Regulation of the Human Topoisomerase IIβ Promoter

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Melanie Willingham

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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).

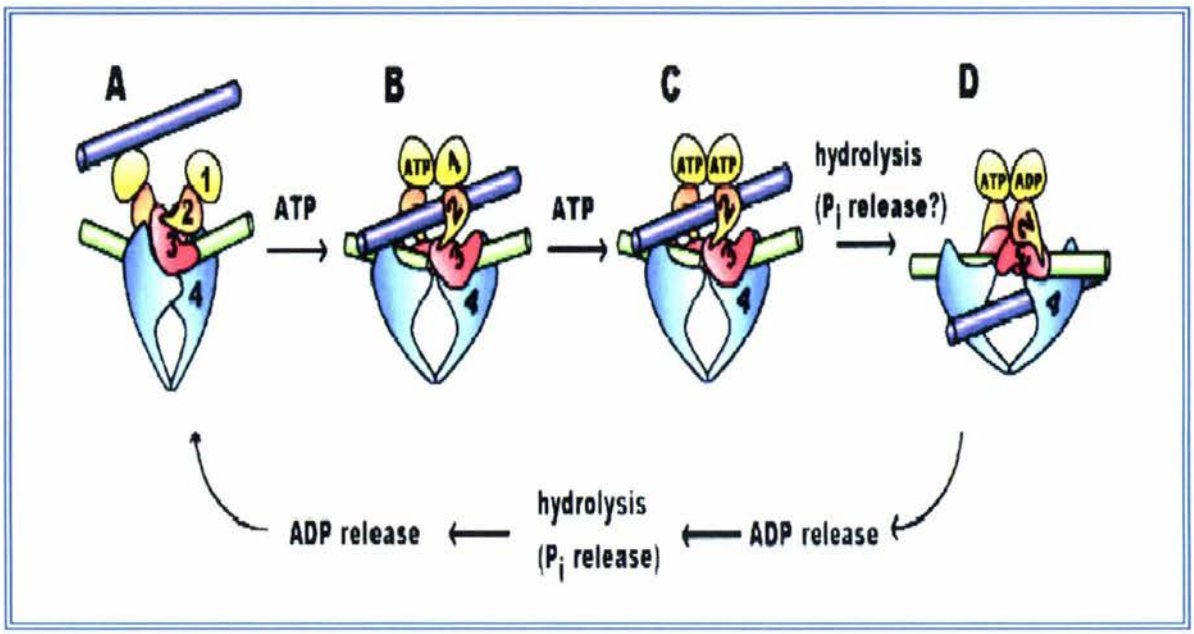

Figure 1.1: Schematic model of the catalytic cycle of topoisomerase II.

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).

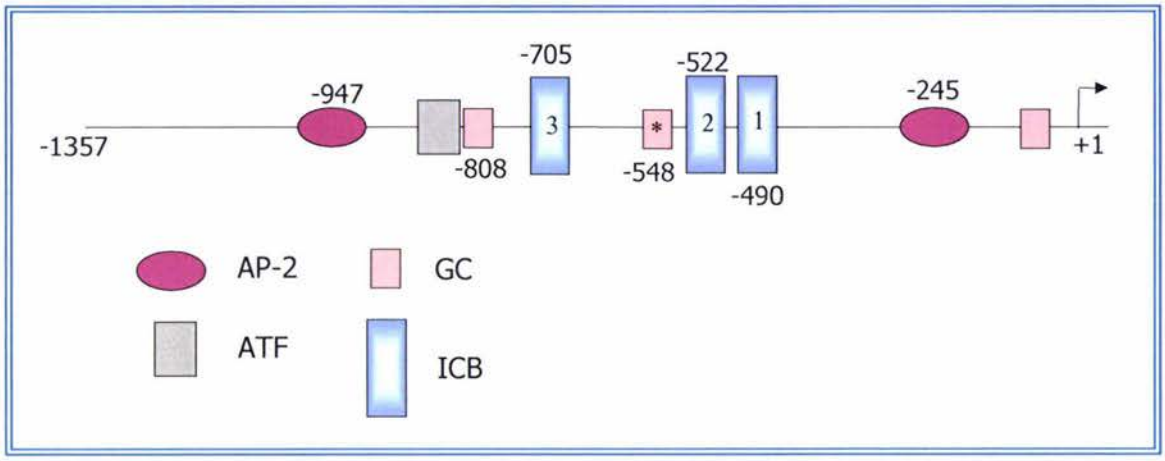

Figure 1.2: Schematic representation of the -1357 topoisomerase IIβ promoter.
Positions of the putative regulatory elements are shown and numbered with respect to the major transcription start site, +1, indicated by the arrow. Of particular interest is a cluster including ICB elements, ICB1 and ICB2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