Regulation of the Human Topoisomerase IIβ Promoter

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Abstract

Eukaryotic DNA topoisomerase II is a ubiquitous nuclear enzyme essential for maintaining the correct conformation of DNA. The enzyme acts to catalyse changes in the tertiary structure of DNA, via the introduction of transient double-stranded breaks. Mammalian cells express two isoforms of type II topoisomerase, designated topoisomerase IIα and topoisomerase IIβ, which display differential expression and intracellular localisation. Levels of topoisomerase IIα gene expression are elevated in rapidly proliferating cells, whereas the β isoform is expressed at approximately equal levels throughout the cell cycle.

Protein products of the two isoforms of topoisomerase II found in human cells are the primary intracellular targets of many common, effective chemotherapy drugs. The development of drug resistance, however, is a major clinical problem caused by both enzymes. The levels of topoisomerase IIα and topoisomerase IIβ are important determinants for the sensitivity of cells to the cytoxicity of drugs, with down-regulation of topoisomerase II thought to be a major factor involved in drug resistance.

The rate of transcription is the main mechanism for controlling topoisomerase II expression and activity, and this is achieved by the binding of transcription factors to specific regulatory elements within the promoter sequence. Molecular mechanisms responsible for the regulation of expression of the topoisomerase II enzymes are thought to be associated with resistance to chemotherapy drugs, and therefore understanding these mechanisms is an important research focus.

This study reports the cloning and characterisation of a 1.5 kb fragment of the 5'-flanking and untranslated region of the topoisomerase IIβ promoter (-1357 to +122). Analyses of 5'-serially and internally deleted luciferase reporter constructs revealed a region upstream of the transcription start site (-1357 to -1228), which could have a negative regulatory role, and suggested 55% of topoisomerase IIβ promoter activity could be attributed to the region between -654 and -456. Mutational analysis of putative regulatory elements indicated that the two inverted CCAAT box (ICB1 and ICB2) within the latter region were important for regulation of topoisomerase IIβ promoter activity. Gel mobility shift assays indicated that both inverted CCAAT boxes in the promoter bound the transcription factor NF-Y, while ICB2 and a GC element were capable of binding transcription factors Sp1 and Sp3.

Abbreviations

Amp	Ampicillin
Amsacrine	Topoisomerase II poison
Ap-2	Activator protein 2
ATF	Activating transcription factor
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
β-Gal	β-Galactosidase
bp	Base pairs (DNA)
BSA	Bovine serum albumin
CDE	Cell-cycle dependent element
cDNA	Synthetic DNA, generated from RNA
cpm	counts per minute
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EMSA	Electrophoretic mobility shift assay
FCS	Foetal calf serum
GCG	Genetics computer group
G segment	Gated segment (DNA)
IPTG	Isopropyl thiogalactoside
HAT	Histone acetyl transferases
HeLa	Human cervical carcinoma cells
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]
HFM	Histone fold motif
ICB	Inverted CCAAT box
ICBP90	Inverted CCAAT box binding protein Mr 90 kDa
IgG	Immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases (DNA)

LB	Luria Bertani bacteriological media
MCS	Multiple cloning site
MDR	Multidrug resistance
MDR1	Multidrug resistance gene
MRP	Multidrug resistance-associated protein
MEM	Eagle's minimal essential media
mt	mutated/mutant
NEB	New England Biolabs
NF-Y	Nuclear factor Y
ONPG	o-Nitrophenol β-D-Galacto-pyranoside
PAGE	Polyacrylamide gel electrophoresis
p53	Tumour suppressor protein p53
PBS	Phosphate buffered saline
PBSE	Phosphate buffered saline plus EDTA
pBS SK+	pBluescript SK+ vector
PEG	Polyethylene glycol
pGL3B	pGL3Basic vector
PIC	Pre-iniation complex
Pol II	RNA polymerase II
Q-rich	Glutamine-rich
Rb	Retinoblastoma protein
RNase	Ribonuclease
RT	Room temperature
Sp1	Specificity protein 1
Sp3	Specificity protein 3
STET	Sucrose, Tris, EDTA and triton-X buffer
SV40	Simian virus 40
T segment	Transport segment (DNA)
TAE	Tris acetate EDTA buffer
TAF's	TBP associated factors
TATA	TATA box; conserved A/T rich septameter transcription sequence
TBE	Tris borate EDTA
TBP	TATA binding protein

TE	Tris-EDTA buffer (10 mM Tris pH 8.0, 1 mM EDTA)
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEN	Tris-EDTA buffer with sodium (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl)
TIF's	Transcription initiation factors
TFIID	Transcription initiation factor complex; TBP and TAFs
TF	Transcription factor
TMTC	To many to count
UV	Ultra-violet light
VM-26	Etoposide
X-gal	5-bromo-4 chromo-3-indolyl-β-D-galactopyranoside
Wt	Wild type

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Chapter 1: Introduction.

1.1 Overview.

DNA topoisomerases are essential nuclear enzymes which catalyse topological genomic changes via the introduction of transient single or double-stranded breaks. Topoisomerases are classified as either type I or type II, based on their mechanism of action. In humans, two closely related but genetically distinct isoforms of topoisomerase II are found, topoisomerase IIα and β. The two isoforms share the same catalytic function, but differences in biochemical properties, nuclear localisation and expression patterns suggest different, as yet unknown, roles for each enzyme (Chung *et al.,* 1989; Zandvliet *et al.,* 1996). Topoisomerase IIα is localised in the nucleoplasm and its expression is cell cycle modulated (Grue *et al.,* 1998), whereas topoisomerase IIβ is localised in the nucleolus, with a relatively constant transcription rate throughout the cell cycle (Cowell *et al.,* 1998).

A number of effective anti-cancer drugs target type II topoisomerases in mammalian cells. These drugs, termed topoisomerase II poisons or topoisomerase II inhibitors, act in different ways to inhibit the normal action of the topoisomerase II enzyme. Topoisomerase II poisons act to stabilise enzyme-DNA complexes, leading to an accumulation of double-stranded DNA breaks, which result in cell death. Topoisomerase II inhibitors can inhibit enzyme activity by various mechanisms, which do not involve the stabilisation of the DNA-enzyme complex. The development of resistance to these drugs is a major clinical problem, as illustrated by the development of drug resistance in approximately 60% of breast cancers treated using the topoisomerase II poison, doxorubicin. Topoisomerase IIα and β show different patterns of drug sensitivity and the level of topoisomerase II protein in cells correlates with sensitivity to killing by these drugs.

The down-regulation of topoisomerase II is one of several mechanisms implicated in the resistance to topoisomerase II drugs. A number of studies have determined that decreased levels of topoisomerase IIα and β are present in resistant cell lines and tumours (Son *et al.,* 1998; Dingemans *et al.,* 1998; Lage *et al.,* 2000). The ratio of topoisomerase IIα and β also appears to be a significant factor in the development of drug resistance (Withoff *et al.,* 1996; Padget el al, 2000). Other cellular events

implicated in the development of drug resistance include; mutations in topoisomerase II proteins (Dingemans *et al.,* 1998; Robert and Larsen, 1998), the phosphorylation state of the enzyme (Burden and Sullivan, 1994), and topoisomerase II RNA stability (Goswami *et al.,* 1996).

A number of studies focusing on the Topoisomerase IIα promoter have used binding and functional assays to partially characterise the promoter (Hochhauser *et al.,* 1992; Isaacs *et al.,* 1996a; reviewed in Isaacs *et al.,* 1998; Bakshi *et al.,* 2001). In contrast, the topoisomerase IIβ promoter has been the subject of limited investigations (Ng *et al.,* 1997; Lok *et al.,* 2002), therefore little is known about the clinical relevance or regulation of this isoform. This thesis focuses on the transcriptional regulation of human topoisomerase IIβ as a means to understanding the molecular mechanisms responsible for drug resistance.

1.2 Topoisomerases.

Topoisomerases share the common role of relaxing DNA to relieve torsional stress that occurs during DNA replication, transcription, recombination and cell division. These enzymes act by introducing transient breaks into the DNA helix and passing a second intact DNA strand through the break, before the original strand is religated (refer to figure 1.1). An enzyme-bridged intermediate (cleavable complex) is produced during the cleavage reaction, in which a tyrosine residue in the active site of each protein monomer becomes covalently linked to a 5'-phosphate group of one of the DNA strands (reviewed by Capranico and Binaschi, 1998). The catalytic cycle ends with the release of the DNA and the enzyme returns to its original confirmation (Champoux, 2001).

Three types of topoisomerase are found in eukaryotes: I, II and III. Topoisomerase I enzymes are able to relax both positively and negatively supercoiled DNA. These enzymes catalyse a conformational change in DNA by introduction of a transient single-stranded DNA break, thereby relieving one supercoil for each catalytic cycle. Topoisomerase I activity is generally associated with transcription and DNA replication in humans (Isaacs *et al.,* 1995), and is ATP independent. Topoisomerase III is a novel enzyme in humans, which is thought to partially relax negative DNA supercoiling in an

ATP-dependent process during replication (Nitiss, 1998), and appears to have a higher affinity for single-stranded DNA (Kim *et al.,* 1998).

Of the three topoisomerases only type II is essential for cell viability. Topoisomerase II is a homodimeric enzyme involved in many aspects of cellular metabolism, where catenation, knotting and positive supercoiling of DNA are common consequences of normal cellular activity. The enzyme can relax both negatively and positively supercoiled DNA by introducing transient double-stranded breaks in the DNA duplex at a specific site, and passing a second intact DNA duplex through the gap (figure 1.1). The enzyme is also a significant structural component of the nuclear matrix scaffold and plays a key role in folding and organisation of nuclear chromatin (Kimura *et al.,* 1996).

The two mammalian isoforms of topoisomerase II, α and β, are encoded by two separate genes on different chromosomes (Tsai-Pflugfelder *et al.,* 1988; Jenkins *et al.,* 1992; Tan *et al.,* 1992). Although the two isoforms share strong sequence identity, being 68% identical at the amino acid level, and have closely related functions, the two enzymes appear to be differentially regulated (Austin *et al.,* 1993). Topoisomerase IIβ, localised in the nucleolus, is expressed ubiquitously in all tissues with a relatively constant transcription rate throughout the cell cycle (Austin *et al.,* 1995). In contrast, the α isozyme, localised in the nucleoplasm, is highly expressed in tissues which are composed of rapidly proliferating cells, and its expression is cell cycle modulated (Woessner *et al.,* 1990).

The topoisomerase II protein consists of three principle domains: an ATPase activity is found at the amino-terminal, a central catalytic core domain, and the primary dimerisation interface and regulatory domain at the carboxy-terminus (reviewed by Robert and Larsen, 1998). The central catalytic core introduces staggered double-stranded breaks within the DNA phosphodiester backbone, and an enzyme-DNA intermediate is formed. The process is ATP-dependent, with a conformational change in the dimeric enzyme stimulated by ATP binding (Champoux, 2001), and a concomitant movement of the transported helix through the break in the gated helix, followed by

religation of the gate helix. Hydrolysis of ATP is essential for the enzyme to return to its original conformation ready for the next catalytic cycle (figure 1.1).

The C-terminal regions of the topoisomerase II isoforms are the least conserved (Austin *et al.,* 1995; Cowell *et al.,* 1998), and these regions are thought to be responsible for localisation of the protein within the cell (Adachi *et al.,* 1997). Phosphorylation occurs at the C-terminus of both isoforms, which is suggested to be involved in controlling nuclear localisation of the topoisomerase IIβ isoform (Cowell *et al.,* 1998).

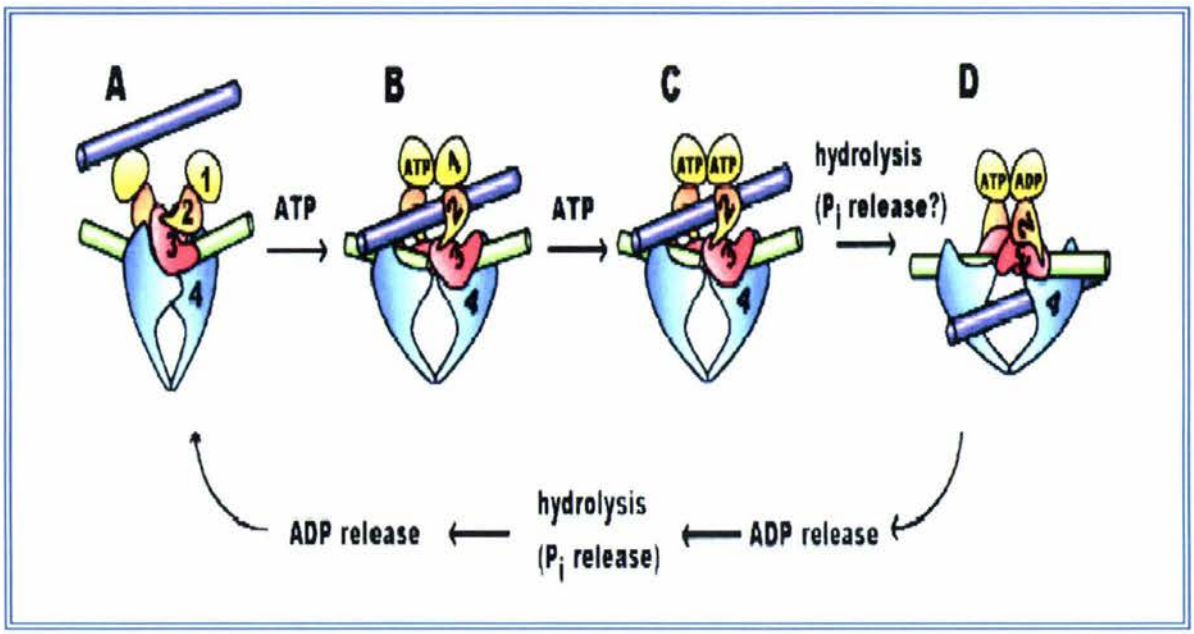


Figure 1.1: Schematic model of the catalytic cycle of topoisomerase II.

Topoisomerase II is shown as a dimer and the domains of one monomer are numbered starting from the N terminus. The C-terminal domain, which is neither conserved nor required for catalytic activity, is not shown. The G and T segments of DNA are shown as green and purple rods, respectively. A) Topoiosomerase II binds the DNA duplex at a region of incorrect topology, the G strand. B) ATP binds, and the enzyme captures the second helix, the T strand. C) A second molecule of ATP binds, and the G strand is cleaved. D) The T strand is passed through the G strand with the hydrolysis of ATP. The step at which the T segment exits the topoisomerase remains unclear and therefore is not shown (Baird *et al.,* 1999).

1.3 Topoisomerase II as a target for chemotherapeutic drugs.

High levels of topoisomerase II present in proliferating cells are essential for the relief of torsional stress that occurs during DNA replication and cell division. This, and the ability of the enzyme to generate double-stranded breaks, makes the enzyme a key target for a

number of commonly used anti-cancer drugs in mammalian cells. Both isoforms of topoisomerase II are targets of anticancer drugs, some of which are isoform specific (reviewed by Kaufmann, 1997). Anti-cancer drugs targeting topoisomerase II fall into two classes: catalytic inhibitors and topoisomerase II poisons. In the presence of the drugs the normal cellular activity of the topoisomerase enzymes is inhibited, and as a consequence the enzyme becomes a cellular toxin (reviewed by Robert and Larsen, 1998). The topoisomerase II poisons have been associated with the down-regulation of topoisomerase II expression, and thereby the development of drug resistance (reviewed by Larsen and Skladanowski, 1998).

1.3.1 Mechanism of action of topoisomerase II drugs.

Topoisomerase II poisons, such as the anthracyclines, etoposide and doxorubicin, act by stabilising the topoisomerase II enzyme-DNA intermediate complex, to form what is termed the 'cleavable complex', and thus inhibiting religation (Burden and Osheroff, 1998). It is thought that cellular processes such as transcription and replication convert the drug-stabilised complexes into irreversible double-stranded DNA breaks (refer to figure 1.1), which are toxic to the cell. Stabilisation of this complex is suggested to be sufficient to inhibit cell proliferation, possibly by being interpreted as a lethal signal, which subsequently triggers the apoptotic pathway (Walker *et al.*, 1991). The drug-stabilised complex can only be reversed if the drug is removed before the activation of the cellular replication and transcription machinery (reviewed by Baguley and Ferguson, 1998).

The level of topoisomerase II protein in cells correlates with sensitivity to killing by these drugs, with high levels conferring relative sensitivity and low levels resistance. Decreased levels of topoisomerase II, and the subsequent reduction in frequency of 'cleavable complex' formation, has been suggested to decrease the number of double-stranded breaks to a level which can be adequately repaired by cellular mechanisms. In contrast, high levels of topoisomerase II provide numerous drug targets, a cellular condition in which a high degree of interaction between the drug and the active enzyme induces high cellular toxicity.

Topoisomerase II inhibitors act by a number of different mechanisms to inhibit the enzymes activity without stabilising a cleavable complex (Andoh and Ishida, 1998).

Topoisomerase II inhibitors, such as bisdioxopiperazines (for example ICRF-193), stabilise the circular clamp conformation of DNA topoisomerase II, therefore preventing strand passage (Roca *et al.,* 1994). Other topoisomerase II inhibitors act by directly inhibiting the ATPase domain of the enzyme, for example Novobiocin (reviewed by Isaacs *et al.,* 1995), and Suramin interferes with topoisomerase II activity by inhibiting enzyme phosphorylation. Overall the action of the topoisomerase II inhibitors result in cell death via an accumulation of topological problems in cells, a consequence of an inability of the topoisomerase II enzyme to carry out its normal functions.

1.3.2 Topoisomerase II changes and chemosensitivity in tumours.

Although many factors have been implicated in the development of drug resistance, modification of topoisomerase II expression is thought to represent the predominant mechanism underlying acquired drug resistance. Many reported cases of drug resistance have been attributed to a down-regulation in topoisomerase IIα expression (Robert and Larsen, 1998), however the topoisomerase IIβ is yet to be investigated in detail and therefore cannot be ruled out as a factor in this effect. Of interest, is the finding that the two isoforms can interact with drugs via the same DNA sequences (Austin and Marsh, 1998). Etoposide-resistant melanoma cells exhibited reduced topoisomerase II activity corresponding to an increasing degree of drug resistance, indicating that modulation of topoisomerase II activity contributes to drug-resistance (Lage *et al.,* 2000).

The levels of topoisomerase IIβ have been shown to be decreased or even absent in some resistant cell lines, including human small lung cancer cells resistant to mitoxantrone (Gao *et al.,* 1999), Chinese hamster lung cells (DC-3F) resistant to 9-OH-ellepticine (Dereuddre *et al.,* 1997), and human leukemia cell line (HL-60) resistant to amsacrine (Herzog *et al.,* 1998). Topoisomerase IIβ expression also appears to be up-regulated in a number of different tumours (Turley *et al.,* 1997), which also could affect the response of patients to the topoisomerase II poisons. These findings suggest that topoisomerase IIβ is a target for many of the same drugs which target topoisomerase IIα, and topoisomerase IIβ is also thought to be in part responsible for the development of resistance to drugs that target these enzymes.

The reduced levels of topoisomerase IIα present in resistant cell lines and tumours has been directly correlated with a decrease in topoisomerase IIα mRNA, which occurs as a result of transcriptional down-regulation rather than reduced mRNA stability (Kubo *et al.*, 1995; Asano *et al.*, 1996). Anti-sense RNA fragments, which act to suppress normal topoisomerase IIα mRNA processing, were used to show that the down-regulation of topoisomerase IIα expression can generate etoposide-resistance in mammalian cells (reviewed by Isaacs *et al.*, 1998).

Phosphorylation of the topoisomerase IIα enzyme has been shown to inhibit the enzymes catalytic activity and thereby influence drug susceptibility (Osheroff *et al.*, 1991; reviewed by Isaacs *et al.*, 1998). Different phosphorylation states of the topoisomerase IIα and β isoforms have been suggested to affect the ability of the enzyme to bind to chromosomes, and in this way inhibit enzyme activity (Burden and Sullivan, 1994; Kimura *et al.*, 1996). Phosphorylation of the topoisomerase IIβ enzyme is also thought to be associated with the trafficking of the enzyme out of the nucleus during mitosis (Burden *et al.*, 1994). In a study involving topoisomerase IIα, the translocation of the enzyme from nucleus to cytoplasm was shown to result in etoposide-resistance in human leukemic cells, without any change in relative amounts of topoisomerase IIα (Valkov *et al.*, 2000). This suggests that cellular localisation of the topoisomerase, which appears to be regulated by phosphorylation, could be an important factor in drug resistance.

Fibronectin adhesion by means of β1 integrins appears to protect cells from initial drug-induced DNA damage by reducing topoisomerase II activity secondarily to alterations in the nuclear distribution of topoisomerase IIβ (Hazelhurst *et al.*, 2001). Adhesion of human lymphoma cells to fibronectin resulted in a tighter interaction between topoisomerase IIβ and the nucleus and provided a survival advantage with respect to damage induced by several topoisomerase II inhibitors. Mutations in topoisomerase II proteins have been identified in drug-resistant cell lines (Dingemans *et al.*, 1998; Robert and Larsen, 1998), and these mutations have also been implicated in drug resistance (Dingemans *et al.*, 1998). One particular study suggested that mutations found to decrease topoisomerase IIβ binding affinity for magnesium ions, specifically required for DNA cleavage, could contribute to drug resistance (West *et al.*, 2000).

Other studies have implicated a number of other factors in the down-regulation of topoisomerase IIα; including methylation of the gene (Tan *et al.,* 1992), histone deacetylation (Adachi *et al.,* 2000), RNA stability (Goswami *et al.,* 1996), binding of p53 to the promoter (Sandri *et al.,* 1996), and altered expression of multidrug resistance gene (Bredel, 2001). It has also been shown that topoisomerase IIα can form heterodimers with topoisomerase IIβ *in vivo*, an interaction which has been suggested to control topoisomerase II activity by maintaining relative numbers of heterodimers to homodimers, which could be significant in drug resistance (Gromova *et al.,* 1998).

1.4 Transcription in Eukaryotes.

The main mechanism for controlling topoisomerase II expression and activity is thought to be regulation at the transcriptional level. Protein coding genes are transcribed by RNA polymerase II (Pol II), under the control of regulatory DNA elements in the promoter, recognised with high efficiency and specificity by trans-acting transcription factors. Transcription is initiated by formation of a pre-initiation complex (PIC) composed of a set of general transcription factors, termed transcription initiation factors (TIFs), which assemble at the promoter. TIFs generally bind common core promoter elements, while additional regulatory factors bind in a combinatorial fashion at gene-specific upstream or downstream regulatory elements. Transcriptional activity is also dependent on other factors, such as DNA organisation, and the ability of transcriptional activators and repressors to access their target DNA sequences and associated DNA-binding proteins.

In many promoters, a TATA box element is located approximately 25 base pairs (bp) upstream of the transcription start site. This specific sequence is the site of PIC formation, recognised by a TATA-binding protein (TBP) which binds first at the TATA box and then recruits TIFs and Pol II to the promoter, thereby mediating formation of the transcription PIC. Other transcription factors are also known to be recruited to the PIC, with putative roles in facilitating the movement of Pol II away from the promoter or influencing chromatin structure to enable DNA access.

Trans-acting transcription factors can act as repressors or activators by binding directly to cis-acting gene-specific regulatory elements or indirectly via protein-protein

interactions. These cis-acting elements or 'enhancers' can be located upstream or downstream of the transcription start site, and can act at large distances from the gene. The efficiency and frequency of transcription initiation is greatly affected by the carefully orchestrated movements of transcription factors, which bind specifically at the promoter, and associate with the basal transcription complex and Pol II.

Some promoters lacking a TATA box, termed TATA-less promoters, require alternative recognition mechanisms for PIC formation. Basal transcription and promoter specificity is achieved through TBP-associated factors (TAFs), which are recruited to TATA-less promoters through recognition of specific A/T-rich sequences functioning in a similar manner to the TATA box. It is unclear whether TBP binds directly to the DNA or indirectly via protein-protein interactions (White and Jackson, 1992) in a complex, such as with TFIID (a TIF), as a means of enabling Pol II to be correctly positioned at the start site. TFIID is a multiprotein complex composed of TBP and TAFs, which directs PIC assembly and has been shown to interact directly with Pol II (Hoffmann *et al.*, 1997).

The 5'-flanking region of both topoisomerase IIα and β promoters have a very high GC content and contain no canonical TATA box element, characteristic of promoters of genes involved in housekeeping functions (Yoon *et al.*, 1999). Topoisomerase IIα and β promoters appear to exhibit only weak identity suggesting that the expression of the two genes may not be co-ordinated.

1.5 Topoisomerase IIβ.

Human topoisomerase IIβ is an essential 180 kDa nuclear enzyme encoded by a gene located on chromosome 3p24 (Jenkins *et al.*, 1992; Tan *et al.*, 1992). The *in vivo* function of topoisomerase IIβ remains unclear, but the enzyme has been implicated to play a role in neuronal cell survival (Isaacs *et al.*, 1995; Kubo *et al.*, 1995). Elevated levels of topoisomerase IIβ expression in neuronal cells of developing rat brain and the association of active RNA synthesis in neuronal nuclei suggest a role for topoisomerase IIβ in transcription in this tissue (Tsutsui *et al.*, 2000). Analysis of the human topoisomerase IIβ sequence indicates several putative nuclear localisation signals and many potential phosphorylation sites, as well as putative sites for other post-translational modifications.

Topoisomerase IIβ appears to differ from the α isoform in a number of ways. It is therefore possible that DNA damage induced by drugs targeting the two isoforms may be detected differently within the cell, inducing different signal transduction pathways and cell-cycle checkpoints (Gao *et al.,* 1999). In support of this, one study demonstrated that topoisomerase IIβ, but not topoisomerase IIα, is tagged for proteasome degradation by sumoylation, following exposure to topoisomerase II catalytic inhibitor ICRF-193 (Isik *et al.,* 2003). Cellular quantification has shown that levels of topoisomerase IIβ exceed levels of topoisomerase IIα in plateau phase cells (Padget *et al.,* 2000), and levels of the two are comparable even in proliferating cells, which contradicts previous studies of the two isoforms. The presence of topoisomerase IIβ at these significant levels, would be an important consideration when investigating the action of anti-cancer drugs. In fact, topoisomerase IIβ has been shown to be expressed at higher levels than topoisomerase IIα in a greater proportion of tumour cells (Robert and Larsen, 1998), suggesting that proliferation is not the sole reason for the up-regulation of topoisomerase IIβ.

1.5.1 Topoisomerase IIβ promoter.

One previous study of the topoisomerase IIβ promoter (Ng *et al.,* 1997), found two putative transcription start sites located at adenine 193 and guanine 89, upstream from the ATG translation initiation codon. The minimal topoisomerase IIβ promoter encompassing a region approximately -569 bp upstream of the 5'-transcriptional start site has been found to be responsible for about 70% of the topoisomerase IIβ promoter activity (Lok *et al.,* 2002). Analysis of the -569 bp topoisomerase IIβ sequence has revealed a number of important sequences which could be involved in the regulation of topoisomerase IIβ expression. Of particular importance are two inverted CCAAT boxes (ICB1 and ICB2) and a GC box located between 486-533 bases upstream of the major transcription start site (figure 1.2).

Other putative regulatory factors in the topoisomerase IIβ promoter include two additional GC boxes, another ICB element, a consensus sequence for an activating transcription factor (ATF) binding site, an activator protein-2 (AP2) site (figure 1.2), and a potential binding site for p53. Additional consensus elements for transcription factor binding have been identified at the 5'-end of the first intron in the topoisomerase

IIβ promoter. These include, two GC boxes, reverse strand sequences between +267 and +280 that are a close match to the consensus CTF/NF-1 binding site, and an AP2 binding site sequence present on the reverse strand between +305 and +312. The CTF/NF1 site could be important for transcriptional regulation as this DNA sequence has been shown to interact directly with TBP (Xiao *et al.*, 1994).

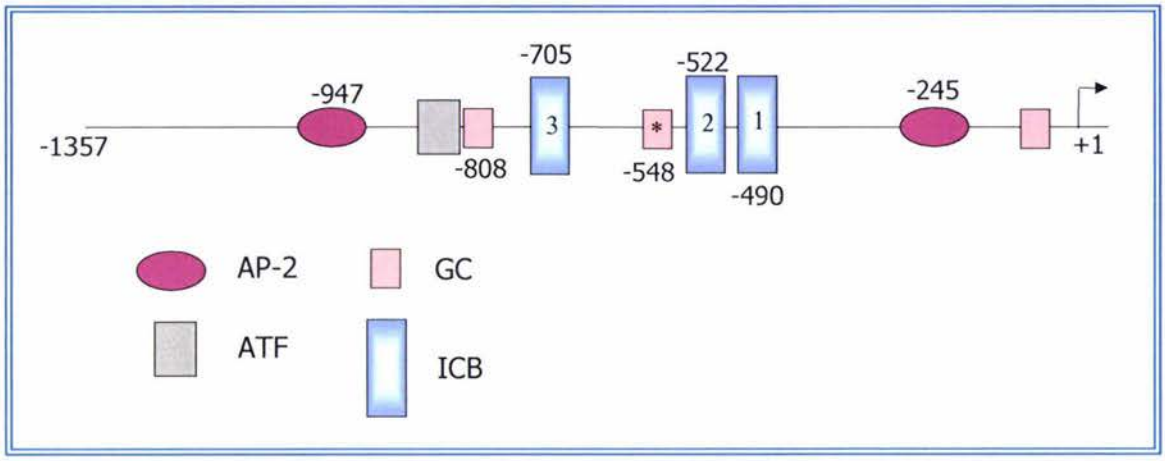


Figure 1.2: Schematic representation of the -1357 topoisomerase IIβ promoter.
Positions of the putative regulatory elements are shown and numbered with respect to the major transcription start site, +1, indicated by the arrow. Of particular interest is a cluster including ICB elements, ICB1 and ICB2 (numbered), and a GC element (*) located within the minimal promoter region (-490 and -548).

Transient transfection assays with different sequences of the topoisomerase IIβ gene were used to identify regions important for transcriptional regulation of the gene. The results indicated the presence of positive element(s) between -1000 and -500 and probable negative element(s) in the region between -500 and -14 (Ng *et al.*, 1997). Sequences downstream of the translation initiation codon, within the first intron, were also found to contribute to the promoter activity. The involvement of intronic sequence elements has been reported in the transcriptional regulation of many other genes (Alder *et al.*, 1992; Zastawny and Ling, 1993; Lazar *et al.*, 1994).

A number of important sequences have also been identified in the topoisomerase IIα promoter, some of which have been implicated in the transcriptional regulation of topoisomerase IIα, including five functional ICB and two functional GC boxes. As with the topoisomerase IIβ, a cluster of two CCAAT boxes (ICB1-2) and one GC

element (GC1) are located in the topoisomerase IIα promoter, but the order of the GC and ICB elements is reversed. The regulation of topoisomerase IIα promoter activity is thought to involve a complex series of interactions between proximal and distal elements of the promoter. The presence of similar elements in the promoters of topoisomerase IIα and β could suggest that regulation of expression of the two enzymes could be coordinated at least in some situations.

Transcriptional control of topoisomerase IIβ is thought to be regulated through the binding of transcription factors to important regulatory elements within the topoisomerase IIβ promoter. Characterising these interactions and determining the consequence of these interactions for topoisomerase IIβ transcriptional regulation could provide information about the molecular mechanisms responsible for drug resistance. Particularly since transcription factors that bind to ICB and GC boxes have been shown to be up- or down-regulated in drug-resistant cells.

1.5.2 CCAAT Elements.

The CCAAT box is a conserved regulatory element, generally located upstream of the transcription start point in many promoters. Over 30% of all eukaryotic promoters have been found to contain CCAAT boxes in either the direct or inverse orientation (Bucher, 1990). CCAAT boxes function as cis-acting regulatory elements and are targets for a variety of protein factors, including ICBP90 (inverted CCAAT box binding protein 90), Y-box binding protein (YB-1), p53 and transcription factor NF-Y (nuclear factor Y). Inverted CCAAT boxes are thought to be important elements for basal-level transcription, and the transcriptional activity of a number of promoters has been found to change when mutations were introduced into the CCAAT motif (Yoon *et al.*, 1999).

ICBP90, a novel CCAAT box binding protein, appears to be involved in cell proliferation processes, with enhanced expression of the protein in tumour cells. The ICBP90 protein has been shown to bind to CCAAT elements in the topoisomerase IIα gene *in vitro* using binding assays, and has been implicated in the activation of topoisomerase IIα expression, although direct evidence of this has not been reported (Hopfner *et al.*, 2000; Hopfner *et al.*, 2001).

The Y-box binding protein (YB-1) is another CCAAT binding protein, which may be involved in the activation of topoisomerase IIα expression. Reduced topoisomerase IIα expression was seen when YB-1 expression decreased due to the expression of antisense YB-1. The co-expression of YB-1 and topoisomerase IIα in human colorectal carcinomas suggested that YB-1 could be involved in regulating DNA topoisomerase IIα gene expression (Shibao *et al.*, 1999). YB-1 is also thought to be responsible for the activation of the MDR1 promoter in response to various environmental stimuli, such as drugs, etoposide and teniposide (reviewed in Kubo *et al.*, 1995).

Protein, p53, is a crucial factor in the regulation of cell-cycle progression in mammals. Expression levels are usually extremely low, however a dramatic increase in expression and activity of p53 is observed in response to DNA damage, where it controls the initiation of cell-cycle arrest and entry into the apoptotic pathway (Reisman and Loging, 1998). Studies using the minimal promoter of topoisomerase IIα have demonstrated that p53 has the ability to significantly decrease topoisomerase IIα expression (Sandri *et al.*, 1996). ICB elements were shown to be essential for the p53-mediated down-regulation of topoisomerase IIα promoter activity, as the deletion of all five topoisomerase IIα ICB elements abolished the effect (Wang *et al.*, 1997). In addition, topoisomerase II proteins were shown to co-precipitate with p53 protein, and therefore a role in the control of p53-mediated apoptosis was suggested (Yuwen *et al.*, 1997).

The ICB1 and ICB2 elements of the topoisomerase IIβ promoter have been shown to be critical for topoisomerase IIβ transcriptional activity, where the simultaneous disruption of both elements resulted in a dramatic decrease in promoter activity (Lok *et al.*, 2002). In contrast, a single deletion in either ICB element had little effect, suggesting each element alone was sufficient to support topoisomerase IIβ promoter activity.

Each ICB element in topoisomerase IIα appears to have a slightly different function, while still working together to influence topoisomerase IIα expression. Several studies have suggested that the binding of negative regulators to the topoisomerase

IIα ICB1 element could be a factor in the down-regulation of topoisomerase IIα activity observed in resistant cells (Furakawa *et al.*, 1998; Takano *et al.*, 1999; Falck *et al.*, 1999). Topoisomerase IIα ICB2 element has been implicated in the down-regulation of topoisomerase IIα activity in confluence-arrested cells due to an interaction with a repressor factor (Isaacs *et al.*, 1996a), while the deletion of ICB2 completely abrogated the down-regulation of topoisomerase IIα activity (Takano *et al.*, 1999). One study showed that the up-regulation of topoisomerase IIα in ICRF-187-resistant cells was mediated in part by altered regulation of the ICB3 element, and suggested a role for ICB3 in the negative regulation of topoisomerase IIα (Morgan and Beck, 2001). Topoisomerase IIα ICB elements appear to be able to bind similar proteins, with the exception of ICB5 (Herzog and Zwelling, 1997), which also did not appear to be important for topoisomerase IIα regulation (Hochhauser *et al.*, 1992).

1.5.3 NF-Y (Nuclear Factor Y).

Among the most prevalent CCAAT box binding protein, NF-Y (also known as CP1, CBF, ACF) is a ubiquitously expressed heterotrimeric protein made up of at least three subunits (NF-YA, NF-YB, NF-YC). Although bipartite activation domains are only located in NF-YA and NF-YC, the presence of all three subunits is necessary to form a functional binding complex at the CCAAT box sequence (reviewed by Isaacs *et al.*, 1998). NF-YB and NF-YC subunits interact tightly to form a stable heterodimer (Bellorini *et al.*, 1997), which is joined by the NF-YA subunit. Each of the three subunits of NF-Y has been shown to contact DNA (Sinha *et al.*, 1996), and a mutation in any one subunit disrupts DNA binding of the NF-Y trimer (Laing and Maity, 1998; Hu and Maity, 2000).

NF-Y protein is highly conserved and has been identified as the CCAAT box activator in over 100 promoters. It is involved in several aspects of transcriptional activation including cell-cycle dependent, inducible, tissue-specific activation, as well as maintaining basal transcription. The ICB sequence is known to be the specific motif to which NF-Y binds (Mantovani, 1998; Lok *et al.*, 2002), however flanking sequences have also been shown to affect NF-Y binding (Dorn *et al.*, 1987; Mantovani, 1998).

CCAAT boxes alone cannot activate transcription and it has been suggested that NF-Y may increase the binding of other transcription factors at their target DNA sequences. NF-Y has been shown to interact with a number of transcription factors and co-activators regulating gene expression, in particular Sp1 (Roder *et al.*, 1999). NF-Y contains two glutamine (Q)-rich activation domains which were shown to be necessary for transcriptional activation (Coustry *et al.*, 2001). These Q-rich domains are considered to be essential in mediating protein-protein interactions between transcriptional activators and other components of the transcription machinery, for example to direct binding of TAFs (Mantovani *et al.*, 1992). This could suggest a role for NF-Y in directing the formation of the PIC at TATA-less promoters. NF-YB/NF-YC dimerisation is mediated through histone fold motifs (HFM) (reviewed by Mantovani, 1999), a motif shared by some of the TBP-associated TAFs that mediate activation as part of the TFIID complex (Burley and Roeder, 1997). NF-YB can associate with TFIID in the absence of NF-YA (Bellorini *et al.*, 1997), which might suggest a role for NF-Y in transcriptional initiation, or as a general promoter organiser.

DNA is condensed into a highly ordered, compact nucleosomal structure, achieved by the association of histones with the DNA, which is inaccessible to transcription factors. Remodelling of this highly compact DNA structure to allow transcriptional activity is achieved by histone acetylation, which weakens the histone interactions with DNA. It has been shown that NF-Y is able to disrupt nucleosomal organisation of the topoisomerase IIα promoter through interaction with CCAAT promoter elements (Coustry *et al.*, 2001), and NF-Y has been implicated in the stimulation of histone acetylation (Adachi *et al.*, 2000). NF-Y was actually found to possess histone acetyl-transferase (HAT) activity *in vivo* through an association with the HATs, GCN5 and PCAF (Jin and Scotto, 1998). It has been speculated that the associated histone acetyl-transferases might serve to modulate NF-Y transactivation potential by aiding disruption of local chromatin structure (Currie, 1997). This ability to induce distortion of the double helix upon binding to DNA *in vitro*, facilitates transcription factor access to DNA binding sites, therefore NF-Y serves as a "promoter organiser" (Ronchi et al 1995; Mantovani *et al.*, 1999).

The Q-rich domains of NF-YA and C have been implicated in the ability of NF-Y to induce distortion of the double helix (Liberati *et al.,* 1999). NF-Y is thought to be capable of bending DNA at CCAAT sites by 62-82°, and rotationally twisting the DNA by 100° (Ronchi *et al.,* 1995). DNA bending by transcription factors is thought to have a significant influence on transcription regulation by bringing promoter elements into close proximity. TBP is also capable of DNA bending, and it is thought that in the absence of TBP-TATA interactions at the promoter, NF-Y could function as a pivotal factor in connecting upstream activators with the general transcription machinery (Mantovani, 1998).

Binding studies have shown that NF-Y is able to bind to the ICB1 and ICB2 elements in the topoisomerase IIβ promoter and significantly influence topoisomerase IIβ gene expression. Several studies have provided evidence of an important functional role for NF-Y in the topoisomerase IIα gene transcription. Specific binding of NF-Y to the ICB1-4 elements of the human topoisomerase IIα promoter has been shown *in vitro* (Wang, *et al.,* 1997a; Isaacs *et al.,* 1996). Higher levels of NF-Y binding at the topoisomerase IIα ICB2, were observed in extracts from proliferating cells, than from confluence-arrested cells (Isaacs *et al.,* 1996). Dominant negative NF-YA has been used to inhibit the binding of NF-Y complex to DNA, and a concurrent reduction in topoisomerase IIα expression was observed (Hu and Maity, 2000). Together these results showed that the differential regulation of topoisomerase IIα is mediated, at least in part, through proliferation-specific binding of factors to an ICB element in the gene promoter. Modification of NF-Y (for example phosphorylation) or an interaction with other factors, could be responsible for the down-regulation of topoisomerase IIα gene expression in confluence-arrested cells. In addition, the topoisomerase IIα promoter was not completely repressed in NF-Y depleted HeLa nuclear cell extracts *in vitro* (Coustry *et al.,* 2001). Thus, in addition to NF-Y, other transcription factors must also be important for the regulation of topoisomerase IIα gene expression.

1.5.4 GC Rich Regions.

GC boxes are another common promoter element, often found in close proximity to CCAAT boxes or binding sites of other transcription factors (Kadonaga *et al.,* 1989), as seen in the promoters of both topoisomerase II isoforms. Multiple GC boxes are

commonly present in a promoter, with each GC box proposed to have a different functional role.

The human topoisomerase IIβ promoter contains three GC boxes; one located at –113 in close proximity to the transcription start site, the second (-548) following two ICB elements just inside the defined minimal topoisomerase IIβ promoter, and the third outside this region. Functional assays showed that mutation or elimination of the second GC element (-548) resulted in only a slight decrease (20%) in topoisomerase IIβ promoter activity (Lok *et al.,* 2002). However, when the same GC box was mutated with either of the two adjacent ICB elements (-522, -490) a much larger decrease was observed, in particular with the first ICB element (-490), suggesting a functional synergy may exist between the GC and ICB elements in the topoisomerase IIβ promoter.

The topoisomerase IIα promoter has two GC boxes, GC1 and GC2. A mutation in GC1 does not result in a decrease in topoisomerase IIα activity, however in drug-resistant cells a mutation in GC1 results in an increase in promoter activity (reviewed by Isaacs *et al.,* 1998). GC2 is considered to be the least important in topoisomerase IIα regulation and is thought to be functional only under certain growth conditions (Hochhauser *et al.,* 1992).

1.5.5 Sp1 (Specificity Protein 1).

The Sp1 protein is a member of a large multi-gene family capable of regulating the transcription of genes via interactions with GC motifs in the promoter, acting on both proximal and distal elements to maintain basal activity (McEwen and Ornitz, 1998). Sp1 typically activates promoters containing multiple GC motifs, although a single Sp1 binding site is sufficient to stimulate promoter activity (Kadonaga *et al.,* 1987). Like NF-Y, Sp1 contains Q-rich activation domains, which are thought to mediate protein-protein interactions, and are required for transcriptional activation (Courey and Tjian, 1988). Sp1 mediated transcriptional regulation also depends on three zinc finger structures responsible for DNA binding (Kadonaga *et al.,* 1998). The Q-rich domain of Sp1 is thought to target one of the TFIID components (Chen *et al.,* 1994),

and therefore activate promoters which are transcribed by Pol II (Dynan and Tjian, 1983; Kadonaga *et al.*, 1986).

Sp1 has been shown to exhibit functional synergy with other transcription factors, including NF-Y, which could explain why a single GC box is sufficient in some promoters. When two or more Sp1 sites are found in a promoter, DNA-bound Sp1 monomers have been shown to self-associate, thereby bringing distant promoter elements into close proximity (Su *et al.*, 1991). This was demonstrated using a modified thymidine kinase (TK) promoter containing up- and down-stream Sp1 binding sites. The DNA was shown to loop *in vitro*, and this was correlated with Sp1-mediated synergistic activation of transcription *in vivo*. Using microscopy, it was determined that individual Sp1 proteins associate to form tetramers, which in turn interact as multiple tetramers stacked at the DNA loop junctions, where each monomer is capable of interacting with a single GC motif (Mastrangelo *et al.*, 1991). Synergistic activation requires the formation of higher order complexes, co-ordinated by Sp1 molecules bound at proximal and distal sites in the promoter (reviewed by Liberati *et al.*, 1999). The presence of GC boxes in such positions in both topoisomerase IIα and β promoters may indicate that DNA looping is required for the regulation of transcription.

Sp1 has been shown to be post-translationally modified. For example, phosphorylation has been shown to allow Sp1 to bind DNA more tightly (Ge *et al.*, 2001), and therefore is thought to increase Sp1 binding to GC boxes and thereby facilitate activation of a number of genes.

Binding studies have shown that Sp1 can bind to the topoisomerase IIβ GC element at position -548, and that Sp1 has a regulatory effect on topoisomerase IIβ expression (Lok *et al.*, 2002). Another study showed specific binding of Sp1 to unidentified downstream elements in the topoisomerase IIβ promoter, which may be involved in transcriptional regulation (Ng *et al.*, 1997). Sp1 has been shown to play an important role in the transcriptional regulation of the topoisomerase IIα promoter, mediated through the binding of GC1 and GC2 elements (Kubo *et al.*, 1995; Magan *et al.*, 2003).

1.5.6 Interactions of NF-Y and Sp1.

An *in vivo* interaction between NF-Y and Sp1 has been revealed using a yeast two-hybrid system (Roder *et al.*, 1999), which could be mediated by the Q-rich domains present in both proteins. Each of the proteins is also able to interact with particular TAFs within TFIID (Coustry *et al.*, 1998). A functional co-operation between NF-Y and Sp1 has been shown previously to play a key role in the transcriptional regulation of a number of genes including human and rat fatty acid synthase (FAS) (Roder *et al.*, 1997), human P-glycoprotein (Hu *et al.*, 2000), human cathepsin L (Jean *et al.*, 2002), and the human MHC II-associated invariant chain (II) (Wright *et al.*, 1995).

In all reported cases of co-operativity between NF-Y and Sp1 the binding sites for the two transcription factors are located in close proximity in the promoter regions of the genes, suggesting that the distance between the two elements is important. The mechanisms of co-operativity may vary. In addition to the direct physical interaction described above, co-operative binding to the promoter region can occur, as has been demonstrated for NF-Y and Sp1 binding to the FAS promoter (Roder *et al.*, 1997). In the case of the human MHC II invariant chain promoter, the CCAAT box is non-consensus, and therefore NF-Y binding is inefficient without Sp1. In addition, both Sp1 and NF-Y have been shown to associate with the co-activator p300 (Faniello *et al.*, 1999; Xiao *et al.*, 2000), therefore co-operativity could involve interactions between Sp1 and NF-Y with the p300 co-activator.

Functional assays using the topoisomerase IIβ promoter suggest a functional synergy may exist between the ICB elements and the GC box in the topoisomerase IIβ promoter (Lok *et al.*, 2002), which could be mediated through an interaction between NF-Y and Sp1 binding at these elements. Binding assays using the topoisomerase IIα promoter have provided important evidence for co-operativity between the two proximal GC and ICB elements in the promoter in order to recruit Sp1 and NF-Y, respectively (Magan *et al.*, 2003). In addition NF-Y, Sp1 and Sp3 have been shown to have a regulatory effect on topoisomerase IIα expression (Magan *et al.*, 2003), where an interaction between the three transcription factors is thought to occur (Roder *et al.*, 1999).

1.5.7 Sp3 (Specificity Protein 3).

Sp3 transcription factor is another member of the Sp family, capable of regulating the transcription of genes via interactions with GC motifs in the promoter. Both Sp1 and Sp3 are ubiquitously expressed in mammalian cells, have similar structures including DNA binding domains which recognise similar target sequences, and can be phosphorylated to increase binding affinity (Ge *et al.,* 2001). However unlike Sp1, Sp3 is capable of dual functions in transcriptional regulation, acting as an activator or a repressor (Noti, 1997). Three isoforms of Sp3 are known, due to the use of alternative translation initiation sites within the mRNA, which has further complicated elucidation of a transcriptional role for the protein. Sp3 is thought to target promoters containing only a single Sp binding site, while promoters containing multiple binding sites are only weakly activated, or fail to be activated by Sp3 (reviewed by Suske, 1999). The relative levels of Sp1 and Sp3 present are thought to dictate the response of the promoter to these factors. It was shown that when Sp1 and Sp3 were equally expressed and Sp3 did not act as a strong activator, it competed with Sp1 for the same binding site and thus decreased Sp1-mediated activation (de Luca *et al.,* 1996; reviewed by Suske, 1999). Therefore, the activity of Sp3 appears to vary depending on structure and arrangement of recognition sites, as well as cellular context.

Sp3 has been found to be present at increased levels in drug resistant cell lines, and therefore may directly down-regulate topoisomerase II promoter activity (reviewed by Isaacs *et al.,* 1998). In one etoposide-resistant cell line derived from human cancer cells, a decrease in topoisomerase IIα gene expression has been correlated to Sp3 up-regulation, however the position at which Sp3 bound the promoter was not determined. (Kubo *et al.,* 1995). Sp3 has also been shown to interact with NF-YA and co-operatively stimulate gene expression (Yamada *et al.,* 2000). This could suggest that an Sp3 repressive role may involve inhibitory protein-protein interactions with components of the general transcription complex or with other transcription factors.

Both Sp1 and Sp3 were found to bind to the GC element in the topoisomerase IIβ promoter. Sp3 has been shown to bind GC2 (Szremska, 2000) in the topoisomerase

IIα promoter, and the binding of Sp3 at GC1 was shown to affect expression levels of topoisomerase IIα (Magan *et al.*, 2003).

1.6 Research Aims.

Although many recent studies have increased our understanding of topoisomerase IIα transcriptional regulation, little is known about the regulation of the topoisomerase IIβ isoform.

The molecular mechanisms responsible for the regulation of expression of topoisomerase IIβ and α are thought to be associated with resistance to chemotherapy drugs. The cloning and characterisation of the topoisomerase IIβ promoter region would allow analysis of the modulation of expression at the molecular level. The identification of cis-acting elements important for transcriptional regulation, and transacting factors interacting with these elements to mediate the control, would be possible. Insight into the modulation of topoisomerase II gene expression would allow characterisation of the mechanism of resistance to anti-cancer drugs. To this end, the regulation of topoisomerase IIβ expression was investigated by isolating and studying a 1.5 kb region of the topoisomerase IIβ promoter.

The specific objectives of this research were as follows:

- To isolate and subclone the topoisomerase IIβ promoter region.
- To generate a series of truncated promoter regions in a luciferase reporter vector.
- To generate mutant promoter constructs containing mutations in topoisomerase IIβ promoter elements, ICB1 and ICB2.
- To carry out preliminary functional assays in tissue culture cells to assess the activity of the cloned promoter regions.
- To carry out a series of DNA-binding assays using oligonucleotides representing the GC, ICB1 and ICB2 elements of the topoisomerase IIβ promoter.

The publication of a parallel study (Lok *et al.*, 2002) occurred half-way through the research carried out for this thesis.

Chapter 2: Materials and Methods.

2.1 Materials.

Restriction endonucleases and buffers, DNA modifying enzymes and buffers, thermosensitive alkaline phosphatase (1 U/μL), DNA quantification standards and agarose LE powder for electrophoresis were obtained from the following sources: New England Biolabs, MA, USA; Boehringer Mannheim, Germany, and Roche, Mt. Wellington, Auckland.

DMSO, DTT, IPTG, EDTA, X-gal, PEG (10%), dNTPs, triton-X-100, ampicillin, lysozyme, ethidium bromide, BSA, mineral oil, ficoll, oligonucleotides for gel shifts, primers RV3, GL2 and topoisomerase IIβ primers were purchased from Sigma Chemical Company, St Louis, MO, USA.

CONCERT™ maxi preparation kit, 10 x trypsin, penicillin-streptomycin (5,000 U/mL), OPTI-MEM with Earle's salts (L-glutamine and non-essential amino acids), bacteriological agar, 1 kb plus ladder, Luria Bertani (LB) broth base, foetal calf serum were purchased from GIBCOBRL, Invitrogen Corporation, Invitrogen NZ Limited, Penrose, Auckland, New Zealand.

PCR purification kit QIAquick was purchased from QIAGEN, New Zealand distributors: Biolab Scientific Ltd, Albany, Auckland, New Zealand.

Bradford protein assay kit and Quantum® Prep Plasmid Miniprep kit were purchased from BioRad Laboratories, CA, USA.

40% (w/v) solution of acrylamide and NN'-methylenebisacrylamide in water (Acrylogel) was from BDH Laboratory Supplies, Poole, England.

γ^{32}P-[ATP] was purchased from Perkin Elmer Life Sciences Inc, Boston, MA, USA.

All primary antibodies (NF-YA, NF-YC, Sp1 and Sp3) were purchased from Santa Cruz Biotechnology, CA, USA.

GCG Version 9.1 was purchased from Wisconsin Genetics Computer Group, USA. FLUOstar galaxy system and software was purchased from BMG Labtechnologies Pty. Ltd, Melbourne, Australia.

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

Poly (dI.dC) was purchased from Amersham Pharmacia Biotech AB, Uppsala, Sweden.

Plasmids pGL3Basic, pCMVSPORT-β-Galactosidase, pGEM®-T vector system, GC-rich PCR system, and the Luciferase Assay System were purchased from Promega Corporation, WI, USA.

The *Escherichia coli* XL-1 blue strain, plasmid pBluescript SK+, and *Pfu* DNA polymerase were purchased from Stratagene, La Jolla, CA, USA.

All sterile tissue culture flasks, plates, cell scrapers and tubes were purchased from Nunc Inc, Naperville, IL, USA.

0.8 μM sterilisation double layer filters were purchased from Drummond, USA.

FuGENE™ 6 transfection reagent, ONPG, Complete™ Mini EDTA free Protease inhibitor cocktail tablets, T4 DNA ligase, and *Taq* polymerase were purchased from Roche Molecular Biochemicals, IN, USA.

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

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

2.2 Methods.

2.2.1 Agarose gel electrophoresis.

Generally, agarose gel electrophoresis was performed using 1% agarose in 1 x TAE buffer (40 mM Tris-acetate, 2 mM ethylene diamine tetra-acetic acid (EDTA) pH 8.0), containing ethidium bromide (0.5 μg/mL). The agarose was melted in a microwave oven and allowed to cool to 55°C, before pouring into the gel apparatus. DNA was loaded into the gel by mixing with DNA loading dye (40% w/v sucrose, 0.25% bromophenol blue). Electrophoresis was carried out at 85 V for approximately 1 hour and visualised by exposure to UV light. The sizes of DNA bands were determined by measuring the distance a fragment had migrated from the well and comparing it with the known size bands of the 1 kb PLUS molecular size standard. If the DNA fragments were expected to be very large or very small in size, the percentage of agarose used was adjusted as indicated in Sambrook et al., (2001).

2.2.2 Oligonucleotides.

Oligonucleotides were synthesised by the Sigma Chemical Company and provided as a dried stock, which was rehydrated in TE to a concentration of 10 μg/μL and stored at –20°C. Prior to use oligonucleotides were further diluted in dH_2O: to a concentration of 50 ng/μL for PCR reactions, 100 ng/μL for electrophoretic mobility shift assays, and 1 μg/μL for competitor oligonucleotides.

2.2.3 Restriction endonuclease digests.

Generally 500-1000 ng of DNA was digested with 5-10 units of restriction endonuclease along with the appropriate buffer at 1 x concentration, as per the manufacturer's recommendation.

For restriction enzyme digestion of plasmid DNA prepared by the rapid boil method, 5 μL of DNA was used with 1 μL (8-10 units) of restriction enzyme along with 3 μL of appropriate (10 x) buffer (as specified by the manufacturer) in a total volume of 30 μL. For digestion of plasmid DNA prepared using the Quantum® Prep Plasmid Miniprep (BioRad) method (30-50 ng/μL), 5-10 μL of DNA was added to the digestion reaction depending on the concentration of the sample. For highly concentrated plasmid DNA

(~1 μg/μL) prepared using the CONCERT Rapid Plasmid Maxiprep Kit (GIBCOBRL, Invitrogen), 1 μL was used in the restriction digest (in 30 μL) total volume.

Each digest was incubated at a temperature optimal for the activity of the enzyme used in the reaction, as per manufacturer specifications. A small aliquot of the digested DNA was checked by agarose gel electrophoresis for completeness of digestion. When large volumes of DNA were required, the total volume of the digestion mixture was made up to 50 μL, and the components were adjusted accordingly.

2.2.4 Alkaline phosphatase treatment of vectors.

Prior to ligation, alkaline phosphatase treatment of the vector DNA was necessary, after restriction endonuclease digestion. Removal of 5'-phosphate groups from linearised vector DNA is essential to prevent vector self-ligation.

1 μL of thermosensitive alkaline phosphatase (1 U/μL, Roche) and 12 μL of thermosensitive alkaline phosphatase buffer were added to 1-5 μg of digested vector and incubated for exactly 15 minutes at 65°C. To stop the reaction, 4 μL of stop buffer was added, and a second incubation of 15 minutes at 65°C was carried out. The vector DNA was ethanol precipitated and quantified using agarose gel electrophoresis and quantification standards.

2.2.5 Generation of blunt ends.

Treatment with the Klenow fragment of DNA Polymerase I (Pol I) was used to end-fill up to 1 μg of DNA per reaction, which consisted of the following: isolated DNA fragment (up to 1 μg), 2 μL React®2 buffer (Invitrogen), 1 μL Klenow enzyme, 1 μL dNTPs (2 mM), and made up to a total volume of 20 μL with water. The reaction mix was incubated at 30°C for 15 minutes and then heat inactivated at 75°C for 10 minutes. If the reaction was performed immediately after a restriction enzyme digest, 1 μL Klenow enzyme and 1 μL dNTPs (2mM), were added directly to the restriction digest.

2.2.6 DNA purification by gel electrophoresis.

Purification of DNA fragments was achieved by using agarose gel electrophoresis. DNA was separated by electrophoresis, using 1% agarose in 1 x TAE buffer containing ethidium bromide (0.5 μg/mL), at 40 V for 4 hours. DNA bands were visualised under UV light using a hand-held UV lamp, and the bands containing DNA to be purified, were excised with a scalpel blade. The DNA was purified from the agarose slice using CONCERT™ gel purification kit (GIBCOBRL, Invitrogen) according to the manufacturer's instructions. This kit uses a system of spin cartridges containing a silica membrane. The agarose gel is dissolved by sodium perchlorate and DNA adheres to the silica support. DNA is released upon elution with TE buffer, after washes with ethanol-based buffers. Alternatively, DNA was purified using the freeze-squeeze method (Thuring et al., 1975) (DNA was quantified by gel electrophoresis using quantification standards).

2.2.7. Phenol-chloroform extraction and ethanol precipitation.

DNA was purified by phenol-chloroform extraction and concentrated by precipitation as described in Sambrook et al., (2001).

2.2.8 PCR purification.

Purification of PCR products was required before they could be used in subsequent ligation, to remove oil, primers, salts and excess dNTPs. This was achieved using QIAquick PCR purification kit (QIAGEN). This system utilises a silica-based spin cartridge to purify double-stranded DNA. PCR products were purified according to manufacturer's instructions and then quantified by agarose gel electrophoresis using quantification standards.

2.2.9 Purification of genomic DNA.

DNA was purified from 3 ml of freshly extracted human blood using the Wizard (Promega) genomic DNA extraction reagents, according to the manufacturers instructions. The DNA isolation protocol makes use of the different stabilities of the red blood cells and white blood cell membranes. Red blood cells are lysed in a hypotonic solution containing NH_4Cl (155 mM), $KHCO_3$ (10 mM) and EDTA (0.1

mM). The white blood cells and their nuclei are then lysed by a proprietary reagent. At high salt concentrations (2.5 M NaCl) the DNP dissolves and dissociates into DNA and protein (mainly histones, which are arginine- and lysine-rich, low molecular weight proteins). The proteins can be denatured and removed by centrifugation. The DNA will remain soluble and can then be precipitated by the addition of ethanol.

2.2.10 Polymerase chain reaction (PCR).

PCR reactions were performed using either *Taq*, *Tgo*, *Pfu* DNA polymerases, or a GC-rich system, which contains a blend of *Taq* and *Tgo* enzymes. The *Taq* DNA polymerase reactions contained 5 µL 10 x PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3, 15 mM $MgCl_2$, Roche), 5 µL dNTPs (3 mM), 5 µL of each primer (50 ng/µL), 0.5-1.5 µL $MgCl_2$ (25 mM), 2 µL DMSO, 0.5 µL of *Taq* DNA polymerase (5 U/µL), 1 µL of the DNA template (100 ng/µL), and sterile H_2O (sH_2O) up to a total volume of 50 µL.

The *Pfu* DNA polymerase reactions contained 5 µL 10 x *Pfu* DNA polymerase buffer (100 mM KCl, 60 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.2, 20 mM $MgCl_2$, 1% Triton X-100, 100 µg/mL BSA, Stratagene), 5 µL of each primer (50 ng/µL), 5 µL dNTPs (3 mM), 0.5-1.5 µL $MgCl_2$ (25 mM), 2 µL DMSO, 0.5 µL of *Pfu* DNA polymerase (5 U/µL), 1 µL of the DNA template (100 ng/µL), and sH_2O up to a total volume of 50 µL.

The *Tgo* DNA polymerase reactions contained 2.5 µL 10 x *Tgo* DNA polymerase buffer (50 mM Tris-HCl pH 8.5, 87.5 mM $(NH_4)_2SO_4$, 6.25 mM $MgCl_2$, 2.5% Tween 20, 7.5% DMSO, Roche), 2.5 µL of each primer (50 ng/µL), 2.5 µL dNTPs (3 mM), 0.25-0.75 µL $MgCl_2$ (25 mM), 2 µL DMSO, 0.25 µL of *Tgo* DNA polymerase (5 U/µL), 0.5 µL of the DNA template (100 ng/µL), and sH_2O up to a total volume of 25 µL.

The GC-rich system reactions contained two mixes: Master mix 1 consisting of 5 µL of each primer (50 ng/µL), 4 µL dNTPs (10 mM), 1 µL DNA template (100 ng/µL), 20 µL GC-rich resolution solution (5 mM) and Master mix 2 consisting of 10 µL 5 x GC-rich reaction buffer, 1 µL GC-rich enzyme (*Taq*/*Tgo* polymerase), and 1.5 – 3.5

μL sH_2O and 0.5 – 2.5 μL $MgCl_2$ (25mM, Sigma) to a total of 4 μL. Each mix was individually prepared and mixed, and then the two were combined and mixed.

The polymerase enzyme was always added last, and the reactions were mixed thoroughly before a drop of mineral oil was added, if necessary. PCR reactions were carried out in a thermal cycler (HYBAID Omn-E, SciTech (NZ) Ltd, or GeneAmp®PCR System 2700, Applied Biosystems, Foster City, USA), programmed as shown below. In addition to the reaction, a negative control with no DNA was included with every PCR experiment to ensure that no contamination was present. A positive control was also used (Factor IX promoter), producing a DNA product of known size.

PCR thermocycling conditions

95°C for 5 minutes	
95°C for 30 seconds	
55°C for 30 seconds	40 x cycles
72°C for 1 minute, 30 seconds	
72°C for 7 minutes	

5 μL of each PCR reaction was analysed for the correct product by agarose gel electrophoresis.

2.2.11 Ligations using pGEM®-T.

PCR products were cloned into the pGEM-T vector (Appendix 2, Promega) to enable the production of amplified insert DNA without the need for further PCR. pGEM-T vector kits contain 2 x rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP) and 10% w/v polyethylene glycol (PEG)) and control insert DNA. The vector was supplied in a linearised form, prepared for ligation by *Eco* RI digestion and the addition of 3'-terminal thymidine at both ends. These single 3'-T overhangs greatly improve the efficiency of ligation and provide compatible overhangs for PCR products generated by certain thermostable polymerases, including *Taq* polymerase.

PCR products were subcloned into pGEM-T, following the manufacturers instructions. The control vector was used with a 1:1 (insert:vector) molar ratio of control DNA and a 3:1 (insert:vector) molar ratio of PCR DNA in a total volume of 10 µL. Ligation mixes were incubated overnight at 4°C and transformation of *Escherichia coli* (*E.coli*) XL-1 blue cells was performed the following day, utilising blue/white selection (Berger and Kimmel, 1987).

2.2.12 Ligations using pGL3Basic and pBluescript SK+ vectors.

The DNA insert fragments and vector DNA were digested with restriction endonucleases to generate compatible ends necessary for successful ligation. Ligation efficiency is dependent on the concentration of DNA ends in the reaction. To ensure complementary ends of the vector and insert are joined, the concentration of insert must be higher than that of the vector (Berger and Kimmel, 1987).

The amount of vector DNA used in the ligations was usually 65 ng for a 3:1 (insert: vector) molar ratio, or 75 ng for a 2:1 (insert:vector) molar ratio. The amount of insert DNA that was added to ligations was calculated using the following formula.

$$\text{Amount of insert DNA (ng)} = \frac{\text{Amount of vector DNA (ng)} \times \text{size of insert (bp)}}{\text{Size of vector (bp)}} \times \frac{\text{insert}}{\text{vector}}_{\text{molar ratio}}$$

Ligation mixes also contained 4 µL (5 x) T4 ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% w/v PEG), 1 µL T4 DNA ligase (1 U/µL, Roche) and dH_2O up to a final volume of 20 µL. Ligation mixes were prepared, mixed thoroughly and incubated overnight at 4°C. The following day, the ligation reactions were used for the transformation of *E.coli* XL-1 blue competent cells.

2.2.13 Transformation of *Escherichia coli* (*E.coli*) XL-1 cells.

E.coli competent XL-1 blue cells were made by inoculating 5 mL LB-tet broths (100 mg/mL tetracycline) with single colonies and growing at 37°C with vigorous shaking. The cells were pelleted at early log phase (A_{600} of 0.4 - 0.5) at 12,000 rpm for 1 minute and resuspended in one-tenth volume of ice cold TSS buffer (10 g/L tryptone, 5 g/L yeast extract, 100 g/L PEG-4000, 5% DMSO, 5% 1M $MgCl_2$, filter sterilised). The competent cells were then stored on ice and used within 2-3 hours.

Typically, half of a ligation mix (10 μL) was added to 100 μL of XL-1 blue competent cells and incubated on ice for 15 minutes. The samples were then heat shocked in a 42°C waterbath for exactly 2 minutes, followed by a second 15 minute incubation on ice. The transformed cells were then diluted 100-fold in LB-amp broth (20 mg/mL, 100 mg/mL ampicillin) and grown at 37°C with vigorous shaking for 30 – 90 minutes. 100 μL of the transformed cells were then plated onto warm LB-amp plates (20 mg/mL, 1.5% agar, 100 mg/mL ampicillin). Transformed clones were selected by growth on ampicillin. Reactions without DNA or without ampicillin were included as controls. The plates were incubated for approximately 16 hours overnight at 37°C, after which colonies were scored. XL-1 blue cells also have the capacity for blue/white selection, which was achieved by plating the transformants on LB-amp plates supplemented with 0.5 mM isopropyl thiogalactoside (IPTG) and 80 μg/mL X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside).

2.2.14 Isolation of Plasmid DNA from *E.coli.*

2.2.14.1 Rapid-boil method.

Plasmid DNA was prepared for screening purposes by the rapid boil method, as described by Holmes and Quigley (1981). Plasmid DNA obtained by this method was of low purity, but was suitable for use in diagnostic digests.

2.2.14.2 Small-scale plasmid isolation.

Small-scale plasmid DNA preparation was used when a small quantity of high quality plasmid DNA was required for subsequent cloning, PCR or sequencing. Generally, 2 mL of a 5 mL overnight culture was pelleted and plasmid DNA isolated using a Quantum® Prep Plasmid Miniprep Kit (BioRad), according to the manufacturers instructions. This system uses an alkaline lysis (containing SDS) method (Birnboim and Doly, 1979) to release plasmid DNA from the cell. Purification of the plasmid DNA is achieved by a patented Quantum prep matrix, which contains a diatomaceous earth that binds DNA.

2.2.14.3 Large-scale plasmid isolation.

Large scale plasmid DNA preparations were carried out to obtain the high quality and quantity required for transfections and automated sequencing. 5 mL LB-amp cultures grown from single transformed *E. coli* XL-1 blue colonies were used to inoculate 500

mL of LB-amp broth (100 mg/mL ampicillin) in 2 L flasks and incubated overnight at 37°C with vigorous shaking. Plasmid DNA was then extracted using the CONCERT™ High Purity Plasmid Maxiprep Kit (GIBCOBRL®, Invitrogen) according to the manufacturers instructions. This system is based on a modified alkaline lysis method (Birnboim and Doly, 1979), where plasmid DNA is purified on an anion exchange resin. Plasmid DNA was stored at -20°C.

2.2.15 DNA quantification.

2.2.15.1 Quantification of DNA by gel electrophoresis

Purified PCR products or inserts for subcloning were quantified by gel electrophoresis. The DNA samples to quantify were loaded onto a 1% agarose gel alongside a series of standards of known DNA concentrations, and were analysed by electrophoresis. The standard used was linearised plasmid Bluescript II, with a range of 10-100 ng/5μL. The intensity of fluorescence of the sample under UV light was compared with the concentration standards.

2.2.15.2 Quantification of DNA by UV spectroscopy

Plasmid DNA prepared on a large scale was quantified using the Pharmacia Biotech Ultraspec 300 UV/Visible Spectrophotometer, by measuring the UV absorption of the DNA sample in quartz cuvettes at 260 nm and 280 nm. The concentration factor 1 E_{260} - 50 μg/mL was used for double-stranded DNA.

The purity of the DNA was assessed by the A_{260}/A_{280} absorbance ratio. A ratio of 1.8 is expected for pure DNA, ratios greater than 1.8 indicate RNA, less than 1.8 indicate protein contamination.

2.2.16 Sequencing of DNA.

Automated sequencing using an ABI 377-18/36 DNA sequencer (PE Biosystems) or an ABI 3730 DNA sequencer (PE Biosystems) was performed on plasmid DNA template by Lorraine Berry, Allan Wilson Centre sequencing facility, Massey University. The sequencing was carried out according to manufacturer's instructions using ABI PRISM® BigDye™ terminator cycle sequencing (PE Biosystems) chemistry. Primers and

templates were diluted to 0.8 pmol/μL and 200 ng/μL, respectively (in sH_2O) for sequencing.

2.2.17 Analysis of DNA sequences.

The DNA sequences were analysed using the Wisconsin Package v.9.1, Genetics Computer Group (GCG), Maddison, WI. Various programs within this suite of software were used to accomplish particular tasks, such as: restriction maps (map), generate contigs of individual sequences (GelMerge), find sequence similarity (bestfit), and find putative binding factors by identifying consensus sequences within a sequence (findpatterns).

The TRANSFAC database (MatInspector V2.2, http://transfac.gbf.de/cgi-bin/matSearch.pl.), which enables an extensive database search (*in silico*) on transcription factors, their binding sites, and DNA-binding profiles, was also used to analyse sequences. The outputs were analysed manually for any relevant sequence motifs.

2.2.18 Tissue culture.

HeLa cells and MDA-MB-231 cells were used for transient transfections and HeLa cell extracts for electrophoretic mobility shift assays. All tissue culture operations prior to harvesting were carried out aseptically and in a laminar flow hood (Crossflow 1800 with HEPA filter, Westinghouse). Cells were incubated at all times in a 37°C, 5% CO_2 incubator (Jouan IG150, France), in humid conditions.

HeLa cells and MDA-MB-231 cells were grown in complete Eagle's OPTI-minimal essential medium (MEM) Reduced Serum Medium, containing non-essential amino acids (GIBCOBRL, Invitrogen) which was prepared as instructed by the manufacturers and filter sterilised through a 0.2 μm filter. MEM was dispensed into sterile bottles in 194 mL volumes, which were supplemented as required with 2% (4 mL) foetal calf serum (FCS) and 1% (2 mL) penicillin/streptomycin (Pen/Strep, 5000 U/mL penicillin G sodium and 5 mg/mL streptomycin sulfate in 0.85% saline). Media was stored at 4°C, but warmed to room temperature before use.

2.2.18.1 Starting HeLa and MDA-MB-231 cell cultures.

Cell cultures were started from 1 mL frozen HeLa or MDA-MB-231 cell stocks (prepared by Kirsty Allen, Institute of Molecular BioSciences, Massey University, Palmerston North). A frozen aliquot of HeLa or MDA-MB-231 cells (in FCS containing 10% DMSO, stored under liquid nitrogen) was thawed and the cells transferred to 5 mL of supplemented OPTI-MEM, then mixed thoroughly. The cells were pelleted by centrifugation for 5 minutes at approximately 100 g and the supernatant removed. The pellet was resuspended in 2 mL of supplemented OPTI-MEM and 1 mL used in each monolayer T80 flask (Nunc) containing 14 mL of supplemented OPTI-MEM. HeLa cells were left to grow for about 2-3 days in the 37°C incubator before passaging. MDA-MB-231 cells were left to grow for about 4-5 days in the 37°C incubator before passaging.

2.2.18.2 Maintenance of HeLa and MDA-MB-231 cells.

HeLa and MDA-MB-231 cells were grown in T80 flasks to 80-90% confluence before passaging the cells into new flasks with fresh media. Passaging the cells initially involved removing the old media, and then washing the cells twice with 9 mL phosphate buffered saline (PBSE) (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4.2H_2O$ pH 7.2 plus 0.5 mM EDTA) plus 1 mL 10 x trypsin (GIBCOBRL). The cells were left lying flat for a few minutes to dislodge them from the flask surface; further detachment was achieved by sharply tapping the side of the flask. The cells were then thoroughly resuspended by aspiration in 5 mL of supplemented OPTI-MEM, where 1 mL of resuspended cells was used to seed a new T80 flask containing 14 mL of fresh supplemented OPTI-MEM and the remainder of the cells were used to seed plates for transfections. Cells were placed back into the 37°C incubator and the HeLa cells were passaged every 2-3 days, while the MDA-MB-231 cells were passaged every 4-5 days.

2.2.18.3 Preparing HeLa and MDA-MB-231 cells for freezing.

Stocks of HeLa and MDA-MB-231 cells were frozen after the first passage. Cells were grown to 80% confluence, passaged and resuspended in 5 mL of FCS containing 10% DMSO. The cells were then dispensed into sterile 1 mL cryotubes (Nunc) and frozen slowly (by wrapping cryotubes in multiple layers of tissue paper) at -70°C, to avoid disruption of the cell membrane. Once frozen the HeLa and MDA-MB-231 cells were stored under liquid nitrogen.

2.2.19 Electrophoretic mobility shift assays (EMSA).

2.2.19.1 Radioactively labelling the oligonucleotide probe for EMSA.

T4 Polynucleotide kinase (PNK) was used to catalyse the transfer of the γ-phosphate of ATP to the 5'-hydroxyl terminus of the DNA strand. Labelling of oligonucleotides was carried out, as indicated below, in 1.5 mL microcentrifuge tubes, mixed, then incubated at 37°C for 45 minutes.

oligonucleotide (100 ng/μL)	1μL
10 x PNK buffer (0.5 M Tris-HCl pH 8.0, 0.1 M $MgCl_2$,	
50 mM DDT, 0.5 mg/mL BSA)	1 μL
γ^{32}P-[ATP] (10 μCi/μL, Perkin Elmer)	4 μL
T4 polynucleotide kinase (10 U/μL, Roche)	1 μL
sH_2O	3 μL

The labelled oligonucleotide was then annealed to its complementary oligonucleotide at 6 x excess. 6 μL complementary oligonucleotide (100 ng/μL) was added to the labelled oligonucleotide, along with 2.5 μL 1 M KCl and 31.5 μL of sH_20. This mixture was heated to 95°C for 5 minutes in a boiling water bath, which was subsequently turned off and the samples were left to cool to room temperature in the water bath (approximately 1 hour). 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, or 10 mM Tris pH 7.8, 1 mM EDTA, 0.1 mM DTT, 5% glycerol) was added to the cooled double-stranded oligonucleotides, which were then gel purified immediately.

2.2.19.2 Purifying the labelled oligonucleotides.

To remove any residual single-stranded oligonucleotides, unincorporated label or other contaminants, the labelled oligonucleotides (total volume 50 μL) were purified by electrophoresis on a 10% polyacrylamide gel (37 cm long with 0.4 mm spacers) in 1 x TBE (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA pH 8.0) at 30 W for 1 ½ hours. The gel was wrapped in saran wrap and exposed to X-ray film for approximately 1 minute, which enabled the visualisation of the double-stranded labelled oligonucleotides within the gel. The appropriate band(s) were excised from the gel and DNA eluted by placing each gel slice into 300 μL 50 mM KCl and incubating at 37°C overnight. The

following day, the gel suspension was mixed by vortexing, centrifuged at 12,000 g for 5 minutes and the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube. 1 µL of each probe was analysed for incorporation of radioactivity using Cerenkov (Beckman LS3801 Scintillation Counter) counting. The samples were stored in a radioactivity safe Perspex container at 4°C, until required.

2.2.19.3 Preparing HeLa cell extracts for EMSA.

HeLa cells were grown to 80-90% confluence in 14 mL supplemented OPTI-MEM in 150 mm diameter plates (Nunc), in a 37°C CO_2 incubator. The media was removed and the HeLa cells were washed twice with 2 mL PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4 2H_2O$ pH 7.2). 1 mL of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl) was then added to each plate and the cells were scraped off and placed into 1.5 mL microcentrifuge tubes. The cell suspension was centrifuged at 12,000 g for 5 minutes and the supernatant discarded. The pellet was resuspended in 300 µL of extraction buffer (containing Complete mini EDTA-free Protease inhibitor cocktail (Roche), 40 mM Hepes pH 7.9, 0.4 M KCl, 1 mM DTT and 10% glycerol). Cells were disrupted using 3 freeze-thaw cycles in liquid nitrogen. The cell extract was centrifuged again at 12,000 g for 5 minutes (at 4°C) and the supernatant dispensed into 30 µL volumes, then immediately snap frozen in liquid nitrogen and stored at -70°C. The proteins present in the HeLa cell extracts were quantified using the Bradford Protein Assay (section 2.2.21).

2.2.19.4 Preparing double-stranded competitors.

Unlabelled double-stranded competitors were generated by adding 5 µL of each complementary single-stranded oligonucleotide (1 µg/µL) together and heating to 95°C for 5 minutes in a Hybaid Omn-E thermal cycler. Samples were cooled stepwise by 10°C every 5 minutes until they reached 25°C. Double-stranded competitors were stored at -20°C until required.

2.2.19.5 Binding reactions for EMSAs.

Poly dI.dC is a non-biological polymeric nucleic acid that acts as a competitor DNA in EMSAs. Excess poly dI.dC was added to the reactions to minimise the occurrence of non-specific DNA-protein interactions.

HeLa extract (~7 μg protein) was added to the following premix; 10 μL gel shift buffer, 1 μL poly dI.dC (1 mg/mL in $MgCl_2$) and water to make up a total volume of 20 μL, then incubated on ice for 10 minutes. Competitors were added before the addition of probe. Approximately 0.3 ng of labelled oligonucleotide (1 μL) was added to the premixes and left at room temperature for 15 minutes.

2.2.19.6 Polyacrylamide gel electrophoresis (PAGE) for EMSAs.

As a control 2 μL of loading dye was loaded in an exterior lane to monitor the progress of electrophoresis; 10 μL of each reaction was loaded onto a non-denaturing 4% polyacrylamide gel in 0.25 x TBE and electrophoresed at 200 V for about 1 hour (BRL V15.17 apparatus fitted with 0.75 mm spacers). The gels were transferred onto DE-81 paper and dried using Bio-Rad Gel Dryer 583 for about 20 minutes at 80°C. The gels were then exposed to X-ray film for at least 20 hours at -70°C using a radioactive safe cassette with intensifying screens. The X-ray (Kodak) films were developed using a 100Plus™ Automatic X-ray film processor in a dark room.

2.2.20 Bradford protein assay.

The protein concentration of HeLa extracts was determined using the Bradford protein assay dye reagent concentrate (BioRad) in 96 well microplates (Nunc) according to manufacturer's instructions. Standards were prepared from a 1 mg/ml stock to a range of 0 – 2.5 μg in water, and 10 μL of HeLa cell extract, diluted 1:2, 1:5, 1:10, 1:20, 1:40 and 1:60, were analysed. Samples were left to develop colour at room temperature for a minimum of 10 minutes and absorbance read at 595 nm. A protein standard curve was constructed using the standard amounts of BSA, from which the amount of protein present in HeLa extract was determined.

2.2.21 Transient transfections.

HeLa and MDA-MB-231 cells were transiently transfected with FuGENE™6; a multi-component lipid-based reagent that complexes with and transports DNA into the cells during transfection. Cells being transfected using FuGENE™6 are required to be at 50-80% confluence on the day of transfection. Thus, the day prior to transfection 800 μL of supplemented OPTI-MEM (in 12 well plates) was seeded with 100 μL of HeLa cell suspension, which had been taken from freshly resuspended (in 5 mL) 80 - 90%

confluent HeLa or MDA-MB-231 cell stocks in T80 flasks. Each transfection was performed in triplicate and using a 3:1 ratio of FuGENE™6 to DNA (3 μL FuGENE™6:1 μg DNA), according to manufacturers instructions. HeLa and MDA-MB-231 cells were harvested 40-48 hours after transfection and assayed for luciferase and β-galactosidase activity as soon as possible.

2.2.22 Harvesting cell extracts for luciferase and β-Galactosidase assays.

The media was removed from the cells, which were then rinsed twice with 1 mL of PBS. 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) was added to each well containing cells, and incubated at room temperature for 15 minutes. The cells were then scraped off the surface of the wells using a cell scraper and transferred to 1.5 mL microcentrifuge tubes. Cell extracts were centrifuged at 12,000 rpm for 30 seconds to remove any cellular debris and the supernatant transferred into fresh microcentrifuge tubes. The cell extracts were stored at 4°C until required.

2.2.23 β-Galactosidase assays.

The plasmid pCMVSPORT-β-Gal was used as an internal control for transfections, which allowed the efficiency of transfection to be determined using a β-galactosidase assay. Cell extract (5 μL) was incubated with 50 μL ONPG (*o*-nitrophenyl-β-D-galactopyranside, 2 mg/mL in 60 mM NaH_2PO_4, 40 mM Na_2HPO_2) and 100 μL β-galactosidase assay buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_2, 10 mM KCl, 1 mM $MgCl_2$) in microtitre (96 well, Nunc) plates, at 37°C for 3-8 hours. After incubation, 50 μL 1 M Na_2CO_3 was added to stop the reaction and develop full yellow colour. Absorbances were measured at 405 nm against a blank of 5 μL cell lysis buffer using a 96 well plate reader (anthos reader HT2 type 12 500, anthos labtech instruments, Salsberg, Austria).

2.2.24 Luciferase assays.

The reporter gene luciferase enabled the measurement of promoter activity. 5 μL of HeLa or MDA-MB-231 cell extract was mixed with 20 μL of luciferase reagent (Promega) and analysed using a FLUOstar galaxy microplate reader (BMG Labtechnologies Pty. Ltd, Melbourne, Australia). Luciferase activity was measured by

the amount of light that was emitted, and fibre optics within the microplate reader detected and conveyed this information as actual photon counts. This information was accessed through Excel (Microsoft 97), which is interfaced with the microplate reader. The maximum photon count obtained (within the 3 minute measuring period) was the value retained for data analysis. 5 μL cell lysis buffer was used as a blank.

2.2.25 GMO approval codes.

This work was carried out with ERMA regulatory authority approval as follows:

- Topoisomerase IIβ clones (pGEM-T and pBluescript): GMO 99 MU 25
- Expression plasmids (Sp1 and Sp3): GMO 00/MU 40
- Topoisomerase IIβ promoter plasmids (pGL3Basic): GMO 98 MU 53

Chapter 3: Isolation and Cloning of the Topoisomerase IIβ promoter sequence.

3.1 Introduction.

The first step in the characterisation of the topoisomerase IIβ promoter was the isolation and cloning of the 5'-flanking sequence of the human topoisomerase IIβ gene. A partial topoisomerase IIβ promoter sequence was published in 1997 (Ng *et al.,* 1997), but was lacking the full upstream regulatory sequence. Since then the entire human topoisomerase IIβ gene sequence has been deposited in GenBank (Accession Number AC093416). This was the extent of the characterisation of the topoisomerase IIβ promoter at the outset of the research described in this thesis, however another group working on the topoisomerase IIβ promoter published their findings prior to the completion of this work (Lok *et al.,* 2002). This study isolated a 1.3 kb genomic fragment corresponding to the 5'-flanking and untranslated regions of the topoisomerase IIβ gene, but still lacking the distal 5'-regulatory region. In the current study a larger 1.54 kb region of the topoisomerase IIβ promoter was isolated and cloned in an attempt to increase our understanding of the mechanisms regulating expression, and to allow a comparison between the regulatory mechanisms of the α and β genes.

Reporter genes are commonly used to examine eukaryotic gene expression *in vivo*. In a controlled assay system the reporter gene can be used to investigate promoter activity and thereby elucidate the mechanisms of gene regulation. A reporter gene construct contains the promoter under investigation linked to a promoter-less reporter gene, such as firefly luciferase (for example in the vector pGL3Basic). The promoter of interest is fused upstream of the reporter gene, so that transcripts initiating at the promoter proceed through the reporter gene. It is common for a reporter gene to encode an easily measurable protein, and the level of transcription initiated at the promoter is thought to be a reflection of the amount of reporter protein produced. In order to be sensitive to changes in transcription due to interactions between the introduced promoter sequence and transcriptional regulators the effect of the reporter protein on cell physiology must be minimal (Ausubel *et al.,* 1991).

In order to investigate regions of the promoter important for topoisomerase IIβ gene expression, a deletion series of the wild type promoter sequence (-1357 to +126) was generated in pGL3Basic vector, to allow a comparison with wild type activities, using functional assays. A total of nine constructs were generated; a series of five 5'-deletion constructs and an additional set of four constructs with an internal deletion introduced within the truncated promoter sequences.

The Lok (2002) study indicated that two inverted CCAAT boxes (ICB1 and ICB2) that bind the NF-Y transcription factor and a GC box that binds the Sp1 transcription factor are important for topoisomerase IIβ promoter activity. PCR mutagenesis was used to introduce mutations into the ICB1 or ICB2 elements in order to investigate the importance of these two elements for topoisomerase IIβ promoter activity. An additional construct was created consisting of the 180 bp region, containing the GC, ICB2 and ICB1 elements, cloned upstream of an SV40 promoter in a luciferase reporter vector. The purpose of this construct was to examine the ability of this putative regulatory region to enhance expression driven by a heterologous promoter, thereby investigating its influence on expression from the topoisomerase IIβ promoter.

3.2 Generating a topoisomerase IIβ promoter clone.

3.2.1 Introduction.

The topoisomerase IIβ promoter sequence was isolated by carrying out polymerase chain reactions (PCR), using specific oligonucleotide primers, and human genomic DNA as a template. The isolated DNA sequence was first cloned into a pGEM-T vector to facilitate cloning of the PCR product. In order to investigate the functional significance of the topoisomerase IIβ promoter region, the cloned DNA was then sub-cloned into a pGL3Basic luciferase reporter vector to be used in transient transfection experiments. This pGL3Basic construct provided the material from which the additional deletion and mutation constructs were generated.

The initial cloning strategy was to remove the 1.54 kb insert from the pGEM-T vector (pGEM-T TIIβ-1357), using a *Sac* I restriction site in the multiple cloning site (MCS) and a *Bgl* II recognition site at the 3'-end of the DNA sequence. This strategy provided the simplest cloning technique, making use of two cohesive DNA ends for re-ligation, and therefore allowing directional cloning. The topoisomerase IIβ promoter sequence needed to be ligated into the pGL3Basic vector in the 5'- to 3'-direction in order to drive transcription of the luciferase gene. However, digestion with these enzymes did not release the insert, which suggested that the recognition sites may have been altered during the cloning process. Subsequently, the less straightforward blunt-ended cloning strategy outlined in figure 3.1 was developed. The need to use restriction endonuclease *Apa* I resulted in the removal of 57 bp from the 3'-end of the topoisomerase IIβ promoter region, due to an internal *Apa* I site, but the 5'-sequence was unchanged. Thus, a 1.5 kb fragment of the topoisomerase IIβ promoter sequence was cloned into pGL3Basic.

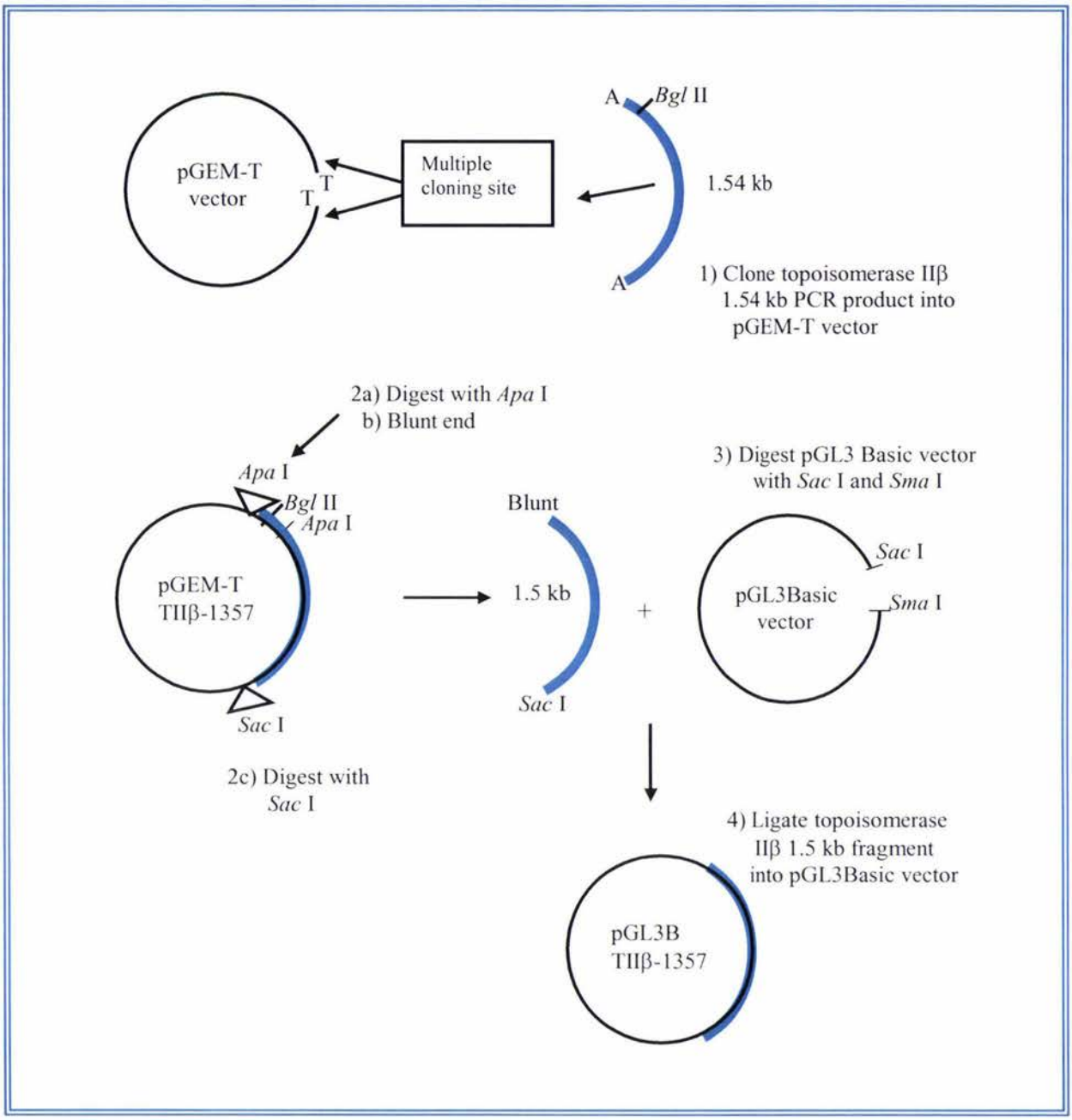


Figure 3.1: Cloning strategy for topoisomerase IIβ-1357 clone.

As an initial step in the cloning process, the topoisomerase IIβ 1.54 kb (-1357 bases upstream of the transcription start site to +183 bases after the transcription start site) PCR product was cloned into the pGEM-T vector. Restriction enzymes *Apa* I (followed by blunt-ending) and *Sac* I were used to remove a 1.5 kb fragment (-1357 to +126) of the original 1.54 kb insert, for subcloning into a *Sma* I/*Sac* I digested pGL3Basic luciferase vector.

3.2.2 Topoisomerase IIβ PCR.

A set of oligonucleotide primers was designed based on the topoisomerase IIβ sequence. These included forward and reverse primers (TIIβ-Fwd and Rev) of 25 nucleotides (nt) in length, flanking approximately 2.7 kb of promoter sequence, and forward internal primers to be used in subsequent sequencing reactions (TIIβ-Seq1-6). Restriction enzyme recognition sites were introduced into the flanking, forward and reverse primer sequences for use in subsequent cloning. The TIIβ-Fwd contained a *Sac* I site and the TIIβ-Rev contained a *Bgl* II site. The 18 nt sequencing primers were designed to anneal at sites approximately 400 bp apart, thereby generating overlapping sequence. Sequences of oligonucleotides used in this study are shown in appendix 1.

Human genomic DNA to be used as a template in the PCR reactions was purified, as described in section 2.2.9, and the concentration was determined by gel electrophoresis using quantification standards and by UV spectrophotometry, as described in section 2.2.15. Due to the high GC content of the topoisomerase IIβ promoter sequence several different PCR polymerase enzymes were tested, to optimise the reproducibility of the PCR products obtained. PCR reactions were performed using *Taq*, *Pfu*, *Tgo*, and a GC-rich system (Promega).

The flanking primers, TIIβ-Fwd and Rev, were first used in an attempt to PCR amplify the 2.7 kb of topoisomerase IIβ upstream sequence. $MgCl_2$ titrations were carried out with each type of polymerase in an attempt to produce conditions optimal for the amplification of this fragment. When these primers failed to produce the desired product, different combinations of the TIIβ-Rev and the sequencing forward primers were used in PCR reactions in an attempt to amplify smaller regions of the promoter. The purpose of these reactions was to test the integrity of the forward and reverse primers, and with a tentative goal of producing smaller overlapping fragments to encompass approximately 2.7 kb of the upstream region.

Additional forward and reverse internal primers were designed to carry out these experiments (TIIβ-mm1-mm3, see Appendix 1). Eventually PCR products were successfully produced for the 5'-end of the promoter of 688 bp in size, using *Taq*

polymerase and the GC-rich system, and for the 3'-end of the promoter of 1.54 kb (figure 3.2), using the GC-rich system (refer to section 2.2.10 for PCR reactions). However, the remaining internal sequence could not be isolated and therefore the focus of the study became the characterisation of the 1.54 kb fragment, corresponding to the topoisomerase IIβ sequence -1357 to +183 (where +1 denotes the major transcription start site.

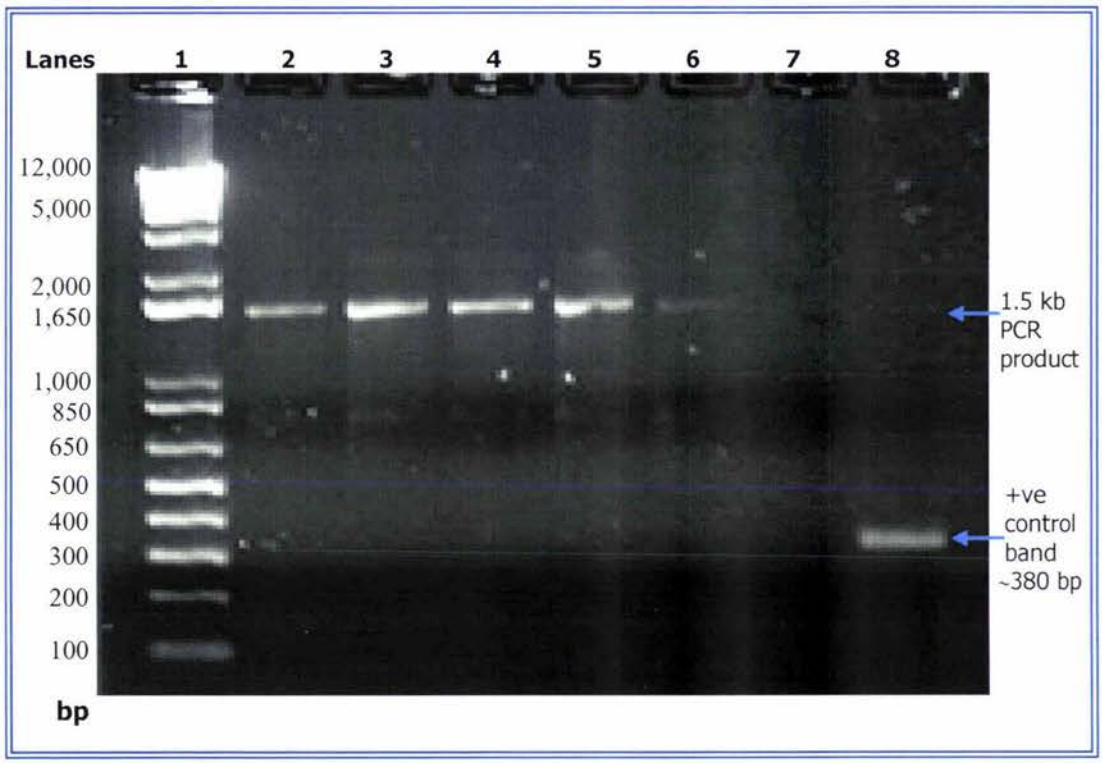


Figure 3.2: PCR reaction to generate topoisomerase IIβ 1.54 kb fragment.

5 μL of each reaction was applied to a 1% agarose gel in 1 x TAE buffer and electrophoresed for about 1 hour at 85 V. Ethidium bromide (0.5 μg/mL) was incorporated into the gel and the DNA was visualised by exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are indicated on the left in base pairs (bp).

Lanes	2	5 μL standard PCR reaction with 1.75 mM $MgCl_2$
	3	5 μL standard PCR reaction with 2.00 mM $MgCl_2$
	4	5 μL standard PCR reaction with 2.25 mM $MgCl_2$
	5	5 μL standard PCR reaction with 2.50 mM $MgCl_2$
	6	5 μL standard PCR reaction with 2.75 mM $MgCl_2$
	7	5 μL negative control (PCR reaction with no template), no product
	8	5 μL positive control (Factor IX promoter), ~380 bp product

Lanes 2-6 contain the correct product, with an optimal $MgCl_2$ concentration of 2.0 - 2.5 mM producing the most product.

3.2.3 Cloning of topoisomerase IIβ 1.54 kb PCR product into pGEM-T vector.

PCR products that required purification (removal of primers, dNTPs, salts and enzymes) for further experiments were purified using QIAquick PCR purification kit (QIAGEN) and the concentration was determined by gel electrophoresis using quantification standards.

3.2.3.1 Ligations into pGEM-T.

Ligations were carried out as described in section 2.2.11, and a representative ligation experiment is illustrated in table 3.1.

Reaction	Vector minus ligase (control)	Vector plus control DNA (positive control)	Vector plus ligase (background control)	Vector plus PCR DNA (3:1 molar ratio)
2x ligation buffer	5 µL	5 µL	5 µL	5 µL
pGEM-T vector (50 ng)	1.0 µL	1.0 µL	1.0 µL	0.7 µL
PCR product (~15 ng)	-	-	-	3.5 µL
Control insert DNA (25 ng/µL)	-	2 µL	-	-
T4 DNA ligase (1 U/µL)	-	1 µL	1 µL	1 µL
H_2O	4 µL	1 µL	3 µL	-
Total	10 µL	10 µL	10 µL	10 µL

Table 3.1 - Ligation reactions with pGEM®-T.

Ligation reactions were carried out to clone the 1.54 kb PCR product into vector pGEM-T. Reactions were incubated at 4°C overnight, and then 5 µL of each ligation was used to transform XL-1 blue (*E.coli*) competent cells. For the PCR DNA, the ligation was carried out using a 3:1 (insert:vector) molar ratio, and for the control DNA the molar ratio was 1:1.

3.2.3.2 Transformations.

Ligation reactions were incubated overnight at 4°C before being used in a transformation reaction. Transformations were carried out as described in section 2.2.13, using blue/white selection to identify correct transformants, and a representative result is illustrated in table 3.2, below.

Reaction	Ligation Mixture	XL-1 competent Cells	LB-broth	Volume plated	Number of colonies	
					white	blue
1) XL-1 competent cells (control) 10^6 dilution	-	100 µL	890 µL	LB plates 100 µL	TMTC	-
10^7 dilution	-	100 µL	890 µL	100 µL	351	-
2) XL-1 competent cells (negative control)	-	100 µL	890 µL	100 µL LB+amp	0	-
3) Vector minus ligase (control)	5µL	100µL	895 µL	100µL	0	5
4) Vector plus control DNA (positive control)	5 µL	100µL	895µL	100 µL	100	18
5) Vector plus ligase (background control)	5 µL	100 µL	895 µL	100 µL	0	9
6) Vector plus PCR DNA	5 µL	100 µL	895 µL	100 µL	135*	20

Table 3.2: Results of XL-1 transformations using pGEM-T ligations.

Transformation of XL-1 competent cells using plasmids generated from ligation of 1.54 kb topoisomerase IIβ fragment or control DNA into pGEM-T vector (table 3.1). Unless indicated, all samples were plated onto LB plus ampicillin plates with 20 µL IPTG (20 mg/mL in water) and 40 µL X-gal (stock solution in 20 mg/mL dimethylformamide). Plates were incubated at 37°C overnight, and colonies were then scored. TMTC indicates colonies were to numerous to count. Colonies were creamy white or blue in colour. White colonies (*) were selected and cultured to isolate plasmid DNA for analysis.

XL-1 competent cells are unable to grow in the presence of ampicillin, however the pGEM-T vector confers ampicillin resistance in those cells successfully transformed. Transformation mixtures were plated onto LB/IPTG/X-gal with or without ampicillin to distinguish which plasmids contained the insert DNA. Successful cloning of an

insert into the pGEM-T vector interrupted the coding sequence of the *lacZ'* gene; therefore the recombinant clones were identified by colour screening of the colonies. Colonies containing the uninterrupted β-galactosidase gene grown on X-gal/IPTG plates are blue, whereas colonies containing the vector with insert are white.

The control consisting of XL-1 cells grown on LB only plates (reaction 1) grew only white colonies, as expected, while the control plate of XL-1 cells in the presence of ampicillin (reaction 2) could not support colony growth. Together these controls provided evidence that the XL-1 cells were viable and that white colonies observed in the other reactions were not the result of contamination or spontaneous ampicillin resistance.

The positive control (reaction 3) used the control insert DNA supplied by the manufacturer, and measured the efficiency of ligation. Typically, colonies observed should be about 10-40% blue and no less than 60% white. Blue colonies could be due to a failure in the ligation reaction. A complete absence of colony growth would suggest the transformation has failed. The results obtained from the positive control, as shown in table 3.2, illustrate 85% of colonies were white, indicating that the ligation and transformations proceeded efficiently. The background blue colonies observed in control reactions 3 and 5, suggest that the presence of blue colonies is mainly due to vector self-ligation or undigested vectors.

The ligation reaction containing pGEM-T vector with the topoisomerase IIβ 1.54 kb PCR product, resulted in approximately 40% white and 60% blue colonies. White colonies were picked for screening to determine the presence of the insert DNA. The selected colonies were maintained in the presence of ampicillin and plasmid DNA was isolated from *E. coli* cultures using the rapid boil DNA extraction method (Holmes and Quigley, 1981).

3.2.3.3 Screen for inserts.

Positive clones were identified by restriction endonuclease digestion of the isolated DNA, as described in section 2.2.3, and the reactions were analysed by gel electrophoresis. The enzymes selected for screening for the presence of the insert

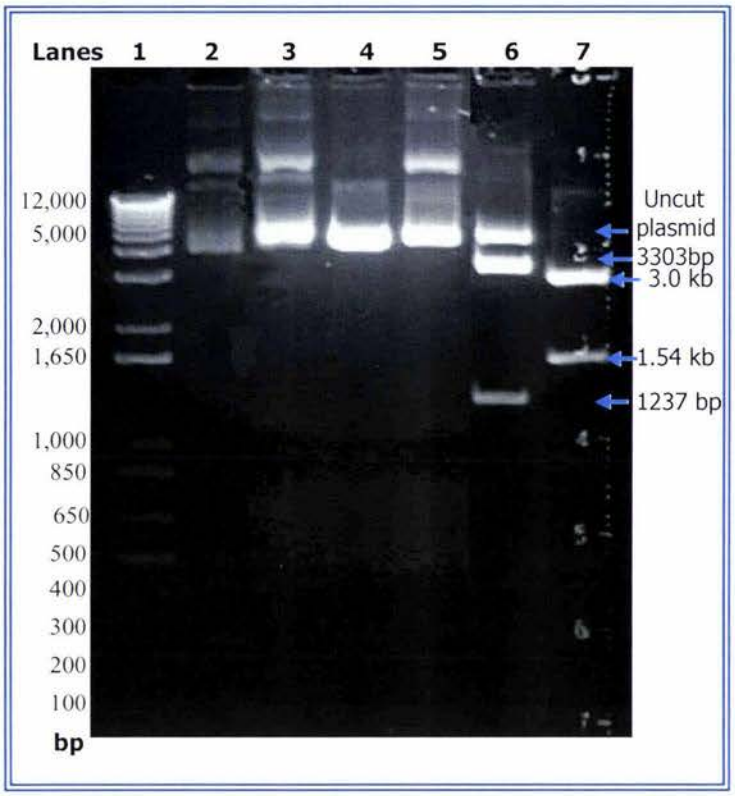
were *Sac* I and *Nco* I, which cut at sites in pGEM-T MCS, releasing the 1.54 kb insert (data not shown). Plasmid DNA isolated from four colonies appeared to have the correct insert, corresponding to the 1.54 kb topoisomerase IIβ promoter region. One of these colonies was maintained and a large scale plasmid preparation was carried out using CONCERT™ High Purity Plasmid Maxiprep Kit (GIBCOBRL®, Invitrogen), as described in section 2.2.14.3, to obtain DNA of high quality and quantity required for subsequent cloning and sequencing. The concentration of the plasmid was determined by gel electrophoresis using quantification standards and UV spectrophotometry.

Diagnostic digests, as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert. Correct orientation was confirmed by digestion with *Nar* I and *Sac* I. The topoisomerase IIβ promoter has a unique restriction site for the enzyme *Nar* I, which was used to positively identify the PCR product. Also, *Nar* I used in conjunction with *Sac* I in the 3'-MCS of the pGEM-T vector produces two fragments of 3303 bp and 1237 bp in size, if the orientation of the insert is correct. The results of these digests, confirming the identity of the topoisomerase IIβ-1357 promoter sequence, are shown in figure 3.4.

3.2.3.4 Sequencing of the plasmids.

Sequencing was carried out, as described in section 2.2.16, to confirm the topoisomerase IIβ promoter sequence and to ensure no undesirable mutations had been introduced (see Appendix 3 for sequence). Analysis of the pGEM-T TIIβ-1357 sequence was carried out using the bestfit program (GCG Version 9.1; Genetics Computer Group, Madison, Wisconsin) against the human topoisomerase IIβ promoter sequence (Accession Number AC093416) (Appendix 3). The resulting sequence was 99.85% identical to the topoisomerase IIβ promoter sequence, and contained only two mutations; one A→ G at -1035 and the second G→ C at +124, neither of which were within putative regulatory elements. While they may be PCR-induced errors, they could equally be naturally occuring polymorphisms.

Figure 3.4: Analysis of pGEM-T vector containing topoisomerase IIβ-1357 fragment.



1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lane	2	1 μL uncut plasmid DNA
	3	30 μL plasmid DNA digested with *Sac* I
	4	30 μL plasmid DNA digested with *Nco* I
	5	30 μL plasmid DNA digested with *Nar* I
	6	30 μL plasmid DNA digested with *Nar* I and *Sac* I
	7	30 μL plasmid DNA digested with *Nco* I and *Sac* I

Nar I cut at -120 in the topoisomerase IIβ promoter sequence -1357 to +183 (1.54 kb), and *Sac* I cuts within the pGEM-T MCS, to produce fragments of 3303 bp and 1237 bp, as seen in lane 6. *Nco* I and *Sac* I cut out the 1.54 kb insert as seen in lane 7. Lower mobility bands present in lane 6 are due to incomplete digestion. The pGEM-T clone was confirmed to have a fragment of the topoisomerase IIβ promoter, approximately 1.54 kb in size.

3.2.4 Subcloning topoisomerase IIβ-1357 into pGL3Basic vector.

3.2.4.1 Preparation of 1.5 kb insert.

Three μg of pGEM-T TIIβ-1357, containing the topoisomerase IIβ 1.54 kb insert, was digested with *Apa* I to cut at +126 of the topoisomerase IIβ promoter sequence. The linearised vector was treated with Klenow enzyme (as described in section 2.2.5) to

fill in the recessed ends produced by enzymatic digestion, thereby generating blunt ends, and purified using the freeze squeeze gel purification method (Thuring *et al.*, 1975). Subsequent digestion with *Sac* I released a 1.5 kb fragment, which was purified and quantified by gel electrophoresis using quantification standards.

3.2.4.2 Preparation of pGL3Basic vector.

Vector pGL3Basic (Appendix 2) was prepared for ligation by digestion with *Sac* I and *Sma* I restriction enzymes to allow cloning of the insert into the MCS in the correct orientation. The enzyme recognition sites are located too close together in the vector to allow distinction between a single or double digest by gel electrophoresis. For this reason, the activity of the individual enzymes was tested in a separate reaction. The linear vector was treated with thermosensitive alkaline phosphatase (TSAP) to remove 5'-phosphate groups (Section 2.2.4), thereby preventing self-ligation, and was purified using the freeze squeeze gel purification method.

3.2.4.3 Ligation into pGL3Basic vector.

Ligations were carried out as described in section 2.2.12, and are shown in table 3.3.

Reactions	Control 1 (no ligase)	Control 2 (no insert)	1.5 kb insert	
			3:1 ratio	2:1 ratio
pGL3Basic vector (10 ng/μL)	5 μL	5 μL	5 μL	6 μL
1.5 kb insert (10 ng/μL)	5 μL	-	5 μL	3.7 μL
T4 5x ligase buffer	4 μL	4 μL	4 μL	4 μL
T4 DNA ligase (1 U/μL)	-	1 μL	1 μL	1 μL
H_2O	6 μL	10 μL	5 μL	5.3 μL
Total	20 μL	20 μL	20 μL	20 μL

Table 3.3: Ligation reactions with 1.5 kb insert into pGL3Basic.

Ligation reactions were carried out to clone the 1.5 kb topoisomerase IIβ promoter fragment into vector pGL3Basic. Reactions were incubated at 4°C overnight, and then 5 μL of each ligation was used to transform XL-1 blue (*E.coli*) competent cells. For the 1.5 kb insert reaction the ligation was carried out using a 3:1 (insert:vector) molar ratio and 2:1 molar ratio.

3.2.4.4 Transformations.

Ligation reactions were incubated overnight at 4°C before being used in a transformation reaction. Transformations were carried out as described in sections 2.2.13 and 3.2.3.2, and the results are shown in table 3.4, below. The pGL3Basic vector confers ampicillin resistance in those XL-1 competent cells successfully transformed, which would otherwise be unable to grow in the presence of ampicillin. pGL3Basic vector does not provide a visual selection method by which putative clones containing the insert can be picked, therefore random colonies were selected for screening.

Reaction	Ligation Mixture	XL-1 competent Cells	LB-broth	Volume plated	Number of colonies
1) XL-1 competent cells (control) 10^6 dilution	-	100 µL	890 µL	LB plates 100 µL	TMTC
10^7 dilution	-	100 µL	890 µL	100 µL	298
2) XL-1 competent cells (negative control)	-	100 µL	890 µL	LB+amp 100 µL	0
3) Transformation control (uncut vector)	2 µL	100 µL	898 µL	100 µL	TMTC
4) Control 1 (no ligase)	5µL	100µL	895 µL	100µL	0
5) Control 2 (no insert)	5 µL	100µL	895µL	100 µL	0
6) pGL3Basic + 1.5 kb insert (3:1)	5 µL	100 µL	895 µL	100 µL	155
7) pGL3Basic + 1.5 kb insert (2:1)	5 µL	100 µL	895 µL	100 µL	132

Table 3.4: Results of XL-1 transformations using pGL3Basic - 1.5 kb insert ligations.

Transformation of XL-1 competent cells using plasmids generated from ligation of 1.5 kb topoisomerase IIβ fragment into pGL3Basic vector (table 3.3). Unless indicated, all samples were plated onto LB plus ampicillin plates. Plates were incubated at 37°C overnight, and the number of colonies on each plate was scored. TMTC indicates colonies were to numerous to count. Colonies were creamy white colour. Eight colonies each from the 3:1 and 2:1 plates were selected and cultured to isolate plasmid DNA for analysis.

Together, control reactions 1 and 2 provided evidence that the XL-1 cells were viable and that colonies observed in the other reactions were not the result of contamination or spontaneous ampicillin resistance. The uncut vector transformation control (reaction 3) also yielded a high number of colonies as expected, and indicated successful transformation at high efficiency. Control reactions 4 and 5, acted as background controls, and the absence of colonies indicated that the digested vectors were unable to self-ligate in the absence of insert and undigested vectors were not present.

The ligation reactions containing pGL3Basic vector with the topoisomerase IIβ 1.5 kb insert yielded a moderate number of colonies, indicating that the ligations had been successful.

3.2.4.5 Screen for inserts.

White colonies were selected and cultured to isolate plasmid DNA for analysis, as described in section 3.2.3.3. The restriction enzymes selected to identify positive clones, were *Bgl* II and *Kpn* I, which cut flanking sites in the pGL3Basic vector MCS, and therefore release the 1.5 kb insert (data not shown). The reactions were analysed by gel electrophoresis, with the results indicating that plasmid DNA isolated from two colonies appeared to have the correct insert corresponding to the 1.5 kb topoisomerase IIβ promoter region. One of the cultures used for these two rapid boil samples was maintained, and a large scale plasmid preparation was carried out (refer to section 2.2.14.3) and the concentration of the plasmid was determined (table 3.5), as described in sections 2.2.15 and 3.2.3.3.

Construct	A260	A280	Ratio	Concentration
PGL3B-TIIβ-1357	0.174	0.067	2.60	435 µg/mL

Table 3.5: DNA Quantification of pGL3B-TIIβ-1357.

Plasmid DNA was purified using CONCERT™ High Purity Plasmid Purification System. The absorbance of plasmid DNA, diluted 1 in 10, was determined by UV-spectrophotometry, and concentrations were calculated as described in section 2.2.15.

Diagnostic digests, as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert. Correct orientation was confirmed by digestion with *Nar* I, as previously described, and *Kpn* I which cuts in the MCS of the pGL3Basic vector to produce three fragments of 4679 bp, 1237 bp and 367 bp in size, if the orientation of the insert is correct (figure 3.5). From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the 1.5 kb fragment corresponding to topoisomerase IIβ promoter sequence -1357, was confirmed to be correct in pGL3Basic.

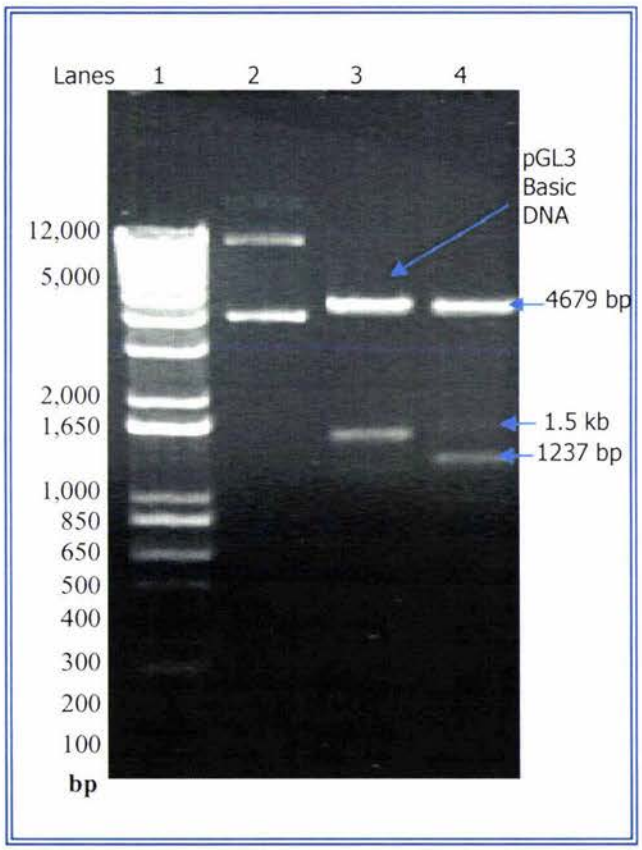


Figure 3.5: Analysis of pGL3Basic vector containing topoisomerase IIβ 1.5 kb fragment.

1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

2 1 μL uncut plasmid DNA

3 30 μL plasmid DNA digested with *Bgl* II *and Kpn* I

4 30μL plasmid DNA digested with *Nar* I and *Kpn* I

Bgl II and *Kpn* I cut out the 1.5 kb insert as seen in lane 3. *Nar* I cut at -120 in the topoisomerase IIβ promoter sequence -1357 to +126 (1.5 kb) and within the vector (121), while *Kpn* I cuts within the pGL3Basic MCS, to produce fragments of 4679 bp, 1237 bp and 367 bp, as seen in lane 4. The 367 bp band was not visible due to the concentration which was optimal for separation of the lower mobility bands. The pGL3Basic clone was confirmed to have a fragment of the topoisomerase IIβ promoter, approximately 1.5 kb in size.

3.3 Generating topoisomerase IIβ promoter deletion constructs.

In order to investigate regions of the topoisomerase IIβ promoter important for transcriptional regulation, the pGL3B-TIIβ-1357 was used to generate a series of truncated promoter regions in the pGL3Basic vector. A series of five 5'-deletion constructs was generated by restriction enzyme digestion and religation, of purified fragments into pGL3Basic vector, as illustrated in figures 3.6 and 3.7.

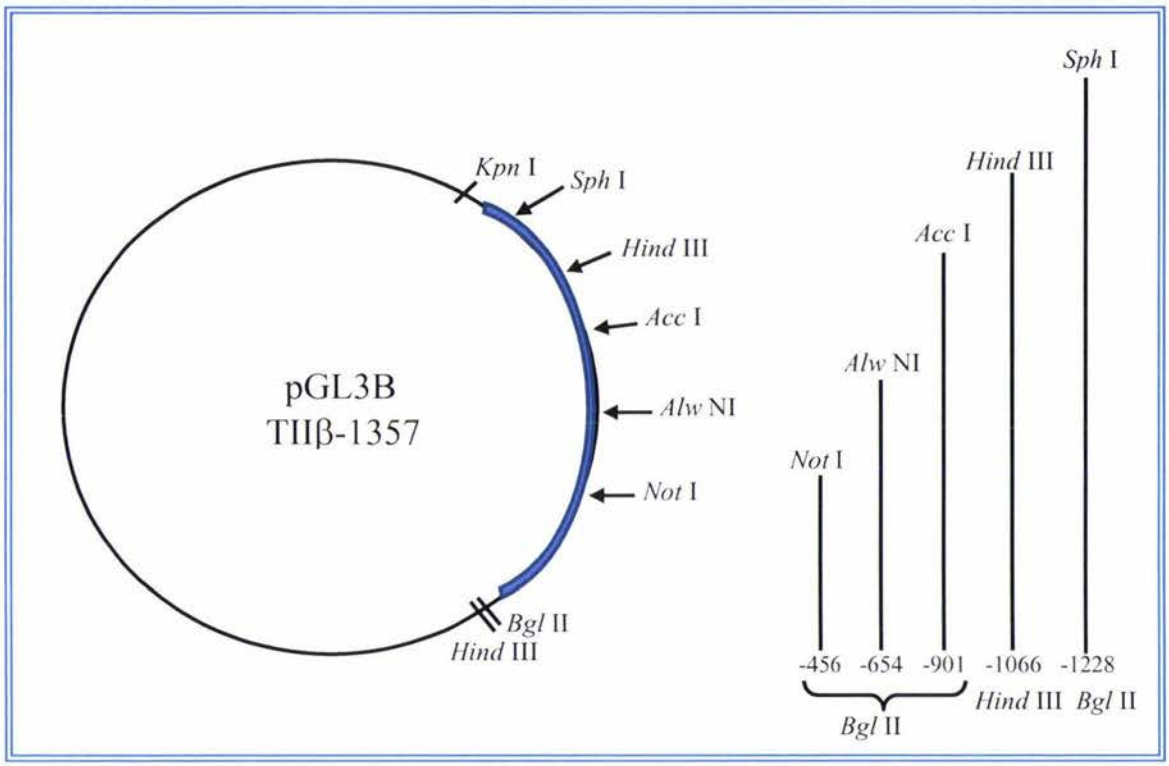


Figure 3.6: Schematic of 5'-serial deletion fragments.

Unique restriction sites within the topoisomerase IIβ-1357 sequence were selected to produce the deletion series. Fragments of 1354, 1192, 1027, 780 and 582 bp in size were isolated from the pGL3B-TIIβ-1357 construct to generate -1228, -1066, -901, -654 and -456 sequence deletions. Refer to figure 3.7 for cloning strategy.

Unique restriction sites within the 1.5 kb insert DNA were required to produce the desired truncations. Unfortunately, appropriate sites were also present in the vector, which excluded the use of the simple cloning technique of a double digest to remove the 5'-sequence, followed by religation. As an alternative method, the unique site selected for each deletion was first cut in a single enzyme digest, followed by the generation of blunt ends, and then a second digest to release the desired fragment. Figure 3.7 shows a representative cloning strategy.

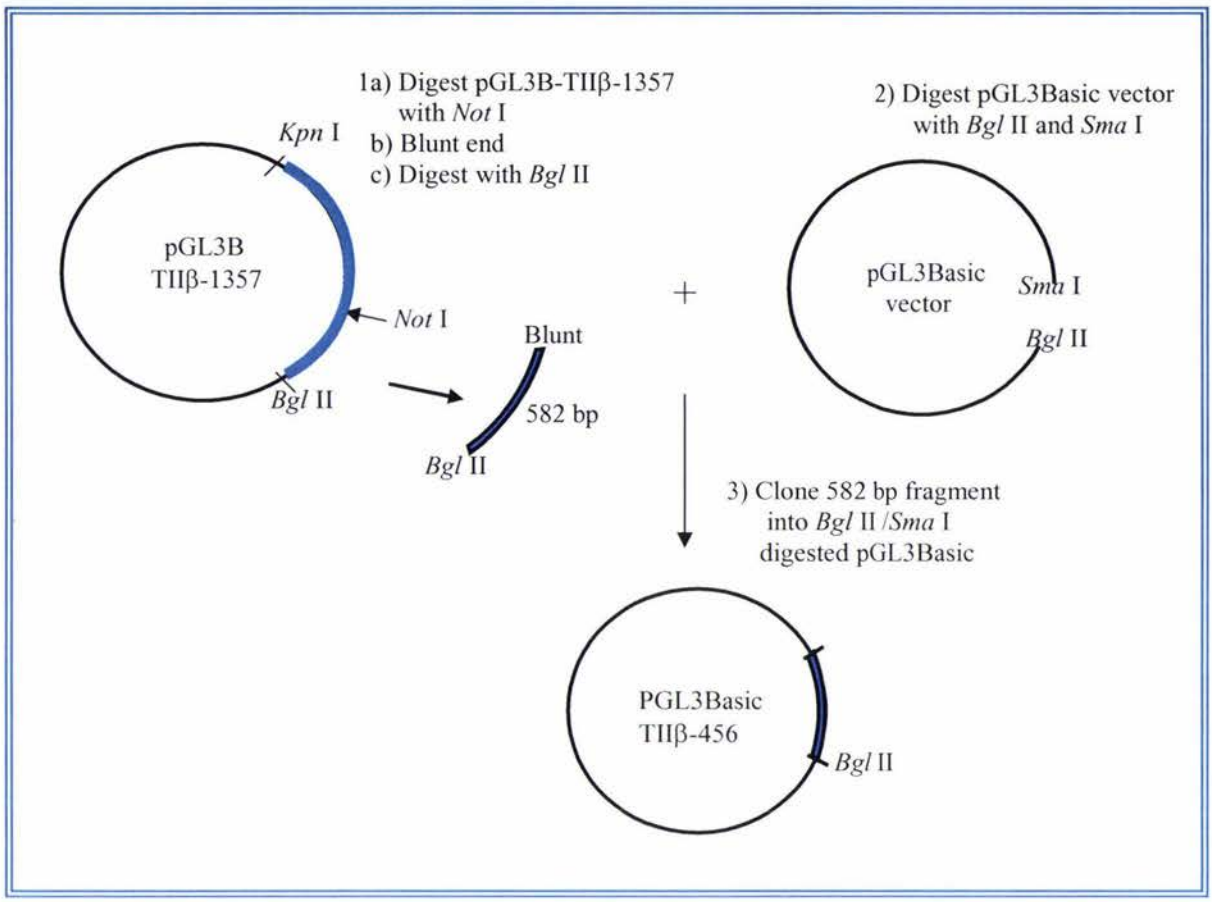


Figure 3.7: Cloning strategy to generate -456 deletion construct.

Subcloning a 582 bp region from the pGL3B-TIIβ-1357 construct back into pGL3Basic to generate the pGL3B-TIIβ-456 construct. For the -1228, -901, -654 and -456 deletions, as the appropriate restriction sites also cut within the vector, single digests were first carried out, followed by the generation of blunt ends. The desired fragment was then released using *Bgl* II in the pGL3Basic vector MCS and gel purified. For the -1066 deletion, the desired fragment was released in a single digest using *Hind* III restriction enzyme, and gel purified. The truncated promoter region was then ligated into the pGL3Basic vector and characterised.

3.3.1 Preparation of deletion constructs.

For the -1228, -901, -654 and -456 deletions, three μg of pGL3B-TIIβ-1357 was digested with the appropriate enzyme (as shown in the cloning strategy), producing two or three fragments, depending on the number of times the enzyme cut both insert and vector (see Appendix 3 for restriction sites). The linear DNA fragments were treated with Klenow enzyme (as described in section 2.2.5) to generate blunt ends. The DNA fragments were ethanol precipitated (Sambrook *et al.*, 2001), to remove unwanted components from the previous reactions, and then digested with *Bgl* II to

remove the insert. For the -1066 deletion, three μg of the pGL3B-TIIβ-1357 was digested with *Hind* III to release the 1192 bp fragment. The restriction endonucleases used and the sizes of DNA fragments generated are displayed in table 3.6. The inserts were purified and quantified, as described in section 3.2.4.1.

Deletion construct	1st restriction enzyme	fragment sizes	2nd restriction enzyme	fragment sizes
pGL3Basic-TIIβ-1228	*Sph* I	3986, 2297	*Bgl* II	1354, 943
pGL3Basic-TIIβ-1066	*Hind* III	4852, 1192, 239	-	1192
pGL3Basic-TIIβ-901	*Acc* I	3245, 3038	*Bgl* II	1027, 2011
pGL3Basic-TIIβ-654	*Alw* NI	3456, 2827	*Bgl* II	2676, 780
pGL3Basic-TIIβ-456	*Not* I	5233, 1050	*Bgl* II	4651, 582

Table 3.6: Restriction enzymes used to generate deletion inserts.

Fragments of 1354, 1192, 1027, 780 and 582 bp in size were isolated from the pGL3B-TIIβ-1357 construct to generate topoisomerase IIβ sequence deletions; -1228, -1066, -901, -654 and -456. For the -1228, -901, -654 and -456 deletions, two separate digests were carried out, with the second digest releasing the desired fragment. Following the first digest, the DNA fragment to be used in the second digest was gel purified (underlined). In the final column the desired cloning fragments are underlined. For the -1066 deletion the desired fragment was released in a single digest using *Hind* III restriction enzyme and gel purified. The truncated promoter region was then ligated into the pGL3Basic vector and characterised.

Vector pGL3Basic (Appendix 2) was prepared for ligation by generating *Bgl* II and *Sma* I cohesive ends for all deletion inserts except the 1192 bp insert, which required *Hind* III cohesive ends. The protocol was the same as described in section 3.2.4.2.

Ligations and transformations were carried out as previously described for cloning into the pGL3Basic vector (refer to sections 2.2.12, 2.2.13 and 3.2.4), using random selection to pick colonies for screening, and similar results were obtained.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Kpn* I and *Hind* III to cut out the 1192 bp insert, or *Kpn* I and *Bgl* II to cut out all other inserts. These enzymes cut flanking sites in the pGL3Basic vector MCS and therefore cut out the truncated inserts. (data not shown). Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), diagnostic digests as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert (figure 3.8). Correct orientation was confirmed using *Not* I for the -1228, -1066, and -901 deletions, as previously described, and *Nar* I and *Kpn* I for the -654 and -456 deletions, as described in section 3.2.3.3 (figure 3.8). The DNA fragment sizes expected if the orientation of the insert is correct are shown in table 3.7, below. The results of these digests, and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), confirmed the identity of the topoisomerase IIβ truncated promoter sequences in pGL3Basic.

Deletion construct	Uncut plasmid DNA (bp)	*Bgl* II/*Kpn* I	*Hind* III	*Nar* I/*Kpn* I	*Not* I
pGL3Basic-TIIβ-1228	6154	4800, 1354	-	-	5233, 921
pGL3Basic-TIIβ-1066	5992	-	4800, 1192	-	5233, 759
pGL3Basic-TIIβ-901	5827	4800, 1027	-	-	5233, 594
pGL3Basic-TIIβ-654	5580	4800, 780	-	4679, 534, 367	-
pGL3Basic-TIIβ-456	5382	4800, 582	-	4679, 367, 336	-

Table 3.7: Restriction enzymes used in deletion construct diagnostic digests.

Kpn I, and *Bgl* II or *Hind* III, were used to cut out the deletion inserts (figure 3.8). The topoisomerase IIβ promoter sequence has unique restriction sites for the enzymes *Nar* I and *Not* I, which were used to positively identify the deletion inserts (figure 3.8). The results of these digests confirmed the identity of the topoisomerase IIβ truncated promoter sequences.

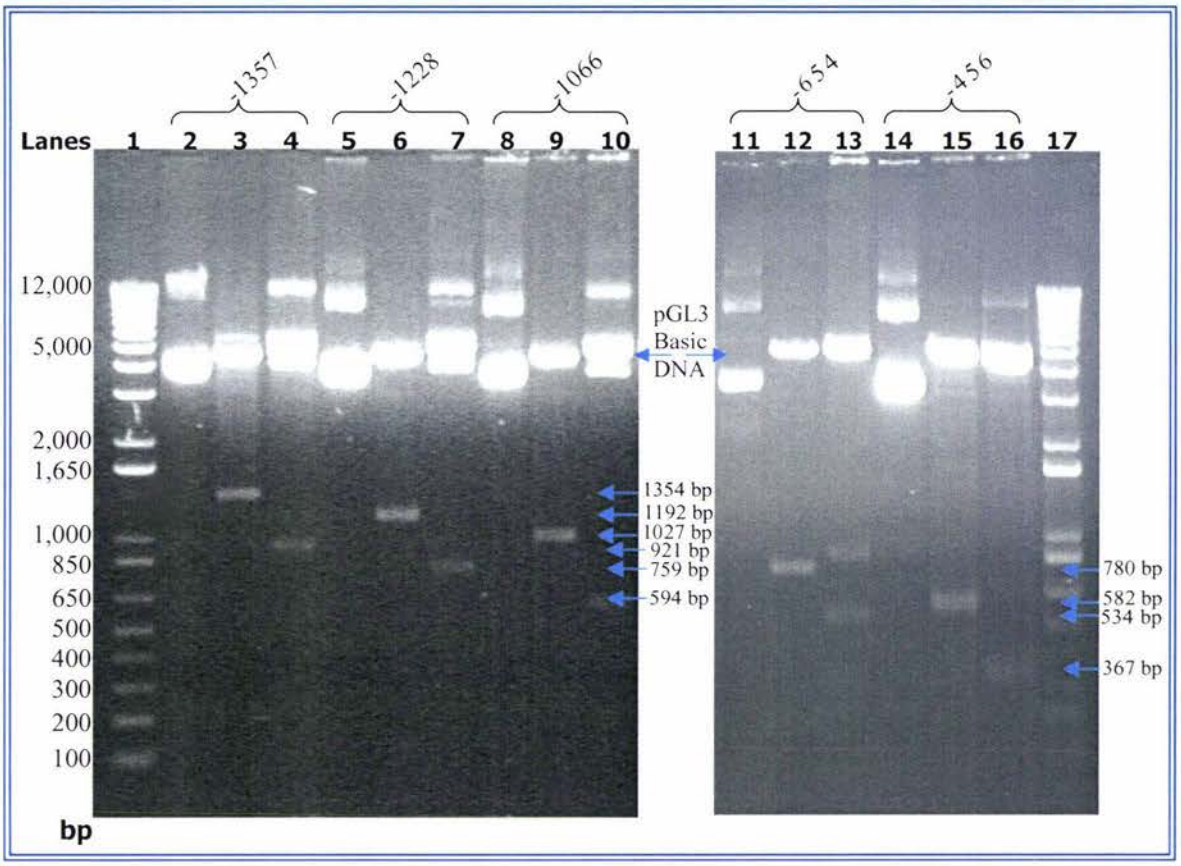


Figure 3.8: Analysis of pGL3Basic vectors containing topoisomerase IIβ promoter truncations.

1-2 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1-2 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lanes 1 and 17, and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lanes 2, 5, 8, 11 & 14	1-2 μL uncut plasmid DNA
Lanes 3, 9, 12 & 15	30 μL plasmid DNA digested with *Bgl* II & *Kpn* I
Lane 6	30 μL plasmid DNA digested with *Hind* III
Lanes 4, 7, & 10	30 μL plasmid DNA digested with *Not* I
Lanes 13 & 16	30 μL plasmid DNA digested with *Nar* I & *Kpn* I

Table 3.7 displays the fragment sizes expected if the insert DNA is correct, and these matched the sizes obtained. Additional bands, above the insert bands, in lanes 4, 7, 10, 13 and 16 are due to incomplete digestion. The pGL3Basic clones were each confirmed to contain truncated fragments of; 1354 bp, 1192 bp, 1027 bp, 780 bp or 582 bp in size, corresponding to the topoisomerase IIβ promoter deletions; -1228, -1066, -901, -654 and -456.

3.4 Generating internal deletions within topoisomerase IIβ promoter deletion constructs.

In order to investigate the importance of the putative GC, ICB1 and ICB2 elements located in the region from -486 to -553 in the topoisomerase IIβ promoter, an internal deletion was introduced into the promoter sequence. An internal deletion construct was produced from the pGL3B-TIIβ-1357 full length sequence, which was then used to generate pGL3B-TIIβ-1228, -1066, -901 deletion constructs with the internal deletion.

Restriction enzyme digests could be used to cut out the sequence flanking the internal region to be deleted, however pGL3Basic did not contain the appropriate restriction sites in the MCS to allow religation of the two fragments without the presence of additional vector sequence between the two. For this reason flanking fragments of 582 bp and 703 bp in size were cut from the pGL3B-TIIβ-1357 construct and subcloned into pBluescript SK+ (pBS SK+) vector (Appendix 2, Promega), as shown in figure 3.9. This resulted in an internal deletion of 198 bp (-654 to -456), which encompassed the sequence containing the putative regulatory elements, and resulted in a 1.3 kb topoisomerase IIβ promoter fragment (-1357-ID). The 1.3 kb insert was then subcloned back into pGL3Basic and a second set of 5'-serial deletion constructs was generated containing the internal deletion. These new plasmids were generated by restriction enzyme digestion, and religation of purified fragments, into pGL3Basic vector.

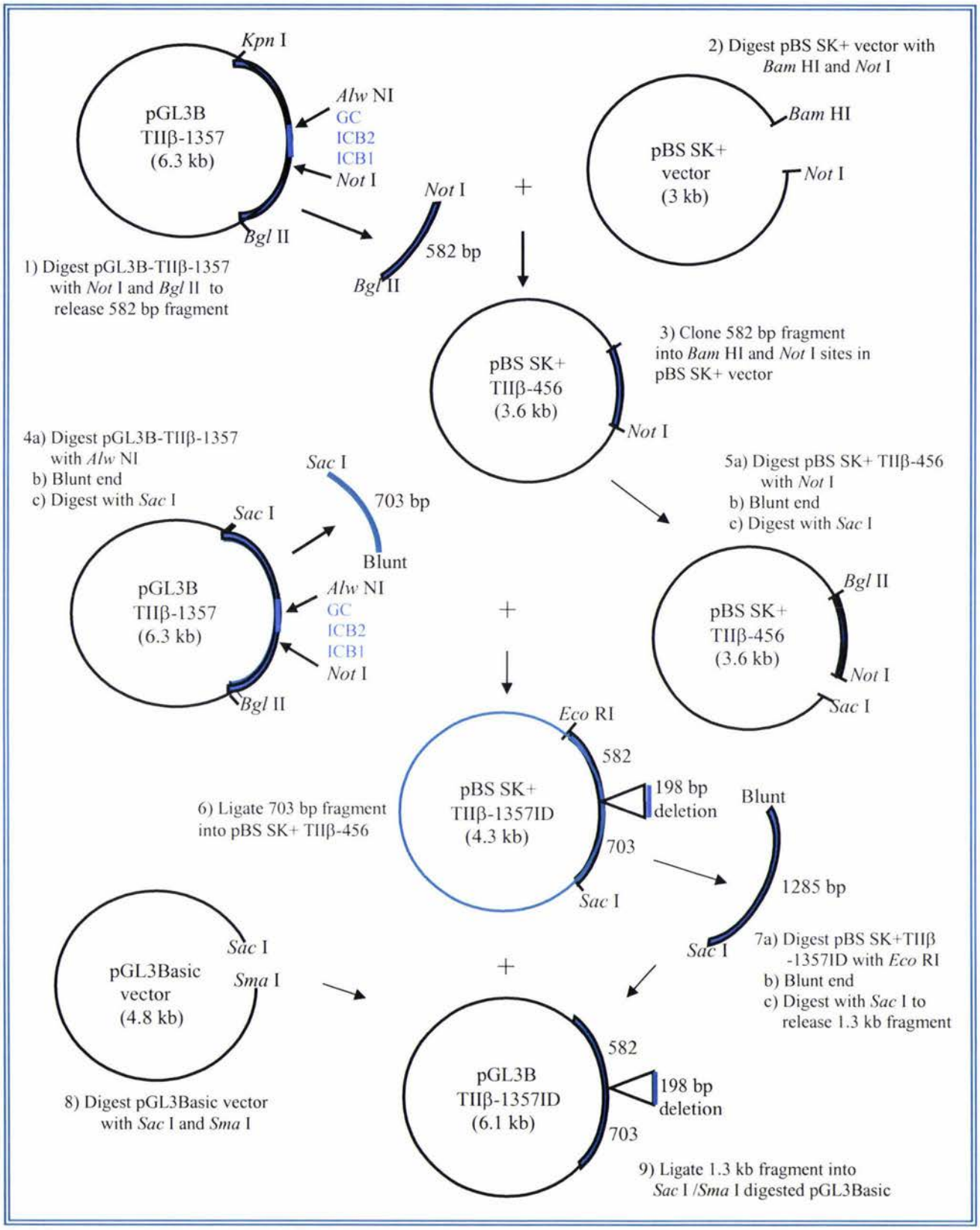


Figure 3.9: Cloning strategy to generate internal deletion constructs.

pBS SK+ was used as an intermediary in the process of generating a pGL3B-TIIβ-1357ID construct. This plasmid was then used to generate 5'-serial deletions, as described in section 3.3, to create pGL3B-TIIβ-1228ID, -1066ID and -901ID constructs.

3.4.1 Generating a pBS SK+ internal deletion plasmid.

3.4.1.1 Generating a pBS SK+ TIIβ-456 bp construct.

Three μg of pGL3B-TIIβ-1357 was digested with the restriction enzymes, *Not* I and *Bgl* II, to release the 582 bp fragment, corresponding to the topoisomerase IIβ sequence -456. The 582 bp fragment was purified and quantified as described in section 3.2.4.1. pBS SK+ was prepared for ligation, as described in section 3.2.4.2, by generating *Not* I and *Bam* HI cohesive ends. *Bam* HI digestion produces *Bgl* II compatible ends.

Ligations were carried out as described in sections 2.2.12 and 3.2.4.3. Transformations were carried out as described in sections 2.2.13 and 3.2.3.2, using blue/white selection to identify correct transformants, as previously described for cloning into the pGEM-T vector (refer to section 3.2.3.2), and similar results were obtained.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Kpn* I and *Sac* I, which cut flanking sites in the pBS SK+ vector MCS and therefore cut out the 582 bp insert (data not shown). Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), additional diagnostic digests were carried out as described above, to confirm that the insert DNA was the desired topoisomerase IIβ sequence (figure 3.10). The correct orientation was confirmed using *Nar* I, as previously described, which cuts once within the insert to linearise the plasmid (figure 3.10). From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the 582 bp fragment corresponding to the topoisomerase IIβ promoter sequence -456, was confirmed to be correct in pBS SK+ vector.

3.4.1.2 Generating a pBS SK+ TIIβ-1357ID construct.

Three μg of pGL3B-TIIβ-1357 was digested with restriction enzyme *Alw* NI. The linear DNA vector was treated with klenow enzyme (as described in section 2.2.5) to generate blunt ends. The DNA fragments were ethanol precipitated (Sambrook *et al.*, 2001), and then digested with *Sac* I to release the 703 bp insert. The insert DNA was purified and quantified as described in section 3.2.4.1.

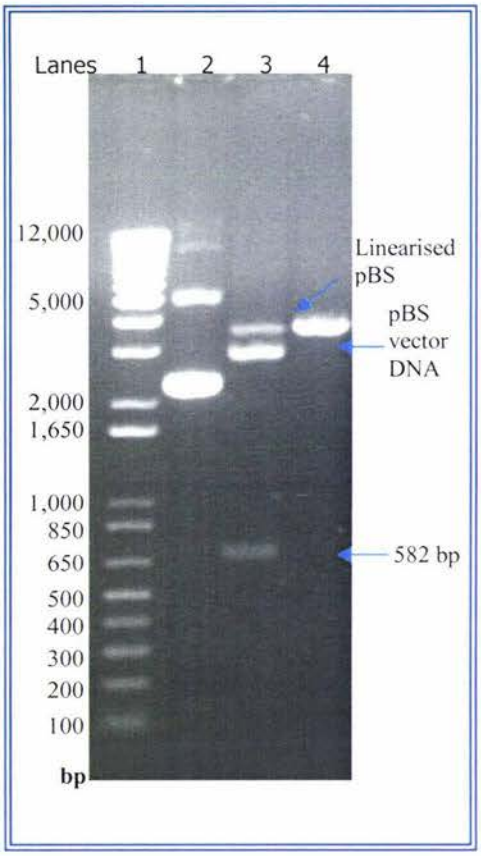


Figure 3.10: Analysis of pBS SK+ vector containing topoisomerase IIβ-456.

1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lane 2	1 μL uncut plasmid DNA
3	30 μL plasmid DNA digested with *Kpn* I and *Sac* I
4	30 μL plasmid DNA digested with *Nar* I

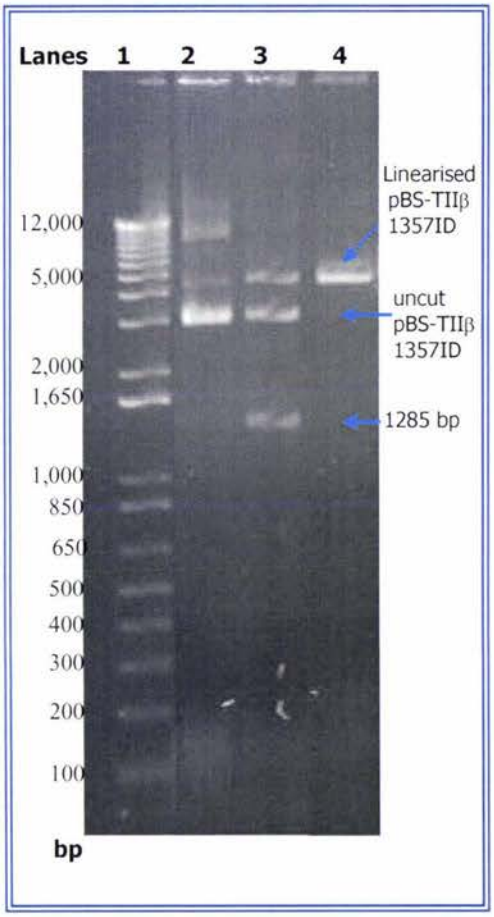
Kpn I and *Sac* I cut out the 582 bp insert, as seen in lane 3. *Nar* I cut at -120 in the topoisomerase IIβ promoter sequence -456 to +126, to produce a single linear fragment of 3582 bp, as seen in lane 4. Uncut pBS SK+ vector present in lane 3 is due to incomplete digestion. The pBS SK+ clone was confirmed to have a fragment of approximately 582 bp in size, corresponding to the topoisomerase IIβ promoter sequence -456.

pBS SK+ TIIβ-456 was prepared for ligation by digesting with restriction enzyme *Not* I, followed by klenow enzyme (as described in section 2.2.5) to generate blunt ends. The DNA fragments were ethanol precipitated (Sambrook *et al.*, 2001), and then digested with *Sac* I, to generate *Sac* I and *Sma* I compatible cohesive ends. The linear vector was treated with TSAP to remove 5'-phosphate groups (Section 2.2.4), followed by purification and quantification as described in section 3.2.4.2.

Ligations and transformations were carried out as previously described for cloning into the pGL3Basic vector (refer to sections 2.2.12, 2.2.13 and 3.2.4), using random selection to pick colonies for screening, and similar results were obtained. As the β-galactosidase gene present in the pBS SK+ vector is already disrupted by the presence of the 582 bp insert, blue/white selection was no longer possible.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Eco* RI and *Sac* I, which cut flanking sites in the pBS SK+ vector MCS and therefore cut out the 1.3 kb insert, corresponding to the topoisomerase IIβ promoter sequence -1357 containing a 198 bp internal deletion (data not shown).

Figure 3.11: Analysis of pBS SK+ vector containing topoisomerase IIβ-1357 with a 198 bp internal deletion.



1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lane 2 1 μL uncut plasmid DNA

3 30 μL plasmid DNA digested with *Eco* RI and *Sac* I

4 30 μL plasmid DNA digested with *Nar* I

Eco RI and *Sac* I cut out the 1285 bp insert, as seen in lane 3. The lower mobility band labelled uncut plasmid in lane 3 is due to incomplete digestion. *Nar* I cut at -120 in the topoisomerase IIβ promoter sequence -1357 to +126, to produce a single linear fragment of 4285 bp, as seen in lane 4. The pBS SK+ clone was confirmed to have a fragment of the topoisomerase IIβ promoter, approximately 1285 bp in size, corresponding to the -1357 sequence with a 198 bp internal deletion.

Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), diagnostic digests as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert (figure 3.11). The correct orientation was confirmed using *Nar* I, as previously described, which cuts once within the insert to linearise the plasmid. From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the 1.3 kb insert corresponding to the topoisomerase IIβ sequence -1357 containing a 198 bp internal deletion, was confirmed to be correct in pBS SK+ (pBS SK+ TIIβ-1357ID).

3.4.2 Generating a pGL3B-TIIβ-1357ID construct.

Three μg of the pBS SK+ TIIβ-1357ID construct was digested with restriction enzyme *Eco* RI. The linear DNA vector was treated with klenow enzyme (as described in section 2.2.5) to generate blunt ends. The DNA fragments were ethanol precipitated (Sambrook *et al.*, 2001), and then digested with *Sac* I to release the 1.3 kb insert. The insert DNA was purified and quantified as described in section 3.2.4.1. Vector pGL3Basic (Appendix 2) was prepared for ligation by generating *Sac* I and *Sma* I cohesive ends, using the same methods described in section 3.2.4.2.

Ligations and transformations were carried out as previously described for cloning into the pGL3Basic vector (refer to sections 2.2.12, 2.2.13 and 3.2.4), using random selection to pick colonies for screening, and similar results were obtained.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Kpn* I and *Bgl* II, which cut flanking sites in the pGL3Basic vector MCS and therefore cut out the 1.3 kb insert (data not shown). Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), diagnostic digests as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert. From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the 1.3 kb promoter insert corresponding to the topoisomerase IIβ sequence -1357 with a 198 bp internal deletion, was confirmed to be correct in pGL3Basic (pGL3B-TIIβ-1357ID) (figure 3.12, lanes 2-4).

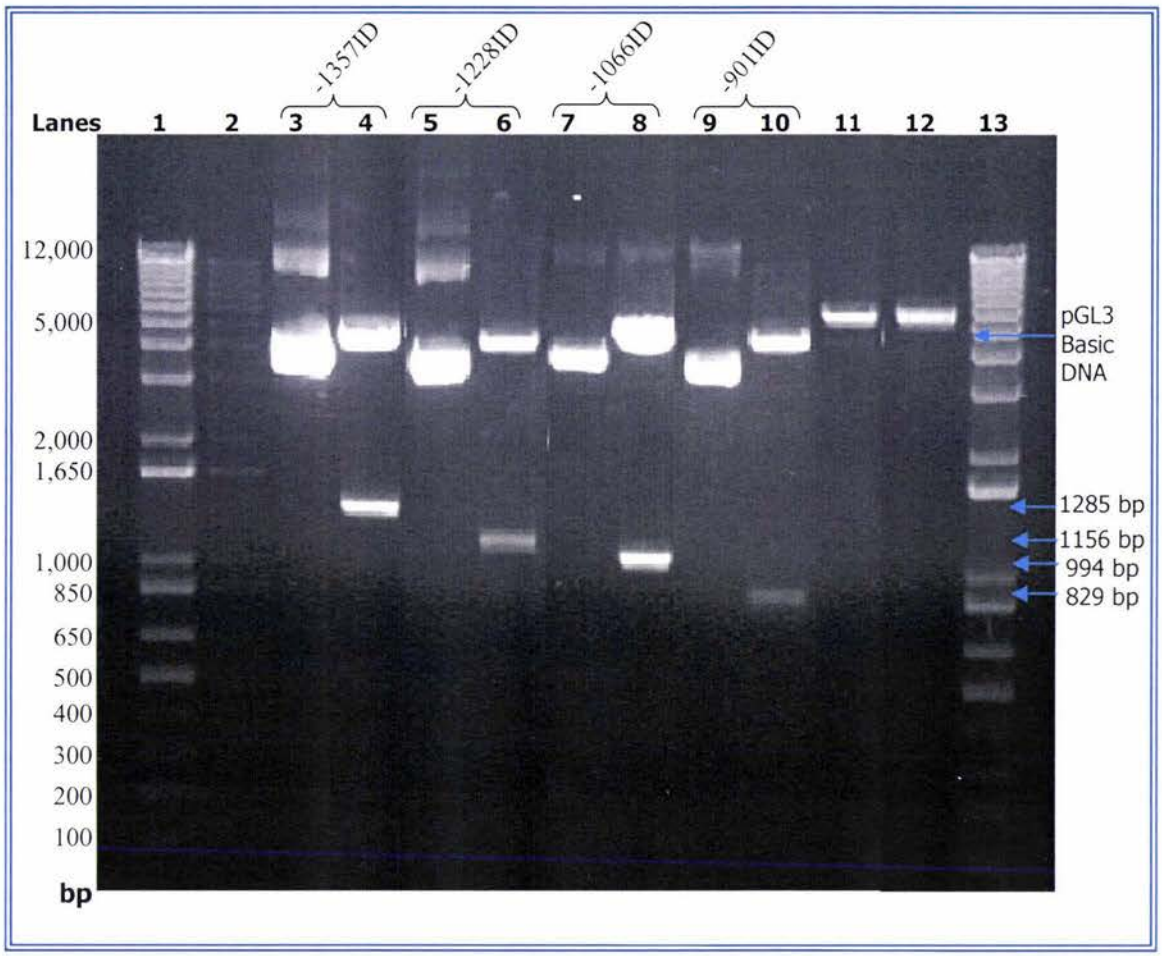


Figure 3.12: Analysis of pGL3Basic vector containing topoisomerase IIβ truncated fragments containing a 198 bp internal deletion.

1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 x TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. Molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lanes 1 & 13	10 μL 1 kb plus ladder (1:10)
Odd Lanes 3-9	1 μL uncut plasmid DNA
Even Lanes 4-10	30 μL plasmid DNA digested with *Kpn* I & *Bgl* II
Lane 11	30μL plasmid DNA digested with *Kpn* I
Lane 12	30μL plasmid DNA digested with *Bgl* II

The pGL3Basic clones were confirmed by DNA sequence analysis to have truncated fragments of the topoisomerase IIβ promoter sequence of 1285 bp, 1156 bp, 994 bp and 829 bp in size.

3.4.3 Generating pGL3B-TIIβ-1228ID, -1066ID and -901ID constructs.

The pGL3B-TIIβ-1357ID construct was then used to generate pGL3B-TIIβ-1228, -1066, -901 deletion constructs with the internal deletion, by the same methods as described in section 3.3. Diagnostic digests were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert using restriction enzymes *Kpn* I and *Bgl* II, which cut out the 1156 bp, 994 bp and 829 bp inserts (figure 3.12). From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the 1156 bp, 994 bp and 829 bp promoter inserts corresponding to the topoisomerase IIβ sequences -1354, -1192 and -1027 with a 198 bp internal deletion, were confirmed to be correct in pGL3Basic.

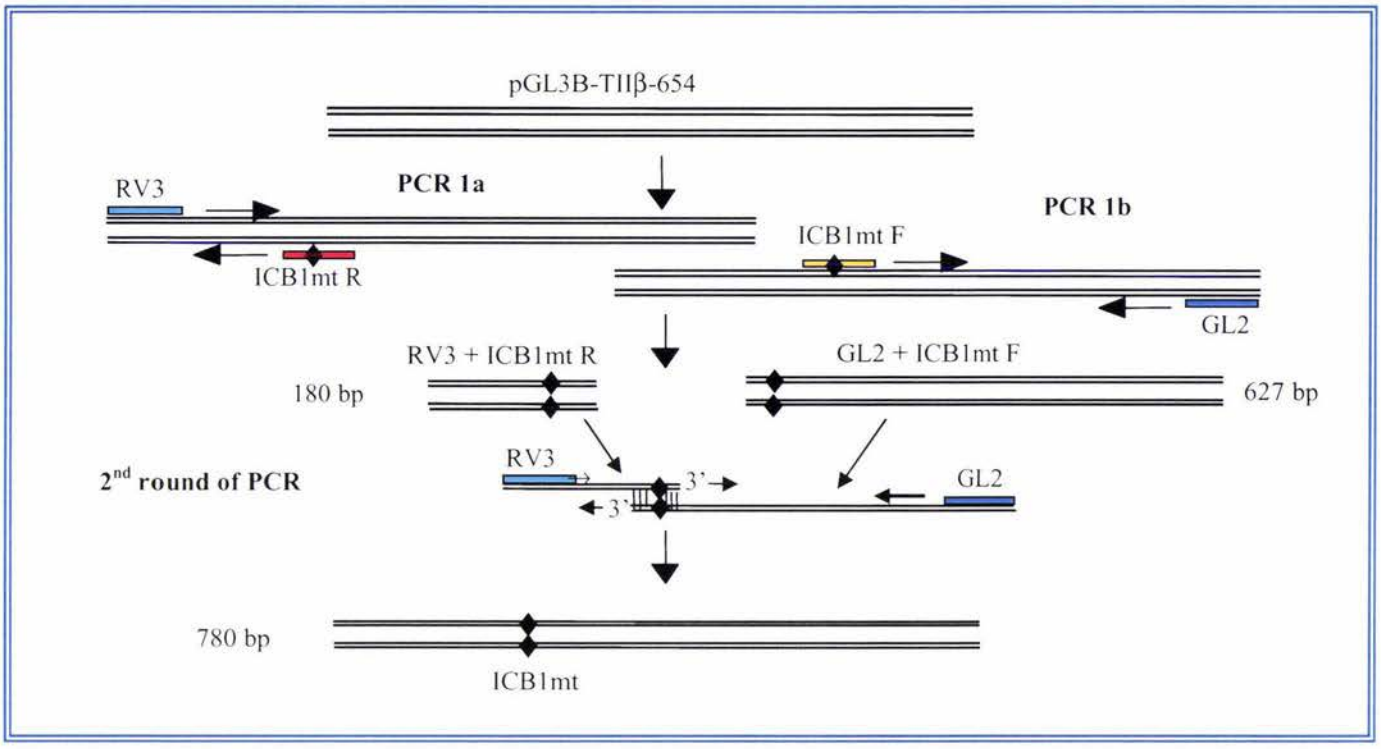
3.5 Generating ICB1 and ICB2 mutant constructs.

In order to investigate roles of ICB1 and ICB2 in topoisomerase IIβ promoter transcriptional regulation, the pGL3B-TIIβ-654 construct, consisting of the minimal promoter region containing the GC, ICB1 and ICB2 elements, was used to generate ICB1 and ICB2 mutant constructs. Specific point mutations were introduced into the -654 bp sequence by PCR mutagenesis (figure 3.13) and the resulting products were cloned into pGL3Basic vector for functional analysis.

3.5.1 PCR mutagenesis.

A set of oligonucleotide primers was designed, 27 nt in length, which encompassed either the ICB1 or ICB2 elements of the topoisomerase IIβ promoter, and containing specific point mutations to abolish the putative regulatory element (refer to Appendix 1 for oligonucleotide sequences). The sequence changes also introduced restriction enzyme recognition sites, a *Xba* I site within the ICB1mt sequence and a *Bgl* II site within the ICB2mt sequence, which allowed confirmation of the mutation. PCR reactions were carried out, as described in section 2.2.10, using pGL3B-TIIβ-654 as a template and *Taq* polymerase (Promega). The PCR reactions were carried out in thermal cyclers (HYBAID Omn-E, SciTech (NZ) Ltd or GeneAmp®PCR System 2700, Applied Biosystems (USA).

a) Generating ICB1mt insert



b) Generating ICB2mt

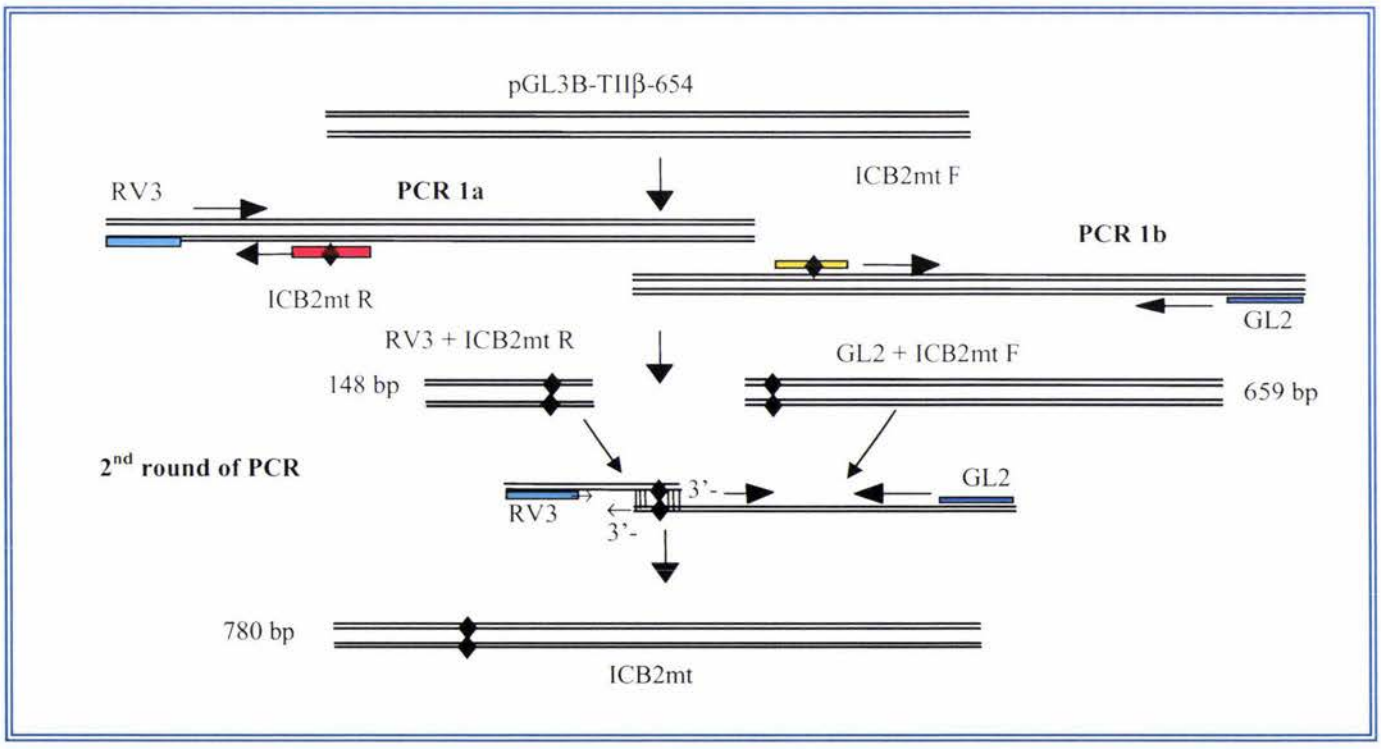


Figure 3.13: Schematic diagram of site-directed mutagenesis by overlap extension.

To generate ICB1 and ICB2 mutant inserts, pGL3B-TIIβ-654 was used as a template. Specific point mutations were introduced within the ICB1 or ICB2 elements using oligonucleotide primers designed to abolish the consensus NF-Y binding site.

The first round of PCR consists of two independent reactions, one containing the flanking forward primer (RV3) and the internal ICB1mt or ICB2mt reverse primer, and the other containing the flanking reverse primer (GL2) and the internal ICB1mt or ICB2mt forward primer. By using two internal primers that overlap, the two products generated in the first PCR could be fused by denaturing and annealing in a subsequent PCR reaction. The overlap allows one strand from each fragment to act as a primer to extend the other fragment, resulting in the mutant product, which is amplified during the second round of PCR.

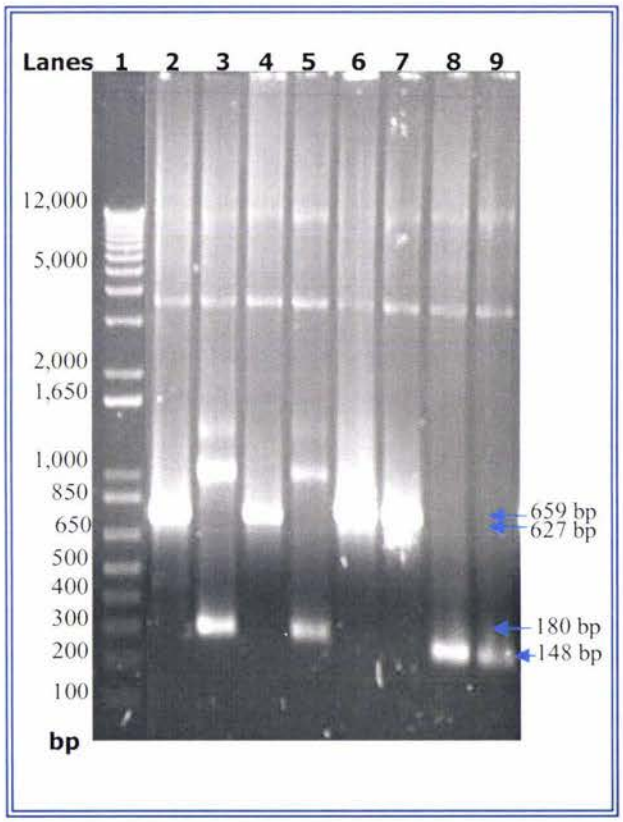


Figure 3.14: First PCR mutagenesis reactions to generate topoisomerase IIβ mutated product.

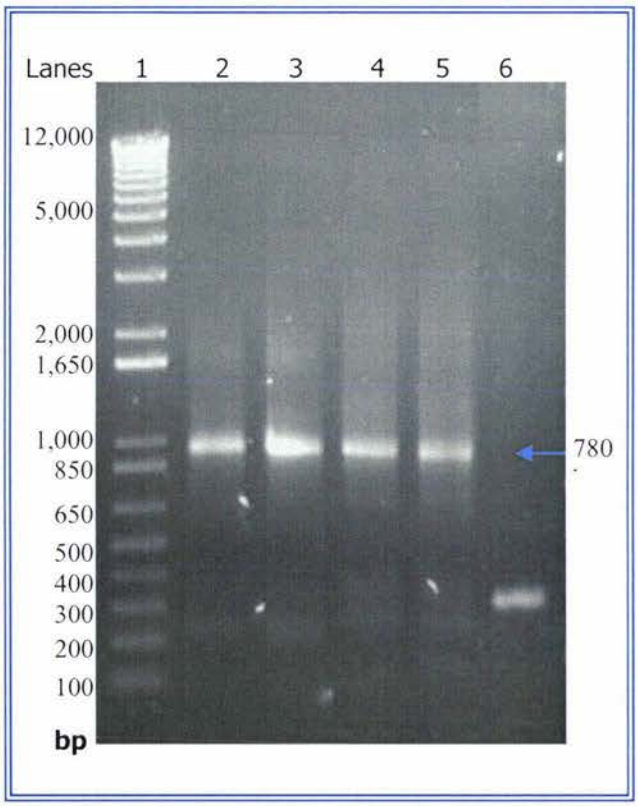
5 µL of each reaction was applied to a 1.0% agarose gel in 1 x TAE buffer and electrophoresed for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 µg/mL) into the gel and exposure to UV light. 10 µL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp).

Lanes 2 & 4 5 µL PCR mutagenesis reaction using ICB1mt Fwd primer, 627 bp product
Lanes 3 & 5 5 µL PCR mutagenesis reaction using ICB1 mt Rev primer, 180 bp product
Lanes 6-7 5 µL PCR mutagenesis reaction using ICB2 mt Fwd primer, 659 bp product
Lanes 8-9 5 µL PCR mutagenesis reaction using ICB2 mt Rev primer, 148 bp product

PCR products of the expected sizes were obtained following the first round of PCR mutagenesis (refer to figure 3.14) to introduce a mutation into either the ICB1 or ICB2 elements in the topoisomerase IIβ promoter sequence –654. Bands of lower mobility visible are due to non-specific annealing of the primers during PCR.

Figures 3.14 and 3.15 show the products generated after the first and second rounds of PCR. Restriction endonuclease digests followed by agarose gel electrophoresis, were used to check the PCR products, using the restriction enzyme sites within the mutated sequence (data not shown).

Figure 3.15: Second PCR mutagenesis reaction to generate topoisomerase IIβ 780 bp mutated product.



5 µL of each reaction was applied to a 1% agarose gel in 1 x TAE buffer and electrophoresed for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 µg/mL) into the gel and exposure to UV light. 10 µL of 1 kb plus ladder (1:10) is present in lane 1, and the molecular sizes of bands are displayed on the left in base pairs (bp).

Lanes	2-3	5 µL second PCR mutagenesis reaction (ICB1mt)
Lanes	4-5	5 µL second PCR mutagenesis reaction (ICB2mt)
Lanes	6	5 µL positive control (Factor IX promoter), ~380 bp product

Lanes 2-5 contain PCR product, which appears to be slightly larger than the expected size (780 bp) following the second round of PCR mutagenesis. DNA sequence analysis confirmed the amplified DNA to be the correct sequence, corresponding to the 780 bp topoisomerase IIβ promoter sequence –654, with a mutation introduced into either ICB1 or ICB2.

3.5.2 Ligation of ICB1mt and ICB2mt into pGL3Basic vector.

PCR products were purified using the freeze squeeze method (Holmes and Quigley, 1981). Restriction enzyme digestions were carried out using *Bgl* II and *Kpn* I for ICB1mt, and *Hind* III and *Kpn* I for ICB2mt. The DNA fragments were ethanol precipitated (Sambrook *et al.,* 2001), and quantified by gel electrophoresis using quantification standards. Vector pGL3Basic was prepared for ligation by generating *Kpn* I, and *Bgl* II or *Hind* III, cohesive ends using the same methods described in section 3.2.4.2.

Ligations and transformations were carried out as previously described for cloning into the pGL3Basic vector (Refer to sections 2.2.12, 2.2.13 and 3.2.4), using random selection to pick colonies for screening, and similar results were obtained.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Kpn* I and *Hind* III, which cut flanking sites in the pGL3Basic vector MCS and therefore cut out the 780 bp insert containing the ICB1 or ICB2 mutant sequence (data not shown). Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), diagnostic digests, as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert. The restriction sites generated in the mutated elements were utilised in a diagnostic digest to confirm the presence of the correct mutation. If the ICB1 mutant sequence has been introduced, restriction enzyme *Xba* I cuts to produce fragments of 3222 bp and 2358 bp in size, and if the ICB2 mutant sequence has been introduced *Bgl* II cuts to produce fragments of 4896 bp and 684 bp in size (figure 3.16). From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the topoisomerase IIβ ICB1 and ICB2 mutated promoter sequences were confirmed to be correct in pGL3Basic. The mutated sequence is shown in figure 3.17.

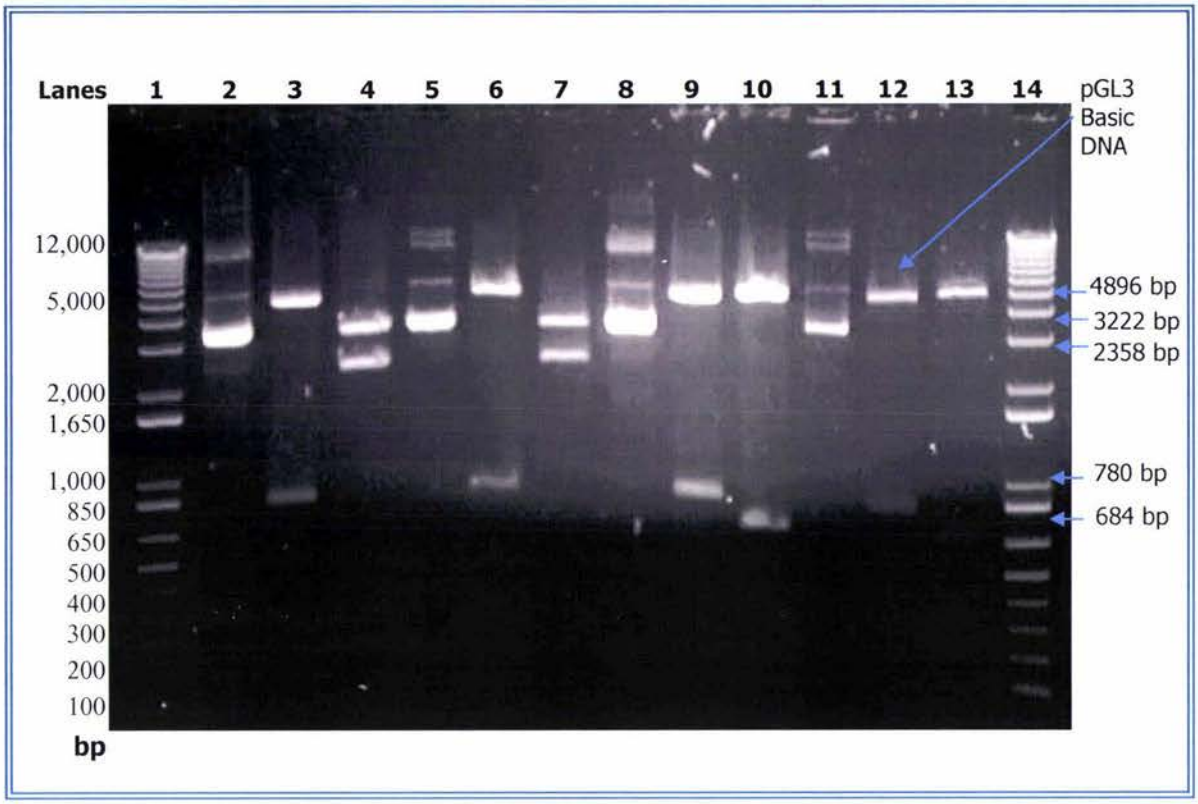


Figure 3.16: Analysis of pGL3Basic vector containing ICB1/2 mutated inserts.

1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (1 μL) samples were analysed on a 1% agarose gel in 1 X TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lanes 1 and 14 and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lanes	2 & 5	1 μL plasmid DNA (ICB1mt) undigested
	3 & 6	30μL plasmid DNA digested with *Hind* III and *Kpn* I
	4 & 7	30μL plasmid DNA digested with *Xba* I
	8 & 11	1 μL plasmid DNA (ICB2mt) undigested
	9 & 12	30μL plasmid DNA digested with *Hind* III and *Kpn* I
	10 & 13	30μL plasmid DNA digested with *Bgl* II

If the 780 bp insert contains a mutation within ICB1 then *Xba* I cuts to produce 3222 bp and 2358 bp fragments, while a mutation in ICB2 generates a second *Bgl* II site, producing fragments of 4896 bp and 684 bp. The pGL3Basic clones were confirmed to have a 780 bp fragment of the topoisomerase IIβ promoter sequence, containing either a ICB1 or ICB2 mutation.

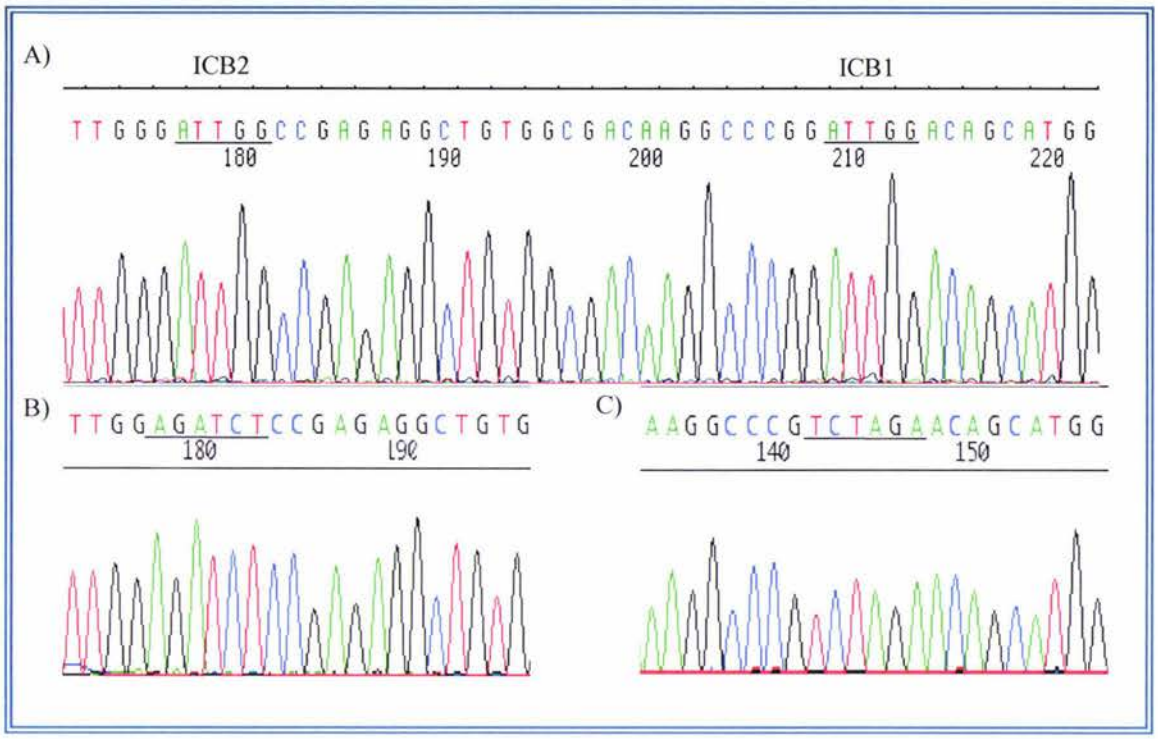


Figure 3.17: Sequence of constructs containing ICB1 or ICB2 mutations.

Representation of sequencing for the topoisomerase IIβ ICB1 and ICB2 mutant constructs. Peak colours: blue = cytosine, red = thymidine, green = adenosine, and black = guanosine. The letters above the peaks represent a computer estimate of the nucleotide at that position based on the peak intensity.

Plasmid DNA, prepared using the CONCERT™ High Purity Plasmid Maxiprep Kit (GIBCOBRL®, Invitrogen), was sequenced from the RV3 primer. A) Sequence shows the wild type topoisomerase IIβ ICB1 and ICB2 elements, B) Sequence shows the mutated topoisomerase IIβ ICB2 element (underlined), C) Sequence shows the mutated topoisomerase IIβ ICB1 element (underlined).

3.6 Generating a pGL3Basic promoter vector TIIβ-180 construct.

In order to investigate roles of GC, ICB1 and ICB2 in topoisomerase IIβ promoter transcriptional regulation, the 180 bp promoter region containing the three elements, was cloned into a pGL3Basic promoter vector. In contrast to the standard pGL3Basic vector, the pGL3Basic Promoter vector (Promega) contains the SV40 promoter with up and downstream MCS to allow the introduction of DNA of interest. PCR was used to isolate the 180 bp region, which was then cloned into a pGL3Basic promoter vector upstream of the SV40 promoter to allow functional analysis.

3.6.1 Generation of PCR products.

PCR reactions were carried out as described in section 2.2.10 using pGL3B-TIIβ-654 as a template, and *Taq* polymerase (Promega). The 180 bp product was amplified using a flanking forward primer (RV3) and the internal ICB1 reverse primer. The PCR reactions were carried out in thermal cyclers (HYBAID Omn-E, SciTech (NZ) Ltd or GeneAmp®PCR System 2700, Applied Biosystems (USA). The 180 bp PCR product does not contain any restriction enzyme sites which could be used to confirm the sequence, therefore agarose gel electrophoresis was used to check the size of the PCR products (data not shown).

3.6.2 Ligation of 180 bp PCR product into pGL3Basic promoter vector.

PCR products were purified using the freeze squeeze method (Thuring *et al.*, 1975), and treated with Klenow enzyme (as described in section 2.2.5) to generate blunt ends. The DNA fragments were ethanol precipitated (Sambrook *et al.*, 2001), and then digested with *Kpn* I, to generate one blunt end and one *Kpn* I cohesive end. The insert DNA was purified using the freeze squeeze gel purification method and quantified by gel electrophoresis using quantification standards. Vector pGL3Basic promoter (Appendix 1) was prepared for ligation by generating *Kpn* I and *Sma* I cohesive ends, using the same methods described in section 3.2.4.2.

Ligations and transformations were carried out as previously described for cloning into the pGL3Basic vector (Refer to sections 2.2.12, 2.2.13 and 3.2.4), using random selection to pick colonies for screening, and similar results were obtained.

Diagnostic digests were carried out to identify positive clones using restriction enzymes *Kpn* I and *Hind* III, which cut flanking sites in the pGL3Basic promoter vector MCS, and therefore cut out the 180 bp insert (figure 3.17). Following DNA extraction (refer to sections 2.2.14.3 and 3.2.4.5), diagnostic digests as described above, were carried out to confirm that the purified plasmid contained the correct topoisomerase IIβ promoter insert. Also, single and double digests were performed to determine a difference in size between the vector linearised by a single digest and the vector with insert DNA removed (data not shown). As expected if the 180 bp insert was present, the vector digested with two enzymes was smaller than the vector digested with only a single enzyme. From the results of these digests and subsequent sequencing (refer to section 2.2.16 and 3.2.3.4), the topoisomerase IIβ 180 bp promoter sequence was confirmed to be correct in the pGL3Basic promoter vector.

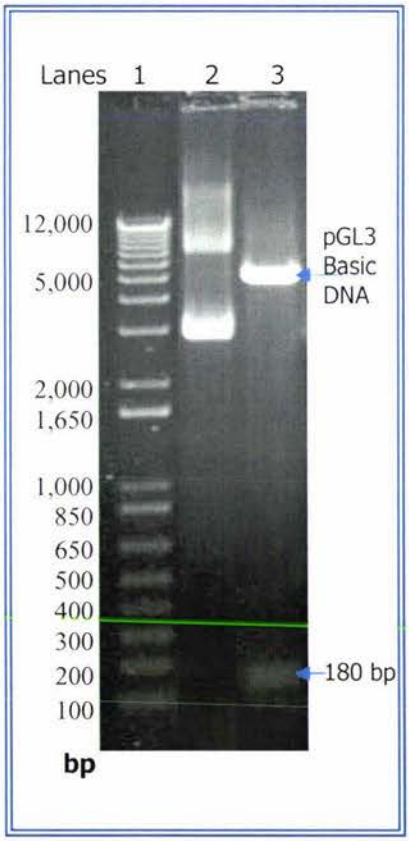


Figure 3.17: Diagnostic digests of putative pGL3BP-TIIβ-180 clones.

1 μL (~1 μg/μL) of DNA was digested with 1 μL (10 U/μL) of each restriction enzyme. Digested (30 μL) and uncut (5 μL) samples were analysed on a 2% agarose gel in 1 X TAE buffer by electrophoresis for about 1 hour at 85 V. DNA was visualised by incorporating ethidium bromide (0.5 μg/mL) into the gel and exposure to UV light. 10 μL of 1 kb plus ladder (1:10) is present in lane 1 and the molecular sizes of bands are displayed on the left in base pairs (bp). Enzymatic digestions were performed as described in section 2.2.3.

Lane 2 1 μL uncut plasmid DNA

Lane 3 30 μL plasmid DNA digested with *Kpn* I & *Hind* III

Hind III and *Kpn* I cut within the MCS of the pGL3Basic vector to release the 180 bp insert as seen in lane 3. The varying high mobility bands visible in the uncut vector lanes represent the different states of the circular plasmid; i.e. relaxed or supercoiled. The pGL3BP vector was confirmed to contain a DNA fragment approximately 180 bp in size.

3.7 Chapter Summary.

A 1.5 kb region of the 5'-flanking and untranslated sequence of the human topoisomerase IIβ gene was isolated and cloned into the pGL3Basic luciferase reporter vector.

In order to investigate regions of the promoter important for topoisomerase IIβ gene expression, a deletion series of the wild type promoter sequence (-1357 to +126) was generated in pGL3Basic vector, for use in functional assays. A total of nine constructs were generated; a series of five 5'-deletion constructs and an additional set of four constructs with an internal deletion introduced within the truncated promoter sequences. The internal deletion removed a 198 bp region containing putative GC, ICB1 and ICB2 regulatory elements and therefore was used to investigate the importance of this region for topoisomerase IIβ promoter activity.

PCR mutagenesis was used to introduce mutations into the ICB1 or ICB2 elements in order to investigate the importance of these two elements for topoisomerase IIβ promoter activity.

An additional construct was created consisting of the 180 bp region, containing the GC, ICB2 and ICB1 elements, cloned upstream of an SV40 promoter in a luciferase reporter vector. The purpose of this construct was to examine the ability of this putative regulatory region to enhance expression driven by a heterologous promoter, thereby investigating its influence on expression from the topoisomerase IIβ promoter.

Chapter 4: GC1, ICB1 and ICB2 binding assays of the topoisomerase IIβ promoter.

4.1 Introduction.

It has been shown that the transcription factors Sp1 and NF-Y bind to putative regulatory elements in the topoisomerase IIβ promoter, GC, and ICB1 and ICB2, respectively (Lok *et al.*, 2002). To further characterise these interactions, electrophoretic mobility shift assays (EMSA) were carried out using sequence specific probes and HeLa nuclear cell extracts as a protein source.

The EMSA is a simple method used for the detection of protein-nucleic acid interactions *in vitro*, using non-denaturing polyacrylamide gel electrophoresis. This technique utilises the differences in electrophoretic mobility of a DNA probe and a DNA probe with protein bound. A double-stranded oligonucleotide probe radioactively labelled on one strand with γ-^{32}P[ATP] is incubated with a protein extract containing the putative DNA-binding proteins. Once the binding reaction is complete, the sample is subjected to electrophoresis using a low ionic strength polyacrylamide gel, and the results are visualised by autoradiography. Free probe runs at a position characteristic of its size, while probe with protein bound will migrate at a much slower rate due to its increased mass, and therefore appears as a "shifted" band of DNA higher up in the gel (figure 4.1). Non-specific DNA-protein interactions are minimised by the addition of poly (dI-dC) to the binding reaction.

Sequence specificity of the DNA-protein interaction can be demonstrated using competitor assays to show that the protein binds to the DNA probe with a substantially higher affinity than to random or mutated DNA sequences. Unlabelled oligonucleotides are added in molar excess to the binding reaction, before the addition of the labelled DNA. If the protein preferentially binds to the competitor DNA, less protein will be available for interaction with the labelled probe. This outcome can be detected visually by a decrease in intensity of the "shifted" band.

The specific proteins present in the protein-DNA complex can be identified by the addition of antibodies in supershift assays. Specific antibodies are added to the binding reaction prior to addition of the labelled DNA, to allow recognition of and binding to proteins which may be involved in complex formation. Subsequent formation of an antibody-protein-DNA ternary complex will result in a further reduction in mobility, and thus a "supershifted" band. Alternatively complex formation could be inhibited if the antibody attaches to the associating protein at a site necessary for the binding reaction to occur.

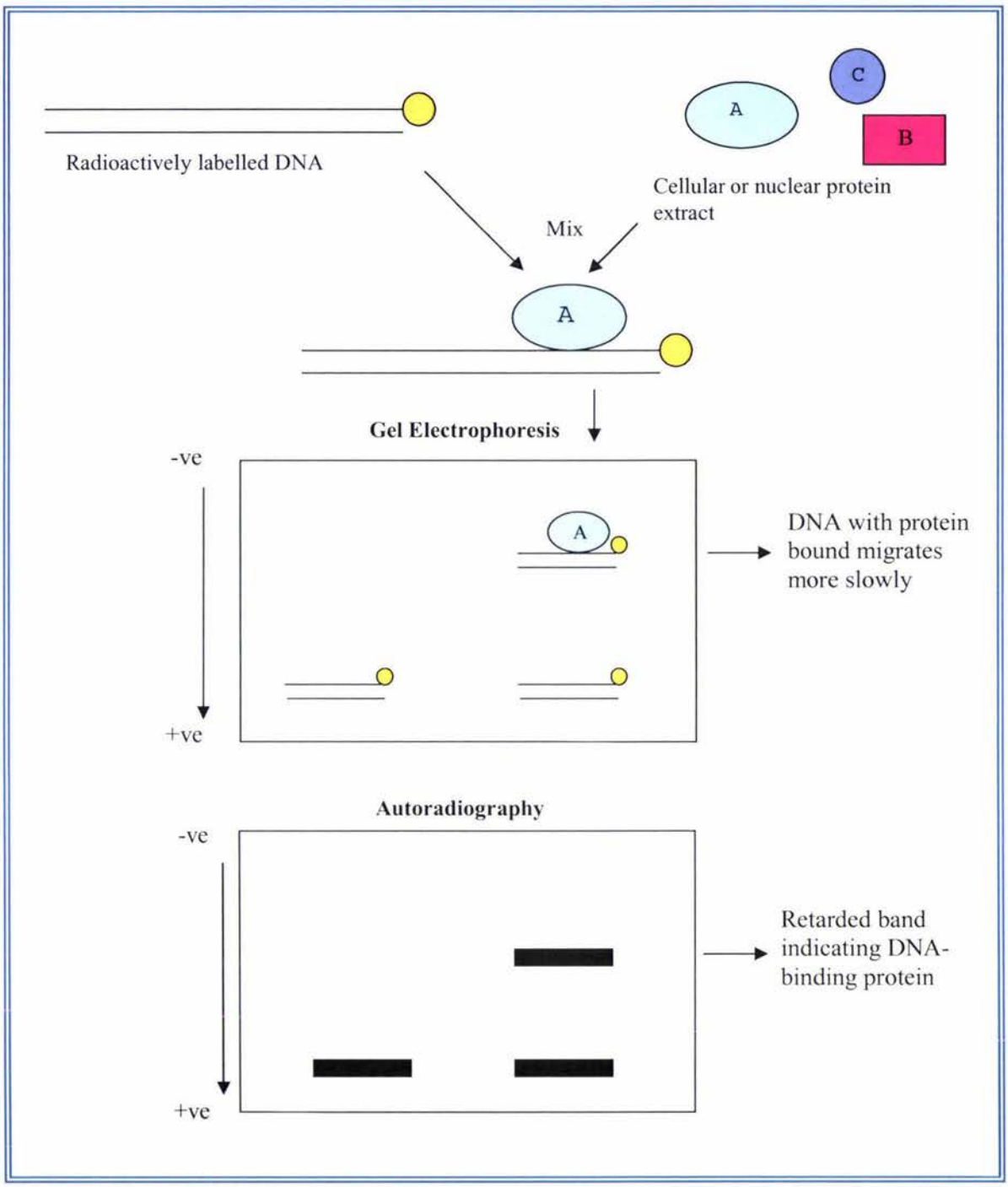


Figure 4.1: Schematic diagram of EMSA.

Binding of a protein to the radioactively labelled DNA causes it to move slowly upon gel electrophoresis, resulting in the appearance of a retarded band detected by autoradiography (Latchman, 1993).

4.2 DNA-protein binding studies of the Topoisomerase IIβ promoter.

Lok et al., (2002) showed the ability of transcription factor Sp1 to bind to the GC element, and transcription factor NF-Y to bind to the ICB1 and ICB2 elements, of the topoisomerase IIβ promoter. Other studies have shown that NF-Y can bind to ICB1-4 elements in the topoisomerase IIα promoter (Isaacs *et al.,* 1996a; Coustry *et al.,* 2001; Morgan and Beck, 2001) and both Sp1 and Sp3 can bind to the GC1 and GC2 elements (Magan *et al.,* 2003). The latter study also provided evidence for co-operativity between ICB1 and GC1 elements of the topoisomerase IIα promoter in order to recruit proteins to each element, therefore it is possible that the same interplay may occur in the topoisomerase IIβ promoter. ICB elements do not contain GC sequences to which Sp1 is known to bind, however it is possible that NF-Y transcription factor bound at ICB2 is responsible for recruitment of Sp1 to the promoter. If this were the case, a functional interaction between Sp1 and NF-Y proteins bound at GC and ICB2 elements respectively, could be important for the transcriptional regulation of the topoisomerase IIβ promoter.

To investigate this possibility, several sets of EMSA experiments were carried out in order to examine the binding interactions occurring with six different oligonucleotide probes. The oligonucleotide probes were designed to encompass both the ICB2 and GC elements of the topoisomerase IIβ promoter, and the single elements GC, ICB2 (refer to figure 4.2) and ICB1 (Appendix 1). To determine sequence specificity of protein-DNA interactions identified, various competitor oligonucleotides were also designed containing mutations in either or both GC and ICB2 (see Appendix 1).

The mutated sequences were analysed using the Findpatterns programme of GCG (Wisconsin Genetics Computer Group, USA) and the MatInspector V2.2 programme of the TRANSFAC4.0 (http://transfac.gbf.decreases/cgibin/matSearch/matsearch.pl). Using both of these programmes, searches of the transcription factor database were carried out to identify any possible binding motifs remaining in the mutated oligonucleotide sequences. This ensured that Sp1 and NF-Y consensus sequences were abolished by the introduced mutations to the GC and ICB2 elements, respectively, and that no additional consensus binding sites were introduced.

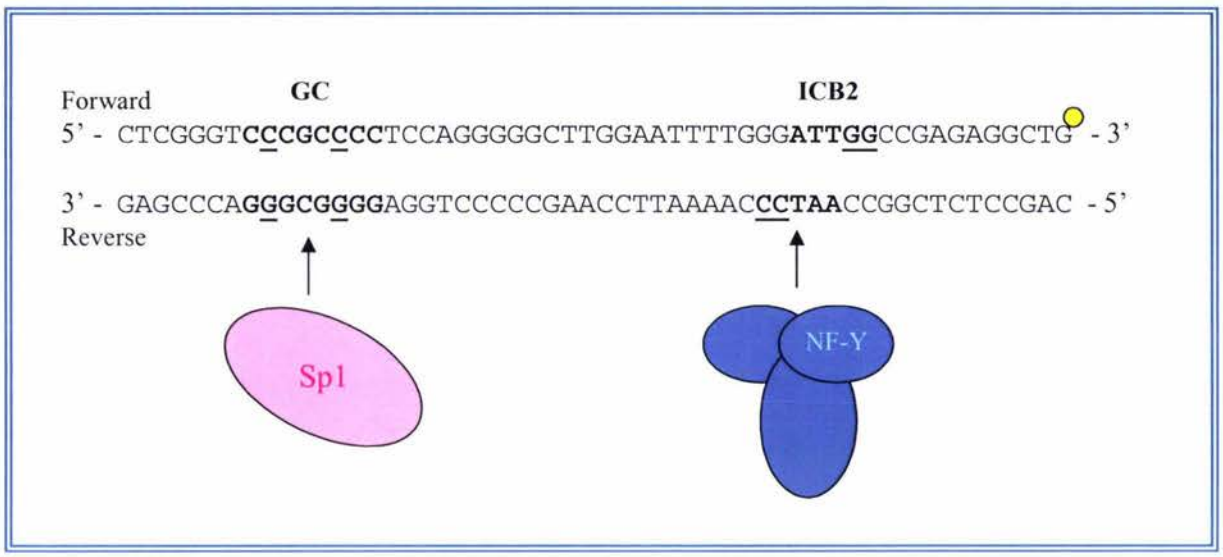


Figure 4.2: Schematic representation of ICB2, GC and putative transcription factors.
NF-Y is shown as the blue trimeric complex on the right associating with the ICB2 element, and Sp1 is shown to associate with GC on the left (pink). The sequence of the double-stranded wild type 54 bp oligonucleotide probe used in electrophoretic mobility shift assays, is shown at the top. The underlined sequence in each element was mutated to inhibit protein binding and yellow signifies the strand labelled with γ-^{32}P[ATP] (adapted from Isaacs, 1996b).

4.2.1 Preparation of HeLa extracts.

Transcription factors: Sp1, Sp3 and NF-Y, are known to be present in HeLa cells (Hagen *et al.,* 1994), therefore the preparation of cell extracts enriched for these proteins was not required. HeLa cell extracts were prepared as described in section 2.2.19.3 and the amount of protein present was quantified as described in section 2.2.20. Protein concentrations for the four different HeLa extract samples prepared, as determined by the Bradford protein assay, were estimated to be 4.12, 3.80, 3.08 and 2.64 μg protein/μL (refer to Appendix 4 for details).

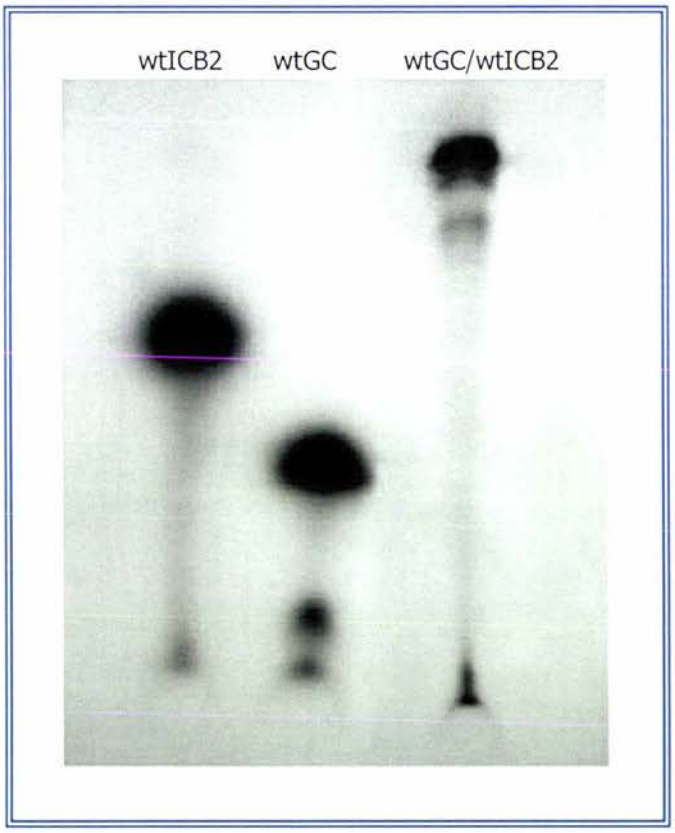
4.2.2 Preparation of the ^{32}P-labelled probes.

Commercially synthesised oligonucleotides were labelled with γ-^{32}P[ATP] on one strand, as described in section 2.2.19.1, and annealed to the complementary unlabelled oligonucleotide. Polyacrylamide gel electrophoresis (PAGE) was used for the purification of double-stranded labelled oligonucleotides (refer to section 2.2.19.6) to

remove any contaminating short or single-stranded DNA fragments, as shown in figure 4.3.

An accurate estimation of the DNA probe concentration was not possible due to immeasurable losses during the purification process, however the amount of radioactivity incorporated into the labelled oligonucleotides was determined using Cerenkov counting (refer to table 4.1). The amount of radioactivity per μL of labelled oligonucleotide ranged from approximately 9,000 to 68,000 counts per minute (cpm). 0.5-1.0 μL of probe was added to each EMSA to give approximately 10-20,000 cpm per binding reaction. Taking into account purification losses of approximately 50% from the 200 ng of oligonucleotide labelled, DNA concentration can be roughly estimated to be 0.3 ng DNA/μL, and therefore approximately 0.15-0.3ng of DNA were added to each EMSA.

Figure 4.3: Gel Purification of the ^{32}P labelled oligonucleotide probes for EMSA.



The oligonucleotides were end labelled with γ-^{32}P[ATP] using T4 polynucleotide kinase. The double-stranded labelled oligonucleotides were purified by electrophoresis on a 10% non-denaturing polyacrylamide gel in 1 x TBE, for approximately 2 hours, at 30 W.

The location of the probe in the gel was detected by autoradiography, allowing excision of the bands. DNA was eluted from the agarose by overnight incubation in 300 μL 50 mM KCl.

Probe	Counts per minute (1 μL)			
	1st set	2nd set	3rd set	4th set
wtGC/wtICB2	53626.40	8539.45	10575.94	19948.12
mtGC/wtICB2	45035.80	10594.32		
wtGC/mtICB2	11264.40	15793.67		
wtGC			28019.96	51974.06
wtICB1			24432.59	
wtICB2			30265.74	68166.80
Blank	31.20	29.54	20.88	22.31

Table 4.1: Incorporation of γ-^{32}P[ATP] into oligonucleotide probes.

Cerenkov counting (Beckman LS3801 Scintillation Counter) was used to estimate the radioactivity incorporated into each double-stranded oligonucleotide probe. Approximately 0.15-0.3 ng of DNA was used for each EMSA.

4.2.3 Binding Reactions.

Multiple factors influence successful binding reactions such as DNA and protein concentrations, salt concentration, temperature, and the intrinsic stability of the complex itself. For this reason, the optimal binding conditions for each DNA probe were essential for accurate and consistent results. Each competition and supershift assay was carried out in duplicate for each probe to establish reproducibility. The experiments are subject to variation in the amount of protein extract, probe and competitor added to the binding reactions.

Binding reactions were prepared in two stages (as described in section 2.2.19.5); the first consisting of HeLa extract, poly dI.dC, gel shift buffer, competitor DNA or antibody if required, and the final step was the addition of labelled probe. For the third set of EMSAs an alternative buffer was tested with the aim of further optimising binding conditions and was found to produce clearer, more consistent results. As illustrated in figure 4.5 onwards, bands are more distinct, possibly due to a more stable interaction between the DNA probe and protein being maintained during electrophoresis.

4.2.4 HeLa extract titration

As the equilibrium between bound and unbound DNA to protein is a crucial factor in the accuracy of EMSA experiments, the amount of nuclear extract and DNA probe added are important. Without an accurate estimate of DNA probe concentration, it was necessary to determine the optimal amount of HeLa extract required to maximise protein-DNA interactions. At the same time, the integrity of the DNA probes could also be determined.

HeLa extract titrations were carried out using 0.5-1.0 μL of DNA probe (approximately 10-20,000 cpm) and varying amounts of HeLa extract from 0-4 μL (0-12 μg of protein depending on the extract used). An extract titration was performed for each set of labelled oligonucleotides prior to EMSA experiments, as the optimal amount of HeLa extract can vary depending on the DNA probe. The results of a representative HeLa extract titration are presented in figure 4.4.

Figure 4.4 illustrates the resulting banding patterns observed with the addition of increasing amounts of HeLa extract to three different oligonucleotide probes representing the double element GC/ICB2 of the topoisomerase IIβ promoter sequence, with either GC or ICB2 mutations or as wild type. 0.5 μL of probe DNA was added to the binding reactions, with the exception of the mtGC/wtICB2 probe, of which 1.0 μL was added due to the lower value of cpm.

Control lanes are included for each probe (lanes 1, 5 and 9), which do not contain HeLa extract and therefore lack protein-DNA interactions as shown by the presence of only a single band, which represents free probe. The addition of HeLa extract to binding reactions shown in subsequent lanes results in a distinct banding pattern, which increases in intensity as the amount of HeLa extract added increases from 1 – 4 μL. The appearance of slightly smeared or distorted bands sometimes observed could be due to partial dissociation of the protein–DNA complex during electrophoresis. The optimal amount of HeLa extract for this assay appears to be 2 μL (~8 μg) as the bands are the most distinct at this concentration. For subsequent EMSA experiments using the same probes, the addition of 6-8 μg of extract was sufficient to optimise binding conditions.

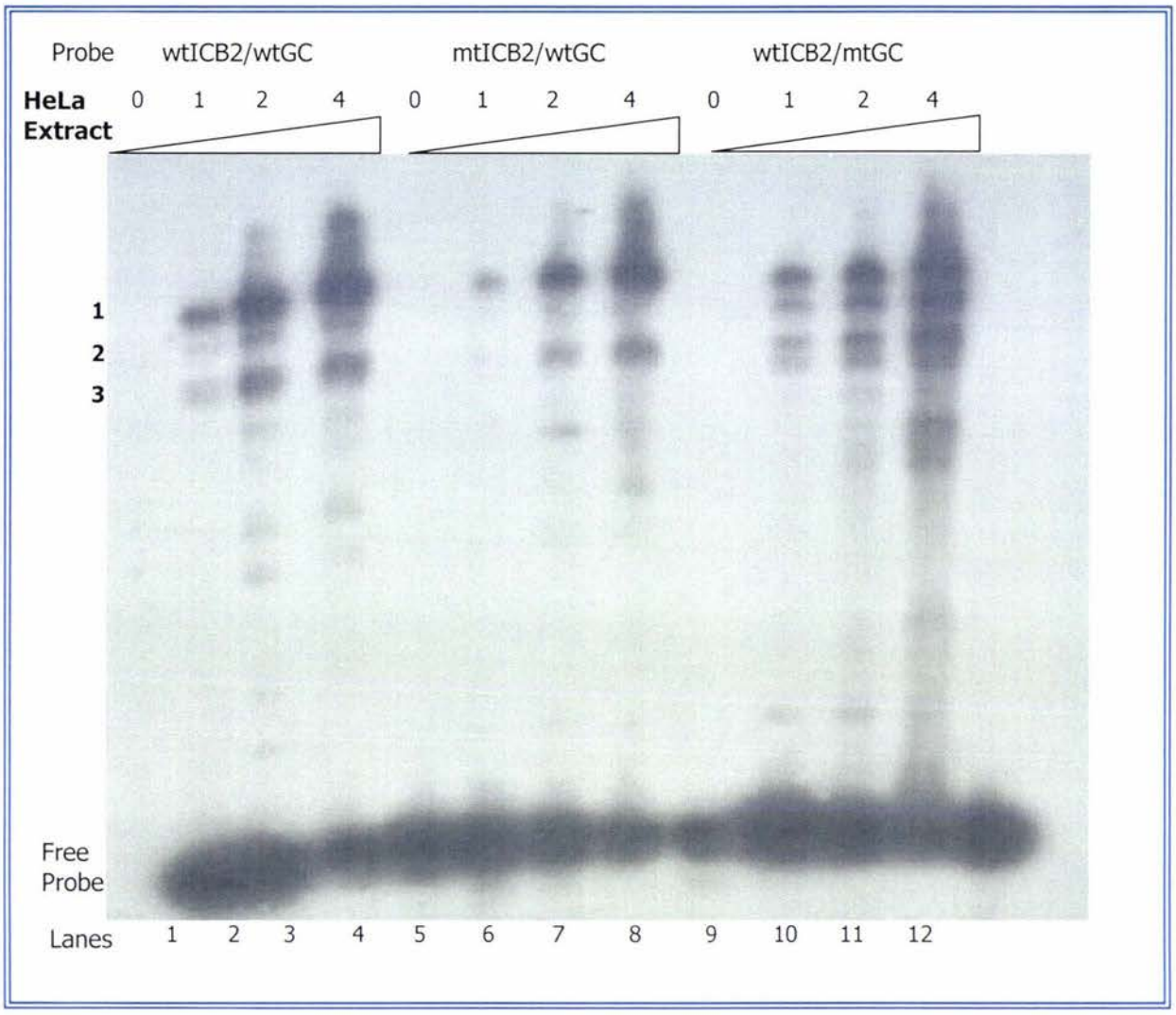


Figure 4.4: HeLa extract titration.

Increasing amounts (0, 1, 2 and 4 μL) of HeLa extract (4.12 μg protein/μL) were added with 0.5 μL of wtGC/wtICB2 or mtGC/wtICB2 probes, and 1.0 μl of the wtGC/mtICB2 probe. 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour, at 200 V. The gel was subsequently dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C, in a radioactive safe cassette with intensifying screens. EMSAs were carried out as described in section 2.2.19.

Control lanes 1, 5, and 9 contain no HeLa extract. The putative specific interactions are labelled 1-3, with the remaining bands representing non-specific interactions. Free probe with no protein bound is visible at the bottom of the gel. This figure is representative of duplicate experiments.

Three distinct protein-DNA complexes (labelled 1-3) were observed in reactions using the wtGC/wtICB2 DNA probe (lanes 2, 3, and 4). The intensity of the three bands varies according to the mutation present in the DNA probe, which suggests some influence on the binding capabilities of proteins due to changes in consensus binding sites. Of the three bands, only band 2 is completely removed due to a mutated DNA sequence, suggesting a strong protein-binding specificity for this interaction only. Other bands of higher mobility appear to occur randomly depending on the probe present in the reaction, and are therefore likely to be the result of non-specific DNA-protein interactions.

A mutation within the ICB2 element (lanes 6-8) resulted in the loss of band 2, while this band was present when GC was mutated (10-12). However, a decrease in intensity of bands 1 and 3 was observed when either GC or ICB elements were mutated, in comparison to the wild type probe. This suggests that the interactions represented by these two bands may not be specific for either element, while band 2 is specific for the ICB2 element as a mutation in ICB2 completely inhibits this interaction.

Extract titrations were carried out for each labelled probe including ICB1, ICB2 and GC single element probes (data not shown). The resulting banding patterns showed the presence of bands 1 and 3 only with the GC single probe. ICB1 and ICB2 single probes both distinctly exhibited band 2, and additional faint bands 1 and 3 were also usually seen (refer to antibody supershift and competitor assay, probe plus extract control lanes, figures 4.5, 4.10 and 4.11). These results suggest that the proteins involved in the complexes represented by bands 1 and 3 preferentially bind the GC element, while proteins present in band 2 preferentially bind the ICB elements with high sequence specificity.

These observed variations in banding patterns provide important preliminary evidence to suggest that some proteins may bind both GC and ICB2, while a unique protein-DNA interaction also occurs involving only ICB1 or ICB2. To further characterise the protein interactions occurring at GC and/or ICB1 and ICB2 oligonucleotides, a series of EMSAs were carried out using antibody supershifts and competitor oligonucleotides.

4.2.5 Antibody supershifts.

To determine the identity of proteins involved in binding interactions with the probes derived from topoisomerase IIβ promoter sequences, antibodies against putative transcription factors Sp1, Sp3, and NF-Y (against either NF-Y subunit A or C) were included in EMSA binding reactions.

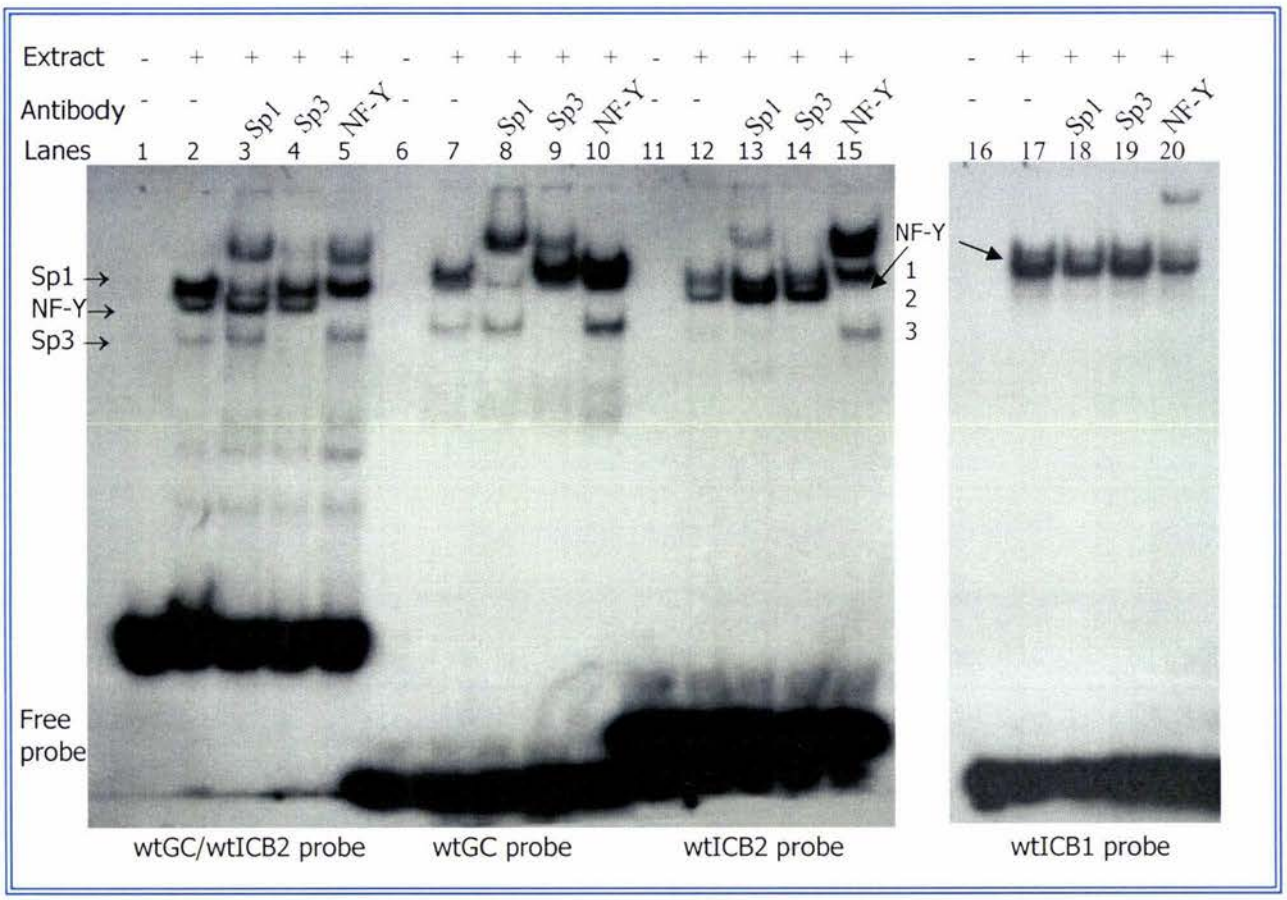


Figure 4.5: Antibody Supershift with double and single element probes.

2μL (200 μg/mL) of antibody was added to each binding reaction with 2 μL of HeLa extract (2.64 μg protein/μL) and 0.5 μL of wtGC, wtICB1 or wtICB2 probes, and 1.0 μl of the wtGC/wtICB2 probe. 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without antibody. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

Figure 4.5 shows the results of an antibody supershift assay carried out using the wtGC/wtICB2 probe, and single element GC, ICB1 and ICB2 probes. Lanes 1, 6, 11, 16 represent controls containing probe only, without the addition of HeLa extract. Lanes 2, 7, 12 and 17 are additional controls containing probe and HeLa extract, without the addition of antibody, as a standard against which to compare the effect of added antibody. The control lane 2 contains the same three bands that were observed for the wtGC/wtICB2 probe in the extract titration (figure 4.4), and control lanes 7, 12 and 17 have the same banding patterns that were observed for the three single element probes, as previously described.

Antibody supershifts were observed with the three antibodies tested with the wtGC/wtICB2 probe (lanes 3-5), allowing three distinct protein-DNA complexes to be identified. Two of the three antibodies induced supershifts with the single GC probe (lanes 8-10), while the three antibodies induced shifts, of varying strengths, for the ICB2 probe (lanes 13-15), and only one of the antibodies induced a supershift with the ICB1 single probe (lanes 18-20).

The addition of antibody against Sp1 (lanes 3, 8, 13 and 18) resulted in a strong supershift of band 1 for both the wtGC/wtICB2 and GC probes, but only a slight shift for the ICB2 probe, and no change was seen for the ICB1 probe. This indicates the presence of Sp1 within complexes formed with all probes, represented by band 1, except for the ICB1 probe. The result however suggests only low levels of Sp1 in association with ICB2, as illustrated by the very low intensity of the shifted band with anti-Sp1 antibody. With the addition of antibody against NF-YA (lanes 5, 10, 15 and 20) a strong shift of band 2 was observed with the wtGC/wtICB2 and ICB2 probes, while only a weak shift was seen for ICB1 probe, and no change in this band was seen with the GC probe. The same result was observed with the addition of antibody against the C subunit of NF-Y. This confirms that band 2 does represent NF-Y, and supports a specific interaction between NF-Y and the ICB elements, as NF-Y was not shown to interact with the single GC probe. In response to the addition of antibody against Sp3 (lanes 4, 9, 14 and 19), a strong supershift of band 3 was observed with the wtGC/wtICB2 and GC probes, while a weak shift was seen for the ICB2 probe, and no change was seen with the ICB1 probe. Therefore, band 3 represents an interaction involving Sp3 protein, which appears to interact to varying degrees, with

both the GC and ICB2 elements, but not with ICB1. The positions of other lower mobility bands were not seen to change, supporting the idea that they are the result of non-specific interactions.

Antibody supershift assays were carried out for each of the labelled probes to confirm the presence of the same three proteins within protein-DNA complexes formed and to identify changes in binding patterns which may be observed in the presence or absence of particular elements. Although a second band was usually visible with the ICB1 probe, at a position corresponding to the Sp1 protein complex identified with the other probes, the anti-Sp1 antibody did not induce a supershift of this band.

Taken together, these data suggest that three distinct protein-DNA complexes are able to form with the wtGC/wtICB2 oligonucleotide from the topoisomerase IIβ promoter. These three complexes observed appear to contain Sp1, Sp3 and NF-Y. Two of the three protein complexes appear able to form in the presence of GC alone, consisting of Sp1 and Sp3. All three protein complexes appear able to form with the ICB2 probe, suggesting that NF-Y, Sp1 and Sp3 are all involved in protein interactions with this element. With the ICB1 probe only a single complex consisting of NF-Y was identified, although an additional complex consistent with the position of Sp1 in other EMSA, was usually observed. Competitor assays were used to characterise the sequence specificity of the binding interactions.

4.2.6 Competitor Assays.

To further investigate the binding interactions taking place and their specificity, competitor assays were performed. Double-stranded competitor oligonucleotides were prepared as described in section 2.2.19.4. Increasing amounts (0-100 ng) of the unlabelled, cold competitor were added to standard EMSA reactions containing labelled DNA probe and HeLa extract. Competitor oligonucleotides used included unlabelled DNA homologous to the labelled counterparts, and GC, ICB1, and ICB2 single element competitors containing either the intact element or specific point mutations to abolish the consensus binding site. Competitor oligonucleotides representing the topoisomerase IIα promoter GC and ICB elements were also tested to determine the specificity of the observed interaction for the topoisomerase IIβ promoter.

4.2.6.1 EMSAs with wtGC/wtICB2 probe.

The wild type double element probe was used in a set of competitor assays. If protein bound at GC was independent of protein bound at ICB2, then the introduction of a mutation within ICB2 of a double element competitor would not affect the strength of competition for binding of the GC associating proteins, Sp1 and Sp3, and vice versa. If however, synergism occurs between the GC and ICB2 elements, a double element competitor containing a mutation should be a less effective competitor than a wildtype double element oligonucleotide.

Double element competitors.

The competitors used in this assay were unlabelled homologues of the wtGC/wtICB2 probe, and probes containing mutations in either one or both of the GC and ICB2 elements. The results are shown in figure 4.6.

The homologous competitor wtGC/wtICB2 was able to compete for binding with all of the protein(s) in the three complexes observed (lanes 2-5), as shown by the uniform reduction in band intensity illustrated as the amount of competitor increases. Results observed with the addition of competitor oligonucleotide containing mutations in both GC and ICB2 (lanes 17-20) suggest a decrease in the intensity of band 1 and band 3, while no significant competition was seen for the protein(s) present in band 2. The

observed competition in the presence of two mutated elements would suggest a loss of non-specific interactions from the protein-DNA complexes represented by bands 1 and 3, while the interactions represented by band 2 are highly specific.

Strong competition was observed for band 2 using the competitor oligonucleotide containing a mutation in the GC consensus binding site (lanes 7-10), which confirms that the protein represented by band 2 (identified as NF-Y in figure 4.5) preferentially binds to ICB2. With the addition of 50 and 100 ng of competitor a weaker binding is also exhibited for the protein complexes represented by bands 1 and 3 (identified as Sp1 and Sp3), as indicated by the reduced intensity of the bands. The reduced binding of Sp1 and Sp3 (bands 1 and 3, lanes 7-10) could be due to an interaction with NF-Y bound at ICB2 of the competitor element.

When competitors containing a mutation in the ICB2 sequence (lanes 12-15) were added, strong competition was observed for the protein(s) in bands 1 & 3, but no significant change occurred in band 2. Comparison between band 2 in lanes 12 and 13 suggests that the more intense band observed in lane 13 is probably due to loading error. Therefore, the protein identified in band 2 (NF-Y) appears to preferentially bind to ICB2. Some residual binding is visible for band 1 (comparing lanes 4 and 5 with lanes 14 and 15) which suggests that NF-Y may be able to recruit a small amount of Sp1 to ICB2, and that Sp1 bound at GC may assist binding of NF-Y at ICB2. Overall these data support an interaction between NF-Y bound at ICB2 and Sp1 (and/or Sp3) bound at GC.

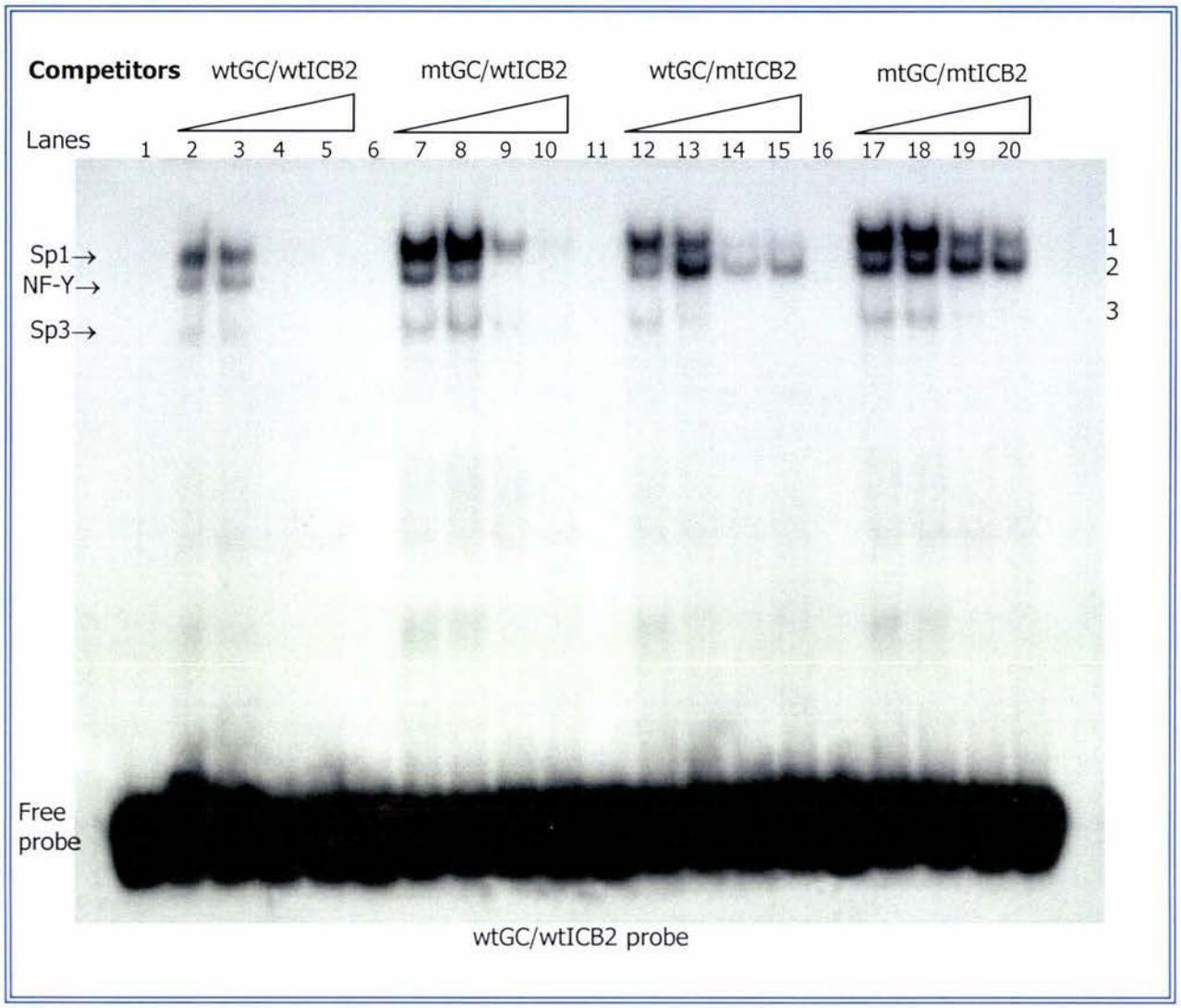


Figure 4.6: Competitor Assays with wtGC/wtICB2 probe (double element competitors). Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 1.0 μL of wtGC/wtICB2 probe with 2 μL of HeLa extract (3.10 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

Single competitors.

To investigate the importance of flanking sequence for protein binding interactions, a set of competitor assays was carried out using shorter competitor oligonucleotides consisting of single elements, including ICB1, ICB2 and GC elements from both topoisomerase IIβ and α (refer to Appendix 1 for oligonucleotide sequences). Determining the ability of single elements alone, to compete for proteins binding within the observed complexes, could also provide an insight into possible synergism occurring between the ICB2 and GC elements of the topoisomerase IIβ promoter. Figures 4.7 and 4.8 show the results of these experiments using ICB and GC competitors, respectively.

If protein bound at GC were independent of protein bound at ICB2, then a GC competitor should only compete for binding of the GC associating proteins, Sp1 and Sp3, and vice versa. If however, synergism occurs between the GC and ICB2 elements, a single element competitor should be a less effective competitor than a double element oligonucleotide. If the flanking sequences between the GC and ICB2 elements are playing a role in protein binding, then that protein would be expected to preferentially bind the longer oligonucleotide probe rather than the shorter competitor.

In both figures 4.7 and 4.8, lanes 1, 5, 10 and 15 are controls containing probe only. As in previous wild type double element probe assays, three protein-DNA complexes were observed, and additional bands attributed to non-specific binding interactions.

In figure 4.7, the topoisomerase IIβ ICB1 (ICB1β) (lanes 2-5) and ICB2 (ICB2β) competitors (lanes 7-10) exhibit the ability to compete strongly for NF-Y, and also showed the ability to compete weakly for Sp1 and Sp3, with ICB2β competing more strongly than ICB1β. This suggests the ability of NF-Y bound at either of the ICB elements to recruit Sp1 and Sp3. The topoisomerase IIα ICB1 (ICB1α) competitor (lanes 12-15) had the ability to weakly compete with all three proteins, suggesting the ability of NF-Y bound at ICB1α to recruit Sp1 and Sp3. The mutated ICB1α competitor was unable to compete for any of the bands observed (lanes 17-20).

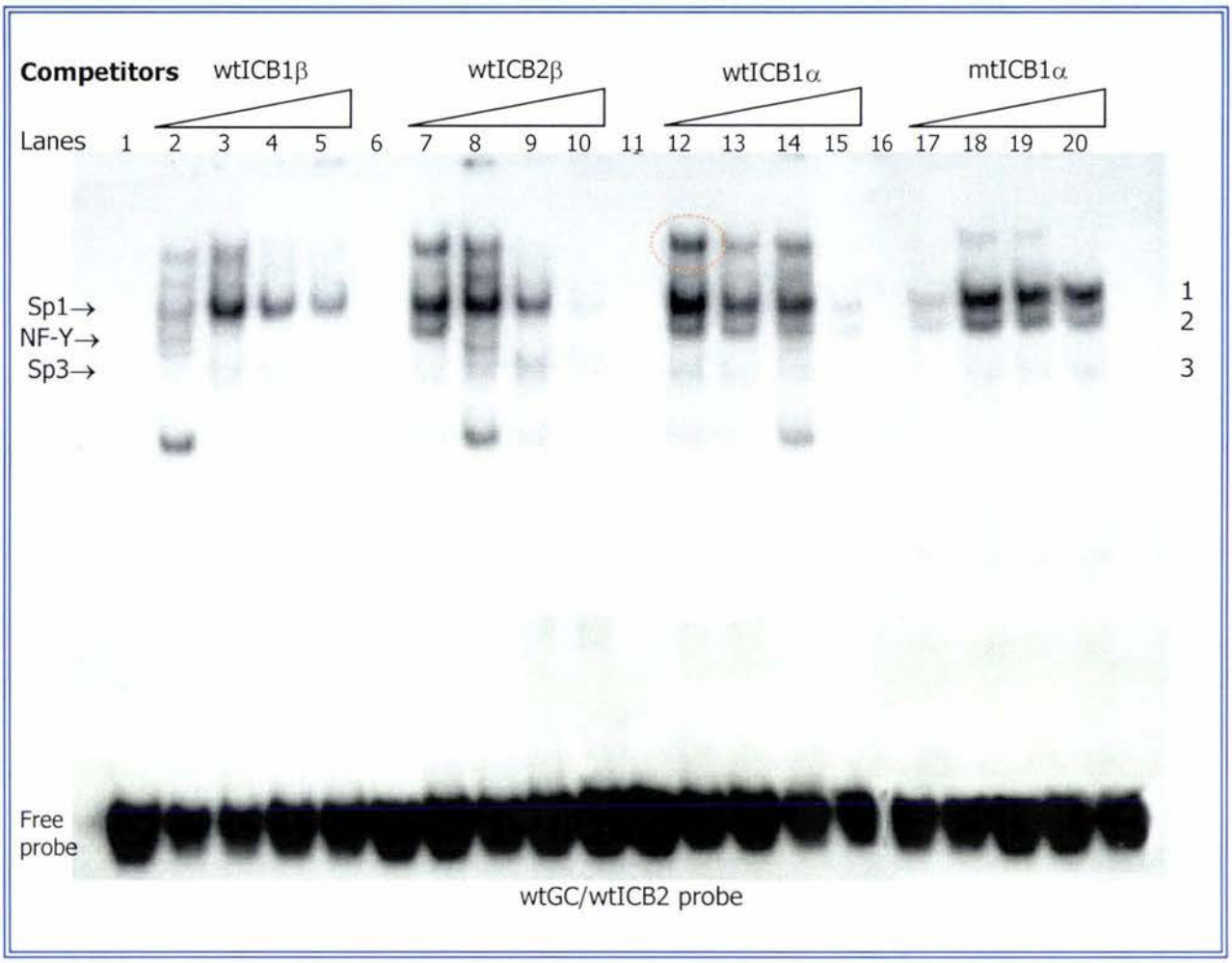


Figure 4.7: Competitor Assays with wtGC/wtICB2 probe.

(ICB single element competitors)

Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 1.0 μL of wtGC/wtICB2 probe with 2 μL of HeLa extract (3.80 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments. The red circle indicates a putative ICB2/NF-Y/Sp1 or Sp3 complex, this multi-protein complex is also visible in lanes 2-3, 7-8, 13-14 and 18-19.

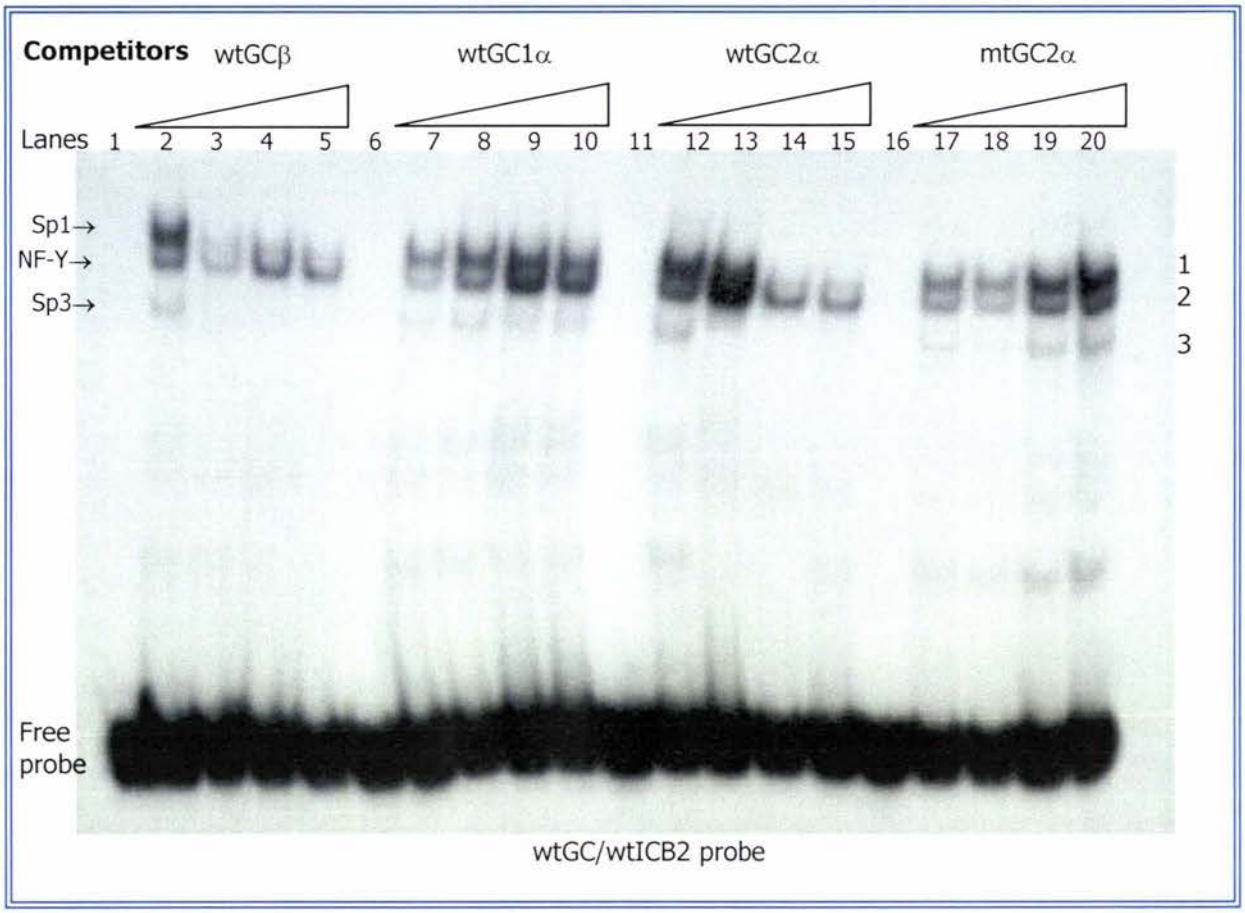


Figure 4.8: Competitor Assays with wtGC/wtICB2 probe

(GC single element competitors).

Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 1.0 μL of wtGC/wtICB probe with 2 μL of HeLa extract (3.10 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

In figure 4.8 topoisomerase IIβ GC element (GCβ) competitor (lanes 2-5) was able to compete for two of the three bands observed, representing Sp1 and Sp3, while no significant competition was seen for NF-Y. The topoisomerase IIα GC1 element (GC1α) competitor (lanes 7-10) could not compete for any of the three bound

proteins, while the topoisomerase IIα GC2 element (GC2α) competitor (lanes 12-15) was able to strongly compete for both Sp1 and Sp3, but not NF-Y. This suggests that the proteins bind with a higher affinity to the single element GC2α, than the wild type double element, and bind with the lowest affinity to the GC1α single element. The GC2α mutant competitor could not compete for any of the bands observed (lanes 17-20).

The collective results of EMSAs thus far (figures 4.6-4.8) indicate that NF-Y preferentially binds the ICB elements independently of Sp1 and/or Sp3 bound at GC, and vice versa. However, an introduced mutation within either the ICB2 or GC element in a GC/ICB2 double element competitor weakens the competition for proteins bound at a double element probe. In addition, the single element competitors exhibited the weakest competition for proteins bound at the double element probes. This suggests that although Sp1 and/or Sp3 are able to bind GC independently of NF-Y bound at ICB2, the protein(s) display a lower binding affinity for the GC element in the absence of the intact ICB2 element, and vice versa. These findings taken with the ability of ICB1 and ICB2 to compete for Sp1 and Sp3 bound at GC, suggest an interaction between the GC and ICB elements.

A series of EMSA experiments using wtGC/mtICB2 and mtGC/wtICB2 probes and homologous and heterologous competitors supported the conclusion that NF-Y preferentially binds ICB2 independently of Sp1 and/or Sp3 bound at GC. This was shown by the ability of the protein complex consisting of NF-Y to bind ICB2 in the absence of an intact GC element, as previously shown in figure 4.6, lanes 7-10 and using single element ICB competitors in figure 4.7. Support was also provided for the conclusion that Sp1 and Sp3 appear to preferentially bind GC independently of NF-Y bound at ICB2. This was shown by the ability of Sp1 and Sp3 to bind GC in the absence of an intact ICB2 element, as previously shown in figure 4.6, lanes 12-15 and using single element GC competitors in figure 4.8. In addition, it was found that NF-Y was unable to bind in the absence of an intact ICB2 element, however Sp1 and Sp3 were able to bind in the absence of an intact GC element. The differences in binding affinities previously observed (figures 4.6-4.8) in the presence of GC and ICB2, or only a single element, was also supported using the double element probe containing a

mutated element with the double or single element competitors. These data suggest an interaction occurs between NF-Y bound at ICB2 and Sp1 and/or Sp3 bound at GC.

4.2.6.2 Competitor Assays with single element probes.

A final set of competitor assays was carried out using single element oligonucleotide probes that consisted of the topoisomerase IIβ GC, ICB1 or ICB2 element. This experiment was designed to further investigate the specificity of protein binding interactions and the importance of flanking sequences, by also using single element competitors. The ICB1 element was labelled to examine binding interactions occurring at this element, and to provide a comparison with the observed binding specificity for the ICB2 element. Figures 4.9 to 4.11 show the results of these experiments using ICB and GC competitors from both the topoisomerase IIβ and topoisomerase IIα promoters.

If the flanking sequences between the GC and ICB2 elements are playing a role in protein binding, then that protein would be expected to be unable to bind or to bind less effectively to the shorter oligonucleotide probe, than the longer oligonucleotide probe. If the formation of a complex at a particular element is dependent upon an association with proteins bound at a second element, then these interactions will be absent from the labelled single element probe. Also, if synergism occurs between the GC and ICB2 elements, a single element competitor should be a more effective competitor of proteins bound at a single element oligonucleotide.

In figures 4.9-11, lanes 1, 5, 10 and 15 are controls containing probe only. As previously described, two protein-DNA complexes were observed with the GC single element probe (figure 4.9), which represent Sp1 and Sp3 proteins, along with additional bands attributed to non-specific binding interactions. The GCβ competitor (lanes 2-5), GC1α competitor (data not shown) and the GC2α competitor (lanes 12-15) all exhibited the ability to strongly compete for both Sp1 and Sp3, showing a similar binding affinity for the same element from the two different isoforms. The ICB2β competitor (lanes 7-10) and ICB1β competitor (data not shown) were able to weakly compete for both Sp1 and Sp3, supporting the ability of NF-Y bound at ICB

to recruit Sp1 and Sp3. The ICBα competitor (data not shown), and the GC2α mutant competitor (lanes 17-20) were unable to compete for either of the bands observed.

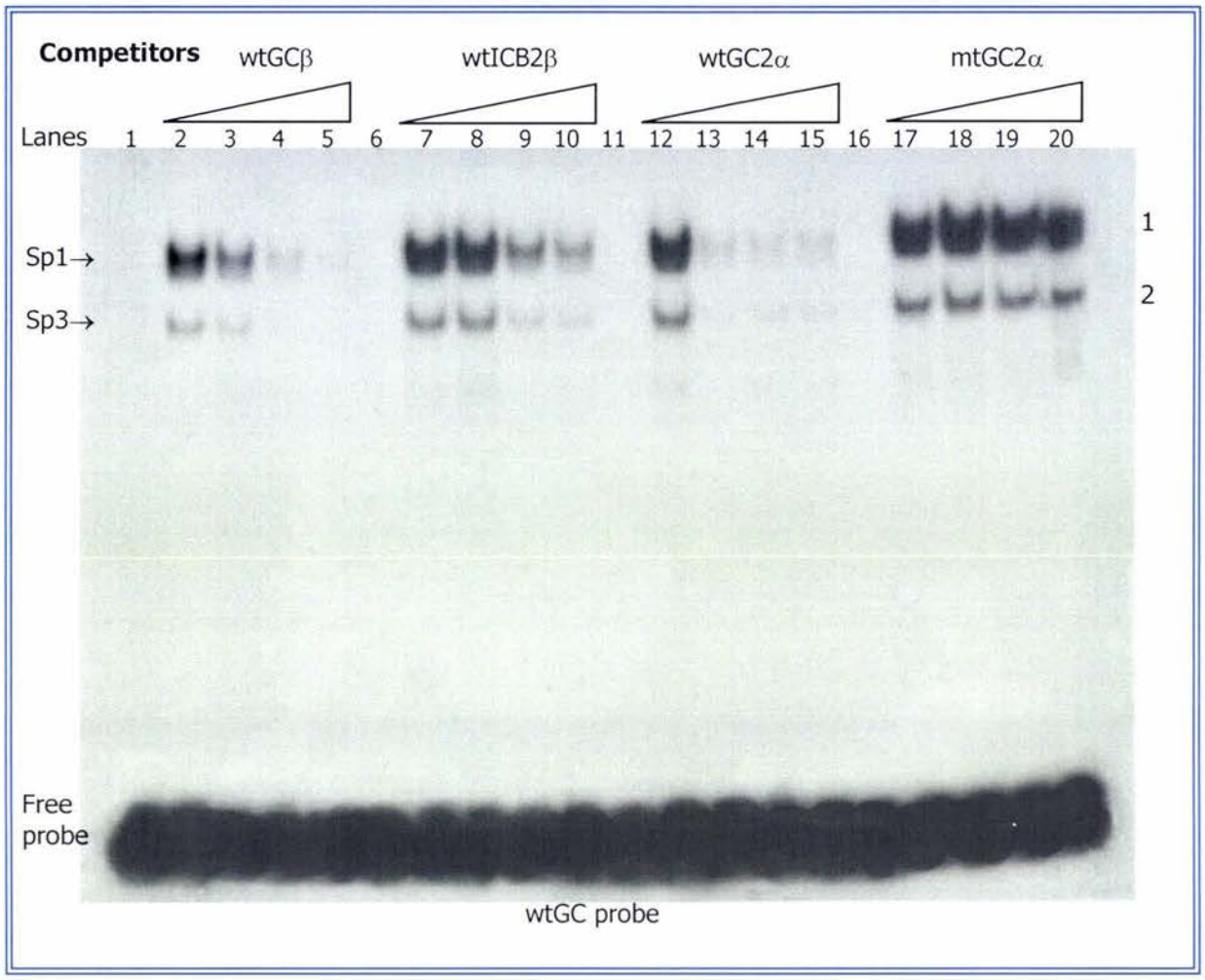


Figure 4.9: Competitor Assays with wtGC probe.

Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 0.5 μL of wtGC probe with 2 μL of HeLa extract (3.10 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

In figure 4.10, two protein-DNA complexes are observed with the ICB1β single element probe, the second representing NF-Y. Although the complex represented by band 1 was not positively identified by an antibody supershift, the position of the band, along with the previously exhibited ability of ICB1 to recruit Sp1, suggest that this protein complex could contain Sp1. The ICB1β competitor (data not shown) and ICB2β competitor (lanes 12-15) were both able to strongly compete for binding of NF-Y and the putative Sp1 complex. ICB1α competitor (lanes 17-20) showed the ability to compete weakly for NF-Y and Sp1, suggesting a lower affinity for NF-Y binding at the topoisomerase IIα ICB1 element. Unexpectedly, GC2α competitor (lanes 2-5) exhibited a moderate ability to compete for NF-Y bound at the ICB1β probe, along with the ability to compete strongly for Sp1. This could suggest a relatively weak interaction between NF-Y and the ICB1β element, which is easily disrupted by Sp1 bound at GC2α. GC1α competitor (data not shown), GCβ competitor (lanes 7-10) and GC2α mutant were each unable to compete for either of the bands observed.

In the final figure 4.11, the two protein-DNA complexes are observed with the ICB2β single element probe, which represent the NF-Y protein and the Sp1 protein, however the Sp3 complex is not visible. The ICB1β (lanes 2-5) and ICB2β competitors (lanes 7-10), were both able to compete strongly for binding of NF-Y and Sp1. The ICB1α competitor (lanes 12-15) showed the ability to compete weakly for NF-Y and Sp1, and therefore suggesting a higher affinity for NF-Y binding at both topoisomerase IIβ ICB elements, than the topoisomerase IIα ICB1 element. The GC2α competitor (lanes 17-20) exhibited a moderate ability to compete for NF-Y and Sp1 bound at the ICB2β probe, which suggests that NF-Y has a strong binding affinity for Sp1 bound at this GCα element. GCβ competitor, GC1α competitor, and GC2α mutant (data not shown) were each unable to compete for either of the bands observed.

Together the results of figures 4.9, 4.10 and 4.11, show a stronger competition of the single element competitor for proteins bound at the single element probe than previously seen with the double element probes. This suggests a lower protein binding affinity at the single element probe, indicating the importance of flanking sequence for strong protein-DNA interactions.

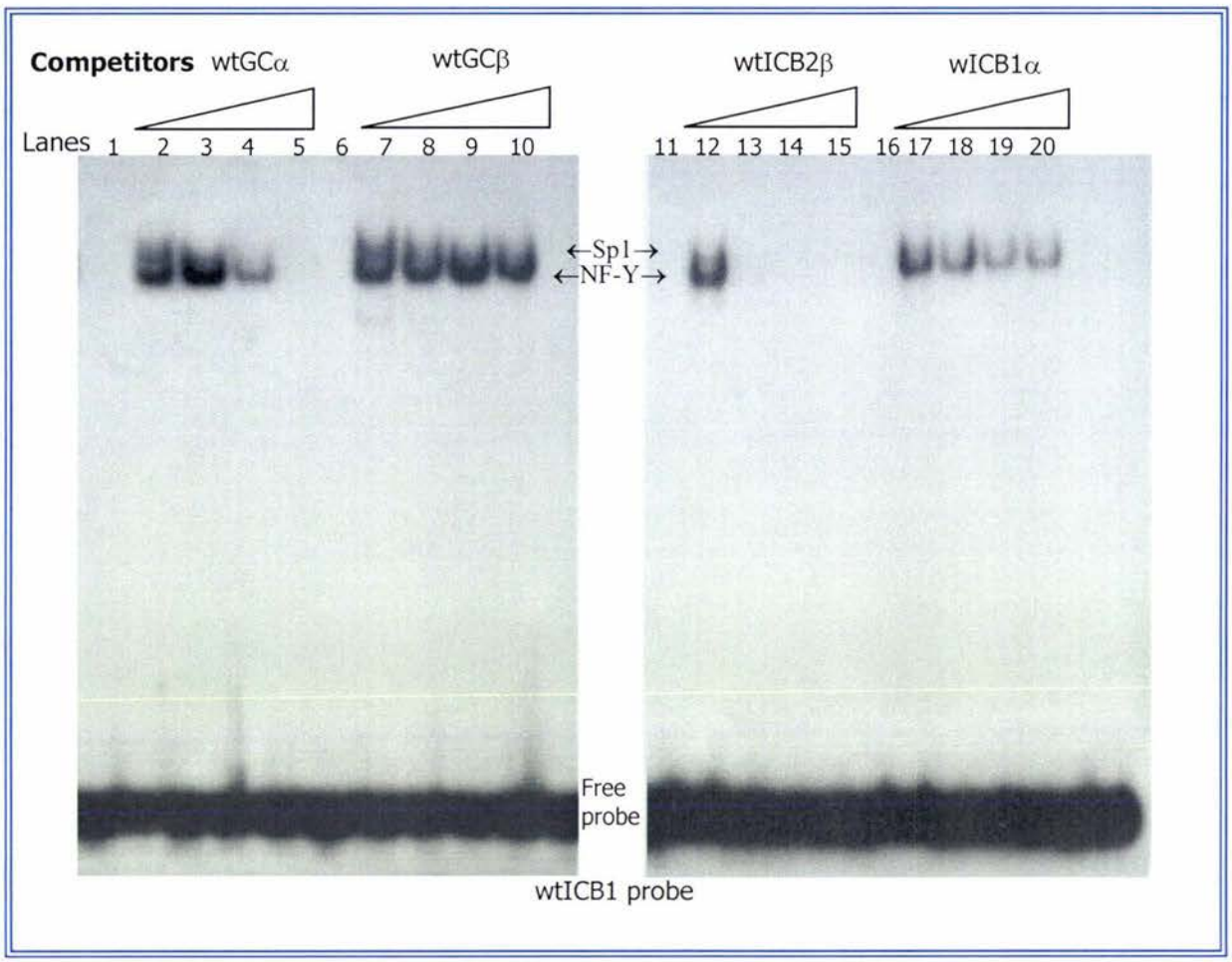


Figure 4.10: Competitor Assays with wtICB1 probe.

Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 0.5 μL of wtICB1 probe with 2 μL of HeLa extract (3.10 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

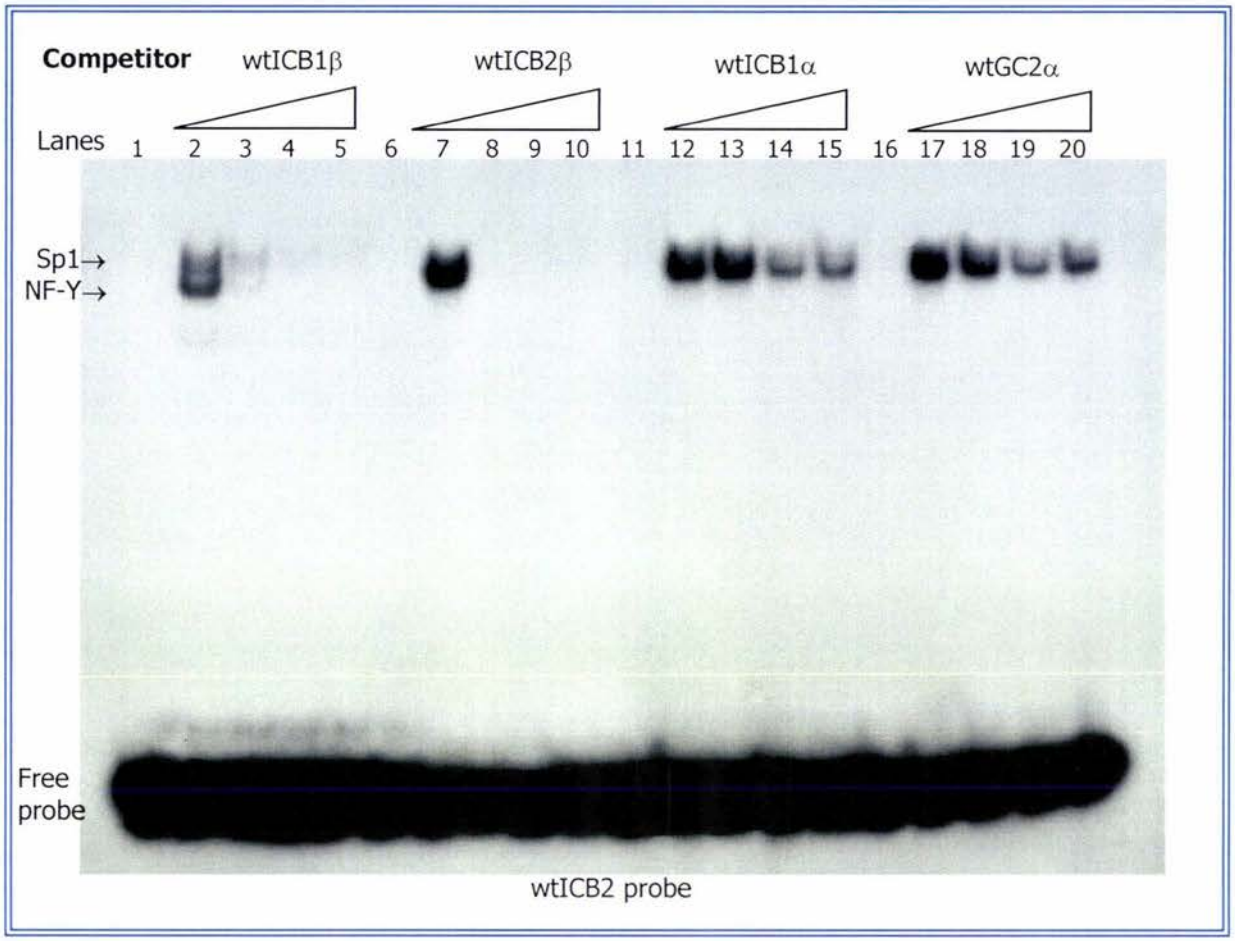


Figure 4.11: Competitor Assays with wtICB2 probe.

Increasing amounts (0, 5, 50 and 100 ng DNA) of double-stranded competitor were added to 0.5 μL of wtICB2 probe with 2 μL of HeLa extract (2.64 μg protein/μL). 10 μL of each binding reaction was loaded onto a 4% polyacrylamide gel in 0.25 x TBE, and electrophoresis was carried out for approximately one hour at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film for approximately 18 hours at -70°C.

Lanes 1, 6, 11 and 16 are controls containing probe only. Lanes 2, 7, 12 and 17 are controls containing extract, but without competitor. Specific interactions are labelled, with the remaining bands representing non-specific interactions. Arrows indicate the bands that identify specific protein(s) in the DNA-protein complexes. This figure is representative of duplicate experiments.

Overall the results suggest that three proteins can associate with the wtGC/wtICB2 promoter sequence of topoisomerase IIβ. Band 1 contains Sp1, and this protein does not appear to bind with high sequence specificity, as the band is still present when the probe contains a mutated GC sequence (figure 4.4, lanes 10-12), and when a single ICB element probe was used (figures 4.10 and 4.11). This suggests that Sp1 is still able to bind in the absence of an intact GC element. The same result was seen for band 3, which contains Sp3 and also binds to the GC region (figure 4.4, lanes 10-12). Band 2 contains an NF-Y protein complex, which binds to the ICB1 and ICB2 elements of the oligonucleotide with high sequence specificity, as this band is not observed when the probe contains a mutated ICB2 sequence (figure 4.4, lanes 6-8). NF-Y appears to bind the ICB elements with different affinities in the order ICB2β>ICB1β>>ICB1α, which suggests a more important role for ICB2 in the protein interactions occurring at the topoisomerase IIβ promoter, than for the ICB1 element, or for ICB1 in the topoisomerase IIα promoter.

Topoisomerase IIβ GC and ICB2 promoter elements have been shown to associate with different proteins, however it appears that the ICB elements have the ability to recruit Sp1 and Sp3 (figure 4.4, lanes 10-12; figure 4.5, lanes 13 and 14; figure 4.10, lanes 2, 7, 12 and 17). In support of this idea, the single elements ICB1/2 had the ability to recruit NF-Y and at the same time decrease the binding affinities of Sp1 and Sp3 (figure 4.7, lanes 2-5 and 7-10). Therefore, it is likely that Sp1 and Sp3 are recruited to ICB2 via a putative interaction between NF-Y bound at ICB2 and Sp1/Sp3 (figure 4.6, lanes 7-10), and the same appears to be true for ICB1 (figure 4.11, lanes 2-5). However, Sp1 and Sp3 bound to GC do not appear to recruit NF-Y (figure 4.6, lanes 12-15 and figure 4.8, lanes 2-5). This suggests that the binding of NF-Y to ICB1/2 could occur first, allowing the subsequent recruitment of Sp1/Sp3 to the complex. Also, the single element competitor assays to investigate sequence specificity suggested that binding affinities of associating proteins were highest with the double element probe, in particular the wild type, and weakest with the single element alone (figures 4.7 and 4.8). This suggests that NF-Y, Sp1 and Sp3 preferentially bind to the longer GC/ICB2 sequence.

The observed patterns of protein binding suggest that the ICB2 sequence is not essential for Sp1 or Sp3 to bind, and the GC sequence is not required for NF-Y to bind. However, the interaction between GC and Sp1 and/or Sp3 is enhanced by an intact ICB2 element, as is the interaction between ICB2 and NF-Y in the presence of GC. This appears to suggest that the identified protein interactions at the GC and ICB2 elements are more stable when all proteins are able to bind. In particular, NF-Y bound at ICB2 appears to be essential to maintain strong interactions between GC and Sp1 and/or Sp3. Therefore, it appears that NF-Y bound at ICB2 may be involved in recruiting Sp1 and Sp3 to GC, and this could occur via a direct protein-protein interaction. Both proteins are known to contain Q-rich domains, which have been implicated in the mediation of protein-protein interactions (Roder *et al*, 1997). Therefore, the recruitment of Sp1 and Sp3 to the ICB2 element could occur via a putative interaction between NF-Y and Sp1/Sp3.

A direct interaction occurring between NF-Y and Sp1 or Sp3 should result in the formation of an additional lower mobility complex consisting of the two proteins bound to DNA. A complex of this description is in fact present with EMSA experiments using the wild type double element probe (figure 4.7, enclosed in red circle). The removal of NF-Y, Sp1 and Sp3 by the addition of competitors consequently resulted in the removal of this putative DNA/NF-Y/Sp1 or Sp3 complex (figure 4.7, lanes 4, 5, 9, 10, 15 and 20). The same complex was observed with other EMSAs (data not shown), and an absence from others could be due to the clarity of the gel photo, or disruption of the complex during electrophoresis. The presence of the lower mobility band correlated with a higher protein concentration (3.8 μg/μL and 4.12 μg/μL) and the second buffer.

4.3 Chapter summary.

Three distinct protein complexes were shown to form with a length of DNA sequence encompassing both the GC and ICB2 elements of the topoisomerase IIβ promoter, and antibody supershifts allowed the positive identification of transcription factors Sp1, Sp3 and NF-Y within these complexes.

Sp1 and Sp3 appear to preferentially bind to GC. NF-Y was shown to bind to ICB1 and ICB2 exhibiting strong sequence specificity. The presence of NF-Y at ICB1/2 may allow Sp1 to be recruited to GC or to a GC element containing a mutation. The presence of an intact GC and ICB2 element facilitates a strong interaction between Sp1 and NF-Y, respectively, bound at these elements. A fourth complex may also form at the GC/ICB2 elements consisting of NF-Y/Sp1/Sp3, where NF-Y binds first and enables binding of the other transcription factors.

The binding of Sp3 to the GC box was highly variable, such that no definite conclusions could be made about the functional significance of this protein.

The functional significance of NF-Y, Sp1 and Sp3 binding to ICB1, ICB2 and GC elements in topoisomerase IIβ expression was addressed by carrying out transient transfection experiments using reporter gene assays, as described in chapter 5.

Chapter Five: Transient Transfections.

5.1 Introduction.

Transfection is a commonly used technique to transfer an altered gene or DNA of interest into mammalian cells, in order to study gene regulation within the intact cellular environment.

Stable transfections involve the integration of transfected DNA into the chromosomal DNA. In contrast, transient transfections do not involve DNA integration, but rather the introduced DNA persists in the nucleus for several days before being degraded. Within the cell a plasmid carrying a cloned promoter is subject to many of the regulatory mechanisms that control the expression of endogenous genes. Assays to detect reporter gene activity are performed on cell extracts 12-72 hours after transfection, before degradation of the introduced DNA occurs. The level of transient expression of the reporter gene within the transfected cells is dependent on the number of cells that take up the vector and the efficiency of expression within each cell. Four techniques are commonly used to perform transfections: lipofection, calcium phosphate precipitation, electroporation and DEAE-dextran-mediated-transfections.

Both the calcium phosphate and DEAE-dextran-induced transfections, utilise a chemical environment for the DNA and the subsequent endocytotic uptake by unknown mechanisms. For electroporation, cells in a solution containing DNA are subjected to a high voltage pulse that causes the opening of transient pores in their membranes. DNA can then enter through the holes directly into the cytoplasm without the aid of endocytotic vesicles, which can sometimes damage the DNA. DNA can also be incorporated into artificial lipid vesicles (liposomes) which fuse with the cell membrane, releasing their contents directly into the cytoplasm. Lipids are small, positively charged and unilamellar (single bilayer) vesicles, which spontaneously form cationic-DNA complexes, when mixed with DNA in solution. In this way lipofection enhances the DNA-cell surface interactions, as does the calcium phosphate and DEAE-dextran-induced methods. Liposome-mediated transfection was used in this study as it has a higher efficiency and greater reproducibility than the other

methods, while only requiring a small amount of DNA to obtain high transfection efficiency.

One commonly used reporter is the firefly luciferase (Photinus pyralis) gene, which provides a quick sensitive assay and requires neither radioactivity nor specific antibodies, as do other reporters. The sensitivity of the luciferase reporter gene allows weak promoters and poorly transfecting cells to be investigated (Ausubel *et al.*, 1991). Smaller amounts of plasmid can also be successfully used (Alam and Cook, 1990), which avoids inconsistancies which can arise from the use of large amounts of plasmid, such as competition between a control plasmid and the promoter construct under investigation, for limited amounts of transcription factors.

The minimal promoter (-569 bp upstream of 5'-transcriptional start site) for topoisomerase IIβ expression had already been defined and previously cloned into a pGL3Basic luciferase vector (Lok *et al.*, 2002). These authors also successfully monitored the transient expression of luciferase under the control of the topoisomerase IIβ promoter (pGL3B-1067 topoisomerase IIβ) in HeLa cells, therefore a similar approach was used in this study. An additional 290 bp (-1357 bp) upstream of the 5'-transcriptional start site was cloned, and the functional significance of this region of the topoisomerase IIβ promoter was investigated *in vivo* using functional assays.

To identify important regulatory elements within the cloned region of the topoisomerase IIβ promoter, the effect of 5'-serial and internal deletions on the ability of topoisomerase IIβ promoter to drive luciferase expression, was also investigated *in vivo*. The significance of several regulatory elements in the topoisomerase IIβ promoter and the functional relevance of putative transcription factors that bind them were investigated using constructs containing mutations in either ICB1 or ICB2 (refer to figure 1.2, chapter 1) in functional assays. The ability of the 180 bp region (-654 to -474) containing the putative ICB1, ICB2 and GC elements to drive expression of a SV40 promoter (without the surrounding sequence) was also investigated using the pGL3BP-180 construct in functional assays.

5.1.1 Luciferase Assay.

The Promega luciferase assay system was chosen as a reporter in this research, as it provides several advantages over other available reporter gene systems. The pGL3Basic reporter vector contains a modified firefly luciferase cDNA and a redesigned vector backbone, which increase luciferase expression and improve *in vivo* vector stability. Luciferase assays are rapid and sensitive, with the reaction catalysed by the firefly luciferase protein synthesised in the transfected cultured cells. The firefly luciferase protein when supplied with substrate can emit light (562 nm), in the presence of ATP:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow[\textbf{Luciferase}]{Mg^{2+}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

The light emitted can be detected as a direct measurement of the amount of luciferase activity, which reflects the activity of the promoter region inserted in front of the luciferase reporter gene. The Promega luciferase assay system produces a greater enzymatic turnover of the luciferase, resulting in an increased light intensity. The sensitivity of the system theoretically allows the detection of less than 10^{-20} mole of luciferase (Promega Technical Manual).

Light emittence was measured using a FLUOstar galaxy (BMG labtechnologies, Germany) detection system. Light emitted is measured by detecting photons released and relaying the actual photon counts through Excel™ (Microsoft Office, 97) for analysis. A photon count was taken every second over three minutes, following injection of the luciferase reagent. Generally, the addition of luciferase to substrate first results in a flash of light proportional to the quantity of enzyme present, followed by a rapid decay which gives an extended period of low-intensity light emission. For this reason, the maximum luciferase reading recorded was used for subsequent calculations.

5.1.2 β-Galactosidase assays.

All transient transfections included the co-transfection of a vector expressing bacterial β-Galactosidase, pCMVSPORT-β-Gal (Invitrogen), along with the luciferase reporter vectors. β-Galactosidase provides an internal standard by which luciferase activity can

be normalised for variations in cell number, extract preparation and transfection efficiency (Alam and Cook, 1990). pCMVSPORT-β-Gal vector is driven by the human cytomegalovirus (CMV) promoter and was chosen as it lacks binding sites for putative transcription factors involved in the regulation of the topoisomerase IIβ promoter. This is important for comparison of transcriptional regulation between different promoter constructs, to avoid expression being influenced by endogenous transcription factors interacting with the control vector.

β-Galactosidase catalyses the hydrolysis of various β-Galactosides, such as lactose, and also the catalysis of synthetic substrate, ONPG (o-nitrophenyl-β-D-galactopyranoside). Hydrolysis of ONPG produces a visible yellow product, o-nitrophenol, which can be measured at 405 nm.

Reaction catalysed by β-Galactosidase:

$$\text{0-Nitrophenyl-β-Galactoside} + H_2O \xrightarrow{\textbf{β-Galactosidase}} \text{o-Nitrophenol} + \text{Galactose}$$

The exogenous amounts of β-Galactosidase present in the transfected cells were determined by measuring the amount of ONPG hydrolysis. Cell extracts were assayed for both luciferase and β-Galactosidase activity, allowing the normalisation of luciferase values. To limit the effect of endogenous β-Galactosidase the assays were performed at pH 8, a pH which is suboptimal for endogenous β-Galactosidase while having minimal effect on bacterial β-Galactosidase produced from the transfected plasmid (Bronstein *et al.*, 1994).

5.1.3 Analysis of Data.

Variability of luciferase assays can be the result of differential degradation due to rapid decay of the luciferase enzyme, which has a half-life of only approximately 3 hours in transfected mammalian cells (Thompson *et al.*, 1991). For this reason each transfection was carried out in triplicate and repeated at least three times to increase accuracy. HeLa cells were cotransfected with appropriate vectors as described in section 2.2.21. Luciferase and β-Galactosidase assays were performed on all cell extracts, as described in sections 2.2.23 and 2.2.24. Luciferase and β-Galactosidase

activities were corrected by subtracting the value of an appropriate blank sample, to correct for any background signals. Luciferase activities were normalised against β-Galactosidase activities by dividing the luciferase value by the β-Galactosidase value for each cell extract.

Normalised luciferase activity = Luc activity/ β-Gal activity

Figure 5.1: Normalisation of luciferase activity.

Luciferase activities were normalised against β-Gal activities as an internal control to correct for any variations in expression within the HeLa cells.

Constructs	Maximum luciferase Values	Corrected maximum luciferase values	β-Gal values	Corrected β-Gal values	Luc/β-Gal ratio	Average Luc/β-Gal ratio	Luciferase activity relative to wt %
wt-1357	7735 7698 7717	7693 7656 7675	0.763 0.760 0.745	0.691 0.688 0.673	11133.14 11127.91 11404.16	11221.74	100
Blank	42	-	0.072	-	-	-	-
wt-1249	51383 44288 45144	51316 44221 45077	0.872 0.801 0.813	0.800 0.729 0.741	64145.00 60659.81 60832.66	61879.16	439
Blank	67	-	0.072	-	-	-	-

Table 5.1: Calculations involved in analysis of one triplicate set of HeLa cell extracts.

Representative results from a transient transfection experiment. For each construct, transfections were carried out in triplicate and analysed as shown above, and each triplicate set of transfections was repeated in at least three independent experiments.

To enable comparison between independent experiments, the results were expressed relative to the pGL3Basic topoisomerase IIβ -1357wt (pGL3B-TIIβ-1357wt) vector in each experiment, with the wild type activity arbitrarily set at 100%. The normalised luciferase activities for all constructs were expressed as a percentage of the wild type by dividing by the average normalised pGL3B-TIIβ-1357wt luciferase values. In this way any deviation from the wild type activity was shown as an increase or decrease relative to wild type. In all cases the average of no less than three normalised luciferase activities from independent transfections were used.

A certain amount of experimental error is unavoidable and for this reason the average deviation was calculated between triplicates samples, and between independent experiments. The average deviation is a measure of the variability within a small data set and is calculated using the following formula:

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

Where n = the number of observations
X = the individual observation
$\overline{x}$ = the average/mean

Figure 5.2: Formula used to calculate the average deviation from the mean.
The calculations for the average deviation were performed using Excel (97, Microsoft); this function returns the average of the absolute deviations of data points from their mean, and therefore shows fluctuations between data sets.

Example average deviation calculation:
((11133.14 - 11221.74) + (11127.91 - 11221.74) + (11404.16 - 11221.74)) x (1/3) = 121.62 luciferase units
Percentage error calculation: (AVEDEV/AVE) x 100
(121.62 ÷ 11221.74) x 100 = 1.08% error within wt triplicates.

The statistical significance of observed differences between two data sets was determined using the statistical *t*-test. The *t*-test overcomes problems commonly encountered when comparisons are being made between small data sets by calculating

a t-distribution, rather than a normal distribution, which is more appropriate for larger data sets.

$$t = \frac{Mean_{grp1} - Mean_{grp2}}{\sqrt{S^2\left[\frac{1}{n_{grp1}} + \frac{1}{n_{grp2}}\right]}}$$

Where the pooled variance (s^2) is estimated by:

$$S^2 = \frac{(n_{grp1} - 1)\, s^2_{grp1} + (n_{grp2} - 1)\, s^2_{grp2}}{N_{grp1} + n_{grp2} - 2}$$

Where n = number of data points within a population
S^2 = variance of a population

Figure 5.3: Formula for 2-sample *t*-test assuming equal variances.

This type of t-test is used to determine whether 2 samples are likely to have come from the same two underlying populations that have the same mean, and was carried out using Excel (97, Microsoft).

The t-test generates a p-value (the probability associated with the t-test), which provides evidence of differences between data sets. If the p-value generated is greater than 0.1 there is at least a 10% chance that the two samples share the same mean, which is observed for similar data sets. When data sets exhibit larger variations and a p-value less than 0.05 is generated, the data sets are statistically significantly different, having a less than 5% chance of being similar.

p-value	Evidence for significant difference between two samples	Difference
$p > 0.10$	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: Strength of evidence provided by the p-values.

The probability associated with the t-test is represented by the p-value, which provides varying degrees of evidence for an observed variation.

5.2 Transient transfections using HeLa cells.

Transfections were carried out as described in section 2.2.21, using the lipofection agent FuGENE™6 (Roche). FuGENE™6 is a unique blend of lipids and additional components which allow high efficiency transfections (Technical manual, Promega). Optimal performance of FuGENE™6 requires cells to be between 50-80% confluent at the time of transfection. The transient reporter gene activity is detected 12-72 hours after transfection for the rapid testing of putative regulatory sequences.

The optimal amount of vector for successful and reproducible transfections was first established and then used for subsequent transfection of HeLa cells with the various reporter gene constructs.

5.2.1 Dosage dependent expression of pGL3B-TIIβ-1357wt .

HeLa cells were transfected with varying amounts of the pGL3B-TIIβ-1357wt vector in order to establish the amount of vector to be used for further experiments (Figure 5.4). As a maximum of 4 μg of DNA is recommended for optimal transfections, a range of 1.5-2.5 μg of plasmid DNA was tested, and proved to be sufficient for successful and reproducible transfections. To minimise cost, without effecting experimental efficiency, 1.0 μg was chosen as an optimal amount of total DNA (pGL3B-TIIβ-1357wt vector + control vectors).

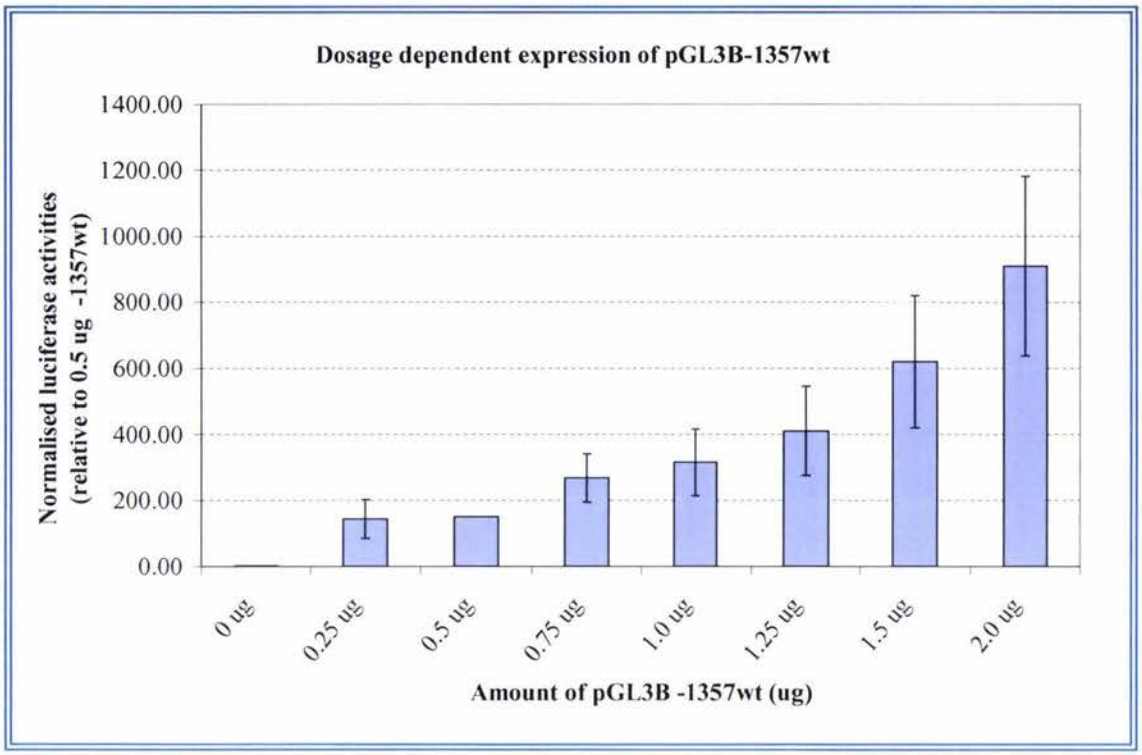
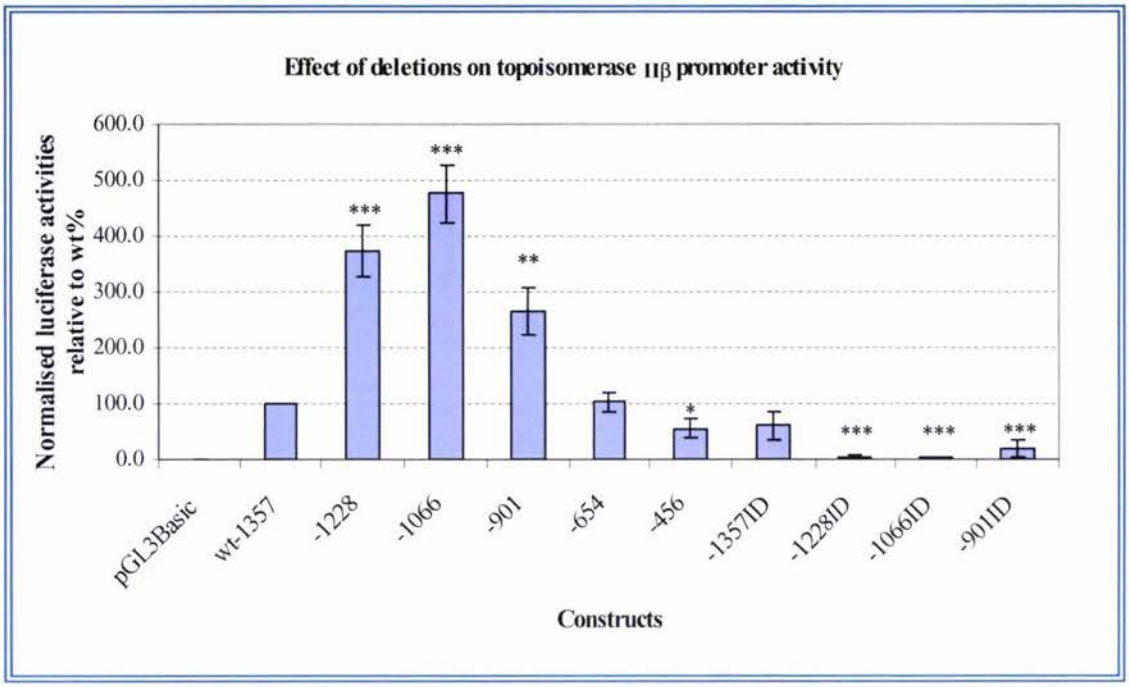


Figure 5.4: Dosage dependent expression of pGL3B-TIIβ-1357wt.

HeLa cells were transiently transfected with a range of 0.25-2 μg pGL3B-TIIβ-1357wt vector plus pGL3Basic control vector up to a total of 2 μg, and including 0.5 μg pCMVSPORT-β-Gal control vector. The normalised luciferase values represent the ratio of luciferase activity to β-Galactosidase activity, as described earlier. The values presented are the average of three independent experiments (see Appendix 5, experiments 1-3) carried out in triplicate. The normalised luciferase activity of 0.5 μg pGL3B-TIIβ-1357wt was chosen as 100% and used to calculate relative values for all other activities.

5.2.2 Expression of topoisomerase IIβ promoter deletion constructs in HeLa cells.

HeLa cells were transfected when they were approximately 70-80% confluent and harvested 40-46 hours after transfection as described in section 2.2.22. Luciferase and β-Galactosidase activities were determined immediately after harvesting the cell extracts (sections 2.2.23 and 2.2.24, respectively); figure 5.5 illustrates the results of these transfections.



Construct Tested	Normalised luciferase activities relative to wt%	p-values	Significant Difference
pGL3Basic	-0.1±0.3%	-	-
wt-1357	100%	-	-
-1228	373.1±45.1%	0.00001	Yes***
-1066	475.4±50.7%	0.0007	Yes***
-901	264.6±42.6%	0.001	Yes**
-654	102.9±17.2%	0.25	No
-456	54.9±17.9%	0.04	Yes*
-1357ID	60.3±24.7%	0.06	No
-1228ID	5.34±3.0%	8.7×10^{-12}	Yes***
-1066ID	2.0±0.9%	1.55×10^{-8}	Yes***
-901ID	20.5±16.0%	1.35×10^{-5}	Yes***

Figure 5.5: The effect of deletions on topoisomerase IIβ promoter activity. Changes in reporter gene expression were observed with the different 5'-serial and internal deletions in comparison to the pGL3B-TIIβ-1357wt expression. HeLa cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA and harvested 42 hours later. The normalised luciferase activities are shown relative to wild type activity, with each construct tested in a minimum of three experiments out of a set of six experiments, each done in triplicate (see Appendix 5, experiments 4-9).

The significance of changes in expression observed, between each construct and the wild type, was calculated using a *t*-test to yield p-values as shown above. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

Figure 5.5 demonstrates that expression of the reporter gene driven by topoisomerase IIβ promoter varies depending on the amount and position of promoter sequence. A significant increase in promoter activity (approximately 373%) is observed with a deletion between -1357 and -1228, suggesting the presence of a repressor element within this region. A further increase (approximately 475% of wild type activity) was observed with the removal of an additional 238bp (-1228 to -1066), suggesting a second important region in the negative regulation of the topoisomerase IIβ promoter. The trend changes with the -901 construct (removing -1357 to -901), which shows a level of activity reduced to nearly 50% of the -1066 construct activity, while still showing an increase (approximately 264%) over wild type expression. This result suggests the presence of an activator element between -1066 and -901. From -901 to -654 a second large decrease in activity (approximately 62%) is observed, bringing the expression level back down to approximately wild type level, suggesting a second important region for the up-regulation of the topoisomerase IIβ promoter. A significant decrease from wild type activity (approximately 55%) was observed with a further deletion of the 198 bp region (-654 to -456), which contains the putative ICB and GC elements, suggesting that this deleted region is sufficient to support 45% of wild type activity.

The -1357wt internal deletion construct (minus putative ICB1, ICB2 and GC elements) showed a decrease, to approximately 60% of wild type activity. This finding is consistent with the expression levels observed for the -456 construct, which also lacks the putative ICB and GC elements. The same internal deletion in the -1228 and -1066 constructs resulted in an almost complete loss of activity (5% and 2%, respectively, of wild type activity), suggesting the removal of important activator elements. The -901ID construct resulted in a decrease to approximately 20% of wild type activity, showing an unexpected difference to the previous two internal deletion constructs.

5.3 Transient transfections using MDA-MB-231 cells.

To provide a comparison of expression levels in a different cancer cell line, the deletion constructs were also tested in MDA-MB-231 cells, which are human breast cancer cells. This allows an insight into whether variations in regulation of gene expression may exist within different cells. The transient transfections were carried out under the same conditions, and following the same protocol, as used for the transient transfections of HeLa cells.

5.3.1 Dosage dependent expression of pGL3B-TIIβ-1357wt in MDA-MB-231 cells.

As the optimal amount of transfected DNA could vary between cell lines, the dosage dependent expression of pGL3B-TIIβ-1357wt in MDA-MB-231 cells was also examined. MDA-MB-231 cells were transfected with varying amounts of the pGL3B-TIIβ-1357wt vector, in order to establish the amount of vector to be used for further experiments (Figure 5.6). A range of 1.5-2.5 μg of plasmid DNA was tested and proved to be sufficient for successful and reproducible transfections. Comparable results were observed, indicating no significant difference in expression between the two cell lines. For consistency between experiments, 1.0 μg was also chosen as an optimal amount of total DNA (pGL3B-TIIβ-1357wt vector + control vectors) for use in MDA-MB-231 transient transfections.

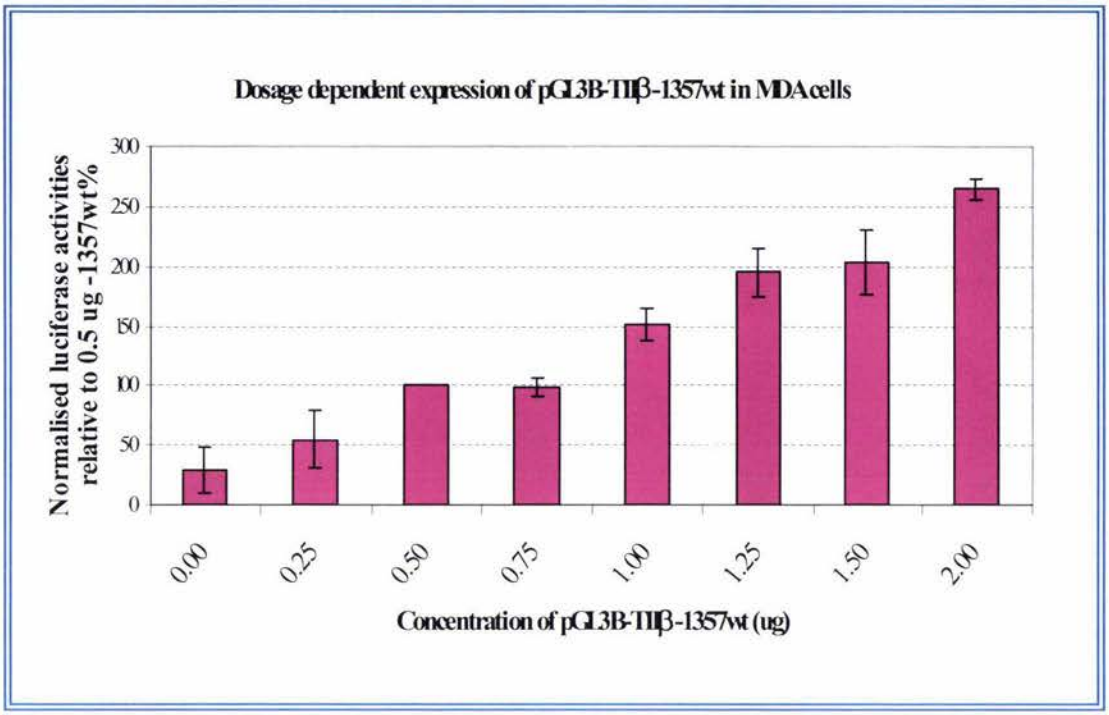
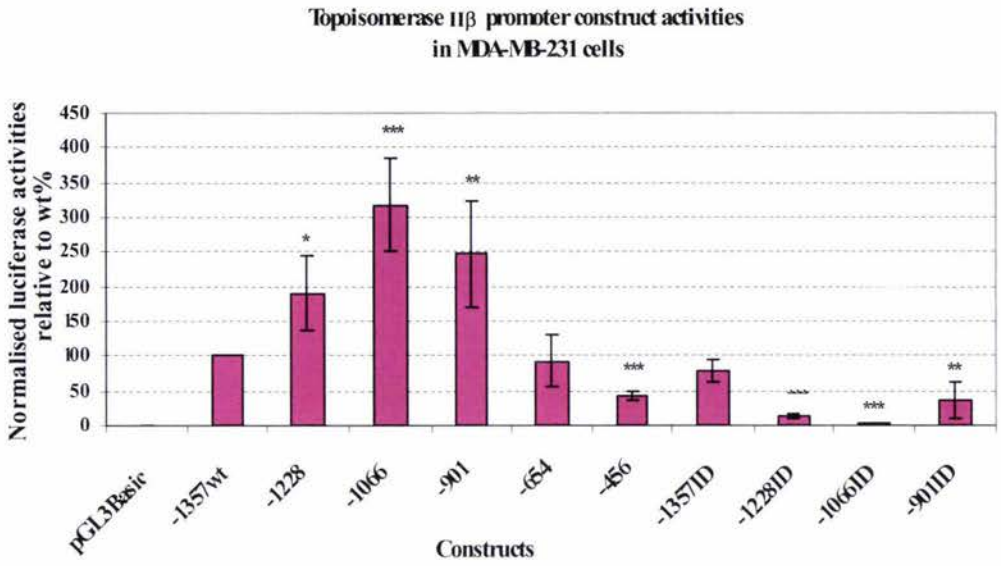


Figure 5.6: Dosage dependent expression of pGL3B-TIIβ-1357wt in MDA-MB-231 cells.

MDA-MB-231 cells were transiently transfected with a range of 0.25-2 μg pGL3B-TIIβ-1357wt vector plus pGL3Basic control vector up to a total of 2 μg, and including 0.5 μg pCMVSPORT-β-Gal control vector. The normalised luciferase values represent the ratio of luciferase activity to β-Galactosidase activity, as described earlier. The values presented are the average of three independent experiments (see Appendix 5, transfections 10-12) carried out in triplicate. The normalised luciferase activity of 0.5 μg pGL3B-TIIβ-1357wt was chosen as 100% and used to calculate relative values for all other activities.

5.3.2 Expression of topoisomerase IIβ promoter deletions constructs in MDA-MB-231 cells.

MDA-MB-231 cells were transfected when they were approximately 70-80% confluent and harvested 40-46 hours after transfection as described in section 2.2.22. Luciferase and β-Galactosidase activities were determined immediately after harvesting the cell extracts (sections 2.2.23 and 2.2.24, respectively); figure 5.7 illustrates the results of these transfections.



Construct Tested	Normalised luciferase activities relative to wt%	p-values	Significant Difference
pGL3Basic	0.7±0.68%	-	-
wt-1357	100%	-	-
-1228	192.8±55.4%	0.03	Yes*
-1066	317.5±66.3%	4.69×10^{-5}	Yes***
-901	246.8±76.0%	0.002	Yes**
-654	92.7±36.7%	0.78	No
-456	42.3±5.3%	3.26×10^{-6}	Yes***
-1357ID	78.0±15.2%	0.08	No
-1228ID	12.9±4.6%	2.14×10^{-12}	Yes***
-1066ID	4.2±0.7%	6.45×10^{-9}	Yes***
-901ID	34.7±26.2%	0.005	Yes**

Figure 5.7: Effect of deletions on topoisomerase IIβ promoter activity in MDA-MB-231 cells.

Transient transfection of topoisomerase IIβ deletion constructs into MDA-MB-231 cells resulted in the same trends in reporter gene expression as observed in HeLa cells (See Figure 5.5 for comparison). MDA-MB-231 cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA and harvested 42 hours later. The normalised luciferase activities are shown relative to wild type activity, with each construct tested in a minimum of three experiments, out of a set of 6 experiments, each done in triplicate (see Appendix 5, experiments 13-18).

The significance of changes in expression observed, between each deletion construct and the wild type was calculated using a test-test, to yield p-values as shown above. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

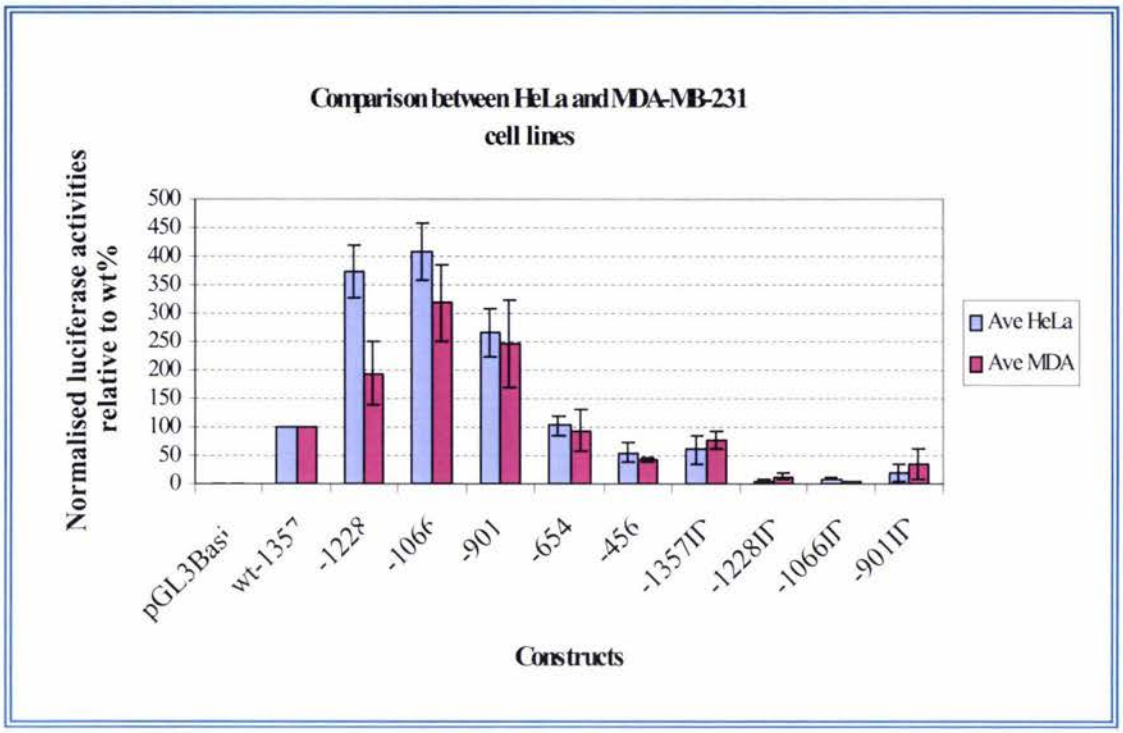


Figure 5.8: Comparing the levels of deletion construct expression between HeLa and MDA-MB-231 cell lines.

Expression levels seen in MDA-MB-231 cells are not significantly different than those observed in HeLa cells, with the same overall trends present in both cell lines, suggesting no significant difference in regulation of expression between the two cell lines.

Overall these results demonstrate that only four of the nine constructs tested produced a significant decrease in expression of the reporter gene, relative to the wild type. Each construct that displayed a decrease contained a deletion, which removed the ICB1, ICB2 and GC elements (-654 to -456), indicating the importance of these elements for basal transcription. In addition, important repressor elements may be present within the region between -1357 and -1066 bp, upstream of the transcription start site, while the region between -1066 and -654 may contain elements important for the up-regulation of the topoisomerase IIβ promoter.

5.4 Transient co-transfections.

The results obtained from EMSA, as described in chapter 4, demonstrate that ICB1 and ICB2 have the ability to bind Sp1 and Sp3, as well as NF-Y. Previous work showed that Sp1 specifically binds the topoisomerase IIβ GC element (Lok *et al.*,

2002). However, this study did not investigate the ability of Sp1 to bind ICB elements, or whether Sp3 was also able to bind either the GC or ICB elements, in the topoisomerase IIβ promoter.

Sp1 and Sp3 have been shown to play an important role in the transcriptional regulation of the topoisomerase IIα promoter, mediated through the binding of GC1 and GC2 elements (Kubo *et al.*, 1995; Szremska, 2001; Magan *et al.*, 2003). It is likely that Sp1 and Sp3 are also important factors in topoisomerase IIβ transcriptional regulation. In order to investigate the roles of the transcription factors on topoisomerase IIβ transcription *in vivo*, Sp1 and Sp3 expression vectors were used in co-transfection experiments with the topoisomerase IIβ reporter constructs.

Co-transfection experiments involve the simultaneous introduction of the reporter plasmid construct along with an expression plasmid (Sp1 or Sp3) driven by a viral promoter. The expression vector enables Sp1 or Sp3 to be over-expressed in HeLa cells, where interaction with regions of the topoisomerase IIβ promoter can occur, thereby influencing luciferase expression (Carey and Smale, 2000).

5.4.1 Addition of Sp1 to topoisomerase IIβ ICBmt promoter constructs.

To investigate the effects of transcription factor Sp1 on topoisomerase IIβ expression *in vivo*, co-transfection experiments were carried out using an expression vector that allows the over-expression of Sp1. The presence of excess Sp1 may result in changes in expression, which occur due to the interaction of the transcription factor with the topoisomerase IIβ promoter. In these experiments the change in expression is reflected by a change in relative luciferase activity driven by the topoisomerase IIβ constructs.

5.4.2 Sp1 titration.

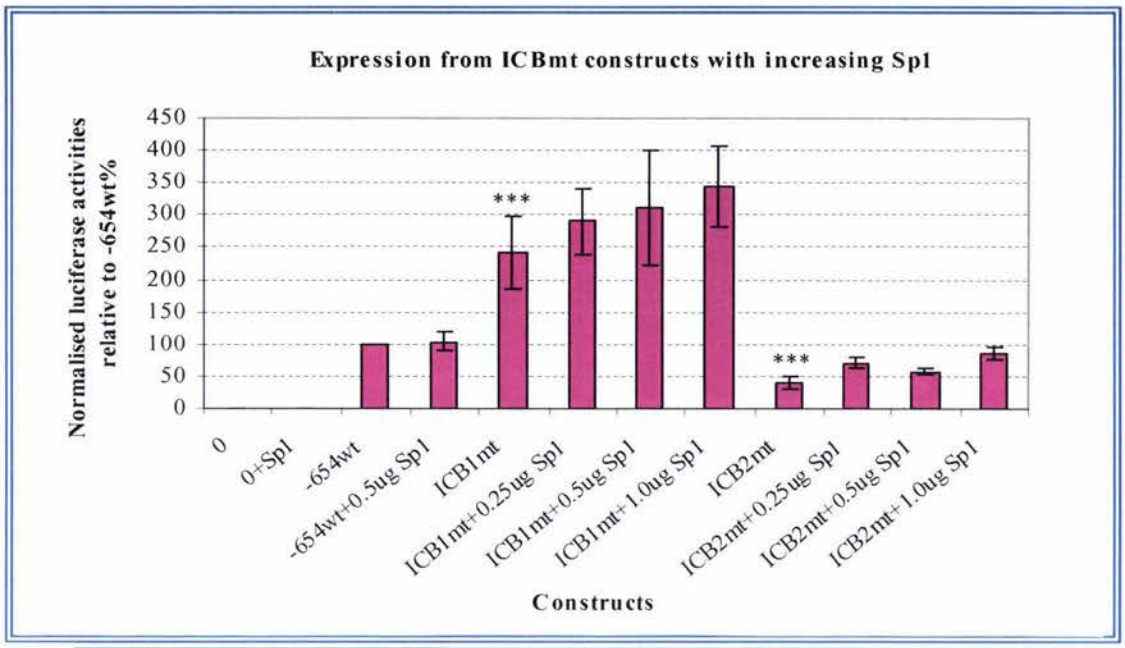
Varying amounts of the Sp1 expression vector were added to transient transfections in order to determine the optimal amount of Sp1 plasmid to use in these experiments. As the mutated constructs were produced from the -654 deletion construct (minimal promoter region), the -654wt construct was tested along with the ICB1mt and ICB2mt constructs. The -654wt construct has the same relative transcriptional activity as that

of the -1357wt, but lacks the putative positive regulatory elements between -1357 and -654.

Figure 5.9 illustrates that the addition of Sp1 has varying effects on luciferase activity, and suggests that Sp1 may have the ability to activate the topoisomerase IIβ promoter in ICBmt constructs, while having no effect on -654wt construct activity.

Expression levels of the -654wt construct did not appreciably change in response to the addition of increasing amounts of Sp1, suggesting that Sp1 was not capable of activating the topoisomerase IIβ promoter within this construct. This finding could suggest the involvement of upstream elements in the Sp1 mediated activation of the topoisomerase IIβ promoter. In contrast however, the addition of Sp1 expression vector to ICB1 and ICB2 mutant constructs resulted in an increase in luciferase activity (approximately 30% and 125%, respectively), with a more significant increase observed in ICB2mt construct activity. This result suggests that the presence of two ICB elements is not essential for the regulation of the topoisomerase IIβ promoter, and that ICB1 and ICB2 are probably not the target elements for Sp1-mediated up-regulation within the promoter. The lack of Sp1 mediated activation of the topoisomerase IIβ promoter in the -654wt construct could indicate an inhibitory effect on Sp1 activation in the presence of both functional ICB elements.

In addition, a mutation in ICB1 appears to result in an increased topoisomerase IIβ activity, in comparison to the -654 wild type activity. A *t*-test to compare the two generated a p-value of 0.0005, which provides very strong evidence (table 5.2) for a significant difference between expression levels of the two constructs. This result indicates that ICB1 could be playing a repressive role in regulation of topoisomerase IIβ promoter activity. The opposite was seen with a mutation in ICB2, which resulted in a decrease in topoisomerase IIβ activity in comparison to the -654 wild type. A comparison between the activities of these two constructs also indicated very strong evidence for a significant difference (p-value = 4.16×10^{-7}). Therefore, ICB2 appears to be necessary to maintain wild type topoisomerase IIβ promoter strength.



Construct Tested	Normalised luciferase activities relative to -654%	p-values	Significant Difference
pGL3Basic	0.7±0.2%	-	-
pGL3Basic + 0.5 μg Sp1	0.6±0.4%	-	-
-654wt	100%	-	-
-654wt + 0.50 μg Sp1	104.1±13.8%	-	-
ICB1mt	243.05±56.37%	0.0005	Yes***
ICB1mt + 0.25 μg Sp1	290.0±51.8%	-	-
ICB1mt + 0.50 μg Sp1	310.5±88.7%	-	-
ICB1mt + 1.00 μg Sp1	343.7±63.2%	-	-
ICB2mt	39.6±9.7%	4.16×10^{-7}	Yes***
ICB2mt + 0.25 μg Sp1	70.3±8.4%	-	-
ICB2mt + 0.50 μg Sp1	57.0±4.6%	-	-
ICB2mt + 1.00 μg Sp1	85.3±9.8%	-	-

Figure 5.9: Sp1 titrations.

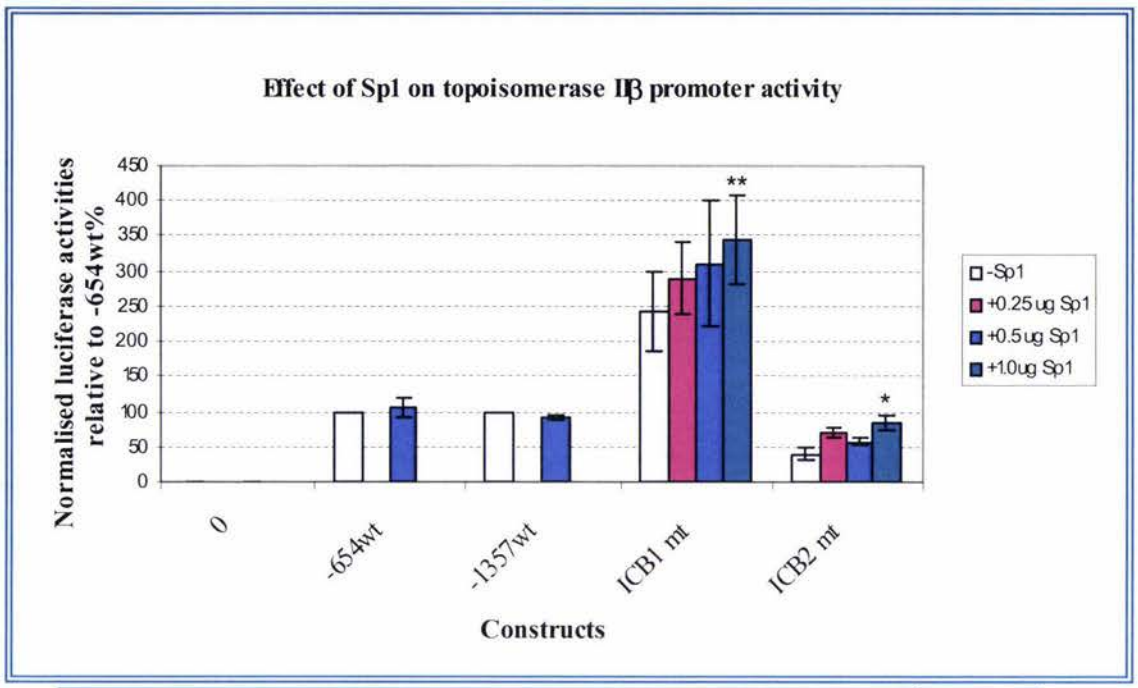
Reporter vector expression changes depending on the amount of Sp1 expression vector added to the HeLa cells along with the deletion constructs. HeLa cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA, with varying amounts (0-1.0 μg) of Sp1 expression plasmid, and harvested 42 hours later. The normalised luciferase activities are shown relative to the -654wt construct activity, the deletion construct from which the ICBmt constructs were created. Each construct was tested in a minimum of three experiments, each done in triplicate (see Appendix 5, experiments 19-23 and 26).

The significance of changes in expression observed, between each deletion construct and the wild type was calculated using a test-test, to yield p-values as shown above. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

To determine whether the changes in expression observed with the addition of Sp1 were of statistical significance, *t*-tests were carried out for each construct to generate p-values as shown in figures 5.10. In addition, to investigate the requirement of elements upstream of the -654 sequence, Sp1 was co-transfected with the -1357wt construct. As Sp1-mediated activation of ICBmt promoter constructs was observed over a range of Sp1 concentrations, with no significant deviation in error, a concentration of 0.5 μg of Sp1 was chosen for testing the effect of Sp1 on the expression from the -1357wt construct. Figure 5.10 illustrates the result of these transfection experiments in relation to the data already presented in figure 5.9.

No significant difference in luciferase activity was observed with the addition of Sp1, with the exception of the ICBmt constructs, which showed only a small increase. This suggests that Sp1 does not usually have the ability to regulate the regions of topoisomerase IIβ promoter within these constructs.

Comparing the p-values, with the exception of the ICB1mt and ICB2mt constructs, Sp1 does not show any significant effect on the topoisomerase IIβ promoter activity. For the ICB1mt construct, p-values indicate strong evidence (table 5.2) for a significant increase in activity with 1.0 μg of Sp1, and for the ICB2mt construct, p-values indicate slight evidence for a significant increase in activity with 0.25μg of Sp1 and moderate evidence for a significant increase in activity with 1.0 μg of Sp1. These results suggest minimal influence of Sp1 on topoisomerase IIβ transcriptional regulation. The ability of Sp1 to influence topoisomerase IIβ activity in the absence of two functional ICB elements, could suggest an inhibitory effect occurring in the presence of both elements, which affects Sp1 activity.



Construct Tested	Normalised luciferase activities relative to -654wt	p-values	Significant Difference
pGL3Basic	0.7±0.2%	-	-
pGL3Basic + 0.5 μg Sp1	0.6±0.4%	0.74	No
-654wt	100%	-	-
-654wt + 0.5 μg Sp1	104.13±13.78%	0.72	No
-1357wt	100%	-	-
-1357wt + 0.5 μg Sp1	92.33±3.89	0.06	No
ICB1mt	243.05±56.37%	-	-
ICB1mt + 0.25 μg Sp1	290.0±51.8%	0.10	No
ICB1mt + 0.50 μg Sp1	310.5±88.7%	0.10	No
ICB1mt + 1.00 μg Sp1	343.7±63.2%	0.01	Yes**
ICB2mt	39.6±9.7%	-	-
ICB2mt + 0.25 μg Sp1	70.3±8.4%	0.07	No
ICB2mt + 0.50 μg Sp1	57.0±4.6%	0.28	No
ICB2mt + 1.00 μg Sp1	85.3±9.8%	0.045	Yes*

Figure 5.10: Effect of Sp1 on topoisomerase IIβ promoter activity relative to -654wt.

HeLa cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA, and 0.5 μg of Sp1 expression plasmid, and harvested 42 hours later. The normalised luciferase activities are shown relative to the -654 deletion construct activity, with the exception of the –1357wt construct. The results for the -1357wt construct plus Sp1 are from three experiments each done in triplicate (see Appendix 5, experiments 20, 22 and 23), and expressed relative to -1357wt. The remaining data is the same as illustrated in figure 5.9, with the difference being, the p-values represent the significance of differences for each construct, with and without Sp1 expression vector, rather than in relation to -654wt construct activity. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

5.4.3 Addition of Sp3 to topoisomerase IIβ ICBmt promoter constructs.

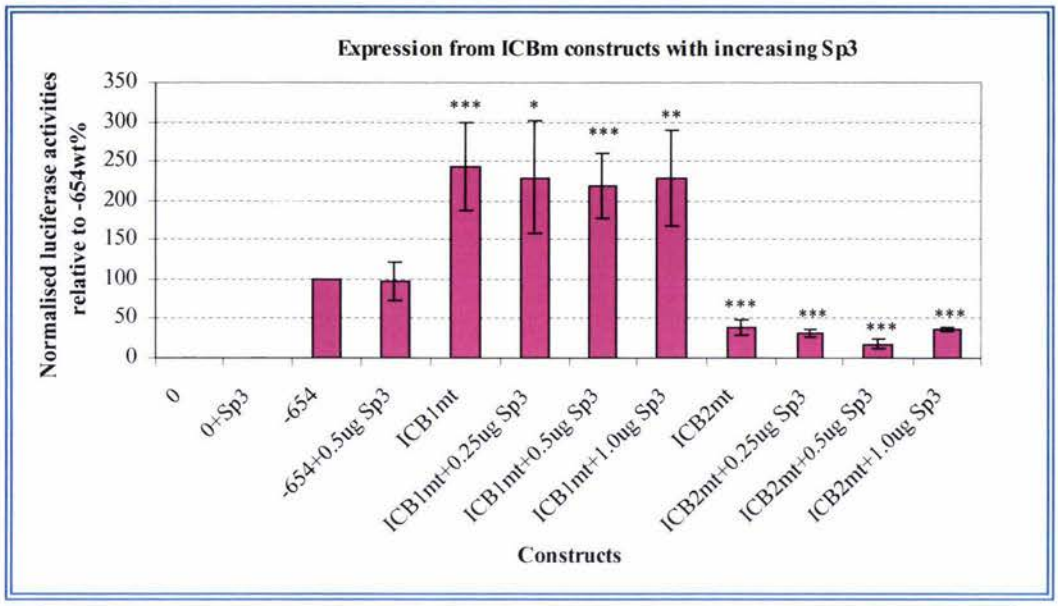
In chapter 4, it was shown that the transcription factor, Sp3, is able to bind GC and ICB elements of the topoisomerase IIβ promoter. In other studies Sp3 has been shown to bind GC2 (Szremska, 2000) and GC1 (Magan *et al.*, 2003) elements in the topoisomerase IIα promoter. The latter study also used co-transfections to show the Sp3 transcription factor is able to affect the expression levels of the topoisomerase IIα promoter. Therefore, the effect of this transcription factor on the expression of topoisomerase IIβ promoter constructs was also investigated.

5.4.4 Sp3 titration.

HeLa cells were co-transfected with a Sp3 expression vector to determine the *in vivo* effects of the Sp3 transcription factor on topoisomerase IIβ expression. Increasing amounts of Sp3 expression vector were added to transient transfection experiments and subsequent changes in luciferase activity were monitored. Constructs tested were the same as those used in previous Sp1 co-transfection experiments.

Figure 5.11 illustrates that generally the addition of Sp3 had no effect on luciferase activity, suggesting that Sp3 does not usually have the ability to regulate the -654wt topoisomerase IIβ promoter construct or constructs with a mutation in either ICB1 or ICB2.

One exception seen was a 40% decrease in expression of the ICB2mt construct relative to wild type activity, with the addition of 0.5 μg Sp3, which suggests that Sp3 is capable of down-regulating the topoisomerase IIβ promoter in the absence of a functional ICB2 element. This result indicates that the ICB1 could play a role in the Sp3-mediated regulation of topoisomerase IIβ promoter activity, while the ICB2 element is probably not a target element for the Sp3-mediated effect. The fact that a decrease was observed only with 0.5 μg of expression vector could be attributed to insufficient Sp3 with the addition of only 0.25 μg of expression vector, while the addition of 1.0 μg could have resulted in an inhibitory effect on Sp3 activity.



Construct Tested	Normalised luciferase activities relative to -654%	p-values	Significant Difference
pGL3Basic	0.63±0.22%	-	-
pGL3Basic + μg Sp3	0.32±0.25%	-	-
-654wt	100%	-	-
-654wt + 0.5 μg Sp3	98.0±24.0%	0.92	No
ICB1mt	243.1±56.4%	0.0005	Yes***
ICB1mt + 0.25 μg Sp3	229.1±72.2%	0.02	Yes*
ICB1mt + 0.50 μg Sp3	219.0±41.3%	0.001	Yes***
ICB1mt + 1.00 μg Sp3	228.9±61.4%	0.01	Yes**
ICB2mt	39.6±9.7%	4.16×10^{-7}	Yes***
ICB2mt + 0.25 μg Sp3	31.8±5.8%	3.52×10^{-6}	Yes***
ICB2mt + 0.50 μg Sp3	25.7±6.7%	1.62×10^{-6}	Yes***
ICB2mt + 1.00 μg Sp3	36.8±1.9%	1.09×10^{-8}	Yes***

Figure 5.11: Sp3 titrations.

Reporter vector expression generally shows no change in response to varying amounts of Sp3 expression vector added to the HeLa cells, along with the deletion constructs. HeLa cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA, with varying amounts (0-1.0 μg) of Sp3 expression plasmid, and harvested 42 hours later. The normalised luciferase activities are shown relative to the -654 deletion construct activity, the wild type construct from which the ICB mutant constructs were created. Each construct was tested in a minimum of three experiments, each done in triplicate (see Appendix 5, experiments 19, 22-26)).

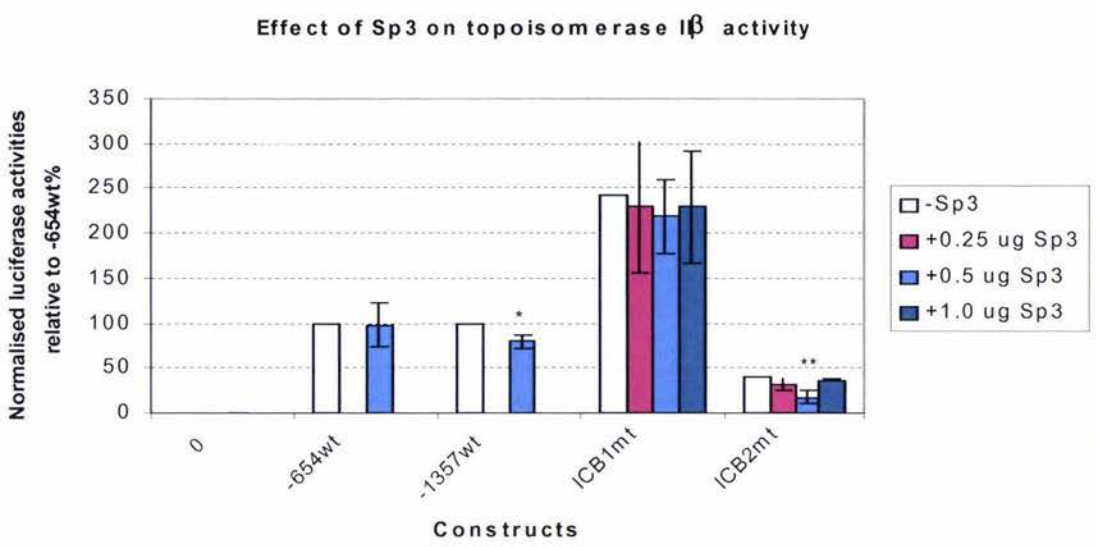
The significance of changes in expression observed, between each deletion construct and the wild type was calculated using a test-test, to yield p-values as shown above. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

The lack of Sp3 mediated activation of the topoisomerase IIβ promoter in the -654wt construct could support the idea of an inhibitory effect on Sp3 activation in the presence of a functional ICB2 element. Another possibility is the requirement of upstream elements for the Sp3-mediated down-regulation of the topoisomerase IIβ promoter in the presence of ICB2. To investigate whether the presence of elements upstream of the -654 sequence were able to influence the effect of Sp3 on the topoisomerase IIβ promoter, the -1357wt construct was co-transfected with the Sp3 expression vector. As the co-transfection of ICBmt constructs with varying amounts of Sp3 expression vector produced no significant deviation in error, a concentration of 0.5 μg of Sp3 was chosen for testing the effect of Sp3 on the expression from the -1357wt construct. Figure 5.12 illustrates the result of these transfection experiments in relation to the data already presented in figure 5.11.

To determine whether the changes in expression observed with the addition of Sp3 were of statistical significance, *t*-tests were carried out for each construct to generate p-values as shown in figures 5.12.

No significant difference in luciferase activity was seen with the addition of Sp3, with the exception of the -1357wt and ICB2mt constructs, which showed only a small decrease. This suggests that Sp3 does not usually have the ability to regulate the regions of topoisomerase IIβ promoter within these constructs.

Comparing p-values, moderate evidence (table 5.2) exists for a significant decrease in -1357wt construct activity, however no significant change is observed in expression levels of the -654wt construct. For the ICB2mt construct, p-values indicate moderate evidence for a significant increase in activity with 0.5 μg of Sp1 only. This result suggests upstream elements present in the -1357wt construct, could be involved in a Sp3-mediated down-regulation of the topoisomerase IIβ promoter, although the effect is minor. These findings suggest that while Sp3 does have some ability to influence on the topoisomerase IIβ promoter activity, it does not appear to play a significant role in terms of transcriptional regulation. It also appears that upstream elements, or the absence of the functional ICB2 element, are necessary for the Sp3-mediated down-regulation of topoisomerase IIβ expression.



Construct Tested	Normalised luciferase activities relative to -654%	p-values	Significant Difference
pGL3Basic	0.6±0.2%	-	-
pGL3Basic + 0.5 μg Sp3	0.2±0.1%	0.23	No
-654wt	100%	-	-
-654wt + 0.5 μg Sp3	98.0±24.0%	0.92	No
-1357wt	100%	-	-
-1357wt + 0.5 μg Sp3	79.1±7.7%	0.02	Yes*
ICB1mt	243.1±56.4%	-	-
ICB1mt + 0.25 μg Sp3	229.1±72.2%	0.52	No
ICB1mt + 0.50 μg Sp3	219.0±41.3%	0.67	No
ICB1mt + 1.00 μg Sp3	228.9±61.4%	0.50	No
ICB2mt	39.6±9.7%	-	-
ICB2mt + 0.25 μg Sp3	31.8±5.8%	0.23	No
ICB2mt + 0.50 μg Sp3	25.7±6.7%	0.008	Yes**
ICB2mt + 1.00 μg Sp3	36.8±1.9%	0.25	No

Figure 5.12: Effect of Sp3 on topoisomerase IIβ promoter activity relative to -654wt.

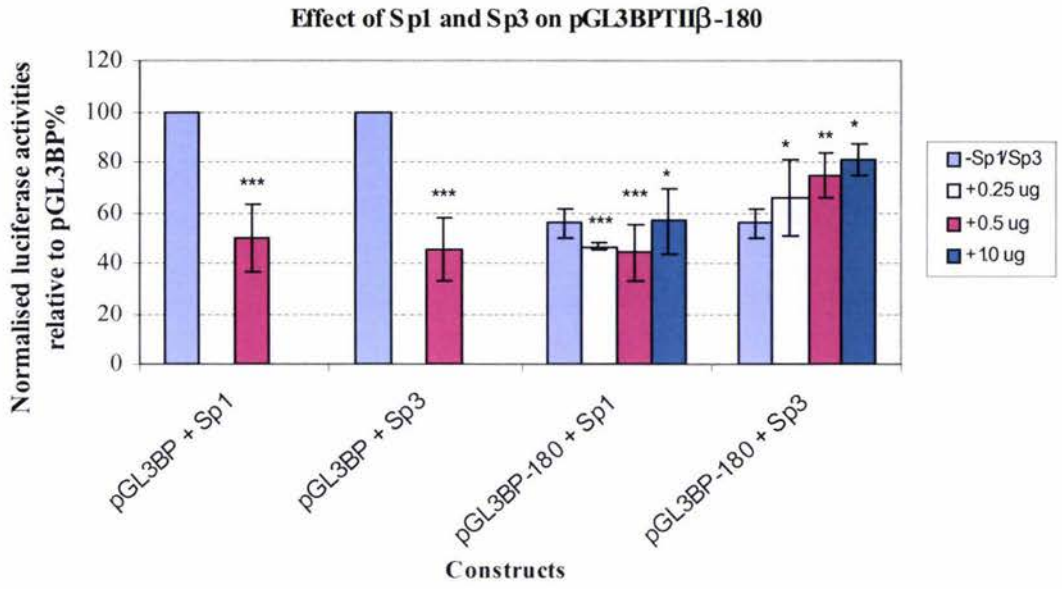
HeLa cells were transfected with 0.5 μg each of a pGL3Basic deletion construct and pCMVSPORT-β-Gal plasmid DNA, and 0.5 μg of Sp3 expression plasmid, and harvested 42 hours later. The normalised luciferase activities are shown relative to the –654 deletion construct activity, with the exception of the –1357wt construct. The results for the -1357wt construct plus Sp3 are from a set of three experiments each done in triplicate (see Appendix 5, experiments 22-24), and are expressed relative to -1357wt. The remaining data are the same as illustrated in figure 5.11, with the difference being, the p-values represent the significance of differences for each construct, with and without Sp3 expression vector, rather than in relation to -654wt construct activity. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

5.5 Effect of Sp1 and Sp3 on pGL3Basic promoter vector 180 bp construct.

Co-transfection experiment data displayed thus far suggests a sequence upstream of the 180 bp region containing the putative GC and ICB elements (-654 to -474), may contain essential elements for the regulation of the topoisomerase IIβ promoter.

To investigate the ability of this 180bp region of the topoisomerase IIβ promoter to drive expression in the absence of surrounding sequence, the pGL3Basic promoter vector construct was used. The pGL3Basic promoter vector contains the topoisomerase IIβ 180 bp sequence upstream of the SV40 promoter (pGL3BP-TIIβ-180), which drives expression of the luciferase gene. As the SV40 promoter contains multiple GC elements to which Sp3 is thought to be able to interact, co-transfection experiments were carried out using Sp1 or Sp3 expression vectors with the pGL3BP-180 construct. The interaction of overexpressed Sp1 or Sp3 transcription factors with the SV40 promoter would result in a change in luciferase activity. By comparing luciferase activities of the pGL3Basic promoter vector alone and the pGL3BP-TIIβ-180 construct, any differences in activity could be determined, and therefore any change in expression due to the interaction of Sp1 or Sp3 with the 180 bp region.

For co-transfections to investigate the effect of the 180 bp region of topoisomerase IIβ in the pGL3Basic promoter vector, varying amounts of the Sp1 and Sp3 expression vector were added to transient transfections in order to optimise the amount of transcription factor present in the cells. To provide a comparison, the empty pGL3Basic promoter vector control was tested with 0.5 μg of Sp1 and Sp3 expression vector, as used in previous co-transfection experiments.



Construct Tested	Normalised luciferase activities relative to pGL3BP%	p-values	Significant Difference
pGL3BP	100%	-	-
pGL3BP + 0.5 μg Sp1	50.0±13.3%	0.0001	Yes***
pGL3BP + 0.5 μg Sp3	45.4±12.7%	$6.4x10^{-5}$	Yes***
pGL3BP-TIIβ-180	55.8±6.0%	$3.0x10^{-6}$	Yes***
pGL3BP-TIIβ-180 + 0.25 μg Sp1	46.5±1.3%	$8.0x10^{-7}$	Yes***
pGL3BP-TIIβ-180 + 0.5 μg Sp1	44.0±11.1%	0.0003	Yes***
pGL3BP-TIIβ-180 + 1.0 μg Sp1	56.9±12.9%	0.013	Yes*
pGL3BP-TIIβ-180 + 0.25 μg Sp3	65.8±15.1%	0.04	Yes*
pGL3BP-TIIβ-180 + 0.5 μg Sp3	74.5±8.8%	0.006	Yes**
pGL3BP-TIIβ-180 + 1.0 μg Sp3	80.9±6.2%	0.02	Yes*

Figure 5.13: Effect of Sp1 and Sp3 on pGL3Basic promoter vector 180 bp construct activity.

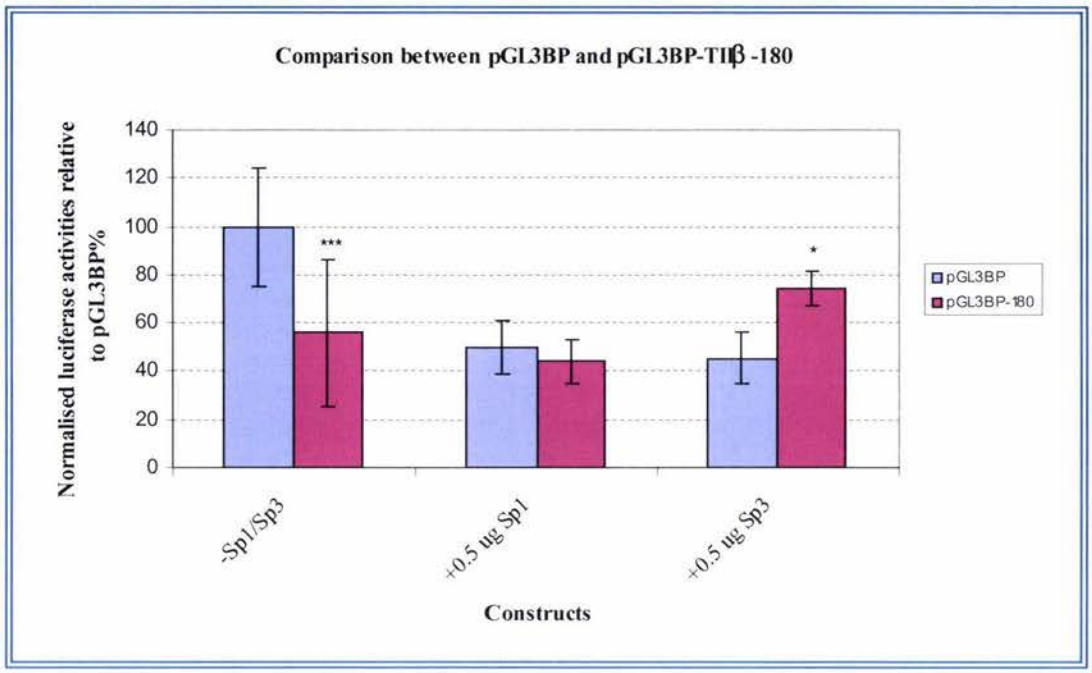
Changes between expression patterns pGL3Basic promoter vector and pGL3BP-TIIβ-180 construct are seen. HeLa cells were transfected with 0.5 μg each of a pGL3BP-TIIβ-180 construct or pGL3BP empty vector and pCMVSPORT-β-Gal plasmid DNA, with varying amounts (0-1.0 μg) of Sp1 or Sp3 expression plasmid, and harvested 42 hours later. The normalised luciferase activities are shown relative to the pGL3Basic promoter vector activity. The results of a set of 5 experiments each done in triplicate (see Appendix 5, experiments 27-31).

The significance of changes in expression observed, between each deletion construct and the wild type was calculated using a test-test, to yield p-values as shown above. The interpretation of the p-values is shown as a yes or no for statistical significance, with the strength of evidence indicated by *s (refer to table 5.2).

Figure 5.13 illustrates that the 180 bp region alone has a small, but significant, effect on expression of the SV40 promoter, and therefore is probably not acting alone in the regulation of the topoisomerase IIβ promoter. A decrease in luciferase expression (approximately 50%) is observed with the pGL3B-TIIβ-180 construct, relative to the pGL3Basic promoter vector alone. This result suggests a negative effect on the promoter activity in the presence of this region of topoisomerase IIβ promoter alone, possibly due to an interaction with endogenous Sp1 and Sp3 transcription factors in the HeLa cells. Overexpression of Sp1 or Sp3 transcription factors with the pGL3Basic promoter vector resulted in a 50% decrease in luciferase activity, which brings the expression down to a level similar to that seen with the pGL3BP-TIIβ-180 construct alone. This could suggest a down-regulation due to the effect of Sp1 and/or Sp3 transcription factors on the SV40 promoter and the 180 bp region, as similar results are seen with both elements in the presence of endogenous transcription factors or with the SV40 alone with overexpressed transcription factors.

With the addition of Sp1 expression vector the expression levels of both the empty vector and pGL3BP-TIIβ-180 construct were comparable, suggesting that the 180 bp region is not involved in an Sp1-mediated effect on promoter activity. In the presence of Sp3 a slight increase in luciferase activity was observed from the pGL3BP-TIIβ-180 construct relative to empty vector, which could suggest an influence of the 180 bp region on up-regulation of expression in the presence of Sp3. As shown below, p-values generated show that all changes in expression of the pGL3BP-TIIβ-180 construct, with and without Sp1 and Sp3, and the pGL3BP with Sp1 and Sp3, are significantly different to the expression of pGL3Basic promoter vector alone.

Figure 5.14 presents data already represented in figure 5.13, with the focus on comparison between the pGL3Basic promoter empty vector and the same vector containing the 180 bp region of the topoisomerase IIβ promoter. *t*-tests were performed to evaluate the statistical significance of changes in expression observed between the two constructs, and the generated p-values are shown below.



Sp1/Sp3	Normalised luciferase Activities relative to pGL3BP% pGL3BP	pGL3BP-180	p-values	Significant Difference
-Sp1/Sp3	100%	55.8±6.0%	2.98x10-6	Yes***
+Sp1	50.0±13.3%	44.0±11.1%	0.78	No
+Sp3	45.4±12.7%	74.5±8.8%	0.04	Yes*

Figure 5.14: Comparing the effects of Sp1 and Sp3 on pGL3Basic promoter vector construct vs empty vector activity.

Data is a subset of that presented in figure 5.13, displayed in this way for clarity and interpretation, of variations in expression of the pGL3BP, observed in the presence of the 180 bp region of the topoisomerase IIβ promoter.

P-values generated indicated strong evidence for a significant decrease in luciferase expression of the pGL3BPTIIβ-180 construct relative to the pGL3Basic promoter vector. No significant change in luciferase activity was observed with the addition of Sp1 to empty vector or the pGL3BP-TIIβ-180 construct, however moderate evidence exists for a significant difference between expression levels in the presence of Sp3 transcription factor. Overall, the 180 bp region appears to act as a repressor, independently of Sp1, and also to be involved in an Sp3-mediated up-regulation of pGL3Basic promoter vector expression levels. This suggests that while the 180 bp region appears to be involved in the regulation of topoisomerase IIβ promoter expression, co-operativity with other important regulatory elements must exist.

5.6 Chapter summary.

Deletions removing the ICB1, ICB2 and GC elements (-654 to -456) resulted in a decrease in luciferase activity, indicating the importance of these elements for basal transcription. In addition, important repressor elements may be present within the region between -1357 and -1066 bp, upstream of the transcription start site, while the region between -1066 and -654 may contain elements important for the up-regulation of the topoisomerase IIβ promoter.

A mutation in ICB1 has the ability to increase wild type activity, suggesting that ICB1 is normally a repressive element. In contrast the loss of ICB2 resulted in a decrease in wild type activity, suggesting an important role for maintaining wild type promoter activity.

The presence of Sp1 expression vector, appeared to have no significant effect on the luciferase activities of the topoisomerase IIβ promoter constructs, with the exception of the ICB mt constructs, which showed an increase in luciferase activity. As Sp1 was unable to influence expression of the -654 construct, the ICB elements are probably not the targets of Sp1-mediated regulation of the topoisomerase IIβ promoter and the presence of a two functional ICB elements may have an inhibitory effect.

Addition of Sp3 with the -1357wt construct displayed a small decrease in luciferase activity, while no change in expression levels of the -654 construct were seen, indicating the Sp3-mediated effect may require upstream regulatory elements. In the absence of upstream elements, a mutation in the ICB2, but not ICB1, appears to allow Sp3-mediated down-regulation, although the effect is minimal. This could suggest a low level of Sp3-mediated regulation of the topoisomerase IIβ promoter, via ICB1.

In general, the 180 bp region of the topoisomerase IIβ promoter appears to act as a repressor of pGL3Basic promoter vector expression levels. However, the 180 bp exhibited only a limited effect, which shows that while this region appears to be involved in the negative regulation of topoisomerase IIβ promoter expression, other important regulatory elements must be involved.

Chapter 6: Discussion and Future work.

The regulation of topoisomerase IIβ gene expression was investigated using a series of EMSAs and functional assays. Two inverted CCAAT boxes and a GC box within the topoisomerase IIβ promoter, located between 486-533 bases upstream of the major transcription start site, have previously been shown to be responsible for about 70% of the topoisomerase IIβ promoter activity (Lok *et al.,* 2002). For this reason the three elements were the focus for EMSAs carried out in this study. Deletion constructs of a PCR-generated topoisomerase IIβ 5'-flanking sequence, and constructs containing specific point mutations introduced into the ICB1 and ICB2 elements of the topoisomerase IIβ promoter, were used in transient transfections to identify regions of importance for topoisomerase IIβ transcriptional regulation.

6.1 Isolation of topoisomerase IIβ promoter sequence.

In an attempt to increase understanding of the regulation of human topoisomerase IIβ expression, a 1.5 kb region encompassing 5'-flanking and untranslated sequence (-1357 to +122) of topoisomerase IIβ gene, was cloned and sequenced. Previously, a 1.3 kb 5'-flanking region (-1067 to +193 bp) of the topoisomerase IIβ gene has been cloned and preliminary investigations undertaken (Lok *et al.,* 2002). The initial aim was to clone ~2.5 kb of upstream sequence, however problems with PCR were prohibitive. Nevertheless, an investigation into an additional 0.29 kb 5'-region in this study adds to our knowledge of topoisomerase IIβ gene expression.

6.2 Electrophoretic Mobility Shift Assays.

The topoisomerase IIβ promoter elements GC, ICB1 and ICB2 were studied by electrophoretic mobility shift assays to investigate protein-binding interactions at these regions. Three proteins were found to bind in the presence of GC and ICB2, which are in close proximity in the topoisomerase IIβ promoter. The three proteins were identified using antibodies against NF-Y, Sp1 and Sp3 (figure 4.5). This finding is of particular significance, as these three transcription factors have been found to be modulated during the development of drug resistance (Kubo *et al.,* 1995; Yoon *et al.,* 1999; Wang *et al.,* 1997a). Previous work has shown that transcription factor NF-Y can bind to ICB1 and ICB2 elements and transcription factor Sp1 can bind to GC in

the topoisomerase IIβ promoter (Lok *et al.*, 2002). The ability of transcription factor Sp3 to bind the promoter was not investigated by these authors.

In this study a composite GC-ICB2 oligonucleotide and single element GC, ICB1 and ICB2 oligonucleotides were used, and transcription factors Sp1 and Sp3 were shown to bind to GC and transcription factor NF-Y was shown to bind to ICB1 and ICB2. The finding that Sp3 can also bind the GC element in the topoisomerase IIβ promoter is novel information. In addition, this study provided important evidence for co-operativity between the GC and ICB2 elements of the topoisomerase IIβ promoter in order to recruit proteins to the GC element.

It was shown that Sp1 and Sp3 could be recruited to ICB2 in the absence of an intact GC element, however no evidence of the reverse occurring was found, suggesting that an intact ICB2 element is required to observe co-operativity between NF-Y and Sp1/3. Sp1, but not Sp3, antibodies supershifted the complex formed with the ICB2 element, as well as the GC element. This suggests an interaction between the two transcription factors and DNA to form NF-Y/Sp1/DNA complexes at the ICB2 and GC elements of the topoisomerase IIβ promoter. The addition of antibodies against NF-YA or NF-YC did not result in a supershift of the complex formed at the GC element. Although the observed binding patterns were not reciprocal, the findings are still indicative of functional synergy between GC and ICB2 through a physical interaction between NF-Y and Sp1. NF-Y and Sp1 both contain Q-rich domains, which are proposed to mediate protein-protein interactions. In fact, an *in vivo* interaction between NF-Y and Sp1 has previously been shown using a yeast two-hybrid system (Roder *et al.*, 1999).

In previous work, the results of functional assays suggested that a functional synergy may exist between the ICB elements and the GC box in the topoisomerase IIβ promoter (Lok *et al.*, 2002). A functional co-operation between NF-Y and Sp1 has been shown previously to play a key role in the transcriptional regulation of the promoter of a number of genes (Roder *et al.*, 1997; Hu *et al.*, 2000; Jean *et al.*, 2002), including human topoisomerase IIα (Magan *et al.*, 2003). The mechanisms of cooperativity may vary. In addition to the direct physical interaction described above,

co-operative binding to the promoter region can occur, as has been demonstrated for NF-Y and Sp1 binding to the FAS promoter (Roder *et al.,* 1997).

Although NF-Y has been the focus of numerous studies, complete understanding of the mechanism by which NF-Y regulates gene expression is yet to be achieved. NF-Y has been implicated in the stimulation of histone acetylation (Adachi *et al.,* 2000) and in fact, a physical association between NF-Y and histone acetyltransferase enzymes has been shown (Currie, 1997). NF-Y has been shown to possess histone acetyl-transferase (HAT) activity *in vivo* through an association with HATs, GCN5 and PCAF (Jin and Scotto, 1998). It has been speculated that the associated histone acetyltransferases might serve to modulate NF-Y transactivation potential by aiding disruption of local chromatin structure, which is a widely recognised capability of NF-Y (Currie, 1997). This ability to induce distortion of the double helix upon binding to DNA *in vitro*, facilitates transcription factor access to DNA binding sites, therefore NF-Y serves as a "promoter organiser" (Ronchi et al 1995; Mantovani *et al.,* 1999). As well as playing an important role in transcription reinitiation and activation, NF-Y has been implicated to have a role in basal transcription (Mantovani *et al.,* 1992).

Together with previous knowledge of NF-Y, the ability of NF-Y bound at ICB2 to recruit Sp1/3 shown in this study, could suggest that NF-Y initially binds to ICB2 and enables the recruitment of Sp1/3 to GC. In this scenario Sp1/3 should be unable to bind GC in the absence of NF-Y, which is not supported by the results of EMSA experiments illustrated in figures 4.6 (lanes 7-10) and figure 4.9. However the significance of these findings requires further investigation, as the oligonucleotides used in EMSAs were not organised into nucleosome structures, and therefore the access of transcription factors was not restricted by the structural organisation of the DNA.

In all reported cases of co-operativity between NF-Y and Sp1 the binding sites for the two transcription factors are located in close proximity in the promoter regions of the genes, suggesting that the distance between the two elements is important. In order to investigate this idea, EMSAs could be carried out using oligonucleotides which contain GC and ICB2 elements located at varying distances apart, to determine the

effect on NF-Ys ability to recruit Sp1 to ICB2. The functional significance of the degree of separation between the two elements could be investigated using reporter gene assays.

Competition assays indicated that the two ICB elements of topoisomerase IIβ bound NF-Y with different affinities, in the order ICB2β>ICB1β and both exhibited higher affinities than the topoisomerase IIα ICB1 for NF-Y. These results indicate that the topoisomerase IIβ ICB elements have different protein binding capabilities, and therefore are likely to have different functions. Individual ICB elements in the topoisomerase IIα promoter were also found to bind NF-Y with different affinities, in the order of ICB2>ICB3>ICB1 (Szremska, 2001). The ICB sequence is known to be the specific motif to which NF-Y binds (Mantovani, 1998; Lok *et al.,* 2002), however flanking sequences have also been shown to affect NF-Y binding (Dorn *et al.,* 1987; Mantovani, 1998). ICB1 and ICB2 elements in the topoisomerase IIβ promoter have different sequences flanking the CCAAT box (table 6.1), as do the topoisomerase IIα ICB elements, which could explain the observed differences in NF-Y binding.

Element	Sequence
ICB1	CCCGG**ATTGG**ACAGC
ICB2	TTGGG**ATTGG**CCGAG

Table 6.1: DNA sequences of the ICB elements from topoisomerase IIβ promoter.

This result is inconsistent with that of Lok *et al.,* (2002), where ICB1 was demonstrated by EMSAs to have the highest affinity for complex formation. This suggests that other key factors affect NF-Y binding, such as other transcription factors or assay binding conditions.

Transcription factors NF-Y, Sp1 and Sp3 have been shown to have a regulatory effect on topoisomerase IIα expression (Magan *et al.,* 2002), where an interaction between the three transcription factors is thought to occur (Roder *et al.,* 1999). Previously, NF-Y and Sp1 (Lok *et al.,* 2002), and in this study (chapter 5) NF-Y, Sp1 and Sp3, have also been shown to modulate topoisomerase IIβ expression. A decrease in NF-Y

activity has been correlated with a down-regulation of topoisomerase IIα in drug-resistant myeloma cells (Wang *et al.,* 1997a). However evidence contrary to this finding indicates the importance of other transcription factors in the regulation of topoisomerase IIα transcription, which may be cell-type specific (Isaacs *et al.,* 1995; Isaacs et at, 1996a). This could suggest that other transcription factors may also be important in regulating topoisomerase IIβ gene expression.

6.2.1 Additional EMSA experiments.

It would be of interest to investigate protein binding interactions at the -1357 to -1066, and -1066 to -654 regions of the topoisomerase II*β* promoter, to identify important regulatory elements, which may be involved in the observed up- and down-regulation of promoter activity. This would aid the elucidation of molecular mechanisms involved in the regulation of the topoisomerase II*β* expression.

As each EMSA in this study was conducted using HeLa cell extract, it would be of interest to investigate the binding patterns generated using other cancer cell lines in EMSA experiments, to see if they are cell-line specific or general.

6.2.2 Identifying other protein interactions with ICB1 and ICB2 elements of the topoisomerase II*β* promoter.

EMSA experiments could first be carried out using topoisomerase II*β* ICB1 or ICB2 labelled probes in Sp1 and Sp3 depleted HeLa cell lines, to determine if proteins in addition to NF-Y are seen to bind. If NF-Y is the only protein found to bind the ICB elements in the absence of Sp1/3, then it is likely that the uncharacterised protein(s) are Sp1 and/or Sp3. In this case the amounts of antibody and HeLa extract need to be optimised to ascertain the concentration needed to produce a detectable result.

Isolation of the uncharacterised protein(s) (Sp1/3?) binding at ICB1 and ICB2 topoisomerase II*β* promoter elements could be achieved using NF-Y depleted HeLa cell lines. In this way, a ^{32}P labelled or biotinylated oligonucleotide, containing either the ICB1 or ICB2, could be used in a binding reaction to isolate proteins other than NF-Y bound at these elements. The bound protein could then be separated using

denaturing polyacrylamide gel electrophoresis (SDS-PAGE), and then isolated from the gel to allow further analysis or purification.

If the preliminary EMSA experiments show that NF-Y is necessary to bind the uncharacterised protein(s), then NF-Y could be used to isolate the uncharacterised protein. For example, a pull-down assay could be conducted, using a glutathione-S-transferase (GST)-NF-Y fusion protein. The uncharacterised protein could be identified using N-terminal microsequencing to determine the amino acid sequence, which can then be matched against existing sequences (such as the NCBI, Entrez protein sequence database), and thereby determine protein identity and putative function.

6.2.3 DNA footprinting assays.

DNA footprinting assays could be conducted to locate regions of the topoisomerase IIβ promoter to which proteins bind. In DNA footprinting, regions of importance for protein binding are revealed by protection of the DNA sequence from digestion by endonuclease DNase I (refer to figure 6.1). DNase I cuts DNA molecules randomly, which results in a mixture of DNA subfragments of varying lengths, with the exception of DNA associated with proteins, which will be protected. When analysed by electrophoresis this appears as a relatively uniform ladder of bands, each arising from cuts at a single position in the fragment. Where protein is bound to the DNA, thereby preventing nicking of the DNA, the ladder is interrupted by a gap representing the sequence protected from DNase cleavage by bound protein. A sequencing ladder of the same region used in the footprinting assay can be used to identify the nucleotide sequence to which proteins bind. Using this DNA sequence information, cognate transcription factors can often be identified using programmes such as TRANSFAC database (MatInspector V2.2, http://transfac.gbf.de/cgi-bin/matSearch.pl).

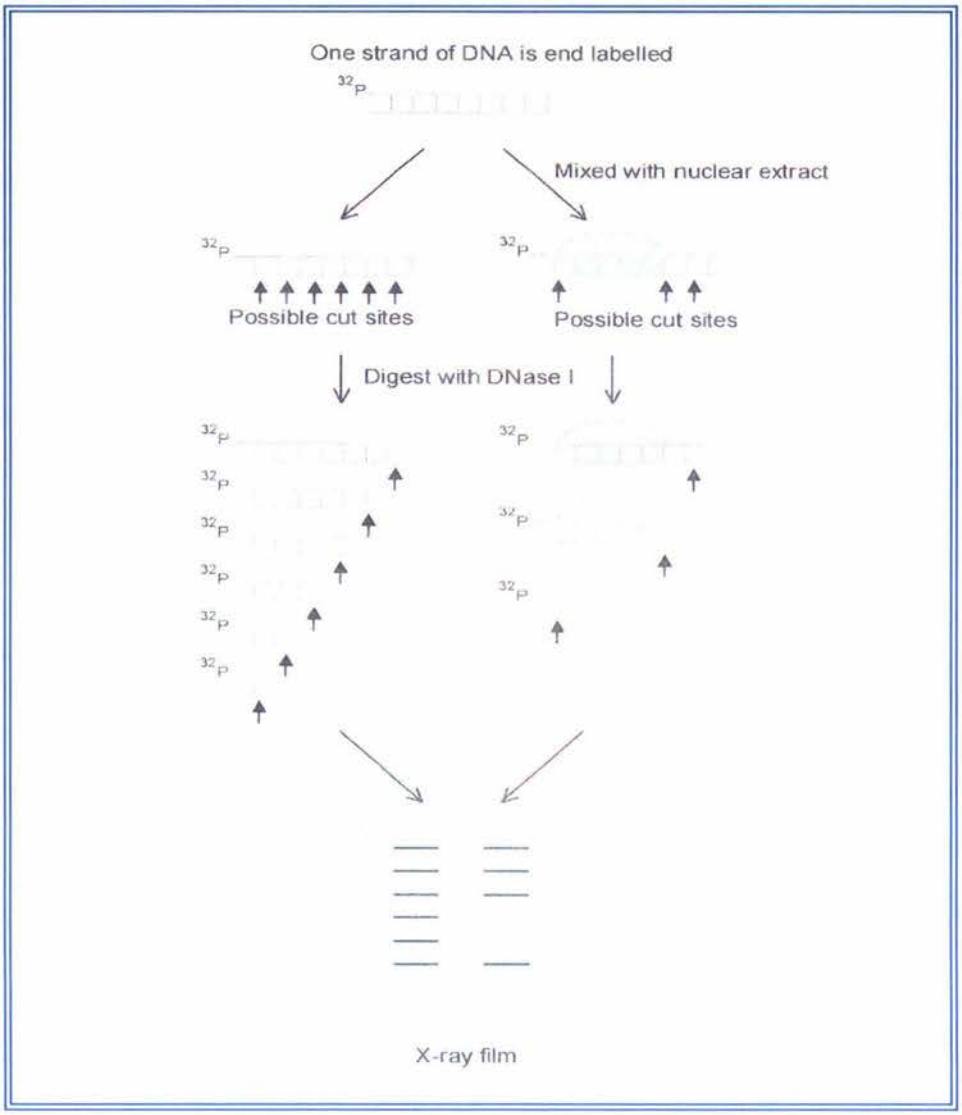


Figure 6.1: Schematic representation of a DNA footprinting assay.

A restriction fragment or oligonucleotide containing a consensus binding site for a protein is end-labelled with ^{32}P. As in EMSA, the DNA fragment is incubated with cellular proteins, allowing DNA-protein complexes to form. The reaction mixture is then treated with limiting amounts of DNase I, and in the absence of bound protein, the enzyme cuts throughout the fragment, generating a mixture of labelled subfragments of varying sizes. The fragments are separated on a polyacrylamide gel and bands are visualised by exposure to X-ray film. A relatively uniform ladder of bands will be visible, which is interrupted by a "footprint" representing the sequence protected from DNase cleavage by bound protein. A sequencing ladder of the same region used in the footprinting assay will enable the identification of the exact binding site of the protein to the oligonucleotide. (Figure adapted from Sambrook and Russell, 2001).

6.2.4 Chromatin-immunoprecipitation (CHIP) assay.

CHIP assays could be conducted to investigate regions of the topoisomerase IIβ promoter to which proteins bind, including whether Sp1 and/or Sp3 can bind at GC and/or ICB2. CHIP assays enable investigation of the direct or indirect association of chromosomal proteins with chromosomal DNA *in vivo*. CHIP assays involve two straightforward steps; first, *in vivo* formaldehyde cross-linking of whole cells that freezes protein-protein and protein-DNA interactions, followed by immunoprecipitation of protein-DNA complexes with specific antibodies, from sonicated extracts. In this way specific antibodies against Sp1 or Sp3 could be used to precipitate Sp1/3 proteins and any DNA fragment cross-linked to them. Thus, DNA sequence elements associated with Sp1/3 in the context of the cellular environment are enriched in the immunoprecipitated sample. After reversal of the formaldehyde cross-links and purification of the DNA, the precipitated DNA fragments can be cloned into a vector for isolation and further characterisation of binding sites and functional relevance.

6.3 Transient Transfections.

The ability of the -1357 to +122 topoisomerase IIβ promoter fragment to drive expression of a luciferase reporter gene *in vivo* was investigated first. The effect of 5'-serial, and internal deletions (removing GC, ICB1 and ICB2 elements) or specific mutations introduced into the ICB1 or ICB2 elements, on the ability of the topoisomerase IIβ promoter to drive luciferase expression, was then investigated. The ability of the 180 bp region (-654 to -474) containing the ICB1, ICB2 and GC elements to drive luciferase expression from an SV40 promoter (without the surrounding sequence) was also investigated.

6.3.1 Transcriptional regulation of topoisomerase IIβ 1.5 kb promoter region.

The minimal promoter (-569 bp upstream of 5'-transcriptional start site) for topoisomerase IIβ expression has previously been defined and ICB1, ICB2 and GC elements were found to be responsible for about 70% of the topoisomerase IIβ activity (Lok *et al.*, 2002).

In the current study, deletions removing the ICB1, ICB2 and GC elements (-654 to -474) resulted in an approximately 55% decrease in luciferase activity, indicating the importance of these elements for basal transcription, and supporting the results of Lok *et al.,* (2002). In addition, important repressor elements may be present within the region between -1357 and -1066 bp, upstream of the transcription start site, a region of the promoter which until this time had not been investigated. Also the region between -1066 and -654 may contain elements important for the up-regulation of the topoisomerase IIβ promoter.

6.3.2 Role of isolated ICB1, ICB2 and GC in the transcriptional regulation of topoisomerase IIβ.

The presence of the 180 bp region of the topoisomerase IIβ promoter containing GC, ICB1 and ICB2 resulted in an approximately 50% decrease in activity of the SV40 promoter, suggesting that this region may act as a repressor within this construct. These results suggest that although this 180 bp region is able to influence transcriptional activity, the effect is the opposite to that previously indicated by the deletion of this region, as described above. Therefore the effect on transcriptional activity appears to change in the absence of surrounding topoisomerase II*β* promoter sequence, suggesting that other important regulatory elements must also be involved in the regulation of topoisomerase II*β* gene expression.

Sp1 and Sp3 bound at multiple GC elements within the SV40 promoter would also influence the changes in transcriptional activity observed with this construct, and could be involved in the down-regulation of promoter activity. It has been suggested that an observed synergy between proximal and distal elements of a promoter could be due to interactions between distally and proximally bound Sp1 (Su *et al.,* 1991). Sp1 has been shown to form multiple tetramers, which enable DNA looping to occur, thereby bringing proximal and distal elements closer together (Mastrangelo *et al.,* 1991; Su *et al.,* 1991). As the SV40 promoter contains multiple GC boxes, it is possible that Sp1/3 bound at the topoisomerase II*β* promoter GC element and Sp1/3 bound at the SV40 promoter interact with each other, and thereby influence transcriptional activity.

6.3.3 Role of ICB elements in the transcriptional regulation of topoisomerase IIβ.

By introducing specific mutations into either the ICB1 or ICB2 element, the two inverted CCAAT boxes were found to play key roles in the topoisomerase IIβ promoter activity. A mutation in ICB1 has the ability to significantly increase wild type activity (2.5-fold), suggesting that ICB1 is normally a repressive element. In contrast the loss of ICB2 resulted in a decrease in wild type activity (~0.5 fold), which could suggest an important role for maintaining wild type levels of topoisomerase IIβ promoter activity.

ICB1 and ICB2 have previously been shown to be critical for topoisomerase IIβ promoter activity, with simultaneous disruption of both elements resulting in a loss of activity, while the mutation of a single ICB element had little effect (Lok *et al.*, 2002). This is inconsistent with the current findings, which indicated significant and reciprocal changes in promoter activity when either element was mutated. Individual ICB elements in topoisomerase IIα promoter activity have been reported as both stimulatory and repressive in various cellular backgrounds (Isaacs *et al.*, 1996; Furakawa *et al.*, 1998; Falck *et al.*, 1999; Adachi *et al.*, 2000). The same may be true for topoisomerase II*β*.

6.3.3.1 ICB1.

NF-Y activity is known to be down-regulated in a number of drug-resistant cells (Wang *et al.*, 1997a). It has been suggested that a negative regulatory protein binding to ICB1 could be responsible for decreased topoisomerase IIα promoter activity, and when this repressor can no longer bind to ICB1 the down-regulation is no longer observed (Takano *et al.*, 1999; Falck *et al.*, 1999; Furakawa *et al.*, 1998). Significantly, the latter study showed that the protein released from ICB1 was not NF-Y. The research described in this thesis established that a mutation in ICB1 resulted in an approximately 2.5 fold increase in luciferase activity compared to wild type, suggesting that topoisomerase IIβ promoter activity is normally down-regulated via interactions occurring at this element. This could explain the lower affinity of NF-Y for ICB1 than ICB2 (as suggested in EMSA experiments).

6.3.3.2 ICB2.

The ICB2 element of the topoisomerase IIα promoter has an important regulatory role, showing down-regulation of topoisomerase IIα expression in confluence-arrested cells, which is thought to be the result of a factor binding to this element (Isaacs *et al.,* 1996a). Conversely deletion of the ICB2α element resulted in down-regulation of topoisomerase IIα promoter activity (Takano *et al.,* 1999). Deletion of the ICB2 element of topoisomerase IIβ resulted in a 60% decrease in luciferase activity relative to wild type activity, a similar decrease to that seen with the removal of GC, ICB1 and ICB2. This suggests that ICB2 is a critical factor in topoisomerase IIβ promoter activity, and that the binding of NF-Y to the ICB2 element in the topoisomerase IIβ promoter could be critical in maintaining basal levels of transcriptional activity. The residual activity observed could be due to other functional interactions within the -654 region of the topoisomerase IIβ promoter, which could involve the ICB1 and GC elements.

Together these results suggest that the binding of an activator (NF-Y) and a repressor protein binding at ICB1/2 may modulate the transcriptional regulation of topoisomerase IIβ, in normal and drug-resistant cells. This idea was supported by EMSA experiments, which indicate the presence of a second protein binding the ICB1/2 elements, in addition to NF-Y. Basal transcription could be maintained by an equilibrium between the activator protein bound at ICB2 and the repressor protein bound at ICB1. Therefore, the regulation of topoisomerase IIβ gene expression could be achieved by a displacement of NF-Y binding at ICB1/2, in exchange for a repressor protein (refer to figure 6.2), with NF-Y binding with higher affinity at ICB2, while the repressor protein binds with higher affinity at ICB1.

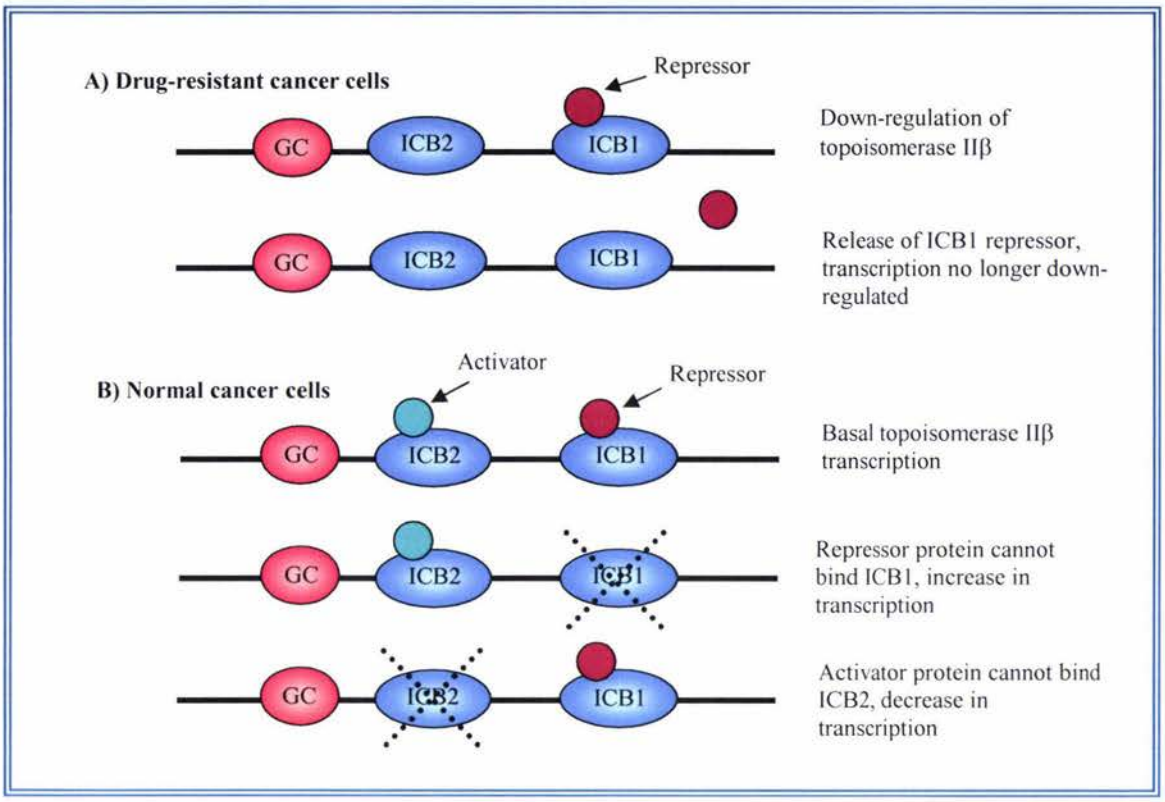


Figure 6.2: Schematic representation of transcriptional regulation of topoisomerase IIβ promoter through ICB1, ICB2 and GC.

The transcriptional regulation of topoisomerase IIβ could be achieved by reducing the ability of a repressor protein to bind to ICB1, and inducing the binding of NF-Y to ICB1/2.

A CCAAT box binding protein, termed CCAAT displacement protein (CDP), has been isolated which does appear to negatively regulate the binding of NF-Y to CCAAT boxes (Mantovani, 1998). It would be of interest to determine whether an association occurs between the CDP protein and the topoisomerase IIβ ICB elements. Additional EMSA experiments could also be carried out using a composite ICB1/ICB2 oligonucleotide, or a longer ICB1 oligonucleotide, in an attempt to identify the putative repressor protein. Alternatively, NF-Y is capable of both stimulatory and repressive roles depending on the promoter context.

6.3.3.3 Sp1 and ICB1/2.

Sp1 is widely recognised as a transcriptional activator shown to be responsible for the up-regulation of transcription in a variety of promoters (Ge *et al.*, 2001; Keates *et al.*, 2001), as well as topoisomerase IIα (Magan *et al.*, 2003). Sp1 had no significant effect on transcriptional regulation of the topoisomerase IIβ, with the possible

exception of the constructs containing a mutation in either the ICB1 or ICB2 element (95% confidence). Approximately 30% and 125% increases in luciferase activity were seen with the ICB1 and ICB2 mutant constructs, respectively, in the presence of Sp1, which could suggest the involvement of the ICB elements in this Sp1-mediated increase. Significantly, both ICB1 and ICB2 elements have been shown to be capable of recruiting Sp1. As Sp1 was unable to influence expression of the -654 construct, the ICB elements are unlikely to be the targets of Sp1-mediated regulation of the topoisomerase IIβ promoter. The presence of two functional ICB elements could in fact have an inhibitory effect.

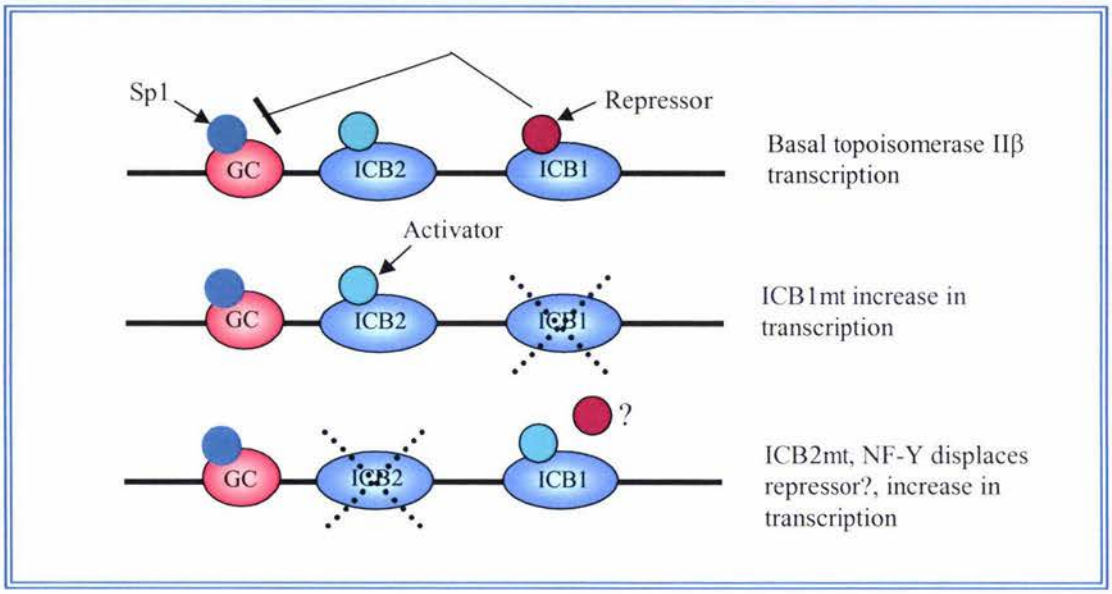


Figure 6.3: Schematic representation of Sp1-mediated transcriptional regulation of topoisomerase IIβ promoter.

Transient co-transfection experiments suggested that GC and ICB2 may act co-operatively to regulate topoisomerase IIβ transcription. The ICB elements were implicated in an Sp1-mediated up-regulation of topoisomerase IIβ promoter, possibly involving NF-Y bound at ICB1/2 recruiting Sp1 to GC. An Sp1-mediated up-regulation of topoisomerase IIβ promoter in the absence of ICB2 could be due to an increase in binding of NF-Y at ICB1 in the absence of ICB2, thereby resulting in the displacement of repressor protein bound at ICB1 and/or NF-Y bound at ICB1 recruiting Sp1 to GC.

These findings could suggest that either ICB element can influence an Sp1-mediated increase in promoter activity. In the absence of ICB1 negative regulation, ICB2 could enhance an Sp1-mediated up-regulation of topoisomerase IIβ promoter activity by

recruiting Sp1 to GC. Whereas, in the absence of ICB2, NF-Y may bind ICB1 with a higher affinity and thereby displace repressor protein bound at ICB1 (figure 6.3). Therefore, the effect of Sp1 regulation could be modulated by the ratio of repressor protein bound at ICB1, and NF-Y bound at ICB2.

6.3.3.4 Sp3 and ICB1/2.

There was no consistent significant difference in transcription with the addition of Sp3, in any of the constructs tested. This suggests that Sp3 is not likely to be involved in transcriptional regulation of the topoisomerase IIβ promoter.

The role of upstream elements in an Sp1/3-mediated regulation could be investigated by generating the ICB mutations in the full-length -1357wt construct and determining the effect on promoter activity in co-transfection experiments. As, Sp1 and Sp3 proteins are constitutively expressed at high levels in HeLa cells (Hagen *et al.*, 1994), the addition of ectopic Sp1/3 may not have a significant additional effect. A better indication of the effect of Sp1/3 on topoisomerase IIβ activity could be determined by carrying out co-transfections in a cell line, which contains low levels, or no endogenous Sp1/3.

RNA_i interference is one possible method of removing endogenous Sp1 and/or Sp3 protein or other protein of interest. In this technique interference RNA (RNA_i) is introduced into the cell where it binds to homologous regions of the targeted mRNA strand (Sp1/3 mRNA), hence inducing mRNA degradation. In this way a particular protein of interest can be effectively removed from the cell.

6.3.4 Comparison between HeLa and MDA-MB-231 cell lines.

The same trends were observed in HeLa cells and MDA-MB-231 breast cancer cells, in response to changes in the topoisomerase IIβ promoter. This suggests that breast cancer cells are likely to show the same response to mutations introduced within the topoisomerase IIβ promoter elements, and overexpression of Sp1 or Sp3 transcription factors, as observed with HeLa cells. Additional transient transfections and co-transfections using Sp1/3 expression vectors could be carried out in MDA-MB-231 cell lines to determine the effect.

6.3.5 Additional transient transfections.

It has been shown that mutation of GC and either one of the ICB elements results in a significant loss in topoisomerase IIβ promoter activity (Lok *et al.*, 2002), supporting the idea of a functional synergy between the ICB and GC elements. This study failed to address the effect of Sp1 or Sp3 in the absence of GC. Future work could include generating a GC mutant construct to further investigate the importance of this element for topoisomerase IIβ promoter transcriptional regulation. The introduction of combinatorial mutations; GC and ICB1, GC and ICB2, ICB1 and ICB2, and GC, ICB1 and ICB2 elements, would also allow a more in-depth investigation into the importance of these regulatory elements in topoisomerase IIβ transcriptional regulation, as well as the existence of functional redundancy between elements. Co-transfection experiments could be carried out using these mutant constructs and Sp1 or Sp3 to investigate the role of these transcription factors at GC, ICB1 and ICB2 elements in topoisomerase IIβ promoter regulation.

Determining the effect NF-Y transcription factor has on topoisomerase IIβ promoter activity is also necessary for full understanding of regulatory mechanisms. A dominant negative form of NF-Y, with mutations in the DNA binding domain, has previously been used to confirm the involvement of NF-Y in topoisomerase IIβ promoter activity (Lok *et al.*, 2002). Co-transfection experiments using both Sp1 and Sp3, or NF-Y and Sp1 or Sp3, in $Sp1^-$ or $Sp3^-$ cell lines, could provide information about the interactions occurring between these transcription factors at the topoisomerase IIβ promoter. The observed changes in topoisomerase IIβ promoter activity in response to altered levels of NF-Y and Sp1/3 could then be compared to the changes observed in topoisomerase IIα promoter under the same conditions.

Investigation into the -1357 to -1066 and -1066 to -654 regions would also be of interest to identify regulatory elements, which may be involved in the observed up- or down-regulation of the topoisomerase IIβ promoter activity. This could be achieved by introducing specific mutations within putative regulatory elements, identified by EMSA or DNAse I footprinting as previously described, and determining the effect on promoter activity.

6.4 Protein-protein interactions at the topoisomerase IIβ promoter.

ICB1α and GC1α have been implicated to act antagonistically in the regulation of topoisomerase IIα gene in drug-resistant cells (Takano *et al.*, 1999). In the topoisomerase IIβ promoter, a functional co-operation between GC and the ICB elements has been suggested in the regulation of promoter activity (Lok *et al.*, 2002). The current findings suggest that this could be due to an interaction between the transcription factors that bind to these elements. The results of EMSA experiments suggested that NF-Y, Sp1 and Sp3 may interact to form a multi-protein complex (figure 4.7, indicated by red circle), which will require further investigation. On the other hand any functional effects of Sp1 and Sp3 were minimal using the constructs and sequences available. Functional assays suggested that the two ICB elements may act antagonistically to regulate topoisomerase IIβ gene expression.

A number of promoters contain multiple CCAAT boxes, which are located at variable distances apart. The distance between the ICB1 and ICB2 elements in the topoisomerase IIβ promoter is 30 bp (centre to centre), which is very close to 3 times a helical turn of DNA (10.4 bp) (Liberati *et al.*, 1998). This could mean that proteins, such as transcription factor NF-Y or a repressor protein, bound at the two ICB elements are close together in the topoisomerase IIβ promoter. This close proximity of DNA-protein interactions at ICB1 and ICB2 could assist their antagonistic roles in the regulation of the topoisomerase IIβ.

6.5 Transcriptional regulation of topoisomerase IIβ.

In summary, the results of this thesis indicate that the ICB1 and ICB2 elements are critical for topoisomerase IIβ transcription, and thus topoisomerase IIβ expression may be modulated by the levels or activities of transcription factors binding at these elements. In particular ICB2 appears to be critical for maintaining basal levels of topoisomerase IIβ transcription, and binds NF-Y with a higher affinity than does ICB1. The ICB1 element was implicated as a key factor in the negative regulation of the topoisomerase IIβ promoter, which appears to be mediated by the binding of an unknown repressor protein. The residual activity observed in the absence of ICB1,

ICB2 and GC is likely to be due to remaining elements in the topoisomerase IIβ promoter and their cognate proteins.

The role of NF-Y in the regulation of topoisomerase IIβ gene expression may be in maintaining the correct spatial conformation of the promoter. Three lines of evidence support this hypothesis. The first, transactivation by Sp1 was only observed with a construct containing a mutation in ICB2. Secondly, Sp1 transcription factor appeared to interact with the protein complexes formed with the ICB elements. Lastly, current literature supports an architectural role of NF-Y in transcriptional regulation (Mantovani, 1998; Mantovani, 1999).

It has been suggested that DNA looping is mediated by NF-Y-DNA interactions (Liberati *et al.*, 1999). Therefore, NF-Y bound at ICB1/2 could induce DNA bending in the topoisomerase IIβ promoter (figure 6.4). DNA bending induced by NF-Y bound at ICB2, could act to bring a repressor bound at ICB1 closer to ICB2 and GC elements, thereby disrupting or inhibiting interactions occurring at the two elements. In the absence of repressor bound at ICB1, closing the distance between ICB2 and GC, could enhance co-operativity between the two elements, resulting in an up-regulation of promoter activity. DNA bending experiments could be carried out to investigate the capacity of a complex consisting of either of the topoisomerase IIβ ICB elements to induce DNA bending.

The findings revealed in this study add to the knowledge of a complex series of interactions, which occur in the regulation of the human topoisomerase IIβ gene. Whether interactions described here play a role in the down-regulation of topoisomerase IIβ in drug resistant cells, will require further investigation. A more detailed analysis into how these, and other unidentified transcription factors, interact with the topoisomerase IIβ and α promoters is required to elucidate the mechanisms involved in the development of drug resistance.

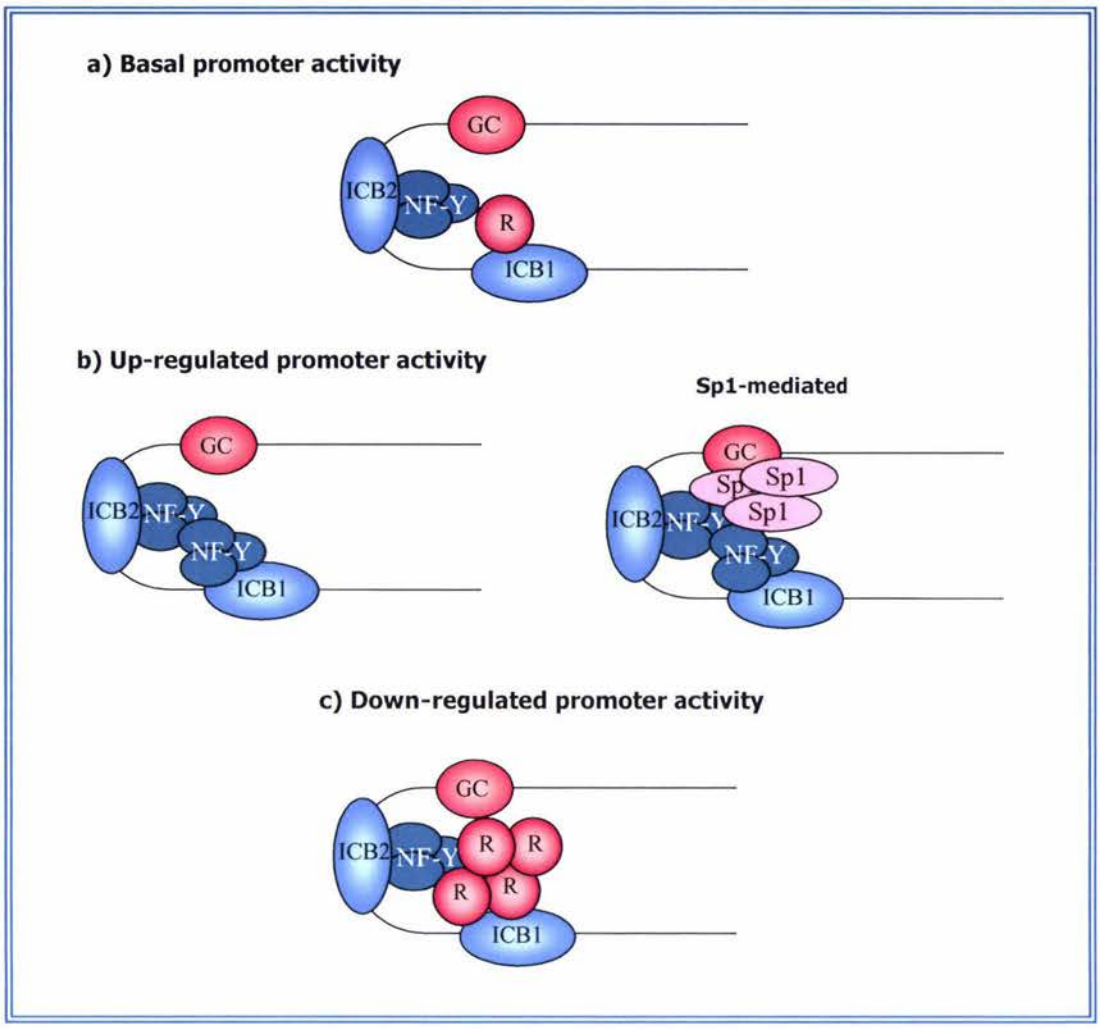


Figure 6.4: Models for the transcriptional regulation of topoisomerase IIβ.

Each figure represents putative interactions between transcription factors binding target regulatory elements in the topoisomerase IIβ promoter. NF-Y is shown to bend DNA at ICB2.

A) Basal transcription levels may be maintained due to the levels of activator and repressor proteins present. B) Increased levels of NF-Y could displace the repressor protein bound at ICB1, increasing the level of transcription; Recruitment of Sp1 to GC by NF-Y could enhance the transcriptional up-regulation. C) Increased levels of a repressor protein bound at ICB1 could inhibit the stimulatory effect of the NF-Y, decreasing the level of transcription.

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Appendix

Appendix 1: Primer and Oligonucleotide sequences.

Sequences of oligonucleotides used in this study. The underlined sequences represent the underlined and bold sequences represent the binding elements and mutations respectively. The experiments in which the oligonucleotides were used is displayed, with Seq = Sequencing primer and PCR mut = PCR mutagenesis.

Oligo Name	Sequence 5' - 3'	Experiment
TIIBpromo.for	CGCG GAGCTC TGA GGC AAG TGA AAA GAA C	PCR
TIIBpromo.rev	GGCC TCTAGA TCC AGC TCA CAG GCC CTG AGG CC	PCR
TIIBpromo.int	GCGC GAGCTC CGG AAG ATA AAG AGC TTT GAC AAC T	PCR
TIIBseq1	TGA CAG AGC AAG ACT CTG	Seq & PCR
TIIBseq2	GTT TCA GAG TTC TCC ACC	Seq & PCR
TIIBseq3	CCA TCT GCC AAA GAC TGT	Seq & PCR
TIIBseq4	TTG CAC CAG TGG TCT AC	Seq & PCR
TIIBseq5	TTG GGA TTG GCC GAG AGG	Seq & PCR
TIIBseq6	CTC CCT GCT TTC TCC TCA G	Seq & PCR
MM1	GAC TTC AGA ACA GTG AAG CCT T	PCR
MM2	CTG AGG AGA AAG CAG GGA G	PCR
MM3	GTA GAC CAC TGG TGC AAG	PCR
MM4	CGCC GCTAGC CCA TCT GCC AAA GAC TGT	PCR
GCwtF	CGGGTCCCGCCCCTCCAG	EMSA
GCwtR	CTGGAGGGGCGGGACCCG	EMSA
ICB2wtF	TTGGGATTGGCCGAG	EMSA
ICB2wtR	CTCGGCCAATCCCAA	EMSA
ICB1wtF	ACAAGGCCCGGATTGGACAGCATGGCG	EMSA
ICB1wtR	CGCCATGCTGTCCAATCCGGGCCTTGT	EMSA & PCR
ICB2wtF	GGAATTTTGGGATTGGCCGAGAGGCTG	EMSA
ICB2wtR	CAGCCTCTCGGCCAATCCCAAAATTCC	EMSA
ICB1mtF	ACAAGGCCCG**TCTAGA**ACAGCATGGCG	EMSA & PCR Mut.
ICB1mtR	CGCCATGCTGT**TCTAGA**CGGGCCTTGT	EMSA & PCR Mut.
ICB2mtF	GGAATTTTGG**AGATCT**CCGAGAGGCTG	EMSA & PCR Mut.
ICB2mtR	CAGCCTCTCGG**AGATCT**CCAAAATTCC	EMSA & PCR Mut.
GCmtF	CTCGGGTC**GAGCTC**CTCCAGG	EMSA
GCmtR	CCTGGAGG**AGCTCG**ACCCGAG	EMSA

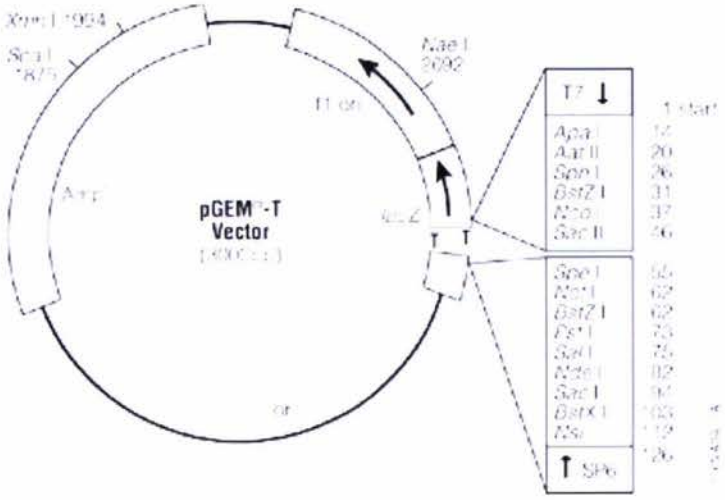
Oligo Name	Sequence 5' - 3'	Experiment
GCIwtICB2wtF	CTCGGGTCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTG	EMSA
GCwtICB2wtR	CAGCCTCTCGGCCAATCCCAAAATTCCAAGCCCCCTGGAGGGGCGGGACCCGAG	EMSA
GCmtICB2wtF	CTCGGGTC**GA**GC**T**CCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTG	EMSA
GCmtICB2wtR	CAGCCTCTCGGCCAATCCCAAAATTCCAAGCCCCCTGGAGG**A**GC**TC**GACCCGAG	EMSA
GCwtICB2mtF	CTCGGGTCCCGCCCCTCCAGGGGGCTTGGAATTTTGG**AGATCT**CCGAGAGGCTG	EMSA
GCwtICB2mtR	CAGCCTCTCGG**AGATCT**CAAAATTCCAAGCCCCCTGGAGGGGCGGGACCCGAG	EMSA
GCmtICB2mtF	CTCGGGTC**GA**GC**T**CCTCCAGGGGGCTTGGAATTTTGG**AGATCT**CCGAGAGGCTG	EMSA
GCmtICB2mtR	CAGCCTCTCGG**AGATCT**CCAAAATTCCAAGCCCCCTGGAGG**A**GC**TC**GACCCGAG	EMSA

Topoisomerase IIα competitor oligonucleotides.

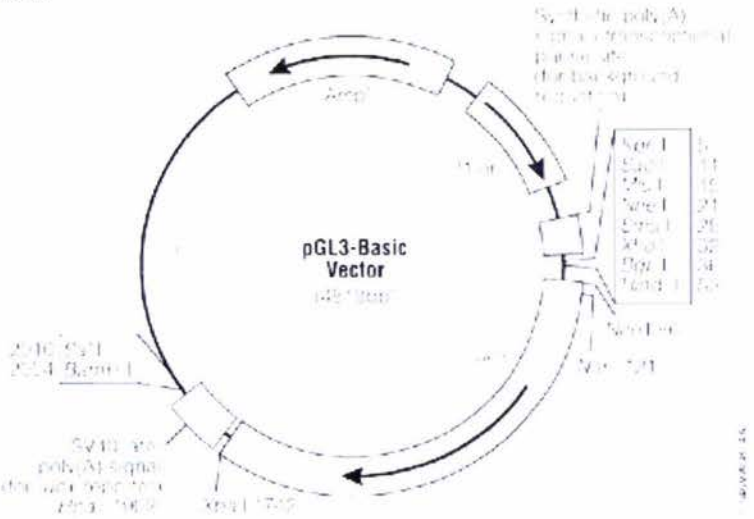
ICB1wtF	GAGTCAGGGATTGGCTGGTCTGC	EMSA
ICB1wtR	GCAGACCAGCCAATCCCTGACTC	EMSA
ICB1mtF	GAGTCAGGGATT**CC**CTGGTCTGC	EMSA
ICB1mtR	GCAGACCAG**GG**AATCCCTGACTC	EMSA
GC1wtF	CTGCTTCGGGCGGGCTAAAG	EMSA
GC1wtR	CTTTAGCCCGCCCGAAGCAG	EMSA
GC1mtF	CTGCTTCG**T**GCG**T**GCTAAAG	EMSA
GC1mtR	CTTTAGC**A**CGC**A**CGAAGCAG	EMSA

Appendix 2: Vector Maps.

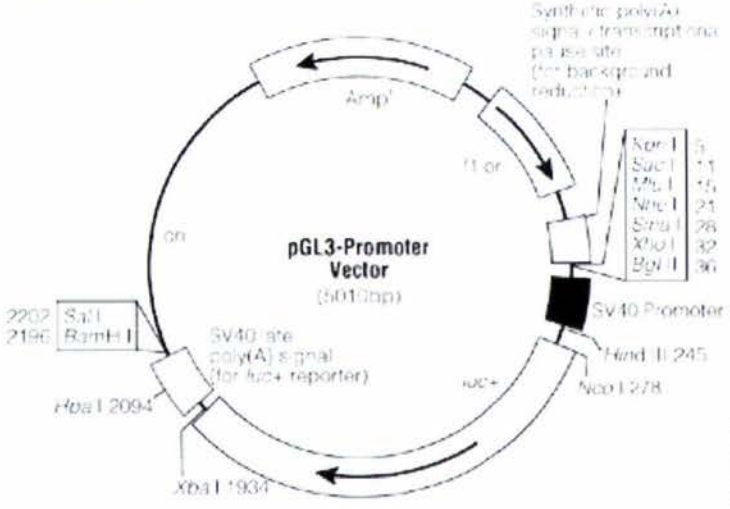
2.1 pGEM-T Vector.



2.2 pGL3Basic Vector.

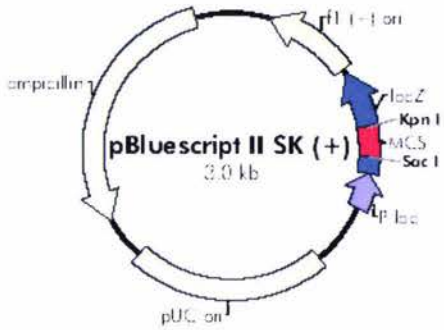


2.3 pGL3Basic Promoter Vector.



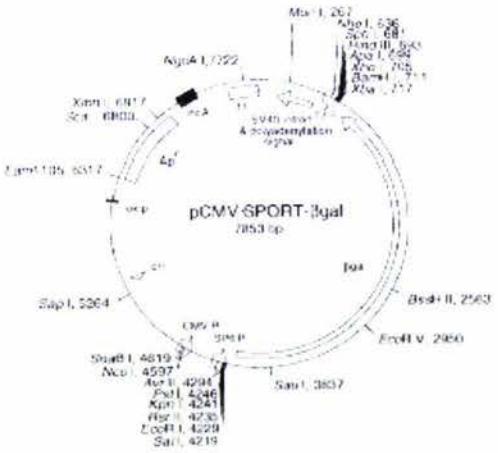
2.4 pBluescript SK(+)

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
***lac* promoter** 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript II SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)

2.5 pCMV-SPORTβGal



MAP of pCMV•SPORT-βgal

Appendix 3: Topoisomerase IIβ sequences and analysis.

3.1 Sequencing of pGEMT TIIβ-1357.

Sequence results using primer T7
Length 647 bases (TIIBSeq4 primer sequence shown in bold)

```
-1357                                                CCATC TGCCAAAGAC
-1342  TGTGATGTTG CTGTATTGGT AATTTTCATT CTTAGAAAGC TTTAATGTGA
-1292  ATGAAATTAT TCCTTACTGT GCATTTGAAA ATTCCACTGA AAACAGTTTT
-1242  AAAAATTAAG CATGCCCTCT TAGAGGCAGG GGGCACAGTT CTGGACAGAT
-1192  GAGAAAAGTG ACAGTGACAT CTCTGCATTG TTCAGAGAAG AAAGGTCAAC
-1142  AAAGAGTAGA AATAGAACAC TCAACCCACC CTGCATTCTT TCCCTAGAAC
-1092  ATTCTCTGTG TGTCCCTTAT CATTTAAGCT TTTCATATGT AGTTTTCTTG
-1042  AAACTGACCA TATGCTCTTT TGGGGTGAGG GGAGGAGACG GAAGAAGGAG
 -992  ATGGAGAAGG TCTGAGATGA TTTTTTTCGA CAGCAGCGTC CACTTAGGCC
 -942  CTTGGGGAGG CCGGGTCACG GGGCCTCCTT GCACCAGTGG TCTACAACGT
 -892  CTTGCCTGCG GTTTACAACC GCTCTTTTAA AATAATTTCC CTTTGGGACG
 -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
 -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
 -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA C
```

Sequence results using primer TIIBSeq4
Length 498 bases (TIIBSeq5 primer sequence shown in bold)

```
 -865                                      TAA AATAATTTCC CTTTGGGACG
 -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
 -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
 -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA CCCGAGGCCA ATTCGACCCT
 -692  TTCCCTTTGC CGCCCTCAAT TGACCCTTGA AGCAGCCCCT GCTCTCCCTT
 -642  CAAATGGAAA ACCCACAGAC ACACACACAA ACAAAAACCC CAAGTCTTCC
 -592  TTTCGGTTGC TACCCGCAAT GACGTTTCCC CCCTCGGGTC CCGCCCCTCC
 -542  AGGGGGCTTG GAATTTTGGG ATTGGCCGAG AGGCTGTGGC GACAAGGCCC
 -492  GGATTGGACA GCATGGCGCT GACTGACAGC GGGGGCGGCC GCCGCGCCCT
 -442  CCCTCTCTCC CCGGTGTGCA AATGTGTGTG TGCGGTGTTA TGCCGGACAA
 -392  GAGGGAGGTG ACCGTGGCGG CGGCGG
```

Sequence results using primer TIIBSeq5
Length 497 bases

```
 -370                          GCGGCGGC GGCTCTGTTT ATTGTCCCTC
 -342  TCGGTGTGTG TGTGTGAGGA AATCGGGGCT GCAGCGAGGC TAAGGCTGCC
 -292  TTTGAAGCAG CGGCGGCGAC CGGGACGACT ACTCTGGCGA CTCGAGTGGC
 -242  TGGCCTTCGC GGAGTGTGAG AAGGACAAGG CACCTCTGCG TCCTCGCCAC
 -192  GTCCGAGCGC CTCGGGCTCC CCGGCCGCCC TCGCGGCTCG CACGCCCGGG
 -142  CTTCAGCCCG GCCTGCAGCG GCGCCCGCGG GCGGGCGAGA AGGCAACGCC
  -92  GCCGCTCGGC CGCCGCCGGT CGCTCCCTGC TTTCTCCTCA GCCGCCGCGC
                                                  +1
  -42  TAGGCCCGGG CGACGCGGAC GCCGCGCCTC GAGTTTGAGG GCAGCCGGCG
   +8  GCGCGGCCTC CTCAGCGGGC TCGGCTGGAC GTCCGCTCCG GATCTTCGCG
  +58  ATGGGGCGCG GGGGTCGGCG CGGCTAGGAG TGCGGCGAGT GGAGCGGTGG
 +108  GTGCGGAGCG GCGGGGCCCA GCGGCCCGCA GGGAGGCGGG AGCGGCGGCT
 +158  GCGGCCTCAG GGCCTGTGAG CTGGA
```

Contig of Topoisomerase IIβ promoter sequences:
1540 bases

```
-1357                                                CCATC TGCCAAAGAC
-1342  TGTGATGTTG CTGTATTGGT AATTTTCATT CTTAGAAAGC TTTAATGTGA
-1292  ATGAAATTAT TCCTTACTGT GCATTTGAAA ATTCCACTGA AAACAGTTTT
-1242  AAAAATTAAG CATGCCCTCT TAGAGGCAGG GGGCACAGTT CTGGACAGAT
-1192  GAGAAAAGTG ACAGTGACAT CTCTGCATTG TTCAGAGAAG AAAGGTCAAC
-1142  AAAGAGTAGA AATAGAACAC TCAACCCACC CTGCATTCTT TCCCTAGAAC
-1092  ATTCTCTGTG TGTCCCTTAT CATTTAAGCT TTTCATATGT AGTTTTCTTG
-1042  AAACTGACCA TATGCTCTTT TGGGGTGAGG GGAGGAGACG GAAGAAGGAG
 -992  ATGGAGAAGG TCTGAGATGA TTTTTTTCGA CAGCAGCGTC CACTTAGGCC
 -942  CTTGGGGAGG CCGGGTCACG GGGCCTCCTT GCACCAGTGG TCTACAACGT
 -892  CTTGCCTGCG GTTTACAACC GCTCTTTTAA AATAATTTCC CTTTGGGACG
 -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
 -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
 -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA CCCGAGGCCA ATTCGACCCT
 -692  TTCCCTTTGC CGCCCTCAAT TGACCCTTGA AGCAGCCCCT GCTCTCCCTT
 -642  CAAATGGAAA ACCCACAGAC ACACACACAA ACAAAAACCC CAAGTCTTCC
 -592  TTTCGGTTGC TACCCGCAAT GACGTTTCCC CCCTCGGGTC CCGCCCCTCC
 -542  AGGGGGCTTG GAATTTTGGG ATTGGCCGAG AGGCTGTGGC GACAAGGCCC
 -492  GGATTGGACA GCATGGCGCT GACTGACAGC GGGGGCGGCC GCCGCGCCCT
 -442  CCCTCTCTCC CCGGTGTGCA AATGTGTGTG TGCGGTGTTA TGCCGGACAA
 -392  GAGGGAGGTG ACCGTGGCGG CGGCGGCGGC GGCTCTGTTT ATTGTCCCTC
 -342  TCGGTGTGTG TGTGTGAGGA AATCGGGGCT GCAGCGAGGC TAAGGCTGCC
 -292  TTTGAAGCAG CGGCGGCGAC CGGGACGACT ACTCTGGCGA CTCGAGTGGC
 -242  TGGCCTTCGC GGAGTGTGAG AAGGACAAGG CACCTCTGCG TCCTCGCCAC
 -192  GTCCGAGCGC CTCGGGCTCC CCGGCCGCCC TCGCGGCTCG CACGCCCGGG
 -142  CTTCAGCCCG GCCTGCAGCG GCGCCCGCGG GCGGGCGAGA AGGCAACGCC
  -92  GCCGCTCGGC CGCCGCCGGT CGCTCCCTGC TTTCTCCTCA GCCGCCGCGC
                                                  +1
  -42  TAGGCCCGGG CGACGCGGAC GCCGCGCCTC GAGTTTGAGG GCAGCCGGCG
   +8  GCGCGGCCTC CTCAGCGGGC TCGGCTGGAC GTCCGCTCCG GATCTTCGCG
  +58  ATGGGGCGCG GGGGTCGGCG CGGCTAGGAG TGCGGCGAGT GGAGCGGTGG
 +108  GTGCGGAGCG GCGGGGCCCA GCGGCCCGCA GGGAGGCGGG AGCGGCGGCT
 +158  GCGGCCTCAG GGCCTGTGAG CTGGA
```

3.2 Sequencing of pGL3Basic TIIβ-1357.

Sequence results using primer RV3
Length 647 bases (TIIBSeq4 primer sequence shown in bold)

```
-1357                                                CCATC TGCCAAAGAC
-1342  TGTGATGTTG CTGTATTGGT AATTTTCATT CTTAGAAAGC TTTAATGTGA
-1292  ATGAAATTAT TCCTTACTGT GCATTTGAAA ATTCCACTGA AAACAGTTTT
-1242  AAAAATTAAG CATGCCCTCT TAGAGGCAGG GGGCACAGTT CTGGACAGAT
-1192  GAGAAAAGTG ACAGTGACAT CTCTGCATTG TTCAGAGAAG AAAGGTCAAC
-1142  AAAGAGTAGA AATAGAACAC TCAACCCACC CTGCATTCTT TCCCTAGAAC
-1092  ATTCTCTGTG TGTCCCTTAT CATTTAAGCT TTTCATATGT AGTTTTCTTG
-1042  AAACTGACCA TATGCTCTTT TGGGGTGAGG GGAGGAGACG GAAGAAGGAG
 -992  ATGGAGAAGG TCTGAGATGA TTTTTTTCGA CAGCAGCGTC CACTTAGGCC
 -942  CTTGGGGAGG CCGGGTCACG GGGCCTCCTT GCACCAGTGG TCTACAACGT
 -892  CTTGCCTGCG GTTTACAACC GCTCTTTTAA AATAATTTCC CTTTGGGACG
 -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
 -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
 -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA C
```

Sequence results using primer TIIBSeq4
Length 498 bases (TIIBSeq5 primer sequence shown in bold)

```
 -865                                     TAA AATAATTTCC CTTTGGGACG
 -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
 -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
 -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA CCCGAGGCCA ATTCGACCCT
 -692  TTCCCTTTGC CGCCCTCAAT TGACCCTTGA AGCAGCCCCT GCTCTCCCTT
 -642  CAAATGGAAA ACCCACAGAC ACACACACAA ACAAAAACCC CAAGTCTTCC
 -592  TTTCGGTTGC TACCCGCAAT GACGTTTCCC CCCTCGGGTC CCGCCCCTCC
 -542  AGGGGGCTTG GAATTTTGGG ATTGGCCGAG AGGCTGTGGC GACAAGGCCC
 -492  GGATTGGACA GCATGGCGCT GACTGACAGC GGGGGCGGCC GCCGCGCCCT
 -442  CCCTCTCTCC CCGGTGTGCA AATGTGTGTG TGCGGTGTTA TGCCGGACAA
 -392  GAGGGAGGTG ACCGTGGCGG CGGCGG
```

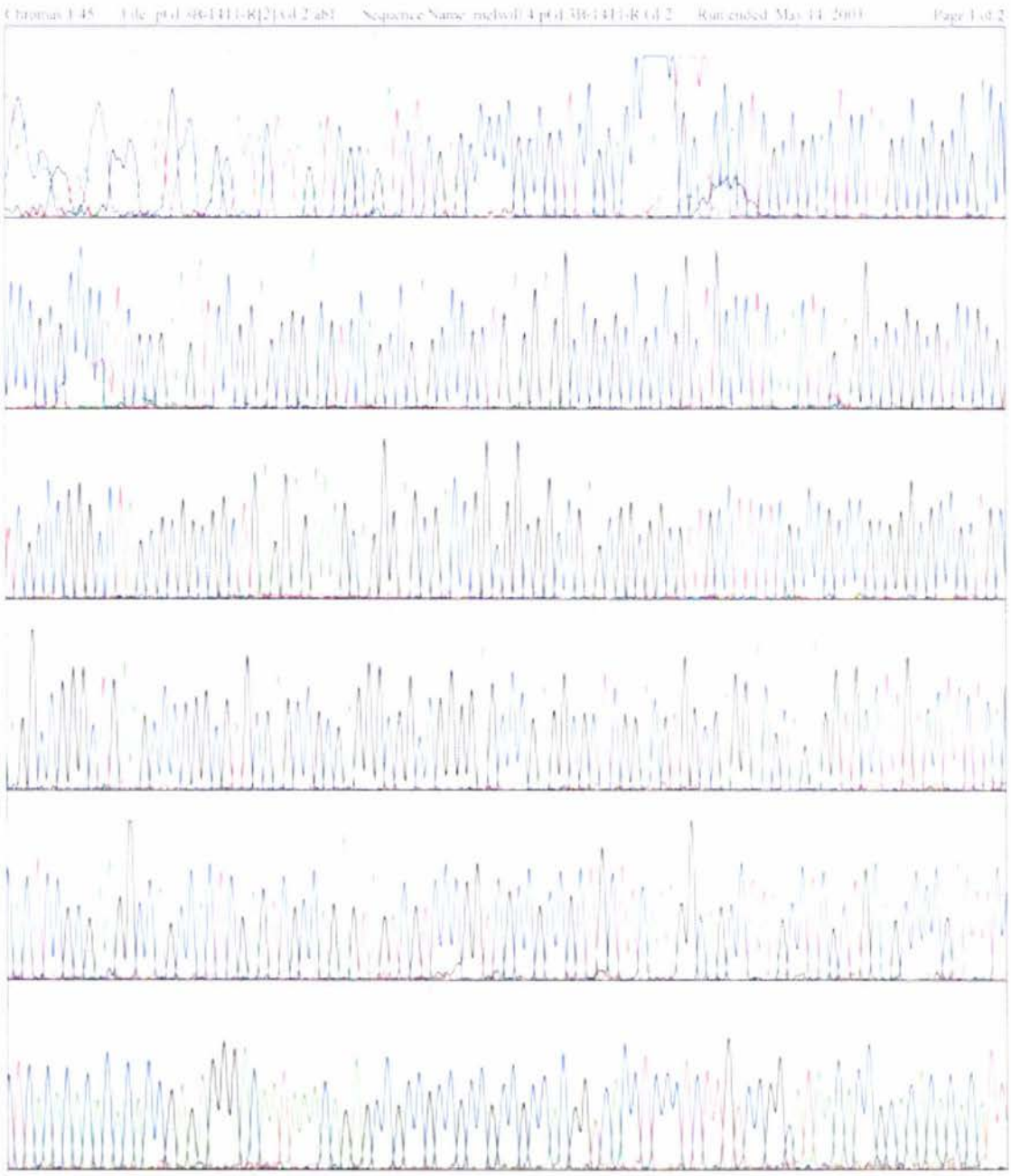
Sequence results using primer TIIBSeq5
Length 497 bases

```
 -370                          GCGGCGGC GGCTCTGTTT ATTGTCCCTC
 -342  TCGGTGTGTG TGTGTGAGGA AATCGGGGCT GCAGCGAGGC TAAGGCTGCC
 -292  TTTGAAGCAG CGGCGGCGAC CGGGACGACT ACTCTGGCGA CTCGAGTGGC
 -242  TGGCCTTCGC GGAGTGTGAG AAGGACAAGG CACCTCTGCG TCCTCGCCAC
 -192  GTCCGAGCGC CTCGGGCTCC CCGGCCGCCC TCGCGGCTCG CACGCCCGGG
 -142  CTTCAGCCCG GCCTGCAGCG GCGCCCGCGG GCGGGCGAGA AGGCAACGCC
  -92  GCCGCTCGGC CGCCGCCGGT CGCTCCCTGC TTTCTCCTCA GCCGCCGCGC
                                                  +1
  -42  TAGGCCCGGG CGACGCGGAC GCCGCGCCTC GAGTTTGAGG GCAGCCGGCG
   +8  GCGCGGCCTC CTCAGCGGGC TCGGCTGGAC GTCCGCTCCG GATCTTCGCG
  +58  ATGGGGCGCG GGGGTCGGCG CGGCTAGGAG TGCGGCGAGT GGAGCGGTGG
 +108  GTGCGGAGCG GCGGGGCCC
```

Contig of Topoisomerase IIβ promoter sequences:
1483 bases

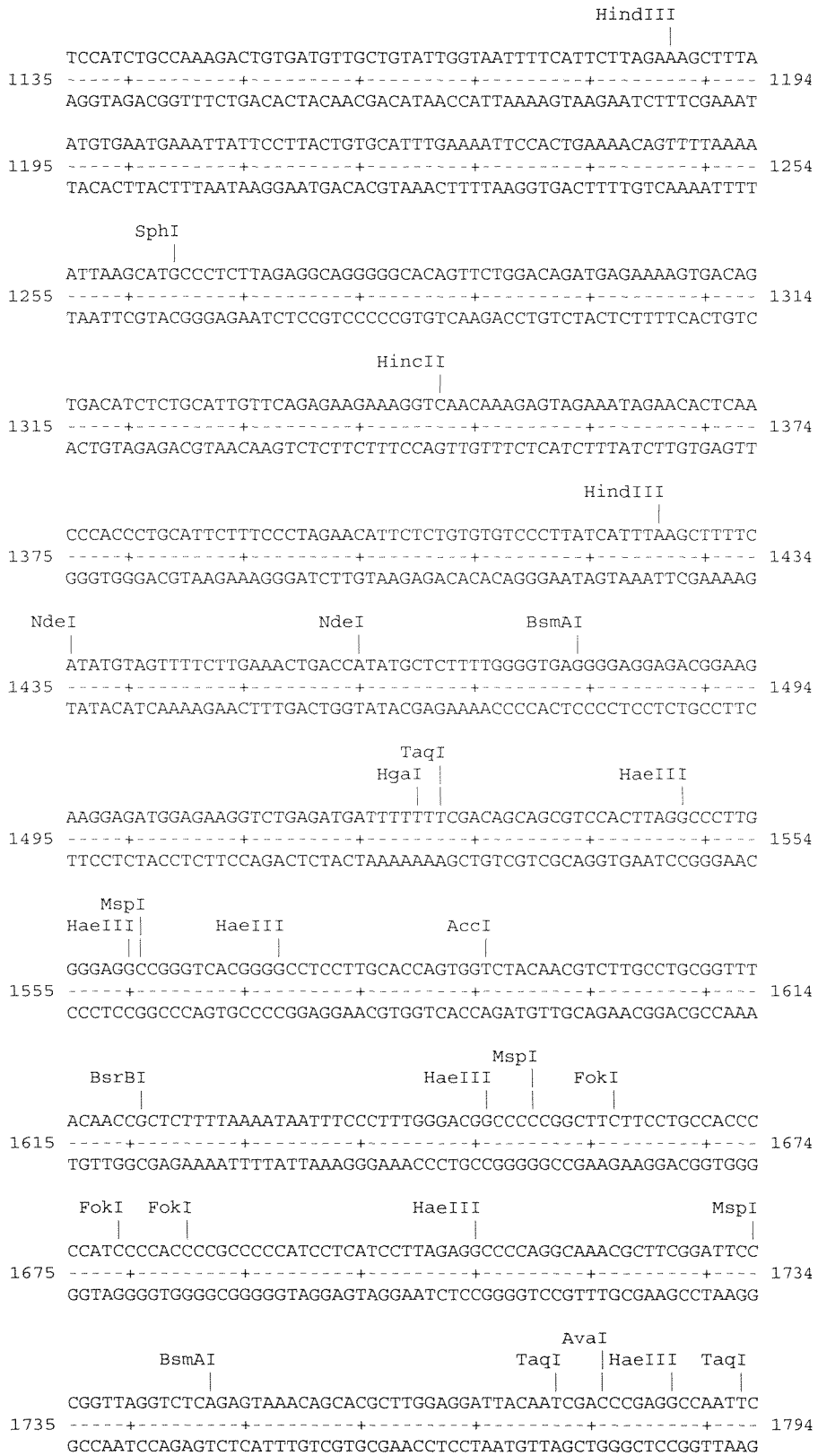
```
 -1357                                                CCATC TGCCAAAGAC
 -1342  TGTGATGTTG CTGTATTGGT AATTTTCATT CTTAGAAAGC TTTAATGTGA
 -1292  ATGAAATTAT TCCTTACTGT GCATTTGAAA ATTCCACTGA AAACAGTTTT
 -1242  AAAAATTAAG CATGCCCTCT TAGAGGCAGG GGGCACAGTT CTGGACAGAT
 -1192  GAGAAAAGTG ACAGTGACAT CTCTGCATTG TTCAGAGAAG AAAGGTCAAC
 -1142  AAAGAGTAGA AATAGAACAC TCAACCCACC CTGCATTCTT TCCCTAGAAC
 -1092  ATTCTCTGTG TGTCCCTTAT CATTTAAGCT TTTCATATGT AGTTTTCTTG
 -1042  AAACTGACCA TATGCTCTTT TGGGGTGAGG GGAGGAGACG GAAGAAGGAG
  -992  ATGGAGAAGG TCTGAGATGA TTTTTTTCGA CAGCAGCGTC CACTTAGGCC
  -942  CTTGGGGAGG CCGGGTCACG GGGCCTCCTT GCACCAGTGG TCTACAACGT
  -892  CTTGCCTGCG GTTTACAACC GCTCTTTTAA AATAATTTCC CTTTGGGACG
  -842  GCCCCCGGCT TCTTCCTGCC ACCCCCATCC CCACCCCGCC CCCATCCTCA
  -792  TCCTTAGAGG CCCCAGGCAA ACGCTTCGGA TTCCCGGTTA GGTCTCAGAG
  -742  TAAACAGCAC GCTTGGAGGA TTACAATCGA CCCGAGGCCA ATTCGACCCT
  -692  TTCCCTTTGC CGCCCTCAAT TGACCCTTGA AGCAGCCCCT GCTCTCCCTT
  -642  CAAATGGAAA ACCCACAGAC ACACACACAA ACAAAAACCC CAAGTCTTCC
  -592  TTTCGGTTGC TACCCGCAAT GACGTTTCCC CCCTCGGGTC CCGCCCCTCC
  -542  AGGGGGCTTG GAATTTTGGG ATTGGCCGAG AGGCTGTGGC GACAAGGCCC
  -492  GGATTGGACA GCATGGCGCT GACTGACAGC GGGGGCGGCC GCCGCGCCCT
  -442  CCCTCTCTCC CCGGTGTGCA AATGTGTGTG TGCGGTGTTA TGCCGGACAA
  -392  GAGGGAGGTG ACCGTGGCGG CGGCGGCGGC GGCTCTGTTT ATTGTCCCTC
  -342  TCGGTGTGTG TGTGTGAGGA AATCGGGGCT GCAGCGAGGC TAAGGCTGCC
  -292  TTTGAAGCAG CGGCGGCGAC CGGGACGACT ACTCTGGCGA CTCGAGTGGC
  -242  TGGCCTTCGC GGAGTGTGAG AAGGACAAGG CACCTCTGCG TCCTCGCCAC
  -192  GTCCGAGCGC CTCGGGCTCC CCGGCCGCCC TCGCGGCTCG CACGCCCGGG
  -142  CTTCAGCCCG GCCTGCAGCG GCGCCCGCGG GCGGGCGAGA AGGCAACGCC
   -92  GCCGCTCGGC CGCCGCCGGT CGCTCCCTGC TTTCTCCTCA GCCGCCGCGC
                                                   +1
   -42  TAGGCCCGGG CGACGCGGAC GCCGCGCCTC GAGTTTGAGG GCAGCCGGCG
    +8  GCGCGGCCTC CTCAGCGGGC TCGGCTGGAC GTCCGCTCCG GATCTTCGCG
   +58  ATGGGGCGCG GGGGTCGGCG CGGCTAGGAG TGCGGCGAGT GGAGCGGTGG
  +108  GTGCGGAGCG GCGGGGCCC
```

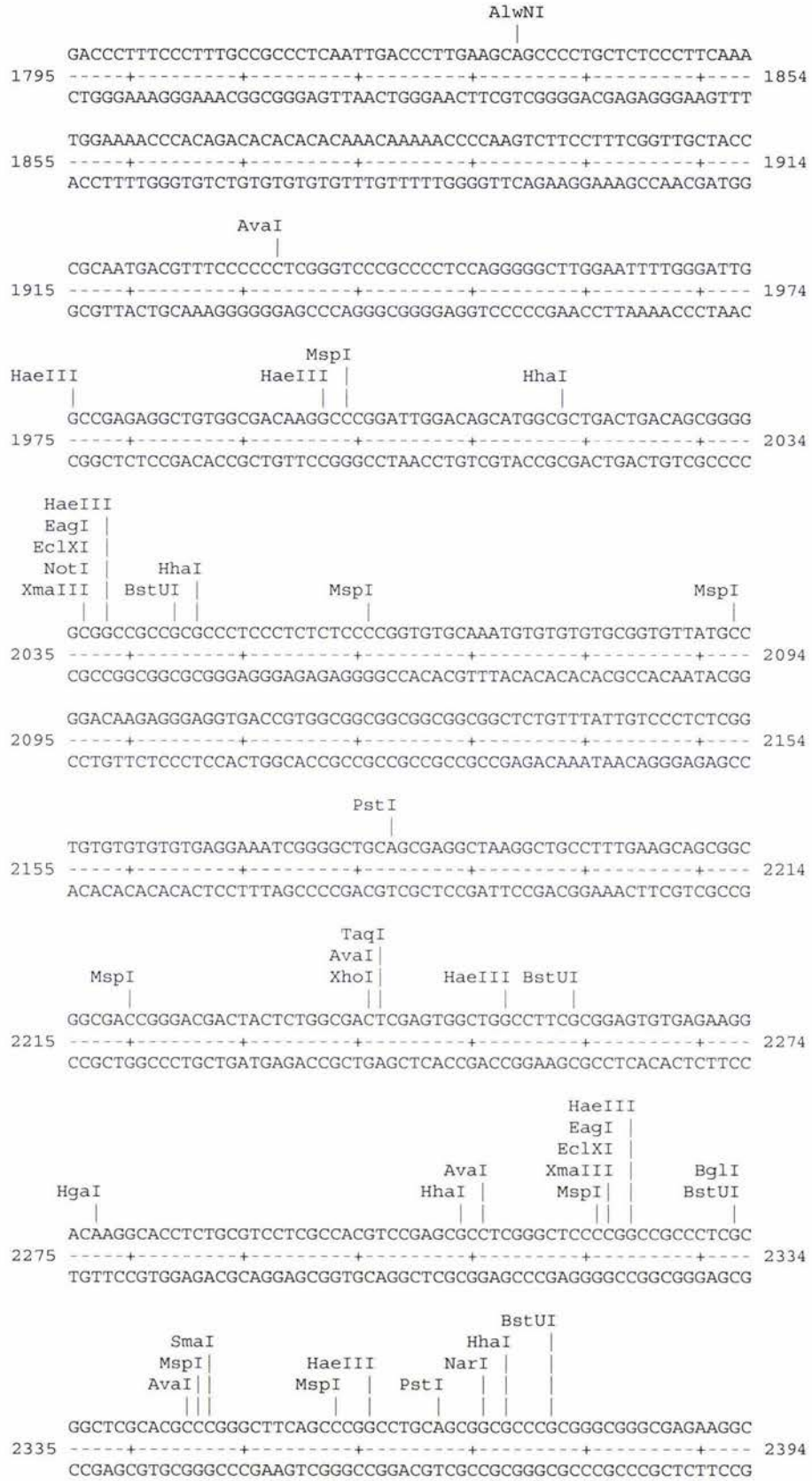
3.3 Chromatogram of pGL3B-TIIβ-1411 construct sequence.



3.4 - GCG Sequence Analysis

Restriction endonuclease MAP (GCG, Wisconsin Package version 9.1) of the 1.54 kb topoisomerase IIβ promoter sequence.




AlwNI
GACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCAGCCCCTGCTCTCCCTTCAAA
1795 -----+---------+---------+---------+---------+---------+---------+---- 1854
CTGGGAAAGGGAAACGGCGGGAGTTAACTGGGAACTTCGTCGGGGACGAGAGGGAAGTTT
TGGAAAACCCACAGACACACACACAAACAAAAACCCCAAGTCTTCCTTTCGGTTGCTACC
1855 -----+---------+---------+---------+---------+---------+---------+---- 1914
ACCTTTTGGGTGTCTGTGTGTGTTTGTTTTTGGGGTTCAGAAGGAAAGCCAACGATGG
AvaI
CGCAATGACGTTTCCCCCCTCGGGTCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTG
1915 -----+---------+---------+---------+---------+---------+---------+---- 1974
GCGTTACTGCAAAGGGGGGAGCCCAGGGCGGGGAGGTCCCCCGAACCTTAAAACCCTAAC
MspI
HaeIII HaeIII HhaI
GCCGAGAGGCTGTGGCGACAAGGCCCGGATTGGACAGCATGGCGCTGACTGACAGCGGGG
1975 -----+---------+---------+---------+---------+---------+---------+---- 2034
CGGCTCTCCGACACCGCTGTTCCGGGCCTAACCTGTCGTACCGCGACTGACTGTCGCCCC
HaeIII
EagI
EclXI
NotI HhaI
XmaIII BstUI MspI MspI
GCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTGTGTGCGGTGTTATGCC
2035 -----+---------+---------+---------+---------+---------+---------+---- 2094
CGCCGGCGGCGCGGGAGGGAGAGAGGGGCCACACGTTTACACACACACGCCACAATACGG
GGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCTCTGTTTATTGTCCCTCTCGG
2095 -----+---------+---------+---------+---------+---------+---------+---- 2154
CCTGTTCTCCCTCCACTGGCACCGCCGCCGCCGCCGCCGAGACAAATAACAGGGAGAGCC
PstI
TGTGTGTGTGTGAGGAAATCGGGGCTGCAGCGAGGCTAAGGCTGCCTTTGAAGCAGCGGC
2155 -----+---------+---------+---------+---------+---------+---------+---- 2214
ACACACACACACTCCTTTAGCCCCGACGTCGCTCCGATTCCGACGGAAACTTCGTCGCCG
TaqI
AvaI
MspI XhoI HaeIII BstUI
GGCGACCGGGACGACTACTCTGGCGACTCGAGTGGCTGGCCTTCGCGGAGTGTGAGAAGG
2215 -----+---------+---------+---------+---------+---------+---------+---- 2274
CCGCTGGCCCTGCTGATGAGACCGCTGAGCTCACCGACCGGAAGCGCCTCACACTCTTCC
HaeIII
EagI
EclXI
AvaI XmaIII BglI
HgaI HhaI MspI BstUI
ACAAGGCACCTCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCCGCCCTCGC
2275 -----+---------+---------+---------+---------+---------+---------+---- 2334
TGTTCCGTGGAGACGCAGGAGCGGTGCAGGCTCGCGGAGCCCGAGGGGCCGGCGGGAGCG
BstUI
SmaI HhaI
MspI HaeIII NarI
AvaI MspI PstI
GGCTCGCACGCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCGCGGGCGGGCGAGAAGGC
2335 -----+---------+---------+---------+---------+---------+---------+---- 2394
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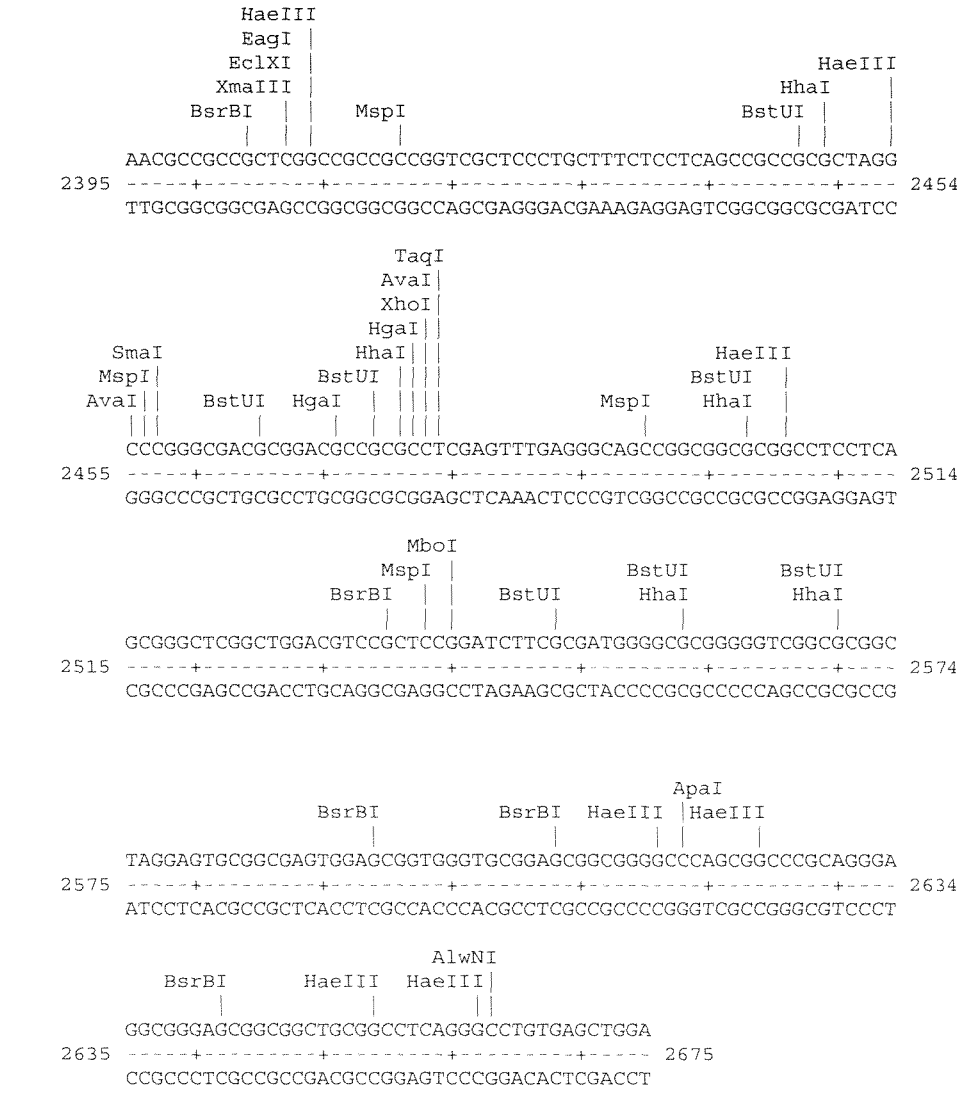


Enzymes that do cut:

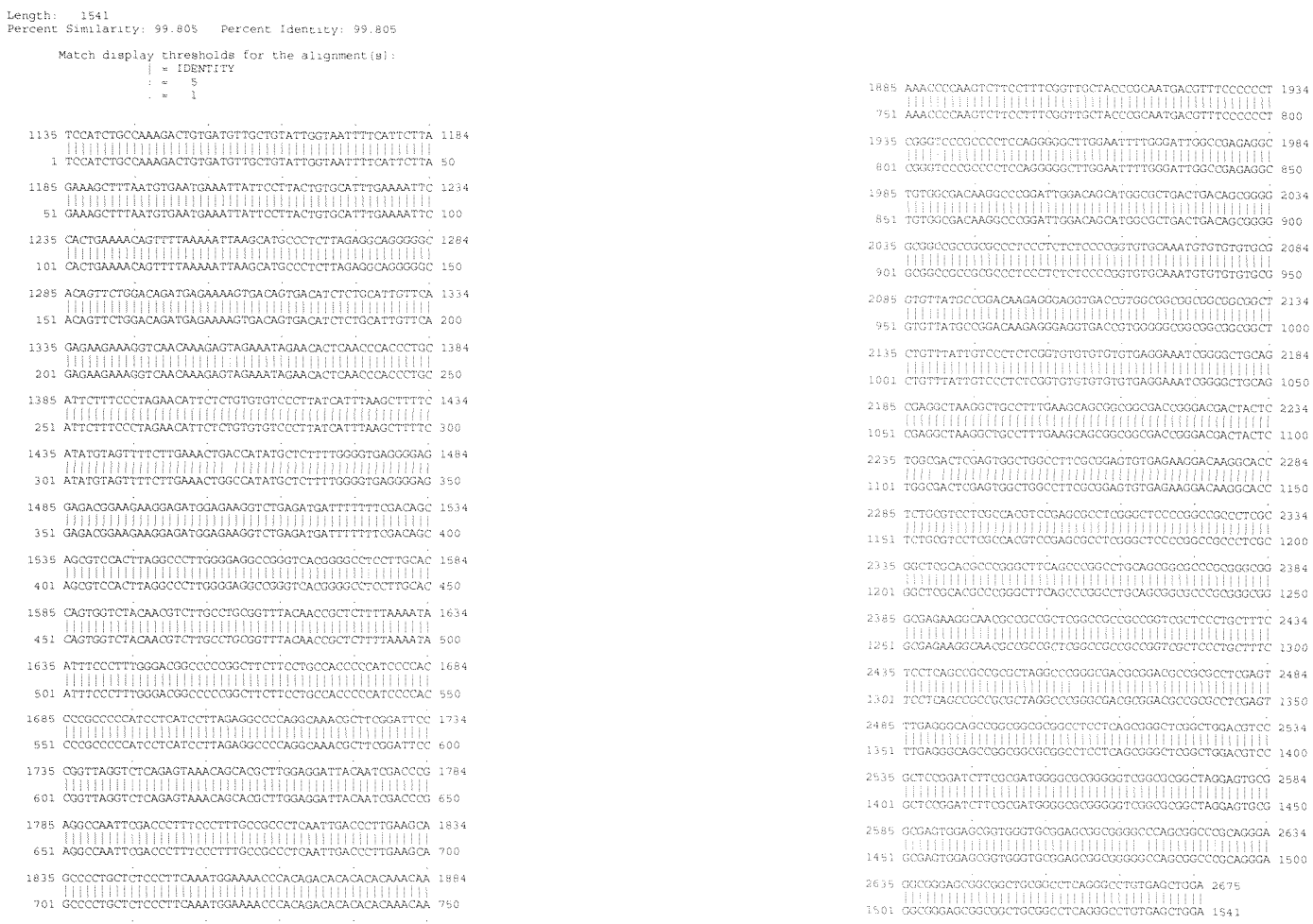
AccI	AlwNI	ApaI	AvaI	BglI	BsmAI	BsrBI	BstUI
EagI	EclXI	FokI	HaeIII	HgaI	HhaI	HincII	HindIII
MboI	MspI	NarI	NdeI	NotI	PstI	SmaI	SphI
TaqI	XhoI	XmaIII					

Enzymes that do not cut:

BamHI	BglII	BlnI	BstBI	BstXI	ClaI	EcoRI	KpnI
MluI	MscI	NcoI	NheI	NsiI	PvuI	PvuII	RsaI
SacI	SalI	ScaI	SfiI	SnaBI	SspI	XbaI	

3.5 Bestfit

Comparison between the topoisomerase IIβ promoter sequence and pGEMT-TIIβ-1357 construct.



Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1357 *construct sequence*

```
Length:   1484
Percent Similarity: 99.865   Percent Identity: 99.865

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1


1135 TCCATCTGCCAAAGACTGTGATGTTGCTGTATTGGTAATTTTCATTCTTA 1184
     |||||||||||||||||||||||||||||||||||||||||||||||||||
   1 TCCATCTGCCAAAGACTGTGATGTTGCTGTATTGGTAATTTTCATTCTTA 50

1185 GAAAGCTTTAATGTGAATGAAATTATTCCTTACTGTGCATTTGAAAATTC 1234
     |||||||||||||||||||||||||||||||||||||||||||||||||||
  51 GAAAGCTTTAATGTGAATGAAATTATTCCTTACTGTGCATTTGAAAATTC 100

1235 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 1284
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 101 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 150

1285 ACAGTTCTGGACAGATGAGAAAAGTGACAGTGACATCTCTGCATTGTTCA 1334
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 151 ACAGTTCTGGACAGATGAGAAAAGTGACAGTGACATCTCTGCATTGTTCA 200

1335 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACACCCTGC 1384
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 201 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACACCCTGC 250

1385 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 1434
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 251 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 300

1435 ATATGTAGTTTTCTTGAAACTGACCATATGCTCTTTTGGGGTGAGGGGAG 1484
     |||||||||||||||||||| ||||||||||||||||||||||||||||||
 301 ATATGTAGTTTTCTTGAAACTGGCCATATGCTCTTTTGGGGTGAGGGGAG 350

1485 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTCGACAGC 1534
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 351 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTCGACAGC 400

1535 AGCGTCCACTTAGGCCCTTGGGGAGGCCGGGTCACGGGGCCTCCTTGCAC 1584
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 401 AGCGTCCACTTAGGCCCTTGGGGAGGCCGGGTCACGGGGCCTCCTTGCAC 450

1585 CAGTGGTCTACAACGTCTTGCCTGCGGTTTACAACCGCTCTTTTAAAATA 1634
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 451 CAGTGGTCTACAACGTCTTGCCTGCGGTTTACAACCGCTCTTTTAAAATA 500

1635 ATTTCCCTTTGGGACGGCCCCCGGCTTCTTCCTGCCACCCCCATCCCCAC 1684
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 501 ATTTCCCTTTGGGACGGCCCCCGGCTTCTTCCTGCCACCCCCATCCCCAC 550

1685 CCCGCCCCCATCCTCATCCTTAGAGGCCCCAGGCAAACGCTTCGGATTCC 1734
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 551 CCCGCCCCCATCCTCATCCTTAGAGGCCCCAGGCAAACGCTTCGGATTCC 600

1735 CGGTTAGGTCTCAGAGTAAACAGCACGCTTGGAGGATTACAATCGACCCG 1784
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 601 CGGTTAGGTCTCAGAGTAAACAGCACGCTTGGAGGATTACAATCGACCCG 650

1785 AGGCCAATTCGACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCA 1834
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 651 AGGCCAATTCGACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCA 700

1835 GCCCCTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACAAACAA 1884
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 701 GCCCCTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACAAACAA 750

1885 AAACCCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCT 1934
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 751 AAACCCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCT 800

1935 CGGGTCCCGCCCCTCCAGGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGC 1984
     |||||||||||||||||||||||||||||||||||||||||||||||||||
 801 CGGGTCCCGCCCCTCCAGGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGC 850

1985 TGTGGCGACAAGGCCCGGATTGGACAGCATGGCGCTGACTGACAGCGGGG 2034
     |||||||||||||||||||||||||||||||||||||||||||||| ||||||
 851 TGTGGCGACAAGGCCCGGATTGGACAGCATGGCGCTGACTGACAGCGGGG 900

2035 GCGGCCGCCCGCCCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTGTGTGCG 2084
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 901 GCGGCCGCCCGCCCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTGTGTGCG 950

2085 GTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCT 2134
     |||| |||||||||||||||||||||||||||||||||||||||||||||||
 951 GTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCT 1000

2135 CTGTTTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAG 2184
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1001 CTGTTTATTGTCCCTCTCGGTGTGTGTGTGAGGAAATCGGGGCTGCAG 1050

2185 CGAGGCTAAGGCTGCCTTTGAAGCAGCCGGCGGCGACCGGGACGACTACTC 2234
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1051 CGAGGCTAAGGCTGCCTTTGAAGCAGCCGGCGGCGACCGGGACGACTACTC 1100

2235 TGGCGACTCGAGTGGCTGGCCTTCGGGGAGTGTGAGAAGGACAAGGCACC 2284
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1101 TGGCGACTCGAGTGGCTGGCCTTCGCGGAGTGTGAGAAGGACAAGGCACC 1150

2285 TCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCGCCCCTCGC 2334
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1151 TCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCGCCCCTCGC 1200

2335 GGCTCGCACGCCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCCGCGGGGCGG 2384
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1201 GGCTCGCACGCCCCGGGCTTCAGCCCGGCCTGCAGCGGCGCCCCGCGGGGCGG 1250

2385 GCGAGAAGGCAACGCCGCCGGCTCGGCCCGCCCCCGCCGGTCGCTCCCTGCTTTC 2434
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1251 GCGAGAAGGCAACGCCGCCGGCTCGGCCCGCCCCCGCCGGTCGCTCCCTGCTTTC 1300

2435 TCCTCAGCCGCCCCCCTAGGCCCGGGCGACGCCGACGCCCGCGCCTCGAGT 2484
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1301 TCCTCAGCCGCCCCCCTAGGCCCGGGCGACGCCGACGCCCGCGCCTCGAGT 1350

2485 TTGAGGGCAGCCCGGCGGCGCCGGCCTCCTCAGCCGGCTCCGGCTGGACGTCC 2534
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1351 TTGAGGGCAGCCCGGCGGCGCCGGCCTCCTCAGCCGGCTCCGGCTGGACGTCC 1400

2535 GCTCCGGATCTTCGCGATGGGGCGCGGGGGTCGGCGCGGCTAGGAGTGCG 2684
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2585 GCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC 2618
     |||||||||||||||||||||||||||||||| |
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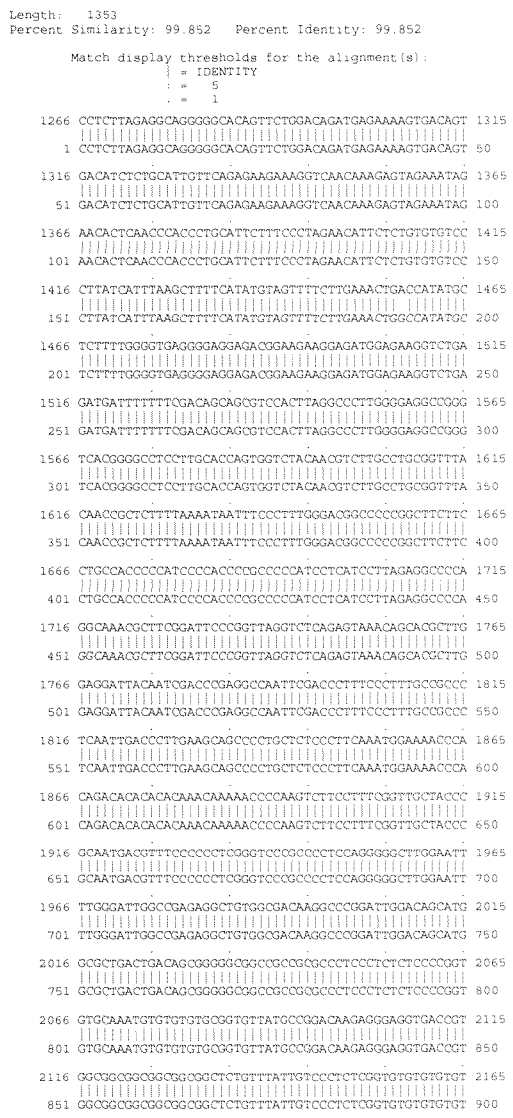
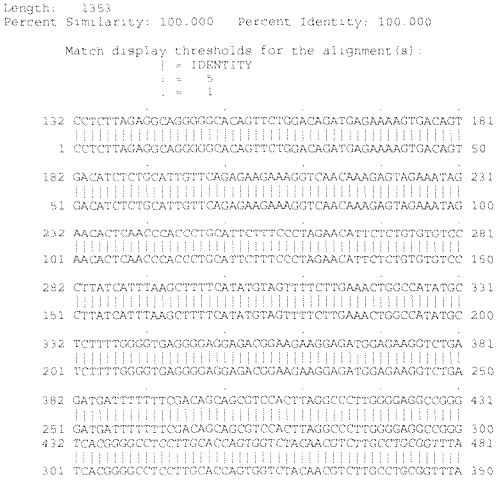
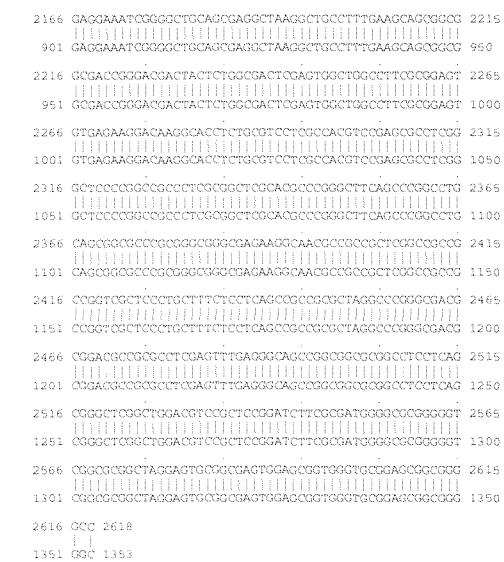
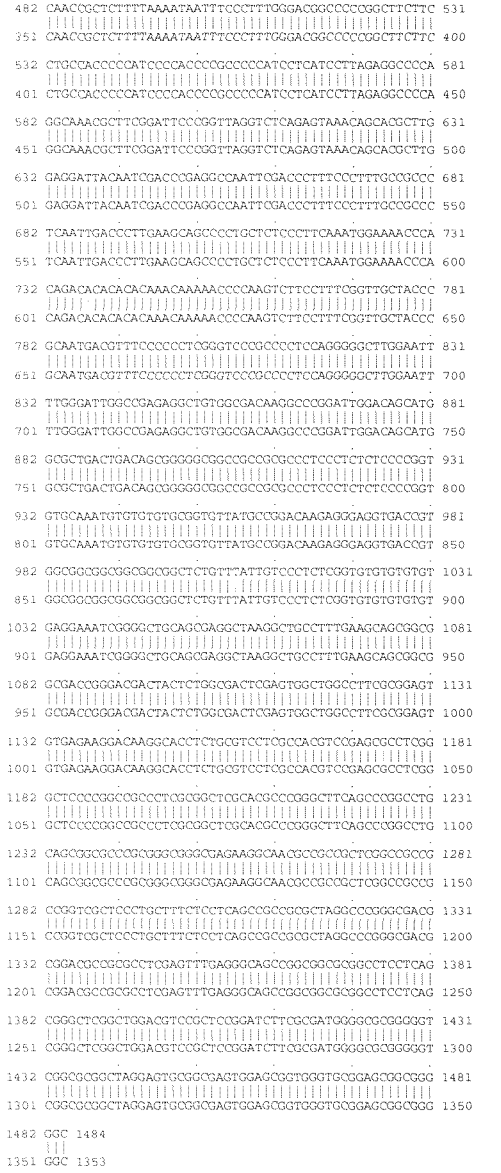
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1228 construct sequence



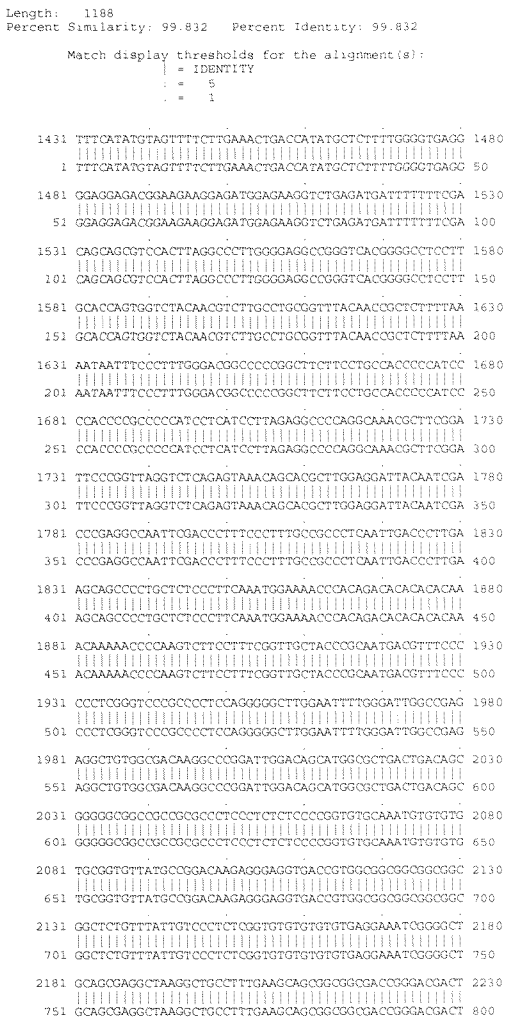
Comparison between pGL3B-TIIβ construct sequence and pGL3B-TIIβ-1228 construct sequence

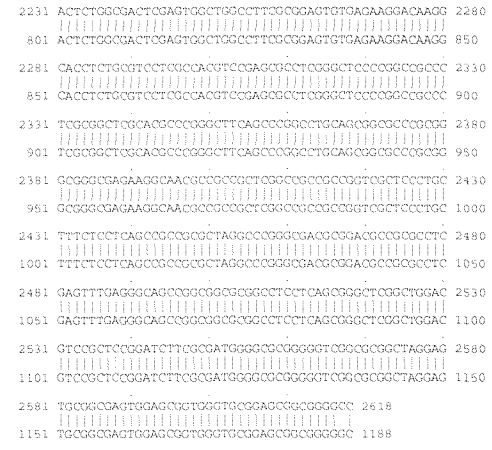




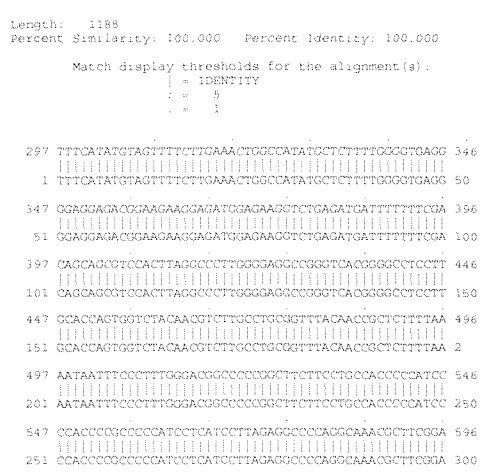


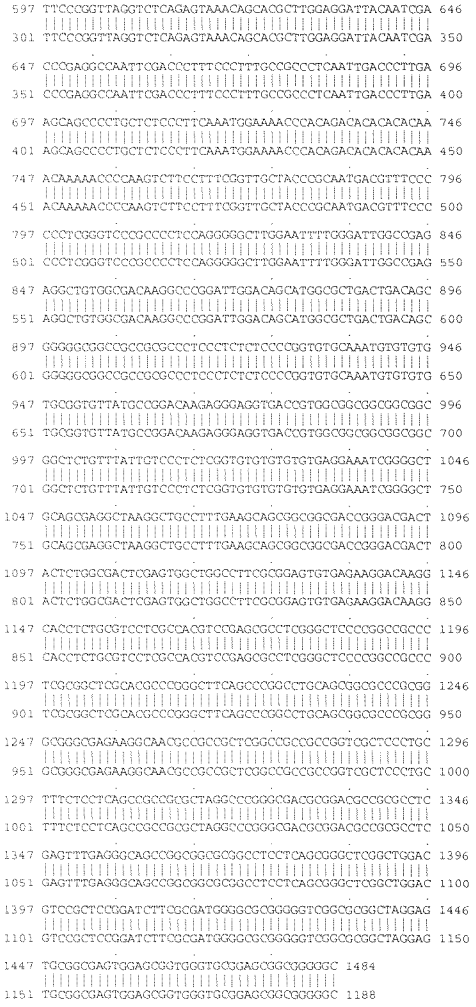
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1051 construct sequence





Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-1051 construct sequence

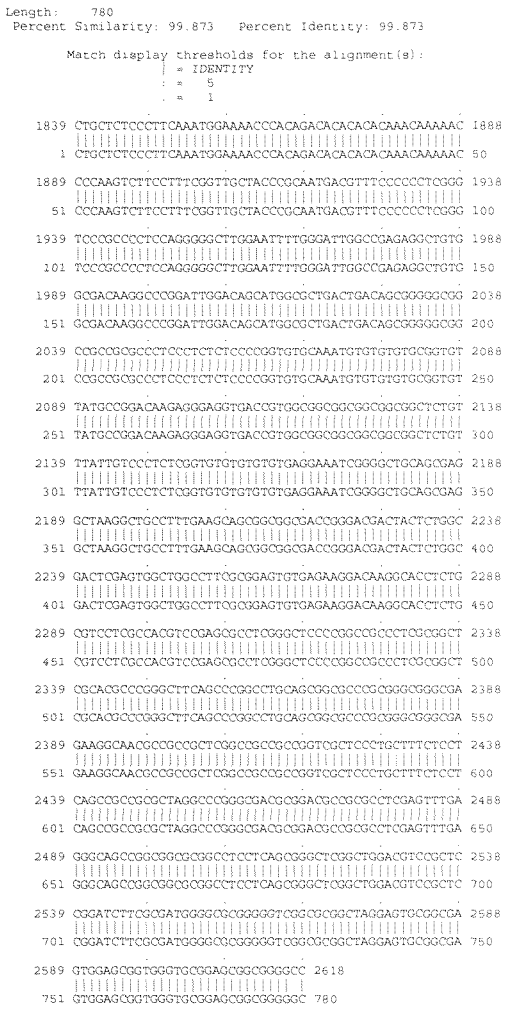




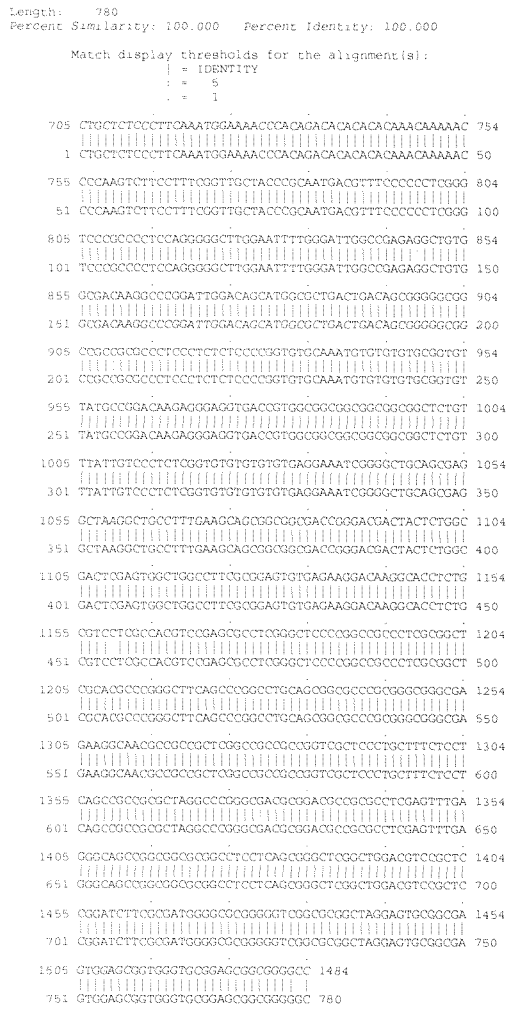
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-901 construct sequence

Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-901 construct sequence

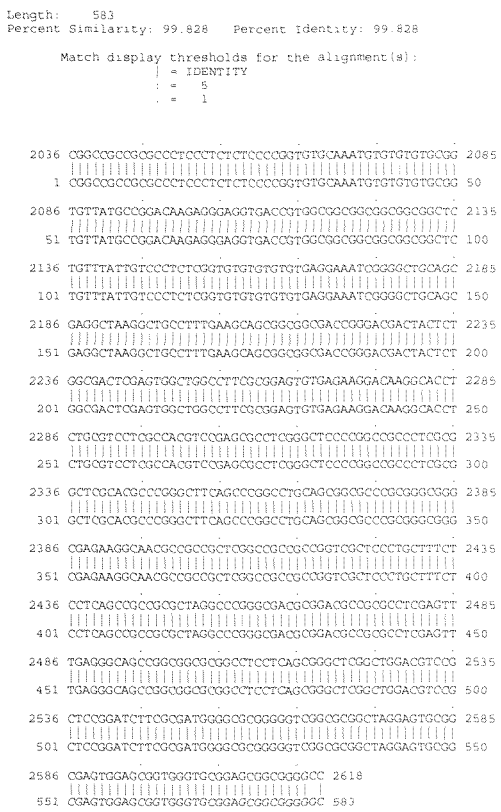
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-654 construct sequence



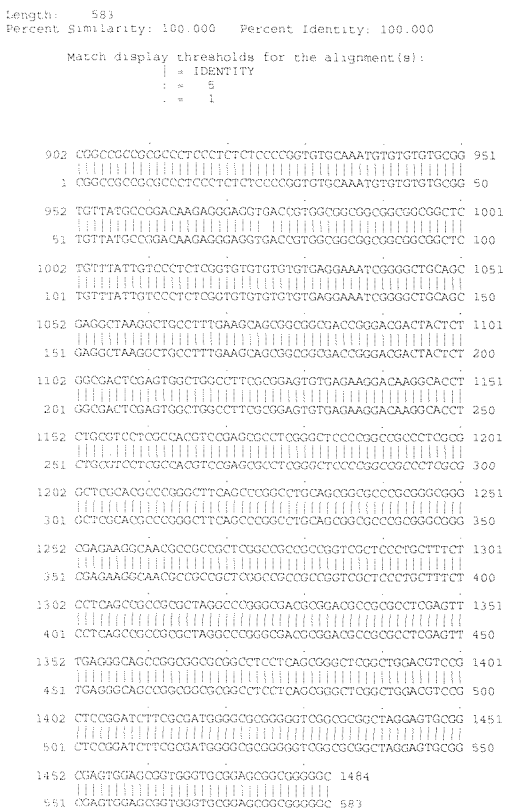
Comparison between pGL3B-TIIβ-1357 promoter sequence and pGL3B-TIIβ-654 construct sequence



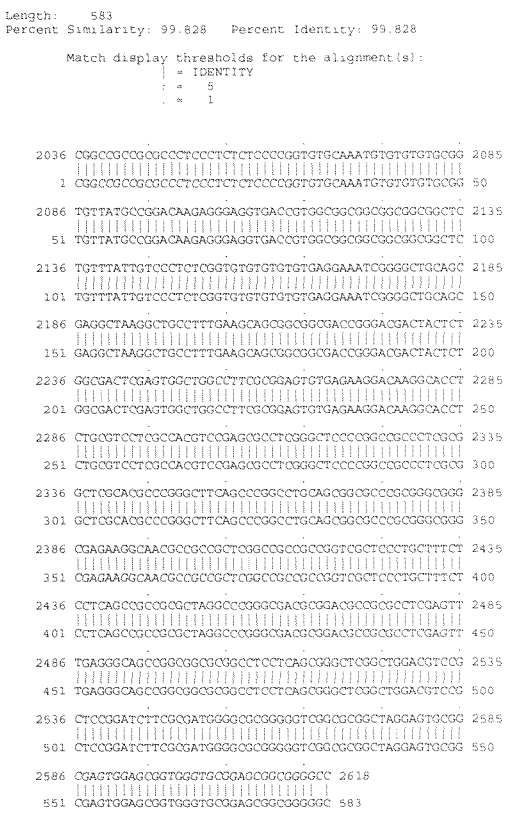
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-456 construct sequence



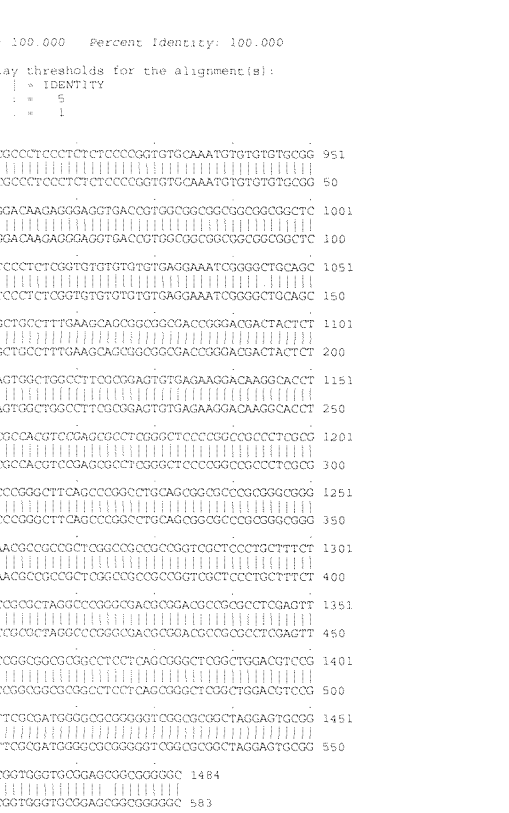
Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-456 construct sequence



Comparison between the topoisomerase IIβ promoter sequence and pBS-TIIβ-456 construct sequence



Comparison between pGL3B-TIIβ-1357 construct sequence and pBS-TIIβ-456 construct sequence



Comparison between pGL3B-TIIβ-1357 construct sequence and pBS-TIIβ-1357ID construct sequence

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Match display thresholds for the alignment(s):
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1185 GAAAGCTTTAATGTGAATGAAATTATTCCTTACTGTGCATTTGAAAATTC 1234
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1235 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 1284
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 101 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 150

1285 ACAGTTCTGGACAGATGAGAAAAGTGACAGTGACATCTCTGCATTGTTCA 1334
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1335 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACCCTGC 1384
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 201 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACCCTGC 250

1385 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 1434
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 251 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 300

1435 ATATGTAGTTTTCTTGAAACTGACCATATGCTCTTTTGGGGTGAGGGGAG 1484
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 301 ATATGTAGTTTTCTTGAAACTGGCCATATGCTCTTTTGGGGTGAGGGGAG 350

1485 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTTCGACAGC 1534
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 351 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTTCGACAGC 400

1535 AGCGTCCACTTAGGCCCTTGGGGAGGCCGGGTCACGGGGCCTCCTTGCAC 1584
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 401 AGCGTCCACTTAGGCCCTTGGGGAGGCCGGGTCACGGGGCCTCCTTGCAC 450

1585 CAGTGGTCTACAACGTCTTGCCTGCGGTTTACAACCGCTCTTTTAAAATA 1634
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 451 CAGTGGTCTACAACGTCTTGCCTGCGGTTTACAACCGCTCTTTTAAAATA 500

1635 ATTTCCCTTTGGGACGGCCCCCGGCTTCTTCCTGCCACCCCCATCCCCAC 1684
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1685 CCCGCCCCCATCCTCATCCTTAGAGGCCCCAGGCAAACGCTTCGGATTCC 1734
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1735 CGGTTAGGTCTCAGAGTAAACAGCACGCTTGGAGGATTACAATCGACCCG 1784
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1785 AGGCCAATTCGACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCA 1834
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 651 AGGCCAATTCGACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCA 700

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 701 G..................................................  701


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2085 GTGTTATGCCGGACAAGAGGGAGGTGACCGTGGCGGCGGCGGCGGCGGCT 2134
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2135 CTGTTTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAG 2184
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 800 CTGTTTATTGTCCCTCTCGGTGTGTGTGTGTGAGGAAATCGGGGCTGCAG 849
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2235 TGGCGACTCGAGTGGCTGGCCTTCGCGGAGTGTGAGAAGGACAAGGCACC 2284
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 900 TGGCGACTCGAGTGGCTGGCCTTCGCGGAGTGTGAGAAGGACAAGGCACC 949

2285 TCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCCGCCCTCGC 2334
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1050 GCGAGAAGGCAACGCCGCCGCTCGGCCGCCGCCGGTCGCTCCCTGCTTTC 1099

2435 TCCTCAGCCGCCGCGGCTAGGCCCGGGCGACGGGACGCCGGCGCCTCGAGT 2484
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1100 TCCTCAGCCGCCGCGGCTAGGCCCGGGCGACGGGACGCCGGCGCCTCGAGT 1149

2485 TTGAGGGCAGCCGGCGGGGCGGCGCTCCTCAGCGGGCTCGGCTGGACGTCC 2534
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1150 TTGAGGGCAGCCGGCGGGGCGGCGCTCCTCAGCGGGCTCGGCTGGACGTCC 1199

2535 GCTCCCGATCTTCGCGATGGGGCGCGGGGGTCGGCGCGGCTAGGAGTGCG 2584
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1200 GCTCCGGATCTTCGCGATGGGGCGCGGGGGTCGGCGCGGCTAGGAGTGCG 1249

2585 GCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGCC 2618
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1250 GCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGGC 1283
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Comparison between pGL3B-TIIβ-1357 construct sequence and pBS-TIIβ-1357ID construct sequence

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Length:   1484
Percent Similarity: 100.000   Percent Identity: 100.000
Match display thresholds for the alignment(s):
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   1 TCCATCTGCCAAAGACTGTGATGTTGCTGTATTGGTAATTTTCATTCTTA 50
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   1 TCCATCTGCCAAAGACTGTGATGTTGCTGTATTGGTAATTTTCATTCTTA 50

  51 GAAAGCTTTAATGTGAATGAAATTATTCCTTACTGTGCATTTGAAAATTC 100
     ||||||||||||||||||||||||||||||||||||||||||||||||||
  51 GAAAGCTTTAATGTGAATGAAATTATTCCTTACTGTGCATTTGAAAATTC 100

 101 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 150
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 101 CACTGAAAACAGTTTTAAAAATTAAGCATGCCCTCTTAGAGGCAGGGGGC 150

 151 ACAGTTCTGGACAGATGAGAAAAGTGACAGTGACATCTCTGCATTGTTCA 200
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 151 ACAGTTCTGGACAGATGAGAAAAGTGACAGTGACATCTCTGCATTGTTCA 200

 201 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACCCTGC 250
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 201 GAGAAGAAAGGTCAACAAAGAGTAGAAATAGAACACTCAACCCACCCTGC 250

 251 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 300
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 251 ATTCTTTCCCTAGAACATTCTCTGTGTGTCCCTTATCATTTAAGCTTTTC 300

 301 ATATGTAGTTTTCTTGAAACTGGCCATATGCTCTTTTGGGGTGAGGGGAG 350
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 301 ATATGTAGTTTTCTTGAAACTGGCCATATGCTCTTTTGGGGTGAGGGGAG 350

 351 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTTCGACAGC 400
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 351 GAGACGGAAGAAGGAGATGGAGAAGGTCTGAGATGATTTTTTTTCGACAGC 400

 401 AGCGTCCACTTAGGCCCTTGGGGAGGCCGGGTCACGGGGCCTCCTTGCAC 450
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 501 ATTTCCCTTTGGGACGGCCCCCGGCTTCTTCCTGCCACCCCCATCCCCAC 550

 551 CCCGCCCCCATCCTCATCCTTAGAGGCCCCAGGCAAACGCTTCGGATTCC 600
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 551 CCCGCCCCCATCCTCATCCTTAGAGGCCCCAGGCAAACGCTTCGGATTCC 600

 601 CGGTTAGGTCTCAGAGTAAACAGCACGCTTGGAGGATTACAATCGACCCG 650
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 601 CGGTTAGGTCTCAGAGTAAACAGCACGCTTGGAGGATTACAATCGACCCG 650

 651 AGGCCAATTCGACCCTTTCCCTTTGCCGCCCTCAATTGACCCTTGAAGCA 700
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 701 GCCCCTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAA 750
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 701 G..................................................  701


 901 GCGGCCGCCGCGCCCTCCCTCTCTCCCCGGTGTGCAAATGTGTGTGTGCG 950
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1151 TCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCCGCCCTCGC 1200
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 950 TCTGCGTCCTCGCCACGTCCGAGCGCCTCGGGCTCCCCGGCCGCCCTCGC 999

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1351 TTGAGGGCAGCCGGCGGGGCGGCGCTCCTCAGCGGGCTCGGCTGGACGTCC 1400
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1150 TTGAGGGCAGCCGGCGGGGCGGCGCTCCTCAGCGGGCTCGGCTGGACGTCC 1199

1401 GCTCCGGATCTTCGCGATGGGGCGCGGGGGTCGGCGCGGCTAGGAGTGCG 1450
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1200 GCTCCGGATCTTCGCGATGGGGCGCGGGGGTCGGCGCGGCTAGGAGTGCG 1249

1451 GCGAGTGGAGCGGTGGGTGCGGAGCGGCGGGGGC 1484
     |||||||||||||||||||||||||||||||||
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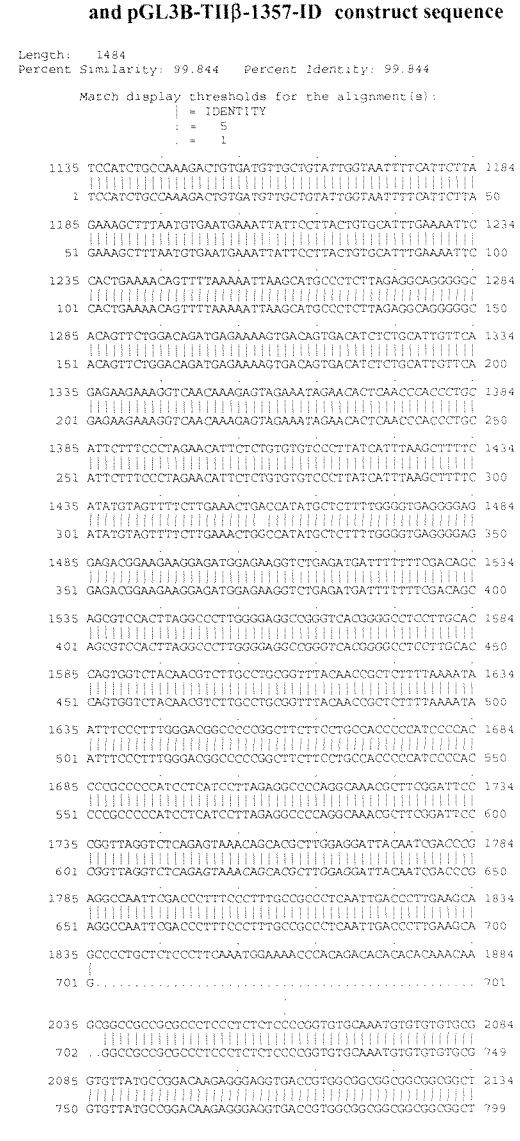
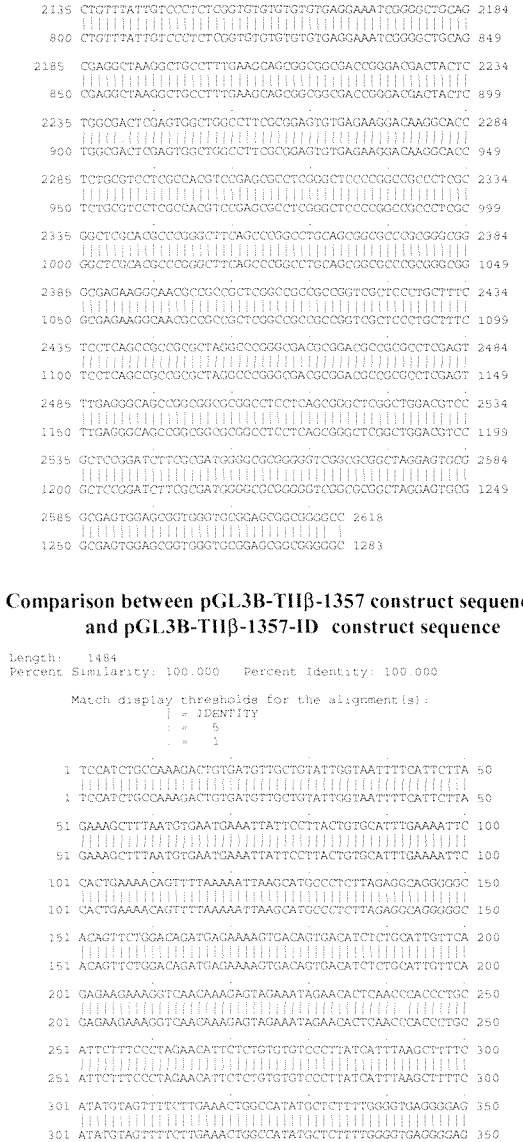
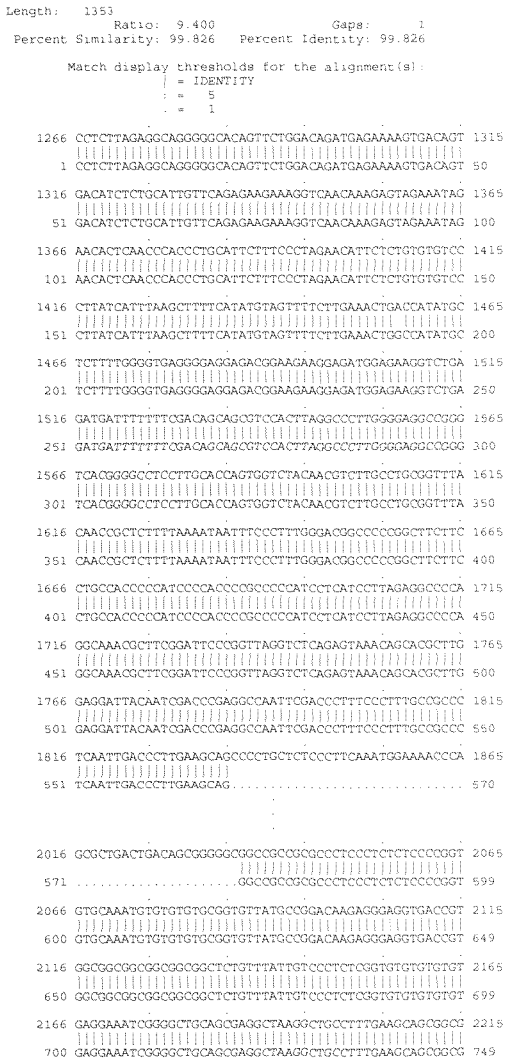
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1357-ID construct sequence



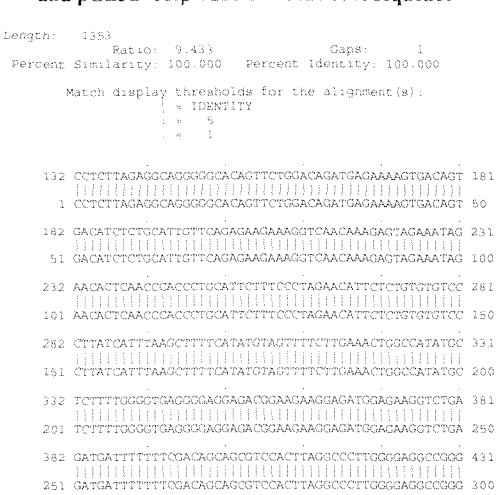


Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-1357-ID construct sequence

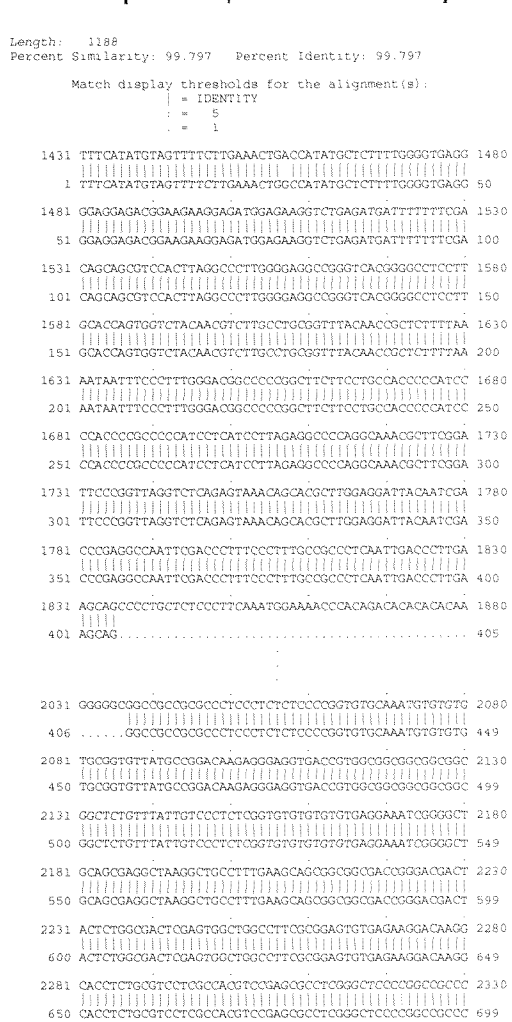
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1228-ID construct sequence



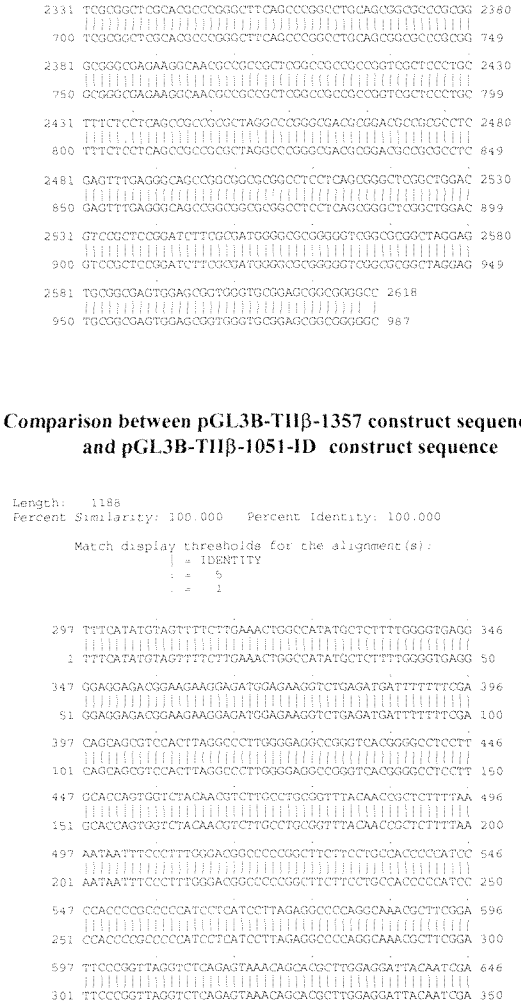
Comparison between pGL3B-TIIβ construct sequence and pGL3B-TIIβ-1228-ID construct sequence

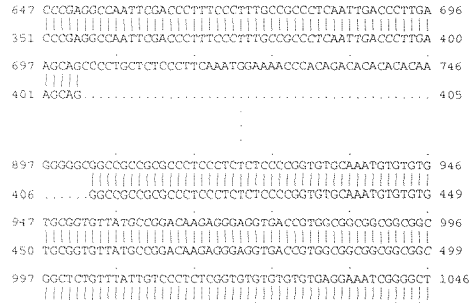


Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-1051-ID construct sequence

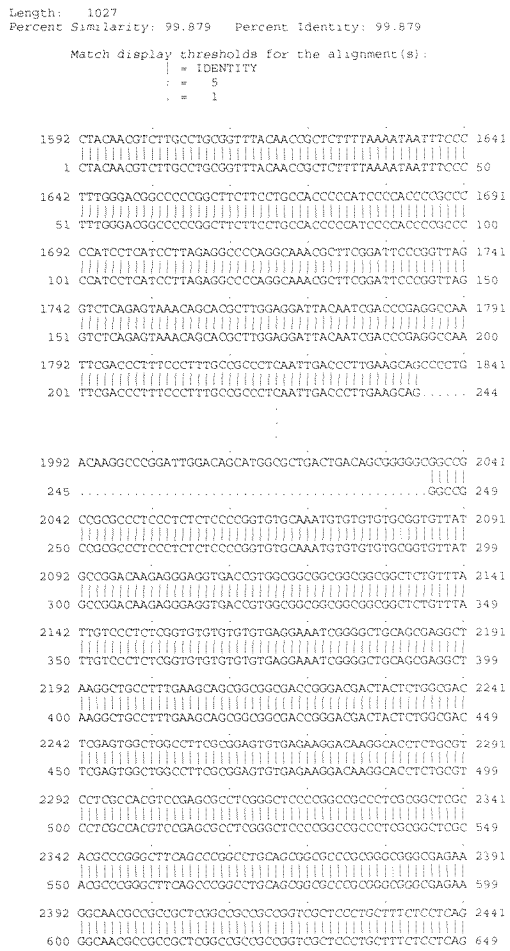


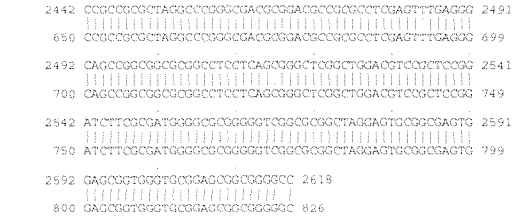
Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-1051-ID construct sequence



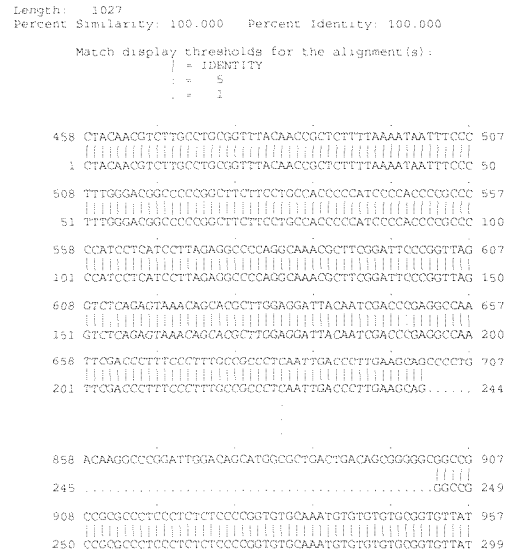


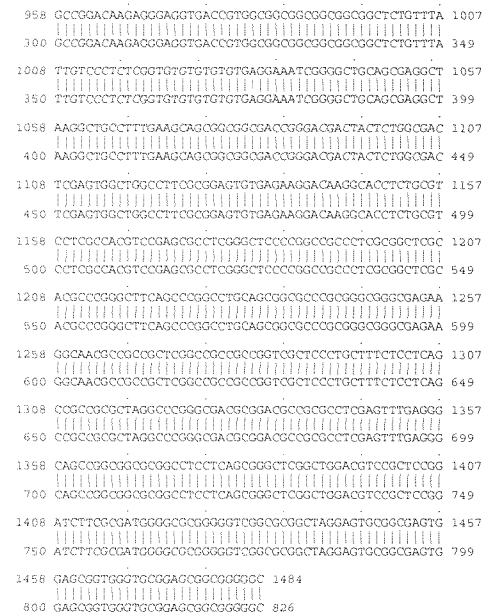
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-901-ID construct sequence



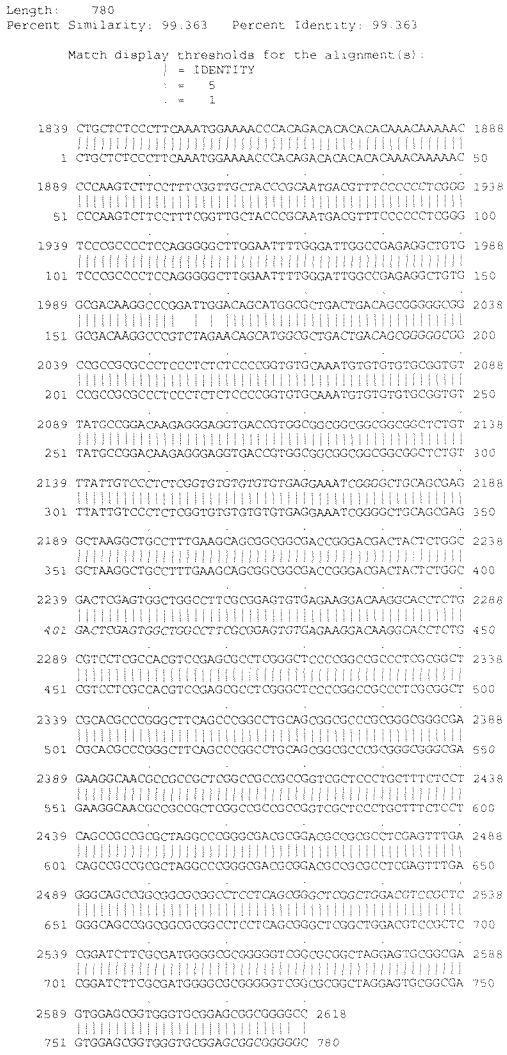


Comparison betweenpGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-901-ID construct sequence

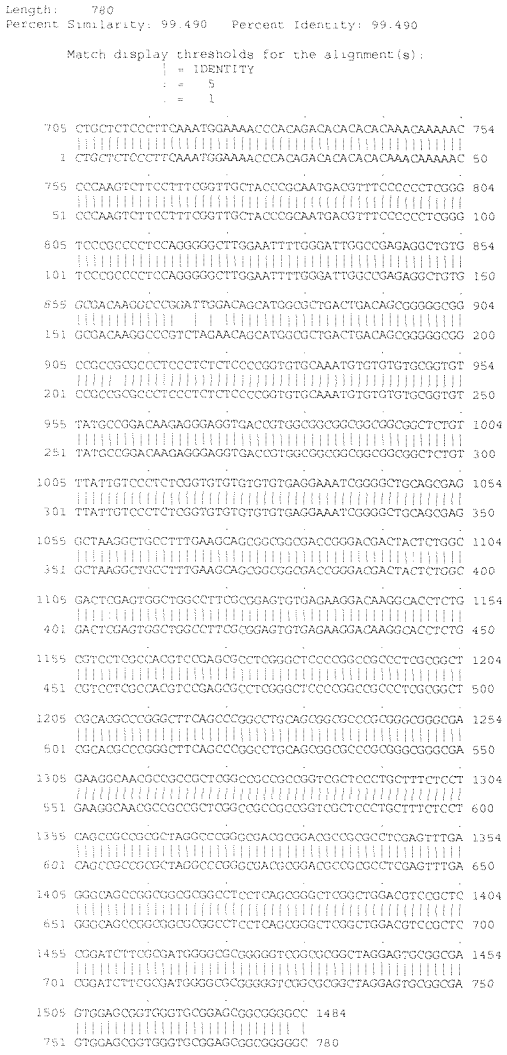




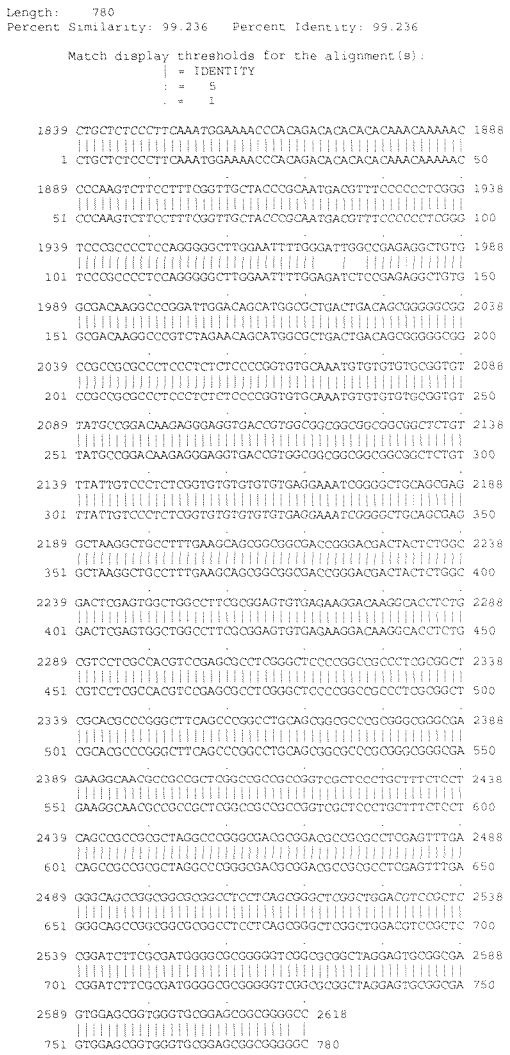
Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-ICB1mt construct sequence



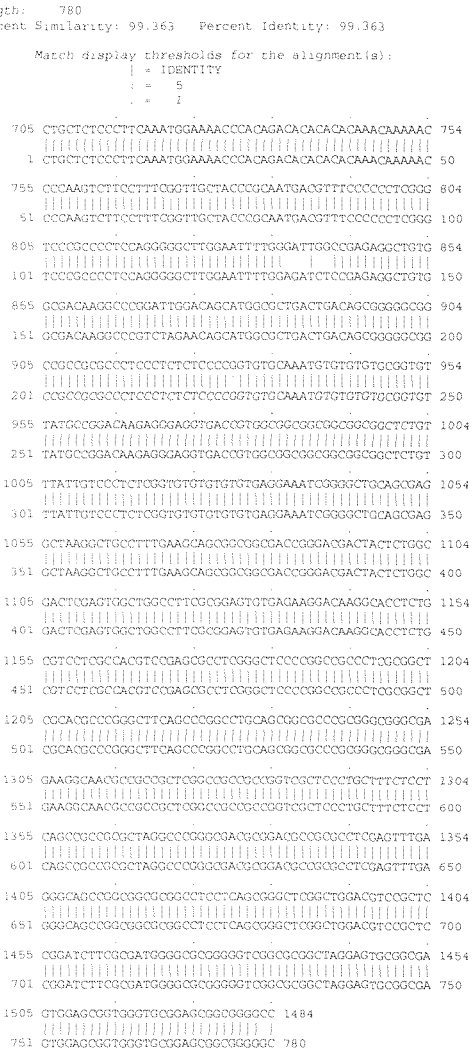
Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-ICB1mt construct sequence



Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-ICB2 mt construct sequence



Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-ICB2mt construct sequence



Comparison between topoisomerase IIβ promoter sequence and pGL3B-TIIβ-180 construct sequence

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Percent Similarity: 100.000   Percent Identity: 100.000

        Match display thresholds for the alignment(s):
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         |||||||||||||||||||||||||||||||||||||||||||||||||||
       1 CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAAC 50

    1889 CCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGG 1938
         |||||||||||||||||||||||||||||||||||||||||||||||||||
      51 CCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGG 100

    1939 TCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTGTG 1988
         |||||||||||||||||||||||||||||||||||||||||||||||||||
     101 TCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTGTG 150

    1989 GCGACAAGGCCCGGATTGGACAGCATGGCG 2018
         ||||||||||||||||||||||||||||||
     151 GCGACAAGGCCCGGATTGGACAGCATGGCG 180
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Comparison between pGL3B-TIIβ-1357 construct sequence and pGL3B-TIIβ-180 construct sequence

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Length:    180
Percent Similarity: 100.000   Percent Identity: 100.000

        Match display thresholds for the alignment(s):
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         |||||||||||||||||||||||||||||||||||||||||||||||||||
       1 CTGCTCTCCCTTCAAATGGAAAACCCACAGACACACACACAAACAAAAAC 50

    1889 CCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGG 1938
         |||||||||||||||||||||||||||||||||||||||||||||||||||
      51 CCCAAGTCTTCCTTTCGGTTGCTACCCGCAATGACGTTTCCCCCCTCGGG 100

    1939 TCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTGTG 1988
         |||||||||||||||||||||||||||||||||||||||||||||||||||
     101 TCCCGCCCCTCCAGGGGGCTTGGAATTTTGGGATTGGCCGAGAGGCTGTG 150

    1989 GCGACAAGGCCCGGATTGGACAGCATGGCG 2018
         ||||||||||||||||||||||||||||||
     151 GCGACAAGGCCCGGATTGGACAGCATGGCG 180
```

3.6 Search of potential transcription factor binding sites.

```
! FINDPATTERNS on
/bionavigator/cell/girlzone/151825/nuc.gcg_n
uc allowing 0 mismatches

! Using patterns from:
GenMoreData:tfsites.dat

        nuc.gcg_nuc  ck: 2564  len: 2,734 !
null

CACA                    CACACACACA
          1,870: ACAGA CACACACACA AACAA

CACA /Rev               TGTGTGTGTG
          2,073: GCAAA TGTGTGTGTG CGGTG
          2,155: CTCGG TGTGTGTGTG TGAGG
          2,157: CGGTG TGTGTGTGTG AGGAA

Pit-1_CS1               WTATYCAT
             60: TTGTT TTATTCAT TTCAA

LVa_RS /Rev             CTGTTC
            676: CTTCA CTGTTC TGAAG

Sp1_CS1 /Rev            RYYCCGCCCM
          1,683: TCCCC ACCCCGCCCC CATCC
          1,938: CTCGG GTCCCGCCCC TCCAG

MRE_CS2                 TGCRCNC
            643: AAGTT TGCACCC CAAGT
          1,075: GAGTG TGCGCCC AATAA

MRE_CS2 /Rev            GNGYGCA
            304: GTGGA GGGTGCA GTGAG
          2,064: CCCCG GTGTGCA AATGT

TATA-box.2              TATAAA
            899: ATCTT TATAAA AAGCT
          1,000: CAGGT TATAAA TGGTT

TATA-box.2 /Rev         TTTATA
            441: TGTAT TTTATA GCTTA
            897: TCATC TTTATA AAAAG

FSE2.1                  GAGAGGA
            830: CCCTT GAGAGGA AATGC

AP-2_CS2                CCCCAGGC
          1,711: AGAGG CCCCAGGC AAACG

CP2-gamma-FBG /Rev      AGTGGCT
          2,245: ACTCG AGTGGCT GGCCT

BGP1_RS1                GGGCGG
            138: AAGAT GGGCGG CTCAC
          2,033: AGCGG GGGCGG CCGCC
          2,379: CCCGC GGGCGG GCGAG
          2,724: GGCGT GGGCGG CGGCA

BGP1_RS1 /Rev           CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG

C/EBP_CS1               TKNNGYAAK
                1:      TGAGGCAAG TGAAA

C/EBP_CS1 /Rev          MTTRCNNMA
          1,578: GCCTC CTTGCACCA GTGGT

his3-Tr-TATA            TATAAA
            899: ATCTT TATAAA AAGCT
          1,000: CAGGT TATAAA TGGTT

his3-Tr-TATA /Rev       TTTATA
            441: TGTAT TTTATA GCTTA
            897: TCATC TTTATA AAAAG

Sp1_CS2                 GGGCGG
            138: AAGAT GGGCGG CTCAC
          2,033: AGCGG GGGCGG CCGCC
          2,379: CCCGC GGGCGG GCGAG
          2,724: GGCGT GGGCGG CGGCA

Sp1_CS2 /Rev            CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG

E4TF1_CS                RTGACGT
          1,919: CCGCA ATGACGT TTCCC

AP-2_CS3                CCSCRGGC
          1,711: AGAGG CCCCAGGC AAACG
          2,375: GGCGC CCGCGGGC GGGCG

AP-2_CS3 /Rev           GCCYGSGG
          1,804: GTCTT GCCTGCGG TTTAC
          2,373: GCGGC GCCCGCGG GCGGG

GH-CSE1                 TAAATTA
            743: ATGTT TAAATTA TTTCC

GH-CSE2                 AATAAAT
          1,082: CGCCC AATAAAT ACTTG

APRT-mouse_US /Rev      GGCGGGGC
          2,610: GGAGC GGCGGGGC CCAGC

HC3 /Rev                TGGTGG
            231: AGGCA TGGTGG CCGGT
            925: CTCTT TGGTGG CACTT

NFI.2 /Rev              GATTGGC
          1,970: TTTGG GATTGGC CGAGA

SP1-1E3/2 /Rev          ACCCCGCCCC
          1,683: TCCCC ACCCCGCCCC CATCC

SP1-SV/6 /Rev           GTCCCGCCCC
          1,938: CTCGG GTCCCGCCCC TCCAG

ETF_RS2                 CCCCCGGC
          1,852: GACGG CCCCCGGC TTCTT

AP-2_CS4                YCSCCMNSSS
          1,672: TGCCA CCCCCATCCC CACCC
          1,678: CCCCA TCCCCACCCC GCCCC
          1,925: CGTTT CCCCCCTCGG GTCCC
          2,325: CCCGG CCGCCCTCGC GGCTC
          2,681: GGCAC TCGCCATGGC CAAGT

AP-2_CS4 /Rev           SSSNKGGSGR
          1,549: TTAGG CCCTTGGGGA GGCCG
          2,029: TGACA GCGGGGGCGG CCGCC
          2,375: GGCGC CCGCGGGCGG GCGAG
          2,379: CCCGC GGGCGGGCGA GAAGG
          2,454: GCTAG GCCCGGGCCA CGCGG
          2,720: AGCCG GCGTGGGCGG CGGCA

PEA3_RS                 AGGAAG
          1,115: AAAAC AGGAAG AAGTA

PEA3_RS /Rev            CTTCCT
          1,662: GGCTT CTTCCT GCCAC
          1,896: CAAGT CTTCCT TTCGG

XRE_CS1                 CACGCW
          1,758: AACAG CACGCT TGGAG

XRE_CS1 /Rev            WGCGTG
          1,061: GGCAC AGCGTG GCAGA

NF-E1_CS1               MYWATCWY
          1,416: TGTCC CTTATCAT TTAAG

NF-E1.5 /Rev            AAGATA
            390: AACGG AAGATA AAGAG

NF-E1.7                 TCATCAC
            911: AGCTT TCATCAC AACTC

CREB_CS1 /Rev           TGACGT
          1,920: CGCAA TGACGT TTCCC

CuE1.1 /Rev             GCCATCTT
          1,128: GTAAA GCCATCTT CCATC

AT-Alu /Rev             GYCTGTAATCCCAGCT
              242: CCGGT GCCTGTAATCCCAGCT ACCTG

MPa1.1 /Rev             AATTACAC
          1,099: AGTGG AATTACAC TGAAA

MPa2.1                  ATGTATTT
            435: CAGCT ATGTATTT TATAG

MPa2.1 /Rev             AAATACAT
            516: AAACA AAATACAT GTGTA

a-actin_US /Rev         GGCGAC
          1,988: GCTGT GGCGAC AAGGC
          2,215: GCGGC GGCGAC CGGGA
          2,236: ACTCT GGCGAC TCGAG
          2,459: GCCCG GGCGAC GCGGA

actin_5c_US             TATAAAA
            899: ATCTT TATAAAA AGCTT

actin_5c_US /Rev        TTTTATA
            440: ATGTA TTTTATA GCTTA

Ad2MLP_US.3             TATAAA
            899: ATCTT TATAAA AAGCT
          1,000: CAGGT TATAAA TGGTT

Ad2MLP_US.3 /Rev        TTTATA
            441: TGTAT TTTATA GCTTA
            897: TCATC TTTATA AAAAG

Ad2MLP_US.4             TATAAAA
            899: ATCTT TATAAAA AGCTT

Ad2MLP_US.4 /Rev        TTTTATA
            440: ATGTA TTTTATA GCTTA

Ad2MLP_US.5             TATAAAA
            899: ATCTT TATAAAA AGCTT

Ad2MLP_US.5 /Rev        TTTTATA
            440: ATGTA TTTTATA GCTTA

Adh1_US2                CCCCGG
          1,653: ACGGC CCCCGG CTTCT
          2,059: TCTCT CCCCGG TGTGC
          2,319: GGGCT CCCCGG CGGCC

NFI_CS5                 TCGNNNNNGCCAA
          2,681: GGCAC TCGCCATGGCCAA GTCGG

bA-globin.1 /Rev        CCCGCCCC
          1,685: CCCAC CCCGCCCC CATCC
          1,940: CGGGT CCCGCCCC TCCAG

GRE_CS8 /Rev            WCTGWTCT
            675: GCTTC ACTGTTCT GAAGT

GCN4-HIS3.4 /Rev        GAGTAA
          1,748: TCTCA GAGTAA ACAGC

GCN4-HIS4.3 /Rev        TGACTG
          2,020: GGCGC TGACTG ACAGC

H4TF-1hist /Rev         GAAATC
          1,012: GGTTT GAAATC AGGGG
          2,169: GTGAG GAAATC GGGGC

MLTF-HMGCoAred /Rev     GTCACG
          1,565: GCCGG GTCACG GGGCC

CTF/CBP-hs              GATTGG
          1,970: TTTGG GATTGG CCGAG
          2,002: GCCCG GATTGG ACAGC

hsp70.5                 GATTGG
          1,970: TTTGG GATTGG CCGAG
          2,002: GCCCG GATTGG ACAGC

Sp1-hsp70_(1)           GGCGGG
          2,380: CCGCG GGCGGG CGAGA
          2,610: GGAGC GGCGGG GCCCA
          2,635: AGGGA GGCGGG AGCGG

Sp1-hsp70_(1) /Rev      CCCGCC
          1,685: CCCAC CCCGCC CCCAT
          1,940: CGGGT CCCGCC CCTCC

hsp70.2                 GGCGGG
          2,380: CCGCG GGCGGG CGAGA
          2,610: GGAGC GGCGGG GCCCA
          2,635: AGGGA GGCGGG AGCGG

hsp70.2 /Rev            CCCGCC
          1,685: CCCAC CCCGCC CCCAT

(TFIID/TBF)-RS          TATAAA
            899: ATCTT TATAAA AAGCT
          1,000: CAGGT TATAAA TGGTT

(TFIID/TBF)-RS /Rev     TTTATA
            441: TGTAT TTTATA GCTTA
            897: TCATC TTTATA AAAAG

IE1.2                   CTTTCC
          1,388: GCATT CTTTCC CTAGA
          1,799: CGACC CTTTCC CTTTG

Sp1-IE-3.1              CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG

Sp1-IE-3.1 /Rev         GGGCGG
            138: AAGAT GGGCGG CTCAC
          2,033: AGCGG GGGCGG CCGCC
          2,379: CCCGC GGGCGG GCGAG
          2,724: GGCGT GGGCGG CGGCA

Sp1-IE-3.2              GGGCGG
            138: AAGAT GGGCGG CTCAC
          2,033: AGCGG GGGCGG CCGCC
          2,379: CCCGC GGGCGG GCGAG
          2,724: GGCGT GGGCGG CGGCA

Sp1-IE-3.2 /Rev         CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG

Sp1-IE-3.3              CCCGCC
          1,685: CCCAC CCCGCC CCCAT
          1,940: CGGGT CCCGCC CCTCC

Sp1-IE-3.3 /Rev         GGCGGG
          2,380: CCGCG GGCGGG CGAGA
          2,610: GGAGC GGCGGG GCCCA
          2,635: AGGGA GGCGGG AGCGG

Sp1-IE-3.4              CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG

Sp1-IE-3.4 /Rev         GGGCGG
            138: AAGAT GGGCGG CTCAC
          2,033: AGCGG GGGCGG CCGCC
          2,379: CCCGC GGGCGG GCGAG
          2,724: GGCGT GGGCGG CGGCA

Sp1-IE-3.5              CCGCCC
          1,686: CCACC CCGCCC CCATC
          1,810: CTTTG CCGCCC TCAAT
          1,941: GGGTC CCGCCC CTCCA
          2,325: CCCGG CCGCCC TCGCG
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Sp1-IE-3.5 /Rev       GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

Sp1-IE-4/5            CCCGCC
        1,685: CCCAC CCCGCC CCCAT
        1,940: CGGGT CCCGCC CCTCC

Sp1-IE-4/5 /Rev       GGCGGG
        2,380: CCGCG GGCGGG CGAGA
        2,610: GGAGC GGCGGG GCCCA
        2,635: AGGGA GGCGGG AGCGG

Sp1-IE-4/5.2          CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

Sp1-IE-4/5.2 /Rev     GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

GCN4-ILV1.2           TGAGTG
        1,092: ATACT TGAGTG GAATT

GCN4-ILV1.2 /Rev      CACTCA
        1,368: TAGAA CACTCA ACCCA

IgHC.20 /Rev          GCCATCTT
        1,128: GTAAA GCCATCTT CCATC

IgHC.19 /Rev          ATTTTCAT
        1,172: TGGTA ATTTTCAT TCTTA

lysozyme-TATA /Rev    TTTTAAC
          566: GATCA TTTTAAC AAGCA

MT-I.1                GCACTC
        2,677: TGGAG GCACTC GCCAT

MT-I.1 /Rev           GAGTGC
        2,578: GCTAG GAGTGC GGCGA

(Sp1)-MT-I.1          GGGGGCGG
        2,031: ACAGC GGGGGCGG CCGCC

(Sp1)-MT-I.1 /Rev     CCGCCCCC
        1,686: CCACC CCGCCCCC ATCCT

MT-I.2 /Rev           CGGTGTGCA
        2,062: CTCCC CGGTGTGCA AATGT

GR-MT-IIA /Rev        AGGACA
        2,272: TGAGA AGGACA AGGCA

LVa-Mo-MuLV /Rev      CTGTTC
          676: CTTCA CTGTTC TGAAG

c-mos_DS1             TGGTTTG
        1,006: ATAAA TGGTTTG AAATC

c-mos_DS3             GTTTTAA
        1,246: AAACA GTTTTAA AAATT

LSF-SV40_(1)          CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

LSF-SV40_(1) /Rev     GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

Sp1-SV40.4            GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

Sp1-SV40.4 /Rev       CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

Sp1-SV40.1 /Rev       CCCGCCCC
        1,685: CCCAC CCCGCCCC CATCC
        1,940: CGGGT CCCGCCCC TCCAG

U2snRNA.1             STGCAAAT
        2,066: CCGGT GTGCAAAT GTGTG

(Sp1)-U2snR.1         GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

(Sp1)-U2snR.1 /Rev    CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

(Sp1)-U2snR.2         ACGCCC
        2,342: CTCGC ACGCCC GGGCT

(Sp1)-U2snR.3         GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

(Sp1)-U2snR.3 /Rev    CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

GR-uteroglobin.1      TGTTCT
          677: TTCAC TGTTCT GAAGT

GR-uteroglobin.1 /Rev  AGAACA
           15: TGAAA AGAACA TTATT
          552: CAGTA AGAACA CAAGA
        1,364: GAAAT AGAACA CTCAA
        1,396: TCCCT AGAACA TTCTC

GR-uteroglobin.2      TGTTCT
          677: TTCAC TGTTCT GAAGT

GR-uteroglobin.2 /Rev  AGAACA
           15: TGAAA AGAACA TTATT
          552: CAGTA AGAACA CAAGA
        1,364: GAAAT AGAACA CTCAA
        1,396: TCCCT AGAACA TTCTC

WAP_US5               CCAAGT
          649: GCACC CCAAGT TCAAG
        1,890: AAACC CCAAGT CTTCC
        2,690: CATGG CCAAGT CGGGT

WAP_US5 /Rev          ACTTGG
          528: TGTGT ACTTGG AGATG
          620: TGGAT ACTTGG TCAGG

WAP_US6               TTTAAA
          416: TTAAG TTTAAA AAAGG
          492: TTGCC TTTAAA ATTAC
          741: CTATG TTTAAA TTATT
          793: TGCAG TTTAAA GATGT
        1,248: ACAGT TTTAAA AATTA
        1,626: GCTCT TTTAAA ATAAT

ATF_RS                TGACGT
        1,920: CGCAA TGACGT TTCCC

ADH_US2 /Rev          GGGAGGTG
          294: AACCT GGGAGGTG GAGGG
        2,103: CAAGA GGGAGGTG ACCGT

EARLY-SEQ1            YYCCGCCC
        1,684: CCCCA CCCCGCCC CCATC
        1,939: TCGGG TCCCGCCC CTCCA

SP1_CS3               GGGCGG
          138: AAGAT GGGCGG CTCAC
        2,033: AGCGG GGGCGG CCGCC
        2,379: CCCGC GGGCGG GCGAG
        2,724: GGCGT GGGCGG CGGCA

SP1_CS3 /Rev          CCGCCC
        1,686: CCACC CCGCCC CCATC
        1,810: CTTTG CCGCCC TCAAT
        1,941: GGGTC CCGCCC CTCCA
        2,325: CCCGG CCGCCC TCGCG

Ad-conserved-sequence-e  TGACGT
        1,920: CGCAA TGACGT TTCCC

malT_CS               GGAKGA
        1,481: TGAGG GGAGGA GACGG
        1,765: CGCTT GGAGGA TTACA

malT_CS /Rev          TCMTCC
        1,698: CATCC TCATCC TTAGA

alpha-factor_stim_element /Rev  TGTTTCA
          756: TTCCA TGTTTCA GAGTT

TTR_inverted_repeat   CGCCGCG
        2,640: GCGGC CGCCGCG CCCTC
        2,443: TCAGC CGCCGCG CTAGG
        2,470: GCGGA CGCCGCG CCTCG

malT-malPp /Rev       GGAGGA
        1,481: TGAGG GGAGGA GACGG
        1,765: CGCTT GGAGGA TTACA

H2B-CCAAT             CCAATNA
        1,080: TGCGC CCAATAA ATACT

histone_H4_CS.2 /Rev  TCAGGR
          155: TGAGG TCAGGA GTTCG
          590: GAGGC TCAGGG AAGTT
        1,016: TGAAA TCAGGG CCCTT
        2,657: CGGCC TCAGGG CCTGT

H2A_conserved_US      YCATTC
        1,176: AATTT TCATTC TTAGA

H2A_conserved_US /Rev  GAATGR
        1,199: AATGT GAATGA AATTA

IEmRNA-4/5_start /Rev  GACCGGGACG
        2,218: GCGGC GACCGGGACG ACTAC

IEmRNA-4/5_DS         GACCGGGACG
        2,218: GCGGC GACCGGGACG ACTAC

INF.1                 AAGTGA
            7: GAGGC AAGTGA AAAGA
        1,306: GAGAA AAGTGA CAGTG

INF.1 /Rev            TCACTT
          145: GCGGC TCACTT GAGGT

(Sp1)-TK.1            CCCCGCCC
        1,684: CCCCA CCCCGCCC CCATC

JCV_repeated_sequence  GGGNGGRR
        1,480: GTGAG GGGAGGAG ACGGA

JCV_repeated_sequence /Rev  YYCCNCCC
        1,679: CCCAT CCCCACCC CGCCC
        1,684: CCCCA CCCCGCCC CCATC
        1,926: GACGT TTCCCCCC TCGGG
        1,939: TCGGG TCCCGCCC CTCCA

ol11_DS               ATTCTTA
        1,178: TTTTC ATTCTTA GAAAG

yeast.term.2 /Rev     TATAAAAA
          899: ATCTT TATAAAAA GCTTT

lambda.c              GGYGTRYG
        2,153: CTCTC GGTGTGTG TGTGT

lambda.c /Rev         CRYACRCC
        2,339: CGGCT CGCACGCC CGGGC

lambda.d              GGTGTGTG
        2,153: CTCTC GGTGTGTG TGTGT

E4F1-E2 /Rev          ATGACGT
        1,919: CCGCA ATGACGT TTCCC

zeste-Ubx             CGAGCG
        2,304: ACGTC CGAGCG CCTCG

zeste-Ubx /Rev        CGCTCG
        2,403: GCCGC CGCTCG GCCGC

zeste-white           TGAGTG
        1,092: ATACT TGAGTG GAATT

zeste-white /Rev      CACTCA
        1,368: TAGAA CACTCA ACCCA

AP-1_CS3 /Rev         TKANTCA
           60: TTGTT TTATTCA TTTCA

NFkB_CS4 /Rev         GRRANYCCC
          188: ATGGT GAAAACCCC GTCTC

Pu_box                GAGGAA
          832: CTTGA GAGGAA ATGCA
        2,166: TGTGT GAGGAA ATCGG

TATA-box-CS           TATAWAW
          899: ATCTT TATAAAA AGCTT
        1,000: CAGGT TATAAAT GGTTT

TATA-box-CS /Rev      WTWTATA
          440: ATGTA TTTTATA GCTTA

GC-box_(1) /Rev       RYYMCGCCYM
        1,683: TCCCC ACCCCGCCCC CATCC
        1,938: CTCGG GTCCCGCCCC TCCAG

AP-2_CS5              GSSWGSCC
          125: ATTTT GGGAGGCC AAGAT
          172: GACCA GGGTGGCC AACAT
        1,565: CCTTG GGGAGGCC GGGTC
        2,248: CGAGT GGCTGGCC TTCGC

AP-2_CS5 /Rev         GGSCWSSC
        2,314: GCCTC GGGCTCCC CGGCC
        2,489: TTTGA GGGCAGCC GGCGG

SDR_RS                GRGSGGTG
        2,592: GAGTG GAGCGGTG GGTGC

NF-E1_CS2 /Rev        CTTATC
        1,416: TGTCC CTTATC ATTTA

bicoid_CS             BBTAATCYV
          108: ATGCC TGTAATCCC AGCAT
          245: GTGCC TGTAATCCC AGCTA

GRE_CS7               WCTGWTCT
          675: GCTTC ACTGTTCT GAAGT

HNF1-HP1 /Rev         CAATNANNANNNG
        1,081: GCGCC CAATAAATACTTG AGTGG
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KROX24_CS              GCGSGGGCG
        2,029: TGACA GCGGGGGCG GCCGC

KROX24-F /Rev          GCGGGGGCG
        2,029: TGACA GCGGGGGCG GCCGC

RAD_CS /Rev            TKCNWCCWCG
        2,287: ACCTC TGCGTCCTCG CCACG

junB-US2               GGCCAAT
        1,786: CCCGA GGCCAAT TCGAC

junB-US2 /Rev          ATTGGCC
        1,971: TTGGG ATTGGCC GAGAG

E1A-F_CS               NGGAYGT
        2,526: TCGGC TGGACGT CCGCT

E1A-F_CS /Rev          ACRTCCN
        2,299: TCGCC ACGTCCG AGCGC
        2,529: GCTGG ACGTCCG CTCCG

TFII-I-HIV-1-Inr2 /Rev  AGAGAGCT
          717: CCATC AGAGAGCT AATTA

H-2RIIBP/T3R-alpha-region-II  GAGGTC
          151: CACTT GAGGTC AGGAG

E2A_CS                 RCAGNTG
          842: AATGC ACAGTTG GAAGT
        1,295: TCTGG ACAGATG AGAAA

E2A_CS /Rev            CANCTGY
        1,137: TCTTC CATCTGC CAAAG

EGR-1_CS /Rev          GCGSGGGCG
        2,029: TGACA GCGGGGGCG GCCGC

EGR-2_RS /Rev          GCGGGGGCGG
        2,029: TGACA GCGGGGGCGG CCGCC

F-ACT1_RS              TGGCGA
        1,987: GGCTG TGGCGA CAAGG
        2,235: TACTC TGGCGA CTCGA

F-ACT1_RS /Rev         TCGCCA
        2,294: CGTCC TCGCCA CGTCC
        2,681: GGCAC TCGCCA TGGCC

HNF-5_CS /Rev          RCAAAYA
        1,877: CACAC ACAAACA AAAAC

MBF-1_CS /Rev          GYGYGCA
        2,064: CCCCG GTGTGCA AATGT

Oct-R_CS               ATGCAAAY
          536: TGGAG ATGCAAAC TCACA

PEA3_CS                AGGAAR
          833: TTGAG AGGAAA TGCAC
        1,115: AAAAC AGGAAG AAGTA
        2,167: GTGTG AGGAAA TCGGG

PEA3_CS /Rev           YTTCCT
        1,662: GGCTT CTTCCT GCCAC
        1,896: CAAGT CTTCCT TTCGG

PuF_RS /Rev            CCCACCC
        1,375: CTCAA CCCACCC TGCAT
        1,680: CCATC CCCACCC CGCCC

SIF_core_RS            CCCGTC
          194: AAAAC CCCGTC TCTAG

Sp1_CS4                KRGGCKRRK
           83: GAACC TAGGCTGGG TGCGG
          264: CCTGG GAGGCTGAG GCAGG
        2,186: GCAGC GAGGCTAAG GCTGC

Sp1_CS4 /Rev           MYYMGCCYM
        1,684: CCCCA CCCCGCCCC CATCC

TGA1b-hex1             TGACGT
        1,920: CGCAA TGACGT TTCCC

CyIIIa-P3A.3 /Rev      AGCGCCTC
        2,306: GTCCG AGCGCCTC GGGCT

P2II_CS /Rev           TAMCCCT
          500: AAAAT TACCCCT TTTCA

p53_CS                 RRRCWWGYYY
        1,258: AAATT AAGCATGCCC TCTTA

AntP_CS                ANNNNCATTA
           14: GTGAA AAGAACATTA TTTTT

AntP_CS /Rev           TAATGNNNNT
        1,193: AGCTT TAATGTGAAT GAAAT

CREB_CS2               TGACGTYW
        1,920: CGCAA TGACGTTT CCCCC

F1S_CS                 CAANANNTGANC
           70: CATTT CAAAATCTGAAC CTAGG

IBP-1_CS2              AAGTGA
            7: GAGGC AAGTGA AAAGA
        1,306: GAGAA AAGTGA CAGTG

IBP-1_CS2 /Rev         TCACTT
          145: GCGGC TCACTT GAGGT

GATA-1_CS2 /Rev        SKTATCW
        1,416: TGTCC CTTATCA TTTAA

T-antigen_RS           GAGGCC
          127: TTTGG GAGGCC AAGAT
        1,557: TTGGG GAGGCC GGGTC
        1,707: CCTTA GAGGCC CCAGG
        1,784: GACCC GAGGCC AATTC

T-antigen_RS /Rev      GGCCTC
        1,572: CAGGG GGCCTC CTTGC
        2,505: GGCGC GGCCTC CTCAG
        2,653: GCTGC GGCCTC AGCGC

TFIIIA_CS /Rev         YTCNRNCCNG
          335: CTGTA CTCCAGCCTG GGTGA
        2,352: CGGGC TTCAGCCCGG CCTGC

AluA                   GGAGGCTGAGGCA
          263: ACCTG GGAGGCTGAGGCA GGAGA

CAP-box                CTTYTG
        1,467: ATGCT CTTTTG GGGTG

histone_CAP_box /Rev   YGAATGR
        1,198: TAATG TGAATGA AATTA

GSG_element_(2)        GCGGGGGCG
        2,029: TGACA GCGGGGGCG GCCGC

CNBP-SRE               STGSSGYG
           91: GCTGG GTGCGGTG GCTCA
        2,080: TGTGT GTGCGGTG TTATG
        2,580: TAGGA GTGCGGCG AGTGG
        2,703: GGTGG CTGCGGCG CGGGA

IRE_(1) /Rev           CAGGCGGG
        2,633: GCAGG GAGGCGGG AGCGG

HNF-5_site             RCAAAYA
        1,877: CACAC ACAAACA AAAAC

Keratinocyte_enhancer  AARCAAA
          511: TTTTC AAACAAA ATACA
        1,879: CACAC AAACAAA AACCC

Keratinocyte_enhancer /Rev  TTTGYTT
          817: ACATT TTTGTTT TCCCT

ZRE5                   TGTGCAA
        2,065: CCCGG TGTGCAA ATGTG

CCAAT_site_4           GATTGG
        1,970: TTTGG GATTGG CCGAG
        2,002: GCCCG GATTGG ACAGC

undefined_site_2       TTTCCA
          750: AATTA TTTCCA TGTTT

undefined_site_2 /Rev  TGGAAA
        1,855: TCAAA TGGAAA ACCCA

UBR                    ACTGTTCT
          679: GCTTC ACTGTTCT GAAGT

TATA_(1)               TATAAAT
        1,000: CAGGT TATAAAT GGTTT

betaP-F1               GATAAA
          392: CGGAA GATAAA GAGCT

betaE-F3 /Rev          CCCCACCC
        1,679: CCCAT CCCCACCC CGCCC

unknown-3'-UTR.1 /Rev  TGGAAAAC
        1,855: TCAAA TGGAAAAC CCACA

site_k /Rev            GACCTG
          804: GATGT GACCTG AGACA

D6 /Rev                TATTCAT
           61: TGTTT TATTCAT TTCAA

D1 /Rev                TTTTCAT
          889: TACTG TTTTCAT CTTTA
        1,173: GGTAA CTTTCAT TCTTA
        1,430: TAAGC TTTTCAT ATGTA

CSS_(2)                GGGCACA
        1,281: GCAGG GGGCACA GTTCT

site_A_(8) /Rev        CCCCGCCCC
        1,684: CCCCA CCCCGCCCC CATCC

CAAT_site(1)           GCCAAT
        1,787: CCGAG GCCAAT TCGAC

CAAT_site(1) /Rev      ATTGGC
        1,971: TTGGG ATTGGC CGAGA

Zif_(1)                GCGGGGGCG
        2,029: TGACA GCGGGGGCG GCCGC

MRE6 /Rev              GTGTGCA
        2,064: CCCCG GTGTGCA AATGT

RIPE3b                 CTTCAGCC
        2,351: CCGGG CTTCAGCC CGGCC

GR-intron-site-2 /Rev  GGCACA
        1,056: AGCCT GGCACA GCGTG
        1,282: CAGGG GGCACA GTTCT

GR-intron-site-3       TGTCCC
        1,411: CTGTG TGTCCC TTATC
        2,143: TTTAT TGTCCC TCTCG

GR-intron-site-4       GACACA
        1,868: CCACA GACACA CACAC

GR-intron-site-4 /Rev  TGTGTC
        1,409: CTCTG TGTGTC CCTTA

DHS_E_(2)              CCTTATCATT
        1,415: GTGTC CCTTATCATT TAAGC

UAS1_(5)               CGCCCCC
        1,687: CACCC CGCCCCC ATCCT

UAS1_(5) /Rev          GGGGGCG
        2,031: ACAGC GGGGGCG GCCGC

T3RE/half /Rev         TGACCTGA
          803: AGATG TGACCTGA GACAT

HCR_(1)                GTTTTATT
           57: ATATT GTTTTATT CATTT

repressing_elements    CCWTNTTNNNW
        1,023: AGGGG CCTTGTTTTTA CTTTG
        1,129: TAAAG CCATCTTCCAT CTGCC

repressing_elements /Rev  WNNNAANAWGG
          416: TTAAG TTTAAAAAAGG AGACA
          601: AAGTT AAATAACAAGG CTATG
        1,488: AGGAG ACGGAAGAAGG AGATG

3'_enhancer/HM         CATCTG
        1,137: TCTTC CATCTG CCAAA

3'_enhancer/HM /Rev    CAGATG
        1,296: CTGGA CAGATG AGAAA

F5                     ACTCGA
        2,240: TGGCG ACTCGA GTGGC

P5 /Rev                TCGAGT
        2,242: GCGAC TCGAGT GGCTG
        2,479: GCGCC TCGAGT TTGAG

Adf-1-CS /Rev          RCGRCRRCRGC
        2,117: CCGTG GCGGCGGCGGC GGCGG
        2,120: TGGCG GCGGCGGCGGC GGCTC
        2,123: CGGCG GCGGCGGCGGC TCTGT

A4_amyloid_protein-undefined-s  GGGCGCGGG
        2,554: CGATG GGGCGCGGG GGTCG

Sp1-apoE /Rev          GGGCGGGC
        2,379: CCCGC GGGCGGGC GAGAA

alphaA-crystallin-undefined-s2 /Rev  GAGAGGC
        1,978: TGGCC GAGAGGC TGTGG

DHFR-undefined-site-1  GGATTGGC
        1,969: TTTTG GGATTGGC CGAGA

window_8 /Rev          GAGAAGAA
        1,335: GTTCA GAGAAGAA AGGTC

IgHC-undefined-site-2  ATTTTCAT
        1,172: TGGTA ATTTTCAT TCTTA

phaseolin-undefined-site  TCCACCT
          769: AGTTC TCCACCT CTAGA

phaseolin-undefined-site /Rev  AGGTGGA
          297: CTGGG AGGTGGA GGGTG

Tg-undefined-site-1 /Rev  ACTTGAGT
        1,089: TAAAT ACTTGAGT GGAAT

motif_IIb              CCGCCGCGCT
        2,442: CTCAG CCGCCGCGCT AGGCC

Sp1-NPY                CCCCTCC
        1,944: TCCCG CCCCTCC AGGGG

GAGA-eve_(6)           CGCTCCC
        2,421: CCGGT CGCTCCC TGCTT

GAGA-eve_(6) /Rev      GGGAGCG
        2,638: GAGGC GGGAGCG GCGGC

PEA3-uPA               AGGAAA
          833: TTGAG AGGAAA TGCAC
        2,167: GTGTG AGGAAA TCGGG

LyF-1-consensus        YYTGGGAGR
          122: AGCAT TTTGGGAGG CCAAG
          259: AGCTA CCTGGGAGG CTGAG
          291: TTGAA CCTGGGAGG TGGAG

MEF-2-consensus        YTWWAAATAR
        1,626: GCTCT TTTAAAATAA TTTCC

PPAR-consensus         AGGTCA
          152: ACTTG AGGTCA GGAGT
        1,343: AAGAA AGGTCA ACAAA
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PPAR-consensus /Rev   TGACCT
          803: AGATG TGACCT GAGAC

SIF-consensus         CCCGTM
          194: AAAAC CCCGTC TCTAG

Ttk-consensus         AGGGYGG
          171: AGACC AGGGTGG CCAAC

Ttk-consensus /Rev    CCRCCCT
        1,376: TCAAC CCACCCT GCATT
        1,810: CTTTG CCGCCCT CAATT
        2,325: CCCGG CCGCCCT CGCGG

MAZ-c-myc /Rev        CCCTCCC
        2,047: CCGCG CCCTCCC TCTCT

Box_4_+_5             ACCCCGCCC
        1,683: TCCCC ACCCCGCCC CCATC

GCF-consensus         NNGCGGGGCN
        2,609: CGGAG CGGCGGGGCC CAGCG

GG-II/GG-I            GGAAAT
          834: TGAGA GGAAAT GCACA
        2,168: TGTGA GGAAAT CGGGG

GG-II/GG-I /Rev       ATTTCC
          749: AAATT ATTTCC ATGTT
        1,635: AAATA ATTTCC CTTTG

TFIID-MBP             TTCAAA
           68: TTCAT TTCAAA ATCTG
          508: CCCTT TTCAAA CAAAA
          869: ACCTC TTCAAA AAACA
        1,849: CTCCC TTCAAA TGGAA

TFIID-MBP /Rev        TTTGAA
           45: TGAGT TTTGAA TATAT
        1,009: AATGG TTTGAA ATCAG
        1,035: TTTAC TTTGAA CCCAT
        1,224: GTGCA TTTGAA AATTC
        2,201: CTGCC TTTGAA GCAGC

3'_enhancer/_CD2E6    TGAGGTCA
          150: TCACT TGAGGTCA GGAGT

Sp1-TPI_(4)           AGGCGG
        2,634: CAGGG AGGCGG GAGCG

GATA-1-EpoR_(1) /Rev  AGATAA
          391: ACGGA AGATAA AGAGC

c-myc_responsive_region  CAGTTG
          843: ATGCA CAGTTG GAAGT

Thy-1-undefined-site-2 /Rev  TTGCTG
        1,158: TGATG TTGCTG TATTG

MMTV-undefined-site   ATCATTTT
          562: ACAAG ATCATTTT AACAA

epsilon-globin-undefined-site2 /Rev  TTTATTC
           59: ATTGT TTTATTC ATTTC

gERE/_gastrin_EGF-reponse_elem /Rev
CCACCCCGCC
        1,681: CATCC CCACCCCGCC CCCAT

Yi-consensus          CCCNCNNNCT
        1,689: CCCCG CCCCCATCCT CATCC
        2,047: CCGCG CCCTCCCTCT CTCCC

Yi-consensus /Rev     AGNNNGNGGG
          297: CTGGG AGGTGGAGGG TGCAG
        1,274: CTTAG AGGCAGGGGG CACAG
        2,482: CCTCG AGTTTGAGGG CAGCC

TATA_box_(1)          TTCATAAA
          470: ACATC TTCATAAA AAAAA

EcR-consensus_(2)     KNTCANTNNMM
          561: CACAA GATCATTTTAA CAAGC

EcR-consensus_(2) /Rev  KKNNANTGANM
            4:    TGA GGCAAGTGAAA AGAAC
          306: GGAGG GTGCAGTGAGC CGAGA
        1,101: TGGAA TTACACTGAAA AACAG
        1,196: TTTAA TGTGAATGAAA TTATT
        1,232: GAAAA TTCCACTGAAA ACAGT
        1,309: AAAAG TGACAGTGACA TCTCT
        1,449: TTTCT TGAAACTGACC ATATG

PAI-2-undefined-site-2 /Rev  TGAGGTCA
          150: TCACT TGAGGTCA GGAGT

Sp1-junD              CCGGCCCC
        1,685: CCCAC CCGGCCCC CATCC
        1,940: CGGGT CCGGCCCC TCCAG

sevenless-undefined-site-5 /Rev  CAAGAGGGA
        2,098: CCGGA CAAGAGGGA GGTGA

USP-MLP /Rev          CATGTG
          521: AAATA CATGTG TACTT

TATA_box_(4)          CAATAAATA
        1,081: GCGCC CAATAAATA CTTGA

OMP-undefined-site    TGGCAC
          928: TTTGG TGGCAC TTCAT
        1,055: GAGcC TGGCAC AGCGT

SEF4-beta-conglycinin_alpha'82  CATTTTTGT
          813: TGAGA CATTTTTGT TTTCC

SEF4-consensus        RTTTTTR
           23: ACATT ATTTTTA TTAAT
          814: GAGAC ATTTTTG TTTTC
        1,027: GCCTT GTTTTTA CTTTG

SEF4-consensus /Rev   YAAAAAY
          369: GTCTC TAAAAAC AACAA
          381: AACAA CAAAAAC GGAAG
          857: GTTCA CAAAAAC ACCTC
        1,250: AGTTT TAAAAAT TAAGC
        1,882: ACAAA CAAAAAC CCCAA

phyA3-undefined-site-2 /Rev  TTCTTTC
        1,386: CTGCA TTCTTTC CCTAG

phyA3-undefined-site-6  GGTAAAGAAA
          659: TTCAA GGTAAAGAAA GGCTT

Egr-1-ADA             GCGTGGGCG
        2,720: AGCCG GCGTGGGCG GCGGC

abaA-consensus        CATTCY
        1,177: ATTTT CATTCT TAGAA
        1,384: CCCTG CATTCT TTCCC
        1,400: TAGAA CATTCT CTGTG

abaA-consensus /Rev   RGAATG
          784: GACTC AGAATG CAGTT

GATA-1-MC-CPA         AGATAA
          391: ACGGA AGATAA AGAGC

NGFI-B-artificial_sequence  AAAGGTCA
        1,341: AGAAG AAAGGTCA ACAAA

TBP-ADA               TAAAAAA
          211: AAAAG TAAAAAA AATTA
          418: AAGTT TAAAAAA GGAGA
          474: CTTCA TAAAAAA AACTC
          728: CTAAT TAAAAAA ACTAT

REB1-consensus        YNNYYACCCG
        1,907: TTCGG TTGCTACCCG CAATG

REB1-consensus /Rev   CGGGTRRNNR
        2,696: CAAGT CGGGTGGCTG CGGCG

NP-TCII-consensus     ANANTTTCC
        1,632: TTAAA ATAATTTCC CTTTG

MAF-consensus         GRRGSAAGK
            2:     T GAGGCAAGT GAAAA

Sp1-gamma-globin_(3)  GGGGCC
        1,019: AATCA GGGGCC TTGTT
        1,570: GTCAC GGGGCC TCCTT
        2,613: GCGGC GGGGCC CAGCG

Sp1-gamma-globin_(3) /Rev  GGCCCC
        1,650: GGGAC GGCCCC CGGCT
        1,709: TTAGA GGCCCC AGGCA

          Total finds:       537
         Total length:     2,734
    Total sequences:           1
             CPU time:     05.52
```
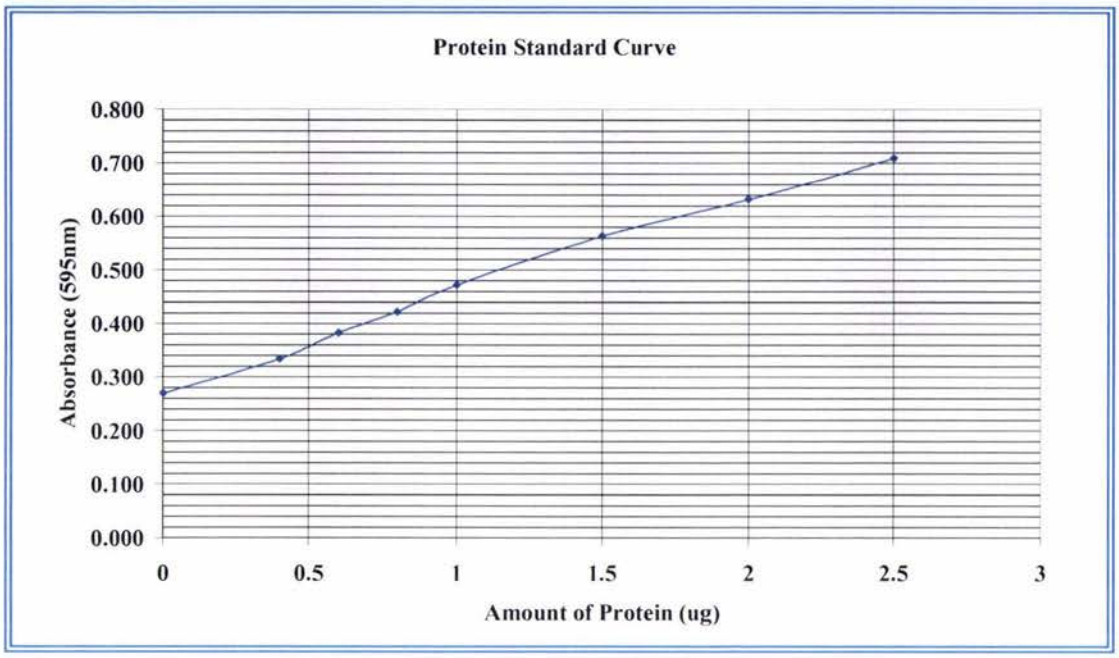

Appendix 4: Protein Standard Curves.



A)

BSA Standard (µg)	Absorbance (595nm)
0	0.269
0.4	0.334
0.6	0.382
0.8	0.422
1.0	0.472
1.5	0.568
2.0	0.657
2.5	0.705

B)

1st HeLa extract		2nd HeLa extract		3rd HeLa extract	
Absorbance 595 nm	Amount of protein (µg)	Absorbance 595 nm	Amount of protein (µg)	Absorbance 595 nm	Amount of protein (µg)
0.466	0.97	0.457	0.89	0.429	0.79
0.487	1.05	0.475	0.98	0.417	0.73
0.493	1.08	0.477	0.99	0.423	0.78
Average	1.03	Average	0.95	Average	0.77
Concentration 4.12 µg/µL		Concentration 3.80 µg/µL		Concentration 3.08 µg/µL	

Results of the Protein Standard Curve.

The Bradford protein assay was carried out as described in section 2.2.21. A) Absorbance values generated with different amounts of BSA protein standard mixed with Bradford reagent, were used to generate the protein standard curve. B) HeLa extracts were mixed with Bradford reagent and the absorbance values were used to relate the amount of protein from the graph shown above. The amounts of protein present in triplicate samples was averaged and used to calculate the concentration of protein present in each HeLa extract. Each concentration was corrected for the dilution (x 40) and volume (÷10). The concentration of the fourth HeLa extract was estimated in the same way (data not shown).

Appendix 5: Transient transfections

5.1 pGL3B-1357 concentration titrations in HeLa

Transfection #1

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	137	122	0.184	0.144	847.22		
0ug	144	129	0.149	0.109	1183.49	1015.35	168.13
	21732	21717	0.173	0.133	163285.71		
	17139	17124	0.156	0.116	147620.69		
0.25ug	15905	15890	0.157	0.117	135811.97	148906.12	9586.39
	45717	45702	0.163	0.123	371560.98		
	43317	43302	0.143	0.103	420407.77		
0.5ug	30550	30535	0.136	0.096	318072.92	370013.89	34627.31
	47774	47759	0.114	0.074	645391.89		
	39501	39486	0.115	0.075	526480.00		
0.75ug						585935.95	59455.95
	51585	51570	0.106	0.066	781363.64		
	62499	62484	0.136	0.096	650875.00		
1.0ug	62224	62209	0.124	0.084	740583.33	724273.99	48932.66
	61940	61925	0.109	0.069	897463.77		
	60739	60724	0.108	0.068	893000.00		
1.25ug	58562	58547	0.105	0.065	900723.08	897062.28	2708.19
	60384	60369	0.106	0.066	914681.82		
	61924	61909	0.121	0.081	764308.64		
1.5ug	57603	57588	0.095	0.055	1047054.55	908681.67	96248.68
	60714	60699	0.078	0.038	1597342.11		
	61655	61640	0.085	0.045	1369777.78		
2.0ug						1483559.9	113782.16
Blank	15		0.040				

Summary

Construct added (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	1015.4	168.1	0.3	23.4
0.25ug	148906.1	9586.4	40.2	9.3
0.5ug	370013.9	34627.3	100.0	13.8
0.75ug	585935.9	59456.0	158.4	14.4
1.0ug	724274.0	48932.7	195.7	9.2
1.25ug	897062.3	2708.2	242.4	0.4
1.5ug	908681.7	96248.7	245.6	15.6
2.0ug	1483559.9	113782.2	400.9	10.8

Transfection #2

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	240	51	0.454	0.379	134.56		
0ug	283	94	0.364	0.289	325.26	229.91	95.35
	3643	3454	0.413	0.338	10218.93		
	3387	3198	0.305	0.23	13904.35		
0.25ug	4050	3861	0.356	0.281	13740.21	12621.17	1601.49
	5562	5373	0.322	0.247	21753.04		
0.5ug	4173	3984	0.288	0.213	18704.23	20228.63	1524.41
	2396	2207	0.18	0.105	21019.05		
0.75ug	3148	2959	0.281	0.206	14364.08	17691.56	3327.48
	3492	3303	0.343	0.268	12324.63		
	4350	4161	0.346	0.271	15354.24		
1.0ug	3373	3184	0.278	0.203	15684.73	14454.53	1419.94
	3300	3111	0.212	0.137	22708.03		
	3255	3066	0.243	0.168	18250.00		
1.25ug						20479.01	2229.01
	6450	6261	0.203	0.128	48914.06		
	7176	6987	0.188	0.113	61831.86		
1.5ug	6909	6720	0.188	0.113	59469.03	56738.32	5216.17
	6279	6090	0.162	0.087	70000.00		
	7392	7203	0.152	0.077	93545.45		
2.0ug						81772.73	11772.73
Blank	189		0.075				

Summary

Construct added (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	229.9	95.3	1.1	58.6
0.25ug	12621.2	1601.5	62.4	16.5
0.5ug	20228.6	1524.4	100.0	10.7
0.75ug	17691.6	3327.5	87.5	26.6
1.0ug	14454.5	1419.9	71.5	12.8
1.25ug	20479.0	2229.0	101.2	15.4
1.5ug	56738.3	5216.2	280.5	12.1
2.0ug	81772.7	11772.7	404.2	20.4

Transfection #3

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	270	133	0.511	0.443	300.23		
	232	95	0.658	0.590	161.02		
0ug						230.62	69.60
	13440	13303	0.513	0.445	29894.38		
	14418	14281	0.504	0.436	32754.59		
0.25ug	14384	14247	0.598	0.530	26881.13	29843.37	1974.82
	6140	6003	0.485	0.417	14395.68		
	6858	6721	0.521	0.453	14836.64		
0.5ug	6421	6284	0.388	0.320	19637.50	16289.94	2231.70
	19520	19383	0.516	0.448	43265.63		
	24263	24126	0.547	0.479	50367.43		
0.75ug	24532	24395	0.584	0.516	47277.13	46970.06	2469.63
	31090	30953	0.639	0.571	54208.41		
	35574	35437	0.637	0.569	62279.44		
1.0ug	31752	31615	0.593	0.525	60219.05	58902.30	3129.26
	32626	32489	0.515	0.447	72682.33		
	25567	25430	0.378	0.310	82032.26		
1.25ug	27316	27179	0.415	0.347	78325.65	77680.08	3331.83
	38665	38528	0.440	0.372	103569.89		
	39030	38893	0.416	0.348	111761.49		
1.5ug	29010	28873	0.284	0.216	133671.30	116334.23	11558.05
	28278	28141	0.254	0.186	151295.70		
	33352	33215	0.245	0.177	187655.37		
	40058	39921	0.323	0.255	156552.94	165168.00	14991.58
2.0ug	137		0.068				
Blank	137		0.068				

Summary

Construct added (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	230.62	98.44	1.42	42.68
0.25ug	29843.37	2937.06	183.20	9.84
0.5ug	16289.94	2907.44	100.00	17.85
0.75ug	46970.06	3560.85	288.34	7.58
1.0ug	58902.30	4193.54	361.59	7.12
1.25ug	77680.08	4708.28	476.86	6.06
1.5ug	116334.23	15562.97	714.15	13.38
2.0ug	165168.00	19651.23	1013.93	11.90

5.2 Topoisomerase IIβ promoter construct activities in HeLa cells

Transfection #4

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	185	-13	0.804	0.750	-17.33		
	217	19	0.686	0.632	30.06		
pGL3Basic	0	-198	0.636	0.582	-340.21	-109.16	154.03
Blank	198		0.054				
	9134	8936	0.549	0.495	18052.53		
	10488	10290	0.582	0.528	19488.64		
-1357	9877	9679	0.590	0.536	18057.84	18533.00	637.09
Blank	198		0.054				
	20599	20401	0.319	0.265	76984.91		
	22366	22168	0.367	0.313	70824.28		
-1228	21026	20828	0.301	0.247	84323.89	77377.69	4630.80
Blank	198		0.054				
	14792	14594	0.581	0.527	27692.60		
	14717	14519	0.637	0.583	24903.95		
-1051	14242	14044	0.657	0.603	23290.22	25295.59	1598.01
Blank	198		0.054				
	18417	18219	0.592	0.538	33864.31		
	19705	19507	0.568	0.514	37951.36		
-901	17391	17193	0.493	0.439	39164.01	36993.23	2085.94
Blank	198		0.054				
	20598	20400	0.767	0.713	28611.50		
	20130	19932	0.809	0.755	26400.00		
-654	17541	17343	0.868	0.814	21305.90	25439.13	2755.49
Blank	198		0.054				
	2493	2295	0.477	0.423	5425.53		
	2462	2264	0.423	0.369	6135.50		
-456	2123	1925	0.392	0.338	5695.27	5752.10	255.60
Blank	198		0.054				
	1076	878	0.560	0.506	1735.18		
	1225	1027	0.512	0.458	2242.36		
-1357ID	1173	975	0.527	0.473	2061.31	2012.95	185.18
Blank	198		0.054				
	286	88	0.262	0.208	423.08		
-1228ID	291	93	0.253	0.199	467.34	445.21	22.13
Blank	198		0.054				
	262	64	0.351	0.297	215.49		
	267	69	0.370	0.316	218.35		
-1051ID						216.92	1.43
Blank	198		0.054				
	1671	1473	0.282	0.228	6460.53		
	1615	1417	0.285	0.231	6134.20		
-901ID	1886	1688	0.268	0.214	7887.85	6827.53	706.88
Blank	198		0.054				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-109.2	154.0	-0.6	-141.1
-1357	18533.0	637.1	100.0	3.4
-1228	77377.7	4630.8	417.5	6.0
-1051	25295.6	1598.0	136.5	6.3
-901	36993.2	2085.9	199.6	5.6
-654	25439.1	2755.5	137.3	10.8
-456	5752.1	255.6	31.0	4.4
-1357ID	2012.9	185.2	10.9	9.2
-1228ID	445.2	22.1	2.4	5.0
-1051ID	216.9	1.4	1.2	0.7
-901ID	6827.5	706.9	36.8	10.4

Transfection #5

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	45	3	0.720	0.648	4.63		
	51	9	0.633	0.561	16.04		
pGL3Basic						10.34	5.71
Blank	42		0.072				
	7735	7693	0.763	0.691	11133.14		
	7698	7656	0.760	0.688	11127.91		
-1357	7717	7675	0.745	0.673	11404.16	11221.74	121.62
Blank	42		0.072				
	37229	37187	0.830	0.758	49059.37		
	40276	40234	0.914	0.842	47783.85		
-1228	39573	39531	0.848	0.776	50942.01	49261.74	1120.18
Blank	42		0.072				
	51383	51316	0.872	0.800	64145.00		
	44288	44221	0.801	0.729	60659.81		
-1051	45144	45077	0.813	0.741	60832.66	61879.16	1510.56
Blank	67		0.072				
	22916	22849	0.777	0.705	32409.93		
	23761	23694	0.771	0.699	33897.00		
-901	21524	21457	0.706	0.634	33843.85	33383.59	649.11
Blank	67		0.072				
	7008	6941	0.824	0.752	9230.05		
	6407	6340	0.788	0.716	8854.75		
-654	6174	6107	0.794	0.722	8458.45	8847.75	259.53
Blank	67		0.072				
	3939	3897	0.795	0.723	5390.04		
	4186	4144	0.749	0.677	6121.12		
-456	4078	4036	0.752	0.680	5935.29	5815.49	283.63
Blank	42		0.072				
	6338	6338	0.773	0.701	9041.37		
	5816	5816	0.695	0.623	9335.47		
-1357ID	5648	5648	0.754	0.682	8281.52	8886.12	403.07
Blank	67		0.072				
	160	118	0.551	0.479	246.35		
	188	146	0.574	0.502	290.84		
-1228ID	142	100	0.430	0.358	279.33	272.17	17.22
Blank	42		0.072				
	4784	4742	0.666	0.594	7983.16		
	4709	4667	0.655	0.583	8005.15		
-1051ID	4176	4134	0.590	0.518	7980.69	7989.67	10.32
Blank	67		0.072				
	48	6	0.449	0.377	15.92		
	52	10	0.370	0.298	33.56		
-901ID						24.74	8.82
Blank	42		0.072				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	10.3	5.7	0.2	55.2
-1357	11221.7	121.6	193.0	1.1
-1228	49261.7	1120.2	847.1	2.3
-1051	61879.2	1510.6	1064.0	2.4
-901	33383.6	649.1	574.0	1.9
-654	8847.8	259.5	152.1	2.9
-456	5815.5	283.6	100.0	4.9
-1357ID	8886.1	403.1	152.8	4.5
-1228ID	272.2	17.2	4.7	6.3
-1051ID	7989.7	10.3	137.4	0.1
-901ID	24.7	8.8	0.4	35.7

Transfection #6

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	20	-32	0.423	0.366	-87.43		
	26	-26	0.326	0.269	-96.65		
pGL3Basic	47	-5	0.364	0.307	-16.29	-66.79	33.67
Blank	52		0.057				
	9544	9492	0.371	0.314	30229.30		
	8431	8379	0.296	0.239	35058.58		
-1357	7322	7270	0.279	0.222	32747.75	32678.54	1632.83
Blank	54		0.057				
	34684	34632	0.346	0.289	119833.91		
	32041	31989	0.326	0.269	118918.22		
-1228	31190	31138	0.341	0.284	109640.85	116130.99	4326.76
Blank	54		0.057				
	34070	34018	0.292	0.235	144757.45		
	34040	33988	0.273	0.216	157351.85		
-1051	34114	34062	0.287	0.230	148095.65	150068.32	4855.69
Blank	54		0.057				
	20915	20863	0.260	0.203	102773.40		
	21179	21127	0.259	0.202	104589.11		
-901	20737	20685	0.257	0.200	103425.00	103595.84	662.18
Blank	54		0.057				
	8151	8099	0.287	0.230	35213.04		
	8196	8144	0.315	0.258	31565.89		
-654	8721	8669	0.341	0.284	30524.65	32434.53	1852.34
Blank	52		0.057				
	5643	5591	0.259	0.202	27678.22		
	5255	5203	0.267	0.210	24776.19		
-456	5956	5904	0.270	0.213	27718.31	26724.24	1298.70
Blank	54		0.057				
	6858	6806	0.317	0.260	26176.92		
	6396	6344	0.274	0.217	29235.02		
-1357ID	5841	5789	0.277	0.220	26313.64	27241.86	1328.77
Blank	54		0.057				
	1075	1023	0.349	0.292	3503.42		
	1119	1067	0.365	0.308	3464.29		
-1228ID	753	701	0.254	0.197	3558.38	3508.70	33.12
Blank	52		0.057				
	8142	8090	0.330	0.273	29633.70		
	7811	7759	0.314	0.257	30190.66		
-1051ID	7556	7504	0.332	0.275	27287.27	29037.21	1166.63
Blank	54		0.057				
	126	74	0.265	0.208	355.77		
-901ID	100	48	0.269	0.212	226.42	291.09	64.68
Blank	54		0.057				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-66.8	33.7	-0.2	-50.4
-1357	32678.5	1632.8	100.0	5.0
-1228	116131.0	4326.8	355.4	3.7
-1051	150068.3	4855.7	459.2	3.2
-901	103595.8	662.2	317.0	0.6
-654	32434.5	1852.3	99.3	5.7
-456	26724.2	1298.7	81.8	4.9
-1357ID	27241.9	1328.8	83.4	4.9
-1228ID	3508.7	33.1	10.7	0.9
-1051ID	29037.2	1166.6	88.9	4.0
-901ID	291.1	64.7	0.9	22.2

Transfection #7

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	212	158	0.549	0.490	322.45		
	39	-15	0.535	0.476	-31.51		
pGL3Basic	171	117	0.556	0.497	235.41	175.45	137.97
Blank	54		0.059				
	23997	23871	0.558	0.499	47837.68		
	27748	27622	0.641	0.582	47460.48		
-1357	25602	25476	0.510	0.451	56487.80	50595.32	3928.32
Blank	126		0.059				
	45634	45508	0.409	0.350	130022.86		
	54432	54306	0.453	0.394	137832.49		
-1228	49625	49499	0.380	0.321	154202.49	140685.95	9011.03
Blank	126		0.059				
	62848	62722	0.621	0.562	111604.98		
	62675	62549	0.677	0.618	101211.97		
-1051	62762	62636	0.698	0.639	98021.91	103613.96	5328.02
Blank	126		0.059				
	58763	58637	0.533	0.474	123706.75		
	51803	51677	0.471	0.412	125429.61		
-901	54371	54245	0.504	0.445	121898.88	123678.41	1186.36
Blank	126		0.059				
	30004	29878	0.697	0.638	46830.72		
	31702	31576	0.668	0.609	51848.93		
-654	27594	27468	0.640	0.581	47277.11	48652.25	2131.12
Blank	126		0.059				
	13360	13234	0.265	0.206	64242.72		
	15239	15113	0.281	0.222	68076.58		
-456	13491	13365	0.263	0.204	65514.71	65944.67	1421.27
Blank	126		0.059				
	15059	15005	0.525	0.466	32199.57		
	15950	15896	0.514	0.455	34936.26		
-1357ID	13082	13028	0.410	0.351	37116.81	34750.88	1700.87
Blank	126		0.059				
	16136	16082	0.210	0.151	106503.31		
	15137	15083	0.234	0.175	86188.57		
-1228ID	15836	15782	0.213	0.154	102480.52	98390.80	8134.82
Blank	54		0.059				
	1297	1243	0.245	0.186	6682.80		
	1270	1216	0.261	0.202	6019.80		
-1051ID	1448	1394	0.262	0.203	6867.00	6523.20	335.60
Blank	54		0.059				
	6789	6663	0.230	0.171	38964.91		
	4717	4591	0.177	0.118	38906.78		
-901ID	6164	6038	0.210	0.151	39986.75	39286.15	467.07
Blank	126		0.059				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	175.4	138.0	0.3	78.6
-1357	50595.3	3928.3	100.0	7.8
-1228	140685.9	9011.0	278.1	6.4
-1051	103613.0	5328.0	204.8	5.1
-901	123678.4	1186.4	244.4	1.0
-654	48652.3	2131.1	96.2	4.4
-456	65944.7	1421.3	130.3	2.2
-1357ID	34750.9	1700.9	68.7	4.9
-1228ID	98390.8	8134.8	194.5	8.3
-1051ID	6523.2	335.6	12.9	5.1
-901ID	39286.1	467.1	77.6	1.2

Transfection #8

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	1469	169	0.312	0.251	673.31		
	1396	96	0.422	0.361	265.93		
pGL3Basic	1363	63	0.524	0.463	136.07	358.43	209.91
Blank	1300		0.061				
	3736	2301	0.525	0.464	4959.05		
	3967	2532	0.507	0.446	5677.13		
-1357	3893	2458	0.625	0.564	4358.16	4998.11	452.68
Blank	1435		0.061				
	8735	8179	0.515	0.454	18015.42		
	12387	11831	0.662	0.601	19685.52		
-1228	10945	10389	0.619	0.558	18618.28	18773.07	608.30
Blank	556		0.061				
	11534	10856	0.564	0.503	21582.50		
	11942	11264	0.610	0.549	20517.30		
-1051	11825	11147	0.613	0.552	20193.84	20764.55	545.30
Blank	678		0.061				
	25860	25052	0.717	0.656	38189.02		
	23752	22944	0.674	0.613	37429.04		
-901	26549	25741	0.767	0.706	36460.34	37359.47	599.42
Blank	808		0.061				
	11703	11255	0.395	0.334	33697.60		
	10611	10163	0.337	0.276	36822.46		
-654	14044	13596	0.475	0.414	32840.58	34453.55	1579.28
Blank	448		0.061				
	3534	2205	0.461	0.400	5512.50		
	3257	1928	0.440	0.379	5087.07		
-456	3213	1884				5299.79	212.71
Blank	1329		0.061				
	2863	1302	0.354	0.293	4443.69		
	2990	1429	0.373	0.312	4580.13		
-1357ID	2874	1313	0.366	0.305	4304.92	4442.91	92.00
Blank	1561		0.061				
	1691	228	0.624	0.563	404.97		
	1702	239	0.767	0.706	338.53		
-1228ID	1477					371.75	33.22
Blank	1463		0.061				
	1861	156	0.207	0.146	1068.49		
	1813	108	0.226	0.165	654.55		
-1051ID	1852	147	0.183	0.122	1204.92	975.99	214.29
Blank	1705		0.061				
	2726	901	0.724	0.663	1358.97		
	2511	686	0.620	0.559	1227.19		
-901ID	2540	715	0.621	0.560	1276.79	1287.65	47.55
Blank	1825		0.061				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	358.4	209.9	7.2	58.6
-1357	4998.1	452.7	100.0	9.1
-1228	18773.1	608.3	375.6	3.2
-1051	20764.5	545.3	415.4	2.6
-901	37359.5	599.4	747.5	1.6
-654	34453.5	1579.3	689.3	4.6
-456	5299.8	212.7	106.0	4.0
-1357ID	4442.9	92.0	88.9	2.1
-1228ID	371.8	33.2	7.4	8.9
-1051ID	976.0	214.3	19.5	22.0
-901ID	1287.7	47.5	25.8	3.7

Transfection #9

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	509	39	0.379	0.318	122.64		
	501	31	0.364	0.303	102.31		
pGL3Basic	490	20	0.340	0.279	71.68	98.88	18.13
Blank	470		0.061				
	1391	913	0.342	0.281	3249.11		
	1513	1035	0.390	0.329	3145.90		
-1357	1299	821	0.351	0.290	2831.03	3075.35	162.88
Blank	478		0.061				
-456	1021	534	0.165	0.104	5134.62		
	1047	560	0.181	0.120	4666.67	4900.64	233.97
Blank	487		0.061				
-1357ID	1218	661	0.203	0.142	4654.93		
	1103	546	0.199	0.138	3956.52		
	1185	628	0.202	0.141	4453.90	4355.12	265.73
Blank	557		0.061				
-1228ID	527	35	0.360	0.299	117.06		
	541	49	0.373	0.312	157.05		
	517	25	0.332	0.271	92.25	122.12	23.29
Blank	492		0.061				
-1051ID	529	17	0.176	0.115	147.83		
	521	9	0.206	0.145	62.07	104.95	42.88
Blank	512		0.061				
-901ID	963	428	0.395	0.334	1281.44		
	948	413	0.419	0.358	1153.63		
	909	374	0.388	0.327	1143.73	1192.93	59.00
Blank	535		0.061				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	98.9	18.1	3.2	18.3
-1357	3075.3	162.9	100.0	5.3
-456	4900.6	234.0	159.4	4.8
-1357ID	4355.1	265.7	141.6	6.1
-1228ID	122.1	23.3	4.0	19.1
-1051ID	104.9	42.9	3.4	40.9
-901ID	1192.9	59.0	38.8	4.9

5.3 PgL3B-1357 concentration titrations in MDA-MB-231 cells

Transfection #10

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	1056	136	0.271	0.224	607.14		
	1075	155	0.309	0.262	591.60		
0ug	1030	110	0.252	0.205	536.59	578.44	27.91
	1319	399	0.310	0.263	1517.11		
	1399	479	0.367	0.320	1496.88		
0.25ug						1004.66	10.12
	1702	782	0.491	0.444	1761.26		
	1718	798	0.541	0.494	1615.38		
0.5ug	1703	783	0.553	0.506	1547.43	1641.36	79.93
	1895	975	0.622	0.575	1695.65		
	1918	998	0.634	0.587	1700.17		
0.75ug	1987	1067	0.685	0.638	1672.41	2534.12	11.33
	2212	1292	0.472	0.425	3040.00		
	2122	1202	0.557	0.510	2356.86		
1.0ug	2282	1362	0.508	0.461	2954.45	2783.77	284.60
	1978	1058	0.427	0.380	2784.21		
	1829	909	0.337	0.290	3134.48		
1.25ug	1843	923	0.466	0.419	2202.86	2707.19	336.21
	1953	1033	0.371	0.324	3188.27		
	1956	1036	0.465	0.418	2478.47		
1.5ug	1974	1054	0.455	0.408	2583.33	2750.02	292.16
	2933	2013	0.446	0.399	5045.11		
	3134	2214	0.587	0.540	4100.00		
2.0ug	2831	1911	0.522	0.475	4023.16	4389.42	437.13
Blank	920		0.047				

Summary

Construct ıdded (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	578.4	27.9	35.2	4.8
0.25ug	1507.0	10.1	91.8	0.7
0.5ug	1641.4	79.9	100.0	4.9
0.75ug	1689.4	11.3	102.9	0.7
1.0ug	2783.8	284.6	169.6	10.2
1.25ug	2707.2	336.2	164.9	12.4
1.5ug	2750.0	292.2	167.5	10.6
2.0ug	4389.4	437.1	267.4	10.0

Transfection #11

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	1129	72	0.598	0.523	137.67		
	1325	268	0.681	0.606	442.24		
0ug						289.96	152.29
	1962	905	0.565	0.490	1846.94		
	1861	804	0.668	0.593	1355.82		
0.25ug	1884	827	0.672	0.597	1385.26	1529.34	211.73
	3300	2243	0.524	0.449	4995.55		
	2897	1840	0.548	0.473	3890.06		
0.5ug	2582	1525	0.579	0.504	3025.79	3970.47	683.39
	3003	1946	0.615	0.540	3603.70		
	2994	1937	0.690	0.615	3149.59		
0.75ug	3312	2255	0.689	0.614	3672.64	3475.31	217.15
	3289	2232	0.523	0.448	4982.14		
	3961	2904	0.588	0.513	5660.82		
1.0ug	3260	2203	0.536	0.461	4778.74	5140.57	346.83
	4398	3341	0.387	0.312	10708.33		
	3669	2612	0.384	0.309	8453.07		
1.25ug	3413	2356	0.409	0.334	7053.89	8738.43	1313.27
	4870	3813	0.382	0.307	12420.20		
	3887	2830	0.425	0.350	8085.71		
1.5ug	4311	3254	0.462	0.387	8408.27	9638.06	1854.76
	5824	4767	0.468	0.393	12129.77		
	5156	4099	0.530	0.455	9008.79		
2.0ug	5377	4320	0.568	0.493	8762.68	9967.08	1441.79
Blank	1057		0.075				

Summary

Construct ıdded (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	2025.6	152.3	51.0	7.5
0.25ug	1529.3	211.7	38.5	13.8
0.5ug	3970.5	683.4	100.0	17.2
0.75ug	3475.3	217.1	87.5	6.2
1.0ug	5140.6	346.8	129.5	6.7
1.25ug	8738.4	1313.3	220.1	15.0
1.5ug	9638.1	1854.8	242.7	19.2
2.0ug	9967.1	1441.8	251.0	14.5

Transfection #12

-1357wt construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	85	25	0.578	0.495	50.5050505		
0ug	62	2	0.6	0.517	3.86847195	27.19	23.32
	484	424	0.568	0.485	874.226804		
	538	478	0.582	0.499	957.915832		
0.25ug						916.07	41.84
	1228	1168	0.486	0.403	2898.26303		
	1320	1260	0.574	0.491	2566.19145		
0.5ug	1287	1227	0.551	0.468	2621.79487	2695.42	135.23
	1847	1787	0.696	0.613	2915.17129		
	1784	1724	0.705	0.622	2771.70418		
0.75ug	1906	1846	0.747	0.664	2780.12048	2822.33	61.89
	2575	2515	0.607	0.524	4799.61832		
	2648	2588	0.714	0.631	4101.42631		
1.0ug	2475	2415	0.781	0.698	3459.88539	4120.31	452.87
	2226	2166	0.469	0.386	5611.39896		
	2353	2293	0.533	0.45	5095.55556		
1.25ug	2257	2197	0.487	0.404	5438.11881	5381.69	190.76
	2285	2225	0.471	0.388	5734.53608		
	2396	2336	0.576	0.493	4738.33671		
1.5ug	2483	2423	0.519	0.436	5557.33945	5343.40	403.38
	3415	3355	0.514	0.431	7784.22274		
	3493	3433	0.542	0.459	7479.30283		
2.0ug	3555	3495	0.581	0.498	7018.07229	7427.20	272.75
Blank	60		0.083				

Summary

Construct ıdded (ug)	Average	Ave Dev	Relative Activity	%Error
0ug	27.2	23.3	1.0	85.8
0.25ug	916.1	41.8	34.0	4.6
0.5ug	2695.4	135.2	100.0	5.0
0.75ug	2822.3	61.9	104.7	2.2
1.0ug	4120.3	452.9	152.9	11.0
1.25ug	5381.7	190.8	199.7	3.5
1.5ug	5343.4	403.4	198.2	7.5
2.0ug	7427.2	272.8	275.5	3.7

5.4 Topoisomerase IIβ promoter construct activities in MDA-MB-231 cells.

Transfection #13

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
pGL3Basic	48	6	0.469	0.405	14.81	24.74	14.21
	63	21	0.520	0.456	46.05		
	48	6	0.513	0.449	13.36		
Blank	42		0.064				
-1357	1118	1076	0.537	0.473	2274.84	2260.61	167.67
	1226	1184	0.538	0.474	2497.89		
	924	882	0.503	0.439	2009.11		
Blank	42		0.064				
-1228	2408	2366	0.376	0.312	7583.33	7122.16	307.45
	2484	2442	0.412	0.348	7017.24		
	2383	2341	0.410	0.346	6765.90		
Blank	42		0.064				
-1051	4043	4001	0.462	0.398	10052.76	9606.07	297.79
	4221	4179	0.501	0.437	9562.93		
	4404	4362	0.538	0.474	9202.53		
Blank	42		0.064				
-901	2352	2310	0.354	0.290	7965.52	7732.82	155.13
	2201	2159	0.346	0.282	7656.03		
	2209	2167	0.350	0.286	7576.92		
Blank	42		0.064				
-654	1042	1000	0.769	0.705	1418.44	1694.92	184.32
	1296	1254	0.740	0.676	1855.03		
	1261	1219	0.737	0.673	1811.29		
Blank	42		0.064				
-456	356	314	0.470	0.406	773.40	833.18	115.01
	395	353	0.415	0.351	1005.70		
	310	268	0.436	0.372	720.43		
Blank	42		0.064				
-1357ID	930	888	0.589	0.525	1691.43	1830.45	92.68
	968	926	0.564	0.500	1852.00		
	977	935	0.544	0.480	1947.92		
Blank	42		0.064				
-1051ID	117	75	0.412	0.348	215.52	316.29	67.18
	186	144	0.444	0.380	378.95		
	171	129	0.428	0.364	354.40		
Blank	42		0.064				
-901ID	140	98	0.335	0.271	361.62	342.11	13.01
	127	85	0.322	0.258	329.46		
	100	58	0.237	0.173	335.26		
Blank	42		0.064				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	24.7	14.2	1.1	57.4
-1357	2260.6	167.7	100.0	7.4
-1228	7122.2	307.5	315.1	4.3
-1051	9606.1	297.8	424.9	3.1
-901	7732.8	155.1	342.1	2.0
-654	1694.9	184.3	75.0	10.9
-456	833.2	115.0	36.9	13.8
-1357ID	1830.4	92.7	81.0	5.1
-1051ID	316.3	67.2	14.0	21.2
-901ID	342.1	13.0	15.1	3.8

Transfection #14

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	64	25	0.279	0.222	112.61		
	52	13	0.186	0.129	100.78		
pGL3Basic	65	26	0.247	0.190	136.84	116.74	13.40
Blank	39		0.057				
	939	900	0.189	0.132	6818.18		
	1051	1012	0.176	0.119	8504.20		
-1357	1063	1024	0.241	0.184	5565.22	6962.53	1027.78
Blank	39		0.057				
	2037	1983	0.216	0.159	12471.70		
	2180	2126	0.213	0.156	13628.21		
-1228	2873	2819	0.261	0.204	13818.63	13306.18	556.32
Blank	54		0.057				
	2369	2315	0.178	0.121	19132.23		
	2198	2144	0.186	0.129	16620.16		
-1051	2067	2013	0.172	0.115	17504.35	17752.24	919.99
Blank	54		0.057				
	1242	1188	0.143	0.086	13813.95		
	1239	1185	0.144	0.087	13620.69		
-901	1377	1323	0.168	0.111	11918.92	13117.85	799.29
Blank	54		0.057				
-456	535	481	0.212	0.155	3103.23		
	412	358	0.189	0.132	2712.12		
	689	635	0.253	0.196	3239.80	3018.38	204.17
Blank	54		0.057				
	914	875	0.189	0.132	6628.79		
	793	754	0.194	0.137	5503.65		
-1357ID	999	960	0.202	0.145	6620.69	6251.04	498.26
Blank	39		0.057				
	655	601	0.212	0.155	3877.42		
	486	432	0.181	0.124	3483.87		
-1051ID	660	606	0.211	0.154	3935.06	3765.45	187.72
Blank	54		0.057				
	56	17	0.157	0.100	170.00		
	47	8	0.131	0.074	108.11		
-901ID						30.95	139.05
Blank	39		0.057				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	116.7	13.4	1.7	11.5
-1357	6962.5	1228.5	100.0	17.6
-1228	13306.2	556.3	191.1	4.2
-1051	17752.2	920.0	255.0	5.2
-901	13117.9	799.3	188.4	6.1
-456	3018.4	204.2	43.4	6.8
-1357ID	6251.0	498.3	89.8	8.0
-1051ID	1194.0	97.6	17.1	8.2
-901ID	139.1	30.9	2.0	22.3

Transfection #15

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	180	59	0.353	0.297	198.65		
	82	-39	0.398	0.342	-114.04		
pGL3Basic	99	-22	0.428	0.372	-59.14	8.49	126.77
Blank	121		0.056				
	5435	5314	0.354	0.298	17832.21		
	5717	5596	0.407	0.351	15943.02		
-1357	4801	4680	0.459	0.403	11612.90	15129.38	2344.32
Blank	121		0.056				
-1051	12180	12059	0.345	0.289	41726.64		
	13550	13429	0.390	0.334	40206.59		
	13528	13407	0.402	0.346	38748.55	40227.26	999.59
Blank	121		0.056				
-901	5674	5553	0.259	0.203	27354.68		
	6134	6013	0.311	0.255	23580.39		
	5891	5770	0.345	0.289	19965.40	23633.49	2480.79
Blank	121		0.056				
-654	4035	3914	0.508	0.452	8659.29		
	4693	4572	0.569	0.513	8912.28		
	4216	4095	0.461	0.405	10111.11	9227.56	589.03
Blank	121		0.056				
-456	2239	2118	0.410	0.354	5983.05		
	2497	2376	0.468	0.412	5766.99		
	2536	2415	0.516	0.460	5250.00	5666.68	277.79
Blank	121		0.056				
-1357ID	2395	2274	0.352	0.296	7682.43		
	2310	2189	0.346	0.290	7548.28		
	2526	2405	0.436	0.380	6328.95	7186.55	571.74
Blank	121		0.056				
-1228ID	477	356	0.451	0.395	901.27		
	573	452	0.425	0.369	1224.93		
	447	326	0.401	0.345	944.93	1023.71	134.15
Blank	121		0.056				
-1051ID	275	154	0.338	0.282	546.10		
	264	143	0.386	0.330	433.33		
	268	147	0.311	0.255	576.47	518.63	56.87
Blank	121		0.056				
-901ID	3001	2880	0.394	0.338	8520.71		
	3003	2882	0.386	0.330	8733.33		
	2714	2593	0.395	0.339	7648.97	8301.00	434.69
Blank	121		0.056				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	8.5	126.8	0.1	1492.7
-1357	15129.4	1228.5	100.0	8.1
-1051	40227.3	999.6	265.9	2.5
-901	23633.5	2480.8	156.2	10.5
-654	9227.6	589.0	61.0	6.4
-456	5666.7	277.8	37.5	4.9
-1357ID	7186.6	571.7	47.5	8.0
-1228ID	1023.7	134.1	6.8	13.1
-1051ID	518.6	56.9	3.4	11.0
-901ID	8301.0	434.7	54.9	5.2

Transfection #16

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	85	31	0.318	0.259	119.69		
	86	32	0.322	0.263	121.67		
pGL3Basic	36					120.68	0.99
Blank	54		0.059				
	8700	8646	0.375	0.316	27360.76		
	8083	8029	0.423	0.364	22057.69		
-1357	6927	6873	0.300	0.241	28518.67	25979.04	2614.23
Blank	54		0.059				
	12098	12044	0.279	0.220	54745.45		
	16146	16092	0.365	0.306	52588.24		
-1228	9736	9682	0.266	0.207	46772.95	51368.88	3063.95
Blank	54		0.059				
	15722	15668	0.301	0.242	64743.80		
	14251	14197	0.284	0.225	63097.78		
-1051	13881	13827	0.317	0.258	53593.02	60478.20	4590.12
Blank	54		0.059				
	9181	9127	0.271	0.212	43051.89		
	10331	10277	0.300	0.241	42643.15		
-901	8126	8072	0.239	0.180	44844.44	43513.16	887.52
Blank	54		0.059				
	5413	5359	0.394	0.335	15997.01		
	6788	6734	0.452	0.393	17134.86		
-654	4599	4545	0.279	0.220	20659.09	17930.32	1819.18
Blank	54		0.059				
-1357ID	6773	6719	0.348	0.324	20737.65		
	7826	7772	0.383	0.256	30359.38		
	6455	6401	0.315	0.289	22148.79	24415.27	3962.73
Blank	54		0.059				
-1228ID	1385	1331	0.342	0.283	4703.18		
	999	945	0.250	0.191	4947.64		
	873	819	0.260	0.201	4074.63	4575.15	333.68
Blank	54		0.059				
-1051ID	330	276	0.288	0.229	1205.24		
	321	267	0.273	0.214	1247.66		
	320	266	0.288	0.229	1161.57	1204.83	28.84
Blank	54		0.059				
	4058	4004	0.270	0.211	18976.30		
	4528	4474	0.370	0.311	14385.85		
-901ID	3739	3685	0.255	0.196	18801.02	17387.73	2001.25
Blank	54		0.059				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	120.7	1.0	0.5	0.8
-1357	25979.0	1228.5	100.0	4.7
-1228	51368.9	3064.0	197.7	6.0
-1051	60478.2	4590.1	232.8	7.6
-901	43513.2	887.5	167.5	2.0
-654	17930.3	1819.2	69.0	10.1
-1357ID	24415.3	3962.7	94.0	16.2
-1228ID	4575.2	333.7	17.6	7.3
-1051ID	1204.8	28.8	4.6	2.4
-901ID	17387.7	2001.2	66.9	11.5

Transfection #17

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	35	-268	0.320	0.221	-1212.67		
	178	-125	0.211	0.112	-1116.07		
pGL3Basic	167	-136	0.393	0.294	-462.59	-930.44	311.90
Blank	303		0.099				
	4008	3705	0.302	0.203	18251.23		
	3776	3473	0.289	0.190	18278.95		
-1357	4483	4180	0.271	0.172	24302.33	20277.50	2683.22
Blank	303		0.099				
	8108	7805	0.449	0.350	22300.00		
	9335	9032	0.437	0.338	26721.89		
-1228	9404	9101	0.380	0.281	32387.90	27136.60	3500.87
Blank	303		0.099				
	26246	25943	0.454	0.355	73078.87		
	28409	28106	0.537	0.438	64168.95		
-1051	27751	27448	0.500	0.401	68448.88	68565.57	3008.87
Blank	303		0.099				
	27412	27109	0.571	0.472	57434.32		
	29761	29458	0.651	0.552	53365.94		
-901	29127	28824	0.596	0.497	57995.98	56265.41	1932.98
Blank	303		0.099				
	882	579	0.377	0.278	2082.73		
	1020	717	0.389	0.290	2472.41		
-1228ID	841	538	0.345	0.246	2186.99	2247.38	150.02
Blank	303		0.099				
	593	290	0.378	0.279	1039.43		
	544	241	0.368	0.269	895.91		
-1051ID	518	215	0.377	0.278	773.38	902.91	91.01
Blank	303		0.099				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-930.4	311.9	-4.6	-33.5
-1357	20277.5	1228.5	100.0	6.1
-1228	27136.6	3500.9	133.8	12.9
-1051	68565.6	3008.9	338.1	4.4
-901	56265.4	1933.0	277.5	3.4
-1228ID	2247.4	150.0	11.1	6.7
-1051ID	902.9	91.0	4.5	10.1

Transfection #18

construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	714	205	0.107	0.043	4767.44		
pGL3Basic	605	96	0.105	0.041	2341.46	3554.45	1212.99
Blank	509		0.064				
	4709	3887	0.110	0.046	84500.00		
	5157	4335	0.105	0.041	105731.71		
-1357	4821	3999	0.100	0.036	111083.33	100438.35	10625.56
Blank	822		0.064				
	7614	7383	0.131	0.067	110194.03		
	7267	7036	0.123	0.059	119254.24		
-1228	7453	7222	0.126	0.062	116483.87	115310.71	3411.12
Blank	231		0.064				
	20359	20016	0.118	0.054	370666.67		
	25122	24779	0.127	0.063	393317.46		
-1051	28813	28470	0.133	0.069	412608.70	392197.61	14353.96
Blank	343		0.064				
	13399	12985	0.097	0.033	393484.85		
	16410	15996	0.112	0.048	333250.00		
-901	16870	16456	0.114	0.050	329120.00	351951.62	27688.82
Blank	414		0.064				
	7595	7022	0.105	0.041	171268.29		
	7999	7426	0.108	0.044	168772.73		
-654	8101	7528	0.111	0.047	160170.21	166737.08	4377.91
Blank	573		0.064				
	2681	2527	0.114	0.050	50540.00		
	3032	2878	0.117	0.053	54301.89		
-456	2575	2421	0.111	0.047	51510.64	52117.51	1456.25
Blank	154		0.064				
	1438	443	0.125	0.061	7262.30		
	1394	399	0.121	0.057	7000.00		
-1228ID	1417	422	0.127	0.063	6698.41	6986.90	192.33
Blank	995		0.064				

Summary

Construct	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	3554.5	1213.0	3.5	34.1
-1357	100438.3	14060.0	100.0	14.0
-1228	115310.7	3411.1	114.8	3.0
-1051	392197.6	14354.0	390.5	3.7
-901	351951.6	27688.8	350.4	7.9
-654	166737.1	5822.3	166.0	3.5
-456	52117.5	1456.3	51.9	2.8
-1228ID	6986.9	282.2	7.0	4.0

5.5 Addition of Sp1 and Sp3 with topoisomerase IIβ ICB1 and ICB2 mutant constructs.

Transfection #19 construct	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
	179	101	0.352	0.290	348.28		
	101	23	0.434	0.372	61.83		
pGL3Basic	101	23	0.418	0.356	64.61	158.24	126.69
Blank	78		0.062				
	13263	12813	0.431	0.369	34723.58		
	14352	13902	0.447	0.385	36109.09		
-654	13144	12694	0.443	0.381	33317.59	34716.75	932.78
Blank	450		0.062				
	17903	17727	0.441	0.379	46773.09		
	21991	21815	0.448	0.386	56515.54		
ICB1m	23426	23250	0.477	0.415	56024.10	53104.24	4220.77
Blank	176		0.062				
ICB1m	28211	27309	0.381	0.319	85608.15		
+ 0.25 ug Sp3	34904	34002	0.394	0.332	102415.66		
	31984	31082	0.352	0.290	107179.31	98401.04	8528.59
Blank	902		0.062				
ICB1m	16354	15551	0.258	0.196	79341.84		
+ 0.5 ug Sp3	16480	15677	0.287	0.225	69675.56		
	18560	17757	0.294	0.232	76538.79	75185.40	3673.23
Blank	803		0.062				
ICB1m	7041	5840	0.162	0.100	58400.00		
+ 1.0 ug Sp3	6673	5472	0.167	0.105	52114.29		
	6424	5223	0.169	0.107	48813.08	53109.12	3527.25
Blank	1201		0.062				
	5689	5487	0.493	0.431	12730.86		
	6935	6733	0.516	0.454	14830.40		
ICB2m	5992	5790	0.456	0.394	14695.43	14085.562	903.14
					9901	9231	0.598	0.536	17222.01		
					11482	10812	0.639	0.577	18738.30		
ICB2m	0.25	-			11351	10681	0.604	0.542	19706.64	18555.65	889.09
Blank					670		0.062				
					13754	12854	0.650	0.588	21860.54		
					15450	14550	0.661	0.599	24290.48		
ICB2m	0.5	-			14126	13226	0.656	0.594	22265.99	22805.67	989.87
Blank					900		0.062				
					21651	20577	0.720	0.658	31272.04		
					28067	26993	0.804	0.742	36378.71		
ICB2m	1	-			27899	26825	0.796	0.734	36546.32	34732.35	2306.88
Blank					1074		0.062				
					5273	4057	0.374	0.312	13003.21		
					4621	3405	0.352	0.290	11741.38		
ICB2m	-	0.25			3834	2618	0.295	0.233	11236.05	11993.55	673.11
Blank					1216		0.062				
					3813	2535	0.262	0.200	12675.00		
					4105	2827	0.344	0.282	10024.82		
ICB2m	-	0.5			3825	2547	0.306	0.244	10438.52	11046.12	1085.92
Blank					1278		0.062				
					2661	1378	0.160	0.098	14061.22		
					2530	1247	0.174	0.112	11133.93		
ICB2m	-	1			2595	1312	0.162	0.100	13120.00	12771.72	1091.86
Blank					1283		0.062				

Summary

Construct	Sp1 (uc	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	138.5	88.7	0.4	64.0
-654	-	-	34716.8	932.8	100.0	2.7
ICB1m	-	-	53104.2	4220.8	153.0	7.9
ICB1m	-	0.25	98401.0	8528.6	283.4	8.7
ICB1m	-	0.5	75185.4	3673.2	216.6	4.9
ICB1m	-	1	53109.1	3527.3	153.0	6.6
ICB2m	-	-	14085.6	903.1	40.6	6.4
ICB2m	0.25	-	18555.7	889.1	53.4	4.8
ICB2m	0.5	-	22805.7	989.9	65.7	4.3
ICB2m	1	-	34732.4	2306.9	100.0	6.6
ICB2m	-	0.25	11993.5	673.1	34.5	5.6
ICB2m	-	0.5	11046.1	1085.9	31.8	9.8
ICB2m	-	1	12771.7	1091.9	36.8	8.5

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			179	101	0.352	0.290	348.28		
			101	23	0.434	0.372	61.83		
pGL3Basic	-	-	101	23	0.418	0.356	64.61	158.24	164.58
Blank			78		0.062				
			1445	1140	0.431	0.369	3089.43		
			1529	1224	0.447	0.385	3179.22		
-654	-	-	1430	1125	0.443	0.381	2952.76	3073.80	114.04
Blank			305		0.062				
			1992	1703	0.441	0.379	4493.40		
			2330	2041	0.448	0.386	5287.56		
ICB1m	-	-	2573	2284	0.477	0.415	5503.61	5094.86	531.96
Blank			289		0.062				
			3259	2937	0.538	0.476	6170.17		
			3230	2908	0.512	0.450	6462.22		
ICB1m	0.25	-	3427	3105	0.510	0.448	6930.80	6521.06	383.72
Blank			322		0.062				
			23030	22669	0.700	0.638	35531.35		
			27773	27412	0.755	0.693	39556.56		
ICB1m	0.5	-	22019	21658	0.698	0.636	34053.46	36380.12	2847.56
Blank			361		0.062				
			5236	4890	0.573	0.511	9569.47		
			5831	5485	0.622	0.560	9794.64		
ICB1m	1	-	5057	4711	0.535	0.473	9959.83	9774.65	195.95
Blank			346		0.062				

Summary

Construct	Sp1 (ug)	Sp3 (ug)	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	138.5	88.7	4.5	64.0
-654	-	-	3073.8	80.7	100.0	2.6
ICB1m	-	-	5094.9	401.0	165.8	7.9
ICB1m	0.25	-	6521.1	273.2	212.1	4.2
ICB1m	0.5	-	36380.1	2117.0	1183.6	5.8
ICB1m	1	-	9774.6	136.8	318.0	1.4

Transfection #20 construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			95	17	0.288	0.217	78.34		
pGL3Basic	-	-	87	9	0.294	0.223	40.36	59.35	18.99
Blank			78		0.071				
			75	26	0.512	0.441	58.96		
			63	14	0.506	0.435	32.18		
pGL3Basic	0.5	-	57	8	0.450	0.379	21.11	37.42	14.36
Blank			49		0.071				
			5319	5221	0.355	0.284	18383.80		
			5272	5174	0.376	0.305	16963.93		
-654	-	-	4722	4624	0.391	0.320	14450.00	16599.25	1432.83
Blank			98		0.071				
			7446	7143	0.547	0.476	15006.30		
			7468	7165	0.580	0.509	14076.62		
-654	0.5	-	7479	7176	0.646	0.575	12480.00	13854.31	916.21
Blank			303		0.071				
			8179	7929	0.357	0.286	27723.78		
			8553	8303	0.360	0.289	28730.10		
ICB1m	-	-	8042	7792	0.307	0.236	33016.95	29823.61	2128.89
Blank			250		0.071				
			11703	11593	0.414	0.343	33798.83		
			14546	14436	0.400	0.329	43878.42		
ICB1m	0.25	-	12103	11993	0.343	0.272	44091.91	40589.722	4527.26
Blank			110		0.071				
			14755	14326	0.538	0.467	30676.66		
			15650	15221	0.523	0.452	33674.78		
ICB1m	0.5	-	14536	14107	0.495	0.424	33271.23	32540.89	1242.82
Blank			429		0.071				
			16207	15634	0.412	0.341	45847.51		
			18107	17534	0.415	0.344	50970.93		
ICB1m	1	-	15417	14844	0.429	0.358	41463.69	46094.04	3251.26
Blank			573		0.071				

	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			1708	992	0.300	0.229	4331.88		
			1653	937	0.286	0.215	4358.14		
ICB2m	-	-	1786	1070	0.295	0.224	4776.79	4488.93	191.90
Blank			716		0.071				
			4491	3598	0.365	0.294	12238.10		
			4883	3990	0.415	0.344	11598.84		
ICB2m	0.25	-	5258	4365	0.411	0.340	12838.24	12225.056	417.48
Blank			893		0.071				
			5236	4512	0.536	0.465	9703.23		
			5076	4352	0.492	0.421	10337.29		
ICB2m	0.5	-	4450	3726	0.501	0.430	8665.12	9568.54	602.29
Blank			724		0.071				
			6153	5067	0.427	0.356	14233.15		
			7047	5961	0.476	0.405	14718.52		
ICB2m	1	-	6040	4954	0.493	0.422	11739.34	13563.67	1216.22
Blank			1086		0.071				
			890	811	0.292	0.221	3669.68		
			1015	936	0.336	0.265	3532.08		
-1357	-	-	885	806	0.307	0.236	3415.25	3539.00	87.12
Blank			112		0.072				
			1243	1194	0.456	0.385	3101.30		
			1417	1368	0.442	0.371	3687.33		
-1357	0.5	-	1271	1222	0.445	0.374	3267.38	3352.00	223.55
Blank			79		0.072				

Summary

Construct	Sp1 (ug)	Sp3 (ug)	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	59.3	19.0	0.4	32.0
pGL3Basic	-	0.5	37.4	14.4	0.2	38.4
-654	-	-	16599.2	1432.8	100.0	8.6
-654	0.5	-	13854.3	916.2	83.5	6.6
ICB1m	-	-	29823.6	2128.9	179.7	7.1
ICB1m	0.25	-	40589.7	4527.3	244.5	11.2
ICB1m	0.5	-	32540.9	1242.8	196.0	3.8
ICB1m	1	-	46094.0	3251.3	277.7	7.1
ICB2m	-	-	4488.9	191.9	27.0	4.3
ICB2m	0.25	-	12225.1	417.5	73.6	3.4
ICB2m	0.5	-	9568.5	602.3	57.6	6.3
ICB2m	1	-	13563.7	1216.2	81.7	9.0
Relative to -1357%						
-1357	-	-	3539.0	87.1	100.0	2.5
-1357	0.5	-	3352.0	223.6	94.7	6.7

Transfection #21 construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			848	85	0.323	0.257	330.74		
			787	24	0.371	0.305	78.69		
pGL3Basic	-	-	781	18	0.373	0.307	58.63	156.02	116.48
Blank			763		0.066				
			794	9	0.515	0.449	20.04		
			866	81	0.549	0.483	167.70		
pGL3Basic	0.5	-	824	39	0.564	0.498	78.31	88.69	52.68
Blank			785		0.066				
			8210	7359	0.428	0.362	20328.73		
			9610	8759	0.425	0.359	24398.33		
-654	-	-	7005	6154	0.385	0.319	19291.54	21339.53	2039.20
Blank			851		0.066				
			12440	11389	0.568	0.502	22687.25		
			14419	13368	0.594	0.528	25318.18		
-654	0.5	-	14839	13788	0.686	0.620	22238.71	23414.71	1268.98
Blank			1051		0.066				
			18130	17147	0.287	0.221	77588.24		
			17808	16825	0.336	0.270	62314.81		
ICB1m	-	-	17129	16146	0.335	0.269	60022.30	66641.78	7297.63
Blank			983		0.066				
			29683	28839	0.460	0.394	73195.43		
			27242	26398	0.398	0.332	79512.05		
ICB1m	0.25	-	26564	25720	0.413	0.347	74121.04	75609.51	2601.69
Blank			844		0.066				
			37090	35716	0.514	0.448	79723.21		
			39275	37901	0.501	0.435	87128.74		
ICB1m	0.5	-	37947	36573	0.518	0.452	80913.72	82588.56	3026.79
Blank			1374		0.066				
			44785	43418	0.549	0.483	89892.34		
			49982	48615	0.529	0.463	105000.00		
ICB1m	1	-	40213	38846	0.486	0.420	92490.48	95794.27	6137.15
Blank			1367		0.066				
			3448	2035	0.250	0.184	11059.78		
			4429	3016	0.306	0.240	12566.67		
ICB2m	-	-	4459	3046	0.328	0.262	11625.95	11750.80	543.91
Blank			1413		0.066				
			8255	6602	0.459	0.393	16798.98		
			8072	6419	0.428	0.362	17732.04		
ICB2m	0.25	-	7596	5943	0.440	0.374	15890.37	16807.13	616.61
Blank			1653		0.066				
			7087	5508	0.599	0.533	10333.96		
			6846	5267	0.564	0.498	10576.31		
ICB2m	0.5	-	7331	5752	0.605	0.539	10671.61	10527.29	128.89
Blank			1579		0.066				
			4921	3461	0.645	0.579	5977.55		
			5199	3739	0.659	0.593	6305.23		
ICB2m	1	-	4612	3152	0.687	0.621	5075.68	5786.15	473.65
Blank			1460		0.066				

Summary

Construct	Sp1 (ug	Sp3 (ug	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	156.0	116.5	0.7	74.7
pGL3Basic	0.5	-	88.7	52.7	0.4	59.4
-654	-	-	21339.5	2039.2	100.0	9.6
-654	0.5	-	23414.7	1269.0	109.7	5.4
ICB1m	-	-	66641.8	7297.6	312.3	11.0
ICB1m	0.25	-	75609.5	2601.7	354.3	3.4
ICB1m	0.5	-	82588.6	3026.8	387.0	3.7
ICB1m	1	-	95794.3	6137.2	448.9	6.4
ICB2m	-	-	11750.8	543.9	55.1	4.6
ICB2m	0.25	-	16807.1	616.6	78.8	3.7
ICB2m	0.5	-	10527.3	128.9	49.3	1.2
ICB2m	1	-	5786.2	473.6	27.1	8.2

Transfection #22

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			224	180	0.270	0.198	909.09		
			252	208	0.268	0.196	1061.22		
pGL3Basic	-	-	108	64	0.278	0.206	310.68	760.33	299.77
Blank			44		0.072				
			79	6	0.456	0.384	15.63		
pGL3Basic	0.5	-	80	7	0.445	0.373	18.77	17.20	1.57
Blank			73		0.072				
			4792	4577	0.486	0.414	11055.56		
			5464	5249	0.514	0.442	11875.57		
-654	-	-	5198	4983	0.552	0.480	10381.25	11104.12	514.29
Blank			215		0.072				
			6409	6080	0.519	0.447	13601.79		
			6475	6146	0.549	0.477	12884.70		
-654	0.5	-	6307	5978	0.524	0.452	13225.66	13237.38	242.94
Blank			329		0.072				
			11831	11552	0.415	0.343	33679.30		
			12738	12459	0.442	0.370	33672.97		
ICB1m	-	-	12590	12311	0.472	0.400	30777.50	32709.92	1288.28
Blank			279		0.072				
			10933	10797	0.387	0.315	34276.19		
			8981	8845	0.317	0.245	36102.04		
ICB1m	0.25	-	10631	10495	0.339	0.267	39307.12	36561.78	1830.22
Blank			136		0.072				
			13122	12594	0.388	0.316	39854.43		
			10171	9643	0.319	0.247	39040.49		
ICB1m	0.5	-	14580	14052	0.403	0.331	42453.17	40449.36	1335.87
Blank			528		0.072				
			18695	18000	0.582	0.510	35294.12		
			17435	16740	0.524	0.452	37035.40		
ICB1m	1	-	15150	14455	0.492	0.420	34416.67	35582.06	968.89
Blank			695		0.072				
			17489	16794	0.567	0.495	33927.27		
			15343	14648	0.519	0.447	32769.57		
ICB1m	-	0.5	14271	13576	0.599	0.527	25760.91	30819.25	3372.23
Blank			670		0.060				
			2686	1919	0.414	0.342	5611.11		
			2756	1989	0.418	0.346	5748.55		
ICB2m	-	-	2589	1822	0.385	0.313	5821.09	5726.92	77.20
Blank			767		0.072				
			3226	2160	0.325	0.253	8537.55		
			3936	2870	0.442	0.370	7756.76		
ICB2m	0.25	-	6367	5301	0.675	0.603	8791.04	8361.78	403.35
Blank			1066		0.072				
			3573	2704	0.516	0.444	6090.09		
			4117	3248	0.550	0.478	6794.98		
ICB2m	0.5	-	3688	2819	0.575	0.503	5604.37	6163.15	421.22
Blank			869		0.072				
			5210	4306	0.637	0.565	7621.24		
			5946	5042	0.648	0.576	8753.47		
ICB2m	1	-	5166	4262	0.583	0.511	8340.51	8238.41	411.45
Blank			904		0.072				
			2863	1302	0.278	0.217	6000.00		
			2990	1429	0.250	0.189	7560.85		
-1357	-	-	2874	1313	0.266	0.205	6404.88	6655.24	603.74
Blank			1561		0.061				
			2988	1540	0.290	0.229	6724.89		
			2710	1262	0.259	0.198	6373.74		
-1357	0.5	-	2718	1270	0.282	0.221	5746.61	6281.74	356.76
Blank			1448		0.061				
			3359	1486	0.354	0.293	5071.67		
			3173	1300	0.373	0.312	4166.67		
			3585	1712	0.366	0.305	5613.11	4950.48	522.55
-1357	-	0.5	1873		0.61				
Blank									

Summary

Construct	Sp1 (ug)	Sp3 (ug)	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	760.3	299.8	6.8	39.4
pGL3Basic	-	0.5	17.2	1.6	0.2	9.1
-654	-	-	11104.1	514.3	100.0	4.6
-654	0.5	-	13237.4	242.9	119.2	1.8
ICB1m	-	-	32709.9	1288.3	294.6	3.9
ICB1m	0.25	-	36561.8	1830.2	329.3	5.0
ICB1m	0.5	-	40449.4	1335.9	364.3	3.3
ICB1m	1	-	35582.1	968.9	320.4	2.7
ICB1m	-	0.5	30819.3	3372.2	277.5	10.9
ICB2m	-	-	5726.9	77.2	51.6	1.3
ICB2m	0.25	-	8361.8	403.4	75.3	4.8
ICB2m	0.5	-	6163.1	421.2	55.5	6.8
ICB2m	1	-	8238.4	411.4	74.2	5.0
Relative to -1357%						
-1357	-	-	6655.2	201.2	100.0	3.0
-1357	0.5	-	6281.7	118.9	94.4	1.9
-1357	-	0.5	4950.5	174.2	74.4	3.5

Transfection #23

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			499	28	0.368	0.307	91.21		
pGL3Basic			496	25	0.380	0.319	78.37	84.79	6.42
Blank			471		0.061				
			331	12	0.292	0.231	51.95		
			341	22	0.237	0.176	125.00		
pGL3Basic		0.5						88.47	36.53
Blank			319		0.060				
-654			3864	2687	0.277	0.216	12439.81		
			4359	3182	0.272	0.211	15080.57		
			3570	2393	0.256	0.195	12271.79	13264.06	1211.01
Blank			1177		0.061				
-654			4338	4116	0.292	0.220	18709.09	17744.12	1001.13
			4510	4288	0.336	0.264	16242.42		
		0.5	4518	4296	0.307	0.235	18280.85		
Blank			222		0.072				
ICB1m			7552	7034	0.261	0.200	35170.00	31247.98	2614.68
			6901	6383	0.282	0.221	28882.35		
			6872	6354	0.275	0.214	29691.59		
Blank			518		0.061				
ICB1m			9072	8751	0.352	0.291	30072.16	30764.601	1064.34
			13136	12815	0.457	0.396	32361.11		
	0.25		11668	11347	0.441	0.380	29860.53		
Blank			321		0.061				
ICB1m			11533	10953	0.489	0.428	25591.12	26931.96	2173.57
			10635	10055	0.463	0.402	25012.44		
	0.5		12355	11775	0.451	0.390	30192.31		
Blank			580		0.061				
ICB1m			19076	18556	0.579	0.518	35822.39	36467.43	2132.83
			17775	17255	0.496	0.435	39666.67		
	1		17714	17194	0.568	0.507	33913.21		
Blank			520		0.061				
ICB1m			3448	3036	0.202	0.141	21531.91	22019.48	487.56
			3872	3460					
		0.25	3608	3196	0.203	0.142	22507.04		
Blank			412		0.061				
ICB1m			3490	3118	0.182	0.121	25768.60	26213.888	2099.83
			3279	2907	0.160	0.099	29363.64		
		0.5	2864	2492	0.167	0.106	23509.43		
Blank			372		0.061				
ICB1m			1993	1573	0.124	0.063	24968.25	24144.90	1086.18
			1942	1522	0.122	0.061	24950.82		
		1	1861	1441	0.125	0.064	22515.63		
Blank			420		0.061				
ICB2m			1324	817	0.224	0.163	5012.27	4284.77	485.00
			1361	854	0.269	0.208	4105.77		
			1187	680	0.243	0.182	3736.26		
Blank			507		0.061				
ICB2m			1047	572	0.197	0.136	4205.88	4250.20	44.32
			1102	627	0.207	0.146	4294.52		
		0.25							
Blank			475		0.061				
ICB2m			728	285	0.147	0.086	3313.95	2850.53	463.43
			665	222	0.154	0.093	2387.10		
		0.5							
Blank			443		0.061				
ICB2m			826	274	0.093	0.032	8562.50	5320.83	581.25
		1	1033	481	0.126	0.065	7400.00		
Blank			552		0.061				
-1357			8208	7408	0.252	0.150	49386.67	47497.04	2707.70
			8002	7202	0.247	0.145	49668.97		
			6186	5386	0.226	0.124	43435.48		
Blank			800		0.102				
-1357			6339	5499	0.228	0.126	43642.86	41085.96	2176.85
			6733	5893	0.243	0.141	41794.33		
	0.5		6324	5484	0.247	0.145	37820.69		
Blank			840		0.102				
-1357			6448	5082	0.252	0.150	33880.00	34300.40	944.90
			6195	4829	0.247	0.145	33303.45		
		0.5	5795	4429	0.226	0.124	35717.74		
Blank			1366		0.102				

Summary

Construct	Sp1 (ug)	Sp3 (ug)	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	84.8	6.4	0.6	7.6
pGL3Basic	-	0.5	88.5	36.5	0.7	41.3
-654	-	-	13264.1	1211.0	100.0	9.1
-654	-	0.5	17744.1	1001.1	133.8	5.6
ICB1m	-	-	31248.0	2614.7	235.6	8.4
ICB1m	0.25	-	30764.6	1064.3	231.9	3.5
ICB1m	0.5	-	26932.0	2173.6	203.0	8.1
ICB1m	1	-	36467.4	2132.8	274.9	5.8
ICB1m	-	0.25	22019.5	487.6	166.0	2.2
ICB1m	-	0.5	26213.9	2099.8	197.6	8.0
ICB1m	-	1	24144.9	1086.2	182.0	4.5
ICB2m	-	-	4284.8	485.0	32.3	11.3
ICB2m	-	0.25	4250.2	44.3	32.0	1.0
ICB2m	-	0.5	2850.5	463.4	21.5	16.3
ICB2m	-	1	5320.8	581.3	40.1	10.9
Relative to 1540%						
-1357	-	-	47497.0	3520.2	100.0	7.4
-1357	0.5	-	41086.0	2975.0	86.5	7.2
-1357	-	0.5	34300.4	1260.9	72.2	3.7

Transfection #24 construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			120	80	0.838	0.767	104.30		
			94	54	0.871	0.800	67.50		
pGL3Basic			104	64	0.828	0.757	84.54	85.45	12.57
Blank			40		0.071				
			78	5	0.436	0.365	13.70		
pGL3Basic		0.5	78	5	0.428	0.357	14.01	13.85	0.15
Blank			73		0.071				
			16010	15846	0.880	0.809	19587.14		
			18832	18668	0.832	0.761	24530.88		
-654			15829	15665	0.854	0.783	20006.39	21374.80	2104.05
Blank			164		0.071				
			6878	6507	0.451	0.380	17123.68		
			7426	7055	0.404	0.333	21186.19		
-654		0.5	6635	6264	0.452	0.381	16440.94	18250.27	1957.28
Blank			371		0.071				
			13365	13208	0.575	0.504	26206.35		
ICB1m		0.25	13857	13700	0.584	0.513	26705.65	26456.00	249.65
Blank			157		0.071				
ICB1m			8267	7708	0.287	0.216	35685.19		
			8343	7784	0.284	0.213	36544.60		
		1	7431	6872	0.280	0.209	32880.38	35036.72	1437.56
Blank			569		0.071				
			5199	4493	0.839	0.768	5850.26		
			4896	4190	0.841	0.770	5441.56		
ICB2m			4351	3645	0.789	0.718	5076.60	5456.14	262.75
Blank			706		0.071				
ICB2m			3026	2248	0.603	0.532	4225.56		
			3071	2293	0.568	0.497	4613.68		
		0.25	2905	2127	0.583	0.512	4154.30	4331.18	188.33
Blank			778		0.071				
ICB2m			2033	1338	0.474	0.403	3320.10		
			2172	1477	0.470	0.399	3701.75		
		0.5	1932	1237	0.422	0.351	3524.22	3515.36	130.17
Blank			695		0.071				
ICB2m			840	238	0.097	0.026	9153.85		
			898	296	0.117	0.046	6434.78		
		1	852	250	0.118	0.047	5531.91	7040.18	1409.11
Blank			602		0.071				
			18608	18341	0.782	0.711	25796.06		
			19891	19624	0.825	0.754	26026.53		
-1357			17570	17303	0.820	0.749	23101.47	24974.69	1248.81
Blank			267		0.071				
			10019	9655	0.481	0.410	23304.88		
			10818	10354	0.533	0.462	22411.26		
-1357		0.5	9574	9210	0.485	0.414	22246.38	22654.17	433.81
Blank			464		0.071				

Summary

Construct	Sp1 (ug)	Sp3 (ug)	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	85.4	12.6	0.4	14.7
pGL3Basic	-	0.5	13.9	0.2	0.1	1.1
-654	-	-	21374.8	2104.1	100.0	9.8
-654	-	0.5	18250.3	1957.3	85.4	10.7
ICB1m	-	0.25	26456.0	249.7	123.8	0.9
ICB1m	-	1	35036.7	1437.6	163.9	4.1
ICB2m	-	-	5456.1	262.7	25.5	4.8
ICB2m	-	0.25	4331.2	188.3	20.3	4.3
ICB2m	-	0.5	3515.4	130.2	16.4	3.7
ICB2m	-	1	7040.2	1409.1	32.9	20.0
Relative to -1357%						
-1357	-	-	24974.7	1248.8	100.0	5.0
-1357	-	0.5	22654.2	433.8	90.7	1.9

Transfection #25

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			558	161	0.437	0.376	428.19		
			479	82	0.380	0.319	257.05		
pGL3Basic			507	110	0.400	0.339	324.48	336.58	61.08
Blank			397		0.061				
			552	85	0.273	0.212	400.94		
			554	87	0.277	0.216	402.78		
pGL3Basic		0.5	518	51	0.278	0.217	235.02	346.25	74.15
Blank			467		0.061				
			13333	12757	0.482	0.421	30301.66		
			13291	12715	0.456	0.395	32189.87		
-654			15132	14556	0.536	0.475	30644.21	31045.25	763.08
Blank			576		0.061				
-654			6411	5835	0.281	0.220	25431.82		
			6221	5405	0.293	0.232	23297.41		
		0.5	6603	5787	0.339	0.278	20816.55	23181.93	1576.92
Blank			816		0.061				
ICB1m			23401	22707	0.584	0.523	43416.83		
			18825	18131	0.450	0.389	46609.25		
			22071	21377	0.538	0.477	44815.51	44947.20	1108.04
Blank			694		0.061				
ICB1m			14946	14351	0.394	0.333	43096.10		
			15359	14764	0.372	0.311	47472.67		
		0.25	16407	15812	0.397	0.336	47059.52	45876.10	1853.33
Blank			595		0.061				
ICB1m			9142	8065	0.265	0.204	39534.31		
			9780	8703	0.248	0.187	46540.11		
		0.5	9825	8748	0.260	0.199	43959.80	43344.74	2540.28
Blank			1077		0.061				
ICB1m			12886	11757	0.189	0.128	91851.56		
			12335	11206	0.193	0.132	84893.94		
		1	12301	11172	0.186	0.125	89376.00	88707.17	2542.15
Blank			1129		0.061				
ICB2m			7107	5863	0.514	0.453	12942.60		
			7150	5906	0.449	0.388	15221.65		
			6663	5419	0.449	0.388	13966.49	14043.58	785.38
Blank			1244		0.061				
ICB2m			4893	3486	0.339	0.278	12539.57		
			5320	3913	0.363	0.302	12956.95		
		0.25	4930	3523	0.354	0.293	12023.89	12506.80	321.94
Blank			1407		0.061				
ICB2m			3379	1954	0.263	0.202	9673.27		
			3254	1829	0.238	0.177	10333.33		
		0.5	3325	1900	0.238	0.177	10734.46	10247.02	382.50
Blank			1425		0.061				
ICB2m			2407	1141	0.165	0.104	10971.15		
			2486	1220	0.159	0.098	12448.98		
		1	2386	1120	0.160	0.099	11313.13	11577.75	580.82
Blank			1266		0.061				

Summary

Construct	Sp1 (ug)	Sp3 (ug	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	336.6	61.1	1.1	18.1
pGL3Basic	-	0.5	346.2	74.2	1.1	21.4
-654	-	-	31045.2	763.1	100.0	2.5
-654	-	0.5	23181.9	1576.9	74.7	6.8
ICB1m	-	-	44947.2	1108.0	144.8	2.5
ICB1m	-	0.25	45876.1	1853.3	147.8	4.0
ICB1m	-	0.5	43344.7	2540.3	139.6	5.9
ICB1m	-	1	88707.2	2542.2	285.7	2.9
ICB2m	-	-	14043.6	785.4	45.2	5.6
ICB2m	-	0.25	12506.8	321.9	40.3	2.6
ICB2m	-	0.5	10247.0	382.5	33.0	3.7
ICB2m	-	1	11577.8	580.8	37.3	5.0

Transfection #26

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			142	69	0.420	0.349	197.71		
			138	65	0.385	0.314	207.01		
pGL3Basic								202.36	4.65
Blank			73		0.071				
			6113	6068	0.340	0.269	22557.62		
			7754	7709	0.414	0.343	22475.22		
-654			6124	6079	0.403	0.332	18310.24	21114.36	1869.41
Blank			45		0.071				
			18531	18496	0.382	0.311	59472.67		
			19959	19924	0.379	0.308	64688.31		
ICB1m			17677	17642	0.363	0.292	60417.81	61526.26	2108.03
Blank			35		0.071				
			47199	46981	0.408	0.337	139409.50		
			49771	49553	0.409	0.338	146606.51		
ICB1m	0.25		47450	47232	0.530	0.459	102901.96	129639.32	17824.91
Blank			218		0.071				
			37266	37210	0.500	0.429	86736.60		
			38789	38733	0.529	0.458	84569.87		
ICB1m	0.5		34718	34662	0.487	0.416	83322.12	84876.19	1240.27
Blank			56		0.071				
			35892	35845	0.523	0.452	79303.10		
			37300	37253	0.459	0.388	96012.89		
ICB1m	1		35589	35542	0.540	0.469	75782.52	83699.50	8208.92
Blank			47		0.071				
			16700	16615	0.305	0.234	71004.27		
			15721	15636	0.294	0.223	70116.59		
ICB1m		0.25	15264	15179	0.320	0.249	60959.84	67360.23	4266.93
Blank			85		0.071				
			11200	11094	0.261	0.190	58389.47		
			11950	11844	0.287	0.216	54833.33		
ICB1m		0.5	10103	9997	0.255	0.184	54331.52	55851.44	1692.02
Blank			106		0.071				
			7379	7273	0.196	0.125	58184.00		
			7566	7460	0.183	0.112	66607.14		
ICB1m		1	7057	6951	0.183	0.112	62062.50	62284.55	2881.73
Blank			106		0.071				

Summary

Construct	Sp1 (u	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3Basic	-	-	202.4	4.6	1.0	2.3
-654	-	-	21114.4	1869.4	100.0	8.9
ICB1m	-	-	61526.3	2108.0	291.4	3.4
ICB1m	0.25	-	129639.3	17824.9	614.0	13.7
ICB1m	0.5	-	84876.2	1240.3	402.0	1.5
ICB1m	1	-	83699.5	8208.9	396.4	9.8
ICB1m	-	0.25	67360.2	4266.9	319.0	6.3
ICB1m	-	0.5	55851.4	1692.0	264.5	3.0
ICB1m	-	1	62284.5	2881.7	295.0	4.6

5.6 Addition of Sp1 and Sp3 with pGL3Basic promoter – topo IIβ 180 bp construct.

Transfection #27

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			966	763	0.213	0.154	4954.55		
pGL3Basic			1140	937	0.188	0.129	7263.57		
Promoter	-	-	1089	886	0.185	0.126	7031.75	6416.62	1271.49
Blank			203		0.059				
			631	566	0.221	0.162	3493.83		
pGL3Basic			631	566	0.214	0.155	3651.61		
Promoter	0.5	-	602	537	0.218	0.159	3377.36	3507.60	137.64
Blank			65		0.059				
			657	525	0.214	0.155	3387.10		
pGL3Basic			774	642	0.215	0.156	4115.38		
Promoter	-	0.5	651	519	0.239	0.180	2883.33	3461.94	619.43
Blank			132		0.059				
			1147	1087	0.355	0.296	3672.30		
			1108	1048	0.360	0.301	3481.73		
pGL3BP-179	-	-	1146	1086	0.349	0.290	3744.83	3632.95	135.89
Blank			60		0.059				
			1699	1596	0.611	0.552	2891.30		
			1892	1789	0.652	0.593	3016.86		
pGL3BP-179	0.25	-	1812	1709	0.638	0.579	2951.64	2953.27	62.80
Blank			103		0.059				
			1960	886	0.514	0.455	1947.25		
			2124	1050	0.576	0.543	1933.70		
pGL3BP-179	0.5	-	1976	902	0.562	0.499	1807.62	1896.19	77.01
Blank			1074		0.059				
			2441	2272	0.589	0.530	4286.79		
			2749	2580	0.602	0.543	4751.38		
pGL3BP-179	1	-	2499	2330	0.558	0.499	4669.34	4569.17	247.96
Blank			169		0.059				
			630	548	0.287	0.157	3490.45		
pGL3BP-179	-	0.25	732	650	0.285	0.194	3350.52		
Blank			664	582	0.299	0.182	3197.80	3346.25	146.37
			82		0.059				
			1935	749	0.223	0.164	4567.07		
pGL3BP-179	-	0.5	1900	714	0.226	0.167	4275.45		
Blank			1774	588	0.209	0.150	3920.00	4254.17	324.06
			1186		0.059				
			890	890	0.216	0.157	5668.79		
pGL3BP-179	-	1	921	921	0.253	0.194	4747.42		
Blank			876	876	0.241	0.182	4813.19	5076.47	514.02
			362		0.059				
			362		0.059				

Summary

Construct	Sp1 (u	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3BP	-	-	6416.6	1271.5	176.6	19.8
pGL3BP	0.5	-	3507.6	137.6	96.5	3.9
pGL3BP	-	0.5	3461.9	619.4	95.3	17.9
pGL3BP-179	-	-	3633.0	135.9	100.0	3.7
pGL3BP-179	0.25	-	2953.3	62.8	81.3	2.1
pGL3BP-179	0.5	-	1896.2	77.0	52.2	4.1
pGL3BP-179	1	-	4569.2	248.0	125.8	5.4
pGL3BP-179	-	0.25	3346.3	146.4	92.1	4.4
pGL3BP-179	-	0.5	4254.2	324.1	117.1	7.6
pGL3BP-179	-	1	5076.5	514.0	139.7	10.1

Transfection #28

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			1908	1619	0.412	0.341	4747.80		
pGL3Basic			2037	1748	0.415	0.344	5081.40		
promoter	-	-	1889	1600	0.429	0.358	4469.27	4766.16	306.47
Blank			289		0.071				
			2534	1074	0.375	0.304	3532.89		
pGL3Basic			2508	1048	0.390	0.319	3285.27		
Promoter	0.5	-	2315	855	0.376	0.305	2803.28	3207.15	371.03
Blank			1460		0.071				
			1809	1523	0.538	0.467	3261.24		
pGL3Basic			1782	1496	0.523	0.452	3309.73		
Promoter	-	0.5	1655	1369	0.495	0.424	3228.77	3266.58	40.74
Blank			286		0.071				
			2306	917	0.328	0.257	3568.09		
			2371	982	0.365	0.294	3340.14		
pGL3BP-179	-	-	2317	928	0.373	0.302	3072.85	3327.03	247.88
Blank			1389		0.071				
			2296	827	0.448	0.377	2193.63		
			2299	830	0.422	0.351	2364.67		
pGL3BP-179	0.25	-	2278	809	0.413	0.342	2365.50	2307.93	98.99
Blank			1469		0.071				
			2851	978	0.481	0.410	2385.37		
			2761	888	0.480	0.409	2171.15		
pGL3BP-179	0.5	-	2698	825	0.473	0.402	2052.24	2202.92	168.82
Blank			1873		0.071				
			2456	791	0.298	0.227	3484.58		
			2550	885	0.401	0.330	2681.82		
pGL3BP-179	1	-	2388	723	0.342	0.271	2667.90	2944.77	467.55
Blank			1665		0.071				
			2606	1112	0.355	0.284	3915.49		
pGL3BP-179	-	0.25	2804	1310	0.376	0.305	4295.08		
Blank			2915	1421	0.391	0.320	4440.63	4217.07	271.12
			1494		0.071				
			2318	266	0.151	0.080	3325.00		
pGL3BP-179	-	0.5	2414	362	0.181	0.110	3290.91		
Blank			2553	501	0.197	0.126	3976.19	3530.70	386.18
			2052		0.071				
			2470	270	0.133	0.062	4354.84		
pGL3BP-179	-	1	2490	290	0.134	0.063	4603.17		
Blank			2476	276	0.141	0.070	3942.86	4300.29	333.52
			2200		0.071				

Summary

Construct	Sp1 (u	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3BP	-	-	4766.2	306.5	143.3	6.4
pGL3BP	0.5	-	3207.1	371.0	96.4	11.6
pGL3BP	-	0.5	3266.6	40.7	98.2	1.2
pGL3BP-179	-	-	3327.0	247.9	100.0	7.5
pGL3BP-179	0.25	-	2307.9	99.0	69.4	4.3
pGL3BP-179	0.5	-	2202.9	168.8	66.2	7.7
pGL3BP-179	1	-	2944.8	467.5	88.5	15.9
pGL3BP-179	-	0.25	4217.1	271.1	126.8	6.4
pGL3BP-179	-	0.5	3530.7	386.2	106.1	10.9
pGL3BP-179	-	1	4300.3	333.5	129.3	7.8

Transfection #29

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			5600	4325	0.403	0.337	12833.83		
pGL3Basic			4418	3143	0.357	0.291	10800.69		
Promoter	-	-	4697	3422	0.373	0.307	11146.58	11593.70	826.75
Blank			1275		0.066				
			2373	1014	0.365	0.299	3391.30		
pGL3Basic			2371	1012	0.376	0.310	3264.52		
Promoter	0.5	-	2439	1080	0.330	0.264	4090.91	3582.24	339.11
Blank			1359		0.066				
			1775	355	0.182	0.116	3060.34		
pGL3Basic			1799	379	0.174	0.108	3509.26		
Promoter	-	0.5	1804	384	0.171	0.105	3657.14	3408.92	232.38
Blank			1420		0.066				
			2861	963	0.199	0.133	7240.60		
pGL3BP-179	-	-	2607	709	0.180	0.114	6219.30		
Blank			2592	694	0.216	0.150	4626.67	6028.86	934.79
			1898		0.066				
pGL3BP-179	0.25	-	3008	1357	0.357	0.291	4663.23		
Blank			3252	1601	0.421	0.355	4509.86	4586.54	76.69
			1651		0.066				
			2990	1541	0.465	0.399	3862.16		
pGL3BP-179	0.5	-	2779	1330	0.367	0.301	4418.60		
Blank			2800	1351	0.374	0.308	4386.36	4222.37	240.15
			1449		0.066				
			2803	456	0.420	0.354	1288.14		
pGL3BP-179	1	-	2939	592	0.418	0.352	1681.82		
Blank			2862	515	0.485	0.419	1229.12	1399.69	188.09
			2347		0.066				
pGL3BP-179	-	0.25	3216	872	0.476	0.410	2126.83		
Blank			3149	805	0.382	0.316	2547.47		
			3335	991	0.430	0.364	2722.53	2465.61	225.85
			2344		0.066				
pGL3BP-179	-	0.5	3416	957	0.260	0.194	4932.99		
Blank			3343	884	0.281	0.215	4111.63		
			3454	995	0.286	0.220	4522.73	4522.45	273.88
			2459		0.066				
pGL3BP-179	-	1	3257	756	0.238	0.172	4395.35		
Blank			3175	674	0.278	0.212	3179.25		
			3163	662	0.253	0.187	3540.11	3704.90	460.30
			2501		0.066				

Summary

Construct	Sp1 (u	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3BP	-	-	11593.7	826.8	192.3	7.1
pGL3BP	0.5	-	3582.2	339.1	59.4	9.5
pGL3BP	-	0.5	3408.9	232.4	56.5	6.8
pGL3BP-179	-	-	6028.9	934.8	100.0	15.5
pGL3BP-179	0.25	-	4586.5	76.7	76.1	1.7
pGL3BP-179	0.5	-	4222.4	240.1	70.0	5.7
pGL3BP-179	1	-	1399.7	188.1	23.2	13.4
pGL3BP-179	-	0.25	2465.6	225.9	40.9	9.2
pGL3BP-179	-	0.5	4522.4	273.9	75.0	6.1
pGL3BP-179	-	1	3704.9	460.3	61.5	12.4

Transfection #30

construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			4517	4473	0.253	0.194	23056.70		
pGL3Basic			4637	4593	0.262	0.203	22625.62		
promoter	-	-	4290	4246	0.278	0.219	19388.13	21690.15	2005.23
Blank			44		0.059				
			4733	4614	0.607	0.548	8419.71		
pGL3Basic			4570	4451	0.708	0.649	6858.24		
promoter	0.5	-	4929	4810	0.653	0.594	8097.64	7791.86	824.42
Blank			119		0.059				
			2324	1975	0.329	0.270	7314.81		
pGL3Basic			2457	2108	0.344	0.285	7396.49		
promoter	-	0.5	1828	1479	0.288	0.229	6458.52	7056.61	519.57
Blank			349		0.059				
			3977	3565	0.401	0.342	10423.98		
			4754	4342	0.502	0.443	9801.35		
pGL3BP-179	-	-	4208	3796	0.429	0.370	10259.46	10161.60	322.64
Blank			412		0.059				

Summary

Construct	Sp1 (u	Sp3 (u	Average	Ave Dev	Relative Activity	%Error
pGL3BP	-	-	21690.1	2005.2	213.5	9.2
pGL3BP	0.5	-	7791.9	824.4	76.7	10.6
pGL3BP	-	0.5	7056.6	519.6	69.4	7.4
pGL3BP-179	-	-	10161.6	322.6	100.0	3.2

Transfection

#31 construct	Sp1 (ug)	Sp3 (ug)	Luciferase Maxima	Luciferase minus Blank	B-Gal (405nm)	B-Gal value minus Blank	Normalised (Luc/B-Gal)	Average	Ave Dev
			1199	72	0.070	0.004	18000.00		
pGL3Basic			1160	33	0.069	0.003	11000.00		
Promoter	-	-						9666.67	4949.75
Blank			1127		0.066				
			3379	2315	0.437	0.371	6239.89		
pGL3Basic			3268	2204	0.445	0.379	5815.30		
Promoter	0.5	-	3427	2363	0.484	0.418	5653.11	5902.77	303.01
Blank			1064		0.066				
			3363	2320	0.608	0.542	4280.44		
pGL3Basic			3178	2135	0.591	0.525	4066.67		
Promoter	-	0.5	3204	2161	0.605	0.539	4009.28	4118.80	142.90
Blank			1043		0.066				
			2542	1436	0.362	0.296	4851.35		
			2508	1402	0.321	0.255	5498.04		
pGL3BP-179	-	-	2408	1302	0.317	0.251	5187.25	5178.88	323.43
Blank			1106		0.066				
			2071	985	0.178	0.112	8794.64		
.3Basic Promoter	0.5	-	2066	980	0.251	0.185	5297.30		
Blank			1969	883	0.264	0.198	4459.60	6183.85	2299.49
			1086		0.066				
			1725	682	0.163	0.097	7030.93		
pGL3BP-179	-	0.5	1675	632	0.164	0.098	6448.98		
Blank			1707	664	0.186	0.120	5533.33	6337.75	754.97
			1043		0.066				

Summary

Construct	Sp1 (uç	Sp3 (uç	Average	Ave Dev	Relative Activity	%Error
pGL3BP	-	-	9666.7	4949.7	186.7	51.2
pGL3BP	0.5	-	5902.8	303.0	114.0	5.1
pGL3BP	-	0.5	4118.8	142.9	79.5	3.5
pGL3BP-179	-	-	5178.9	323.4	100.0	6.2
pGL3BP-179	0.5	-	6183.8	2299.5	119.4	37.2
pGL3BP-179	-	0.5	6337.7	755.0	122.4	11.9