# Transcriptional Regulation of Human Topoisomerase II Beta

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

**Claire Mawson**
**2006**

# Acknowledgments

First and foremost I would like to thank my supervisor Dr Kathryn Stowell for her help, support, and knowledge. I have learnt so much from you and will be forever grateful for the opportunity you provided me with for my Masters.

I would like to express my sincere thanks to Natisha and Lia. Your friendship, support and advice will never be forgotten. You both made the long nights in the lab a very enjoyable time. Thank you also to Robyn, Alice, and Hilbert. I would also like to extend my thanks to everyone else in the lab and to those who worked on the project before me. Dr Richard Isaacs, John Tweedie and Mark Patchett, I really appreciate all you words of encouragement and help along the way.

To my family, Mum, Dad, Sue and Greg, and Graeme, thank you for love and support. To all my friends, especially Lana, thank you for the laughs, fun times, and welcome distraction from the hard work.

Finally I would like to thank John, for his love, support, encouragement, and patience.

# Abstract

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

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

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

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

# Abbreviations

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

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

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

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

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

# List of Figures

# List of Tables

# Table of Contents

# 1. Introduction

## 1.1. Overview

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

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

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

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

## 1.2. Topoisomerases

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

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

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

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

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

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

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

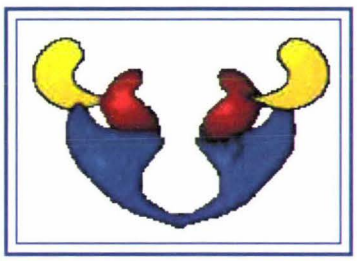

**Figure 1.1: Schematic Representation of Topoisomerase II.**

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

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

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

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

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

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

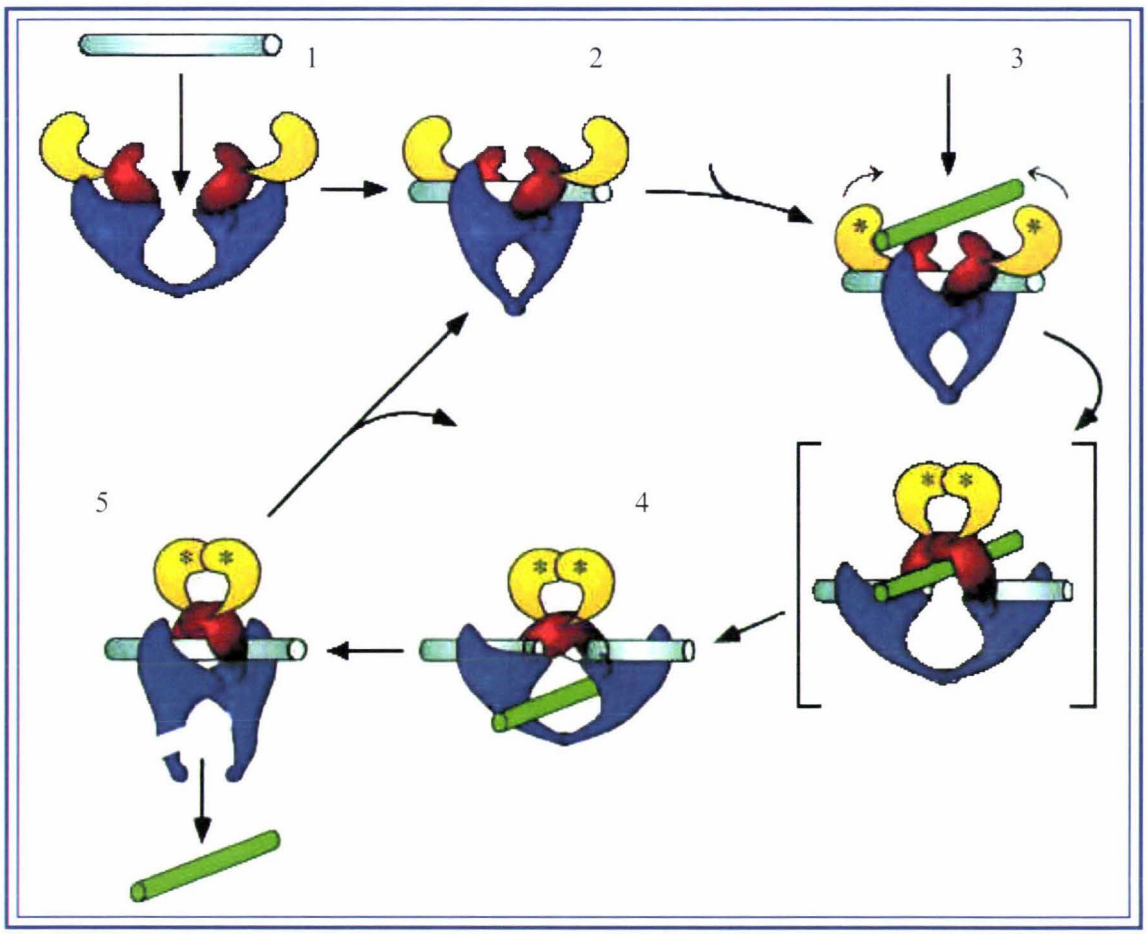


**Figure 1.2: Mechanism of action for Topoisomerase II.**

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

## 1.3. Topoisomerase II as a Chemotherapy Target

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

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

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

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

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

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

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

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

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

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

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

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

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

## 1.4. Transcription in Eukaryotes

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

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

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

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

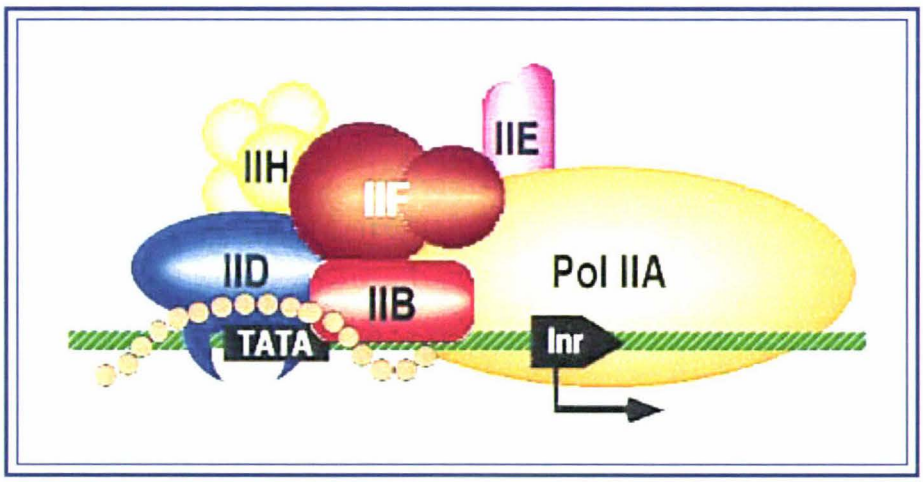


**Figure 1.3: Schematic Representation of the Pre-initiation Complex.**

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

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

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

## 1.5. Topoisomerase II Beta

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

### 1.5.1. The Topoisomerase II Beta Promoter

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

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

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

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

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

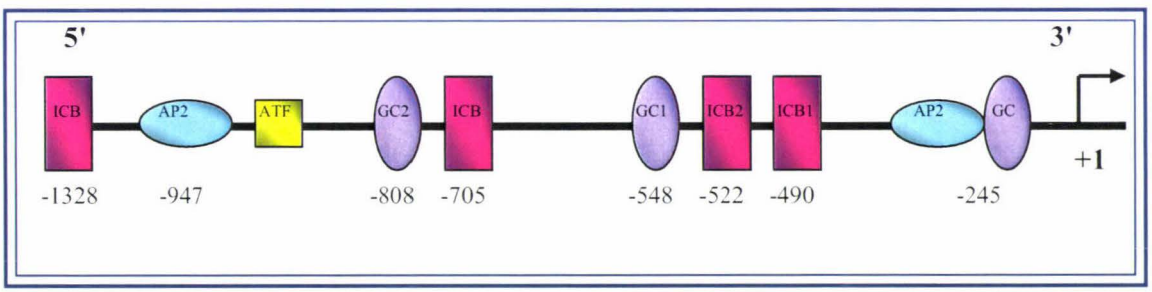


**Figure 1.4: Topoisomerase II Beta Promoter.**

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

## 1.5.2. CCAAT Boxes

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

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

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

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

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

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

### 1.5.3. NF-Y

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

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

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

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

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

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

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

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

### 1.5.4. GC-rich Elements

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

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

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

### 1.5.5. Sp Family of Transcription Factors

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

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

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

### 1.5.6. Sp1

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

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

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

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

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

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

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

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

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

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

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

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

### 1.5.8. Sp3

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

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

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

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

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

## 1.6. Project Outline

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

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

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

Research aims:

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