 | 20511 | 12845 | 3944 | 66 |
| 246 | 271 | 24084 | 38191 | 17625 | 10644 | 3581 | 154 |
| | | | | | | | 99.66667 |

**B-Gal results**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 0.775 | 0.066 | 0.283 | 0.69 | 0.524 | 0.402 | 0.245 | 0.062 |
| 0.733 | 0.064 | 0.277 | 0.714 | 0.488 | 0.407 | 0.29 | 0.062 |
| 0.841 | 0.069 | 0.291 | 0.656 | 0.477 | 0.411 | 0.271 | 0.062 |
| | | | | | | | 0.062 |

**Luciferase minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| -40.6667 | 93.33333 | 23770.33 | 38559.33 | 19412.33 | 11759.33 | 3357.333 |
| 11.33333 | 94.33333 | 20460.33 | 44788.33 | 20411.33 | 12745.33 | 3844.333 |
| 146.3333 | 171.3333 | 23984.33 | 38091.33 | 17525.33 | 10544.33 | 3481.333 |

**B-Gal minus blank**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.713 | 0.004 | 0.221 | 0.628 | 0.462 | 0.34 | 0.183 |
| 0.671 | 0.002 | 0.215 | 0.652 | 0.426 | 0.345 | 0.228 |
| 0.779 | 0.007 | 0.229 | 0.594 | 0.415 | 0.349 | 0.209 |

**Corrected: luciferase/B-gal**

| | | | | | | |
|---|---|---|---|---|---|---|
| -57.036 | 23333.33 | 107558.1 | 61400.21 | 42018.04 | 34586.27 | 18346.08 |
| 16.89021 | 47166.67 | 95164.34 | 68693.76 | 47913.93 | 36943 | 16861.11 |
| 187.8477 | 24476.19 | 104735.1 | 64126.82 | 42229.72 | 30212.99 | 16657.1 |

**Averages**

| | | | | | | |
|---|---|---|---|---|---|---|
| 49.23396 | 31658.73 | 102485.8 | 64740.27 | 44053.89 | 33914.09 | 17288.1 |

**Corrected average relative to -1357**

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.04804 | | 100 | 63.16997 | 42.98535 | 33.09149 | 16.86877 |

**Average deviation from the mean**

| | | | | | | |
|---|---|---|---|---|---|---|
| 92.40914 | 10338.62 | 4880.993 | 2635.664 | 2573.355 | 2467.398 | 705.3243 |

**Percentage error**

| | | | | | | |
|---|---|---|---|---|---|---|
| 187.6939 | 32.65647 | 4.762603 | 4.071136 | 5.84138 | 7.275437 | 4.079826 |

Collated Data

| pcmvSPORT | pGL3Basic | -1357 | 0 ug | 0.25 ug | 0.5 ug | 1 ug |
|---|---|---|---|---|---|---|
| | | | **Expt 1 Corrected: luciferase/B-gal** | | | |
| -449.296 | -159500 | 16996.44 | 12643.17 | 7825.822 | 5715.008 | 1040.419 |
| -497.744 | -24909.1 | 17891.12 | 12808.66 | 9196.822 | 5611.801 | 995.8275 |
| -407.507 | -68000 | 16467.16 | 12317.39 | 7174.533 | 5533.546 | 894.1655 |
| | | | **Average** | | | |
| -451.516 | -84136.4 | 17118.24 | 12589.74 | 8065.726 | 5620.119 | 976.8041 |
| | | | **Corrected average relative to -1357** | | | |
| **-2.63763** | **-491.501** | **100** | **73.54574** | **47.11772** | **32.83117** | **5.706217** |
| | | | **Average deviation from the mean** | | | |
| 30.81917 | 50242.42 | 515.2543 | 181.5679 | 754.0639 | 63.25984 | 55.09236 |
| | | | **Percentage error** | | | |
| -6.82571 | -59.7155 | 3.009972 | 1.442189 | 9.34899 | 1.125596 | 5.640063 |
| | | | | | | |
| | | | **Expt 2 Corrected: luciferase/B-gal** | | | |
| 14764.37 | 387333.3 | 42899.69 | 31255.85 | 28541.79 | 18646.67 | 16378.67 |
| 8858.156 | 534333.3 | 36719.09 | 29100.6 | 25674.62 | 19992.35 | 12679.09 |
| -5858.76 | 500333.3 | 32359.72 | 23057.78 | 26093.91 | 17626.1 | 10918.8 |
| | | | **Average** | | | |
| 5921.256 | 474000 | 37326.17 | 27804.74 | 26770.11 | 18755.04 | 13325.52 |
| | | | **Corrected average relative to -1357** | | | |
| **15.86355** | **1269.887** | **100** | **74.49129** | **71.71942** | **50.24636** | **35.70021** |
| | | | **Average deviation from the mean** | | | |
| 7853.342 | 57777.78 | 3715.683 | 3164.643 | 1181.122 | 824.8757 | 2035.431 |
| | | | **Percentage error** | | | |
| 132.6297 | 12.1894 | 9.954634 | 11.38167 | 4.412093 | 4.398154 | 15.27469 |
| | | | | | | |
| | | | **Expt 3 Corrected: luciferase/B-gal** | | | |
| -57.036 | 23333.33 | 107558.1 | 61400.21 | 42018.04 | 34586.27 | 18346.08 |
| 16.89021 | 47166.67 | 95164.34 | 68693.76 | 47913.93 | 36943 | 16861.11 |
| 187.8477 | 24476.19 | 104735.1 | 64126.82 | 42229.72 | 30212.99 | 16657.1 |
| | | | **Average** | | | |
| 49.23396 | 31658.73 | 102485.8 | 64740.27 | 44053.89 | 33914.09 | 17288.1 |
| | | | **Corrected average relative to -1357** | | | |
| **0.04804** | **30.89084** | **100** | **63.16997** | **42.98535** | **33.09149** | **16.86877** |
| | | | **Average deviation from the mean** | | | |
| 92.40914 | 10338.62 | 4880.993 | 2635.664 | 2573.355 | 2467.398 | 705.3243 |
| | | | **Percentage error** | | | |
| 187.6939 | 32.65647 | 4.762603 | 4.071136 | 5.84138 | 7.275437 | 4.079826 |
| | | | | | | |
| | | | **SUMMARY OF ALL EXPTS THIS SERIES** | | | |
| -2.63763 | -491.501 | 100 | 73.54574 | 47.11772 | 32.83117 | 5.706217 |
| 15.86355 | 1269.887 | 100 | 74.49129 | 71.71942 | 50.24636 | 35.70021 |
| 0.04804 | 30.89084 | 100 | 63.16997 | 42.98535 | 33.09149 | 16.86877 |
| | | | **Average (graphed)** | | | |
| 4.424654 | 269.7588 | 0 | 70.40233 | 53.94083 | 38.72301 | 19.42507 |
| | | | **Percentage error(graphed)** | | | |
| 7.625931 | 666.7519 | 0 | 4.821576 | 11.85239 | 7.682238 | 10.8501 |

**Effect of Mutations on Topoisomerase II Beta Promoter Activity**

Experiment 15

**Luciferase maxima**

| pcmvSPORT | pGL3Basic | WT -1357 | WT -654 | -654 ICB1m | -654 ICB2m |
|---|---|---|---|---|---|
| 38 | 111 | 7643 | 5039 | 4310 | 351 |
| 70 | 137 | 7995 | 4982 | 5028 | 487 |
| 109 | 133 | 7098 | 4190 | 4387 | 414 |

**B-Gal results**

| | | | | | |
|---|---|---|---|---|---|
| 0.607 | 0.088 | 0.46 | 0.545 | 0.367 | 0.368 |
| 0.567 | 0.09 | 0.475 | 0.503 | 0.404 | 0.39 |
| 0.632 | 0.1 | 0.478 | 0.472 | 0.389 | 0.407 |

**Luciferase minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 38 | 111 | 7643 | 5039 | 4310 | 351 |
| 70 | 137 | 7995 | 4982 | 5028 | 487 |
| 109 | 133 | 7098 | 4190 | 4387 | 414 |

**B-Gal minus blank**

| | | | | | |
|---|---|---|---|---|---|
| 0.607 | 0.088 | 0.46 | 0.545 | 0.367 | 0.368 |
| 0.567 | 0.09 | 0.475 | 0.503 | 0.404 | 0.39 |
| 0.632 | 0.1 | 0.478 | 0.472 | 0.389 | 0.407 |

**Corrected: luciferase/B-gal**

| | | | | | |
|---|---|---|---|---|---|
| 62.60297 | 1261.364 | 16615.22 | 9245.872 | 11743.87 | 953.8043 |
| 123.4568 | 1522.222 | 16831.58 | 9904.573 | 12445.54 | 1248.718 |
| 172.4684 | 1330 | 14849.37 | 8877.119 | 11277.63 | 1017.199 |

**Averages**

| | | | | | |
|---|---|---|---|---|---|
| 119.5094 | 1371.195 | 16098.72 | 9342.521 | 11822.35 | 1073.24 |

**Corrected average relative to -1357**

| | | | | | |
|---|---|---|---|---|---|
| 0.742353 | 8.517417 | 100 | 58.03268 | 73.43657 | 6.666618 |

**Average deviation from the mean**

| | | | | | |
|---|---|---|---|---|---|
| 37.9376 | 100.6846 | 832.9003 | 374.7011 | 415.4633 | 116.985 |

**Percentage error**

| | | | | | |
|---|---|---|---|---|---|
| 31.74446 | 7.342836 | 5.173704 | 4.010707 | 3.51422 | 10.90017 |

**Luciferase maxima**

| -654 GC1m | -654 ICB1/ICB2m | -654 ICB1/GC1m | -654 ICB2/GC1m | -654 ICB1/ ICB2/GC1m | Blank |
|---|---|---|---|---|---|
| 908 | 356 | 460 | 753 | 480 | 57 |
| 1164 | 383 | 511 | 920 | 495 | 67 |
| 753 | 363 | 530 | 893 | 525 | 121 |
| | | | | | 81.66667 |

**B-Gal results**

| | | | | | |
|---|---|---|---|---|---|
| 0.291 | 0.319 | 0.247 | 0.327 | 0.283 | 0.07 |
| 0.308 | 0.307 | 0.251 | 0.373 | 0.319 | 0.075 |
| 0.3 | 0.33 | 0.259 | 0.4 | 0.3 | 0.075 |
| | | | | | 0.073333 |

**Luciferase minus blank**

| | | | | |
|---|---|---|---|---|
| 826.3333 | 274.3333 | 378.3333 | 671.3333 | 398.3333 |
| 1082.333 | 301.3333 | 429.3333 | 838.3333 | 413.3333 |
| 671.3333 | 281.3333 | 448.3333 | 811.3333 | 443.3333 |

**B-Gal minus blank**

| | | | | |
|---|---|---|---|---|
| 0.217667 | 0.245667 | 0.173667 | 0.253667 | 0.209667 |
| 0.234667 | 0.233667 | 0.177667 | 0.299667 | 0.245667 |
| 0.226667 | 0.256667 | 0.185667 | 0.326667 | 0.226667 |

**Corrected: luciferase/B-gal**

| | | | | |
|---|---|---|---|---|
| 3796.325 | 1116.689 | 2178.503 | 2646.518 | 1899.841 |
| 4612.216 | 1289.586 | 2416.51 | 2797.553 | 1682.497 |
| 2961.765 | 1096.104 | 2414.722 | 2483.673 | 1955.882 |

**Averages**

| | | | | |
|---|---|---|---|---|
| 3790.102 | 1167.46 | 2336.578 | 2642.581 | 1846.073 |

**Corrected average relative to -1357**

| | | | | |
|---|---|---|---|---|
| 20.09066 | 6.1885 | 12.38579 | 14.00786 | 9.785711 |

**Average deviation from the mean**

| | | | | |
|---|---|---|---|---|
| 552.2247 | 81.41765 | 105.3836 | 105.9386 | 109.0511 |

**Percentage error**

| | | | | |
|---|---|---|---|---|
| 14.57018 | 6.973915 | 4.510168 | 4.008905 | 5.907195 |

# Errata

Transcriptional regulation of human topoisomerase II beta
Claire Mawson

Figures 3.5, page 55; 3.6, page 57; 3.7, page 59; 3.9, page 64; 3.11, page 68; and 4.5, page 87 have suffered formatting errors in the production of the pdf file. This has shifted arrows slightly up the page so that they do not line up with the intended bands.

Section 5.3.3, page 118, line #4 should read as follows. "... and suggests that ICB1 is a negative element,..."