The Role of NF-Y in the Transcriptional Regulation of Human Topoisomerase IIα

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ABSTRACT

DNA topoisomerases are ubiquitous enzymes that catalyse reactions that alter the topological state of DNA during the various processes of DNA metabolism including transcription, recombination, replication and chromosome segregation. Human cells exhibit a Type II enzyme termed DNA topoisomerase IIα. This enzyme is expressed at higher levels in proliferating cells due to an increased demand for chromosome separation. This is advantageous with respect to some of the drugs used in chemotherapy. These drugs can specifically target cancer cells by only being effective at high levels of topoisomerase IIα gene expression. However, the use of such drugs has been limited by both toxicity and the development of resistance. This resistance has been associated with a decrease in topoisomerase IIα at both protein and mRNA levels.

The topoisomerase IIα minimal promoter is 650 base pairs in length and includes promoter elements such as inverted CCAAT boxes (ICBs) and GC rich regions. It has been determined that the ICB elements are of the most interest in terms of regulation of the topoisomerase IIα gene expression. Several studies have shown that the transcription factor NF-Y binds to ICB1-4 of the topoisomerase IIα promoter and regulates transcription through these elements.

This study aimed to determine the importance of NF-Y in the transcriptional regulation of topoisomerase IIα and to investigate the molecular mechanisms by which NF-Y associates with the topoisomerase IIα promoter with a particular focus on the inverted CCAAT box elements. The binding of NF-Y to oligonucleotides containing selected consensus elements of the topoisomerase IIα promoter was analysed (*in vitro*) by electrophoretic mobility shift binding assays. The importance of NF-Y in the regulation of topoisomerase IIα expression was analysed by functional assays, using reporter gene constructs in transiently transfected HeLa cells.

The binding studies indicated that the flanking sequences affect the affinity of the transcription factor NF-Y for ICB1 and ICB2 and that a regulatory element flanking ICB2 may aid in NF-Y binding to that element. Functional assays showed that NF-Y appears to act a negative regulator of topoisomerase IIα with its effect being entirely due to interaction with ICB2.

ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
CAT	chloroamphenicol acetyl transferase
cDNA	complementary DNA
CDP	CCAAT displacement protein
C/EBP	CCAAT enhancer binding protein
CEM	human leukemic (cell line)
CHO	Chinese hamster ovary (cell line)
CO_2	carbon dioxide
cpm	counts per minute
DMSO	dimethyl sulphoxide
DNase I	deoxyribonuclease I
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
G segment	gate segment
GCG	Genetics Computer Group
HADC	histone deacetylase
HAT	histone acetyl-transferase
HeLa	human cervical carcinoma cell line
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid]
HL-60	human promyelocytic leukaemia cells
ICB	inverted CCAAT box
ICBP90	inverted CCAAT box binding protein M_r 90 kDa
IgG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertani
MEM	Eagle's minimal essential medium
MW	molecular weight

NF-Y	Nuclear factor-Y
oligo	oligonucleotide
ONPG	o-nitrophenol β-D-galacto-pyranoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSE	phosphate buffered saline EDTA
PCAF	p300/CBP-associated factor
PEA3	phenylmethylsulfonyl fluoride
pGL2C	pGL2Control
pGL3B	pGL3Basic
PNK	polynucleotide kinase
poly(dI-dC)	poly (dI-dC) poly (dI-dC)
PSV-βgal	pSV-β-galactosidase expression vector
RT	reverse transcriptase
RT-PCR	polymerase chain reaction coupled reverse transcriptase
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sp1	Specificity protein 1
Sp3	Specificity protein 3
SV40	simian virus 40
T segment	transport segment
T80	80 cm^2 flasks
TAE	tris acetate EDTA
TBE	tris boric acid EDTA
TBST	tris buffered saline triton X-100
TBP	TATA-box binding protein
TEMED	N,N,N',N'-Tetramethylethylenediamine
TK	thymidine kinase
Tris	tris (hydroxymethyl)-aminomethane
UV	ultra violet light
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YB-1	Y-box binding protein

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1.1 DNA Topoisomerases

DNA Topoisomerases are ubiquitous enzymes that catalyse reactions that alter the topological state of DNA during the various processes of DNA metabolism; including transcription, recombination, replication and chromosome segregation (Watt and Hickson, 1994). Consequently, these enzymes play an important role in the physiological functions of DNA (Watt and Hickson, 1994).

Two types exist, type I and type II, classified due to their catalytic mechanism of action. Type I enzymes introduce single stranded breaks in DNA, pass an intact strand through the broken strand then reseal the break. Type II enzymes, in contrast make transient double stranded breaks in one segment of DNA and pass an intact duplex through the broken strand before resealing the break (Roca, 1995; Watt and Hickson, 1994).

1.1.1 Prokaryotic DNA Topoisomerases

Escherichia coli contains two type I DNA topoisomerases and two type II DNA topoisomerases. The two type I topoisomerases are topoisomerase I (encoded by the *topA/supX* gene) and topoisomerase III (encoded by the *topB* gene). Mutational analysis indicates that topoisomerase I is involved in transcription acting with DNA gyrase (a prokaryotic type II DNA topoisomerase) to catalyse changes in supercoiling ahead of and behind the translocating transcription machinery (Watt and Hickson, 1994; Austin and Fischer, 1990). Similar analysis of topoisomerase III suggests that it is involved in chromosomes stability and plasmid segregation (Watt and Hickson, 1994; Austin and Fischer, 1990).

DNA gyrase (encoded by *gyrA* and *gyrB* genes) and topoisomerase IV (encoded by par C and par E genes) are the *E.coli* type II DNA topoisomerases. DNA gyrase appears to have multiple roles *in vivo*, including general supercoiling homeostasis, the initiation phase of DNA replication and chromosome partitioning (Watt and Hickson, 1994; Austin and Fischer, 1990). DNA gryase also appears to exhibit a specialised role in

removing knots and catenanes generated by recombination (Watt and Hickson, 1994; Austin and Fischer, 1990).

Unlike DNA gyrase, topoisomerase IV exhibits no supercoiling activity - its role appears to be in the resolution of catenanes generated by replication (Watt and Hickson, 1994; Austin and Fischer, 1990).

1.1.2 Yeast Topoisomerases

Saccharomyces cerevisae exhibits two type I DNA topoisomerases and one type II DNA topoisomerase (Watt and Hickson, 1994; Austin and Fischer, 1990). The yeast topoisomerase I (the *TopI* gene product) is functionally homologous to *E. coli topA* and is able to relax both negatively and positively supercoiled DNA (Watt and Hickson, 1994; Austin and Fischer, 1990). The second type I enzyme topoisomerase III (the *Top3* gene product) shares high sequence homology with the *E. coli* topoisomerase III protein. Like its *E. coli* counter part, the yeast enzyme only relaxes negatively supercoiled DNA and forms a 5' phosphoryl end of cut DNA (Berger *et al.*, 1996; Watt and Hickson, 1994). This mode of action is in contrast to all other type I enzymes, which form a 3' phospho-tyrosyl bond (Berger *et al.*, 1996; Watt and Hickson, 1994). Topoisomerase III enzymes appear to have a significant role in repressing recombination and the maintaining of genome stability (Watt and Hickson, 1994).

Yeast cells exhibit one type II DNA topoisomerase this single type II enzyme is topoisomerase II (*Top2* gene product). Topoisomerase II lacks the supercoiling ability of DNA gyrase but like its prokaryotic counterpart, yeast topoisomerase II is essential for chromosome segregation and therefore cell survival (Berger *et al.*, 1996; Watt and Hickson, 1994).

1.1.3 Human DNA Topoisomerases

Human cells express one type I DNA topoisomerase and two type II DNA topoisomerases. Human topoisomerase I appears to have a structure and function similar to the yeast type I DNA topoisomerase. Topoisomerase I relaxes negatively

supercoiled DNA and is thought to have roles in the initiation and elongation phases of DNA transcription and replication (Isaacs *et al.*, 1998; Watt and Hickson, 1994). Type II DNA topoisomerases in human cells exist in two ubiquitous forms – topoisomerase IIα (170 kDa) and topoisomerase IIβ (180 kDa). These are the products of different genes encoded on separate chromosomes (17q21-22 and 3p24 respectively). An analysis of the genomic sequences indicates that the two genes have arisen via a recent gene duplication event (Isaacs *et al.*, 1998; Watt and Hickson, 1994).

Although these enzymes show similar catalytic activity, they differ biochemically in particular with respect to their thermal stabilities and differences in cell-cycle regulation and tissue-specific expression patterns (Isaacs *et al.*, 1998; Watt and Hickson, 1994). The topoisomerase IIβ product is found in a wide range of tissues including non-proliferating tissues such as the brain, its transcription is constant throughout the cell cycle and is ubiquitously expressed *in vitro* and *in vivo* (Isaacs *et al.*, 1998; Watt and Hickson, 1994). In contrast, topoisomerase IIα is found predominantly in proliferating tissues such as the spleen and bone marrow. Levels of topoisomerase IIα expression change during the cell cycle with low levels in Go/G1 which increase throughout G1 and G2 and reach a maximum in S phase (Isaacs *et al.*, 1998).

These differences between topoisomerase IIα and topoisomerase IIβ indicate different functions with the β isoform thought to play a general role in DNA metabolism, whereas the strict cell cycle regulation of the α isoform suggest that it is likely to play a major role at mitosis (Isaacs *et al.*, 1998; Watt and Hickson, 1994). Mutations in topoisomerase II which affect catalytic function, cause a loss of cell viability due to a failure to segregate newly replicated chromosomes at mitosis (Isaacs *et al.*, 1998; Watt and Hickson, 1994).

In mammalian cells, topoisomerase IIα and topoisomerase IIβ are among the most important cellular targets for anticancer drugs. The topoisomerase IIα protein is of importance due to the enzyme being one of the best available markers of the proliferative state *in vitro* and *in vivo* (Isaacs *et al.*, 1996). High levels of topoisomerase II α gene expression correlate with relative sensitivity of cells to anticancer drugs whereas low levels confer drug resistance. For this reason, the understanding of topoisomerase IIα gene expression and its regulation is of great importance.

Information of such significance could lead to specific modulation of tumour chemosensitivity (Isaacs *et al.*, 1996).

1.2 Human Topoisomerase IIα

Human topoisomerase IIα acts as a homodimer and catalyses a range of reactions on double-stranded DNA including relaxation, decatenation and unknotting (Watt and Hickson, 1994). The proposed mechanism for catalytic activity of topoisomerase IIα involves several steps. The catalytic cycle presented in Figure 1.1 from Berger *et al* 1996.

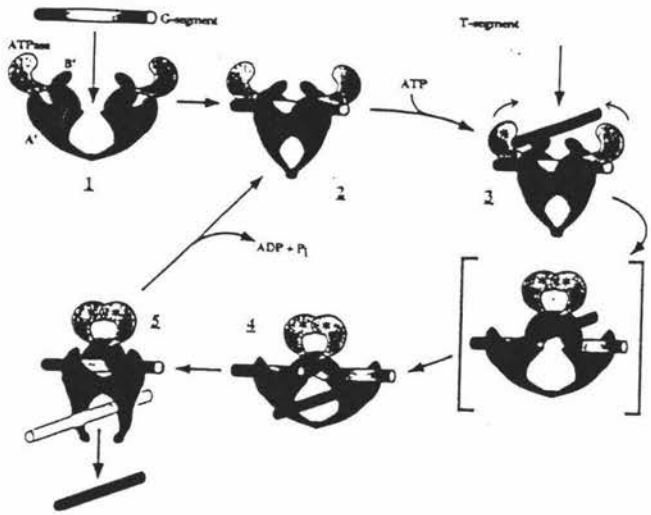

Figure 1.1 – The catalytic cycle of human topoisomerase IIα taken from Berger et al., 1996.

The cycle begins when the unliganded enzyme binds the G or gate segment (1) which induces a conformational change (2). Upon the binding of ATP (*) and the T or transported segment a series of further conformational changes occur and the G segment is cleaved (3). Following these changes the ATPase segments of the enzyme dimerise and the T segment is transported through the break (4). Following transport of the T segment through the break the G segment is resealed and the T segment is released from the enzyme.

The action of the catalytic cycle and the proliferation-dependent regulation of topoisomerase IIα along with its ability to generate double-stranded breaks in DNA and the discovery of specific poisons or inhibitors to topoisomerase IIα is advantageous with

respect to chemotherapy agents. A variety of chemotherapeutic agents can specifically target cancer cells by only being effective at high level of topoisomerase IIα. These drugs stabilise the cleaved intermediate in the catalytic cycle of topoisomerase IIα. When there are high levels of topoisomerase IIα in the cell, a greater number of cleavable complexes arise and cell death occurs. Cell death is due to the inability of the DNA repair apparatus to correct the large number of breaks in the DNA. However, in cells with low levels of topoisomerase IIα few cleavable complexes are formed and the DNA repair apparatus is adequate leading to cell survival. The use of drugs that target topoisomerase IIα has been limited by the development of resistance. This resistance has been attributed to a decrease in topoisomerase IIα levels (Isaacs *et al.*, 1996). A number of factors can contribute to the production of high/low levels of topoisomerase IIα. These include gene expression/regulation, phosphorylation and post-transcriptional modification. This thesis focuses on the regulation of expression of human topoisomerase IIα and hence describes a study of some elements of the promoter and associated transcription factors.

1.2.1 Human Topoisomerase IIα Promoter

The 2.5 kb promoter of topoisomerase IIα has been isolated and cloned from a human placental genomic DNA library (Hochhauser *et al*, 1992) (see Appendix 1 for the sequence of the promoter). Analysis of the promoter revealed multiple sequence motifs as potential transcription factor binding sites (**Figure 1.2**). Putative cis-acting elements within the minimal promoter of 617 bp including two GC-rich boxes (GC1 and GC2), five inverted CCAAT boxes (ICB1-5), a consensus sequence for an activating transcription factor (ATF) binding site, a potential site for c-Myb (Myb) binding and a Myc/Max site. This –617 bp region is the minimal unit necessary for full promoter activity as identified by *in vitro* studies (Isaacs *et al.*, 1998; Isaacs *et al.*, 1996; Hochhauser *et al.*, 1992).

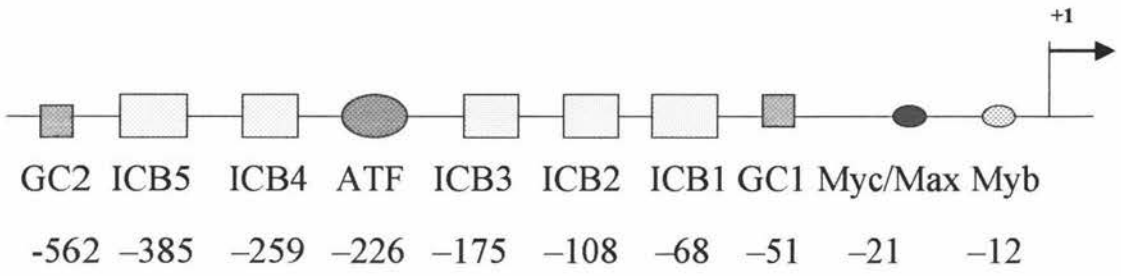

Figure 1.2: The Promoter of Topoisomerase IIα Promoter

Some of these putative transcription factor binding sites have been implicated in both the regulation of topoisomerase IIα expression and in the development of resistance.

Isaacs *et al.*, (1996) used confluence arrest of the cell cycle, which is associated with an active down-regulation of the topoisomerase IIα promoter, as a model system for topoisomerase IIα transcription. Although confluence arrest is not a common characteristic of tumour cells and the regulatory signals involved in confluence induced down-regulation are not likely to be identical to those associated with chemotherapy resistance, it is possible that the mechanism of down-regulation (for example repressor activity) is conserved. Using a 5' deletion series, Isaacs *et al.*, (1996) showed that confluence induced down-regulation of topoisomerase IIα promoter could be supported by a promoter fragment containing only 144 bp upstream of the +1 site. This suggested that the repression event was mediated by transcription factors binding to elements within this region. Using site-specific mutagenesis, Isaacs *et al.*, (1996) showed that down-regulation was driven from the ICB motif located at position –108 relative to the transcription start site (ICB2). When Isaacs *et al.*, (1996) eliminated this ICB2 element by mutation in a promoter consisting of 617 bp upstream of the +1 site, down-regulation at confluence no longer occurred. Mutations at the other putative transcription factor binding sites in the –144 region (ICB1 and GC1) had no effect on confluence arrest induced down-regulation of the topoisomerase IIα promoter. This evidence strongly suggested that ICB2 is the primary binding site involved in down-regulation of the topoisomerase IIα promoter governed by cell cycle arrest signalling.

1.2.2 Transcriptional Regulation of Human Topoisomerase IIα

The most proximal binding motif relative to the transcription start site is a Myb binding site at position –12 (very close to the transcription start site). Brandt *et al* (1997) showed that the *c-myb* proto-oncogene product caused cellular trans-activation of the topoisomerase IIα promoter via the Myb binding site in HL-60 cells. However, B-Myb a more widely expressed member of the Myb family was found to cause promoter up-regulation in HeLa cells as well as in haematopoietic cells (Isaacs *et al*, 1998).

Another transcription factor NF-M (a myeloid-specific member of the CCAAT enchancer-binding protein family required for activation of c-myb regulated target genes), activated the topoisomerase II α promoter in transfection studies using HL-60 cells. However, the use of a dominant negative NF-M construct indicated that NF-M did not contribute to the endogenous regulation of topoisomerase IIα in these cells (Brandt *et al*, 1997). These results suggest that although NF-M is a potent and efficacious trans-activator of topoisomerase IIα (~38-fold over basal), NF-M does not appear to be involved in the endogenous transcriptional regulation of topoisomerase IIα.

Another feature of the topoisomerase IIα promoter is a partial Myc/Max site. C-Myb acts as a heterodimer with a second transcription factor named Max. Together they have a role in growth regulation and have been implicated in activating a number of genes involved in cell cycle regulation (Isaacs *et al.*, 1998, Hochhauser *et al*, 1992).

The ATF transcription factor has been shown to bind to the topoisomerase IIα ATF site *in vitro*. Decreased levels of ATF transcription factor have been correlated with transcriptional repression of the topoisomerase IIα gene, during 12-O-tetradecanoyl phorbol 13-acetate (TPA)- induced differentiation of HL-60 cells (Lim *et al.*, 1998).

Recently, a novel CCAAT binding protein has been described called ICBP90 (Inverted CCAAT Box Binding Protein, Mr 90kDa). ICBP90 bound to CCAAT elements from the topoisomerase IIα gene in *in vitro* binding assays (Hopfner *et al.*, 2000). Analysis of the expression pattern of ICBP90 suggested that in tumour cells there is an enhanced expression of ICBP90. It has been suggested that this protein is involved in activation

of topoisomerase IIα gene expression; however direct evidence for this activation has not been shown.

Another CCAAT binding protein suggested to be involved in the transcriptional regulation of topoisomerase IIα (via promoter activation) is the Y-box binding protein (YB-1). The decrease in expression of YB-1 due to the expression of antisense YB-1 reduced activity of the topoisomerase IIα promoter *in vitro*. In addition, YB-1 and topoisomerase IIα were co-expressed in human colorectal carcinomas, supporting possible involvement of YB-1 in topoisomerase IIα regulation (Shibao *et al.*, 1999).

The gene and chromosome structure might also be important in topoisomerase IIα transcriptional regulation. Structural alterations of one allele of the topoisomerase IIα gene resulted in reduced topoisomerase IIα mRNA and catalytic activity levels in a mitoxantrone-resistant HL-60 cell line (Harker *et al.*, 1995). Methylation of the topoisomerase IIα gene (Tan *et al.*, 1989) and histone acetylation (Adachi *et al.*, 2000; Currie *et al.*, 1998) might also be involved in transcriptional regulation. Through histone acetylation, chromatin structures open and thus allow interactions between DNA and proteins (for example transcription factors) to occur, and methylation has been implicated as a mechanism for topoisomerase IIα down-regulation in resistant cancers (Tan *et al.*, 1989).

An Sp1 consensus motif is found in the topoisomerase IIα promoter at two positions GC1 (-45 to –51) and GC2 (-554 to –563). Sp3 however, another Sp transcription factor of the Sp family has been found to bind only the GC1 site. These transcription factors will be discussed in greater depth in section 1.2.3.

The human topoisomerase IIα promoter has five inverted CCAAT (ICB) boxes. This type of motif is found in many human genes with wide-ranging functions. A number of proteins can specifically bind the CCAAT sequence, but their binding is influenced by the nature of flanking sequences. The ubiquitous transcription factor NF-Y is a CCAAT box binding protein that can recognise and bind ICBs. Within the topoisomerase IIα promoter, NF-Y can recognise and bind ICB1-4 but not ICB5 (Herzog and Zwelling, 1997). NF-Y and its association with the ICB elements of the topoisomerase IIα promoter will be discussed in detail in section 1.2.4.

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1.2.3 Sp1 and Sp3 Transcription Factors

Sp1 is a ubiquitously expressed protein, belonging to a large multi-gene family of transcription factors (Sp1, Sp2, Sp3 and Sp4), which binds GC boxes and can modulate gene expression by either activating or repressing transcription. Sp1 contains glutamine-rich trans-activation domains and three Zn (II) finger motifs used to bind DNA (Kadonaga *et al.*, 1987). Sp1 was originally identified as a transcription factor which binds and activates transcription from multiple GC-boxes in the simian virus 40 (SV40) early promoter (Dynan and Tjian, 1983) and the thymidine kinase (TK) promoter (Jones et al., 1985). Since then, Sp1 has been shown to be involved in the activation of expression of many different genes. The topoisomerase IIα promoter contains two GC boxes one distal (GC2) and one proximal (GC1). The classical Sp1 consensus is GGGGCGGGG. Both GC boxes within the topoisomerase IIα promoter share a high level of identity with the above consensus sequence, with the distal and proximal GC boxes being GGGGGCGGGG and GGGCGGG respectively. These differences may be relevant for binding activity such as affinity and specificity of the different members of the Sp transcription factor family. The GC1 box of the topoisomerase IIα promoter is situated in close proximity to the transcription start site, therefore it is likely that the GC1 box is involved in basal regulation of expression of topoisomerase IIα.

To date there has been limited investigation into the role of Sp1 in the regulation of topoisomerase IIα expression. Kubo *et al.*, (1995) showed that Sp1 levels were unchanged in topoisomerase IIα down-regulated etoposide resistant human KB cancer cells (these cells have decreased levels of topoisomerase IIα mRNA and therefore can be used for a study of topoisomerase IIα transcription), indicating that Sp1 is not involved in topoisomerase IIα down-regulation.

Another study conducted by Yoon *et al.*, (1999) using mouse fibroblast NIH3T3 cells, showed that the Sp1 transcription factor was involved in cell proliferation-dependent regulation of the rat topoisomerase IIα gene promoter. Furthermore, transient transfections, with reporter plasmids containing mutations in the promoter region, were used to show that the GC2 box (corresponding to GC1 element of the human topoisomerase IIα promoter) was involved in the up-regulation of topoisomerase IIα

transcription in proliferating cells. Electrophoretic mobility shift assays (EMSAs) with the GC2 element showed specific binding of the Sp1 transcription factor (Yoon *et al.*, 1999).

Other studies provide evidence for species-specific regulation of topoisomerase IIα despite sequence similarities. A study conducted by Adachi *et al.*, (2000) showed that the proximal GC box did not appear to be responsible for the cell cycle-dependent regulation of the mouse topoisomerase IIα promoter. Mutations in this proximal GC box resulted in the induction of promoter activity, which suggests its involvement in the repression of basal activity. This result was further analysed utilising *in vivo* footprinting analysis. The results obtained showed that the protein binding to the GC box was cell cycle-dependent. In another study cell cycle-dependent regulation of human topoisomerase IIα GC1 was independent of the element. However, in contrast with the study discussed above (Adachi *et al.*, 2000), loss of the GC1 box resulted in reduction of S phase-specific activation of the topoisomerase IIα promoter (Falck *et al.*, 1999). The contrast between these results may be due to a number of factors such as experimental systems or species-specific elements and interactions with the topoisomerase IIα promoter.

In all of these experiments the authors found that sequences upstream of the first GC box and the three ICB elements to –700 bp were not required for cell cycle-dependent regulation, however, they were required for maximal topoisomerase IIα expression. The human topoisomerase IIα promoter contains two GC boxes, which indicates interactions between these elements may be possible. Such interactions between distal and proximal Sp1 binding elements have been observed. The presence of a proximal and distal Sp1 binding site may facilitate a looping effect and studies conducted showed that Sp1-Sp1 protein interactions occur via looping of intervening cis-regulatory sequences (Courey *et al.*, 1989).

In Chinese hamster topoisomerase IIα gene expression, GC boxes do not appear to be involved in transcriptional regulation. In support of this, *in vitro* footprinting analysis (Ng *et al.*, 1995) confirmed that proteins do not bind to the GC1 box.

From the experimental evidence described above it is likely that Sp1 acts as an enhancer of transcription and that it regulates topoisomerase IIα expression via the GC boxes. However, its exact role in transcriptional regulation is still unclear.

More evidence for topoisomerase IIα regulation from the GC boxes has been shown for Sp3, another member of the Sp family of transcription factors. Sp3 exhibits similar binding specificity to Sp1 (Hagen *et al.*, 1994), and was originally thought to be a competitive inhibitor of Sp1 activated transcription. However, it was later found to possess stimulating properties as well. Sp3 has since been shown to be capable of both activating (Bigger *et al.*, 1997; Ding *et al.*, 1999; McEwen and Ornitz, 1998) and repressing (Li *et al.*, 1998; Majello *et al.*, 1997) transcription.

Several isoforms of Sp3 exist: 110-115kDa, 70kDa and 60kDa species, which arise via internal translational initiation within the Sp3 mRNA (Kennett *et al.*, 1997). Sp3, in addition to DNA-binding domains and activation domains (similar to those in the Sp1 protein), contains an inhibitory domain (Suske, 1999). Kennett *et al.*, (1997) suggested that the two small isoforms of Sp3 act as repressor molecules whereas the full length Sp3 isoform acts as an activator. However, Dennig *et al.*, (1996) showed that this may not be the case as expression of full length Sp3 also repressed expression.

Whether Sp3 acts as a repressor or an activator of transcription could depend on the context of binding. The structure and the arrangement of the recognition sites also appear to be important. Promoters containing a single binding site were activated whereas promoters containing multiple binding sites were not (Majello *et al.*, 1997).

Kubo et al., (1995) analysed topoisomerase IIα expression in etoposide/teniposide-resistant human epidermoid KB cells. A 300 bp upstream region of the topoisomerase IIα promoter which contains the GC1 box alone as the Sp3 consensus site was used to drive the expression of the chloramphenicol acetyl transferase (CAT) reporter gene. Using northern blots and CAT reporter gene assays Kubo *et al.*, (1995) demonstrated a 3-fold increase in Sp3 levels in resistant cell lines compared with parental KB cells. The CAT activity was lower in resistant cell lines. These results suggest that Sp3 might may be involved in repressing the topoisomerase IIα promoter. However, Mo *et al.*, (1997) reported conflicting results using a merbarone-resistant cell line, which exhibits

transcriptional down-regulation of the topoisomerase IIα promoter. Using northern analysis Mo *et al.*, (1997) also showed that Sp3 was transcriptionally down-regulated in this cell line. In addition, they also showed that co-transfection of merbarone sensitive cells with Sp3 and with a topoisomerase IIα promoter-reporter construct resulted in the trans-activation of the topoisomerase IIα promoter by Sp3. Co-transfection of resistant cell lines did not result in trans-activation. As the Sp3 expression vector was under control of a constitutive promoter, this finding cannot be explained by down-regulation of Sp3 levels. This suggests that Sp3 activity is dependent on cellular context. Sp3 may be one of the components required for activation, and in a resistant cell line, the required components may not be available. This cell context-specific gene activation may also explain the contradictory results of Kubo *et al.*, (1995) and Mo *et al.*, (1997). In addition, the reporter vector used by Mo *et al.*, (1997) was under control of a –577 to +60 topoisomerase IIα promoter fragment which contains the distal GC box. The presence of both GC elements might be important in determining the regulatory function of Sp3.

Li *et al.*, (1998) described an example where proximal and distal GC boxes have different fuctions in gene regulation. The human transcobalamin II promoter contains a positive-acting distal GC box and a negative-acting proximal GC box. EMSA analyses showed that both GC boxes were recognised by Sp1 and Sp3, and that co-transfection experiments using Sp1 and Sp3 expression plasmids demonstrated that while Sp1 stimulated transcription, Sp3 acted to repress transcription. It is possible that the relative adundance of Sp1 and Sp3 in a cell regulates transcription of topoisomerase IIα. The dual function of GC boxes can act as a switch to control transcription both positively and negatively, depending on the binding by associating activators and repressors, or Sp1/Sp3 interactions. Li *et al.*, (1998) speculated that Sp3 functions as a repressor through protein-protein interactions with components of the general transcription complex. Sp1 has also been shown to interact physically with Sp3 on the human plasminogen activator inhibitor-1 gene, and this interaction resulted in synergistic activation of transcription (Li *et al.*, 1998).

Similarly to Sp1, Sp3 transcription factor does not appear to be essential for topoisomerase IIα gene expression. Sp3-deficient mouse embryos were growth retarded and died at birth of respiratory failure (Bowman *et al.*, 2000). The fact that the embryos

were able to form suggests that the topoisomerase IIα gene was expressed in null Sp3 mice.

1.2.4 Nuclear Factor –Y (NF-Y)

NF-Y (also known as CP1, CBF and ACF) is another transcription factor suggested to be important in the transcriptional regulation of the human topoisomerase IIα promoter. NF-Y is a CCAAT box binding protein and a heteromeric transcription factor comprised of three subunits: 42kDa NF-YA, 36kDa NF-YB and 40kDa NF-YC. It shares significant homology to the yeast transcriptional activators HAP2, HAP3 and HAP5. All three subunits are necessary for the sequence-specific DNA binding activity of NF-Y. NF-YB and NF-YC subunits interact with each other to form a stable heterodimer (Bellorini *et al.*, 1997). NF-Y contains two glutamine rich activation domains which share homology with each other and with the activation domain of the transcription factor Sp1. NF-YA interacts with the NF-YB/C heterodimer and then binds to a CCAAT element. The hybrid surface that forms as a consequence of this trimerisation comprises the DNA binding domain which recognises the CCAAT box motif. A study conducted by Dorn *et al.*, (1987) of different CCAAT elements revealed that specific flanking sequences are also required for high-affinity NF-Y binding. NF-Y is a ubiquitous transcription factor shown to be involved in the regulation of a number of different genes, including those of the major histocompatibility complex class II gene (Milos and Zaret, 1992), cdc (Zwicker *et al.*, 1995) and thymidine kinase (Chang and Liu, 1994). NF-Y is involved in different types of transcriptional activation: basal transcription, cell cycle-dependent, and inducible, tissue specific activation (Hu and Maity, 2000). In all characterised instances NF-Y has been shown to function as a transcriptional activator (Mantovani *et al.*, 1992; Li *et al.*, 1992; Maity *et al.*, 1998).

NF-Y itself is regulated by the differential expression of its subunits during growth and differentiation of different cell lineages (Maity *et al.*, 1998). Alternatively spliced forms of NF-YA have been identified and although activation by these different isoforms seems to be unaffected, it is unknown how they interact with other factors (Li *et al.*, 1992). This gives NF-Y a potential for dynamic transcriptional regulation.

Mechanisms for NF-Y mediated regulation are poorly understood. One of the best characterised examples of NF-Y regulation involves the activation of sterol dependent transcription of the farnesyl diphosphate synthase and 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) synthase genes (Jackson *et al.*, 1995). The sterol responsive behaviour of the promoters of these genes was mapped by mutational analysis to an NF-Y binding inverted CCAAT box (Spear *et al.*, 1994). In this instance, NF-Y was found to mediate tissue specific sterol responsive element binding proteins (activation by sterol status) by interacting with tissue specific sterol responsive element binding proteins (SREBPs).

Several lines of evidence show that NF-Y is likely to be an important regulator of topoisomerase IIα transcription. In an *in vivo* study, a dominant negative NF-YA mutant was expressed stably in mouse fibroblast cells under the control of a tetracycline-responsive promoter (Hu and Maity, 2000). Dominant negative NF-YA inhibits binding of the NF-Y complex to DNA by sequestering the other components of the NF-Y heterotrimer, NF-YB and NF-YC (Mantovani *et al.*, 1994). This dominant negative analog of NF-YA makes it possible to study the role of NF-Y in different promoter systems in which its involvement is suggested. The expression of the mutant subunit resulted in the inhibition of cell growth. Northern blot analyses revealed a reduction of expression of the topoisomerase IIα gene, suggesting a direct role of NF-Y in the regulation of topoisomerase IIα expression (Mantovani *et al.*, 1994).

The CCAAT box is one of the primary constituents of the topoisomerase IIα promoter. Specific binding of NF-Y to the four most 3' CCAAT elements of the promoter has been shown *in vitro* (Herzog and Zwelling, 1997; Wang *et al.*, 1997; Isaacs *et al.*, 1996), supporting the involvement of this transcription factor in topoisomerase IIα gene regulation. In these studies, the different ICB elements had different binding affinities for NF-Y, with ICB1 having the highest and ICB2 having the lowest, while the most distal ICB (ICB5) bound a different unknown factor and showed virtually no affinity for NF-Y at all (Herzog *et al.*, 1997).

As introduced above, Isaacs *et al.*, (1996) have shown that down-regulation of topoisomerase IIα in growth-arrested human MCF-7 breast carcinoma and mouse fibroblast Swiss 3T3 cells appears to be mediated by NF-Y acting on the ICB2, while

none of the other four ICBs appeared to be important for regulation of topoisomerase IIα at confluence arrest. Mutation of ICB2 completely abolished the down-regulation of the topoisomerase IIα promoter seen in confluence-arrested cells. After having identified the involvement of the ICB2 element in topoisomerase IIα promoter repression, Isaacs *et al.*, (1996) conducted EMSA experiments to identify protein factors that bind this element. Antibody supershifts showed that the transcription factor NF-Y bound ICB2 and that this was the only transcription factor identified to bind *in vitro* to ICB2. Changes in NF-Y binding to ICB2, for example phosphorylation of NF-Y or availability of other factors interacting with NF-Y, may induce conformational changes in the promoter causing down-regulation of topoisomerase IIα gene expression in confluence-arrested cells. NF-Y was suggested to relieve repression by binding to ICB2 elements.

ICB2 binding by NF-Y was shown to be increased in extracts from proliferating cells (Isaacs *et al.*, 1996). Binding did occur in confluence arrested cell extracts, but to a lesser extent, indicating that levels of binding competent NF-Y were probably decreased in arrested cells (Isaacs et al., 1996). It has also been shown that down-regulation of the topoisomerase IIα promoter in drug resistant cell lines is associated with a similar decrease in NF-Y complex formation (Wang H *et al.*, 1997). These observations suggest that NF-Y is indeed involved in down-regulation of the promoter and that ICB2 is a critical element in the promoter for gene regulation.

Isaacs *et al.*, (1996) carried out ribonuclease protection assays on RNA from the ICB2 mutant constructs and found that while the hGH reporter mRNA levels expressed from the promoter fragment carrying a mutated ICB1 element were down-regulated in confluence-arrested cells, no such affect was observed in cells with the mutated ICB2 constructs. These results along with other work of Isaacs *et al.*, (1996) suggest that the ICB2 promoter element is functionally distinct from the other ICB elements.

In contrast, the GC2 box from rat topoisomerase IIα promoter, but not an ICB element, was reported to mediate the regulation of topoisomerase IIα in a cell growth-specific manner. In addition, the binding activity of Sp1 to the GC element increased during growth stimulation (Yoon *et al.*, 1999). The different experimental methods and species may have contributed to this difference. Cell cycle arrest achieved by contact inhibition

(Isaacs *et al.*, 1996) or serum starvation (Yoon *et al.*, 1999) might cause different cellular events. In support of the work of Isaacs *et al.*, (1996), Wang *et al.*, (1997) showed that a decrease in NF-Y activity correlated with the down-regulation of topoisomerase IIα transcription in doxorubicin-resistant multiple myeloma cells. These observations suggest that topoisomerase IIα gene repression in confluence-arrested cells (and drug resistant cells) is due to a loss of NF-Y binding.

The ICB1 element has been implicated in the heat shock-induced transcriptional activation of the topoisomerase IIα gene (Furukawa *et al.*, 1998). Using promoter constructs ICB1 was shown to be a requirement in the activation of topoisomerase IIα gene transcription after heat shock. EMSA analysis revealed reduced binding of a nuclear factor to ICB1 after heat shock. These results suggest that repression of topoisomerase IIα transcription, under these experimental conditions, is mediated by a negative regulatory factor interacting with ICB1. Similiar results were seen using NIH3T3 cells stably transfected with reporter plasmids containing various human topoisomerase IIα promoter fragments (Falck *et al.*, 1999). Mutation of ICB1 caused an increase in topoisomerase IIα transcription, demonstrating a repressive character for that element. Furthermore, EMSA experiments demonstrated a decrease in ICB1 binding activity following serum addition. Antibody against NF-Y did not produce a supershift of the ICB1 probe/protein complex. In another study, the transcriptional activities of luciferase reporter constructs were investigated by transient transfection experiments in two drug resistant human epidermoid KB cancer cell lines, KB/VP2 and KB/VM4. The transcriptional activity of the –295 to +85 promoter was significantly down-regulated in these cells. Introduction of a mutation in ICB1 abolished this down-regulation (Takano *et al.*, 1999).

These experiments suggest repression of topoisomerase IIα is caused by binding of regulatory factor(s) to the ICB1 element, activation of transcription occurs by decreasing the binding of that factor and allowing the binding of NF-Y to the ICB element(s). However, *in vivo* footprinting assays of the topoisomerase IIα gene promoter in KB, KB/VP2 and KB/VM4 cells conducted by Takano *et al.*, (1999), did not reveal any marked differences between the resistant and parental cell lines.

75% of the topoisomerase IIα promoter activity can be attributed to its five CCAAT boxes (Ng *et al.*, 1995). However, these elements cannot be substituted for one another without disrupting promoter activity (Wang *et al.*, 1997). Therefore, regulation from the ICBs in the topoisomerase IIα promoter is complex. To date, only NF-Y has been shown to bind four of the ICBs, including ICB2 which seems to direct confluence-induced down-regulation of the promoter. It is possible that NF-Y may act as a transcriptional repressor of topoisomerase IIα at confluence arrest.

It has been suggested that NF-Y, while generally acting as a transcriptional activator, has an architectural role in appropriately positioning other transcription factors. NF-Y appears to be unable to activate alone, but requires the presence of other activators nearby. Ronchi *et al.*, (1995) showed that NF-Y binds both major and minor grooves of DNA, and that the polarity of NF-Y binding may be essential for protein-protein interactions and may influence general promoter architecture. This suggests that different parts of the complex are specifically able to contact different classes of activators. Upon binding of NF-Y, the DNA is distorted to a degree that depends on the adjacent sequences to the NF-Y binding sites (distortion varies between 62 and 82 degrees). DNA bending is a possible mechanism for allowing distal and proximal regulatory elements to interact, facilitating transcriptional regulation. To date there are no known examples of NF-Y mediating a promoter repression effect, therefore the bending of topoisomerase IIα promoter at ICB2 may allow other protein factors binding further upstream to repress transcription. This would explain how a transcriptional activator such as NF-Y could direct down-regulation through promoter remodelling. Ronchi *et al.*, 1995 suggest that this co-operative function is in part elicited by indirect facilitation of protein-protein interactions brought about by NF-Y-induced DNA distortions at the CCAAT element(s), however the exact mechanism of action is unknown. However, this model fails to account for the observation that a topoisomerase IIα promoter fragment, truncated at –144 (relative to the transcription start site), can still direct confluence-arrest induced down-regulation of topoisomerase IIα transcription (Isaacs *et al.*, 1996). Clearly, more investigation into the role of ICB2 and its interaction with NF-Y is required.

Different mechanisms appear to be in place for the regulation of the mouse topoisomerase IIα gene transcription. *In vivo* footprinting analysis revealed cell cycle-

independent protein binding to ICB elements. Changes in NF-Y binding did not account for the cell cycle-dependent topoisomerase IIα transcription. In addition, multiple ICB elements (at least two) were required for cell cycle-regulated transcription (Adachi *et al.*, 2000).

Although the CCAAT element alone is not able to activate transcription, it may be able to increase the activity of neighbouring promoter elements. NF-Y has been shown to interact with other transcription factors and co-activators regulating gene expression, in particular Sp1 (Roder *et al.*, 1999). Co-operation between NF-Y and Sp1 has been demonstrated in the regulation of several genes including the major histocompatability class II-associated invariant chain (Wright *et al.*, 1995), the $p27^{KIP1}$ gene (Inoue *et al.*, 1999), the hamster thymidine kinase gene (Sorensen and Wintersberger, 1999) and the fatty acid synthase gene (Roder *et al.*, 1997; Roder *et al.*, 1999). The promoters of these genes all have in common one or several Sp1 bindng sites located in close proximity (20 or 30 nucleotides) to an ICB. The molecular mechanism responsible for this co-operation has been partly elucidated by the demonstration of interactions between NF-YA and Sp1, and the presence of specific protein-protein interaction domains in NF-YA and Sp1 (Roder *et al.*, 1999).

In the human topoisomerase IIα promoter, the proximal Sp1 binding site is located 12 nucleotides down-stream of the NF-Y binding site (ICB1 element), making such an interaction possible, although this has not yet been demonstrated.

The tumour suppressor protein p53 has been shown to inhibit the transcription of topoisomerase IIα in a fibroblast cell line by acting on the ICB1 element (Wang *et al.*, 1997). p53 is one of the most important regulators of the cell cycle in mammals, and is responsible for cell cycle arrest in response to DNA damage. Following on from Wang *et al.*, (1997), Suttle *et al.*, (1998) showed that transfection of p53-deficient murine 10(1) cells with wild-type p53 results in a decrease of NF-Y binding to ICB elements. Another study conducted by Sandri *et al.*, (1996) demonstrated that p53 inhibited topoisomerase IIα promoter activity in a human ovarian cell line. However, the action of p53 was independent of all characterised transcription factor binding sites. A possible mechanism for the down-regulation of topoisomerase IIα is that the treatment of cells with topoisomerase IIα targeting drugs causes DNA damage, by increased

incidents of cleavable complexes. The accumulation of these produces many double-stranded breaks in DNA. This induces p53 expression and subsequent down-regulation of topoisomerase IIα. In the cdc promoter, p53 was shown to repress cdc2 transcription via an ICB element. The complex formed at the CCAAT motif contained NF-Y. The mechanism of this repression has been suggested by Agoff *et al.*, (1993), in a study where p53 repressed hsp70 transcription by an interaction with NF-Y. These results suggest that direct protein-protein interactions between p53 and NF-Y may decrease the binding affinity of NF-Y and act to repress the topoisomerase IIα promoter.

The activity of NF-Y is also altered by the treatment of cells with an inhibitor of histone deacetylase (HDAC), trichostatin A (Jin and Scotto, 1998). Chromatin structure is modified by opposing activities: co-activators possessing histone acetyl-transferase (HAT) activity and co-repressors, recruiting HDAC. These HAT and HDAC activities alter histone acetylation status, thus resulting in the alteration of chromatin structure. Recently, NF-Y was shown to possess HAT activity in vivo through association with HATs, GCN5 (general control non-repressed protein 5) and PCAF (p300/CBP-associated factor) (Currie, 1998). The overexpression of PCAF stimulated the human MDR1 gene promoter via a direct interaction between NF-Y and PCAF (Jin and Scotto, 1998). This interaction then resulted in the increase of NF-Y activation potential by opening the local chromatin structure and facilitating the access of other transcription factors to the promoter.

Adachi *et al.*, (2000) suggested the involvement of acetylation in the regulation of mouse topoisomerase IIα as another mechanism for control of expression. These authors suggested that histone deacetylase plays a crucial role in the Go/G1-specific repression of the topoisomerase IIα promoter. NF-Y acts here to recruit histone acetyl-transferases to the promoter region of topoisomerase IIα, stimulate histone acetylation and therefore activate transcription (in G2/M).

1.3 Topoisomerase IIα and Chemotherapy

Beyond its essential physiological functions, topoisomerase IIα is the primary cellular target for a wide variety of anticancer drugs (Corbett and Osheroff, 1993). As mentioned previously the proliferative-dependent regulation of topoisomerase IIα and its catalytic mechanism of action coupled with the discovery of specific inhibitors and poisons make this enzyme a key target for chemotherapy.

1.3.1 Topoisomerase II α as a Drug Target

Although both topoisomerase isoforms interact with anticancer drugs in human cells, topoisomerase IIα is considered the primary target for a number of reasons. First, topoisomerase IIα has been shown to be more susceptible to topoisomerase II targeting drugs (Drake *et al.*, 1989) and second, levels of topoisomerase IIα are elevated in proliferating cells whereas topoisomerase IIβ levels are virtually constant throughout the cell cycle.

Anticancer drugs can be classified into two catagories; topoisomerase II poisons and topoisomerase II catalytic inhibitors. Topoisomerase II poisons act by intercalating DNA close to the active site of topoisomerases, stabilising the transient DNA:enzyme interaction complex forming a cleavable complex and preventing the enzyme from religating the cleaved DNA. The initial cytotoxic event following treatment with a topoisomerase II poison is the formation of this cleavable complex which introduces high levels of protein-associated DNA breaks. These complexes are mutagenic and produce permanent double-stranded breaks in DNA. These permanent breaks in the DNA then induce high levels of recombination with sister chromatid exchange, generation of large insertions and deletions, and the production of chromosomal aberrations and translocations (Chen and Liu, 1994; Corbett and Osheroff, 1993). When the strand breaks are present in high enough concentrations they result in cell death by necrosis or apoptosis (Chen and Liu, 1994). Topoisomerase II catalytic inhibitors function by inhibiting the enzyme function without forming cleavable complexes and as such inhibit other steps of the catalytic cycle (Andoh and Ishida, 1998).

1.3.2 Drug Resistance to Topoisomerase II Targeted Drugs

Drug resistance of cancer cells is one of the major problems in cancer chemotherapy and can arise at any step during the treatment process. Alterations in cell-cycle progression, drug transport, drug metabolism, and drug target as well as the processing of DNA damage have all been implicated in development of resistance to chemotherapeutic agents (Chen and Liu, 1994). Resistance to various topoisomerase targeting drugs has been observed with factors as candidates for the production of resistant cancer cells with respect to topoisomerase II targeted drugs these include MDR1 over-expression, reduced topoisomerase II levels, drug resistant mutant topoisomerase, lengthened cell cycle time, and altered DNA repair functions (Chen and Liu, 1994).

Dingemans *et al.*, (1998) state that both resistant cell lines and tumours show that down-regulation of topoisomerase IIα expression is a factor correlated with resistance to topoisomerase II targeted drugs. Higher topoisomerase IIα levels were found in lines derived from topoisomerase II sensitive tumour types such as testis and cell lung cancer (Dingemans *et al.*, 1998; Isaacs *et al.*, 1995), when compared with resistant tumour types such as bladder and non-small cell lung cancers (Son *et al.*, 1998; Towatari *et al.*, 1998). Direct evidence of a role for down-regulation of topoisomerase IIα expression in drug resistance has been shown by the use of antisense topoisomerase IIα RNA where reduced cellular expression of topoisomerase IIα protein and resistance to etoposide was demonstrated (Gudkov *et al.*, 1993; Towatari *et al.*, 1998). Zhou *et al.*, (1999) also showed that transfer of a recombinant adenovirus expressing human topoisomerase IIα, into a resistant cell line increased sensitivity to the drug.

1.4 Aims of the Research

Human topoisomerase IIα is not just important because of its functional role in the maintenance of DNA topology but also because it is a primary target in chemotherapy. Anticancer drugs can specifically target cancer cells by only being effective at high levels of topoisomerase IIα. However, the use of such drugs has been limited by the development of resistance to topoisomerase II targeting drugs. One possible mechanism for the development of resistance employed by a cancer cell is the decrease of topoisomerase IIα levels at both mRNA and protein levels (Isaacs *et al.*, 1996). The major mechanism controlling topoisomerase IIα expression is transcription. Transcription in eukaryotes is regulated through the binding of transcription factors to *cis*-elements in gene promoters. Current research focussed on the topoisomerase IIα promoter has left many unanswered questions and has suggested a role for the interactions between elements in the promoter in down-regulation which may have links to the development of resistance seen in cancer cells. One problem with published results thus far is the lack of functional studies concerning the interaction between the topoisomerase II α promoter and the transcription factor NF-Y. The primary aim of the research undertaken was to identify the molecular mechanisms involved in the transcriptional regulation of human topoisomerase IIα, with particular emphasis on the importance of ICB2 and its interaction with NF-Y.

This aim was addressed as follows:

1. EMSAs were performed using HeLa cell extracts to analyse the binding of NF-Y to DNA fragments containing elements of the topoisomerase IIα promoter.
2. Functional assays were carried out using promoter-reporter constructs containing various mutations in the topoisomerase IIα promoter
3. Functional assays were carried out using topoisomerase IIα promoter constructs in co-transfection experiments with the NF-YA dominant negative construct.