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

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

Chapter One Introduction

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.

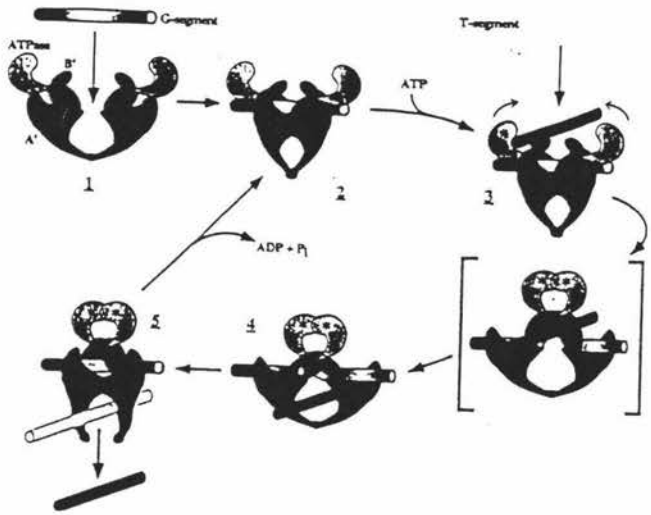

Figure 1.1 – The catalytic cycle of human topoisomerase IIα taken from Berger et al., 1996.

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).

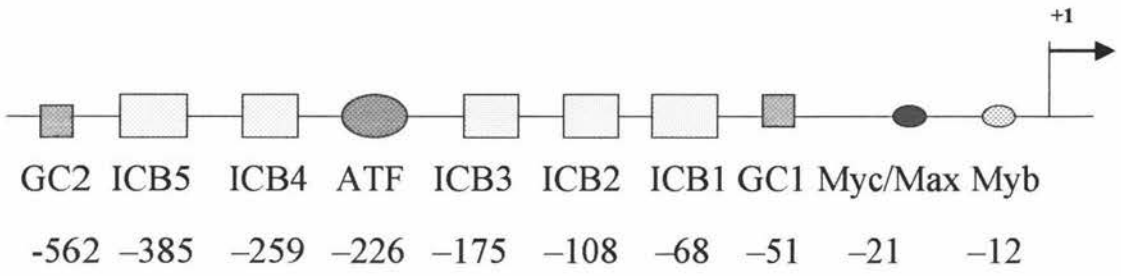

Figure 1.2: The Promoter of Topoisomerase IIα Promoter

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.

Chapter Two: Materials and Methods

2.1 Materials

Restriction endonucleases and accompanying buffers were obtained from the following sources: New England Biolabs Inc., MA, USA; Life Technologies Inc., MD, USA; and Boehringer Mannheim, Germany.

Oligonucleotides were from Sigma-Aldrich, St. Louis, MO, USA or Life Technologies™ Inc., Gaithersburg, MD, USA

Luciferase reporter vectors (pGL3Basic, pGL2Control), the Luciferase assay system (which contained the cell lysis buffer (x5), luciferase assay buffer and the luciferase assay substrate), the pSVβ-galactosidase control plasmid and RQ1 RNase-free DNase were obtained from Promega Corporation, WI, USA.

Trizol™ LS Reagent, PCRx enhancer system, Platinum® *Taq* DNA polymerase high fidelity, the SuperScript™ pre-amplification system for first strand cDNA synthesis, M-MLV reverse transcriptase, oligo dT primer, CONCERT™ high purity plasmid purification system, Luria-Bertani (LB) broth base, bacterial agar, trypsin, penicillin, streptomycin, foetal calf serum and 1 kb PLUS ladder were obtained from Life Technologies, Gaithersburg, MD, USA.

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

Ready-to-go DNA labelling beads (d-CTP), ProbeQuant™ G-50 micro columns and nylon transfer membrane for nucleic acids were from Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England.

[α-^{32}P]dCTP was from NEN™ Life Science Products Inc., Boston, MA, USA.

Adenosine 5'-[γ-^{32}P]triphosphate was purchased from Amersham Pharmacia Biotech Australia Pty Ltd., Australia.

Ampicillin, lysozyme, ethidium bromide, dimethyl sulphoxide (DMSO), ficoll, dithiothreitol (DTT), N,N,N'N'-Tetramethylethylenediamine (TEMED), mineral oil, bovine serum albumin (BSA), *REDTaq*™ DNA polymerase, *Taq* DNA polymerase, including reaction buffer and $MgCl_2$, and Eagle's minimal essential media (MEM media) were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Pfu DNA polymerase was obtained from Stratagene, La Jolla, CA, USA.

FuGENE™ 6 Transfection Reagent, o-nitrophenol-β-D-galacto-pyranoside (ONPG), and the high pure PCR product purification kit were obtained from Roche Molecular Biochemicals, IN, USA.

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

Protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories, CA, USA.

DE-81 and Whatman papers were obtained from Whatman, England.

Cryotubes were obtained from Nunc Inc., Naperville, IL, USA.

T80 tissue culture flasks, T25 tissue culture flasks and 150 mm dishes were obtained from Nunc™, Denmark. 12-well cell culture dishes were obtained from Corning Incorporated, NY, USA.

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

Anti-NF-YA (goat), anti-NF-YB (goat) and anti-NF-YC (goat) were obtained from Santa Cruz Biotechnology, CA, USA.

The X-ray film, photographic developer and fixer were obtained from Eastman Kodak, NY, USA.

NF-Y bacterial expression clones were a generous gift from Dr R Mantovani, Universita Degli Study di Milano, Italy.

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

Scintillation fluid was from Wallac Scintilation Products, UK.

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

2.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments by size for either identification or purification. In solution (pH 8.0), DNA molecules are negatively charged and will migrate towards the positive electrode when an electrical current is applied. Migration is dependent on the size of the DNA fragments and their conformation both of which affect the movement of fragments in the agarose matrix.

Agarose gel electrophoresis was performed using 1% agarose in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA (ethylene diamine tetraacetic acid), pH 8.5) containing ethidium bromide (0.5 μg/mL) at 80-100 V (ethidium bromide is a fluorogenic dye that allows the visualisation of DNA when exposed to UV light using a UV transilluminator). The agarose was melted in a microwave and allowed to cool to approximately 55°C before pouring into the gel casting apparatus. Once the gel was set, DNA was loaded into the gel by mixing with DNA loading dye (10% glycerol, 62.5 mM Tris pH 8.0, 0.1% bromophenol blue). The DNA samples were mixed with the DNA loading dye to raise the density of the solution, this caused the sample to sink to the bottom of the well. The addition of the loading dye also provided a visual measure for progress of electrophoresis (the bromophenol blue dye front runs with the DNA fragments of about 500 bp in 1% agarose gels). The sizes of DNA bands were determined by measuring the distance a fragment had migrated from the well and comparing it with mobility of the 1 kb PLUS molecular size standard.

2.3 Quantitation of DNA by Gel Electrophoresis

Concentrations of homogenous DNA solutions were estimated by gel quantitation. This was achieved by the comparison of the fluoroescent intensity of the DNA band(s) of interest to standards that contained known quantities of DNA. An aliquot (5 μL) of the DNA solution in question was loaded onto an agarose gel alongside DNA standards of 10 ng/5μL, 20 ng/5 μL, 50 ng/5 μL and 100 ng/5 μL to quantify DNA. The standard used was linearised plasmid Bluescript II.

2.4 Quantitation of DNA by UV Spectroscopy

The concentration of plasmid DNA was also determined by UV spectroscopy, using the nucleic acid scan (200-350 nm) programme on the Pharmacia Biotech Ultraspec 3000 UV/Visible spectrophotometer. The purity of DNA was also assessed by this method, using the A_{260}/A_{280} absorbance ratio. Pure DNA has a ratio of 1.8. A ratio greater than 1.8 indicates contamination with RNA, while a ratio of less than 1.8 indicates protein contamination.

2.5 Restriction Digests

A wide range of restriction enzymes were utilised during this study. Typically, digestion reactions were performed in a total volume of 30 μL with 0.4-4.0 μg of DNA. For optimal DNA cleavage, the buffers recommended by the enzyme's manufacturers were used. In a 30 μL digest, 5-10 U of enzyme were generally used. Because the enzyme was stored in glycerol, which could potentially interfere with cleavage activity, digestion reactions were never prepared with more than 10% (v/v) enzyme. The reactions were prepared, vortexed, briefly centrifuged and placed at 37°C for approximately 1 hour. The sample reactions were then placed on ice to cool before an aliquot was analysed by agarose gel electrophoresis (see Section 2.2).

2.6 Annealing Single-stranded Oligonucleotides

Complementary single-stranded oligonucleotides were annealed to produce double-stranded competitor DNA for electrophoretic mobility shift assay (EMSA) experiments. Equal amounts of oligonucleotides were mixed to a final concentration of 100 ng/μL. The annealing reaction was performed in a thermocycler. The sample was heated to 95°C for 5 minutes, then the temperature was dropped by 10°C every two minutes until a final temperature of 25°C was reached. Double-stranded DNA was used directly from the sample.

2.7 Labelling Oligonucleotides for Electrophoretic Mobility Shift Assays

One μL (100 ng/μL) of one of the complementary oligonucleotides was mixed with 2 μL of water, 1 μL 10X kinase buffer (0.5 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 50 mM DTT, 0.5 mg/mL BSA (bovine serum albumin)), 5 μL γ-^{32}P-ATP (10 μL T4 polynucleotide kinase (PNK) (10 U/μL) and incubated at 37°C for 45 minutes. A 6X excess of complementary oligonucleotide (100 ng/μL), 2.5 μL 1 M KCl and 31.5 μL water were added to the mixture. The sample was then heated to 95°C for 5 minutes and allowed to cool slowly to room temperature. An equal volume of 2X gel shift buffer (40 mM Tris pH 7.6, 16% ficoll, 100 mM KCl, 0.4 mM EDTA, 1 mM DTT) was added to the annealed oligonucleotides. The labelled probe was gel purified by non-denaturing 10% polyacrylamide gel electrophoresis using 1X TBE (0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA, pH 8.0) at 30 W. The gel was exposed to X-ray film and the labelled, annealed oligonucleotides were excised from the gel and eluted overnight in 50 mM KCl at 37°C. The mixture was vortexed, centrifuged at 12,000 x g for 5 minutes and the supernatant containing the DNA was transferred to a new tube. The amount of the radioactivity present was determined by Cerenkov counting of 1 μL of the solution, using a Beckman LS3801 Scintillation Counter.

2.8 Ethanol Precipitation

Ethanol precipitation was used to concentrate DNA by precipitation out of solution followed by resuspending in the desired volume. It was also used to partially purify DNA from salts in solution.

DNA was precipitated by mixing with 0.1 volume of 3 M sodium acetate (pH 7.0) and 2.5 volumes of 95% ethanol. The mixture was incubated at -70°C for 30 minutes. The DNA was then pelleted by centrifugation at 12,000 x g for 20 minutes at 4°C. The pellet, containing DNA, was rinsed with ice cold 70% ethanol, dried and resuspended in either H_2O or TE buffer (10 mM Tris-HCl pH 8.0. 1 mM EDTA pH 8.0).

2.9 Isolation of Plasmid DNA

Plasmid DNA was isolated from *E.coli* cultures using the rapid boil DNA extraction method (Holmes and Quigley, 1981). DNA, isolated using this method, contained impurities such as salts, RNA and proteins, however, it was suitable for analysing DNA by restriction endonuclease reactions and PCR. Single-colonies of the ampicillin-resistant bacteria were picked off the LB-amp plates and used for the inoculation of 5 mL LB cultures containing ampicillin. The LB-amp cultures were incubated overnight at 37°C with vigorous shaking. The next day, 1.5 mL aliquots of 5 mL overnight cultures (LB broth) were pelleted at 12,000 x g for 1 minute and the pellets were resuspended in 350 µL STET buffer (8% (w/v) sucrose, 50 mM EDTA, 50 mM Tris pH 8.0, 5% (v/v) Triton X-100) and 25 µL freshly prepared lysozyme (10 mg/mL). The samples were mixed, boiled for 40 seconds in a boiling water bath and immediately centrifuged at 12, 000xg for 10 minutes in a microcentrifuge. The gelatinous precipitate was removed and an equal volume of isopropanol was added to precipitate the DNA. The mixture was placed in the -70°C freezer for 30 minutes then centrifuged at 12,000 x g for 15 minutes at 4°C. DNA pellets were washed with 500 µL 95% ethanol (at -20°C), centrifuged for 1 minute, dried and resuspended in 50 µL TE (10 mM Tris, 1 mM EDTA, pH 8.0).

2.10 Large Scale Preparation of Plasmid DNA

A single colony representing a correct clone was used to inoculate 5 mL LB-amp broth and grown overnight at 37°C. A 2 L flask, containing 500 mL of LB broth, supplemented with ampicillin (100 mg/mL), was then inoculated with the 5 mL culture, and incubated overnight at 37°C with vigorous shaking. Plasmid DNA was isolated using the CONCERT™ High Purity Maxiprep Plasmid Purification System (Life Technologies). This system, which was used to produce high quality pure DNA from the 500 mL culture, is based on a modified alkaline lysis method to break open the cells followed by neutralisation to precipitate the genomic DNA and separate it from the plasmid DNA. The plasmid DNA was then bound to an anion exchange resin under moderate salt conditions. RNA, protein and other contaminants were washed off the column under these conditions. The plasmid DNA was eluted from the column under

high salt conditions, desalted and purified using by isopropanol precipitation and ethanol washing. The dried pellet was resuspended in TE buffer.

2.11 Glycerol Stocks of Recombinant Plasmids

E.coli containing plasmids were preserved by freezing an aliquot of cells in 20% glycerol. A single colony was picked off an LB-amp plate and incubated in 5 mL of LB-amp broth at 37°C overnight with vigorous shaking. A 500 µL aliquot of the overnight culture was mixed with 500 µL of 40% glycerol and snap-frozen using liquid-air or nitrogen and stored at -70°C. The cell lines and plasmids used in this study are listed in Table 2.1.

Cell line or plasmid	Comments	Source or reference
Mammalian Cell Lines		
HeLa	Human adenocarcinoma cervical epithelial cells Low p53 expression	ATCC CCL-2 Akiyama (1987)
Plasmids		
pGL3Basic	4818 kb, see Appendix 3	Promega
pGL2Control	see Appendix 3	Promega
pSV-βgal	see Appendix 3	Promega
pGL3B-617wt -617-ICB1- -617-ICB2-	pGL3B containing 617 bp of the topoisomerase IIα promoter sequence, wildtype or containing specific mutations in ICB1, ICB2.	McLenachan (1998)
ΔYA	Eukaryotic expression vector for NF-YA	R. Mantovani
ΔYA29	Eukaryotic expression vector for NF-YA with a mutation at position 29	R. Mantovani

Table 2.1- Mammalian cell lines and plasmids used in this study.

2.12 DNA Sequencing

Automated sequencing of a plasmid DNA template was carried out using an ABI PRISM (PE Biosystems) sequencer. Sequencing reactions were carried out by Lorraine

Berry, Institute of Molecular BioSciences, Massey University. The sequencing was done according to the manufacturer's instructions using ABI PRISM® BigDye™ terminator cycle sequencing (PE Biosystems) chemistry. The sequencing results were analysed using the Wisconsin Package v. 9.1, Genetics Computer Group (GCG), Madison, WI.

2.13 Isolation of RNA from HeLa cells

RNA was isolated from HeLa cells using Trizol™ LS reagent according to the manufacturers instructions (Life Technologies). The Trizol™ LS reagent contains phenol and guanidine thiocyanate which maintain the integrity of RNA during homogenisation while disrupting cells and cellular components. By adding chloroform, the solution separates the two phases (after centrifugation), an aqueous phase containing RNA and an organic phase. The RNA was removed from the aqueous phase by precipitation with isopropanol. The RNA pellet was washed with -20°C 75% ethanol. The pellet was then allowed to air-dry before being resuspended in RNase-free water and stored at -20°C.

2.14 Quantification and Analysis of RNA

RNA was quantified on a spectrophotometer using the A_{260}/A_{280} ratio. Pure RNA has an A_{260}/A_{280} ratio of 2.0. Following quantification, the RNA was concentrated as follows by ethanol precipitation for loading a small volume on a gel. 0.1 volume of 2 M potassium acetate and 2.5 volumes of 100% ethanol were added to the RNA, mixed well and allowed to stand for 30 minutes at -70°C. The RNA was pelleted by centrifugation at 12,000 x g in a microcentrifuge for 15 minutes at 4°C, allowed to dry and resuspended in 20-50 µL of RNase-free water. 5 µg to 15 µg of RNA was analysed in a 1.5% agarose gel containing 17.6% formaldehyde in a buffer containing 1 x MOPS (1 M MOPS, 250 mM sodium acetate, 5 mM EDTA, pH 7) at 100 mA for 3-4 hours.

2.15 Northern Blot Analysis

Preparation of Northern Blot

After electrophoresis and visualisation with a UV transilluminator to confirm the presence of RNA on the gel, the gel was washed in 20 x SSC (3 M NaCl, 0.3 M trisodium citrate) while the blotting apparatus was being assembled.

Northern blotting uses capillary action to transfer the RNA from an agarose gel onto the membrane. A shallow container was filled to a depth of ~2 cm with 20 x SSC, and a glass plate was placed across the top. Two sheets of Whatman 3MM paper were wet in 20 x SSC and placed across the glass plate to form two wicks into the SSC. The gel was placed upside down on the Whatman paper so the RNA is closer to the membrane for transfer, and a corner was cut off the gel for identification. A piece of nitrocellulose membrane on a nylon base was cut the same size as the gel, wet in 20 x SSC and placed on top of the gel. Two sheets of Whatman 3MM paper wet in 20 x SSC followed by two dry sheets were placed on top of the membrane. The gel was masked with saran wrap to prevent bypassing of the gel. Finally, a stack of paper towels and a weight of approximately 500 g was placed on top of the Whatman 3MM paper to facilitate the transfer of RNA by capillary action.

The apparatus was left for 24-48 hours to allow for sufficient transfer of RNA onto the membrane. Once disassembled, the membrane was dried between blotting paper and exposed to UV radiation in the transilluminator for 30 seconds to strengthen the non-covalent but irreversible attachment of RNA to the membrane by the formation of a small number of cross-links between the bases in the RNA and the membrane (Sambrook *et al.*, 1989). The dried blot was stored desiccated in an air-tight container until used for hybridisation.

Preparation of DNA Probe

A PCR reaction was prepared in a 0.5 mL tube containing 5 µL of 10x buffer (containing magnesium), 5 µL of 3 mM dNTPs, 5 µL of 50 ng/µL of each primer (see Appendix 2), 1 µL of plasmid DNA template, 2.5 µL of *REDTaq*™ DNA polymerase (1

U/μL) and dH_2O to 50 μL. The reagents were mixed well, a drop of mineral oil placed on top to prevent evaporation at high temperatures and the tubes placed in a thermal cycler. The amplification conditions used are described below. The reaction was heated to 95°C for 4 minutes then the PCR was carried out for 30 cycles of 95°C for 30 seconds (denaturing), 55°C for 1 minute (annealing), and 72°C for 1 minute (elongation).

A 5 μL aliquot of the PCR reaction was analysed in a 1% agarose gel at 100 V for 1 hour and subjected to a diagnostic digest to determine the identity of the PCR product. Restriction endonuclease reactions were carried out as described in Section 2.5.

Once the identity of the product was confirmed the PCR reaction was repeated and the product purified using the High Pure PCR purification kit (Roche) according to the manufacturers instructions. This method is based on the binding of DNA molecules to the surface of pre-treated glass fibre fleece columns. Binding of DNA occurs simultaneously as impurities are washed away. The DNA was eluted from the column with a low salt buffer or water.

The purified product was quantified on a 1% agarose gel against a set of known DNA concentration standards (see Section 2.3). Electrophoresis was carried out at 80 V for approximately 1 hour, then visualised using a UV transilluminator. The quantified DNA was stored at -20°C prior to labelling for hybridisation to the northern blot.

Labelling a DNA Probe

The DNA probe was labelled using Ready-to-go™ DNA Labelling Beads (-dCTP) according to the manufacturers instructions (Amersham Pharmacia). This method is based on random-primed DNA synthesis. Approximately 35 ng of DNA (1 μL) was added to 44 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer. This mixture was denatured in a boiling water bath for 3 minutes then plunged into ice for 2 minutes to prevent reannealing of the DNA. The denatured DNA and 5 μL of [α-^{32}P]dCTP (10 μCi/μL) were added to the reaction mix bead (buffer, dATP, dGTP, dTTP, FPLC*pure*™ Klenow Fragment (7-12 units) and random 9-mer oligodeoxyribonucleotides). The mixture was pipetted up and down to mix, pulse centrifuged and incubated at 37°C for 20 minutes.

Unincorporated labelled nucleotides were removed using ProbeQuant™ G-50 size-exclusion Micro Columns (Amersham Pharmacia). The unincorporated nucleotides were retained on the column and the purified probe was recovered in the bottom of the collection tube after centrifugation.

Monitoring the Progress of the Reaction

A 2 µL aliquot of labelled reaction mix (B) and 2 µL aliquot of purified label mix (A) were each diluted in 500 µL of water. 5 µL of each mix was spotted onto Whatman GF/C filter discs and allowed to dry. The filters were placed in scintillation vials and 3 mL of scintillation fluid was added. The amount of radioactivity was determined using a ^{32}P window on a Beckman liquid scintillation counter.

The radioactivity on filter B represented the total amount in 5 µL of the diluted reaction mix. The radioactivity on filter A represented the amount that was incorporated into the DNA.

$$\text{Incorporation of radioactivity} = \frac{\text{cpm on A} \times 100\%}{\text{cpm on B}}$$

$$\text{Specific activity of the labelled probe} = \frac{\text{cpm in A} \times 250\ (\text{DF}) \times 1000}{\text{ng of DNA}}$$

Specific activity units = cpm/ µg

The probe was ready for hybridisation once the success of labelling had been confirmed and the specific activity of the labelled probe had been calculated.

Pre-hybridisation

The dried nitrocellulose membrane was wet by capillary action with 2 x SSC (0.3 M NaCl, 0.03 M trisodium citrate) then pre-hybridised for 3 hours at 68°C in ~20 mL of pre-hybridisation solution (6 x SSC (0.9 M NaCl, 0.09 M trisodium citrate)) with 1 x

Denhardts reagent (0.02% ficoll, 0.02% polyvinylpyrolidone, 0.02% BSA) in a rotary oven.

Hybridisation

Hybridisation was carried out in glass tubes in a rotary oven. The pre-hybridisation solution was removed and 20 mL of hybridisation solution (1 M NaCl, 50 mM phosphate, 2 mM EDTA, 0.5% SDS, 1 x Denhardts reagent, pH 6.5) was added. The labelled probe was boiled for 5 minutes to ensure it remained single-stranded before being added directly to the hybridisation solution. The hybridisation reaction was left to occur at 68°C overnight.

Washings

The labelled probe in the hybridisation solution was poured off and discarded appropriately. The first wash was carried out in glass tubes with subsequent washes carried out in plastic containers in the hybridisation oven. The membrane was washed twice in 6 x SSC with 1% SDS for 1 hour, then washed twice in 6 x SSC with 0.5% SDS for 30 minutes at 68°C. The final high stringency wash was carried out in pre-heated 1 x SSC (0.15 NaCl, 0.015 M trisodium citrate) for exactly 30 minutes at 68°C. The 1 x SSC was poured off and the membrane left to air-dry. The membrane was wrapped in saran wrap and exposed to X-ray film for 48-72 hours in an autoradiography cassette with two intensifying screens at -70°C before being developed in an automated developer.

2.16 Reverse Transcriptase Polymerase Chain Reaction

DNase Treatment of RNA Samples

RNA was treated with the RQ1 RNase-free DNase (Promega) to remove DNA from RNA samples prior to applications such as RT-PCR according to the manufacturers instructions. RQ1 (RNA Qualified) RNase-free DNase is a DNase 1 that degrades both double-stranded and single-stranded DNA endonucleolytically. RQ1 RNase-free DNase is used in applications where maintaining the integrity of RNA is critical. 1 unit of RQ1 RNase-free DNase was used per µg of RNA. The RNA, 10 x Reaction Buffer (provided with the enzyme), enzyme and nuclease-free water were combined to a final volume of 10 µL. The mixture was incubated at 37°C for 30 minutes before 1 µL of RQ1 DNase Stop Solution was added to terminate the reaction. The mixture was incubated at 65°C for 10 minutes to inactivate the DNase. The DNase treated RNA sample was able to be added to an RT-PCR reaction immediately following the treatment or stored at -20°C until required.

First Strand Synthesis

Moloney murine leukemia virus reverse transcriptase (M-MLV RT) contains a DNA polymerase capable of synthesising first strand cDNA from total RNA. It lacks DNA endonuclease activity and therefore does not degrade the DNA once it is made but contains RNase H activity which removes the RNA from RNA:cDNA hybrids once the DNA is synthesised.

All procedures involving RNA were carried out using pipette tips, microfuge tubes, glassware and water which had been treated with 0.01% DEPC by soaking overnight and then autoclaving to remove traces of DEPC. All solutions and equipment were reserved specifically for RNA work to prevent contamination by RNases.

Reactions were prepared as follows in a total volume of 10 µL: 1 µg total RNA, 1 x M-MLV RT buffer, 2 µL of 0.1 M DTT, 2 µL of 10 mM dNTPs, 0.25 µL of 500 ng/µL oligo(dT) primer, 0.1 µL of 200 U/µL M-MLV enzyme and DEPC treated water. The RNA and water were combined, heated to 65°C for 10 minutes to denature the RNA,

pulse centrifuged and placed on ice. The rest of the components were added to the RNA and water, mixed and incubated at 37°C for 1 hour for synthesis of cDNA. The reaction was terminated by heat deactivation of the RT enzyme at 95°C for 5 minutes. The reaction was then placed on ice and used in a PCR reaction or stored at -20°C.

The SuperScript™ Pre-amplification System for First Strand cDNA Synthesis by Life Technologies, was an additional method employed to produce first strand cDNA. The method was carried out according to the manufacturers instructions. The SuperScript™ Pre-amplification System was designed to synthesize first strand cDNA from purified poly(A)+ or total RNA. Following use of this system, target cDNA can be amplified by PCR with specific oligonucleotide primers without the need for intermediate organic extractions or ethanol precipitation. The first strand synthesis reaction is catalysed by SuperScript II RNase H- Reverse Transcriptase (RT). This enzyme has been engineered to eliminate the RNase activity found in other RTs that degrades mRNA during the first strand reaction (SuperScript Life Technologies Instruction Manual).

A first strand cDNA synthesis reaction may be primed using three different methods; random hexamers (for use when mRNA is difficult to copy in its entirety, due to the presence of sequences that cause the RT to abort synthesis), oligo(dT) (more specific and common) and gene-specific primer (GSP) (the most specific priming method, which uses an oligonucleotide containing sequence information of target cDNA).

Oligo(dT) was used for priming first strand synthesis in this study. Each component of the RT reaction was mixed and briefly centrifuged before use. RNA/primer mixtures were prepared as follows: 1-5 µg of total RNA or control RNA, 1 µL of oligo$(dT)_{12-18}$(0.5 µg/µL) and DEPC-treated water up to 12 µL were mixed in sterile 0.5 mL tubes. Note: at the same time as sample reactions were prepared, an RT^- control for sample and a control RNA reaction were also set up. The samples were incubated at 70°C for 10 minutes and then incubated on ice for 1 minute. A reaction mixture cocktail was prepared as follows: 10 x PCR buffer 2 µL/reaction, 25 mM $MgCl_2$ 2 µL/reaction, 10 mM dNTP mix 1 µL/reaction and 0.1 M DTT 2 µL/reaction. The reaction cocktail was prepared in the order indicated. 7 µL of the reaction mixture cocktail was added to each RNA/primer mixture, mixed and pulse centrifuged. The mixtures were incubated at 42°C for 5 minutes. 1 µL (200 units) of SuperScript II RT enzyme was added to each appropriate tube, mixed and incubated at 42°C for 50 minutes. The reactions were

subsequently terminated by placing the tubes at 70°C for 15 minutes then chilling on ice. After a brief centrifugation, 1 µL of RNase H was added to each tube, and the reactions were then incubated at 37°C for 20 minutes. Finally, the reactions were placed on ice and used immediately in a PCR reaction or stored at -20°C until required.

A standard PCR reaction using *REDTaq*™ DNA polymerase and *Taq*™ DNA polymerase (Sigma) was prepared and placed in a thermal cycler at: 95°C for 4 minutes for 1 cycle, 30 cycles of 95°C for 30 seconds, 48.5°C for 30 seconds, 72°C for 1 minute with an extra elongation time of 2 minutes at 72°C. Various methods of optimisation such as the dilution of the RT template (1:10, 1:100 and 1:1000) and magnesium titrations (0.5 mM-3.0 mM) were employed to obtain PCR products. The PCR reactions were analysed on a 1% agarose gel.

Platinum® *Taq* DNA Polymerase High Fidelity (Life Technologies) was also used. This enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D thermostable polymerase and Platinum *Taq* Antibody. *Pyrococcus species* GB-D polymerase possess a proofreading ability, this combined with *Taq* DNA polymerase increases fidelity approximately six times over that of *Taq* DNA polymerase alone, this allows the amplification of simple and complex DNA templates. Anti-*Taq* DNA polymerase antibody complexes with and inhibits polymerase activity. Due to specific binding of the antibody, Platinum® *Taq* DNA Polymerase High Fidelity is provided in an inactive form. This reaction provides an automatic "hot start" for PCR. Hot starts are typically used in PCR to increase sensitivity and yield while, the antibody-bound enzyme is sufficient to eliminate or reduce non-specific amplification in PCR (Platinum® *Taq* DNA Polymerase High Polymerase High Fidelity Instruction Manual).

PCR, using Platinum® *Taq* DNA Polymerase High Fidelity, was carried out according to the manufacturers instruction. The following components were added to a sterile microcentrifuge tube at either ambient temperature, or on ice: 5 µL (1 x) of 10 x High Fidelity PCR Buffer, 1 µL (0.2 mM) 10 mM dNTP mixture, 2 µL (2 mM) 50 mM $MgSO_4$, 1 µL (0.2 µM) Primer mix (10 µM each), ≥ 1 µL template DNA, 0.2-0.5 µL Platinum® *Taq* High Fidelity (1.0-2.5 units as required) and dH_2O to 50 µL. If desired, a master mix can be prepared for multiple reactions, to minimise reagent loss and to

enable accurate pipetting. The tubes were mixed and mineral oil was overlayed on top of the sample before brief centrifugation. The tubes were incubated in a thermal cycler at 94°C for 30 seconds to 2 minute (to denature the template and activate the enzyme), 25-35 cycles of PCR amplification were conducted as follows: 94°C for 15-30 seconds, 55°C for 15-30 seconds and finally 68°C for 1 minute per kb (1 minute for NF-YC ~1 kb in length). The reaction was maintained at 4°C after the previous cycles were completed cycling and the samples stored at -20°C until required. The amplification products were analysed by agarose gel electrophoresis.

PCRx Enhancer System (Life Technologies) was also used for amplification. The PCRx Enhancer System is an optimised buffer and co-solvent system that simplifies PCR amplification of problematic and/or GC-rich templates using standard dNTPs and thermocycling protocols (PCRx Enhancer System Instruction Manual).

The use of the PCRx Enhancer System was carried out according to the manufacturers instructions. The basic PCR optimisation protocol per reaction used is as follows: 5 µL 10X PCRx Amplification Buffer (1X), 1 µL 10 mM dNTP Mixture (0.2 mM each), 1.5 µL 50 mM $MgSO_4$ (1.5 mM), 1 µL Forward Primer (10 µM) (0.2 µM per reaction), 1 µL Reverse Primer (10 µM) (0.2 µM per reaction), 1 µL Template DNA (as required), 3.5 µL Platinum *Taq* DNA Polymerase (5 U/µL) (2.5 units/reaction) and finally autoclaved distilled water to 30 µL. A master mix can be prepared which is the preparation of a cocktail for as many reactions as needed, for example if you require seven reactions, the above reaction mix would be combined in a cocktail for those seven reactions.

		Final PCR	Enhancer	Solution	Concentration	
Component	0	0.5X	1X	2X	3X	4X
Master Mix	30	30	30	30	30	30
10X PCRx Enhancer Soln	-	2.5	5	10	15	20
Autoclaved Water	20	17.5	15	10	5	-

The contents of the tubes were mixed and overlayed with 50 µL of mineral oil. The tubes were incubated in a thermal cycler at 95°C for 2 minutes (to denature the

template). 25-35 cycles of PCR amplification were performed as follows: denature at 95°C for 30-45 seconds, anneal at 55-60°C for 30 seconds and finally extend at 68°C (1 minute/kb). The amplification products were analysed by agarose gel electrophoresis.

Pfu DNA Polymerase was an alternative enzyme used for PCR in this study (Stratagene). *Pfu* DNA Polymerase is a proofreading DNA polymerase isolated from *Pyrococcus furiosus* and exhibits the lowest error rate of any thermostable DNA polymerase (a feature ideal for high-fidelity DNA synthesis). PCR using *Pfu* DNA polymerase was conducted according to the manufacturers instructions. Reaction mixtures were prepared on ice to a final volume of 100 µL. Reaction mixtures were composed of the following: 10 µL10 X buffer, 0.8 µL dNTPs (25 mM each NTP), 1.0 µL of DNA Template (100 ng/µL), 2.5 µL Forward Primer (100 ng/µL), 2.5 µL Reverse Primer (100 ng/µL), 2.0 µL *Pfu* DNA Polymerase (5.0 U) and dH_20 to make up the balance. Immediately before the PCR reaction, 100 µL aliquots of the reaction mixture were placed in sterile tubes, ~50 µL of mineral oil was overlayed on top of the reaction mixture and PCR amplification was performed using optimised conditions. The conditions used were as follows: 1 cycle of 94°C for 45 seconds to denature, 30 cycles of 94°C for 45 seconds, Primer Tm-5°C (NF-YC = 48.5°C-5°C = 43.5°C) for 45 seconds and 72°C for 1-2 minutes/kb of PCR target (NF-YC ~1 kb) and finally 1 cycle of 72°C for 10 minutes. The results were visualised by agarose gel electrophoresis.

In addition to each reaction, both negative and positive control reactions were prepared. A negative control contains no DNA template and a positive control produces an amplification product of known size (~300 bp) under any amplification conditions (DNA template pTG3954, primers 1916 and 1917) (see Appendix 2).

2.17 Oligonucleotide Primers

Each primer was rehydrated to a concentration of 10 µg/µL and stored at -20°C. Primers were used at a stock concentration of 50 ng/µL (unless otherwise stated) for PCR analysis or 100 ng/µL for EMSAs. Sequences of oligonucleotides used in this study are shown in Appendix 2.

2.18 Preparations For Tissue Culture

All the tissue culture manipulations prior to harvesting were performed using asceptic technique in a laminar flow hood.

HeLa cells were grown in MEM (Eagle's minimal essential medium containing non-essential amino acids) media (prepared according to the manufacturers instructions and filter sterilisized through a 0.2 μM filter), supplemented before use with 10 % foetal calf serum (FCS), 1% penicillin and streptomycin (5000 U/mL, 5 mg/mL respectively). Before use with cells, media was warmed to 37°C by placing in hot water (65°C for 5-10 minutes).

2.19 Starting HeLa Cell Cultures

Cell cultures were started from frozen stocks. A 1 mL frozen aliquot of HeLa cells (in 10% dimethyl sulphoxide in FCS, stored under liquid nitrogen) was thawed quickly. Cells were transferred aseptically into 5 mL complete media (MEM with FCS and Pen/Strep) and mixed. Cells were pelleted in a bench top centrifuge for 5 minutes at approximately 100 x g and the supernatant discarded. The pellet was resuspended in 2 mL of media and one mL was used to seed two T80 tissue culture flasks, containing ~14 mL of complete MEM. One of the flasks was used to prepare cells for liquid nitrogen stocks in order to maintain stocks at a low passage number. Once cells became 80% confluent, they were passaged (Section 2.19) and resuspended in 5 mL FCS containing 10% DMSO. These cells were then dispensed into cryotubes in 1 mL aliquots and slowly frozen at -70°C before transferring to liquid nitrogen. Flasks were incubated at all times at 37°C, 5% CO_2 in a humidified atmosphere.

2.20 Tissue Culture and Passage of Tissue Culture Cells

Cells were grown in a monolayer in T80 flasks containing approximately 14 mL complete MEM. Typically, media was replaced every 2 to 3 days with fresh complete MEM media. When cells reached 80% confluence, they were passed into new flasks, to maintain the cell line or for transfections. The media was removed from the flasks and the cells were washed twice with 5 mL 1 x Trypsin solution in PBSE (phosphate

buffered saline EDTA) (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO4.2H_2O$, 0.5 mM EDTA). Once the cells detached from the surface of the flask, the cells were resuspended by aspiration with 10 mL of media. One mL of the suspension was added to a T80 tissue culture flask containing 14 mL of complete media for the maintenance of stocks. If needed for transfections, two drops of cells were added (using a sterile plastic dropper) to each well of a 12-well tissue culture plate, containing 800 µL of complete media.

2.21 Transient Transfections

Transfections were used to introduce DNA constructs into HeLa cells for the purpose of gene expression in mammalian cells. Transfections can be either stable or transient, occurring with or without the integration of vector DNA into the host genome respectively. Transient transfection was used in this study, providing a relatively simple and rapid method of examining gene expression *in vivo*. When a eukaryotic expression vector is introduced into mammalian cells, the regulatory sequences (promoter, terminator, origin of replication) are recognised by the nuclear machinery and processed accordingly, resulting in short term expression. Provided cells are harvested soon after transfection (12-72 hours) this expression can be observed. Without integration into the host genome, the construct is lost after a number of host divisions.

The transfection technology used in this study was FuGENE™ 6 Transfection Reagent (Roche) according to the manufacturers instructions. FuGENE™ 6 Transfection Reagent is a proprietary blend of lipids and other components supplied in 80% ethanol and sterile-filtered that complexes with and transports DNA into the cell during transfection. The benefits of FuGENE™ Reagent include: high transfection efficiency, virtually no cytotoxicity, good function in the presence or absence of serum and it requires minimal optimisation.

The day before transfection, cells were seeded in 12-well tissue culture plates as stated in Section 2.20. FuGENE™ 6 requires cells to be between 50-80% confluent on the day of the experiment. In a sterile tube, 72 µL of FuGENE™ 6 Reagent was added to 1128 µL of serum-free media. 0.5-3 µg DNA solution was set up in sterile tubes as DNA cocktails. After 5 minutes 300 µL of the pre-diluted FuGENE™ 6 Reagent was

added drop wise to each tube containing DNA. Each tube was mixed and incubated for 15 minutes at room temperature. The mixture was added drop wise to the cells, and the cells were placed at 37°C in 5% CO_2. The cells were incubated for 48 hours before harvesting.

2.22 Harvesting Tissue Culture Cells (HeLa Cells)

Media was removed from HeLa cells which were then rinsed twice with 1 mL PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO4.2H_2O$). 80 µL Cell Lysis Buffer (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton-X-100) was added and cells incubated for 15 minutes at room temperature. Cellular debris were scraped from the bottom of the well using a cell scraper and the cell extract collected with a pipette and transferred to a sterile 1.5 mL microcentrifuge tube. Cell extracts were vortexed and centrifuged for 15 seconds at 12,000 rpm to pellet debris. Cell extracts (the supernatant) were stored at -20°C for luciferase and β-galactosidase assays.

2.23 Luciferase Assay

The gene encoding firefly luciferase is a highly effective reporter gene used in the study of gene expression. The luciferase assay is extremely sensitive, rapid, easy to perform and relatively inexpensive. Light production by luciferase has the highest quantum efficiency known of any chemiluminescent reaction. Additionally, luciferase is a monomeric protein that does not require post-translational processing for enzymatic activity. Thus, it can function as a genetic reporter immediately upon translation (luciferase assay system technical bulletin).

The reaction catalysed by firefly luciferase is the oxidation of beetle luciferin with concomitant production of a photon. The conventional assay system for luciferase generates a flash of light that rapidly decays after the enzyme and substrate are mixed. Promega's luciferase assay system allows superior enzymatic turnover of luciferase, this results in greater light intensity that is nearly constant for measurements of up to several minutes (luciferase assay system technical bulletin).

The constant light intensity generated with Promega's luciferase system eliminates the need for rapid mixing protocols. The simplified assay procedure is adaptable and enzyme activity is easily measured with a luminometer (the light intensity measured is proportional to the concentration of the enzyme with a large linear range (10 pg/L – 1 mg/L).

Luciferase activity was measured as follows: 20 µL of HeLa cell extract was mixed with 100 µL of room temperature luciferase assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiotheritol, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP, final pH 7.8). The sample was mixed by pipetting up and down and immediately placed in a luminometer (BioOrbit 1253 Luminometer). Readings of the chemiluminescence were taken every five seconds until a maximum value was reached. The light intensity measured by the luminometer was recorded and used as the measure of luciferase activity. The cell lysis buffer used for harvesting HeLa cells was used to zero the luminometer.

2.24 β-Galactosidase Assay

The enzyme β-galactosidase catalyses the cleavage of various β-galactosides, including the substrate ONPG (o-nitrophenyl-β -D-galactopyranoside). The cleavage of ONPG can be followed in a spectrophotometer due to the release of the o-nitrophenyl group which absorbs light at 420 nm. β-galactosidase assays were carried out as described by Herbomel *et al.*, 1984. 100 µL of β-galactosidase assay buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_2, 10 mM KCl, 1 mM $MgCl_2$) and 50 µL of ONPG buffer (o-nitrophenyl-β- D-galactopyranoside) (2 mg/mL ONPG in 60 mM NaH_2PO_4, 40 mM Na_2HPO_4) were mixed with 20 µL of extract in a 96-well microtitre plate and incubated at 37°C until visibly yellow (1-8 hours). 50 µL of 1 M Na_2CO_3 was then added to terminate the reaction. Absorbances were measured at 405 nm against a blank containing 20 µL of cell lysis buffer.

2.25 HeLa Cell Extract Preparation

HeLa cells were plated onto a 150 mm dish so that they would be 50-80% confluent the next day. Cells were then left for approximately 72 hours. HeLa cells were washed

with 5 mL PBS and 1.5 mL of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl) was added to the plate. After 15 minutes incubation the cells were scraped off the bottom of the plate and transferred to a sterile 1.5 mL microcentrifuge tube. The cells were then pelleted by centrifugation for 5 minutes at 5, 000 x g and resuspended in 300 µL extraction buffer (40 mM HEPES (N-[hydroxehtyl]piperazine-N'-[2-ethane sulfonic acid]) pH 7.9, 0.4 M KCl, 1 mM DTT, 10% glycerol, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 0.1% aprotin). The cells were disrupted by three freeze-thaw cycles in liquid air or nitrogen and centrifuged for 5 minutes at 14,000 x g at 4°C. The supernatant was divided into 30 µL aliquots, snap-frozen in liquid air or nitrogen and stored at -70°C.

2.26 Determination of Protein Concentration of Extracts

Total protein concentration of the extracts was determined using the Bio-Rad protein assay dye reagent concentrate. The Bio-Rad protein assay is based on the Bradford dye-binding procedure (Bradford, 1976). 200 µL of the reagent (1:5 diluted in water) was added to BSA protein standards (0-2.5 µg) or 5 µL of cell extract in a 96-well microtitre plate. The absorbance was determined at 595 nm after 10 minutes. A standard curve was prepared using the protein standards and the concentration of protein samples was determined.

2.27 Binding Reactions For EMSAs

All reactions were carried out in 0.5 mL microfuge tubes.

The general binding reaction mixture (20 µL) contained 10 µL of 2 x gel shift buffer, 1 µg poly(dI-dC), 2 µL of nuclear extract, 0-100 ng of double-stranded competitor DNA, 0-1 µL of antibody (against NF-YA, NF-YB or NF-YC) and sterile water. After 10 minutes incubation at 4°C, 1 µL (0-100 ng) of labelled double-stranded oligonucleotide (~10,000 cpm/µL) was added, and the mixture was incubated at room temperature for 15 minutes prior to polyacrylamide gel electrophoresis (PAGE).

2.28 Polyacrylamide Gel Electrophoresis (PAGE)

Half of each binding reaction was electrophoresed at 200 V on a 4% polyacrylamide gel in 0.25 x TBE at room temperature for ~2 hours. After electrophoresis, the gel was transferred to DE-81 paper, dried at 80°C for 20 minutes using a Gel Dryer 583 (Bio-Rad) and exposed for 10 hours to X-ray film at -70°C using intensifying screens.

Chapter Three: Production of Antibodies to NF-YC via RT-PCR

3.1 Introduction

The purpose of experiments included in this chapter were to isolate RNA from HeLa cells for use in subsequent RT-PCR experiments and ultimately the production of antibodies to the components of the NF-Y complex (NF-YA, YB and YC).

At the planning stage of this research, antibodies to NF-YA and NF-YB, but not NF-YC were available commercially. It was reasoned that as antibodies are expensive and difficult to obtain, if antibodies were produced during the course of this research, overall costs could be reduced and there would be a readily available supply of antibodies to NF-YC for future use.

The antibodies produced would be used in binding assay experiments such as electrophoretic mobility shift assays and western blotting analysis to detect the presence of the NF-Y components (or protein) in extracts.

3.2 RNA Isolation

RNA was isolated from adherent HeLa cells grown in a monolayer in Nunc™ T80 flasks at approximately 80% confluence. Isolation of RNA was achieved using TRIzol™ LS Reagent according to the manufacturers instructions. RNA isolated was then quantified using an Ultraspec 3000 UV/VIS spectrophotometer (Pharmacia Biotech). The results of seven separate RNA isolations were pooled to give an overall RNA concentration of 10 μg/μL. Each individual RNA isolation used to make up the pooled RNA sample had an $A_{260}/_{280}$ of between 1.995 and 2.20 (RNA $A_{260}/_{280}$ ~2.0).

Following quantitation the RNA was analysed visually using denaturing agarose gel electrophoresis (Section 2.2) shown in Figure 3.1. The presence of discrete 28S and 18S rRNA bands with no obvious degradation indicate the RNA was of good quality. The continuous smear present in the sample lane is indicative of mRNA within the preparation.

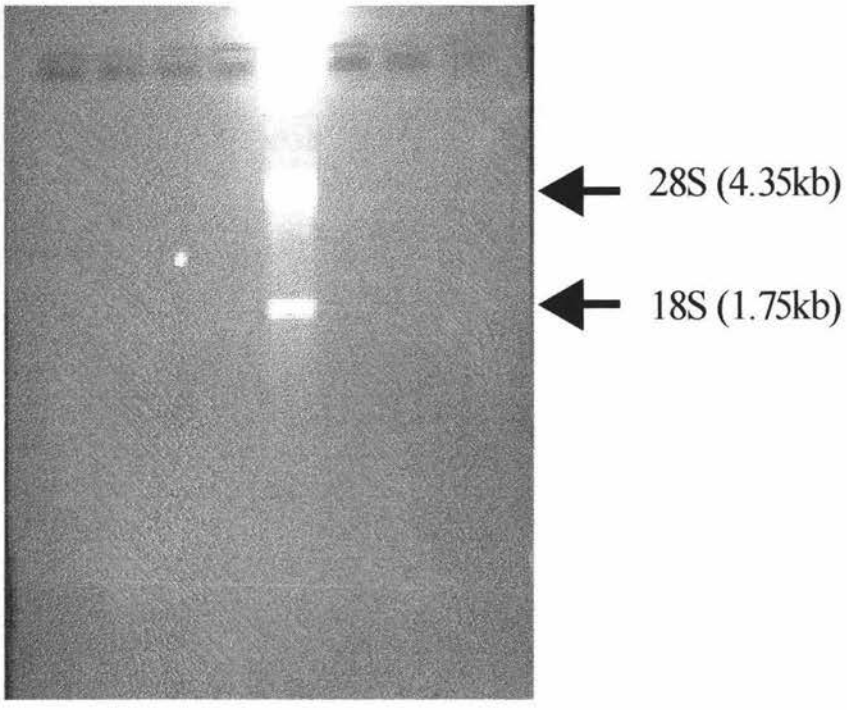


Figure 3. 1: RNA isolated from HeLa cells

This figure shows the 28S (~4.35kb) and the 18S (~1.75kb) rRNA bands characteristic of RNA. A 1.5% agarose gel containing 17.6% formaldehyde in a 1 x MOPS buffer was used to analyse the RNA. Electrophoresis was carried out at 100 mA for 3-4 hours. A sample of approximately 10 μg of RNA with 2 μL of 10 mg/mL ethidium bromide in the sample buffer was loaded in the sample lane.

Having established that the RNA obtained was visibly and quantitatively of sufficient quality and quantity, the gel was then used for northern blotting analysis. This analysis was used to determine the presence of an NF-Y message prior to RT-PCR experiments to obtain cDNA of NF-YB and NF-YC.

3.3 Northern Blotting Analysis

3.3.1 Northern Blot Preliminary Experimentation

For northern blotting analysis the RNA on the Agarose gel was transferred by capillary action onto a nylon membrane (Section 2.15), following which the RNA was fixed to the nylon membrane by exposure to UV light for 20-30 seconds. The membrane was stored in a desiccated environment until the pre-hybridisation step of the northern blotting analysis.

At this stage, a DNA probe was required to detect the presence of mRNA in the RNA sample. A mammalian expression vector ΔYA13 containing the cDNA for NF-YA was used to prepare a probe suitable for northern hybridisation analysis. Oligonucleotide primers for PCR were designed (see Appendix 2) to produce a 573 bp NF-YA product with an asymmetrically placed *Ava* I restriction site for use as a diagnostic tool for the identification of the product. Digestion of the product would give two bands of 377 bp and 198 bp in size.

The PCR reaction (Section 2.15) was carried out using an Omn-E thermal cycler (Hybaid Limited) using conditions shown below (Table 3.1).

Primers Involved	PCR Thermocycling Conditions		
NF-YA probe 5'	95°C	4 minutes	1 cycle
NF-YA probe 3'	95°C	30 seconds	30 cycles
	55°C	30 seconds	
	72°C	1 minute	
	72°C	1 minute	1 cycle

Table 3.1 PCR Thermocycling Conditions Used for the Amplification of the NF-YA Probe Sequence.

In addition to the reactions of interest, a negative control with no template was included to ensure no contamination was present. In addition, a positive control of a known product size was included (~300 bp amplified from primers 1916 and 1917 and template pTG3954). The amplification of the NF-YA probe sequence was carried out using RED*Taq*^{TM} DNA Polymerase from Sigma.

Following completion of the PCR, the products were analysed using agarose gel electrophoresis (Section 2.2). A 1% agarose gel containing ethidium bromide in 1xTAE buffer was run at 80V for approximately one hour. The results obtained were visualised using AlphaImager Gel Doc technology. Figure 3.2 shows the result of the PCR experiment.

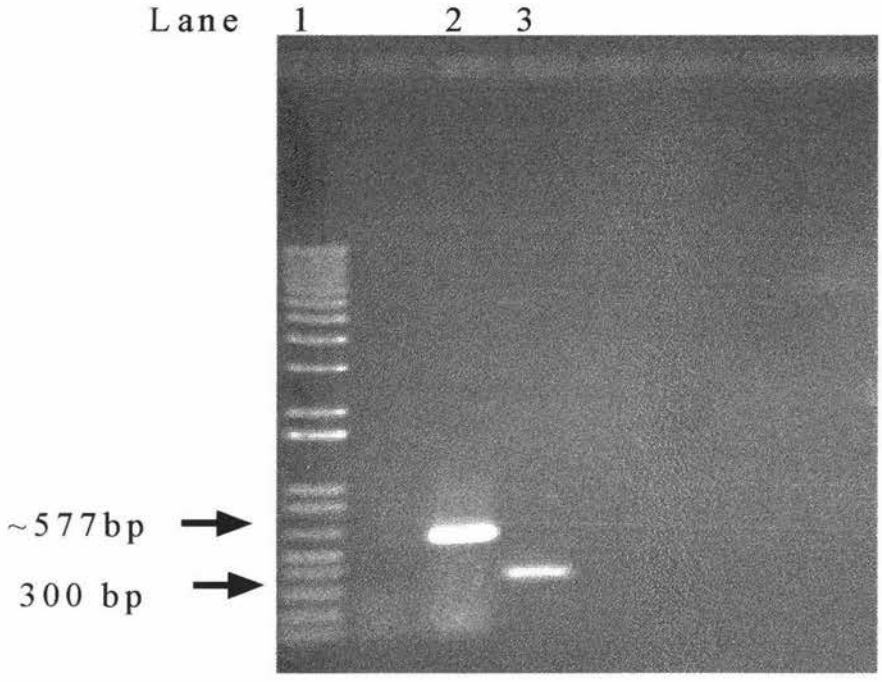


Figure 3.2: Gel Photograph Showing Amplification of NF-YA Probe

The NF-YA probe product was amplified using the conditions indicated in Table 3.1. A 1% agarose gel containing ethidium bromide. The agarose gel was electrophoresed in 1xTAE at 80 V for approximately 1 hour. The bands were visualised using a UV transilluminator. Lane 1 – 1kb ladder, lane 2 – the NF-YA probe (~577bp), lane 3 – the positive control (~300bp).

Having produced a fragment that was the correct size, its identity needed be confirmed. The identity of the PCR product was investigated using a diagnostic digest of 5 µL of the PCR reaction with the restriction enzyme *Ava* I (from New England Biolabs). The digest reactions were prepared and placed at 37°C for approximately one hour. The digest was analysed by agarose gel electrophoresis and visualised on a UV transilluminator and the AlphaImager Gel Doc technology. The PCR product initially produced did not appear to digest with *Ava* I.

The NF-YA probe reactions were subjected to DNA sequence analysis using the ABI 377 automated sequencer and BigDye terminator chemistry. The sequenced analysis showed that the NF-YA probe was authetic and contained the predicted *Ava* I site (see Appendix 4).

Once the identity of the PCR product was confirmed, the PCR experiment was repeated to obtain greater amounts of the NF-YA probe sample. Having done this, PCR purification was carried out using the High Pure PCR purification method (Life Technologies Inc) according to the manufacturers instructions (Section 2.15). The purified product was quantified by agarose gel electrophoresis (Section 2.2). The purified NF-YA product was loaded onto a 1% agarose gel alongside a series of standards of known DNA concentrations, and were analysed by electrophoresis. The intensity of fluorescence of the sample under UV light was compared with the concentration standards and was determined to be approximately 4 ng/µL in concentration. Figure 3.3 shows the results of the quantitation of NF-YA probe by gel electrophoresis.

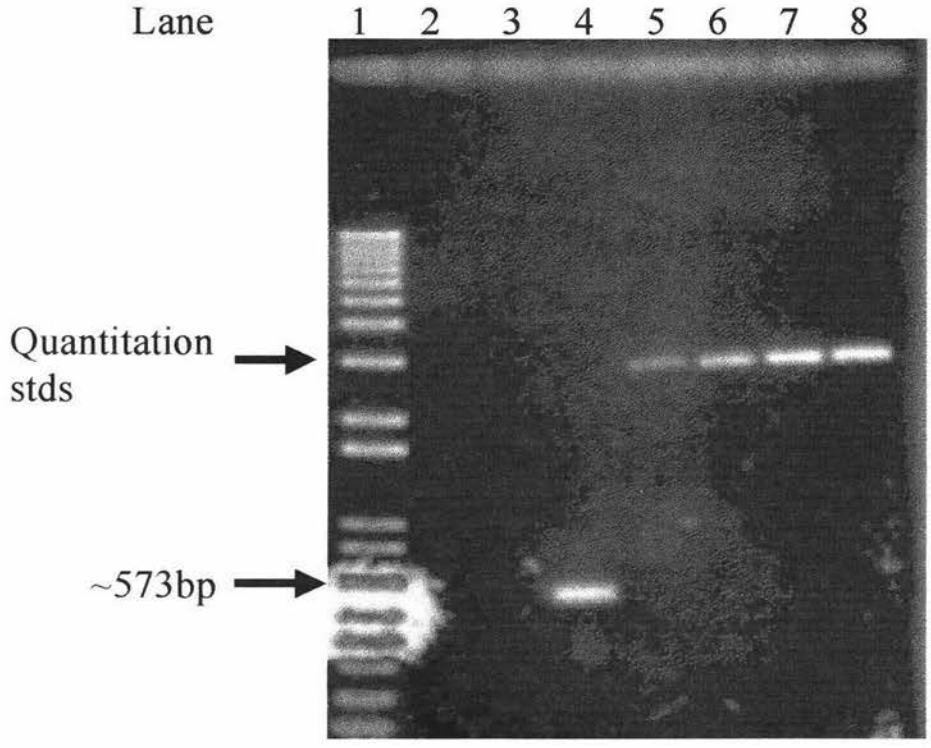


Figure 3.3: Gel Photograph of NF-YA Probe Quantitation

The NF-YA probe was run alongside a series of DNA standards on a 1% agarose gel containing ethidium bromide. The gel was run in 1xTAE at 80 V for approximately 1 hour. Lane 1 – 1 kb ladder, lane 2 and 3 – empty lanes, lane 4 – NF-YA probe, lane 5 – 5 ng/5µL, lane 6 – 10 ng/5µL, lane 7 – 20 ng/5µL, lane 8 – 50 ng/5µL.

Having prepared the NF-YA PCR product, confirmed its identity and determined its concentration, the sample was stored at -20°C until it was required for the labelling step of the northern blotting analysis.

3.3.2 Pre-Hybridisation of Blot and Labelling of Probe

The RNA blot described in Section 3.2 was removed from its desiccated environment and wet by capillary action in water. It was then placed in a hybridisation tube containing pre-hybridisation solution overnight at 68°C in a rotary oven.

The NF-YA probe was labelled using the Ready-To-Go DNA Labelling Beads (dCTP) system from Pharmacia Biotech according to the manufacturers instructions (Section 2.15).

The probe was quantified using ProbeQuant G-50 Microcolumns (Pharmacia Biotech) according to the manufacturers instructions (Section 2.15).

The percentage of incorporation and the counts per minute per microgram were determined as described in Section 2.15. Table 3.2 shows the data obtained from the scintillation counting procedure.

probe reaction	cpm on 'purified'(after)	cpm on 'total'(before)	% incorporation	Specific activity (cpm/µg DNA)
# 1	297223.03	1175632.28	25.3	3.72×10^{8} µg

Table 3.2: The monitoring progress of labelling with [α-^{32}P]dCTP.

The percentage incorporation of radioactivity in the labelled probe although sufficient was lower than optimal. This may have been because the double-stranded DNA was not adequately denatured by boiling prior to the labelling reaction. If the DNA had remained mostly double-stranded, the random primers would have had limited access to single strands and therefore the incorporation of [α-^{32}P]dCTP into the double-stranded DNA would have been low. Successful detection of the desired RNA band on a northern blot analysis relies on a probe with a high specific activity. A specific activity

of $\geq$ 1 x 10^9 cpm/μg of DNA was recommended (Ready-to-go DNA labelling beads Technical Manual). Although the percentage of incorporation and the specific activity obtained was low, the results were sufficient to proceed with the northern blot analysis.

3.3.3 Hybridisation

Having prepared the labelled probe and pre-hybridised the nylon membrane that contains the transferred RNA, hybridisation of the probe to the nylon membrane was carried out at 68°C.

Following hybridisation overnight, excess probe was washed off the membrane, which was then allowed to air dry, it was then placed in plastic wrap and exposed to X-ray film for approximately three days. The film was then developed using an automated developer, however no hybridisation bands were observed.

Although the Northern Blotting Analysis did not give the expected or desired result, it was reasoned that as NF-Y is ubiquitous and constitutive that the HeLa cell RNA should contain the message and that the northern blot should be repeated. The northern blot and hybridisation was repeated several times without success.

3.4 RT-PCR

Due to the reasoning that NF-Y is ubiquitous and constitutive and so the failure for a sufficient result in the northern blot was most likely to be due to experimental error, the RT-PCR experiments were attempted in order to amplify the cDNA for NF-YB and NF-YC. The first step was to take the RNA obtained from HeLa cells, treat it with RQ DNase (Promega) to avoid the possibility of DNA contamination, and use two methods to produce first stand cDNA. Those methods were MMLV (Section 3.4.2) and SuperScript™ (Section 3.4.3) both from Life Technologies. Both methods were carried out according to manufacturers instructions (section 2.16). The advantage of the SuperScript™ system was that a control RNA system was supplied, which enables a check on the validity of results obtained and can aid in troubleshooting. Following the production of the cDNA (the RT step), the PCR phase of the reaction was carried out.

3.4.1 DNase Treatment of RNA

RNA was treated with DNase as described in section 2.16. The treated RNA was then added to the RT-PCR reaction. Because each of the different RT reaction systems required different amounts of RNA, for instance MMLV RT reaction system required 2-10 μg of RNA whereas the SuperScript™ system requires 1-5 μg of RNA separate DNase treatments were carried out to ensure that the correct amounts and instructions were followed.

3.4.2 First Strand Synthesis

RT was carried out using MMLV RT system or SuperScript™ RT system as described in section 2.16.

3.4.3 PCR Experiments

In order to produce the NF-YC product (approximately 1000 bp or 1 kb in size), approximately 35 separate RT-PCR experiments were attempted. Conditions used were appropriate according to the melting temperatures of the primers, that had been designed from the published sequence (see Appendix 2). The production of the cDNA NF-YA and NF-YB was not attempted at this stage, as antibodies to these components of the NF-Y complex were already available. In addition, an NF-YA clone had already been obtained from Mantovani which could be subcloned into a bacterial expression vector for protein production. PCR enzymes used to attempt the production of NF-YC were *REDTaq* DNA polymerase from Sigma, Clear*Taq* DNA Polymerase from Sigma, Platinum® *Taq* DNA Polymerase from Life Technologies and *Pfu* from Life Technologies (Section 2.15). Dilutions of the RT products from the MMLV and the Superscript systems such as 1:10, 1:100 and 1:1000 were attempted as the components of the RT reaction step can inhibit subsequent PCR experiments. Magnesium titrations of 0.5 mM $MgCl_2$, 0.75 mM $MgCl_2$, 1.0 mM $MgCl_2$, 1.5 mM $MgCl_2$, 1.75 mM $MgCl_2$, 2.0 mM $MgCl_2$, 2.5 mM $MgCl_2$ and 3.0 mM $MgCl_2$ were attempted to optimise the PCR reaction step to either obtain a product, clean up products obtained or to obtain a product of the correct size.

Although many different procedures were attempted in order to obtain an NF-YC product, the PCR experiments carried out showed an array of undesirable results from non-specific products ranging from 100 bp-2000 bp in size to no product at all (data not shown). In addition to the NF-YC PCR step, both negative and positive controls were carried out. The negative control (primers alone – no DNA template) showed no result, which is as expected. This indicated that there was no contamination from DNA either from incomplete digestion by the DNase or from cross-contamination during the set-up of the PCR experiments. It was also a check on possible contamination of the primers used. The positive control (a reaction mix which produces a product of known size) produced the expected result, which was a product of approximately 300 bp in size. This was evidence that the PCR conditions, the reaction components (such as the enzyme and buffers) and the reaction itself were all viable. In addition to the above negative and positive controls, a no RT reaction was included, this was a control to check contamination of the RT step by DNA not digested by the DNase. With the Superscript system, the RNA control proved invaluable; subsequent PCR reactions following the RT phase of the RT-PCR reaction provided the expected product, a band of approximately 530 bp in size (see Figure 3.4). This indicated that the RT step was functional. All samples (RNA extracted from HeLa cells, the RNA RT^- reaction, the control RNA and the RT^- control RNA) had been subjected to first strand cDNA production by the two RT systems used (MMLV and Superscript) in the same experiments, at the same time. As the RNA control product was of the expected size, this provided evidence that the RT phase (using Superscript) of the RT-PCR experiment was viable and that all components of the RT step were functional i.e. buffers and enzymes. In addition, it also provided evidence that the first strand cDNA itself was viable and that the conditions used in the PCR reaction were optimal. The RT^- RNA control showed the expected result and provided evidence of no contamination.

A positive control to test the conditions chosen for the production of NF-YC was not available at the time this study started. But towards the end of this phase of the work, antibodies to NF-YC had become commercially available. Also, a generous gift of bacterial expression vectors containing NF-YA, NF-YB and NF-YC had been received from Roberto Mantovani (Universita Degli Study di Milano, Italy). Plasmids containing each of the NF-YA, NF-YB and NF-YC cDNAs had been isolated by Agnieszka Szremska as part of her BSc (Honours) project. A diluted aliquot of each of

these was taken and used as a template in PCR reactions with primers that had been designed previously at the beginning of this project (Appendix 2) to find optimal conditions for the production of PCR products representing the cDNA for NF-YA, NF-YB and NF-YC. The acquisition of these raw materials made it possible to test the primers that had failed to amplify NF-YC cDNA using RT-PCR.

3.5 PCR of NF-YA, NF-YB and NF-YC

The PCR conditions used for the amplification of NF-YA and NF-YB were as follows. The primers that had been designed dictated that a 47°C annealing temperature be used (Appendix 2). For the amplification of NF-YC an annealing temperature of 48.5°C was required. Below in Table 3.3 is a list of the conditions used to amplify NF-YA, NF-YB and NF-YC.

Primers Involved	PCR Thermocycling Conditions		
NF-YA 5' NF-YA 3'	95 °C	4 minutes	1 cycle
	95°C	30 seconds	
	47°C	30 seconds	30 cycles
	72°C	1 minute	
	72°C	2 minute	1 cycle
NF-YB 5' NF-YB 3'	As for NF-YA		
NF-YC 5' NF-YC 3'	95°C	4 minutes	1 cycle
	95°C	30 seconds	
	48.5°C	30 seconds	30 cycles
	72°C	1 minute	
	72°C	2 minutes	1 cycle

Table 3.3 PCR Conditions for the Amplification of NF-YA, NF-YB and NF-YC

The components of the reaction were RED*Taq*™ DNA Polymerase from Sigma, RED*Taq*™ Buffer containing magnesium, 3 mM dNTPS and the plasmid DNA containg the NF-Y cDNAs for the respective templates. Following amplification of these NF-Y templates they were used as positive controls alongside the RT-PCR

reactions using the first strand synthesis obtained using the Superscript method as template with the respective NF-Y primers (see Appendix 2). PCR reactions using the plasmid DNA templates all produced products of the expected size (Figure 3.4).

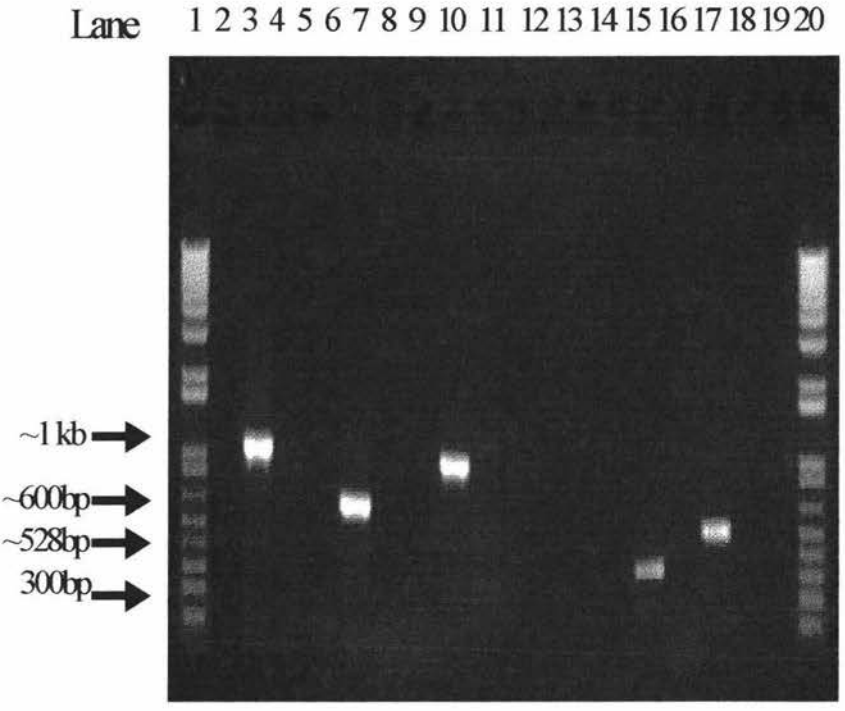


Figure 3.4: Gel Photograph of NF-Y PCR Results

This gel photograph is a compilation of the works described above. 5 µL of each PCR reaction was loaded onto a 1% agarose gel containing ethidium bromide. The gel was electrophoresed in 1xTAE buffer at 80V for approximately 1 hour. Lane 1 – 1 kb ladder, lane 2 – empty lane, lane 3 - NF-YA positive control (~1kb), lanes 4 and 5 - RT and RT dilution 1:10 (respectively) with NF-YA primers, lane 6 – NF-YB positive control (~600bp), lanes 7 and 8 – RT and RT 1:10 (respectively) with NF-YB primers, lane 9 – NF-YC positive controls (~1kb), lanes 10 and 11 – RT and RT 1:10 (respectively) with NF-YC primers, lanes 12-14 – negative controls of NF-YA, YB and YC (primers alone), lane 15 – positive control (~300bp), lane 16 –RT⁻ control, lane 17 – RNA control SuperScript™ Kit, lane 18 –RT⁻ RNA control SuperScript™ Kit.

3.6 Chapter Summary and Conclusions

The identity of the probe as being the desired segment of NF-YA was determined using automated sequencing. The failure to obtain a result with the northern blotting analysis, although questionable is not without explanation, especially when taken with the inability to amplify any of the NF-Y components using RT-PCR. As the positive controls for NF-Y amplification indicate *REDTaq*^{TM} DNA polymerase (from Sigma) was sufficient as the enzyme for amplification, the primers gave the expected products and the PCR conditions were appropriate. Therefore, the results of the RT-PCR experiments and northern blotting analysis suggest that the RNA obtained from HeLa cells may not have been of sufficient quality to be used in the amplification of the components of NF-Y. As a result the production of cDNAs for NF-YA, NF-YB and NF-YC was not continued. Fortunately, during the attempts to produce NF-YC by RT-PCR, commercial antibodies to NF-YC became available. So, in spite of the failure to produce NF-YC, antibodies to all three components of NF-Y were commercially available. In addition, the acquisition of cDNA clones for NF-YA, B and C in bacterial expression vectors will allow production of these proteins in the future. It will be possible to purify each of NF-YA, B and C from *E.coli* and use these proteins to raise the appropriate antibodies should the commercially available ones become inaccessible or too expensive.

Chapter Four: Electrophoretic Mobility Shift Assays

4.1 Introduction

The electrophoretic mobility shift assay (EMSA) or gel shift is a technique that enables the binding interactions between proteins and either DNA or RNA to be analysed. These interactions can be studied by mixing radiolabelled DNA or RNA with proteins and separating any resulting complexes based on electrophoretic mobility using non-denaturing (native) polyacrylamide gel electrophoresis (see Figure 4.1).

Specific binding of the NF-Y transcription factor to inverted CCAAT boxes 1-4 for the topoisomerase IIα promoter has been shown previously (Herzog and Zwelling, 1997; Isaacs *et al.*, 1996; Wang *et al.*, 1997). In this study, EMSAs have been conducted to analyse the efficiency and the specificity of NF-Y binding to the inverted CCAAT Boxes (ICBs) 1 and 2 of the topoisomerase IIα promoter. The particular role of the flanking sequences around these elements was also analysed as the different ICBs of the topoisomerase IIα promoter have been shown to have different functional roles. It is reasonable to suggest that if the actual CCAAT box sequence is the same in all these ICB elements, then any differences in function/role must be due to the flanking sequences around the ICBs or the position of the ICB in the promoter. Therefore, oligonucleotides that contain the ICB1 flanking sequence around the ICB2 element and oligonucleotides that contained the ICB2 flanking sequence around the ICB1 element were designed(see Appendix 2 for oligonucleotide sequences) and used in EMSAs. Wildtype ICB1 and ICB2 oligonucleotides as well as mutant oligonucleotides were used for comparison (see Appendix 2 for oligonucleotide sequences).

In an EMSA experiment a labelled DNA fragment, containing a specific transcription factor-binding site, was mixed with a protein extract. After the binding reaction was complete, the sample was applied to a low ionic strength polyacrylamide gel, electrophoresis is performed, and the gel was then dried and visualised by autoradiography. If an interaction occurs between a protein and the labelled DNA fragment, the resulting electrophoretic pattern will display a band of lower mobility containing the specific DNA-protein complex. Non-specific DNA-protein interactions

were minimised by the addition of poly(dI-dC) to the binding reaction before the addition of the labelled DNA.

To establish sequence specificity of the DNA-protein interactions, competition assays were performed. An unlabelled DNA fragment was added in excess to the binding reaction before the addition of the labelled DNA. If the DNA-protein complex was specific, the majority of the protein bound to the unlabelled DNA, leaving less protein available to be bound to the labelled DNA fragment. This resulted in a reduction or elimination of the band corresponding to the complex formed between the labelled DNA and the protein. Mutant oligonucleotides were used to further analyse sequence specificity. If, due to the presence of a mutation, the protein is unable to bind to the competitor oligonucleotide, the competitor would not be able to compete with the labelled DNA for protein binding, therefore a reduction in complex formation would not be observed.

The proteins present in the DNA-protein complex were be identified by antibody-supershift assays, where antibodies were added to the binding reaction. These antibodies recognised the protein involved in the complex, causing either formation of an antibody-DNA-protein ternary complex, resulting in further reduction in the band mobility, or in the inhibition of complex formation, by binding to an important site within the binding protein.

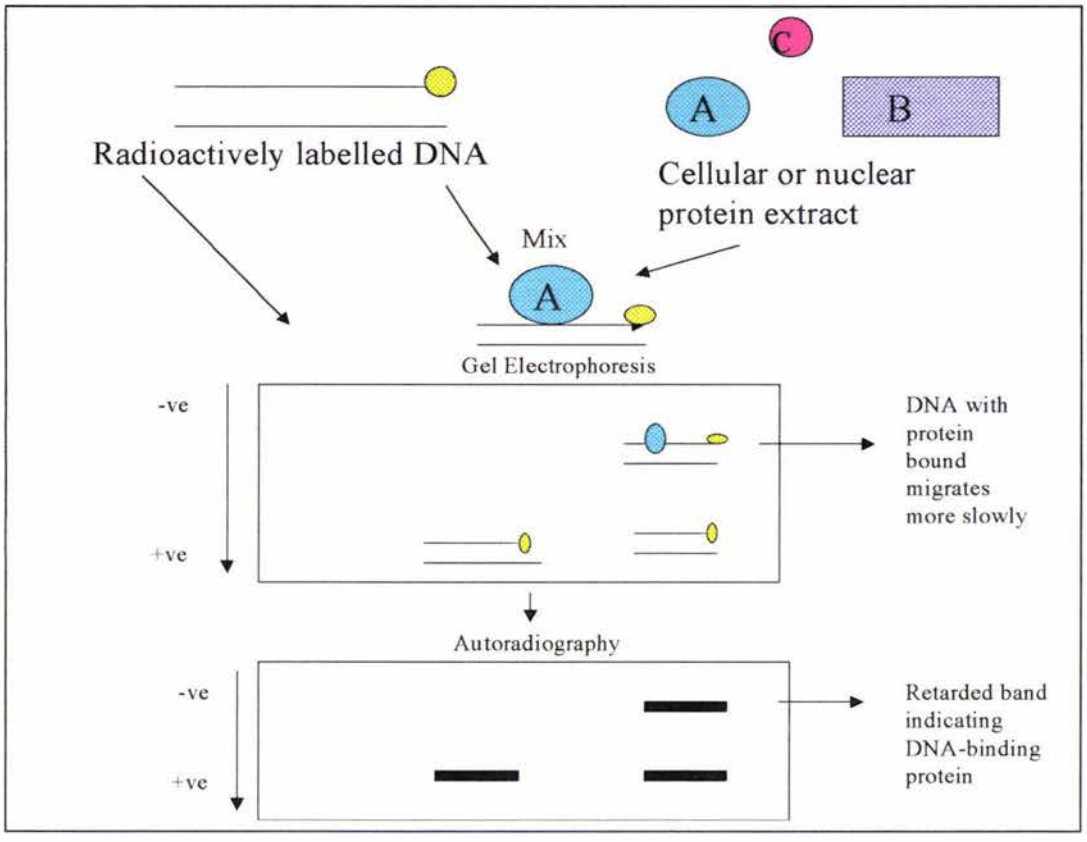


Figure 4.1 – Schematic diagram of EMSA

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

4.2 Preparation of HeLa Cell Extracts

HeLa cell extracts were prepared as described in Section 2.25 and stored at -70°C until required.

4.3 Labelling of Oligonucleotides

ICB1wt, ICB1FICB2ICB1F (ICB1F), ICB2wt, and ICB2FICB1ICB2F (ICB2F) oligonucleotides (see Appendix 2) were labelled with γ-^{32}P-ATP and purified (Figure 4.2) as described in Section 2.7. Table 4.1 shows the activity of the probes purified in the first labelling experiment shown in Figure 4.2.

Probe	Counts per minute (1 μL)
ICB1F	21 035
ICB2 wt	20 029
ICB2 F	22 980
Blank	25

Table 4.1: The radioactivity of each probe (1 μL) in the first labelling experiment determined by Cerenkov Counting.

Table 4.2 shows the values for purification of probes from the second labelling experiment.

Probe	Counts per minute (1 μL)
ICB1wt	41 080
ICB1 F	45 200
ICB2 wt	29 010
ICB2 F	45 098
Blank	25

Table 4.2: The radioactivity (cpm) of each probe (1 μL) in the second labelling experiment determined by Cerenkov Counting.

4.4 EMSA Experiments

After purification and determination of radioactive incorporation, the probes shown in Tables 4.1, 4.2 and Figure 4.2 were subjected to a preliminary EMSA analysis (Figure 4.3 and 4.4). Different amounts of protein extracts (1 μL, 2 μL and 4 μL) were added to determine the appropriate amount of protein extract required for each probe. The protein concentration of the HeLa cell extracts had previously been determined to be ~1.8 μg/μL using a 96-well microtitre Bradford protein assay. From the results observed in Figures 4.3 and 4.4, 2 μL (corresponding to ~3.6 μg of protein) of cell extract was determined to be necessary and sufficient for visualisation of DNA-protein complexes.

Both competition and supershift assays were performed multiple times with HeLa cell extracts for each probe (and each probe except ICB1 was labelled twice) to establish reproducibility of the results. Representative results of the EMSA experiments are shown here in Figures 4.3 and 4.4. Determination of competition efficiency was conducted visually and general trends for the influence of flanking sequences were observed. The experiments are subject to experimental errors such as variations in amounts of protein extract, probe and competitor added to the binding reactions.

Due to the variation of probe labelling efficiency, different exposure times were needed for different probes. A typical exposure was at -70°C overnight unless otherwise indicated in individual figure captions.

In most cases two bands of lower mobility than the free probe were observed. These were labelled S and N. The identity of these bands was investigated using antibodies (Section 4.4.1-2).

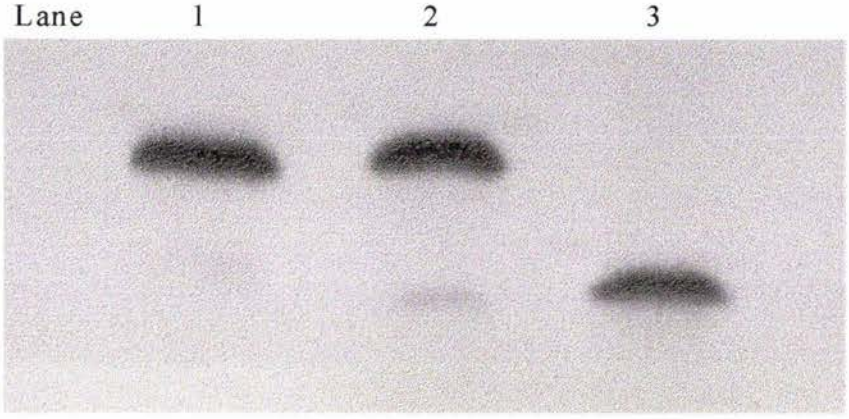


Figure 4.2 PAGE of end-labelled oligonucleotides

100 ng of oligonucleotide was ^{32}P-labelled with T4 polynucleotide kinase (PNK), annealed to its complement and electrophoresed on a 10% polyacrylamide gel in 1X TBE at 30 W for ~2 hours. The gel was exposed to an X-ray film for 1 minute. Bands were excised and eluted overnight in 400 µL of 50 mM KCl prior to Cerenkov counting.

Lane 1: ICB1F labelling reaction.
Lane 2: ICB2F labelling reaction.
Lane 3: ICB2 labelling reaction.

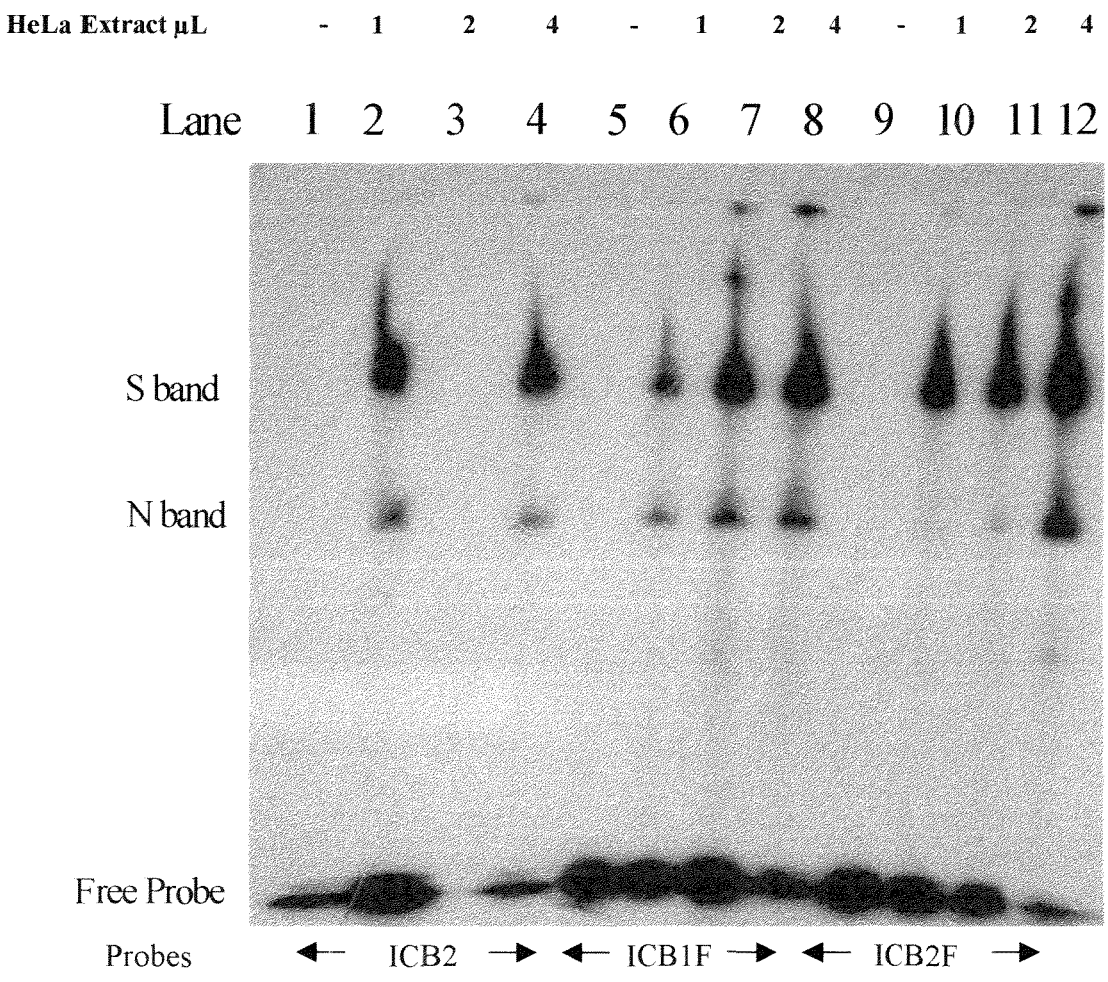


Figure 4.3 EMSA of HeLa extract with probes ICB2, ICB1F and ICB2F

Binding reactions were prepared as described (section 2.27), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lanes 1-4 ICB2 probe. Lanes 5-8 ICB1F probe. Lanes 9-12 ICB2F probe. Lanes 1,5 and 9 are indicative of probe alone. Lanes 2,6 and 10 show respective probes with 1 µL of HeLa cell extract. Lanes 3,7 and 11 show respective probes with 2 µL of HeLa cell extract. Lanes 4,8 and 12 show respective probes with 4 µL of HeLa cell extract.

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

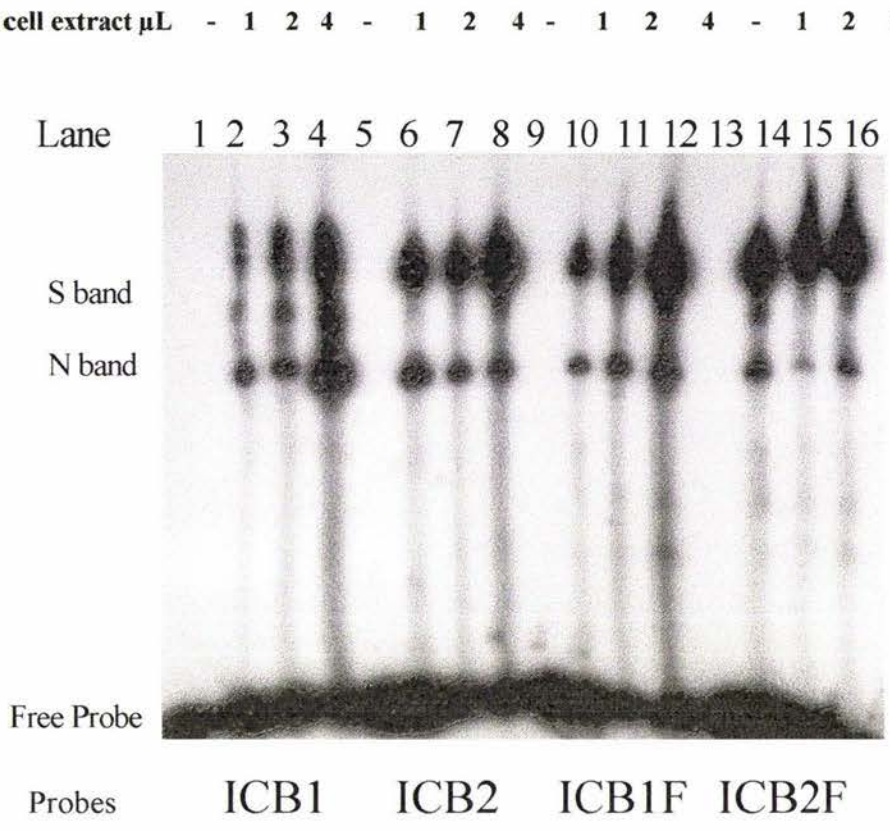


Figure 4.4 EMSA of HeLa extract with probes ICB1, ICB2, ICB1F and ICB2F

Binding reactions were prepared as described (section 2.26), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated below. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lanes 1-4 ICB1 probe. Lanes 5-8 ICB2 probe. Lanes 9-12 ICB1F probe. Lanes 13-16 ICB2F probe. Lanes 1,5,9 and 13 show the respective probes alone. Lanes 2,6,10 and 14 show the respective probes with 1 µL of HeLa cell extract. Lanes 3,7, 11 and 15 show the respective probes with 2 µL of HeLa cell extract. Lanes 4,8,12 and 16 show the respective probes with 4 µL of HeLa cell extract.

4.4.1 ICB2 probe with NF-YA, NF-YB and NF-YC Antibodies

A preliminary EMSA experiment was carried out to determine the optimal antibody dilutions for use in subsequent experiments (see Figure 4.5). Figure 4.5 shows an EMSA of ^{32}P-labelled ICB2 double-stranded probe with HeLa cell extract and antibodies to NF-YA, NF-YB and NF-YC. The antibody supershifts conducted demonstrated that supershifts were only observed with NF-YA and NF-YB antibodies at dilutions of 1:10 (lanes 3 and 7 respectively) and that the S band complex contained the protein NF-Y. These experiments also contained binding reactions (lanes 11, 12 and 13) with dilutions of NF-YC antibody as follows: 1:5, 1 μL undiluted and 2 μL undiluted. However, an NF-YC supershift was not observed under any of these conditions. The failure to obtain a supershift to NF-YC may be due to the fact that an EMSA is a native gel and therefore proteins are not denatured and as NF-Y is a trimer, the epitope that the NF-YC antibody targets may not be available (to bind in the native conformation), it may be buried in the surface where the components of NF-Y bind to each other to form the heterotrimer. However, it must be noted that for NF-Y to bind to DNA all three subunits are required, therefore although the presence of NF-YC was not demonstrated, it was presumed that NF-YC was present in the HeLa cell extracts used in the EMSA experiments conducted during this study.

The S complex was shown to contain the NF-Y transcription factor, as the anti-NF-YA and –NF-YB antibodies altered the migration of this complex (the N complex was not affected). A dilution of 1:10 of antibody to NF-YA was sufficient to produce a supershift, whereas a dilution of 1:5 of antibody to NF-YB was required to produce a supershift. No supershift was observed with antibody to NF-YC.

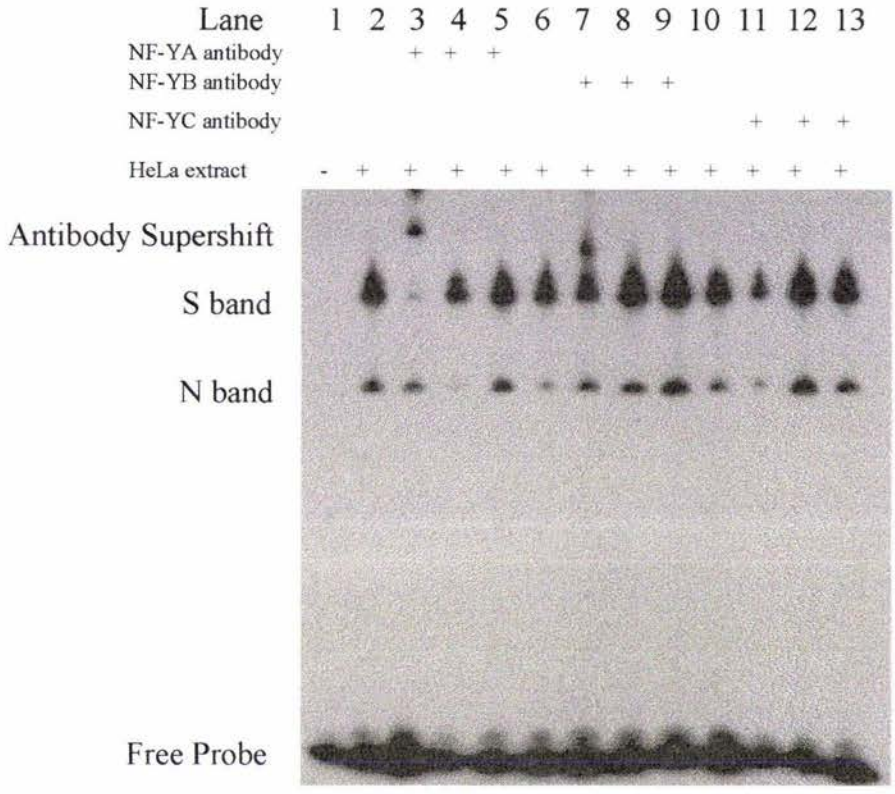


Figure 4.5 EMSA of the ICB2 probe with antibodies to NF-YA, NF-YB and NF-YC

Binding reactions were prepared as described (Section 2.26), with fixed amounts of poly(dI-dC) and HeLa extract. Electrophoresis was carried out at 4% polyacrylamide gel in 0.25X TBE for 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to an X-ray film overnight at -70°C.

Lane 1 contains probe only. Lanes 2-13 contain ICB2 probe with 2 µL of HeLa extract. Lanes 3-5 show the results of addition of antibody to NF-YA to the binding reactions. Lane 3, 1:10 dilution of NF-YA antibody. Lane 4, 1:100 dilution of NF-YA antibody and Lane 5, 1:1000 dilution of NF-YA antibody. Lanes 7-9 show the results of the addition of antibody to NF-YB to the binding reactions. Lane 7, 1:10 dilution of NF-YB antibody. Lane 8, 1:100 dilution of NF-YB antibody. Lane 9, 1:1000 dilution of NF-YB antibody. Lanes 11-13 show the results of the addition of antibody to NF-YC to the binding reactions. Lane 11, 1:10 dilution of NF-YC antibody. Lane 12, 1:100 dilution of NF-YC antibody. Lane 13, 1:1000 dilution of antibody to NF-YC.

The antibodies used were of an original concentration of 200 µg IgG in 0.1 mL PBS. All antibodies were purchased from Santa Cruz Biotechnology.

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

4.4.2 ICB2, ICB1F and ICB2F probes with Antibodies to NF-YA and YB

It was necessary to confirm that the protein binding to the other probes, ICB1F and ICB2F was also NF-Y. Figure 4.6 shows the EMSA result with the ^{32}P-labelled ICB2, ICB1F and ICB2F double-stranded probes with antibodies to NF-YA and NF-YB. Figure 4.7 shows the EMSA result with the ^{32}P-labelled ICB1, ICB2, ICB1F and ICB2F double-stranded probes with antibodies to NF-YA and NF-YB. The antibody supershifts conducted demonstrated that supershifts were only observed with NF-YA (lanes 3, 7 and 11 and lanes 3, 7, 11 and 15 in Figures 4.6 and 4.7 respectively), and NF-YB (lanes 4, 8 and 12 and lanes 4, 8, 12 and 16 in Figures 4.6 and 4.7 respectively) antibodies at dilutions of 1:10 (antibody concentration of 0.2 μg/μL) and that the S band complex was the protein NF-Y.

The results of Figure 4.6 and 4.7 clearly show with all probes used in these EMSA experiments (ICB1, ICB2, ICB1F and ICB2F) that NF-Y is present in the HeLa cell extracts used with positive identifications for NF-YA and NF-YB by the presence of antibody supershifts. Because NF-YA and NF-YB are confirmed to be present, NF-YC must also be present as all three components of NF-Y are required for the binding of NF-Y to DNA.

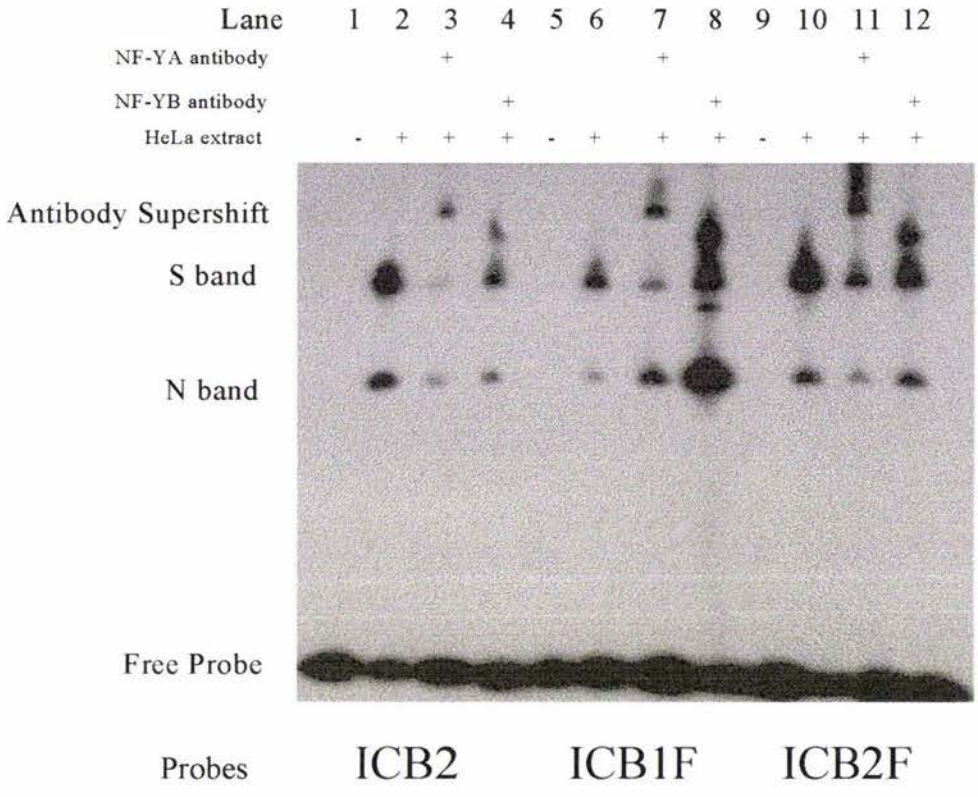


Figure 4.6 EMSA ICB2, ICB1F and ICB2F probes with anti-NF-YA and -NF-YB.

Binding reactions were prepared as described (Section 2.26), with fixed amounts of poly(dI-dC) and HeLa extract. Electrophoresis was carried out using a 4% polyacrylamide gel in 0.25X TBE for 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 contains ICB2 probe only. Lanes 2, 6 and 10 contain probes with 2 µL of HeLa extract alone. Lanes 3-4 show the results of addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 3, 1:10 dilution of NF-YA antibody. Lane 4, 1:5 dilution of NF-YB antibody. Lane 5 contains ICB1F probe alone. Lanes 7-8 show the results of the addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 7, 1:10 dilution of NF-YA antibody. Lane 8, 1:5 dilution of NF-YB antibody. Lane 9 contains the ICB2F probe alone. Lanes 11-12 show the results of the addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 11, 1:10 dilution of NF-YA antibody. Lane 12, 1:5 dilution of NF-YB antibody.

The antibodies used were of an original concentration of 200 µg IgG in 0.1 mL PBS. All antibodies were purchased from Santa Cruz Biotechnology.

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

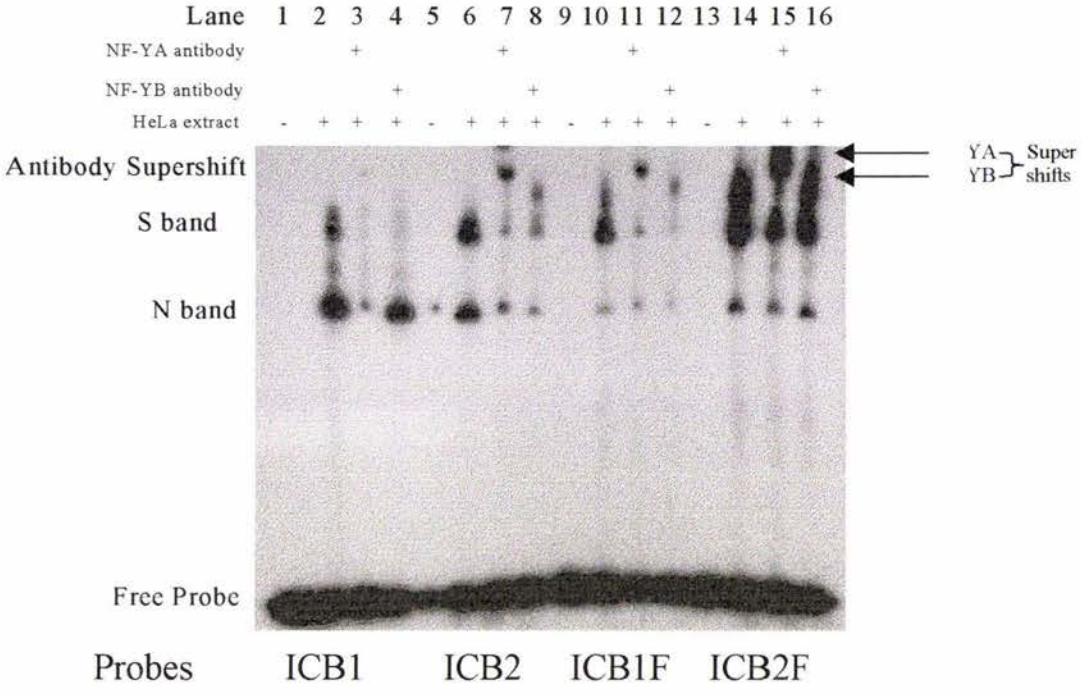


Figure 4.7 EMSA with ICB1, ICB2, ICB1F and ICB2F probes with antibodies to NF-YA and NF-YB

Binding reactions were prepared as described (Section 2.27), with fixed amounts of poly(dI-dC) and HeLa extract. Electrophoresis was carried out using a 4% polyacrylamide gel in 0.25X TBE for 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 contains ICB1 probe only. Lanes 2, 6, 10 and 14 contain probes with 2 µL of HeLa extract. Lanes 3-4 show the results of addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 3, 1:10 dilution of NF-YA antibody. Lane 4, 1:5 dilution of NF-YB antibody. Lane 5 contains ICB2 probe alone. Lanes 7-8 show the results of the addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 7, 1:10 dilution of NF-YA antibody. Lane 8, 1:5 dilution of NF-YB antibody. Lane 9 contains the ICB1F probe alone. Lanes 11-12 show the results of the addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 11, 1:10 dilution of NF-YA antibody. Lane 12, 1:5 dilution of NF-YB antibody. . Lane 13 contains the ICB2F probe alone. Lanes 15-16 show the results of the addition of antibody to NF-YA and NF-YB to the binding reactions. Lane 15, 1:10 dilution of NF-YA antibody. Lane 16, 1:5 dilution of NF-YB antibody.

The antibodies used were of an original concentration of 200 µg IgG in 0.1 mL PBS. All antibodies were purchased from Santa Cruz Biotechnology.

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

EMSAs with ICB1 probe

To confirm and analyse the interaction of NF-Y with the ICB1 element of the topoisomerase IIα promoter, competition and supershift assays were performed as described in section 2.26.

Figure 4.8 shows the result of an EMSA experiment: ICB1 ^{32}P-labelled probe with competitors. This EMSA experiment showed the presence of multiple DNA-protein complexes, formed after incubation with HeLa cell extract. The nature of these complexes was examined by the addition of different unlabelled competitors. Amounts of 5 ng, 10 ng and 100 ng of each competitor yielded approximately 5x, 100x or 500x excess respectively.

The complex of the lowest mobility (labelled S) was not eliminated by the addition of the ICB1mt competitor, however it was affected by the addition of ICB1wt, ICB1F and ICB2F unlabelled competitors. The ICB2F competitor was observed to reduce the S band DNA-protein complex significantly at 5x excess (lane 15), and upon the addition of 100x or 500x of this competitor the S band was eliminated (lanes 16 and 17 respectively). The ICB1wt and ICB1F competitors showed the next greatest competition efficiency, with the S band being reduced by the addition of 5x, 50x or 100x excess (lanes 3-5 and 11-13 respectively) but not abolished. The ICB1mt competitor (lanes 7-9) had little effect on the S band complex. The complexes with the higher mobility (labelled N) were affected in a similar manner by each competitor. This suggests that the protein responsible for complex S formation is specific for the CCAAT box, as it appears not to bind the mutant competitor. The protein responsible for the N-band is likely to be binding non-specifically to the DNA.

This experiment indicates that the ICB1F competitor was able to compete for the protein (NF-Y) from the labelled ICB1 probe with the greatest efficiency. This indicates a high binding affinity of protein to this oligonucleotide sequence, which represents the ICB2 flanking sequence around the ICB1 element of the topoisomerase IIα promoter. The competition efficiency of the ICB1F and the ICB1wt was virtually identical, while the ICB1mt showed the lowest (nil) competition efficiency.

Therefore the order of competition for Figure 4.8: ICB2F>ICB1wt≥ICB1F>ICB1mt

The S complex was shown to contain the NF-Y transcription factor, as the anti-NF-YA and –NF-YB antibodies altered the migration of this complex (see Figures 4.5-4.7)

One point that requires clarification is the inconsistencies between lanes 5 and 9, in Figure 4.8. It appears that the competition seen with the ICB1mutant at 500x excess (lane 9) is greater than that seen with ICB1wildtype. As this effect was not seen on any of the subsequent repeat experiments, it is likely that this is due to pipetting errors and problems associated with the delivery of 2 μL of HeLa cell extract which is viscous by nature.

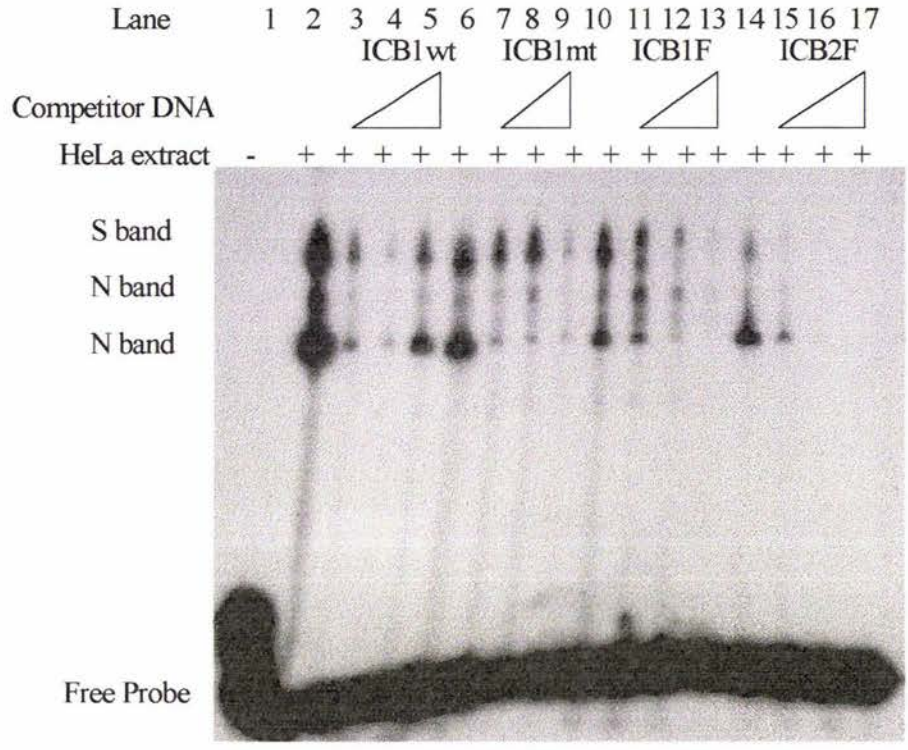


Figure 4.8 EMSA of ICB1 probe with ICB1wt, ICB1mt, ICB1F and ICB2F competitors

Binding reactions were prepared as described (section 2.27), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated below. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 ICB1 probe alone. Lanes 2, 6, 10, and 14, ICB1 probe and 2 µL of HeLa cell extract. All remaining lanes contain probe and extract with lanes 3-5, 7-9, 11-13 and 15-17 containing competitors ICB1wt, ICB1mt, ICB1F and ICB2F respectively. Competitor amounts are in increasing order of 5 ng (10x excess), 10 ng (100x excess) and 100 ng (500x excess).

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

4.4.4 EMSAs with the ICB2 probe

To confirm and analyse the interaction of NF-Y with the ICB2 element of the topoisomerase IIα promoter, competition and supershift assays were performed (as described in Section 2.27).

Figure 4.9 shows the result of an EMSA experiment with ICB1 ^{32}P-labelled probe and competitors. This EMSA experiment showed the presence of multiple DNA-protein complexes, formed after incubation with HeLa cell extract. The nature of these complexes was examined by the addition of different unlabelled competitors. Amounts of 5 ng, 10 ng and 100 ng of each competitor yielded 5x, 100x or 500x excess respectively.

The complex of the lowest mobility (labelled S) was not eliminated by the addition of the ICB2mt competitor (lanes 8-10), however it was affected by the addition of ICB2wt (lanes 3-5), ICB1F (lanes 11-13) and ICB2F (lanes 15-17) unlabelled competitors. The ICB2F competitor was observed to reduce the S band DNA-protein complex significantly at 5x excess (lane 15) and upon the addition of 100x or 500x of this competitor the S band was eliminated (lanes 16 and 17 respectively). The ICB2wt and ICB1F competitors showed the next greatest competition efficiency, with the S band being reduced by the addition of 5x excess (lanes 3 and 11 respectively) and almost abolished with a 100x excess of unlabelled competitor (lanes 4 and 12 respectively). This effect appeared to be maintained upon the addition of 500x excess of competitor (lanes 5 and 13 respectively). The complexes with the higher mobility (labelled N) were affected in a similar manner by each competitor. This reinforces the conclusion from the previous experiment shown in Figure 4.8, that the protein responsible for complex S formation is specific for the CCAAT box, as it does not appear to bind the mutant competitor.

This experiment clearly indicates that the ICB2F competitor was able to compete for the protein (NF-Y) from the labelled ICB2 probe at an excess of unlabelled competitor between 5x and 100x. This indicates high binding affinity of protein for the oligonucleotide sequence which was the ICB2 flanking sequences around the ICB1 element of the topoisomerase IIα promoter. The competition efficiency of the ICB1F

and the ICB2wt oligonucleotides were approximately the same, with the ICB2mt showing the lowest (nil) competition.

Therefore the order of competition shown in Figure 4.9 is ICB2F>ICB2wt≥ICB1F>ICB2mt.

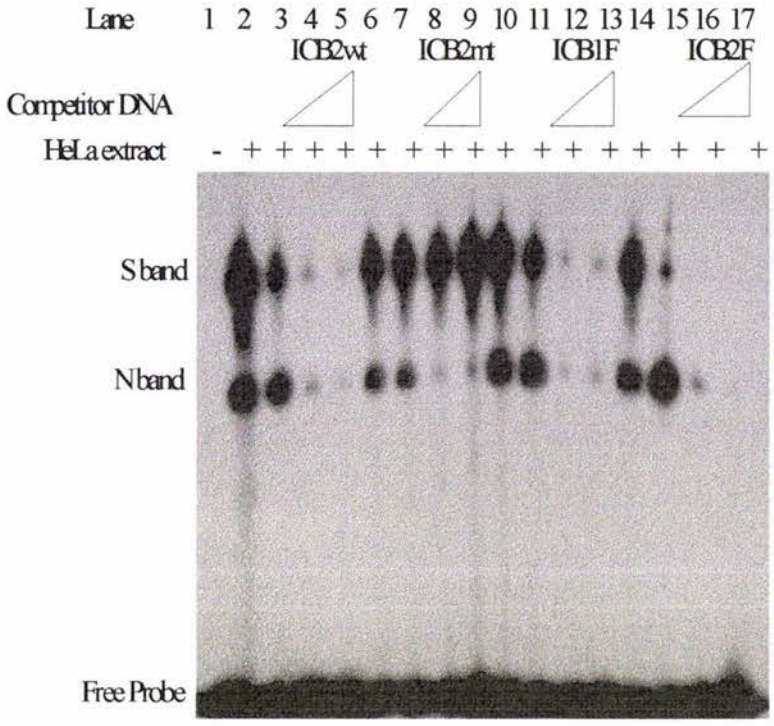


Figure 4.9 EMSA of ICB2 with competitors ICB2wt, ICB2mt, ICB1F and ICB2F

Binding reactions were prepared as described (section 2.26), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated below. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 ICB2 probe alone. Lanes 2, 6, 10, and 14, ICB2 probe and 2 µL of HeLa cell extract. All remaining lanes contain probe and extract with lanes 3-5, 7-9, 11-13 and 15-17 containing competitors ICB2wt, ICB2mt, ICB1F and ICB2F respectively. Competitor amounts are in increasing order of 5 ng (10x excess), 10 ng (50x excess) and 100 ng (500x excess).

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

4.4.5 EMSAs with the ICB1F probe

Figure 4.10 shows the result of an EMSA experiment with ICB1F ^{32}P-labelled probe and competitors. This EMSA experiment showed the presence of multiple DNA-protein complexes, formed after incubation with HeLa cell extract. The nature of these complexes was examined by the addition of different unlabelled competitors. Amounts of 5 ng, 10 ng and 100 ng of each competitor yielded 5x, 100x or 500x excess respectively.

The complex of the lowest mobility (labelled S) was not eliminated by the addition of the ICB2mt competitor, however it was affected by the addition of ICB2wt, ICB1F and ICB2F unlabelled competitors. The ICB2F competitor was observed to abolish the S band DNA-protein complex at 5x excess (lane 15), this effect was maintained upon the addition of 100x or 500x of this competitor (lanes 16 and 17 respectively). The ICB2wt and ICB1F competitors showed the next greatest competition efficiency, with the S band being reduced by the addition of 5x excess (lanes 3 and 11 respectively) and almost abolished with a 100x excess of unlabelled competitor (lanes 4 and 12 respectively). Upon the addition of 500x excess of competitor the S band was completely abolished with both the ICB2wt and ICB1F competitors (lanes 5 and 13 respectively). The ICB2mt competitor did not appear to have an effect on the S band DNA-protein complex (lanes 7-9). The complexes with the higher mobility (labelled N) were affected in a similar manner by each competitor. This suggests that the protein responsible for complex S formation is specific for the CCAAT box, as it appears not to bind the mutant competitor.

This experiment clearly indicates that the ICB2F competitor was able to compete for the protein from the labelled ICB1F probe at an excess of unlabelled competitor of 5x. This indicates high binding affinity of protein for the oligonucleotide sequence which was the ICB2 flanking sequences around the ICB1 element of the topoisomerase IIα promoter. The competition efficiency of the ICB1F and the ICB2wt oligonucleotides were approximately the same, with the ICB2mt showing the lowest (nil) competition.

The complex of lowest mobility (labelled S) was not eliminated by the addition of the ICB2mt competitor. However, it was eliminated by the addition of ICB2wt, ICB1F and

ICB2F unlabelled competitors, suggesting that the complex formed was CCAAT box specific. All other complexes were eliminated by the addition of all competitors.

Order of competition was ICB2F>ICB1F≥ICB2wt>ICB2mt

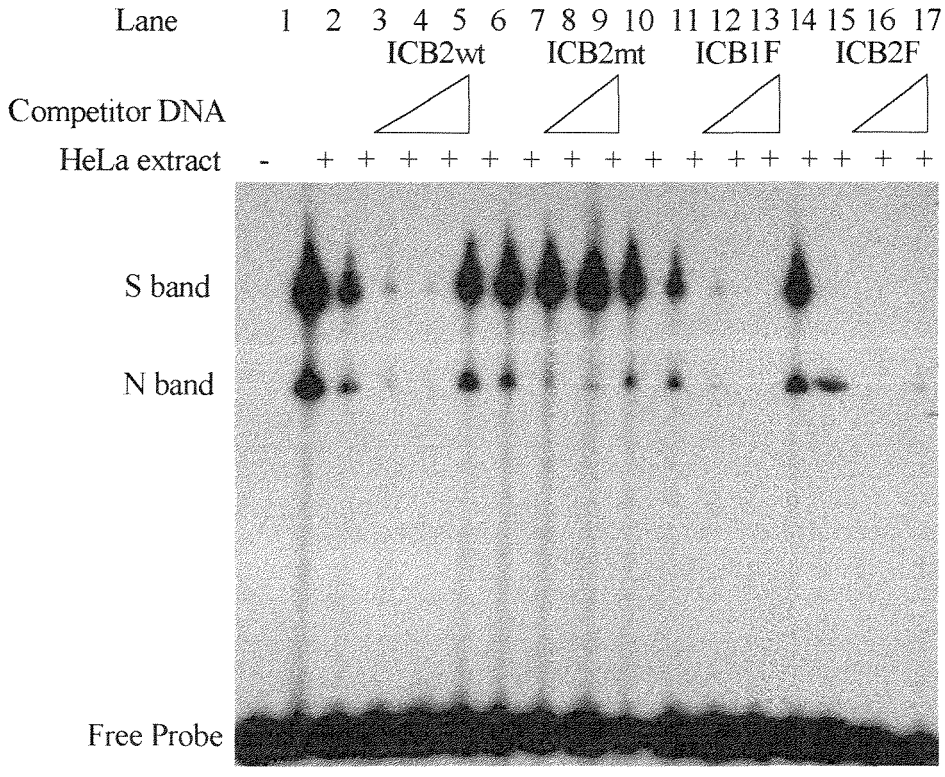


Figure 4.10 EMSA of ICB1F probe with competitors ICB2wt, ICB2mt, ICB1F and ICB2F

Binding reactions were prepared as described (section 2.27), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated below. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 ICB1F probe alone. Lanes 2, 6, 10, and 14 ICB1F probe and 2 µL of HeLa cell extract. All remaining lanes contain probe and extract with lanes 3-5, 7-9, 11-13 and 15-17 containing competitors ICB2wt, ICB2mt, ICB1F and ICB2F respectively. Competitor amounts are in increasing order of 5 ng (10x excess), 10 ng (100x excess) and 100 ng (500x excess).

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

4.4.6 EMSAs with the ICB2F probe

Figure 4.11 shows the results of EMSA with the ^{32}P-labelled ICB2F double-stranded probe. Multiple DNA-probe complexes are formed after the incubation with HeLa cell extract (Lanes 2, 6, 10 and 14). The nature of these complexes was examined by the addition of different unlabelled competitors. Amounts of 5 ng, 10 ng and 100 ng of each competitor yielded 5x, 100x or 500x excess respectively.

The complex of the lowest mobility (labelled S) was not eliminated by the addition of the ICB2mt competitor, however it was affected by the addition of ICB2wt, ICB1F and ICB2F unlabelled competitors. The ICB2F competitor was observed to reduce the S band complex at 5x competitor excess (lane 15) and abolish the S band DNA-protein complex at 100x excess, this elimination was maintained upon the addition of 500x of this competitor (lanes 16 and 17 respectively). The ICB2wt and ICB1F competitors showed the next greatest competition efficiency, with the S band being incrementally reduced by the addition of 5x, 100x and 500x excess of unlabelled competitor (lanes 3-5 and 11-13 respectively). The ICB2mt competitor did not appear to have an effect on the S band DNA-protein complex (lanes 7-9). The complexes with the higher mobility (labelled N) were affected in a similar manner by each competitor. This suggests that the protein responsible for complex S formation is specific for the CCAAT box, as it appears not to bind the mutant competitor.

This experiment clearly indicates that the ICB2F competitor was able to compete for the protein from the labelled ICB2F probe at an excess of unlabelled competitor of 100x. This indicates high binding affinity of protein for the oligonucleotide sequence which was the ICB2 flanking sequences around the ICB1 element of the topoisomerase IIα promoter. The competition efficiency of the ICB1F and the ICB2wt oligonucleotides were approximately the same, with the ICB2mt showing the lowest (nil) competition.

The complex of lowest mobility (labelled S) was not eliminated by the addition of the ICB2mt competitor. However, it was eliminated by the addition of ICB2wt, ICB1F and ICB2F unlabelled competitors, suggesting that the complex formed was CCAAT box specific. All other complexes were eliminated by the addition of all competitors. This

suggests that the protein responsible for complex S formation is specific for the CCAAT box, as it appears not to bind the mutant competitor.

Figure 4.11 shows that the ICB2F competitor was able to compete for the protein in complex S from the labelled ICB2F probe at 5x unlabelled competitor excess, with the S band DNA-protein complex being abolished at 100x and 500x competitor excess. The competition efficiencies of the ICB1Fcompetitor and the ICB2 wildtype competitor are virtually identical with both competitors reducing the intensity of the DNA-protein complexes at a 5x excess but not eliminating the complexes even at a 100x or excess. The mutant ICB2 competitor had no effect on the S band DNA-protein complex, however the N band DNA-protein complex was effected indicating that the S band is CCAAT specific.

Order of Competition Figure 4.11 was ICB2F>ICB1F=ICB2wt>ICB2mt

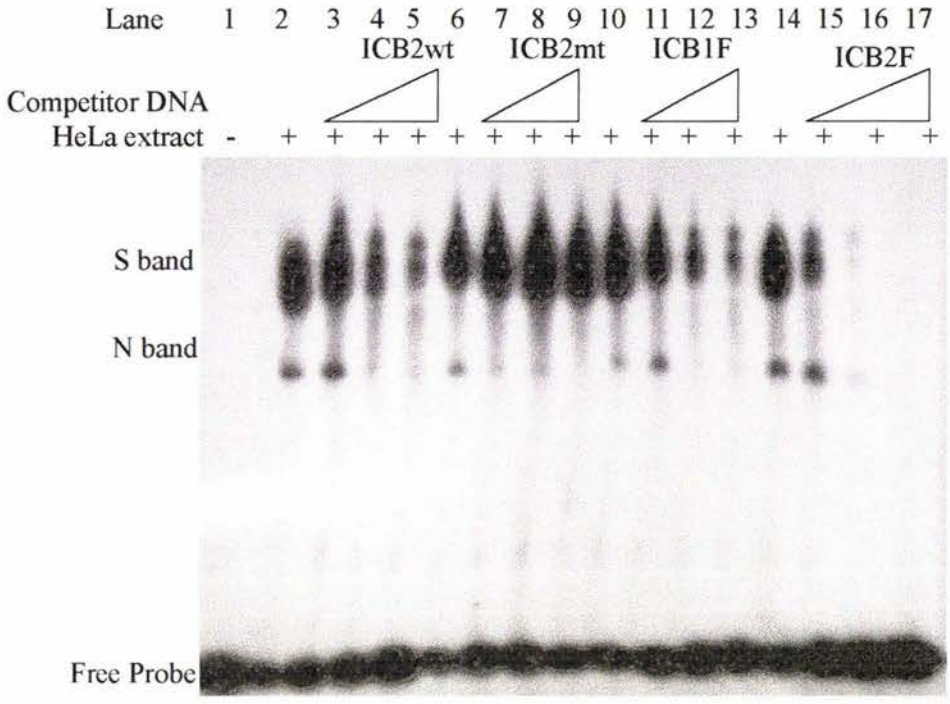


Figure 4.11 EMSA of ICB2F probe with ICB2wt, ICB2mt, ICB1F and ICB2F competitors

Binding reactions were prepared as described (section 2.27), with fixed amounts of poly(dI-dC) and different amounts of extracts as indicated below. Electrophoresis was carried out on a 4% polyacrylamide gel in 0.25X TBE for approximately 2 hours at 200 V. The gel was dried onto DE-81 paper and exposed to X-ray film overnight at -70°C.

Lane 1 ICB1F probe alone. Lanes 2, 6, 10, and 14 ICB1F probe and 2 µL of HeLa cell extract. All remaining lanes contain probe and extract with lanes 3-5, 7-9, 11-13 and 15-17 containing competitors ICB2wt, ICB2mt, ICB1F and ICB2F respectively. Competitor amounts are in increasing order of 5 ng (10x excess), 10 ng (100x excess) and 100 ng (500x excess).

S-specific DNA-protein binding. N-non-specific DNA-protein binding.

4.4 Chapter Summary

The different ICB elements of the topoisomerase IIα promoter appear to have different functions/roles for example, the ICB2 element only is implicated during confluence arrest, but the actual ICB element sequences are all identical (CCAAT) but reversed. This suggests that the flanking sequences around the individual elements of the topoisomerase IIα promoter may determine these individual functions/roles. EMSAs were carried out to analyse proteins that could bind the ICB1 and ICB2 elements in the topoisomerase IIα promoter. In addition the influence of the flanking sequences around the ICB1 and ICB2 elements of the topoisomerase IIα promoter were analysed using oligonucleotides designed that contained the flanking sequence of ICB1 around the ICB2 element and the flanking sequence of ICB2 around the ICB1 element. The protein source for these assays was HeLa cell extracts.

Taken together, the data obtained by competition and antibody supershift analyses confirm that NF-Y is a component of protein complexes interacting with the ICB1 and ICB2 elements. The competition assays performed indicated that the flanking sequence around the ICB2 element is important in determining the binding affinity of the transcription factor NF-Y. The order of affinity for the competition analyses carried out in this study is as follows: **ICB2F>ICB1F≥ICB2wt >ICB1wt>ICB2mt≥ICB1mt.**

The flanking sequences of the ICB2 element rendered the ICB1 inverted CCAAT box a strong binding site for NF-Y.

NF-Y appears to bind to the ICB elements with different affinities and the flanking sequences around the ICB elements of the topoisomerase IIα promoter affect binding. These data indicate that ICB2 may be important in transcriptional regulation and that a regulatory element flanking the ICB2 element may aid in NF-Y binding.

In these *in vitro* assays additional proteins bound to the ICB elements however, binding does not appear to be specific for the CCAAT box. Further investigation is needed to determine whether the flanking sequences which affect binding have a functional role. If a functional role is confirmed, then there may be another protein involved in

stabilising binding, which may in turn have an important role in modulating topoisomerase IIα gene expression.

Chapter Five: Functional Assays of the Human Topoisomerase IIα Promoter

5.1 Introduction

Reporter gene assays using transiently transfected HeLa cells to study the regulatory features of the human topoisomerase IIα promoter. Reporter vectors carrying the minimal topoisomerase IIα promoter (pGL3B-617wt) and various promoter element mutations were used in this study (pGL3B-617ICB1- and pGL3B-617ICB2-), along with co-expression vectors containing NF-YA and a dominant negative NF-YA mutant.

5.2 Transfection Method

HeLa cells were cultured (Section 2.19) and then transfected with plasmids using FuGENE™ 6 Transfection Reagent (Roche) according to the manufacturers instructions. FuGENE™ 6 Transfection Reagent is a multi-component lipid based transfection reagent that complexes with and transports DNA into the cell during transfection. This method involves liposomes, which can fuse with the cell membrane to facilitate the movement of the DNA into cells (FuGENE™ 6 Transfection Reagent Instruction Manual, Version 1, April 1999, Roche Molecular Biochemicals).

Other methods of transfection include calcium phosphate co-precipitation, electroporation and the use of viral vectors. These methods which have produced variable results in a variety of cell types are time consuming, requiring specialised equipment or large amounts of DNA.

5.3 Luciferase Assay

Luciferase was selected as the reporter gene to measure the activity of the human topoisomerase IIα promoter, as the assay is relatively inexpensive, sensitive and fast. In the Luciferase Assay System (Promega) used in this study, the reaction is catalysed by the firefly luciferase protein synthesised in the transfected cultured cells. The firefly luciferase protein, when supplied with the substrate emits light (562 nm) in the presence of ATP:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{Mg^{2+}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

The light emission is proportional to the luciferase activity of the sample, which in turn provides an indirect estimate of the transcription of the luciferase reporter gene.

Promega's Luciferase Assay System allows a greater enzymatic turnover of luciferase, resulting in increased and nearly constant (up to several minutes) light intensity. The system is very sensitive, theoretically allowing detection of less than 10^{-20} moles of luciferase (Luciferase Assay System Technical Manual, Promega, 1993). Luciferase enzyme decays rapidly, with a half-life of approximately 3 hours in transfected mammalian cells. As a result, luciferase activities tend to be more variable for example than chlorophemical transferase assay (CAT) activities. Differential degradation may occur during sample preparation. In order to decrease variability of the luciferase assay, each transfection was performed in triplicate in at least three separate experiments.

The pGL2Control vector (Appendix 3), which has the luciferase gene under the control of the SV40 promoter, was used as a control for the luciferase assay and reagents.

5.4 β-Galactosidase assay

Because variations in cell number, extract preparation and transfection efficiency are difficult to control between experiments an expression vector for the bacterial β-galactosidase gene was included as an internal control. Expression of β-galactosidase is under the control of strong SV40 promoter, making it ideal as an internal control. HeLa cells were transfected with a constant amount of β-galactosidase expression vector

(pSVβ-Gal) and the luciferase reporter. The expression was then quantified by a spectrophotometric assay that measures ONPG hydrolysis. Although mammalian cells may contain endogenous β-galactosidase, this has little effect on the measured β-galactosidase activity as the assay is conducted at pH 8.0, which is inhibitory for mammalian β-galactosidase but has little effect on the activity of the bacterial enzyme expressed from pSVβ-Gal (Bronstein *et al.*, 1994).

5.5 Analysis of Transfections

HeLa cells were co-transfected with appropriate vectors as described in Section 2.20. Transiently transfected HeLa cells were harvested and luciferase and β-galactosidase assays were performed on cell extracts. Luciferase activities were normalised against β-galactosidase activities by dividing luciferase activity by β-galactosidase activity for each cell extract.

To enable the comparison of independent experiments, the results were stated as relative to the expression of pGL3Basic-617wt vector in each experiment. Table 5.1 shows an example of raw data and the calculations involved in the analysis.

Constructs	Luciferase units	B-gal values $A_{405\ nm}$	Ratio (Luc/β-gal)	Average	Relative Luciferase Activity %
0.5µg pSVβ-gal	0.081	0.135	0.600	2.858	38%
+ 1.0µL pGL3B	0.088	0.146	0.600		
	0.059	0.008	7.375		
	0.050	0.610	0.082	0.088	1.3%
	0.066	0.819	0.080		
	0.080	0.794	0.101		
	0.000	0.250	0.000	0.008	0.14%
	0.000	0.235	0.000		
	0.005	0.230	0.023		
0.5µg pSVβ-gal	1.022	0.142	7.197	7.406	100%
+1.0µgpGL3B	1.254	0.244	4.189		
-617wt	1.419	0.131	10.832		
	2.282	0.333	2.751	6.910	100%
	2.961	0.423	2.045		
	2.731	0.397	2.114		
	1.483	0.321	4.621	5.647	100%
	1.881	0.353	5.330		
	2.517	0.360	6.992		
0.5µg pSVβ-gal	5.235	0.624	8.389	7.628	102.9%
+1.0µgpGL3B	5.187	0.669	7.753		
-617ICB1$^-$	5.037	0.747	6.743		
	1.211	0.168	2.625	7.110	102.9%
	2.030	0.290	2.569		
	1.723	0.242	1.914		
	3.242	0.521	6.223	5.816	102%
	2.945	0.530	5.556		
	3.016	0.532	5.670		
0.5µg pSVβ-gal	9.825	0.580	16.940	28.551	385%
+1.0µgpGL3B	11.28	0.237	47.595		
-617ICB2$^-$	12.84	0.608	21.118		
	9.333	0.208	29.490	47.170	682.6%
	9.553	0.214	9.664		
	8.218	0.158	8.016		
	9.130	0.250	36.52	30.14	533.65%
	6.641	0.275	24.15		
	8.271	0.278	29.75		

Table 5.1 – Example of raw data and calculations involved.

All experiments were performed in triplicate within each experiment and an average was calculated. Where the experiment was repeated, an average and standard error of the mean were calculated as follows:

$$SEM = \sqrt{(\sigma^2/n)}$$

where σ – standard deviation and n – number of samples.

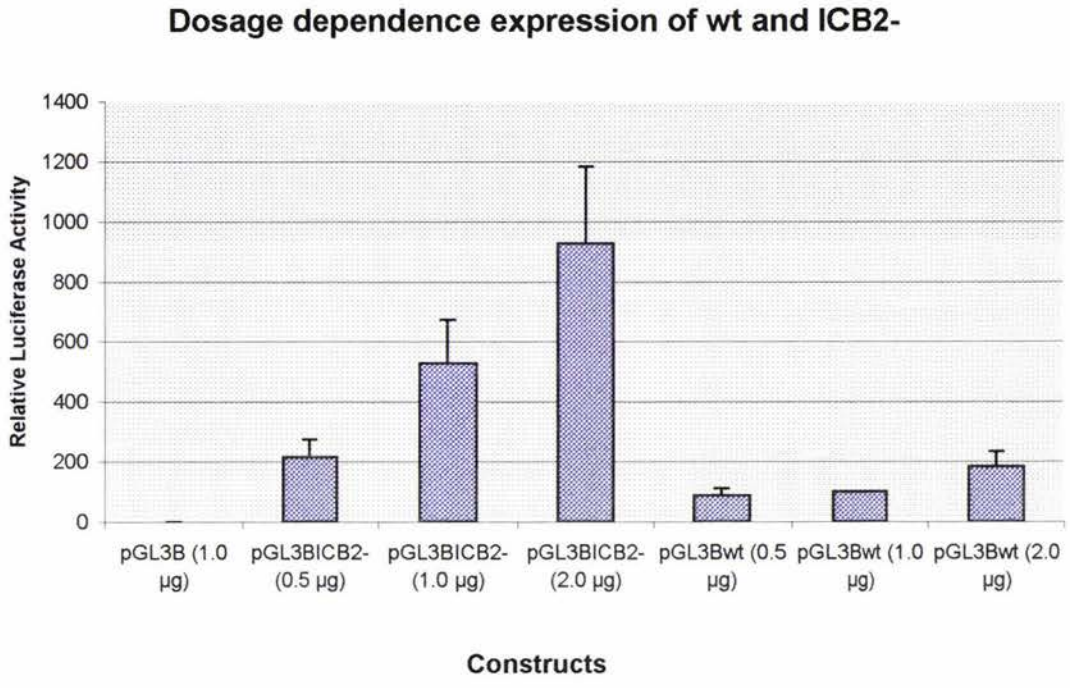
5.6 Transient Transfection Results

5.6.1 Determination of Amount of pSVβ-gal Vector to be used in Experiments

HeLa cells were transfected with three different amounts of pSVβ-gal (0.5 μg, 1.0 μg and 2.0 μg), using FuGENE™ 6 Transfection Reagent (Roche). The results of this experiment determined that 0.5 μg vector DNA was sufficient to yield optimal β-galactosidase activity (data not shown). The results of individual transfection experiments are shown in Appendix 5 – Transfections 1A-1C.

5.6.2 Dosage dependent expression of pGL3B-617w t and ICB2- expression

HeLa cells were transfected with varying amounts of the pGL3B-617wt and ICB2- vector (Figure 5.1). The maximum amount of DNA recommended for transfections is 4 μg, therefore 1.0 μg pGL3B-617wt vector was chosen for use in subsequent experiments to be used to allow for the addition of the pSVβ-gal and expression vectors. A particular focus in this study was ICB2 and its role in topoisomerase IIα promoter expression.



Constructs	Standardised Luciferase Activities
pL3Basic	0.096±0.05%
pL3B-617ICB2- (0.5µg)	215±59.8%
pL3B-617ICB2- (1.0 µg)	529±144.2%
pL3B-617ICB2- (2.0 µg)	927.3±255.4%
pL3B-617wt (0.5 µg)	88±23.6%
pL3B-617wt (1.0 µg)	100%
pL3B-617wt (2.0 µg)	184.3±50.4%

Figure 5.1 – Dosage dependence expression of wt and ICB2-

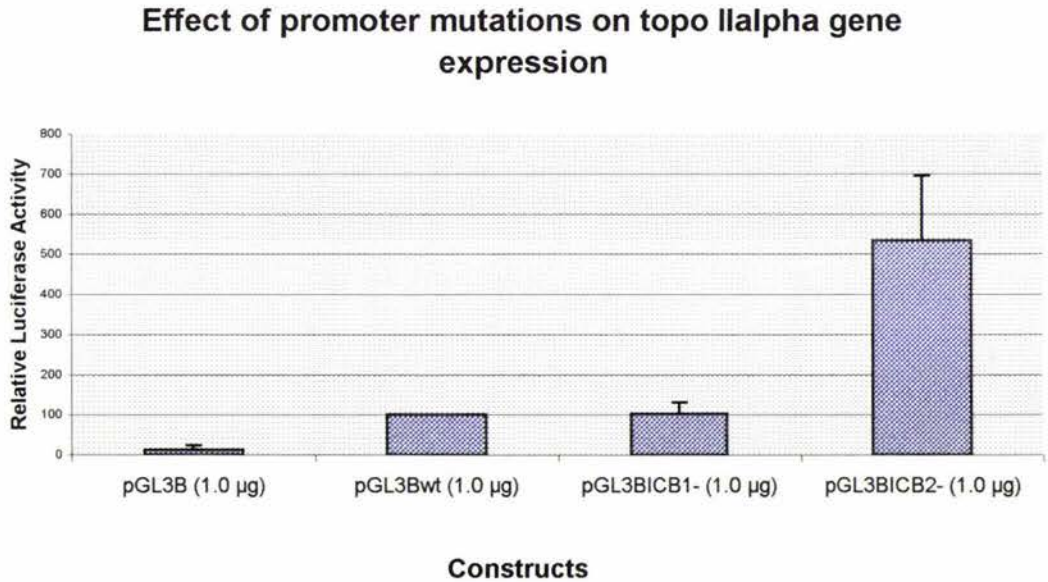
HeLa cells were transiently transfected with 0.5-2.0 µg of pGL3B-617wt and 0.5 µg of pSVβ-gal vectors. The 0.5 µg pSVβ-gal vector was included as an internal control. The standardised luciferase levels represent ratios of luciferase units to β-galactosidase absorbance at 405 nm. The values presented are the average of one experiment (see Appendix 5 – transfection 3A-3C); each transfection was carried out in triplicate. The standardised luciferase activity of 1.0 µg was chosen as 100% and all other activities were adjusted accordingly. This experiment shows an incremental increase of activity for each construct (Figure 5.1). This provides evidence of dosage dependence of

expression with pGL3Bwt and pGL3BICB2- vectors. Therefore it was important to standardise the amount of reporter vector for consistency of data.

5.6.3 Effect of the topoisomerase IIα promoter mutations on reporter vector expression

The ICB1 and ICB2 elements had been shown to bind the NF-Y transcription factor. Mutations of these sites either abolished or reduced binding of the respective transcription factors to these elements (Chapter 4). Reporter gene assays were used to assess the effects of these mutations in either ICB1- or ICB2- on the ability of the topoisomerase IIα promoter to initiate transcription.

Normal and mutant reporter gene constructs were introduced into HeLa cells by transient transfection. Cell extracts were prepared and assayed for luciferase and β-galactosidase activities. Figure 5.2 shows a comparison of results from transient transfections of HeLa cells with topoisomerase IIα promoter constructs.



Constructs	Standardised Luciferase Activities
pGL3Basic	13.15±10.6%
pGL3B-617wt	100%
pGL3B-617ICB1-	102.6±28%
pGL3B-617ICB2-	533.75±161.32%

Figure 5.2 – Effect of the promoter mutations on the topoisomerase IIα expression

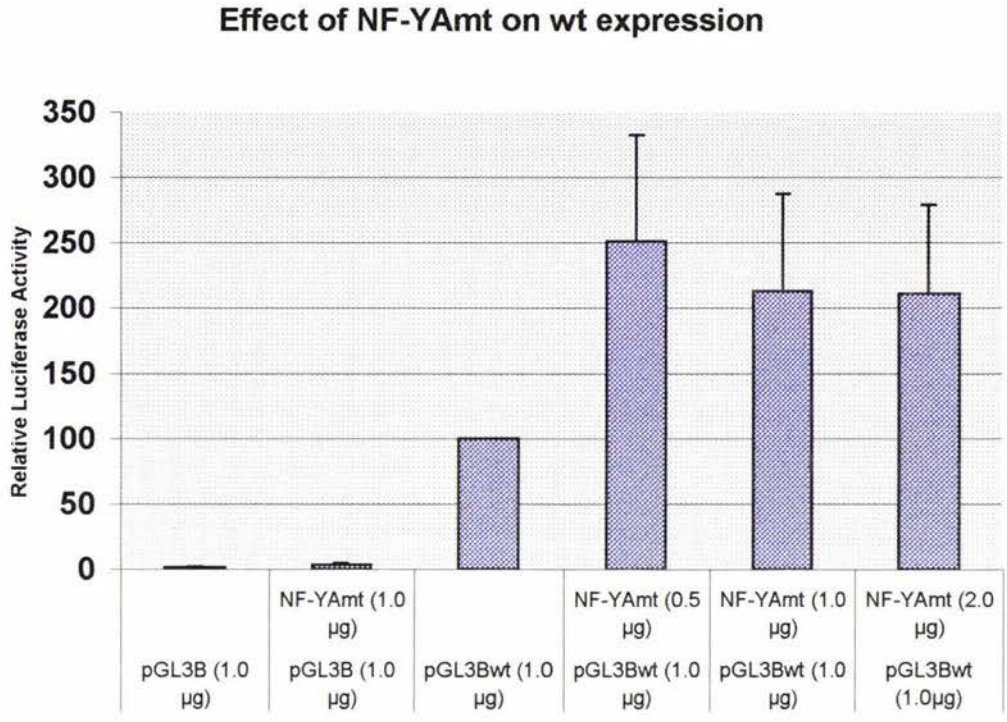
HeLa cells were transiently transfected with 1.0 μg of each pGL3B-617 reporter vector and 0.5 μg of pSVβ-gal vector. The standardised luciferase levels represent the ratios of luciferase units to β-galactosidase absorbance at 405 nm. The values presented for pGL3B-617wt, pGL3B-617ICB1- and pGL3B-617ICB2- are the results of a number of experiments (Appendix 5 – transfections 2A-2C). Transfections in each experiment were carried out in triplicate. The standard deviation of the mean is shown. The standardised luciferase activity of 1.0 μg pGL3B-617wt was chosen as 100% and all other activities were adjusted accordingly.

5.6.4 Effect of the NF-YA dominant negative mutant transcription factor on the topoisomerase IIα promoter constructs-reporter vector expression

NF-Y transcription factor has been shown to bind to the topoisomerase IIα promoter *in vitro*. HeLa cells were co-transfected with the topoisomerase IIα-luciferase reporter and the NF-YA dominant negative mutant ΔYA13 expression vector in order to study the effects of these transcription factors on the topoisomerase IIα promoter activity. The NF-YA dominant negative mutant acts to sequester NF-YB and NF-YC subunits and thus inhibit binding to DNA. The reporter gene expression levels were used to analyse the effect of the transcription factor on the promoter of interest.

Transient co-transfection experiments conducted in this study focused on the effect of the NF-YA dominant negative mutant expression vector on the transcription from the topoisomerase IIα promoter. The topoisomerase IIα-luciferase vectors that were used in the following experiments were pGL3B-617wt and pGL3B-617ICB2-.

HeLa cells were co-transfected with an NF-YA dominant negative expression vector, at varying concentrations, and 1.0 μg pGL3B-617wt vector. As shown in Figure 5.3, NF-YA dominant negative mutant expression vector induced a maximum of 2.5-fold of expression compared with pGL3B-617wt expression.



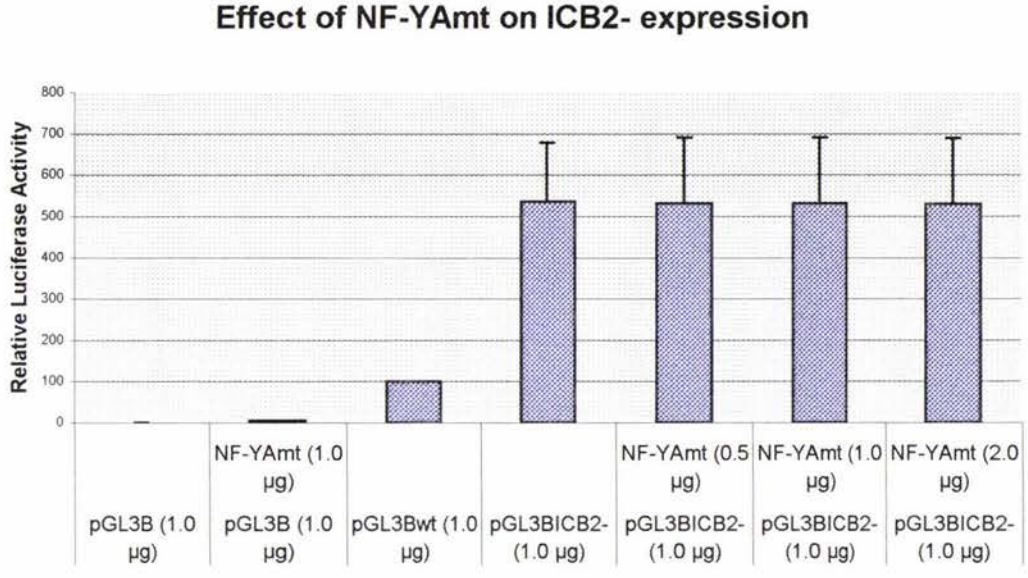
Constructs	Standardised Luciferase Activities
pL3Basic	1.37±0.75%
pL3Basic + 1.0 µg NF-YAmt	3.43±1.37%
pL3B-617wt	100%
pL3B-617wt + 0.5 µg NF-YAmt	251±81.3%
pL3B-617wt + 1.0 µg NF-YAmt	213±74.3%
pL3B-617wt + 2.0 µg NF-YAmt	211±68.1%

Figure 5.3 – Effect of NF-YA dominant negative mutant on pGL3B-617wt expression

HeLa cells were transiently transfected with 0.5-2.0 µg of NF-YA dominant negative expression vector and 1.0 µg pGL3B-617wt vector and 0.5 µg pSVβ-gal vector. The standardised luciferase levels represent the ratios of luciferase units to β-galactosidase absorbance at 405 nm. The results shown in Figure 5.3 clearly show that upon the addition of the NF-YA dominant negative mutant, wildtype topoisomerase IIα expression increases by approximately 1.5-2.0 fold. The values presented are the average of three experiments (Appendix 5 – transfection 4A-4C); each transfection was

conducted in triplicate. The standard deviation of the mean is shown. The standardised luciferase activity of 1.0 µg pGL3B-617wt was chosen as 100% and all other activities were adjusted accordingly.

Because the focus of these studies was on the role of ICB2 in topoisomerase IIα promoter activity, the next experiments addressed the effect of the NF-YA dominant negative mutant expression vector on the pGL3B-617ICB2- vector. Figure 5.5 shows the results obtained.



Constructs	Standardised Luciferase Activities
pGL3Basic	0.05±0.02%
pGL3Basic + 1.0 µg NF-YAmt	4.99±0.27%
pGL3B-617wt	100%
pGL3B-617ICB2-	535±142.7%
pGL3B-617ICB2- + 0.5 µg NF-YAmt	530.7±159.6%
pGL3B-617ICB2- + 1.0 µg NF-YAmt	530±159.7%
pGL3B-617ICB2- + 2.0 µg NF-YAmt	529.3±159.4%

Figure 5.4 – Effect of NF-YA dominant negative vector on pGL3B-617ICB2- expression

HeLa cells were transiently transfected with 0.5-2.0 μg of the NF-YA dominant negative mutant expression vector, 1.0 μg pGL3B-617ICB2- vector and 0.5 μg pSVβ-gal vector. The results presented in Figure 5.4 show that the expression from topoismerase IIα with ICB2- mutation is increased ~5-fold. No further increase is observed in the presence of the NF-YA dominant negative mutant. The standardised luciferase levels represent the ratios of luciferase units to β-galactosidase absorbance at 405 nm. The values presented are the average of three separate experiments (Appendix 5 – transfections 5A-5C); each experiment was conducted in triplicate. The standard deviation of the mean is shown. The standardised luciferase activity of 1.0 μg pGL3B-617wt was chosen as 100% and all other activities were adjusted accordingly.

5.7 Chapter Summary

Mutations in ICB1 and ICB2 boxes revealed that both have a role in basal-level transcription of topoisomerase IIα. In all experiments with pGL3B-617ICB1-, a small but significant increase (~1.2 fold) in expression was observed from this construct relative to the wild-type promoter construct. In all experiments, a significant activation of luciferase expression from pGL3B-617ICB2- was observed (maximum ~ 4 fold). Expression from this mutant was markedly higher than that of the wild-type promoter.

Co-transfection experiments with the NF-YA dominant negative mutant expression vector and the pGL3B-617wt vector resulted in a maximum of 2.0 fold induction of transcription. Co-transfections with the NF-YA dominant negative mutant expression vector and the pGL3B-617ICB2- vector did not result in the induction of activity, suggesting that this element is required for NF-Y-mediated activation. These results suggest that ICB2 is important in transcriptional regulation and that NF-Y appears to act as a negative regulator of transcription.

Chapter Six: Discussion and Further Work

6.1 Production of Antibodies to NF-YC via RT-PCR

In order to produce antibodies against NF-YC, RNA was isolated from HeLa cells and used in RT-PCR to produce the cDNA for NF-YC. NF-YC cDNA would have then been introduced into a bacterial expression vector for the production of NF-YC protein. After purification the protein would have been used for the production of antibodies. Unfortunately, work undertaken during this study to reach this aim was not successful. However, during the attempts to produce NF-YC by RT-PCR, commercial antibodies to NF-YC became available. In addition, cDNA clones for NF-YA, NF-YB and NF-YC in bacterial expression vectors were acquired towards the end of this study. Therefore, if necessary NF-Y protein could be produced in the future. Purification of the protein could be achieved by utilising the His-tag contained in the NF-YC expression vector. NF-YC containing a His-tag could be overexpressed in a suitable *E.coli* strain and purified by affinity chromatography using an NTA-agarose resin. This same approach could be used for the production of NF-YA and NF-YB proteins.

The bacterial expression vectors containing NF-YA and NF-YB do not contain the His-tag. Therefore it would be necessary to subclone NF-YA and NF-YB cDNAs into a His-tag expression vector or a GST-fusion vector and then purify these components individually using nickel or GST affinity resins respectively. Purification of the individual protein subunits would provide the source of antigen in the production of antibodies to the components of NF-Y. These antibodies could then be purified from anti-serum for use in western blotting or electrophoretic mobility shift assays. Obtaining antibodies to the components of NF-Y would be useful not only in the terms of experiments that could be conducted but also because there would be an alternative to commercially produced antibodies.

6.2 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were carried out to analyse proteins that could bind the inverted CCAAT box (ICB) elements 1 and 2 in the topoisomerase IIα

promoter. In addition, the influence of the sequences flanking the ICB1 and ICB2 elements of the topoisomerase IIα promoter were analysed using synthetic oligonucleotides that contained the flanking sequence of ICB1 around the ICB2 element (ICB1FICB2ICB1F) and the flanking sequence of ICB2 around the ICB1 element (ICB2FICB1ICB2F) (see Appendix 2). These oligonucleotides were used in competition assays to determine whether the flanking sequences had an effect on NF-Y binding. Oligonucleotides representing the wildtype and mutant forms of ICB1 and ICB2 were also used in these experiments. The binding assays were carried out to test the hypothesis that the different ICB elements of the topoisomerase IIα promoter have different functions/roles in transcriptional regulation. For example, the ICB2 element only is implicated during confluence arrest, but the actual ICB element sequences are all identical. This suggests that the flanking sequences of the topoisomerase IIα promoter may determine individual functions/roles of each ICB element. The presence of the NF-Y trimer in DNA-protein complexes was confirmed using antibodies against NF-YA, NF-YB and NF-YC in EMSA supershift assays.

Results from this study using competition and antibody supershift analyses have demonstrated and confirmed that NF-Y is a component of protein complexes interacting with the ICB1 and ICB2 elements. The flanking sequences of ICB2 rendered the ICB1 inverted CCAAT box a stronger binding site for NF-Y than the ICB1 element in its normal context. This observation confirms that the sequences around the ICB2 element are important in determining the binding affinity of the transcription factor NF-Y.

These results indicate the ICBs have different efficiencies and specificities for the binding of proteins such as the transcription factor NF-Y, which in turn implies different functions for each ICB element studied within the topoisomerase IIα promoter. These experiments also suggest that flanking sequences affect binding and that a regulatory element may be present within the ICB2 flanking sequence. The flanking sequences may influence binding by a) conformational interactions or b) assisting other proteins to interact.

In order to investigate the possibility of a regulatory factor binding to the flanking sequences of ICB2, ICB2FICB1ICB2F and ICB1FICB2ICB1F sequences were

analysed, a FINDPATTERNS (Wisconsin Package v. 9.1, Genetics Computer Group (GCG), Madison, WI) search was carried out against the transcription factor database using Bionavigator (www.bionavigator.com). The results obtained were generally speaking what would be expected of transcription factors binding to the CCAAT box itself for example; NF-Y and CTF/NF-1. This result suggests a number of things. 1) that the transcription factor that could bind here is novel; 2) a known transcription factor may recognize an alternative sequence or 3) the flanking sequences stabilize binding but there is no direct interaction between DNA and protein in the absence of NF-Y.

The transcription factor NF-Y is a CCAAT box binding protein (CBP) that recognizes and binds to CCAAT sequences with an absolute requirement for all nucleotides in the CCAAT sequence (Mantovani, 1998). 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-YC 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 recognizes the CCAAT box motif. However, is has previously been shown that the sequences around the CCAAT box (the flanking sequences) do affect the binding of NF-Y (Dorn et al, 1987; Mantovani, 1998). In a study conducted by Agnieszka Szremska (Honours Project, 2000), the individual ICB elements bound the NF-Y transcription factor with different affinities, in the order ICB2>ICB3>ICB1, as determined by competition studies and densitometry (Szremska, 2000). This is another example indicating that the ICBs have both quantitatively and qualitatively different capacities to determine protein binding and suggests different functions for each ICB element (Szremska, 2000).

The two ICB elements studied, ICB1 and ICB2 have different sequences flanking the CCAAT box (shown in Table 6.1), supporting this as a reason for the differences in NF-Y binding observed in competition assays.

Element	Sequence
ICB1	CAGGGATTGGCTGGT
ICB2	CTACGGATTGGTTCTT

Table 6.1 – DNA sequences of the ICB elements ICB1 and ICB2 from the human topoisomerase IIα promoter.

This result is in contrast to that published by Herzog and Zwelling (1997) where ICB2 was demonstrated by EMSAs to have the lowest affinity for complex formation. This suggests that there are other factors that affect NF-Y binding, for example, additional transcription factors and assay binding conditions. The results of EMSAs conducted in this study did show the presence of other protein(s) or protein complex members that bound to the probes as several other DNA-protein complexes were observed. These were represented by the N bands illustrated in Figures 4.3-4.11. These DNA-protein complexes did not appear to be CCAAT specific as they showed approximately the same behaviour with each competitor (including the mutant CCAAT elements ICB1 and ICB2).

There are a number of examples of other transcription factors associating with NF-Y. Co-operativity between NF-Y and Sp1 has been demonstrated in the regulation of the major histocompatibility complex class II-associated invariant chain gene (Wright *et al.*, 1995) expression, in the transcriptional regulation of the farnesyl diphosphate synthase gene (Jackson *et al.*, 1995). More recently co-operativity has been demonstrated in the regulation of the fatty acid synthase Insulin-responsive Element 1 (Roder *et al.*, 1997) and the Growth-dependent regulation of the Hamster Thymidine Kinase Promoter (Soresen and Wintersberger, 1999). Such co-operation could affect the binding affinity of NF-Y to the ICB1 element, as a putative Sp1 binding site is adjacent to it. In a study conducted by Agnieszka Szremska (Honours Project, 2000), Sp1 antibodies supershifted the complex formed with the ICB1 element, as well as with the GC1 element. This suggests formation of NF-Y/Sp1/DNA complexes rather than only NF-Y/DNA or Sp1/DNA on the ICB1 and GC1 elements of the topoisomerase IIα promoter respectively (Szremska, 2000). The addition of antibodies to NF-YA or NF-YB did not result in supershifting the complex formed on the GC1 element. However, it is possible that the interactions are dependent on the DNA sequences flanking the ICB1 box, and are not strong enough to be detected with the GC1 probe (Szremska, 2000). The

competition and antibody analyses conducted by Szremska, 2000 suggest that the protein complex formed specifically with the GC1 box contains Sp1 but not Sp3, while both Sp1 and Sp3 were shown to bind specifically to the GC2 box. The GC2 box appears to bind Sp1 with a higher affinity than the GC1 box does. GC2 appears to bind Sp1 and Sp3 with equal affinity (Szremska, 2000). Co-transfection experiments with the Sp1 expression vector resulted in a 4.6-fold induction of transcription. Co-transfection with Sp1 and promoter constructs containing mutations did not result in an increase of activity, suggesting that all three elements are required for Sp1-mediated activation. Taken together, the results of the binding and functional assays suggest an interaction between NF-Y and Sp1 that modulates transcription of topoisomerase IIα. The protein that helps to stabilise NF-Y at ICB2 could be Sp1 which may be brought into close proximity by either GC1 or GC2.

Other proteins have been shown to bind NF-Y, a recent study conducted by Yamada *et al.*, 1999 determined through the use of the yeast two-hybrid system that the serum response factor (SRF) and zinc-fingers and the homeobox (ZHXI) proteins interact with the A subunit of NF-Y. The authors found that these interactions were due to two different domains of the NF-YA. A glutamine-rich region and a serine/threonine-rich region are necessary for the interactions with ZHXI and SRF, respectively (Yamada *et al.*, 1999). The C-terminal region of NF-YA contains domains that interact with NF-YB and NF-YC. Thus, NF-YA has several domains required for protein-protein interactions (Yamada et al., 1999). Yamada et al., 1999 also highlighted recent reports that show that Sp1 and hepatocyte nuclear factor 4 (HNF4) are NF-YA interacting proteins and although the minimal domain region for protein interaction was not determined, it is known that the glutamine-rich region of NF-YA is necessary for interaction with Sp1. How these interactions are linked or how they might influence transcription are still questions that need to be addressed.

Interactions between NF-Y and the high mobility group protein HMG-I(Y) have also been suggested (Currie, 1997). HMG-I(Y) belongs to a group of abundant low mass non-histone chromosomal proteins that have been shown to be important regulators of gene transcription as well as being involved in chromatin structure. Currie (1997) reported that HMG-I(Y) was able to activate NF-Y in transient transfections *in vivo* and provided evidence that the NF-YA subunit alone was able to stably interact with HMG-

I(Y) *in vitro*. This interaction was mapped to the highly conserved DNA binding subunit interacting domain (DBD) of the NF-YA subunit and to a single AT-hook motif in HMG-I(Y). Currie (1997) also found that recombinant HMG-I(Y) stabilised CCAAT binding activity of recombinant NF-Y and native NF-Y in vitro. This protein-protein interaction site may function to modulate NF-Y activity through stabilisation of NF-Y binding to its CCAAT box DNA-binding site.

6.3 Functional Assays of the Human Topoisomerase IIα Promoter

Reporter gene assays using transiently transfected HeLa cells were used to study the regulatory features of the human topoisomerase IIα promoter. Transcriptional activity of the topoisomerase IIα promoter containing a mutated ICB1 (**CC**AAT to **GG**AAT) was not significantly different to the wildtype activity. The activity of the ICB2 (**CC**ATT to **GG**AAT) mutant was 500% (5-fold) of the wildtype activity. This difference is significant in terms of regulation of transcription. Co-transfection experiments with the NF-YA dominant negative mutant expression vector and the pGL3B-617wt vector resulted in a maximum of 2.0-fold induction of transcription. This affect did not appear to be influenced by the amount of NF-YA dominant negative mutant added as the same increase was observed with 0.5 μg, 1.0 μg and 2.0 μg of NF-YA dominant negative co-expression vector. Co-transfections with the NF-YA dominant negative expression vector and pGL3B-617ICB2- vector preserved the 5-fold increase seen with the pGL3B-617ICB2- mutant alone, but did not alter transcription with all amounts of NF-YA dominant negative mutant (0.5 μg, 1.0 μg or 2.0 μg) used. These results suggest that the ICB2- element alone is important in transcriptional regulation and that NF-Y appears to function as a negative regulator.

Three independent groups have investigated the basal activitiy of the topoisomerase IIα promoter in three different cell lines. The results are summarised in Table 6.2.

Topoisomerase IIα construct	Loflin *et al* (1996) % Activity Human erytholeukemic cells	Hochhauser *et al* (1992) % Activity HeLa cells	Furukawa *et al* (1998) % Activity Bladder cells
-2	1.5%	4.9%	-
-20	-	-	1.6%
-32	16%	4.8%	-
-74	-	-	9.3%
-90	11.7%	58%	-
-154	-	-	40.1%
-197	-	-	90.5%
-295	282%	97%	100%
-557	100%	100%	-
-1200	34.3%	72%	-
-2400	75%	35%	-

Table 6.2 – Transient transfection data from three separate studies.

The –2 construct which exhibits negligible activity contains none of the identified putative promoter elements for transcriptional activation. However, the –30 construct contains the Myb and Myc/Max proximal elements of the topoisomerase IIα promoter. The –74 and –90 constructs contain the GC1 and ICB1 proximal promoter elements as well as the Myc/Max and Myb sites of the topoisomerase IIα promoter. The maximum activity for any of these constructs was 58%. The -154 construct contains elements from ICB2 towards the transcription start site and –197 elements from ICB3 towards the transcription start site. The low level of activity exhibited by the –154 construct suggests that upstream elements other than ICB2 are important for transcriptional regulation. The –295 construct contains ICB4, the ATF, ICB3, ICB2, ICB1,GC1, Myc/Max and Myb elements of the topoisomerase IIα promoter. The –557 construct contains elements from ICB5 towards the transcription start site and –1200 and –2400 contain all of the putative promoter elements identified in the topoisomerase IIα promoter (GC2, ICB5, ICB4, ATF, ICB3, ICB2, ICB1, GC1, Myc/Max and Myb).

Transient co-tansfections conducted by Sam McLenachan (Honours Project, 1998), showed results similar to those shown in this study. McLenachan observed a small increase in transcription with pGL3B-617ICB1- vector (1.5-fold) and with pGL3B-617ICB2- expression was markedly (8-10-fold) higher than that of the wildtype topoisomerase IIα promoter.

The involvement of ICB2 in topoisomerase IIα down-regulation is now strongly supported. Previous experiments, described in Chapter One, identified ICB2 as being involved in confluence arrest induced down-regulation of the topoisomerase IIα promoter (Isaacs *et al.*, 1996). The research described here further supports this role for ICB2 in that mutation of this element resulted in a significant activation of the promoter relative to the wild-type promoter (2-10-fold), suggesting the abrogation of repressor binding.

Transient co-transfections conducted by Agnieszka Szremska (Honours Project, 2000) are in contrast to those described above. Szremska, (2000) described a 30% decrease in expression from the ICB1- mutant compared to the wildtype topoisomerase IIα promoter. The activity of the ICB2- mutant was reported as 76% of the wildtype construct, suggesting that this difference is not significant in terms of major regulation of transcription (Szremska, 2000). Szremska, (2000) compares the results concerning ICB2- above to a previous study (Isaacs *et al.*, 1996), which implicates ICB2 in the down-regulation of topoisomerase IIα expression at confluence. Szremska, (2000) suggests that the differences in data are not inconsistent as Isaacs et al., (1996) studied confluence arrest down-regulation, whereas the study conducted by Szremska, (2000) investigated the basal activity of the topoisomerase IIα gene in freely proliferating cells. However, although valid as that may be, the experimental procedure used by Szremska, 2000 (Lipofectamine 2000 by Life Technologies) specified that adherent cells be ~80-90% confluent prior to transfection. Topoisomerase IIα is regulated by the cell cycle, and if the cells are 80-90% confluent it is likely that a portion of the population analysed by Szremska would be in arrest and would not be freely proliferating cells. The transfection method used in this study was FuGENE 6 (Roche) which requires cells to be ~50-80% confluent prior to transfection, this is likely to give a better representation of the regulation of topoisomerase IIα in freely proliferating cells.

Before any further work can be attempted, it would be valuable to repeat the functional assays carried out in this study. This is because there are a variety of factors which need to be considered when using transient transfections of mammalian cell lines to monitor reporter gene activity. There is an assumption that equivalent numbers of the different plasmids are taken up by the cell during transfection, and that one plasmid (e.g. the reporter plasmid) is not preferentially absorbed in favour of another (e.g. the β-galactosidase expression vector). Also, the effects of plasmid size, structure or sequence on the transfecton process is not known. It is also assumed that transcription and translation from the genes of the different plasmids occurs independently, and that the genes of one plasmid are not preferentially expressed over the genes of another.

The potential problems of using adherent cell culture include non-homogenous cell growth causing the production of gradients of supply of certain nutrients. Also, optimal pH and oxygen levels may form gradients of cell culture growth. It is because of variables such as these that it is necessary to carry out many replicates of transient transfection experiments in order to increase the significance of the result obtained. There are also a number of potential concerns with using reporter genes to measure transcriptional activity. Firstly, the reporter system may measure enzyme activity which is only an indirect measure of transcriptional activation. There is an assumption that enzyme activity correlates with the enzyme protein levels, and that these results correlate to activity of the promoter construct. As it is possible for regulation to occur at both the RNA processing and stability stages as well as the translation, processing or activity steps, this assumption, in some cases, may not be correct. A second concern is that there may be potential elements within a gene that may regulate the activity of its own promoter. In the case of a reporter construct, a promoter is separated from these potential regulatory elements, and this may affect the normal activity of the promoter (Ausubel *et al.*, 1991). The effect of the removal of promoter elements from their natural chromatin environment into the unnatural environment of vector DNA is not known (Alam and Cook, 1990). Another concern is that the reporter gene itself and the cell line used in the transient transfections may itself contain elements that affect the activity of promoters linked to it. Also, other factors which should be considered include spurious transcription of the reporter gene induced by vector sequences and specific cellular activators. The potential result of this is that the reporter gene activity levels may not truly reflect the actual activity of the promoter constructs. These

potential effects indicate the importance of controls such as: the reporter gene without the promoter, which could indicate vector-induced activation and the use of a reference constant to all transient transfection experiments which can reduce any variation seen between individual experiments.

All of these factors should be considered when analyzing the results of transfection and reporter gene assays. Nethertheless, transient transfections of reporter gene constructs remain a very useful and simple assay system with which to analyse promoter activity, and provide a starting point for further studies.

In order to discover more about the interactions between NF-Y and the promoter elements of the topoisomerase IIα gene the set of co-transfection experiments with NF-YA dominant negative vector need to be repeated with pGL3B-617ICB1- and the double mutant vector pGL3B-617ICB1-/ICB2-. Also as the results from the EMSAs described in Chapter Four suggest that the flanking sequences around ICB2 are important for NF-Y binding and that a regulatory element may be present in the adjacent sequences to the ICB2 element of the topoisomerase IIα promoter, it is necessary to determine if this observation has any functional significance. This could be achieved by subcloning the oligonucleotides designed for use in the EMSA experiments (ICB2FICB1ICB2F and ICB1FICB2ICB1F) into the pGL3B-617 wildtype reporter vector and use these additional reporter vectors in functional assays (transient co-transfections) alone and with NF-YA dominant negative expression vector. It would also be important to include experiments with the mutated elements (ICB1, ICB2 and the double mutant) with the different flanking sequences to further investigate the role of individual ICB elements within the topoisomerase IIα promoter.

6.4 Mechanism of Transcriptional Regulation of Human Topoisomerase IIα

The results discussed above suggest that the ICB2 element directs a repressional effect on the topoisomerase IIα promoter. This result is supported by previous data, which show that ICB2 directs topoisomerase IIα promoter down-regulation at confluence-induced arrest (Isaacs *et al.*, 1996). However, how ICB2 directs repression is still unclear. From the results discussed above and the reported stimulatory role of NF-Y (Jackson *et al.*, 1995; Maity *et al.*, 1998), it is unlikely that NF-Y itself mediates

promoter repression; it is more likely that NF-Y acts with a co-repressor through the ICB2 element.

The negative effect of NF-Y may be brought about by binding the co-repressor. In the presence of a mutation at ICB2, (or if NF-Y is sequestered by the dominant negative) the co-repressor cannot bind and transcription is activated constitutively.

The role of NF-Y in the regulation of topoisomerase IIα gene expression may simply be in the maintenance of correct spatial conformation of the promoter. Firstly, constructs containing mutations in either ICB1 or ICB2 were not transactivated by the Sp1 transcription factor. Secondly, the Sp1 transcription factor appeared to interact with the protein complex formed with the ICB1 element. In addition, the review of current knowledge about NF-Y and CCAAT boxes also support the architectural role of NF-Y in promoter activation.

Firstly, it is known that NF-Y introduces distortions in the double helix (Ronchi *et al.*, 1995) and creates an environment for recruitment of other transcription factors. Secondly, chromatin structure is modified by opposing activities: co-activators possessing histone acetyl-transferase (HAT) activity and co-repressors, recruiting HDAC (histone deacetylases). These HAT and HDAC activities alter histone acetylation status, thus resulting in the alteration of chromatin structure. Recently the transcription factor NF-Y was shown to possess HAT activity *in vivo* through association with HATs, GCN5 and PCAF (p300/CBP-associated factor). The over-expression of PCAF stimulated the human multidrug resistance 1 gene promoter through a direct interaction between NF-Y and PCAF (Jin and Scotto, 1998). This interaction 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. An involvement of acetylation in the regulation of the mouse topoisomerase IIα gene has been proposed (Adachi et al., 2000). These authors suggested a mechanism by which histone deacetylase plays a crucial role in the G_0/G_1-specific repression of the topoisomerase IIα promoter. Transcriptional activation in G_2/M is mediated by recruitment of HATs by NF-Y to the promoter region which stimulated histone acetylation (Adachi *et al.*, 2000). It remains to be determined whether a similar mechanism might be involved in the regulation of human topoisomerase IIα. However,

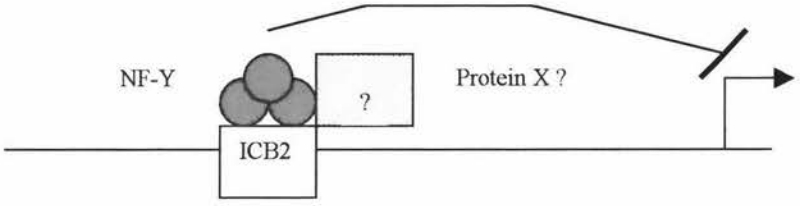
it is plausible that NF-Y, by recruiting HATs to the ICB1 element, increases binding affinity of transcription factors to elements of the topoisomerase IIα promoter. Thirdly, NF-Y can prevent promoters from being inactivated by nucleosome assembly, and can bind to sites that need to be activated but are embedded in nucleosomes (Caretti *et al.*, 1999; Motta *et al.*, 1999). Fourthly, CCAAT elements are not able to activate transcription alone, even if multimerised (Mantovani, 1998). Fifthly, NF-Y facilitated *in vivo* recruitment of upstream DNA binding transcription factors (Wright *et al.*, 1994). Sixthly, there is a strong position preference of the CCAAT element at the –60 position in TATA-less promoters (Mantovani, 1998). The ICB1 element is at position –68 in the human topoisomerase IIα promoter and the position of this element is conserved in the human, rat and hamster topoisomerase IIα promoters. Seventhly, Isaacs *et al.*, 1996 showed that the position of the ICB2 elements was critical in promoter activity, as changing the position of ICB2 in the promoter relative to downstream elements abrogated the down-regulation seen in confluence-arrested cells. This is consistent with NF-Y having a role in altering conformation of the promoter.

Both ICB1 and ICB2 (Furukawa *et al.*, 1998 and Isaacs *et al.*, 1996) elements have been proposed to be involved in the down-regulation of topoisomerase IIα gene expression. One model that explains these observations involved the binding of another regulatory protein to these elements. CCAAT displacement (CDP) protein has been isolated and characterized as a CCAAT binding protein, which appears to negatively regulate the binding of NF-Y to the CCAAT box (Mantovani, 1998). It would be of interest to determine whether CDP can bind to the ICB1 or ICB2 elements of the topoisomerase IIα promoter and negatively regulate topoisomerase IIα transcription.

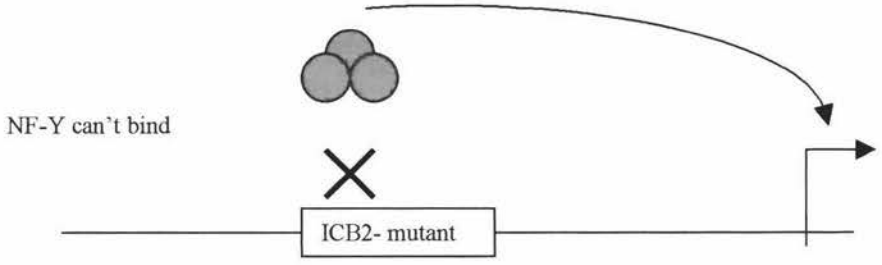
It is possible that an interaction with ICB1 is required for activation via an NF-Y/Sp1 complex and ICB2 for repression with NF-Y/X. Cross talk between ICB1 and ICB2 and transcriptional activation is also likely to depend on the position in the cell cycle.

As mentioned above, Sp1 and NF-Y have been shown to interact. Sp1 may modulate topoisomerase IIα transcription in various ways. The glutamine-rich domains of Sp1 have been shown to interact with a TBP-associated factor, $TAF_{II}110$, via hydrophobic residues present in these domains, and mediate transcriptional activation. Interactions have also been shown to occur between the DNA-binding domain of Sp1 and $TAF_{II}55$

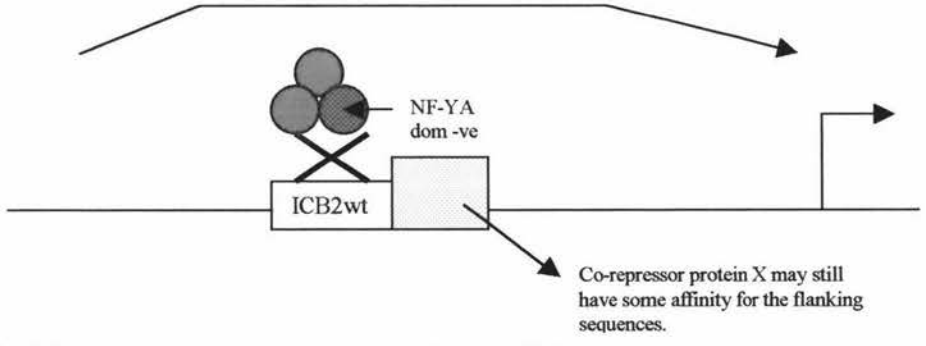
(Parnaik, 1999). These interactions can lead to direct interaction between Sp1 and the initiation complex. This type of interaction may be important in TATA-less promoters, such as topoisomerase IIα. The major activation domains of both Sp1 (Lania *et al.*, 1997) and NF-Y (Maity and de Crombrugghe, 1998) are glutamine rich and both are therefore likely to contact a similar set of basal transcription factors, TAFs or other components of the preinitiation complex.



Normal growth and confluence-arrest (ICB2 and NF-Y (possibly protein X) acting to repress transcription).



Mutation of ICB2, NF-Y cannot bind therefore transcription can be activated (~5-fold increase in transcriptional activation observed in this study).



Mutation in NF-Y such as the presence of the NF-YA dominant negative expression vector, transcription is activated (~2.5-fold increase in transcriptional activation observed in this study).

Figure 6.1 Model for the regulation of topoisomerase IIα

6.5 Analysis of Interactions between NF-Y and other proteins

There are a number of techniques that could be employed to investigate the interactions of other proteins with NF-Y. One such technique is co-immunoprecipitation (see Figure 6.2 for an example of a co-immunoprecipitation strategy). Immunoprecipitation involves the precipitation of a molecule, usually a protein, from a crude mixture of other proteins and biological molecules, often cell or tissue homogenate, using an antibody to the protein of interest and a means of precipitating the complex to allow its separation from the initial mixture (Clegg, 1998). Co-immunoprecipitation is a powerful technique that can detect interactions between two factors. It relies on the specificity of antibodies to recognize one of the components and protein A-Sepharose or G-Sepharose to precipitate the cross-linked immunocomplexes. The use of either protein A or protein G depends on the species in which the immunoprecipitating antibody was raised. Protein G binds strongly to IgG of most species, but Protein A has a more limited selectivity. If the interaction between two factors is sufficiently strong, immunoprecipitation of one will bring down the other (Latchman, 1993). Two basic strategies are available for such co-immunoprecipitation experiments. The first involves testing the hypothesis that two or more known proteins interact. The second strategy involves a search for unknown cellular proteins capable of interacting with a known protein. In this case, cellular proteins are first radioactively labeled with either [^{35}S]methionine (to screen for interactions with cellular proteins) or [^{32}P]phosphate (to screen for interactions with phosphoproteins). Proteins that interact with the known protein are identified initially by gel electrophoresis and autoradiography (Clegg, 1998).

A drawback with co-immunoprecipitation is that non-specific entrapment of proteins can occur, especially when using polyclonal antibodies, which may recognise multiple epitopes leading to highly cross-linked aggregates. Controls are required to distinguish between specific co-immunoprecipitation and non-specific entrapment. The immunoprecipitated pellets can be washed several times to remove non-specific proteins and the stringency of these washes can be increased by adding small amounts of non-ionic detergent (for example Triton X-100). In order to identify the co-immunoprecipitating factor, an antibody raised against it is required for western blotting analysis, unless a clear identification can be made on the basis of molecular size, or the

cloned protein can be labelled with [^{35}S]methionine during *in vitro* translation of its mRNA (Latchman, 1993) and N-terminal sequencing carried out.

Co-immunoprecipitation is not without limitations. It may not be able to detect very weak interactions, or, if the proteins are of low abundance, the immunological probes may not be of sufficient sensitivity. A major limitation is the inability of the method to unequivocally prove that two or more co-immunoprecipitated proteins actually interact in the intact cell and despite the possible controls, the possibility of postlysis artifactual interactions occurring can not be ruled out.

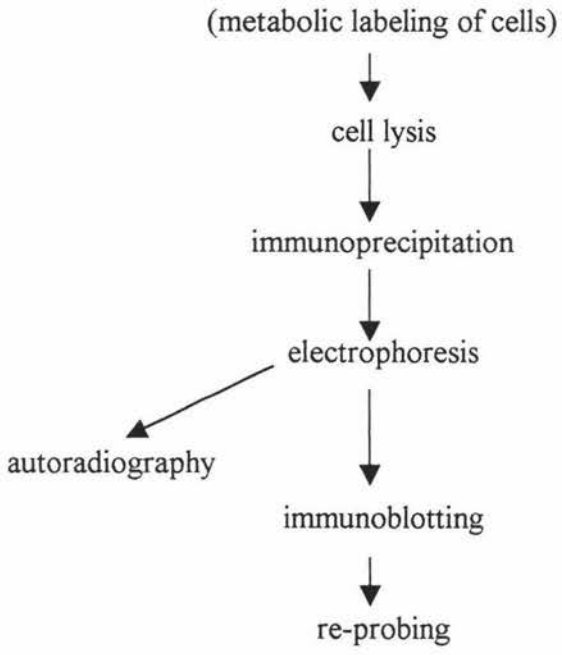


Figure 6.2 Strategy for co-immunoprecipitation of proteins from cultured cells.

To confirm physical interactions between NF-Y and Sp1 complexes detected by Szremska, (2000), co-immunoprecipitation analyses could be conducted using HeLa or COS cell extracts. The complexes could be incubated with Sp1 or NF-YA antibodies for example and the antibody/protein complex captured and purified using protein G-Sepharose beads. Following separation on SDS-PAGE, the proteins could be transferred to a membrane and probed by Western Blotting with antibodies against Sp1 and NF-YA. If Sp1 can interact with the NF-YA subunit present in the cell extracts, the experiment would show that the NF-YA immunoprecipitate is recognized by Sp1 and that the Sp1 immunoprecipitate is recognized by NF-YA antibodies. This would also confirm the presence of Sp1 in the NF-Y precipitate and vice versa.

A limitation in above approach is that it is carried out in the absence of the natural context of the promoter, where neighbouring proteins and the chromatin environment may play significant roles. To study Sp1 and NF-Y binding in the context of chromatin environment, ligation-mediated PCR *in vivo* foot-printing (Rigg *et al.*, 1998) could be used. *In vivo* footprinting allows detection of the interdependence of transcription factors bound to a promoter. Firstly, cell lines that maintain stable integrated human topoisomerase IIα promoter-reporter constructs with mutations in relative elements such as ICB1, GC1 and ICB2 would need to be prepared. If Sp1 and NF-Y bind co-operatively to the topoisomerase IIα promoter, the results would show that mutation in either binding motif eliminates binding from the other element.

Another technique that could be used to determine the nature of proteins interacting with NF-Y is that of the glutathione-S-transferase (GST) fusion expression and purification system (see Figure 6.3 for diagram off a GST-X fusion protein experiment). This system requires the in-frame fusion of cDNA for a protein of interest to the cDNA for the GST protein. This allows isopropyl-β-thiogalactopyranoside (IPTG)-inducible high-level expression of a stable protein, from the lac promoter, in *E.coli*. This GST-X fusion protein can be purified to near homogeneity using a one-step glutathione agarose batch or column purification procedure. It can be released from the glutathione agarose by addition of glutathione (Latchman, 1993).

Prepare the GST-X fusion extracts from *E.coli*
(subclone the cDNA of interest, in-frame into a GST expression vector and grow to required specifications)

↓

Add *E.coli* lysate containing the GST-factor fusion protein to the glutathione agarose gel, incubate and centrifuge

↓

Set up GST-X fusion-glutathione agarose ppt, partly purified non-DNA binding transcription factor and buffer reaction mix

↓

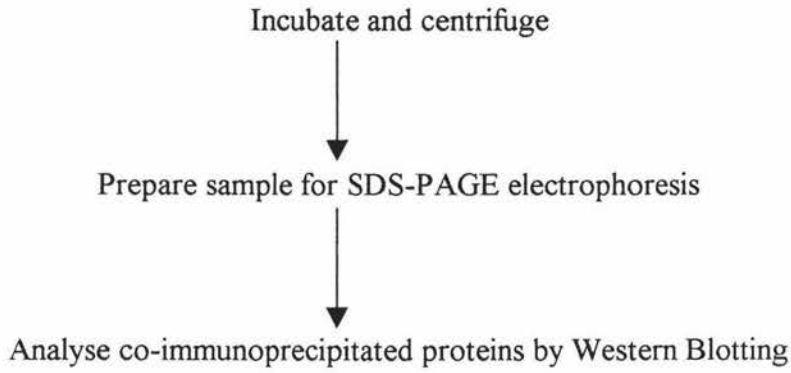


Figure 6.3 Diagram of a GST-X fusion protein experiment.

The various components of NF-Y could be individually subcloned into a GST expression vector (such as pGEX) and a cell extract applied (containing protein(s)). Results obtained could be run on an SDS-PAGE gel and if sufficient in clarity and intensity, a band of interest could be excised from the gel and sent for protein microsequencing to determine the nature of the product bound to the particular component of NF-Y.

Another technique employed to study protein-protein interactions is a molecular genetic approach called the yeast two-hybrid system. The yeast two-hybrid system has enabled investigators to isolate and characterize numerous protein-protein interactions, and to isolate novel interacting partners for many biologically important enzyme complexes, signaling proteins and transcription factors (Clegg, 1998). The development of the yeast two-hybrid system came about by research performed in transcriptional activators. Transcriptional regulatory proteins such as the yeast GAL4 protein consist of 2 independent domains: a DNA-binding domain and an activation domain. The DNA-binding domain binds to a specific nucleotide sequence in the promoter of a gene but alone is insufficient for activation of transcription. The activation domain cannot bind DNA but can activate transcription when brought in close proximity with the DNA-binding domain. The pairing of DNA-binding and activation domains need not be specific, in that a particular DNA-binding domain can be paired with a number of different activation domains and vice versa. The DNA-binding and activation domains of the GAL4 protein were found to be able to be separated yet still retain their biological function (Clegg, 1998).

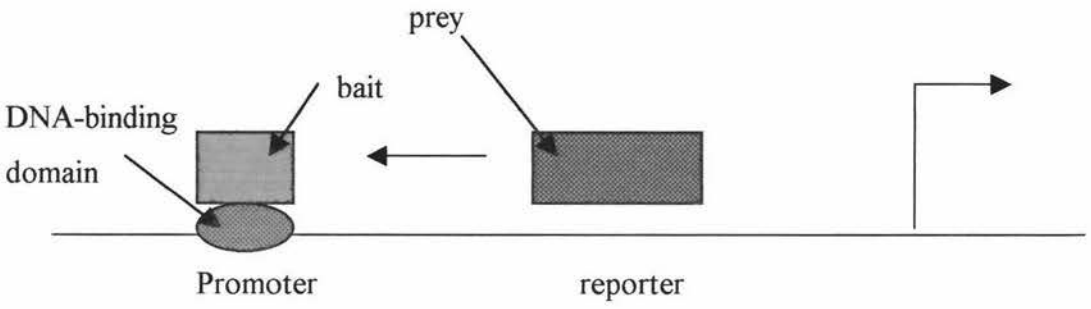


Figure 6.4 – Schematic of a yeast two-hybrid experiment.

The yeast two-hybrid system (depicted in Figure 6.4) requires the development of a DNA-binding domain hybrid (the bait) which contains the cDNA that encodes the protein of interest into a DNA-binding domain plasmid such as pGT13T9 (GAL4) (Clegg, 1998) and a prey library is transformed with the bait vector in appropriate two-hybrid yeast host such as YB2 and a prey library (a prey library contains fusions between activation domains and members of cDNA libraries). If the expressedbait and prey proteins interact transcriptional activation occurs; this is represented by blue colonies forming on selectable plates. Assays of positives is conducted using the β-galactosidase assay.

The individual protein binding domains of the NF-Y subunits; NF-YA, NF-YB and NF-YC could be subcloned into a yeast-two hybrid construct and the protein-protein interaction analysed by the results obtained from the bait and prey reactions.

In conclusion, the transcription factor NF-Y (through its interaction with the ICB2 element of the topoisomerase IIα promoter) has the potential to regulate the *in vivo* transcription of the topoisomerase IIα gene. Further investigation of the interaction(s) between NF-Y and any associated factors in the regulation of topoisomerase IIα gene expression will aid in our understanding. This has greater importance in the context of chemotherapy. One mechanism of drug resistance is suggested to occur due to a down-regulation of topoisomerase IIα transcription. The data presented here suggests that

under wildtype conditions (no mutations within the promoter), NF-Y binds to the ICB2 element and exerts a repressional effect (perhaps a co-repression through an interaction with another factor) on the topoisomerase IIα promoter. If drug resistance is due to down-regulation of topoisomerase IIα then it is conceivable that this mechanism is being exploited. The data presented here suggests that a mutation in ICB2 would render NF-Y unable to bind to the ICB2 element and thus unable to exert its repressional effect and significant activation of transcription is observed. If this mechanism could be exploited by designers of chemotherapeutic agents, then a mutation in ICB2 introduced into a cancerous cell could increase transcription of topoisomerase IIα and due to the nature of topoisomerase IIα poisons, increase sensitivity to such chemotherapeutic agents. However, more investigation is needed into the transcriptional regulation of topoisomerase IIα and its associated transcription factors.

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APPENDIX 1 – THE HUMAN TOPOISOMERASE IIα PROMOTER SEQUENCE

-721 TGGGTAGAGA CGGGGTCTCG CTATGTTGCC CAGGCTGGTC TCCAACTCCT

-671 AGGCTCCAGC GATCCTCCCG CCTCGGCCTC CCAATGTGCT GCGAATACAG

-621 ACTCCAGCCA CCGCACACAG CCTACTTTTA TTTCTTTGAA AAATGAATTC

GC2

-571 GAGGGTAAAG GGGGCGGGGT TGAGGCAGAT GCCAGAATCT GTTCGCTTCA

-521 ACCAAGCAGC CAGGCTGCCT GTCCAGAAAG CCGGCACTCA GTTTCCTCAG

-471 GAAAACGAAG CTAAGGCTCC CATTCCCCTC GCTAACAACG TCAGAACAGA

ICB5

-421 GGACAGTTTT TAGATTTCAG GGATCTTAAA TAGATTGGCA GTTCCTGGAG

-371 AATAAACATC CTTTGCTTTT CTCCTGCACA CTTTTGCCTC AGGCCACCCC

-321 TTCCCGCTTC CAAAGCCCAT CTCTTCCAAG CTTTCCGCAC GAGAAAACAA

ICB4 **ATF**

-271 GTGAGCCCTT CTCATTGGCC AGATTCCCTG TCAATCTCTC CGCTATGACG

ICB3

-221 CCGAGTGGTG CCTTTTGAAG CCTCTCTAGT CCCGCCTCCC TAACCTGATT

-171 GGTTTATTCA AACAAACCCC GGCCAACTCA GCCGTTCATA GGTGGATATA

ICB2

-121 AAAGGCAAGC TACGATTGGT TCTTCTGGAC GGAGACGGTG AGAGCGAGTC

ICB1 **GC1** **Myc/max**

-71 AGGGATTGGC TGGTCTGCTT CGGGCGGGCT AAAGGAAGGT TCAAGTGGAG

Myb

-21 CTCTCCTAAC CGACGCGCGT CTGTGGAGAA GCGGCTTGGT CGGGGGTGGT

+31 CTCGTGGGGT CCTGCCTGTT TAGTCGCTTT CAGGGTTCTT GAGCCCCTTC

+90

+81 ACGACCGTCA CCATGG

APPENDIX 2 - OLIGONUCLEOTIDE SEQUENCES

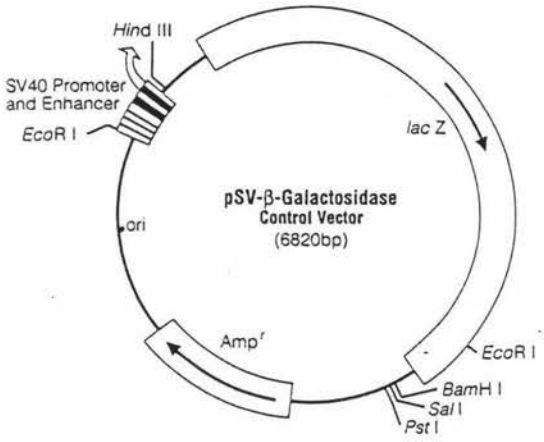
Sequences of oligonucleotides used in this study. The underlined and bold sequences represent the binding elements and mutations respectively.

Oligo Name	5' – sequence - 3'	Purpose
ICB1 F	GAG TCA GGG ATT GGC TGG TCT GC	EMSA
ICB1 R	GCA GAC CAG CCA ATC CCT GAC	EMSA
ICB1 mt F	GAG TCA GGG ATT **CC**C TGG TCT GC	EMSA
ICB1 mt R	GCA GAC CAG **GGA** ATC CCT GAC TC	EMSA
ICB2 S	GGC AAG CTA CGA TTG GTT CTT CTG GAC G	EMSA
ICB2 A	CGT CCA GAA GAA CCA ATC GTA GCT TGC C	EMSA
ICB2 mt S	GGC AAG CTA CGA TT**C** **C**TT CTT CTG GAC G	EMSA
ICB2 mt A	CGT CCA GAA GAA **GGA** ATC GTA GCT TGC C	EMSA
ICB1FICB2ICB1F F	GAG AGC GAG TCT ACG ATT GGT TCT TCT GCT TCG GG	EMSA
ICB1FICB2ICB1F R	CCC GAA GCA GAA GAA CCA ATG CTA GAC TCG CTC TC	EMSA
ICB2FICB1ICB2F F	AAA AGG CAA GCA GGG ATT GGC TGG TCT GGA CGG AG	EMSA
ICB2FICB1ICB2F R	CTC CGT CCA GAC CAG CCA ATC CCT GCT TGC CTT TT	EMSA

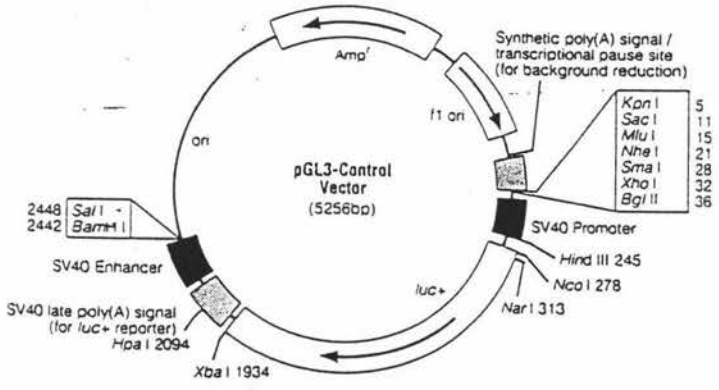
Oligo Name	5' – sequence - 3'	Purpose
Probe NF-YA5'	GCG GGA CAG CAA GTC GCA CAG ACT GCT GAA GGG	PCR
Probe NF-YA3'	GCG CAC TCG GAT GAT CTG TGT CAT TGC TTC ATC GGC	PCR
NF-YA5'	GCG GGA TCC GAG CAG TAT ACA GCA AAC AGC	PCR
NF-YA3'	GCG GGA TTC GGA CAC TCG GAT GAT CTG	PCR
NF-YB5'	GCG GGA TCC ACA ATG GAC GGC GAC	PCR
NF-YB3'	GCG CGA ATT CAT GAA AAC TGA ATT TGC TG	PCR
NF-YC5'	GCG GGA TCC ACA GAA GGA GGG	PCR
NF-YC3'	GCG CGA ATT CAG TCT CCA GTC ACC TGG GG	PCR
1916	GTG CTG CCA CAG TAA ATG TA	PCR
1917	TGA TGA GGC CTG GTG ATT CT	PCR

APPENDIX 3 – VECTOR MAPS

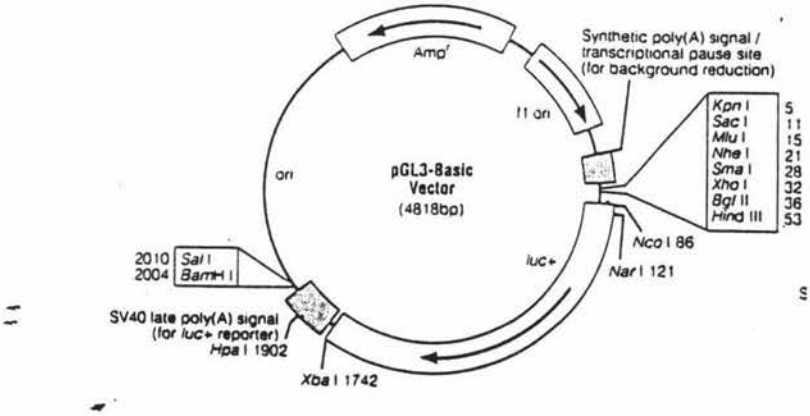
pSV-βgal (Promega)



pGL2Control (Promega)



pGL3Basic (Promega)



APPENDIX 4 – NF-YA SEQUENCE WITH PROBE BESTFIT (GCG)

```
BESTFIT of: YA3  check: 4345  from: 1  to: 427

to: x59711.gb_pr1  check: 3787  from: 1  to: 1383

LOCUS       HSNFYA       1383 bp    RNA             PRI       18-JAN-
1995
DEFINITION  H.sapiens mRNA for CAAT-box DNA binding protein subunit A.
ACCESSION   X59711
NID         g35047
KEYWORDS    CAAT-box DNA binding protein.
SOURCE      human. . . .

 Symbol comparison table:
/usr/local/gcg/gcgcore/data/rundata/swgapdna.cmp
 CompCheck: 2335

        Gap Weight:     50      Average Match: 10.000
     Length Weight:      3   Average Mismatch: -9.000

           Quality:   3447             Length:    415
             Ratio:  8.306              Gaps:        0
 Percent Similarity: 91.084   Percent Identity: 89.639

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

 YA3 x x59711.gb_pr1       March 1, 19101 11:07  ..

     11 CTACCAACATGTTAATGCAGATGGCACAATTCTCCAGCAAGTTACAGTCC 60
        ||| ||||  |||||||||||||||||| |||||||||||||||||||||
    681 CTATCAACCAGTTAATGCAGATGGCACCATTCTCCAGCAAGTTACAGTCC 730

     61 CTGTTTCAGGCATGATCACCATCCCAGCAGCCAGTTTGGCAGGGGCACAG 110
        |||||||||||||||||| |||||||||||||||||||||| ||||||
    731 CTGTTTCAGGCATGATCACTATCCCAGCAGCCAGTTTGGCAGGAGCACAG 780

    111 ATCGGGCAGACAGGAGCCAATACCAACACAACCAGCAGTGGACAAGGGAC 160
        || |  || ||||||||||||||||||||||||||||||||| ||||||||
    781 ATTGTTCAAACAGGAGCCAATACCAACACAACCAGCAGTGGGCAAGGGAC 830

    161 GGTCACTGTGACACTGCCTGTGGCAGGGAATGTGGTCAACTCAGGAGGAA 210
        |||||||||||||| || |||||||| |||||||||| |||||||| |
    831 TGTCACTGTGACACTACCAGTGGCAGGCAATGTGGTCAATTCAGGAGGGA 880

    211 TGGTCATGATGGTACCAGGAGCTGGCTCTGTGCCTGCTATCCAAAGAATC 260
        |||||||||||| || || ||||||||||||||||||||||||||||||
    881 TGGTCATGATGGTTCCTGGGGCTGGCTCTGTGCCTGCTATCCAAAGAATC 930

    261 CCTTTACCTGGAGCANANATGCTGGAANAAAAGCCCCTGTATGTGAATGC 310
        ||| ||||||||||:|:||||| |||:|| |||| || || ||||||||
    931 CCTCTACCTGGAGCAGAGATGCTTGAAGAAGAGCCTCTCTACGTGAATGC 980

    311 CAAACAGTATCACCGCATCCTTAAGAGGAGGCAAGCCCGAGCTAAACTAG 360
        |||||| || ||||| || ||||||||||||||||||||||||||||||||
    981 CAAACAATACCACCGTATTCTTAAGAGGAGGCAAGCCCGAGCTAAACTAG 1030

    361 AGGCANAAGGGAAAATCCCAAAGGAACGAAGGAAATACCTCCATGAGTCT 410
        |||||:|||||||||| |||||||| |||||||||||| |||||||||
   1031 AGGCAGAAGGGAAAATTCCAAAGGAGAGAAGGAAATACCTGCATGAGTCT 1080

    411 CGGCATNGGCACNCC 425
        ||||| :| || :||
```

APPENDIX 5 – TRANSFECTION DATA

Transfection 1A

Constructs	β-gal Values
0.5 μg pSV-βgal	0.839
	0.665
	0.800
1.0 μg pSV-βgal	0.728
	0.850
	0.726
1.5 μg pSV-βgal	0.730
	0.862
	0.714
2.0 μg pSV-βgal	0.855
	0.558
	0.911

Transfection 1B

Constructs	β-gal Values
0.5 µg pSV-βgal	0.829
	0.668
	0.807
1.0 µg pSV-βgal	0.730
	0.849
	0.725
1.5 µg pSV-βgal	0.729
	0.734
	0.841
2.0 µg pSV-βgal	0.825
	0.831
	0.648

Transfection 1C

Constructs	β-gal Values
0.5 µg pSV-βgal	0.900
	0.899
	0.505
1.0 µg pSV-βgal	0.860
	0.592
	0.852
1.5 µg pSV-βgal	0.751
	0.756
	0.797
2.0 µg pSV-βgal	0.821
	0.854
	0.629

Transfection 2A

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.081 0.088 0.059	0.135 0.147 0.008	0.600 0.600 7.375	2.858	38%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	1.022 1.254 1.419	0.142 0.244 0.131	7.197 4.189 10.832	7.406	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB1-	5.235 5.187 5.037	0.624 0.669 0.747	8.389 7.753 6.743	7.628	102.9%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	9.825 11.28 12.84	0.580 0.237 0.608	16.940 47.595 21.118	28.551	385%

Transfection 2B

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.050 0.066 0.080	0.610 0.819 0.794	0.082 0.080 0.101	0.088	1.3%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	2.282 2.961 2.731	0.333 0.423 0.397	6.853 6.999 6.878	6.910	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB1-	1.211 2.030 1.723	0.168 0.290 0.242	7.211 7.000 7.119	7.110	102.9%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	9.333 9.553 8.218	0.208 0.214 0.158	44.86 44.64 52.01	47.170	682.6%

Transfection 2C

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.000 0.000 0.005	0.250 0.235 0.230	0.000 0.000 0.023	0.008	0.14%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	1.483 1.881 2.517	0.321 0.353 0.360	4.621 5.330 6.992	5.647	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB1-	3.242 2.945 3.016	0.521 0.530 0.532	6.223 5.556 5.670	5.816	102%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	9.130 6.641 8.271	0.250 0.275 0.278	36.52 24.15 29.75	30.14	533.73%

Transfection 3A

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.000 0.001 0.001	0.553 0.551 0.552	0.000 0.002 0.002	0.001	0.02%
0.5 µg pSV-βgal + 1.0 µg pGL2C	1.875 2.486 1.724	0.445 0.443 0.442	4.213 5.612 3.901	4.575	53.6%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617ICB2-	6.359 5.900 5.219	0.331 0.335 0.332	19.21 17.61 15.72	17.513	205%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	14.65 11.89 14.48	0.400 0.395 0.399	36.62 30.11 36.29	34.34	402%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617ICB2-	24.75 40.40 44.01	0.250 0.245 0.248	99.01 164.89 177.46	73.56	861%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617wt	2.740 2.660 2.354	0.380 0.379 0.375	7.211 7.018 6.278	6.835	80%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	4.385 3.983 4.519	0.500 0.498 0.510	8.771 7.998 8.861	8.543	100%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617wt	13.20 11.38 11.23	0.780 0.775 0.773	16.92 14.69 14.53	15.38	180%

Transfection 3B

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.009 0.008 0.013	0.445 0.448 0.500	0.021 0.019 0.026	0.022	0.19%
0.5 µg pSV-βgal + 1.0 µg pGL2C	2.976 2.464 2.698	0.470 0.463 0.449	6.331 5.321 6.009	5.887	51.9%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617ICB2-	15.19 11.35 9.725	0.470 0.460 0.450	32.31 24.68 21.61	26.20	232%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	37.44 26.22 23.02	0.400 0.440 0.452	93.61 59.58 50.93	68.04	600%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617ICB2-	46.56 21.81 20.11	0.300 0.229 0.224	155.21 95.23 89.76	113.40	1000%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617wt	2.621 2.438 1.897	0.225 0.222 0.219	11.65 10.98 8.666	10.43	92%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	5.054 4.763 5.498	0.450 0.446 0.454	11.23 10.68 12.11	11.34	100%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617wt	18.55 13.78 11.58	0.635 0.700 0.735	29.21 19.68 15.75	21.55	190%

Transfection 3C

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal +1.0 µg pGL3B	0.003 0.004 0.004	0.442 0.440 0.439	0.006 0.010 0.008	0.008	0.008%
0.5 µg pSV-βgal + 1.0 µg pGL2C	2.264 2.251 2.514	0.439 0.453 0.450	4.998 5.003 5.611	5.204	50.7%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617ICB2-	11.77 9.440 8.639	0.448 0.465 0.458	25.32 20.61 18.78	21.57	210%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	31.37 20.39 12.88	0.460 0.350 0.362	89.62 56.33 34.35	60.10	585%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617ICB2-	44.28 28.74 22.12	0.375 0.350 0.362	140.59 82.11 61.10	94.59	921%
0.5 µg pSV-βgal + 0.5 µg pGL3B -617wt	3.176 2.693 3.053	0.330 0.328 0.329	9.623 8.211 9.279	9.038	88%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	5.242 4.689 4.633	0.480 0.469 0.468	10.92 9.999 9.899	10.27	100%
0.5 µg pSV-βgal + 2.0 µg pGL3B -617wt	14.01 13.48 13.32	0.682 0.721 0.753	20.61 18.70 17.69	18.794	183%

Transfection 4A

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal	0.001 0.001 0.002	0.725 0.603 0.656	0.001 0.002 0.003	0.002	0.03%
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.191 0.155 0.144	0.848 0.798 0.565	0.225 0.194 0.255	0.223	3.0%
0.5 µg pSV-βgal + 1.0 µg pGL2C	2.955 3.419 3.249	0.486 0.401 0.337	6.080 8.526 9.641	8.094	
0.5 µg pSV-βgal + 1.0 µg pGL3B + 1.0 NF-YAmt	0.327 0.337 0.268	0.880 0.740 0.704	0.372 0.455 0.381	0.403	5.3%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	1.110 1.336 1.460	0.214 0.114 0.259	5.187 11.72 5.637	7.512	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 0.5 µg NF-YAmt	7.047 5.436 7.690	0.443 0.368 0.381	15.91 14.77 20.18	16.95	225%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 1.0 µg NF-YAmt	5.995 5.930 6.413	0.283 0.281 0.225	21.18 21.10 28.50	23.59	314%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 2.0 µg NF-YAmt	5.058 5.156 4.308	0.223 0.175 0.434	22.68 29.46 9.926	20.69	275%

Transfection 4B

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal	0.002	0.005	0.400	0.733	3.0%
	0.003	0.003	1.000		
	0.004	0.005	0.800		
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.050	0.535	0.093	0.127	0.57%
	0.043	0.328	0.131		
	0.052	0.405	0.128		
0.5 µg pSV-βgal + 1.0 µg pGL2C	0.746	0.108	6.907	6.124	
	0.545	0.095	5.737		
	0.739	0.129	5.729		
0.5 µg pSV-βgal + 1.0 µg pGL3B + 1.0 NF-YAmt	0.091	0.678	0.134	0.147	0.6%
	0.071	0.426	0.161		
	0.084	0.600	0.140		
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	4.550	0.236	19.28	23.90	100%
	4.186	0.116	36.10		
	5.552	0.340	16.33		
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 0.5 µg NF-YAmt	12.44	0.147	86.99	81.23	339%
	9.791	0.109	89.83		
	9.226	0.138	66.86		
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 1.0 µg NF-YAmt	7.378	0.259	28.49	32.42	135%
	7.684	0.204	37.67		
	7.436	0.239	31.11		
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 2.0 µg NF-YAmt	6.445	0.116	55.56	51.32	214%
	4.304	0.097	44.37		
	5.619	0.104	54.03		

Transfection 4C

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal	0.000 0.001 0.001	0.136 0.284 0.159	0.000 0.003 0.003	0.003	0.03%
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.009 0.010 0.009	0.181 0.328 0.195	0.050 0.032 0.047	0.043	0.57%
0.5 µg pSV-βgal + 1.0 µg pGL2C	1.374 1.280 1.296	0.635 0.640 0.653	2.163 1.999 1.985	2.049	
0.5 µg pSV-βgal + 1.0 µg pGL3B + 1.0 NF-Ymt	0.038 0.034 0.052	0.117 0.133 0.128	0.321 0.258 0.408	0.329	4.4%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	7.790 3.308 2.801	0.638 0.629 0.615	12.21 5.260 4.555	7.462	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 0.5 µg NF-YAmt	5.836 6.821 12.203	0.550 0.555 0.629	10.61 12.29 19.40	14.180	189%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 1.0 µg NF-YAmt	6.166 7.399 11.33	0.553 0.558 0.625	11.15 13.26 18.13	14.180	190%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt + 2.0 µg NF-YAmt	5.319 6.607 5.557	0.532 0.546 0.539	9.999 12.10 10.31	1.802	144%

Transfection 5A

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.000 0.000 0.000	0.440 0.445 0.443	0.000 0.000 0.000	0.000	0%
0.5 µg pSV-βgal + 1.0 µg pGL2C	1.615 1.451 1.549	0.598 0.595 0.596	2.700 2.439 2.599	2.579	
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	3.790 4.452 4.236	0.690 0.715 0.705	5.493 6.227 6.009	5.909	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	16.21 16.06 19.59	0.685 0.683 0.687	23.66 23.51 28.52	25.23	427%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2- + 0.5 µg NF-YAmt	15.79 17.21 17.82	0.679 0.680 0.693	23.25 25.31 25.72	24.76	419%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2- + 1.0 µg NF-YAmt	16.91 17.27 16.90	0.698 0.696 0.679	24.22 24.81 24.89	24.64	417%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2- + 2.0 µg NF-YAmt	13.75 13.76 16.82	0.550 0.554 0.698	24.99 24.83 24.10	24.64	417%
0.5 µg pSV-βgal + pGL3B + NF-YAmt	0.043 0.032 0.039	0.442 0.448 0.446	0.098 0.072 0.086	0.085	1.02%

Transfection 5B

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.003 0.004 0.001	0.329 0.353 0.311	0.000 0.000 0.000	0.000	0%
0.5 µg pSV-βgal + 1.0 µg pGL2C	2.480 2.744 1.360	0.621 0.635 0.618	3.994 4.321 2.200	3.505	
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	5.265 5.364 5.741	0.700 0.732 0.735	7.521 7.329 7.811	7.553	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	31.69 31.41 31.88	0.719 0.714 0.717	44.08 43.99 44.47	44.18	585%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-+ 0.5 µg NF-YAmt	31.41 31.40 31.94	0.719 0.721 0.712	43.68 43.55 44.86	44.03	583%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-+ 1.0 µg NF-YAmt	31.83 31.80 36.86	0.723 0.722 0.850	44.02 44.05 43.36	43.81	580%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2- + 2.0 µg NF-YAmt	36.07 36.24 35.87	0.820 0.832 0.822	43.99 43.56 43.64	43.73	314%
0.5 µg pSV-βgal + pGL3B + NF-YAmt	0.028 0.030 0.032	0.423 0.420 0.421	0.066 0.072 0.075	0.071	0.94%

Transfection 5C

Construct	Luciferase Units	β-gal Values	Ratio	Average	Relative %
0.5 µg pSV-βgal + 1.0 µg pGL3B	0.004 0.001 0.000	0.400 0.321 0.300	0.010 0.002 0.000	0.004	0.05%
0.5 µg pSV-βgal + 1.0 µg pGL2C	3.038 3.273 2.380	0.630 0.625 0.638	5.669 5.237 3.779	4.895	
0.5 µg pSV-βgal + 1.0 µg pGL3B -617wt	5.265 5.364 5.741	0.721 0.719 0.720	7.999 8.499 8.497	8.332	100%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-	27.55 27.51 27.60	0.562 0.560 0.551	49.02 49.12 50.09	49.41	593%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-+ 0.5 µg NF-YAmt	27.19 27.15 27.83	0.560 0.557 0.555	48.56 48.75 50.14	49.15	590%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2-+ 1.0 µg NF-YAmt	30.04 28.42 30.42	0.621 0.562 0.617	48.37 50.56 49.30	49.41	593%
0.5 µg pSV-βgal + 1.0 µg pGL3B -617ICB2- + 2.0 µg NF-YAmt	27.49 26.89 27.14	0.565 0.550 0.538	48.65 48.89 50.45	49.33	592%
0.5 µg pSV-βgal + pGL3B + NF-YAmt	0.160 0.159 0.157	0.192 0.196 0.218	0.832 0.811 0.721	0.788	13%