An investigation into the regulation of the topoisomerase IIα promoter in breast cancer cells exposed to doxorubicin

A thesis presented to Massey University in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry

Kirsty Ann Allen

2003

Dedication

This thesis is dedicated to my parents, Gary and Sandra Allen, and to my twin sister Kim Allen for all their support and encouragement over the last four years.

Acknowledgments

I would like to thank my supervisors Kathryn Stowell, Richard Isaacs and John Tweedie for all their expertise, enthusiasm and support that made this study possible. I would like to also thank the occupants of the Twilight Zone especially Carole Flyger, Robyn Marston, Natisha Magan and Chrissie Down for their patience and tolerance.

I acknowledge the financial support of the New Zealand Cancer Society, the Taranaki Branch of the Cancer Society, the Goodman Family Trust and the Institute of Molecular BioSciences, Massey University that made this research possible.

Abstract

Chemotherapeutic drugs, such as doxorubicin, are some of the most effective agents for the treatment of breast cancer. Acquired resistance to these drugs often develops, however, and may preclude effective treatment. Such resistance is multifactorial in origin, but may include down-regulation of topoisomerase IIα (topo IIα) - an essential enzyme involved in normal DNA metabolism and a target for some of the anticancer drugs. A reduction in the levels of this enzyme is thought to reduce DNA damage induced by the drug-topo IIα complex and so increases the chances of survival.

The mechanisms involved in this down-regulation and the development of resistance to doxorubicin are the focus of this study. Stable breast cancer cell lines, containing deletion constructs of the topo IIα promoter linked to the hGH reporter gene, were exposed to doxorubicin and both the reporter and endogenous gene expression were analysed in the surviving cells. It was shown that the reporter and endogenous topo IIα gene expression in the cell line containing the full length topo IIα promoter construct was no longer correlated in the surviving cells negating the use of this experimental system. Instead the endogenous expression of topo IIα and putative regulatory factors were investigated.

Data suggest that specific cell lines show a down-regulation in the levels of the topo IIα protein. These changes were not due to changes in cellular proliferation rates, cell cycle profile or promoter sequence. Selected cell lines were analysed for changes in the relative amounts of specific transcription factors with putative roles in topo IIα gene regulation and for the expression of proteins proposed to have a role in the development of drug resistance. In specific cell lines, a reduction in topo IIα protein levels correlated with alterations in the relative amounts of NF-YA and/or Sp1. It was shown that the drug efflux pumps MDR1 and MRP1, as well as the heat shock factor Hsp70 were not involved in the survival of cells that were exposed to the drug. *In vivo* footprinting was attempted to detect changes in the *in vivo* binding of proteins to the topo IIα promoter after short term drug exposure.

Abbreviations

A	adenine
a.a	amino acid
AC	adenylyl cyclase
AP-1-4	activator protein-1-4
ATF	activating transcription factor
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CaCO-2	human colon carcinoma cell line
cAMP	cyclic adenosine monophosphate
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
cDNA	complementary DNA
CDK1	cyclin-dependent kinase 1
CDP	CCAAT displacement protein
C/EBP	CCAAT enhancer binding protein
CEM	human leukemic cell line
CKII	casein kinase II
cpm	counts per minute
C-terminal	carboxyl terminal
CTF (NF-1)	CCAAT transcription factor
CTP	cytosine triphosphate
DAG	diacylglycerol
DBD	DNA-binding domain
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNase I	deoxyribonuclease I
dNTPs	deoxynucleoside triphosphates
dox^R	doxorubicin-resistant
DTT	dithiothreitol

E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr-1	early growth response protein-1
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
G	guanine
G418	neomycin sulphate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCG	Genetics Computing Group
GRE	glucocorticoid responsive element
GTP	guanosine triphosphate
HAT	histone acetyltransferase
HeLa	human cervical carcinoma cell line
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
HepG2	human hepatocarcinoma cell line
HFM	histone fold motif
hGH	human growth hormone
HL-60	human promyelocytic leukemia cell line
hr	hour
hRPB11/13	human RNA polymerase II subunit 11/13
HSE	heat shock element
HSF	heat shock factor
Hsp70	Heat shock protein 70
HSP-CBF	Heat shock protein - CCAAT binding protein
ICB	inverted CCAAT box
ICB90	ICB binding protein 90

IgG	immunoglobulin G
IP3	inositol triphosphate
JNK	jun N-terminal kinase
JNKK	JNK kinase
K562	human erythroleukemic cell line
kb	kilobase
LMP	ligation-mediated PCR
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEK	MAPK kinase
MEM	modified Eagle's media
MCF-7	mammary epithelial carcinoma cell line
MDA-MB-231	mammary epithelial carcinoma cell line
MDR1	multidrug resistance protein 1
MDR3	multidrug resistance protein 3
MES-SA	human sarcoma cell line
MMLV	moloney murine leukemia virus
Mnase I	micrococcal nuclease
MRP1	multiple resistance-associated protein 1
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cells
NF-Y	nuclear factor Y
NF-YA	A subunit of NF-Y
NF-YAL	NF-YA long isoform
NF-YAS	NF-YA short isoform
NF-YB	B subunit of NF-Y
NF-YC	C subunit of NF-Y
NIH3T3	murine fibroblast (Swiss 3T3) cell line
NLS	nuclear localization sequence

nt	nucleotide
N-terminal	amino terminal
ONPG	o-Nitrophenol β-D-Galacto-pyranoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSE	phosphate buffered saline EDTA
PCR	polymerase chain reaction
pGL3B	pGL3-Basic
pGL2C	pGL2-Control
P-gp	P-glycoprotein
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenyl methane sulfonyl fluoride
PVDF	polyvinyl difluoride
pSV-β-gal	pSV-β-galactosidase expression vector
RNase	ribonuclease
ROS	reactive oxygen species
RT	reverse transcriptase
RT-PCR	reverse transcription PCR
S/T	serine/threonine
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulphate
SEK	SAPK kinase
SH2	src homology domain 2
SH3	src homology domain 3
Sp1	specificity protein 1
Sp3	specificity protein 3
Stat	Signal Transducers and Activators of Transcription
SV40	Simian virus 40
T	thymine
$t_{½}$	half life

T25/T75	25/75 cm^2 tissue culture vented flasks
TAE	Tris acetate EDTA
TAFs	TBP-associated factors
TBE	Tris Boric acid EDTA
TBP	TATA-box binding protein
TBST	Tris buffered saline triton X-100
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_m	melting temperature
topo IIα	topoisomerase IIα
topo IIβ	topoisomerase IIβ
TPA	12-*O*-tetradecanoylphorbol 13-acetate
Tris	Tris-(hydromethyl) aminomethane
tsp	transcription start point
TTP	thymidine triphosphate
UV	ultra violet light
VP-16^R	etoposide-resistant
VM-26^R	teniposide-resistant
YB-1	Y-box binding protein-1

List of Figures

List of Tables

Table of Contents

Chapter One Introduction

Chapter Two Materials and Methods

Chapter Three Analysis of Drug-exposed Breast Cancer Cells

Chapter Five Other Potential Resistance Mechanisms

Chapter One Introduction

1.1 Introduction

Topoisomerases are ubiquitous enzymes involved in the manipulation of DNA topology. An understanding of the mechanism of action as well as the regulation of topoisomerases (topo) is crucial, not only to understand DNA metabolism within the cell but also as these enzymes have been shown to be the primary target for many commonly used anticancer drugs. The way in which drugs target the topoisomerases to cause cell death and how cells may manipulate topoisomerase activity to develop drug resistance are active areas of research. The subject for this thesis is the modulation of gene expression of the type II topoisomerase, topoisomerase IIα (topo IIα), in cells exposed to doxorubicin, a topo II-targeting drug. In this current study, breast cancer cell lines were exposed to doxorubicin and the transcriptional regulation of topo IIα and other possible resistance mechanisms were investigated in the surviving cells.

The following is an outline of the structure and function of mammalian topoisomerases, with a focus on the type II topoisomerases as well as a summary of the current understanding of the mechanisms governing regulation and expression of the genes encoding the two topo II isoforms.

1.2 Type I and II topoisomerases

There are two broad categories of topoisomerases - type I and type II enzymes. Both types of topoisomerase act by forming transient breaks in the DNA, allowing rotation through or around this break, and then resealing the break. Due to the inherent risk of forming breaks in genomic DNA, the enzymes form covalent bonds with the cleaved DNA to help to maintain genomic integrity (reviewed in Fortune & Osheroff, 2000).

Type I enzymes are monomeric and act by forming transient single-strand breaks, passing the complementary strand through the break, then religating the nick. Eukaryotic topoisomerase I can control the torsional stress on the helix, by either relaxing or supercoiling the DNA (reviewed by Boege, 1996) and may act ahead of the replication or transcriptional machinery to relieve the overwinding that occurs on strand separation. While topoisomerase I (topo I) is a target for a variety of anticancer drugs, this thesis focuses on a type II topoisomerase, human topoisomerase IIα.

Type II enzymes are dimeric and act by forming transient double-stranded breaks in the DNA, passing a DNA double helix through this break and then resealing the break. In mammals, the type II topoisomerases exist as two isoforms α and β. Topo IIα (174 kDa) and topo IIβ (182 kDa) share high amino acid sequence identity (~75%) and have a similar gene structure (Withoff *et al.*, 1996a; Jenkins *et al.*, 1992; Austin & Marsh, 1998; Bakshi *et al.*, 2001) and as the genes are found upon different chromosomes (17q21-22 and 3p21-24 respectively) it has been proposed that the two genes arose from gene duplication of an ancestral gene (Sng *et al.*, 1999; Lang *et al.*, 1998). Although these two isoforms also exhibit similar enzymatic activities, the proteins have been shown to differ in specific biochemical properties and different roles have been proposed for topo IIα and IIβ within the cell.

Topo IIα is an essential enzyme that has been implicated in the segregation of chromosomes during mitosis and meiosis, in chromatin condensation, DNA replication and transcription, recombination, repair and may also act as a chromosome scaffold protein (Saitoh *et al.*, 1995; Earnshaw *et al.*, 1985; Ishida *et al.*, 1994; Barthelmes *et al.*, 2000; Downes *et al.*, 1991; Giocanti *et al.*, 1993; Rattner *et al.*, 1996). Topo IIα has been proposed to resolve any unknotting and untwisting of DNA that can occur during these events (reviewed in Watt & Hickson, 1994; Wang, 1996; Fortune & Osheroff, 2000; Austin & Marsh, 1998; Bakshi *et al.*, 2001). The exact role of topoisomerase IIβ has yet to be defined.

The expression of topo IIα (but not topo IIβ) has been shown to be proliferation-dependent with topo IIα highly expressed in proliferating cells but decreased

expression observed when cells reached plateau growth (Tsutsui *et al.*, 1993; Hsiang *et al.*, 1988; Kimura *et al.*, 1994; Austin & Marsh, 1998; Drake *et al.*, 1989). Isaacs *et al.* (1996) showed the confluence-induced reduction in topo IIα protein was due to the transcriptional down-regulation of the topo IIα gene.

The cellular concentrations of topo IIα protein have been shown to be cell cycle-dependent, with the highest levels in G_2M phase and lowest in G_0/G_1, while topo IIβ appeared to be expressed at a relatively constant level during the cell cycle (Burden *et al.*, 1993; Woessner *et al.*, 1991; Sugimoto *et al.*, 1998; Heck *et al.*, 1988; Kimura *et al.*, 1994; Kaufmann *et al.*, 1991). The cell cycle regulation of topo IIα seems to have both a transcriptional (Falck *et al.*, 1999; Adachi *et al.*, 2000) and a post-transcriptional component through cell cycle-specific alterations to mRNA and protein stability (Goswami *et al.*, 1996; Goswami *et al.*, 2000; Salema *et al.*, 2001).

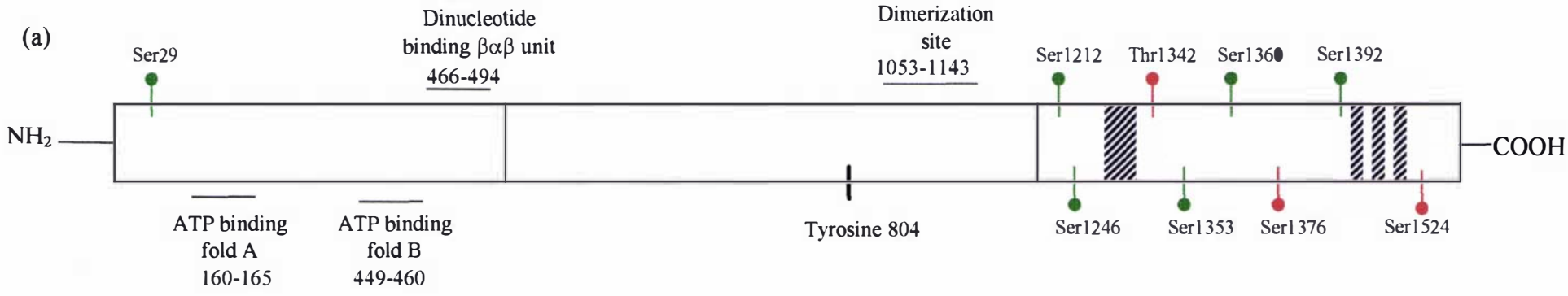
1.3 The structure and function of topo IIα

Each subunit of the topo IIα homodimer has three distinct domains. The N-terminal domain of ~660 amino acids shows homology to the B subunit of *E. coli* DNA gyrase and contains two ATP-binding sequences. The central domain (from amino acids 660 to ~1200) shows homology to the A subunit of *E. coli* DNA gyrase and contains the active site tyrosine (amino acid 804) which is crucial for topo IIα catalytic activity.

The C-terminal domain has a potential but yet undefined regulatory role and contains the putative nuclear localization sequences (NLSs) and a majority of the sites that become phosphorylated (figure 1.1) (reviewed in Fortune & Osheroff, 2000).

The exact mechanism for topo IIα enzyme action has been proposed as follows. The topo IIα homodimer binds to the DNA in a nucleotide-specific cofactor-independent manner. As topo IIα has both general (regulating DNA topology) and specific roles (i.e. at chromosome segregation and condensation) there is still debate about the exact consensus sequence for DNA binding.

The Structure of Topo IIα Protein



(b)

(c)

Residue Position	Base pair change
428	deletion of Ala
429	deletion of Ala
449	Arg → Gln
479	Lys → Glu
486	Arg → Lys
494	Glu → Gly
519	Lys → stop
744	Ala → Thr
797	Lys → Asn

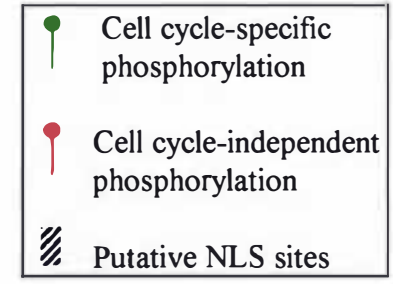


Figure 1.1. (a) Diagram of the ATP-binding sites, the active site tyrosine, phosphorylation sites and putative NLS sites (1279-1290, 1416-1436, 1441-1466 and 1488-1516). (b) and (c) The position of mutations identified in the topo IIα coding region. (Adapted from Isaacs et al.,1998; Robert et al., 1998; Withoff et al., 1996a; Larsen et al., 1998; Vassetzky et al., 1995; Austin and Marsh., 1998; Beck et al., 1999; Bjergbaek et al., 1999; Bakshi et al., 2001).

In the presence of magnesium ions, a DNA cleavage/religation equilibrium is established. The DNA cleavage occurs through a transesterification reaction, while religation occurs via the reverse reaction. The coordinate nicking of opposite strands is performed and each subunit forms a covalent bond between the active site tyrosine and the newly formed 5' termini of the cleaved DNA strand. Upon ATP binding, the topo IIα undergoes a conformational change which allows the passage of a second helix through the double-stranded break. The break is then resealed. A major conformational change occurs upon ATP hydrolysis that allows the opening of the protein clamp to release the DNA. The enzyme is then recycled to be ready for another catalytic cycle (figure 1.2) (Fortune & Osheroff, 2000).

It has been shown that this catalytic reaction occurs by a 'two-gate' mechanism, where the enzyme with DNA bound moves the second DNA helix through one set of "jaws" in the N-terminal region of the enzyme in an ATP-dependent reaction. The second DNA helix then moves through the double-stranded break and is then expelled through a second set of "jaws" in the C-terminal region of the enzyme (Roca & Wang, 1994; Roca *et al.*, 1996; Berger *et al.*, 1996; Roca & Wang, 1992).

1.4 The post translational regulation of topo IIα activity

Topo IIα has been shown to be phosphorylated, with most sites identified in the C-terminal portion of the protein. The protein kinases CKII (Ser1376, Ser1524) , PKC (Ser29), MAPKs (Ser1212, Ser1246) and $p34^{cdc2}$ (Ser1212, Ser1246, Ser1353, Ser1360, Ser1392) have been implicated in these phosphorylations (reviewed in Isaacs *et al.*, 1998; Bakshi *et al.*, 2001) (figure 1.1). The phosphorylation of specific residues has been shown to be cell cycle-dependent. The highest levels of phosphorylation were seen at G_2/M phase where there is an intermediate levels of topoisomerase IIα protein and the greatest enzyme activity (Burden *et al.*, 1993; Burden & Sullivan, 1994; Wells *et al.*, 1995).

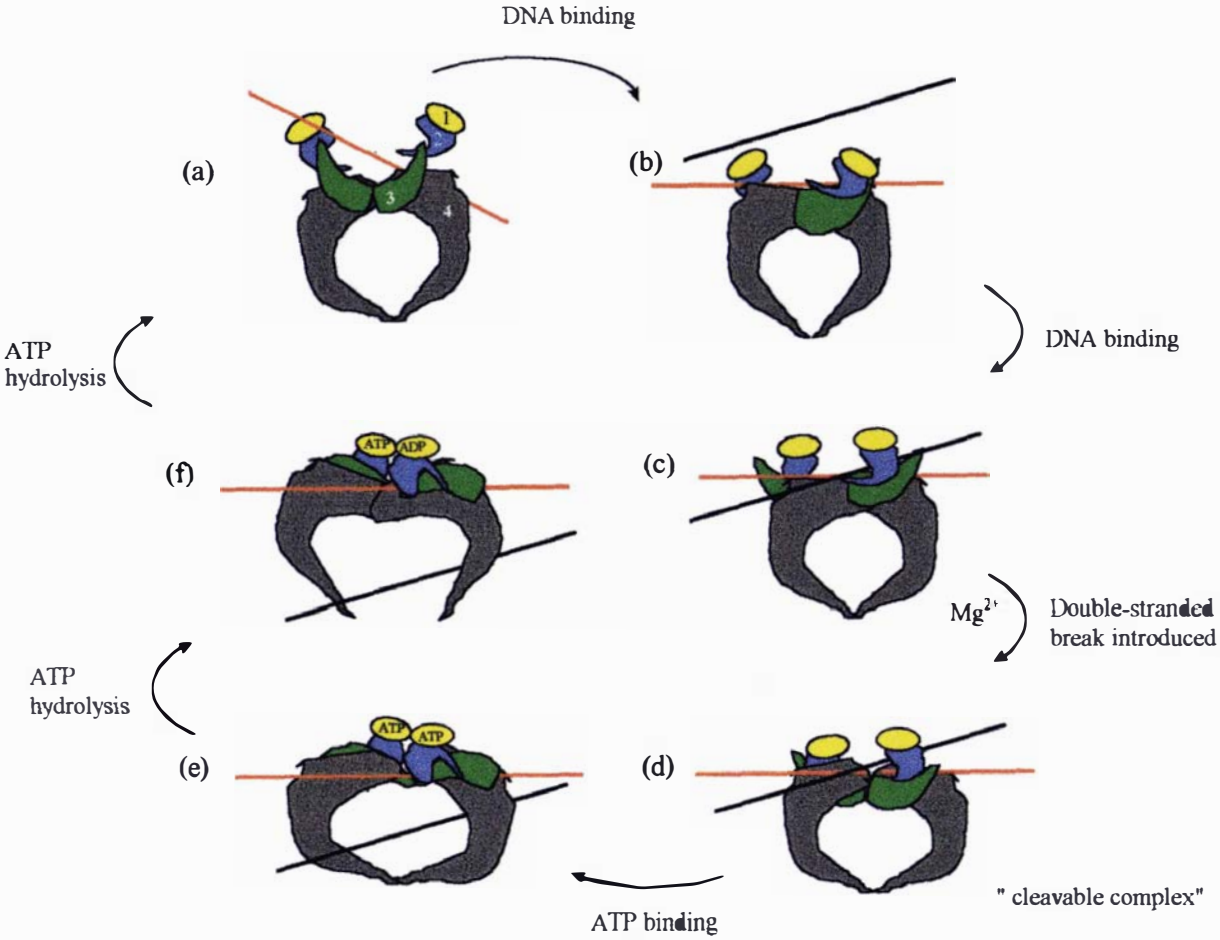


Figure 1.2. Proposed model of the catalytic cycle of topo IIα. Topo IIα is composed of an N-terminal ATPase domain (1), a central domain containing catalytic (2) and dimerization (3) sites and a C-terminal domain (4). (a) the topo II enzyme in an open conformation, (b) the topoisomerase II binds to a region of double-stranded DNA, (c/d/e) a double-stranded break is introduced into the first DNA helix (red line) and upon ATP binding, the second helix (black line) is moved through the double-stranded break. The break is resealed, (e) upon ATP hydrolysis, the second helix is released (f) a further ATP hydrolysis may be required to recycle the enzyme to be ready for the next catalytic cycle (adapted from Magan, 2002; Fortune and Osheroff, 2000; Bakshi et al., 2001).

The role of phosphorylation is still in dispute. Wells *et al.* (1995), DeVore *et al.* (1992), Matsumoto *et al.* (1997) and Matsumoto *et al.* (1999) showed that phosphorylation enhanced catalytic activity, while Escargueil *et al.* (2000) showed that phosphorylation had no effect on catalytic activity and Rzepecki & Fisher (2000) proposed that phosphorylation may regulate interaction with nucleic acids.

1.5 Transcription regulation of eukaryotic promoters

The regulation of gene expression is ultimately reflected in phenotypic changes, due to the regulation of the rate of RNA synthesis and subsequently the cellular concentration of individual proteins. While the pathway that leads to the synthesis of a gene product is a multi-step process, there are many potential points of control in this pathway with the regulation of transcription considered as the primary mechanism in both prokaryotes and eukaryotes (reviewed by Latchman, 1995).

The efficiency of the initiation of transcription by the basal transcription complex is greatly influenced by the binding and activity of specific transcription factors (*trans*-acting factors) to upstream DNA binding elements (*cis*-acting elements) in the promoter of a gene. Distal *cis*-acting elements or "enhancers" that can be found in either orientation many base pairs from the gene can also be targets for *trans*-acting factors. The following is a summary of the putative transcription factors and *cis*-acting elements implicated in the regulation of the topoisomerase IIα gene.

1.5.1 Human topoisomerase IIα promoter

Two kb of the human topoisomerase IIα promoter was isolated from a human placental genomic DNA library by Hochhauser *et al.* (1992). Two GC boxes, 5 inverted CCAAT boxes (ICB1-ICB5), and 1 ATF element were among the putative transcription factor binding sites identified. A classical TATA box was not detected. A high frequency of CpG dinucleotides (~70% GC) within the initial promoter region was noted (figure 1.3).

The human topo IIα promoter has similarities to the promoters from hamster, rat and mouse. All are TATA-less and although the three promoters have a high degree of sequence identity to the human, they vary in both the presence of additional species-specific elements and in the functional significance of shared elements for basal or induced transcription (reviewed in Bakshi *et al.*, 2001).

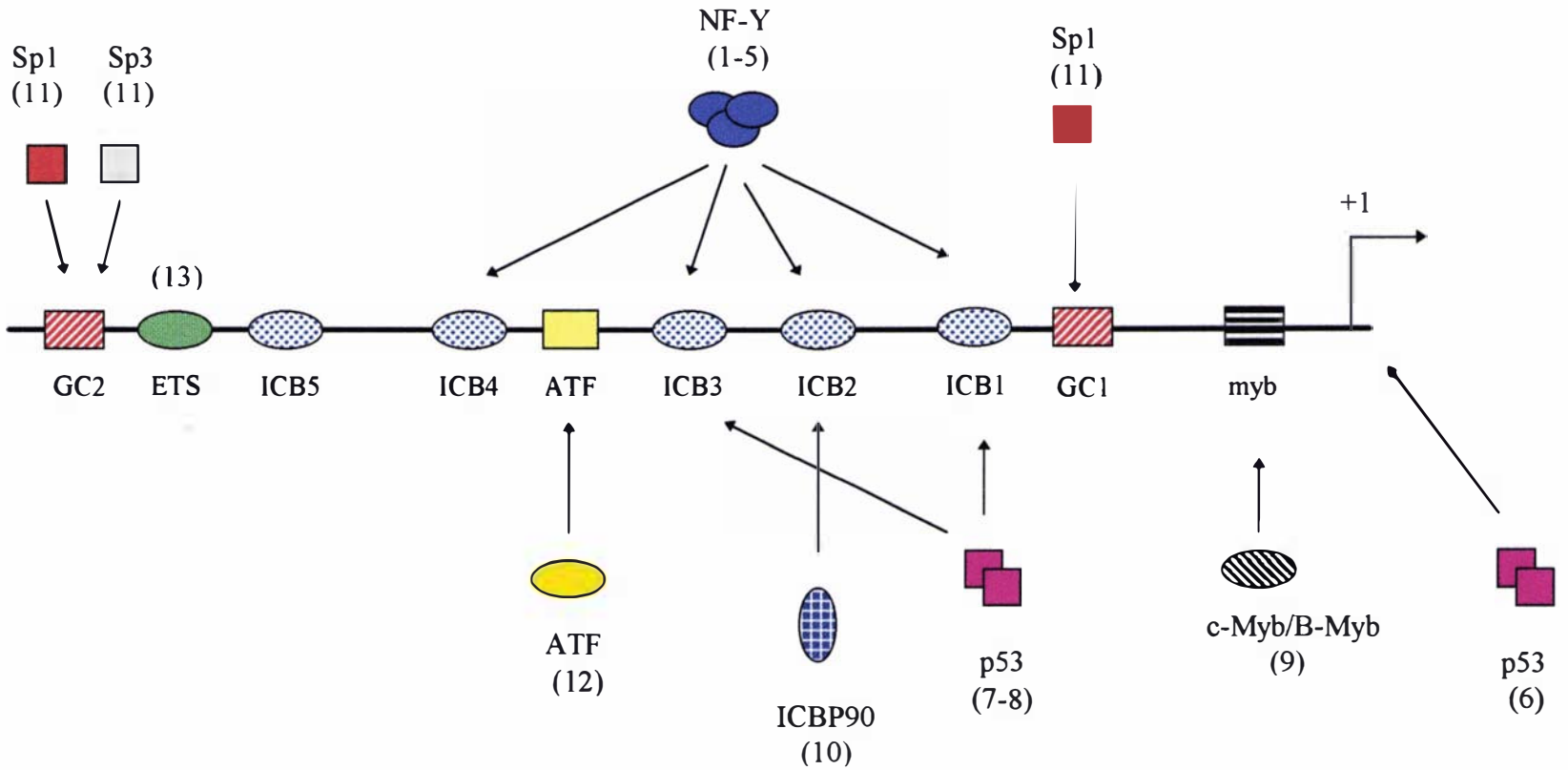


Figure 1.3. Diagram of the transcription factors proposed to interact with specific elements of the topo IIα promoter, labeled are GC2 (-563 to -554), Ets (-475 to -480), ICB5 (-385 to -381), ICB4 (-259 to -255), ATF (-226 to -219), ICB3 (-175 to -171), ICB2 (-108 to -104), ICB1 (-68 to -64), GC1 (-51 to -45) and myb (-16 to -11). ((1) Wang et al., 1997a (2) Morgan and Beck., 2001 (3) Adachi et al., 2000 (4) Isaacs et al., 1996 (5) Hopfner et al., 2000 (6) Sandri et al., 1996 (7) Joshi et al., 2000 (8) Wang et al., 1997b (9) Brandt et al., 1997 (10) Hopfner et al., 2000 (11) Magan, 2002 (12) Lim et al., 1998 (13) Chen et al., 1999).

Since then, the basal transcription of the topo IIα promoter has been investigated in a variety of cell lines with a number of functional promoter elements identified (Appendix 1). Potentially cell type-specific repressive regions have been identified in the extreme 5' of the topo IIα promoter (Hochhauser *et al.*, 1992; Loflin *et al.*, 1996), although their functional significance and cognate DNA binding proteins have yet to be determined.

The importance of specific regions for the basal regulation of the topo IIα promoter have been shown to be cell type-specific. The region containing ICB1 was shown to have a modest activating effect on basal transcription in a number of cell lines (Wang *et al.*, 1997b; Furukawa *et al.*, 1998; Hochhauser *et al.*, 1992). A further increase in transcription was observed on the addition of ICB2 and upstream sequences in most cell lines tested (Furukawa *et al.*, 1998; Chen *et al.*, 1999; Morgan & Beck, 2001; Takano *et al.*, 1999; Wang *et al.*, 1997b).

Collectively these results indicated the importance of the ICB elements in basal transcription although the level of influence of specific ICBs varied. The extent of the effect of each element may be cell-type specific depending on the presence of specific transcription factors and their co-regulators in these cells.

The functional importance of the ICBs and other elements were investigated by their ability to bind specific proteins. It has been shown that the transcription factor NF-Y can bind to ICBs 1-4 although the affinity for binding varies, with ICB3 having the highest binding affinity, ICB1 and 4 an intermediate affinity and ICB2 the lowest. The protein that binds to ICB5 has yet to be identified (Herzog & Zwelling, 1997; Wang *et al.*, 1997a; Tolner *et al.*, 2001; Joshi *et al.*, 2000). NF-Y was implicated as an activator when bound at ICB1 (Magan, 2002). Sp1 has been described as a potential activator of the basal topo IIα promoter activity and both Sp1 and Sp3 have been shown to bind to GC1 and GC2 (Magan, 2002).

1.5.2 Topo IIα regulation during the cell cycle, during differentiation and confluence arrest

The roles of the putative promoter elements in transcriptional regulation of the topo IIα promoter have been investigated during confluence arrest, differentiation of hemapoietic cells, during the cell cycle and under stress conditions.

Isaacs *et al.* (1996) showed that down-regulation of topo IIα promoter activity occurred upon confluence arrest in specific cell lines. Reduced binding of NF-Y to ICB2 in confluence-arrested cells was proposed to be the mechanism for this transcriptional down-regulation. Hopfner *et al.* (2000) showed that ICBP90 could bind to ICB2 and act as a transcriptional activator. This protein has been found to be highly expressed in proliferating tissues and so may be involved in the proliferation-dependent regulation of topo IIα. Yoon *et al.* (1999) showed that Sp1 mediated the up-regulation of the rat topo IIα promoter in proliferating cells.

Concentrations of topo IIα have been shown to vary during the differentiation of hemapoietic cells into monocytes. Brandt *et al.* (1997) showed that c-Myb, a transcription factor involved in cellular proliferation, transactivated the topo IIα promoter through the putative c-Myb element (-16 to -11). This activation was in a hemopoietic-specific manner, and this element was crucial for basal transcription. Lim *et al.* (1998) showed that the reduced binding of ATF to the putative ATF element correlated with the repression of the topo IIα promoter during differentiation. These results differ to that of Loflin *et al.* (1996) where a post-transcriptional mechanism was found to cause the down-regulation of topo IIα levels during the differentiation of K562 cells to megakaryotes.

Falck *et al.* (1999) showed that the transactivation of the human topo IIα promoter during S-phase was due to the reduced binding of a repressive protein to ICB1. This protein was not NF-Y. This differs to the situation of the murine topo IIα promoter where binding of NF-Y to ICB1 was shown to be independent of the cell cycle and it

was proposed that NF-Y may recruit HAT enzymes to the promoter during G_2/M which may result in histone acetylation and transactivation (Adachi *et al.*, 2000).

1.5.3 Regulation of topo IIα during stress conditions

Topo IIα promoter activity has been shown to be modified under stress conditions. An increase in the activity of the topo IIα promoter was observed 6-24 hours after heat shock (Furukawa *et al.*, 1998). The reduced binding of an unknown inhibitory factor to ICB1 was the proposed mechanism for this transactivation. p53 has been shown to have an ICB-dependent inhibitory effect on the topo IIα promoter (Joshi *et al.*, 2000; Wang *et al.*, 1997b). Joshi *et al.* (2000) showed that p53 caused a decrease in the binding of NF-Y to ICB1 and ICB3. These studies differ from that of Sandri *et al.* (1996) where the p53-induced inhibition of the p53 promoter was found to be transcription-factor-binding-site-independent and an interaction of p53 with the basal transcriptional machinery was proposed.

Following DNA damage, it has been seen that the down-regulation of CDK1 (cyclin-dependent kinase 1), the kinase that promotes entry into mitosis occurs resulting in G_2 arrest. This was a transcriptional down-regulation, with the cell cycle-dependent repressive elements CDE (cell-cycle-dependent element) and CHR (cell-cycle genes homology region) implicated. Under these conditions the down-regulation of topo IIα was also seen and it was proposed that putative CDE and CHR elements which are near and overlap GC1 (-37 to -41 and -47 to -51) could be involved (Badie *et al.*, 2000).

1.6 The mechanism of topo II poisons

As topoisomerase II is a major target for many anticancer drugs, alterations in the expression of the gene or to the protein itself may lead to resistance to the drug. A description of the mechanism of drug action follows to provide a framework for understanding the development of resistance.

Chemotherapy is the "chemical warfare" in the battle against cancer, where toxic chemicals are ingested to cause a systemic killing of cancer cells. Chemotherapeutic drugs act on specific cellular targets, with topoisomerase II a common target. Topoisomerase II-targeted drugs tend to fall into two classes, the topo II poisons and the topo II inhibitors. The topo II poisons stabilize the normally transient topo II-DNA cleavage complex to turn the topo II into a cellular toxin, while topo II inhibitors inhibit steps of the topo II catalytic cycle. The descriptions of all the drugs mentioned in this thesis, proposed mechanisms of action and resistance are described in Appendix 2. In this current research the resistance to the topo II poison doxorubicin was investigated.

1.6.1 Doxorubicin

Doxorubicin is a member of the anthracycline class of drugs. Anthracyclines have multiple mechanisms of action, with a major mechanism resulting from the ability of the drug to intercalate between DNA base pairs. When this intercalation occurs near the topo II/DNA complex, it has been proposed that the position of the 3' end of the cleaved DNA strand shifts relative to the covalently-bound 5' end. This would cause misalignment to occur thus preventing religation (reviewed in Topcu, 2001). Another possibility of drug action was described in the 'positional poisoning model' where the drugs enhanced topoisomerase II-mediated DNA cleavage by altering the DNA structure (Marx *et al.*, 1997).

This drug-stabilized complex, where the topo II is covalently bound to the DNA but is unable to religate the double strand cut, is called the cleavable complex (reviewed in Topcu, 2001). It is thought that the collision of the cleavable complexes with the replication fork or transcriptional machinery is required to convert this complex into permanent double-stranded breaks (reviewed in Fortune & Osheroff, 2000; Vassetzky *et al.*, 1995; Zhang *et al.*, 1990). Such double-stranded breaks are not equally distributed throughout the genome and do not occur at all topo II binding sites (Capranico *et al.*, 1997).

At low doses, anthracyclines can halt the cell cycle at G_2M; at higher doses the cell cycle is halted at early S-phase (reviewed in Richardson & Johnson, 1997). Exactly how cells recognise drug-induced damage and cause this cell cycle arrest is not well defined.

The incorrect repair of double-stranded breaks may cause sister chromatid exchange, large insertions or deletions, chromosome aberrations or translocations and if the damage is severe enough, cell death (Froelich-Ammon & Osheroff, 1995; Isaacs *et al.*, 1995; Bassi & Palitti, 2000; Marchetti *et al.*, 2001; Melixetian *et al.*, 2000). Another possible mechanism of DNA damage has been described by Zhou *et al.* (1997) where it was proposed that the reciprocal exchange of topo IIα subunits between cleavage complexes bound at two unrelated sequences caused chromosomal rearrangements, deletions or sister chromatid exchange. The correlation between the amounts of topo IIα protein and drug-induced double-stranded breaks, and the correlation between these breaks and cytotoxicity has been shown in numerous studies (Goldenberg *et al.*, 1986; Zwelling *et al.*, 1991; Eder *et al.*, 1993; Aoyama *et al.*, 1998; Harris *et al.*, 2001), although this relationship has been shown to be equivocal in other systems (Zhou *et al.*, 1999a; Estey *et al.*, 1987).

The other major cytotoxic mechanism involves the ability of doxorubicin to be metabolized. Doxorubicin can be enzymatically reduced by one or two electrons to produce intermediates that can react with oxygen to produce hydrogen peroxide or hydroxyl radicals that can subsequently damage membranes and proteins. The inhibition of topoisomerase I, DNA helicases, DNA and RNA polymerases, DNA repair enzymes, metallothioneine synthesis, mitochondrial oxidative phosphorylation, direct membrane effects and the alkylation and cross linking of DNA are thought to be secondary mechanisms of action (reviewed by Hortobagyi, 1997).

1.7 The role of topo IIα in drug resistance

As in the presence of the drug, topo IIα is converted into a cellular toxin, the levels or activity of topo IIα protein within the cell could determine how much drug-induced

damage occurs and so may determine the cytotoxicity of the treatment. In agreement with this theory, the levels of topo IIα have been shown to determine the sensitivity of cells to specific drugs, with alterations of either the amounts or activity of topoisomerase IIα forming the basis of atypical multidrug resistance.

1.7.1 Alterations in topo IIα catalytic activity in resistant cell lines

Changes to the activity of the topo IIα protein have been seen in resistant cells either through mutations or post-translational modifications. Mutations to the topo IIα coding region have been detected in drug-resistant cells. The topo IIα mutations identified in human topo IIα protein are detailed in figure 1.1. As the mutations cluster around the consensus B ATP binding sequence, the dinucleotide-binding βαβ unit and around the active site tyrosine, it may be proposed that these mutations may inhibit ATP binding or hydrolysis, inhibit catalysis or interfere with the formation of the drug-induced cleavable complexes (reviewed in Vassetzky *et al.*, 1995). While a number of mutations have been seen in *in vitro* studies, the clinical significance of such mutations is unknown as only a few mutations have been detected in clinical samples. For example the Arg486Lys and Glu494Gly mutations have been detected in small cell lung cancers treated with etoposide (Kubo *et al.*, 1996).

Changes to the phosphorylation state of topo IIα have been seen in drug-resistant cell lines. The hypophosphorylation of topo IIα observed in an etoposide-resistant erythroleukemic cell line correlated to a reduction in the amount and stability of the drug-induced cleavable complexes (Ritke *et al.*, 1994; Aoyama *et al.*, 1998). In various etoposide-resistant ($VP\text{-}16^{R}$) cell lines that had reduced levels of topo IIα protein, the hyperphosphorylation of topo IIα was observed. This hyperphosphorylation correlated with the restoration of wild type catalytic activity (Takano *et al.*, 1991; Matsumoto *et al.*, 1997; Matsumoto *et al.*, 1999).

1.7.2 Alterations to the amounts of topo IIα in resistant cell lines

Alterations to the levels of topo IIα have been correlated to modulation of drug sensitivity. Towatari *et al.* (1998) showed that the reduction of topoisomerase IIα mRNA in U937 human monocytic leukemia cells reduced the drug sensitivity of the cells to etoposide and daunorubicin, while McPherson *et al.* (1997) showed that the up-regulation of topo IIα protein enhanced sensitivity to doxorubicin.

The decreased expression of topoisomerase IIα has been observed in a variety of cell lines which have developed resistance to various chemotherapeutic drugs. As outlined in Appendix 2, the down-regulation of topo IIα and to a lesser extent topo IIβ has been observed in a wide range of drug resistant cell lines. Cell types ranging from breast, lung and leukemic cells resistant to doxorubicin, and other topo II poisons such as etoposide and mitoxantrone, all showed decreased topo IIα protein indicating that such a decrease may be a common mechanism of drug resistance to topo II-targeted drugs. This was confirmed by studies which showed that the down-regulation of topo IIα correlated with a reduction of drug-induced DNA damage in a range of cell lines (Webb *et al.*, 1991; Deffie *et al.*, 1989; Qui, *et al.*, 1996; Takano *et al.*, 1991; de Jong *et al.*, 1990). There is also evidence that topo IIβ may also act as a drug target (Austin & Marsh, 1998).

There is significant evidence that alterations to the expression of the topo IIα gene may be a major mechanism for the development of drug resistance at least *in vitro*, but the *in vivo* significance is in dispute. Some correlation has been seen between the response to chemotherapy and topo IIα in specific clinical samples. For example reduced amounts of topo IIα have been observed in specific bladder, ovarian and nephroblastoma tumours after treatment and the amount of topo IIα correlated with clinical response of specific breast, heptacellular and gastric cancers to doxorubicin. Conversely, no correlation has been seen in the hematological, lung, ovarian and lung tumours investigated (reviewed in Dingemans *et al.*, 1998). Potential reasons why a strong correlation was not observed between topo IIα and drug resistance could include the heterogeneity of cells in tumours and the multifactorial resistance that can

occur due to drugs other than topo II poisons (reviewed in Den Boer *et al.*, 1998; Kaufmann *et al.*, 1998).

The observed mechanisms for the down-regulation of topo IIα could include the transcriptional down-regulation of the topo IIα gene or changes to the number of topo IIα genes. While alterations to the topo IIα mRNA or protein stability are possible mechanisms, these have yet to be described in resistant cell lines.

1.7.2.1 Alterations to topo IIα copy number in drug resistant cells

The physical amplification or deletion of the topo IIα gene has been seen in a range of breast tumours. The deletion or amplification of the topo IIα gene has been linked to the amplification of ErbB2 (Her2), a growth factor receptor (Di Leo *et al.*, 2002; Coon *et al.*, 2002; Järvinen *et al.*, 1999; Järvinen *et al.*, 2000; Lehmann *et al.*, 2000). The ErbB2 gene is located near to the topo IIα gene (Hutchinson & Muller, 2000; Bange *et al.*, 2001; Järvinen & Liu, 2000), and the amplification of the ErbB2 gene was seen to be the primary genetic event which was followed by either the telomeric amplification or interstitial deletion of the topo IIα gene (Järvinen *et al.*, 1999). Such changes have been proposed to modify drug sensitivity in specific cell lines. Withoff *et al.* (1996b) showed that in a doxorubicin-resistant (dox^R) lung cancer cell line, a reduction in the topo IIα copy number at least partially accounted for the decrease in topo IIα mRNA. Cells which had amplification of topo IIα had increased topo IIα protein levels and doxorubicin sensitivity while the cells containing topo IIα deletions had increased drug resistance (Järvinen & Liu, 2000).

1.7.2.2 Regulation of the topo IIα promoter in drug-resistant cell lines

Only a limited amount of research has been reported on the regulation of the topo IIα promoter in drug-resistant or drug-exposed cells. Sp3 has been implicated as a potential repressor of the topo IIα promoter in etoposide- or teniposide-resistant human epidermoid cells (Kubo *et al.*, 1995) or an activator in a merbarone-resistant CEM cell line (Mo *et al.*, 1997). In dox^R HL-60 cells, increased Sp1 protein was seen,

although the correlation with topo IIα expression was not investigated (Borellini *et al.*, 1990).

A reduction in NF-Y binding to ICBs 1-4 has been implicated in down-regulation of the topo IIα promoter in drug-resistant cells in several studies (Wang *et al.*, 1997a; Joshi *et al.*, 2000). Conversely an ICRF-8-resistant CEM cell line with increased topo IIα expression exhibited a decrease in NF-Y binding to ICB3 (Morgan & Beck, 2001).

1.8 Transcription factors Sp1, Sp3 and NF-Y

1.8.1 Specificity factor 1 (Sp1)

The GC and GT boxes are widely distributed promoter elements found in eukaryotic promoters of both ubiquitous and tissue-specific genes (reviewed in Suske, 1999). The Sp family of transcription factors are known to bind to these elements. Sp1 and Sp3 are members of this family and while both can bind to GC boxes with similar affinity, the biological functions of these transcription factors are thought to differ (reviewed in Suske, 1999; Philipsen & Suske, 1999; Lania *et al.*, 1997).

Sp1 is a ubiquitously expressed transcription factor with a predominant transactivator activity (reviewed in Parnaik, 1999; Suske, 1999). In TATA-less promoters, Sp1 sites can act to position the transcriptional machinery to the transcription start point and regulate promoter activity (reviewed in Fry & Farnham, 1999) by interacting with components of the transcriptional machinery such as the TATA-box binding protein (Emili *et al.*, 1994) and various TAFs (reviewed in Parnaik, 1999; Suske, 1999; Fry & Farnham, 1999; Lania *et al.*, 1997).

Multiple mechanisms have been proposed to account for the Sp1-induced transactivation including synergistic transactivation through adjacent Sp1 sites; interactions with other transcription factors including NF-Y (Magan, 2002; Roder *et al.*, 1997), Sp3 (Bigger *et al.*, 1997; Tsai *et al.*, 1999), c-Jun (Melnikova & Gardner, 2001), p53 (Borellini & Glazer, 1993; Koutsodontis *et al.*, 2001), estrogen and

progesterone receptors (Wang *et al.*, 1999b; Owen *et al.*, 1998); alterations of chromatin structure (Ding *et al.*, 1999; Parnaik, 1999) and self-association to bring proximal and distal DNA segments together by DNA looping (Mastrangelo *et al.*, 1991; Su *et al.*, 1991).

The post-translational modification of Sp1 may also affect activity, with both phosphorylation and *O*-linked glycosylation of the Sp1 protein observed. These modifications have been proposed to result in a ~105 kDa protein seen above the major 95 kDa protein in immunoblots. The phosphorylation of Sp1 can have either a positive or negative effect on the DNA binding (Zheng *et al.*, 2000; Rohlff *et al.*, 1997; Borellini *et al.*, 1990; Leggett *et al.*, 1995; Daniel *et al.*, 1996; Wang *et al.*, 1999c) and glycosylation of Sp1 appeared to enhance transcriptional activation (Jackson & Tjian, 1988).

1.8.2 Specificity Protein 3 (Sp3)

Sp3 is a ubiquitously expressed protein which has extensive homology to Sp1, but is lacking an N-terminal serine/threonine-rich region. Sp3 is found as three isoforms, a full length 115 kDa protein (due to the initiation of translation from a non-AUG codon) and two smaller isoforms (78 and 80 kDa) which result from differential internal initiation (Kennett *et al.*, 1997; Ammanamanchi & Brattain, 2001a). The shorter isoforms were lacking an N-terminal activation domain and while able to bind to DNA, had little transactivational activity (Kennett *et al.*, 1997).

Sp3 has been shown to contain both activator (glutamine-rich regions) and repressor regions (near to the DNA binding domain). While Sp1 has a predominant activator activity, Sp3 has been found to have both activator and repressor activities depending upon the cellular and promoter context. Sp3 has been shown to be able to repress Sp1-mediated transactivation. This repression may be due to competition for the binding site, interactions through the repressor region, or to interactions with components of the transcriptional machinery (reviewed in Lania *et al.*, 1997). It is thought that the relative abundance of Sp1 and Sp3 can modulate transcription. This ratio can vary under different conditions i.e upon keratinocyte differentiation and hypoxia and in

different cell types. For example, during hypoxia, the level of Sp3 protein decreased while Sp1 protein levels remained unchanged (Discher *et al.*, 1998).

It has also been proposed that the smaller isoforms may act as repressors either through competition for the binding site (Kennett *et al.*, 1997) or due to competition for promoter-specific factors (Kennett *et al.*, 2002). It was suggested that the transcriptional induction by Sp3 may require a mechanism that would relieve this competition possibly by favouring interactions between full length Sp3 and specific TAFs. Like Sp1, Sp3 has been shown to interact with and modulate the activities of other proteins including ER and NF-Y (Galvagni *et al.*, 2001; Stoner *et al.*, 2000; Yamada *et al.*, 2000).

1.8.3 Nuclear Factor Y (NF-Y)

The CCAAT box is one of the most widespread elements found in ~30% of eukaryotic promoters investigated. While a number of CCAAT box binding proteins have been identified (C/EBP, CTF (NF-1), YB-1, HSP-CBF, CDP), NF-Y differs from these in the strict requirement for the pentanucleotide CCAAT sequence for DNA binding (reviewed in Mantovani, 1999; Maity & de Crombrugghe, 1998; Matuoka & Chen, 1999).

NF-Y exists as a trimer containing NF-YA (CBF-B), NF-YB (CBF-A) and NF-YC (CBF-C). NF-YA is a 347 amino acid protein consisting of a near N-terminal glutamine-rich region, a nearby serine/threonine-rich domain, and a near C-terminal DNA binding domain. NF-YB (207 a.a) and NF-YC (335 a.a) both contain a histone fold-like motif, with NF-YC also containing a near C-terminal glutamine-rich domain. The histone-fold motif (HFM) seen in NF-YB and NF-YC were shown to be similar to that seen in histones H2B and H2A respectively. The glutamine-rich regions showed similarity to that seen in Sp1 (reviewed in Mantovani, 1999; Maity & de Crombrugghe, 1998; Matuoka & Chen, 1999).

The NF-Y trimer is assembled in a stepwise manner. Initially a heterodimer is formed between NF-YB and NF-YC due to interactions between the HFMs. This interaction

provides the surface that allows NF-YA to bind. This complex is capable of binding to the CCAAT box (Mantovani, 1999; Maity & de Crombrugghe, 1998).

NF-YA exists as either a long (NF-YAL, 347 amino acids) or short (NF-YAS, 318 amino acids) isoform due to the alternative splicing of the mRNA that removes the exon B in the N-terminal glutamine-rich region. It was found that the short isoform can bind to DNA with the same efficiency and have similar transactivational activity to the long isoform (Bolognese *et al.*, 1999; Morgan & Beck, 2001; Chang & Liu, 1994; Ishimaru *et al.*, 1997; Li *et al.*, 1992), although there is evidence that the different NF-YA isoforms may have a differential effect on transcription on specific promoters (Gu *et al.*, 1999; Ishimaru *et al.*, 1997). There is a strong tissue-specific bias as to which isoform is predominant ; fibroblasts were found to have a majority of the longer isoform while lymphocytes contained a majority of the short isoform (Li *et al.*, 1992). The levels of NF-YAL and NF-YAS have been found to vary under specific conditions, for example increased NF-YAL was observed after serum stimulation (Chang & Liu, 1994).

NF-Y has been shown to be able to increase the affinity and/or stability of nearby bound transcription factors and/or have a synergistic activation on transcription. Examples include Sp1 (Magan, 2002; Roder *et al.*, 1997; Liang *et al.*, 2001; Bigger *et al.*, 1997; Inoue *et al.*, 1999) and p53 (Agoff *et al.*, 1993). Multiple mechanisms have been proposed to explain the actions of NF-Y. NF-Y has been shown to be able to cause distortions or perturbations to the chromatin structure around CCAAT boxes (Liberati *et al.*, 1998; Korner & Muller, 2000; Coustry *et al.*, 2001; Ronchi *et al.*, 1995). It has been proposed that such alterations may alter the alignment of specific factors and enhance or inhibit subsequent interactions. It has been shown that NF-Y can interact directly with nucleosomes (Motta *et al.*, 1999) and NF-Y has been shown to associate with histone acetyltransferases (HATs) (Li *et al.*, 1998; Currie, 1998; Jin & Scotto, 1998).

1.9 Role of topo IIβ in drug resistance

While there is mounting evidence for the role of topo IIα in drug resistance, the role of topo IIβ is not as well defined. The knockout of the topo IIβ gene resulted in cells which had increased resistance to mAMSA and mitoxantrone (Errington *et al.*, 1999). The down-regulation of topo IIβ has been seen in a range of cell lines, with a co-reduction in topo IIα commonly seen (Herzog *et al.*, 1998; Jaffrezou *et al.*, 1994; Lage *et al.*, 2000; Houlbrook *et al.*, 1996; Nielsen *et al.*, 2000b; Harker *et al.*, 1995a; Zhou *et al.*, 1999b; Modrak *et al.*, 1997). As with topo IIα, mutations in the topo IIβ gene have also been detected (Dereuddre *et al.*, 1995; Dereuddre *et al.*, 1997; Herzog *et al.*, 1998) and hyperphosphorylation of topo IIβ has been seen in a dox^R HL-60 cells. As this cell line also contained hypophosphorylated topo IIα, it was proposed that the modification to topo IIβ was a compensatory mechanism to maintain normal topo II catalytic activity (Grabowski *et al.*, 1999).

1.10 The development of tumour cell resistance to chemotherapeutic drugs

There are a variety of mechanisms that cancer cells have developed to increase the chances of survival when treated with anticancer drugs. These include decreasing the amount of intracellular drug either by increasing the drug efflux, decreasing the drug accumulation, or decreasing drug uptake or transport. Increasing the repair of any DNA damage, altering the target of the drugs and other metabolic changes have also been observed. A single tumour cell can therefore become resistant to a wide variety of chemotherapeutic drugs. The roles of MDR1, MDR3, MRP1 and Hsp70 in drug resistance are summarized below.

1.10.1 Multidrug Resistance Protein 1 (MDR1)

MDR1 (P-glycoprotein (P-gp)) is a 170 kDa membrane glycoprotein which, with the related protein MDR3, belongs to the ATP-binding cassette superfamily (ABC family) (reviewed in Cole & Deeley, 1998; Volm, 1998; Ramachandran & Melnick, 1999; Naito *et al.*, 1999; Tan *et al.*, 2000). MDR1 acts as an ATP-dependent drug efflux

pump and is highly expressed in tissues such as the colon, small intestine, liver, kidney and the pancreas (reviewed in Tan *et al.*, 2000; Krishna & Mayer, 2000; Baldini, 1997). The proposed physiological roles of MDR1 include enhancing the secretion or limiting the absorption of xenobiotic compounds in the body, having a role in drug transport across the blood-brain barrier or a role in other protective barriers like the placenta (Tan *et al.*, 2000).

There has been substantial research carried out addressing the role of MDR1 in drug resistance. P-gp is an active transporter of drugs such as anthracyclines, epipodophyllotoxins and taxanes (Arts *et al.*, 2000; Naito *et al.*, 1999; Tan *et al.*, 2000) and the over-expression of P-gp is though to have a major role in both the development and maintenance of multidrug resistance (Slapak *et al.*, 1990; Wurzer *et al.*, 2000; Chen *et al.*, 2000; Wessel *et al.*, 1999; Roninson *et al.*, 1986; Mechetner *et al.*, 1998; Trock *et al.*, 1997; Holzmayer *et al.*, 1992; Bourhis *et al.*, 1989; Marie *et al.*, 1991).

1.10.2 Multidrug Resistance Protein 3 (MDR3)

MDR3 has extensive homology to the C-terminal of MDR1 (~89%) (van der Bliek *et al.*, 1987), with 80-100% identity in transmembrane domains and the ATP-binding region and the greatest divergence seen at the N-terminal (van der Bliek *et al.*, 1988). The exact physiological role of MDR3 is unclear, but MDR3 was shown to be essential for the secretion of phosphatidyl choline into bile (Crawford *et al.*, 1997; Smit *et al.*, 1994; van Helvoort *et al.*, 1996). The role of MDR3 in drug resistance is uncertain, with evidence that the protein cannot enhance drug resistance (Smith *et al.*, 2000; Schinkel *et al.*, 1991).

1.10.3 Multidrug Resistance-associated Protein 1 (MRP1)

MRP1 is a 190 kDa membrane glycoprotein and like MDR1, is a member of the ABC family. Even though MRP1 also acts as an ATP-dependent efflux pump, the protein has only 15% amino acid similarity to MDR1 (reviewed in Naito *et al.*, 1999; Tan *et al.*, 2000; Ramachandran & Melnick, 1999). MRP1 is expressed in a wide range of

tissues including muscle, lung, breast, bladder and spleen and the physiological role for MRP1 is thought to involve the ATP-dependent unidirectional transport of glutathione conjugates like leukotriene C4 (reviewed in Krishna & Mayer, 2000).

MRP1 is thought to have a role in drug resistance through its ability to transport a variety of substrates. It has been shown that MRP1 can transport glutathione drug conjugates, neutral or mildly cationic drugs, small neutral peptides and can co-transport glutathione and unmodified vincristine (Paul *et al.*, 1996; Evers *et al.*, 1996; Leier *et al.*, 1994; Jedlitschky *et al.*, 1996; Loe *et al.*, 1998; de Jong *et al.*, 2001). The over-expression of MRP1 is thought to correlate with drug resistance to doxorubicin, etoposide and daunorubicin (Zaman *et al.*, 1994; Tan *et al.*, 2000; Grant *et al.*, 1994; Cole *et al.*, 1994; Hooijberg *et al.*, 1999; Zaman *et al.*, 1993; Wessel *et al.*, 1997; Cole *et al.*, 1992; Slapak *et al.*, 1994; Diah *et al.*, 2001; Kruh *et al.*, 1994).

1.10.4 Heat shock proteins

Heat shock proteins (hsp) are highly conserved molecules found in nearly every cellular compartment. These molecules are either constitutively expressed or inducible under a variety of physiological conditions such as heat shock, drug exposure or infection. While a number of families have been identified, the Hsp70 family and the small heat shock protein are of special interest in the development of drug resistance.

Hsp70 family members act as ATP-dependent molecular chaperones which are proposed to have roles in the correct folding of newly synthesized proteins, protein sorting and transport between cellular compartments, dissociating protein aggregation and binding to partially denatured proteins to prevent unwanted interactions (reviewed in Creagh *et al.*, 2000; Sarto *et al.*, 2000).

Heatshock proteins may be involved in the development or maintenance of drug resistance. The short term exposure of murine cardiac or HeLa cells to doxorubicin has been shown to induce Hsp70 expression or enhance nucleolar levels (Huber, 1992; Abe *et al.*, 1996), while heat shock proteins seem to have a role in drug resistance in

breast cancer cell lines (Ciocca *et al.*, 1992; Hansen *et al.*, 1999; Oesterreich *et al.*, 1993; Vargas-Roig *et al.*, 1998).

1.11 The current study

While the regulation of the topo IIα promoter has been investigated under various experimental conditions, the mechanisms involved during drug resistance are still unclear with recent research indicating a drug- and cell type-specific component. In this current study, the regulation of the topo IIα promoter in breast cancer cells after doxorubicin exposure was investigated. Breast cancer cell lines that differed in the p53 and ER status were used for these experiments. A summary of the potential importance of p53 and ER is presented below.

1.11.1 p53

p53 has a critical role in the maintenance of genomic integrity in cells exposed to a variety of stressors. In response to DNA damage or drugs such as doxorubicin, the cellular levels of p53 are increased (Tanikawa *et al.*, 2001; Bunz *et al.*, 1999; Zhang *et al.*, 1999; Cowell *et al.*, 2000). p53 can regulate a variety of genes including those involved in cell cycle arrest, apoptosis or genomic stability (reviewed in Somasundaram & El-Deiry, 2000). p53 is tightly regulated in cells under normal conditions through an interaction with MDM2 which targets the p53 for degradation. Under stress conditions, this interaction is inhibited and an up-regulation of p53 is observed. The up-regulation of p53 increased sensitivity to doxorubicin and etoposide (Hochhauser *et al.*, 1999; Seth *et al.*, 1997).

Approximately 50% of cancers contain p53 mutations, with mutant p53 found at elevated levels due to the loss of MDM2-mediated degradation (Somasundaram & El-Deiry, 2000). Breast tumours commonly contain p53 mutations (Aas *et al.*, 1996; van Slooten *et al.*, 1999; Hill & Sommer, 2002), with specific mutations (i.e. in direct DNA contact residues) potentially involved in doxorubicin resistance (O'Connor *et al.*, 1997; Aas *et al.*, 1996).

Another consideration was that the topo IIα promoter may be negatively regulated by p53 (Sandri *et al.*, 1996; Wang *et al.*, 1997b; Joshi *et al.*, 2000). Mutant p53 can act with a gain-of-function action where the mutant protein is able to transactivate or repress specific genes or pathways (reviewed in Sigal & Rotter, 2000; He *et al.*, 2002). Due to these potential effects and as p53 could have a role in drug resistance, two breast cancer cell lines were used which had either wild type or mutant p53 status. The two cell lines chosen were MCF-7 and MDA-MB-231.

The MCF-7 cell line has functional wild type p53 while MDA-MB-231 cells over-express a non-functional mutant p53 protein, with strong nuclear localization observed (Elstner *et al.*, 1995; Gnjatic *et al.*, 1998; O'Connor *et al.*, 1997; Xu & Loo, 2001). This mutant p53 protein contained a missense mutation in the DNA binding domain at codon 280, in exon 8 AGA→ AAA (Arg → Lys) (Bartek *et al.*, 1990). Arg280 is in the H2 helix which binds into the major groove and so this p53 protein was classed as a DNA contact mutation (Cho *et al.*, 1994). While this protein is likely to be folded (Bartek *et al.*, 1990), it is probably unable to transactivate due to the lack of DNA binding, but as other DNA contact mutants retain the ability to transactivate under appropriate conditions, it may be possible for the p53 R280K mutant to transactivate as well (reviewed in Sigal & Rotter, 2000; O'Connor *et al.*, 1997; van Slooten *et al.*, 1999).

1.11.2 Estrogen receptor

It was also noted that the MCF-7 and MDA-MB-231 cell lines differ in estrogen receptor (ER) status, with MCF-7 cells shown to express ER while MDA-MB-231 cells do not (Thompson & Weigel, 1998). This difference may influence cellular response to drugs. While the MCF-7 cells were not specifically grown in the presence of estrogen, it has been observed that the media the cells are grown in may contain contaminants including phenol red which have significant estrogenic activity (Berthosis *et al.*, 1986; Welshon *et al.*, 1988; Bindall *et al.*, 1988).

The two 17β-estradiol (E2) receptors (ERα and ERβ) act as ligand-dependent transcription factors, can bind to estrogen response elements (EREs) in the promoters

of specific genes and through interactions with other factors modify transcription. These factors, through interactions with other transcription factors, can also modulate non-ERE-containing promoters. It has been shown that estrogen and ERs may influence various signaling pathways including PKA, PKC and various MAPK pathways (Segars & Driggers, 2002). It should also be noted that the presence of the estrogen receptor within a cell may modify drug resistance or gene transcription. Teixeira *et al.* (1995) and Zampieri *et al.* (2002) showed that estrogen enhanced resistance to doxorubicin in MCF-7 cells. One mechanism of resistance was thought to be due to the enhancement of the antiapoptotic factor Bcl-2 protein levels by the estrogen. Another proposed mechanism for the estrogen-induced resistance was the interaction of ERα with Sp1 and AP-1. This interaction was proposed to account for the up-regulation of *MDR1* promoter seen in these cells (Zampieri *et al.*, 2002).

1.11.3 Experimental outline

The initial aims of this research were to use an experimental system to identify the specific regions of the topo IIα promoter involved in the regulation seen upon drug exposure of two breast cancer cell lines. To this end, a series of stable cell lines, containing deletions of the topo IIα promoter linked to a reporter gene, were exposed to doxorubicin. It was proposed that the differential expression of the reporter genes attached to these deletion constructs after drug exposure could be used to identify the specific promoter regions involved. If successful, this system could be used in a range of different cell types exposed to different topo II-targeted drugs to identify cell type- or drug-specific mechanisms. This research is detailed in the following chapters. Chapter Three outlines the verification of the use of the experimental system and summarises the analysis of specific cell lines after drug exposure. Chapter Four details the alterations to specific transcription factors in selected cell lines that had alterations to the amounts of topo IIα. Chapter Five investigates other potential resistance mechanisms in the specific cell lines while Chapter Six briefly outlines the use of *in vivo* footprinting as a potential method to observe changes to the *in vivo* binding of proteins to the topo IIα promoter during drug exposure.

Chapter Two Materials and Methods

2.1 Materials

pHGH1964, pHGH617, pHGH144, pHGH101 and pHGH56 were a kind gift from Dr. Richard Isaacs, Palmerston North Hospital.

The pGL3Basic luciferase reporter vector was from Promega Corporation, WI, USA. The SV40:HGH vector was a gift from Richard Isaacs, Palmerston North Hospital. pMAMNeo was from Clontech BD Biosciences, CA, USA. The majority of restriction enzymes, T4 DNA ligase, ligase buffer and LB base medium were from Invitrogen Life Technologies Inc., MD, USA. Proteinase K, calf alkaline phosphatase, the HiPure™ PCR Product Purification Kit and *Taq* polymerase were from Roche Molecular Biochemicals, Mannheim, Germany. Ampillicin, RNase, chicken egg white lysozyme, Red *Taq* polymerase and synthetic oligonucleotides were from Sigma Chemical Company, St. Louis, MO, USA. The QIAGEN Plasmid Preparation Kit and QIAGEN PCR Purification Kit were from Qiagen Pty Ltd., Victoria, Australia. The Quantum® Prep Plasmid Miniprep Kit was from BioRad Laboratories, CA, USA.

The MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection. The CaCO-2, K562, HeLa and HepG2 cell lines were generous gifts.

DMEM and MEM media, sodium pyruvate, and DMSO were all tissue culture grade from Sigma Chemical Company, St. Louis, MO, USA. Crystalline zinc bovine insulin (4 mg/mL), glutamine (200 mM), penicillin (5000 U/mL), streptomycin (5000 μg/mL), 2.5% trypsin, bovine calf serum and G418 (neomycin sulphate) were from Invitrogen Life Technologies Inc., MD, USA. Cryotubes were from Nunc Inc., Naperville, IL, USA. The 0.2 μM SartoLab P media filters were from Sartorius AG, Germany. The filters for sterilization of transfection buffers were Supor Acrodisc 32 0.2 μM filters from Gelman Sciences, MI, USA. The 75 cm^2 and 25 cm^2 filter top 0.2 μM vented

flasks and the 150 mm plates were from Nunc Inc., Naperville, IL, USA. The 60 mm and 100 mm plates were from Falcon, NJ, USA. The 12-well plates with lids were from Corning Costar Corporation, MA, USA. The doxorubicin (2 mg/mL) was a gift from Richard Isaacs, Palmerston North Hospital. The 0.4 % trypan blue and propidium iodide were from Sigma Chemical Company, St. Louis, MO, USA.

Lipofectamine™ was from Invitrogen Life Technologies Inc., MD, USA and Fugene™ 6 was from Roche Molecular Biochemicals, Mannheim, Germany. The Luciferase Assay Kit was from Promega Corporation, WI, USA. This kit contained the cell lysis buffer (x5), luciferase assay buffer and the luciferase assay substrate. The pSV-β-galactosidase control plasmid was from Promega Corporation, WI, USA. The cell scraper (23 cm) was from Nunc Inc., Naperville, IL, USA. The polystyrene luminometer cuvettes were from Crellin B.V., Rotterdam, The Netherlands.

The human growth hormone ELISA kit was supplied by Roche Molecular Biochemicals, Mannheim, Germany.

TRIZOL™ LS Reagent and DEPC were from Applichem, Darmstadt, Germany. The LightCycler-FastStart DNA Master SYBR Green I Kit and glass capillaries were from Roche Molecular Biochemicals, Mannheim, Germany. The MMLV reverse transcriptase and reagents, Deoxyribonuclease I (bovine pancreas, amplification grade) and random hexamers were from Invitrogen Life Technologies MD, USA.

Acrylamide (40% 29:1), Bradford reagent and the SDS-PAGE broad range molecular weight standards were from Bio Rad Laboratories, CA, USA. The nylon membrane (positively charged), the Chemiluminescence Blotting Substrate (POD) Kit and Complete™ Mini protease inhibitors were from Roche Molecular Biochemicals, Mannheim, Germany. The 3 MM paper was from Whatman, England. The PVDF membrane was from BioRad Laboratories, CA, USA. The 0.45 μM nitrocellulose blotting membrane was from Sartorius AG, Germany.

The following primary antibodies were from Santa Cruz Biotechnology, CA, USA; Sp3 (D-20)-G (rabbit polyclonal, antibodies directed against the C-terminal of human Sp3), Sp1 (PEP2)-G (rabbit polyclonal, internal domain of rat/human Sp1), CBF-B (C-18) (goat polyclonal, C-terminal human NF-YA), Topo IIα (K-19) (goat polyclonal, C-terminal human topo IIα), Topo IIβ (F-19) (goat polyclonal, C-terminal human topo IIβ), MDR1 (P-gp)(C-19) (goat polyclonal, C-terminal human MDR1 = MDR3), MRP1 (C-20) (goat polyclonal, C-terminal human MRP1) and Hsp70 (K-20) (goat polyclonal C-terminal human Hsp70). The monoclonal α-tubulin antibody (anti-α-tubulin clone DM 1A) was from Sigma Chemical Company, St. Louis, MO, USA.

The anti-rabbit, anti-mouse or anti-goat IgG (whole molecule) peroxidase conjugates were from Sigma Chemical Company, St. Louis, MO, USA. The X-ray film was from Fuji Photo Film Company, Japan. Developer and fixer were from Eastman Kodak, NY, USA.

Spermine, spermidine, gel purified and biotinylated primers were from Sigma Chemical Company, St. Louis, MO, USA. Sequenase™ version 2.0 DNA sequencing kit (containing enzyme, buffer and nucleotides) was from USB, OH, USA. DNAse I (bovine pancreas, grade II) and *E. coli* tRNA were from Roche Molecular Biochemicals, Mannheim, Germany. Dynabeads® were from Dynal Biotech, ASA, Norway. The Ready-To-Go DNA Labeling Beads (-dCTP) and ProbeQuant™ G-50 microcolumns were from Amersham Pharmacia Biotechnology Inc., Piscataway, New York, USA. [^{32}P]-α-CTP was from Amersham Biosciences, Uppsala, Sweden.

2.2 Methods

2.2.1 Preparation of reporter constructs

2.2.1.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to amplify specific DNA regions. PCR reactions were set up with a final concentration of 1x reaction buffer (10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 3 mM dNTPs, 5 ng/μL of each primer, 0.06 U/μL *Taq* polymerase and 0.02 ng/μL of genomic DNA. Fifty cycles of the following conditions were used; denaturing temperature of 95°C for 30 seconds, annealing temperature of 60°C for 30 seconds and an elongation temperature of 72°C for 2 minutes. The -1964, -1861, -1463, -1163 and -894 to +90 topoisomerase IIα promoter constructs were prepared by this method with the ATG-BAM primer as the reverse primer and the appropriate forward primer (Appendix 7). The forward primers had a *Xba* I site added to the 5' end, while ATG-BAM had a 5' *Bam* HI site added.

2.2.1.2 Restriction endonuclease digests

To perform directional cloning, restriction digests were used to obtain vectors and inserts that have compatible ends. To perform endonuclease digestion, the DNA was digested with 10U of the required endonuclease in the appropriate 1x reaction buffer and incubated at 37°C for 1 hour. By this method 50 μL of the PCR product or 1 μg of the hGH reporter vector (SV40:pHGH (pGEMZf+ with the SV40 promoter linked to the human growth hormone reporter gene)) were digested with 10 U of *Xba* I and *Bam* HI.

2.2.1.3 Purification of the digestion products

The PCR digestion products were purified using the HiPure™ PCR Product Purification Kit according to manufacturers instructions. The HiPure™ Kit consists of

spin columns containing pretreated glass fibres. Nucleic acids are selectively bound to the glass fibres allowing the removal of contaminants from the DNA. The purified DNA is then eluted in low salt conditions. To minimize self ligation, 5' phosphates were removed from the cut ends of the vector using calf alkaline phosphatase and the vector DNA was then purified by phenol/chloroform extraction followed by ethanol precipitation (Sambrook & Russell, 2001). Both insert and vector were gel quantified.

2.2.1.4 Ligation of vector and insert and transformation

50 ng of insert and 48 ng vector (vector : insert ratio 1:3) were added to 2 μL 5x T4 Ligase Buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% w/v polyethylene glycol-800), 1 U T4 ligase and H_2O (to a total of 10 μL) and incubated at 25°C for 1 hour to allow ligation to occur. Ultra-competent XL-1 blue cells were produced by C. Flyger, Institute of Molecular BioSciences, Massey University by the method described in Inoue *et al.* (1990). Transformation of XL-1 cells was carried out according to Pope & Kent (1996).

2.2.1.5 Subcloning of the topoisomerase IIα constructs into pGL3Basic (pGL3B)

The above hGH reporter constructs were digested with *Xba* I *and Bam* HI (pHGH1964, 1861, 1463, 894 and 56) or *Xba* I and *Hind* III (pHGH144 and 101), to subclone the topo IIα promoter constructs into the luciferase reporter vector pGL3Basic. The pGL3-Basic (pGL3B) vector does not contain an SV40 enhancer element (Appendix 6) and so the "expression of luciferase activity.... depends on insertion and proper orientation of a functional promoter upstream of *luc*+". This vector has been modified to enhance luciferase expression and improve the *in vivo* vector stability. These modifications include the removal of the signal for peroxisome targeting in the luciferase cDNA and the removal of transcription factor consensus binding sequences that may cause spurious transcription (Promega pGL3B Luciferase Reporter Vector Technical Manual, 1994).

The inserts were gel purified using the HiPure™ PCR Product Purification Kit and quantified as described previously. The pGL3B vector was prepared as described previously (*Nhe* I and *Hind* III for the -101 and -144 fragment) or *Nhe* I and *Bgl* II (for the other constructs). The ligation of insert and vector was carried out as described above.

2.2.1.6 Isolation of plasmid DNA

Isolation of plasmid DNA for screening purposes was prepared by the rapid-boil procedure of Holmes & Quigley (1981). When high purity DNA was required (e.g sequencing reactions) the Quantum® Prep Plasmid Miniprep Kit was used following the manufacturers' instructions. This system uses alkaline lysis to remove the plasmid DNA from the cell and then the selective binding of the plasmid DNA to the Quantum® prep matrix to purify the DNA.

2.2.1.7 Large scale preparation of plasmid DNA

Five mL of LB broth containing ampicillin (100 μg/mL) was inoculated with a single bacterial colony and grown overnight with shaking at 37°C. All of this culture was used to inoculate a flask containing 500 mL of LB broth containing 100 μg/mL ampicillin which was then incubated overnight at 37°C. The QIAGEN Plasmid Kit was used to produce large scale plasmid DNA for transfections as described in the QIAGEN Plasmid Handbook. This method is based on alkaline lysis of bacteria, followed by neutralization to precipitate chromosomal DNA and protein. Plasmid DNA was then purified by anion exchange chromatography using a modified DEAE-silica gel.

2.2.1.8 Quantification of DNA

The plasmid DNA was quantified using the nucleic acid (200-350 nm) scan programme on the Pharmacia Biotech Ultrospec 300 UV/Visible Spectrophotometer. The purity of the DNA was assessed by the A260/A280 absorbance ratio. A ratio of 1.8 is expected

for pure DNA, ratios greater than 1.8 were expected for RNA, while ratios less than 1.8 indicate protein contamination.

2.2.1.9 Sequencing of DNA

DNA sequencing was carried out by Lorraine Berry (MuSeq facility, Institute of Molecular BioSciences, Massey University). Dideoxy cycle sequencing with Big Dye terminators (version 2.0, PE Biosystems, Foster City, CA, USA) was performed and analysed on a ABI 377-18/36 automated DNA sequencer according to manufacturer's instructions.

2.2.2 The routine maintenance, passage and use of tissue culture cell lines

All tissue culture manipulations prior to harvesting were performed in a laminar flow workstation under sterile conditions. All the cells were grown in a 5% 37°C CO_2 incubator with a humidified atmosphere.

2.2.2.1 Preparation of tissue culture media

Cells were grown in T75 or T25 tissue culture vented flasks. Media was prepared according to the manufacturers instructions. The pH was adjusted to 6.8 so that the final pH would be approximately 7.1 as the pH rises 0.2-0.3 units upon filter sterilization. MCF-7 cells were grown on MEM with 2 mg insulin added to 200 mL of media. MDA-MB-231, HepG2 and HeLa cells were grown on MEM media with 10% FCS while CaCO-2 cells required 20% FCS in MEM. K562 suspension cells required 10% FCS in DMEM media containing 20 mM HEPES. All media was supplemented with penicillin and streptomycin as a 100x stock.

2.2.2.2 Establishment of cell lines from frozen stocks or after initial receipt of the cell lines

All cell lines used were grown from stocks stored in liquid nitrogen. The frozen cells were quickly thawed, added to 5 mL media, centrifuged and the pellet resuspended in media. The cell suspension was added to 6 mL of media in T25 flasks. Once the cells had been passaged once, a new batch of cells were frozen as described below.

2.2.2.3 Freezing of cells for liquid nitrogen storage

Cells for liquid nitrogen storage were prepared by passaging minimally-passaged cells at ~100% confluence and resuspending in bovine calf serum 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.

2.2.2.4 Passage of cells for cell maintenance and transfections

Adherent cells were grown to 80-100% confluence in T75 flasks before passage into new flasks to maintain stocks, or onto plates for transfections, RNA extraction, nuclei or cell extract preparation. Cells were passaged by washing with 1 mL of 0.25% trypsin in PBSE (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 0.5 mM EDTA). The flasks were then left lying flat for ~10 minutes. Flasks were knocked to help dislodge the cells and the cells were resuspended by aspiration with 10 mL media. One mL of this suspension was added to T75 tissue culture flasks containing 12 mL media for the maintenance of stocks. Two or four drops (with a plastic disposable pipette) were added to each well of a 12-well tissue culture plate containing 800 µL media for transfections. Cells were placed in a 37°C, 5% CO_2 incubator in a humidified atmosphere. K562 suspension cells were grown until cell density was ~1 x 10^6 cells/mL. The cells were counted using a haemacytometer as described in Davis (1994) pp 127-130. The average of 3 cell counts was determined. ~10^6 cells were placed into 6 mL of fresh media.

2.2.2.5 Transient transfections

Fresh media was added to the cells two hours prior to transfection. Cells were transfected using a calcium phosphate co-precipitation method as described in Sambrook & Russell (2001). In this method, the DNA is in a chemical environment which induces uptake by endocytosis into the cell by unknown mechanisms. A number of factors such as reagent or media pH and DNA purity can affect the calcium phosphate method, but importantly for transient transfections, the cells transfected by this method tend to contain a representative sampling of the different plasmids in the precipitate (Ausubel *et al.*, 1991). This method is also cost-effective and so was used in this study.

50 µL of Buffer A (0.5 M $CaCl_2$, 0.1 M HEPES, pH 7.05-7.12) was added to 50 µL of DNA and water. 100 µL of Buffer B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM sodium dihydrogen phosphate, 0.75 mM disodium hydrogen phosphate) was added to the plasmid DNA and the mixture was immediately vortexed. After a 15 minute incubation at room temperature, the DNA suspension was dispersed into the medium on the prepared cells. 2 µg of pSV-β-gal control vector and 4 µg of reporter vector were used unless otherwise stated. Each treatment was carried out in triplicate within each transfection experiment. Cells were incubated in the presence of the DNA precipitate for 6 hours and then washed twice with media without additions. The cells were then incubated with fresh complete media for 18 hours in the 37°C, 5% CO_2 incubator.

2.2.2.6 Luciferase assay

The cells were harvested as described in the Luciferase Assay System Technical Bulletin. Luciferase assays are sensitive and rapid and are based on the reaction where the firefly luciferase protein in the presence of ATP and substrate can emit light (562 nm) Luciferin + ATP + O_2 $\xrightarrow{Mg2+}$ oxyluciferin + AMP + PPi + CO_2 + light (reviewed in Bronstein *et al.*, 1994; Gould & Subramani, 1988). The assays for the luciferase reporter gene activity were carried out as described in the Luciferase Kit Technical Bulletin.

2.2.2.7 β-galactosidase assay

β-galactosidase assays were carried out as described in Herbomel *et al.* (1984). 100 μL β-galactosidase assay buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$) and 50 μL ONPG (2 mg/ml ONPG in 60 mM Na_2HPO_4, 40 mM NaH_2PO_4) were mixed with 20 μL of cell extract on a multi-well plate and incubated at 37°C until an adequate colour (3-8 hours) was produced. 50 μL of 1 M sodium carbonate was then added to stop the reaction. Absorbances were measured at 405 nm against a blank containing 20 μL cell lysis buffer. The β-galactosidase values, expressed as total absorbance units, were used to normalize the luciferase values.

2.2.2.8 The production of stable breast cancer cell lines

The topoisomerase IIα reporter vector does not contain a mammalian selectable marker so co-transfection with a vector containing a selectable marker was required. The vector used was pMAMNeo which contains the AHR (aminoglycoside phosphotransferase) gene which confers resistance to neomycin (G418). G418 blocks protein synthesis through interference of ribosomal activity. Both cell lines were transfected with a specific topoisomerase IIα reporter vector and the pMAMNeo vector using either the calcium phosphate or liposomal methods (Fugene™ or Lipofectamine™). The reporter vector was added in 5x excess of pMAMNeo, so that the cells would have a higher chance of taking up the reporter vector that did not have a selectable marker. Upon selection by G418, the chances of random integration of both the pMAMNeo and the topoisomerase IIα-hGH into the genome increase. Cells which integrate only the pMAMNeo vector and the cells which integrate both vectors will survive selection by G418 so additional screening for the expression of hGH protein was required. After transfection, the media was replaced with fresh media supplemented with 800 μg/mL G418. This media was changed every 3-4 days until individual colonies were seen.

2.2.2.9 Assay for human growth hormone (hGH)

To detect the presence of human growth hormone, the hGH ELISA Kit was used as per manufacturers instructions. This is a colorimetric enzyme immunoassay which allows the quantitative determination of human growth hormone. hGH is captured through interactions with anti-hGH antibodies bound at a surface of a microtitre plate. Anti-hGH-DIG antibodies then bind to the hGH followed by DIG antibodies conjugated to peroxidase. The peroxidase then catalyses the cleavage of the substrate to produce a coloured product. The multi-well plate reader was used to measure absorbance at 405 nm with a reference wavelength of 495 nm. A standard curve was produced and the unknown hGH values were obtained by linear regression.

2.2.2.10 Screening for the topoisomerase IIα stables

After 4-6 weeks under constant G418 selection, individual colonies derived from single G418-resistant cells were transferred into separate wells of a 12 well plate. After a week, the media was tested for the presence of hGH by hGH ELISA. Positive stable cell lines were then frozen and/or maintained in media containing 400 μg/mL G418.

2.2.2.11 Exposure of the cells to doxorubicin

Cells were plated on 12-well plates and left overnight. Concentrations of doxorubicin (Adriamycin®) were added to the media for a specified time. The media was then removed and after 3 washes with media (no additions), fresh complete media was added. After 3-6 weeks, single colonies were isolated, amplified and frozen down for storage.

2.2.2.12 Determining the hGH:cell ratio

To determine the hGH:cell ratio, media on control and surviving cell lines was replaced with new media. After 6 hours the medium was removed and hGH concentration determined. To count the cell number, the cells were harvested with trypsin (1x in

PBSE), and 50 μL of the cell suspension added to 50 μL 0.4% trypan blue. The number of cells which excluded the dye was used to determine the % of viable cells. Cells were then counted using a haemacytometer as described in Davis (1994) pp 127-130. The average of 3 cell counts was determined. The ratio of hGH concentration to cell number for the control (cells not exposed to drug) was arbitrarily set at 100% and the sample's ratio compared to this value.

2.2.2.13 Preparation of cells for FACS analysis

Cells were plated ~50% confluence and after 24 hours exposed to doxorubicin. After specified times the cells were harvested in trypsin and centrifuged at 4,000 rpm for 30 seconds. The cells were resuspended in 200 μL 70% ethanol in PBS and left at room temperature for 30 minutes, to fix the cells. After this time the cells could either be stored at 4°C for maximum of a week or stained for immediate FACS analysis. Staining was accomplished by pelleting the cells by centrifugation at 4,000 rpm for 30 seconds, resuspending in 1 mL staining solution (100 μg/mL RNase, 40 μg/mL propidium iodide in PBS) and incubating for 30 minutes at 37°C. FACS analysis using the Becton Dickinson Flow Cytometer was then used to determine the cell cycle profile of the cells.

2.2.2.14 Determination of population doubling time (PDT)

To determine the doubling time of a cell line, a specific number of cells (N_H) was plated, left for a specified time (t_2-t_1) and the number of cells counted (N_I). The population doubling time was determined using the formula from Davis (1994) pg. 126.

$- r = 3.32 (\log N_H - \log N_I)/ (t_2-t_1)$ $\quad$ $PDT = 1/r$

where N_H is the number of cells plated, N_I is the number of cells after time (t_2-t_1) has elapsed.

2.2.3 RT-PCR and real time RT-PCR analysis

2.2.3.1 The isolation of RNA

Cells used for the isolation of RNA were grown on 100 mm plates with 9 mL media. RNA extraction was carried out on cells that were 40-60% confluent. All solutions, pipettor tips and microfuge tubes used in RNA manipulations were treated with 0.05% DEPC overnight and autoclaved prior to use. Trizol™ was used to extract the RNA as per manufacturers' instructions. Trizol™ is a monophasic solution of phenol and guanidine isothiocyanate. This solution maintains the integrity of the RNA while disrupting the cells. Upon addition of chloroform, the sample separates into aqueous and organic phases, with the RNA found in the aqueous phase. The RNA was then isolated and precipitated with isopropanol. The RNA was quantified by A260/A280 absorbance on the Pharmacia Biotech Ultrospec 300 UV/Visible Spectrophotometer.

2.2.3.2 The production of cDNA products by reverse transcription

DNA was removed from the RNA samples prior to reverse transcription as follows; 6 μg of RNA was incubated with 6 U DNAse I (bovine pancreas, amplification grade) in 1x DNAse reaction buffer (20 mM Tris-HCl pH 8.4, 2 mM $MgCl_2$, 50 mM KCl) in a total volume of 50 μL at room temperature for 15 minutes. EDTA to a final volume of > 2 mM was then added. To inactivate the DNase I, the sample was heated to 65°C for 10 minutes and then chilled.

25 μL of the treated RNA was added to 15 μL of a cocktail mix so that the final concentration was 1x MMLV first strand buffer (50 mM Tris-HCl pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithioerythritol), 1 mM dNTPs, 0.01 M DTT, 0.625 ng/μL random hexamers and either 2 U/μL MMLV reverse transcriptase or water. This mixture was incubated for 1 hour at 37°C and subsequently for 5 minutes at 95°C to inactivate the enzyme. PCR using the cDNA as a template was then carried out. 1 μL of this reaction mix was added to each PCR reaction.

2.2.3.3 Primers and PCR conditions

The primers used for either RT-PCR or real time RT-PCR are detailed in Appendix 4. This table includes primer sequence, primer position and diagnostic digest details.

The PCR conditions used for the RT-PCR were

96°C	3 min	
96°C 50-55°C 72°C	30 sec 30 sec 1 min	40-50 cycles
72°C	3 min	

2.2.3.4 Real time RT-PCR using the LightCycler™

The primers used in the real time RT-PCR reactions had either been designed previously for RT-PCR or were designed using the Roche LightCycler Probe Design Software Version 1.0. The primers were designed to produce products with Tm ~55-60°C and GC content ~50% if possible; to avoid sequences which could have possible negative effects on PCR such as direct repeats, homopolymeric runs or inverse repeats and to span introns where possible to distinguish between products derived from genomic DNA and cDNA (Appendix 4).

Real time RT-PCR reactions were carried out as described in the FastStart SYBR Green I Kit instructions. 0.5 µM of each primer, 3-4 mM $MgCl_2$ in 1x master mix (containing *Taq* DNA polymerase, dUTP, dATP, dGTP, dCTP, SYBR Green I dye, 1 mM $MgCl_2$) was added to a final volume of a 15 µL. 5 µL of appropriately diluted first strand cDNA was then added. The cycling conditions were 1 cycle of 95°C 10 minutes, then 25-40 cycles of 95°C for 0 seconds, 55°C for 5 seconds and 72°C 16 seconds. The PCR efficiency of each primer set was determined from the slope of a standard curve of cycle number vs. log concentration (Efficiency = $10x^{-1/slope}$), see Appendix 5 (a) for an example.

The PCR efficiencies under various different $MgCl_2$ concentrations were determined. Primer sets with similar efficiencies (< 0.1 difference) could be quantified using the same standard curve (see Appendix 4), for example a 18S rRNA (3 mM $MgCl_2$, Efficiency 2.04) standard curve could be used in the quantification of topo IIα (4 mM $MgCl_2$, 1.98). 18S rRNA (3mM $MgCl_2$, 2.04) could be used as an internal control for topo IIα (4 mM $MgCl_2$, 1.98), hGH (4 mM $MgCl_2$, 1.95), MRP1 (3 mM $MgCl_2$, 2.04) and α-globin (4 mM $MgCl_2$, 1.97). As another control, the ratio of GAPDH/18S rRNA should be constant in cell lines derived from the same parental cell line.

To quantify a sample, real time RT-PCR reactions of the unknown (i.e. topo IIα) and the appropriate internal control (i.e. 18S rRNA) were performed for the RNA samples. A standard curve control reaction was also performed. This control had been used previously to prepare the standard curve and had a pre-assigned value. The crossover number of this control was used to calibrate the standard curve between different experiments. By using the standard curve, the "concentrations" of the samples can be worked out from the cross-over cycle number of the real time PCR reactions. The ratio of the "concentrations" of the unknown vs. the RNA control were then determined for each sample, with the control cell line ratio was arbitrarily set at 100%. See Appendix 5 (b) for an example. Diagnostic digests and the observed Tm were used to confirm the identity of the PCR product.

2.2.4 The immunoblot procedure

2.2.4.1 The preparation of cell extracts

Several methods for the preparation of cell extracts were used. For cell extracts prepared in a manner similar to that described in Ladias *et al.* (1992), cells were plated on 150 mm plates until 50% confluent. The cells were then washed with PBS and harvested in 1.5 mL of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl). After centrifugation at 12,000 rpm for 1 minute, the pellet was resuspended in 300 μL extraction buffer (40 mM Hepes pH 7.9, 0.4 M KCl, 1 mM DTT, 10% glycerol, 1x Complete™ Mini protease inhibitors). The cell suspension was freeze-

thawed three times using liquid nitrogen to disrupt the cells. The cell extract was centrifuged at 4°C at 14,000xg for 5 minutes and the supernatant was dispensed into 30 μL aliquots, snap-frozen in liquid nitrogen and stored at -70°C. This method was suitable for cytosolic and nuclear proteins.

Other methods were used that differ from that described in Ladias *et al.* (1992) in that detergents were used to lyse the cells and assist in the extraction of membrane-bound proteins. The cells were plated and harvested as above and the pellets resuspended in (a) 10 mM Tris pH 7.4, 150 mM NaCl, 0.5% triton X-100, 0.25% sodium azide and 1x Complete™ mini protease inhibitors (Epsztejn *et al.*, 1999); (b) 50 mM Hepes pH 7.4, 1% triton X-100, 10% glycerol and 1x Complete™ mini protease inhibitors (Montgomery *et al.*, 2000); (c) 50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% nonidet P-40 and 1x Complete™ mini protease inhibitors. The samples were then incubated on ice for 30 minutes (Meschini *et al.*, 2000); (d) 120 mM Tris pH 6.8, 200 mM DTT, 20% glycerol, 2.5% SDS and 1x Complete™ mini protease inhibitors. The samples were then heated at 85°C for 30 minutes and cooled (Nakajima *et al.*, 1995) and (e) 50 mM Tris pH 8.0, 425 mM NaCl, 1% nonidet P-40, 0.1% SDS, 10 μM β-mercaptoethanol and 1x Complete™ mini protease inhibitors (Covacci *et al.*, 2000). After extraction, the samples were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant aliquoted and stored at -70°C.

2.2.4.2 The immunoblotting methods used for the separation and transfer of proteins onto membrane

2.2.4.2 Protein gel and transfer protocols for topo IIα, Sp1, Sp3, NF-YA, α-tubulin, topo IIβ, P-gp, MRP1 and Hsp70 proteins

Protein concentrations of the cell extracts were determined using the Bradford reagent. Proteins in the cell extract (5-20 μg) were separated by polyacrylamide (8%) gel electrophoresis in the presence of SDS (Laemmli, 1970) using the buffer system described by Ornstein (1964) and Davis (1964). Proteins were transferred to nylon membrane by electroblotting with transfer buffer (25 mM Tris, 192 mM Glycine pH

8.3) for 90 minutes at 450 mA. Gels were then stained with Coomassie Blue (0.1% R-250 40% methanol, 10% acetic acid) and destained with Destain I (50% methanol, 10% acetic acid) and Destain II (5% methanol, 7% acetic acid) to determine if efficient transfer had occurred.

A range of different transfer conditions were attempted to detect the topo IIβ, P-gp and MRP1 proteins including alternative transfer buffer (50 mM CAPS pH 11.0), the manipulation of the length or amperage of transfer, or alternate membranes PVDF and nitrocellulose.

2.2.4.3. Immunodetection

The membrane was cut so the simultaneous probing of the same membrane with multiple antibodies could be carried out. The membrane was blocked for 2-3 hr at room temperature using 1% blocking reagent (Chemiluminescence Blotting Substrate (POD) Kit) in TBST (130 mM Tris base, 25 mM NaCl, pH 7.6, 1% triton X-100). The primary antibodies were diluted in 0.5% blocking solution (1:1,000 for topo IIα, Sp3 and Sp1, 1:500 for NF-YA, Hsp70, topo IIβ, P-gp and MRP1, 1:2,000 for α-tubulin). Membranes were incubated overnight at 4°C with the primary antibodies, and after washing with TBST and 0.5% blocking solution, were incubated with peroxidase-conjugate IgG secondary antibodies (1:10,000 dilution for topo IIα (goat) and Sp1 (rabbit), 1:5,000 α-tubulin (mouse), 1:4,000 for Sp3 (rabbit), 1:2,500 for NF-YA (goat), Hsp70 (goat), P-gp (goat) and MRP1 (goat)) for 30 minutes at room temperature. The membranes were then washed for an hour with TBST. Bands were detected using the chemiluminescent reagents as described in the manufacturers instructions. Both X-ray film and the Fujifilm Intelligent Dark Box were used to detect the chemiluminescent signals. The program Image Gauge was used to quantify the signals.

2.2.5 In vivo footprinting

The method for *in vivo* footprinting was adapted from that described in Carey & Smale (2000) pp 350-353 for the nuclei isolation and DNase I digestion and Gould (1998) pp 80-107 for the rest of the protocol.

2.2.5.1 Isolation of nuclei

To isolate nuclei, the cells (50% confluent, T25 flask) were harvested in media and pelleted at 2,000 rpm for 10 minutes at 4°C. The pellet was washed in ice cold PBS and the cells centrifuged at 2,000 rpm for 10 minutes at 4°C. The cells were resuspended in NP-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM Mg Cl_2, 0.5% nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine) and incubated on ice for 5 minutes. To pellet the nuclei, the sample was centrifuged at 2,000 rpm for 10 minutes at 4°C.

2.2.5.2 DNase I digestion

The nuclei were then exposed to DNase I. The nuclei pellet was initially washed with Buffer A (100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 0.15 mM spermine, 0.5 mM spermidine), centrifuged at 2,000 rpm for 10 minutes at 4°C and the supernatant carefully removed. The nuclei were then resuspended in 100 µL of Buffer A (containing 1 mM $CaCl_2$). To 100 µL of nuclei, 3 µg of DNAse I (bovine pancreas, grade II) was added and the samples incubated for 7 minutes at 37°C. To stop the DNase I digestion, 2 µL of 0.5 M EDTA and 100 µL of Buffer A was added. This was followed by the addition of 3 µL of proteinase K (25 mg/mL) and 100 µL of 20% SDS. The samples were then incubated overnight at 37°C. The DNA was treated with 2 µL RNase (10 mg/mL) at 37°C for 2 hours, purified using phenol/chloroform extraction, ethanol precipitated and the pellet resuspended in TE.

2.2.5.3 Alkaline gel electrophoresis

To confirm that the DNase I digests produced single strand DNA fragments between 300-600 bp long, alkaline gel electrophoresis was performed as described in Sambrook & Russell (2001). PCR products sized from 180-450 bp were also electrophoresed on the gel as size markers.

2.2.5.4 Primer extension

To perform primer extension, 0.5-2 μg DNA was added to 0.6 pmol of biotinylated primer #1 (5' GTGACACTTCCATGGTGAC 3') and 1x Sequenase™ buffer (40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) in H_20 to a total volume of 15 μL. The sample was heated to 95°C for 3 min, and then to 45°C for 30 min. The sample was then chilled on ice. 7.5 μL of Mg-dNTP stock (20 mM $MgCl_2$, 20 mM DTT, 0.25 mM each dNTP) was then added to each reaction followed by 5 U Sequenase™ version 2.0 and the samples were incubated at 48°C for 15 min. The samples were then placed on ice, Tris-HCl pH 7.7 was added to a final concentration of 60 mM, and the samples heated at 67°C for 15 min to inactivate the enzyme. The cooled samples were ready for ligation.

2.2.5.5 The ligation reaction

A linker was then blunt end ligated to the primer extension products. To prepare the linker stock (20 pmol/μL), 20 pmol/μL 25-mer (5'GCGGTGACCCGGGAGA TCTGAATTC 3') and 20 pmol/μL 11-mer (5' GAATTCAGATC 3') were mixed in 250 mM Tris-HCl pH 7.7. The solution was heated for 95°C for 3 min, then over two hours, the solution was allow to cool gradually from 70°C to 4°C. The ligation mix was prepared (13.33 mM $MgCl_2$, 30 mM DTT, 1.66 mM ATP, 83.3 μg/mL BSA, 100 pmol Linker stock, 3 U/reaction T4 DNA Ligase) and 45 μL of the ligation mix was added to 30 μL primer extension product, and incubated overnight at 18-20°C.

2.2.5.6 Extension product capture

Magnetic streptavidin beads were used to purify the biotinylated ligation product. The beads were washed with 2x BW buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl), resuspended in 2x BW buffer and an equal volume of ligation mix was added. The samples were then incubated for 15 minutes at room temperature. The beads were washed with 2x BW buffer. Freshly prepared 0.15 M NaOH (37 µL) was then added to the beads and the mixture incubated at 37°C 10 min. The supernatant was transferred into new tubes and firstly 3.75 µL 2 M Tris pH 7.7 then 37 µL 0.15 M HCl was added to neutralize the solution. 9.4 µL ligation stop mix (2.7 M Na acetate pH 5.2, 1 mg/ml *E coli*. tRNA) was then added. To ethanol precipitate the ligation product, 220 µL 100% EtOH was added to the samples which were placed on dry ice for 20 minutes, and then centrifuged at 15,000xg at 4°C for 15 min. The pellet was washed with 75% EtOH, dried and resuspended in 50 µL H_2O.

2.2.5.7 PCR amplification

To the 50 µL of DNA (from section 2.2.5.6), 50 µL of 2x *Taq* polymerase mix (2x *Taq* buffer (20 mM Tris-HCl pH 8.3, 100 mM KCl), 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 pmol/µL primer #2 (5' CTTATGGTGACGGTCGTGAAGGGGC 3'), 0.2 pmol/µL linker primer, 0.06 U/µL *Taq* polymerase) was added.

The PCR conditions used were :

1 min 95°C 2 min 60°C 3 min 76°C	⎪	18-20 cycles

Once PCR was completed, 10 µL booster mix (1 U *Taq* polymerase/10 µL of 1x *Taq* polymerase mix) was added and the samples incubated at 74°C for 10 min. 15.5 µL *Taq* stop solution (2.4 M sodium acetate pH 5.2, 0.08 M EDTA pH 7.7, 0.32 mg/mL

tRNA) was then added and phenol/chloroform extraction and ethanol precipitation were performed as before. The pellet was stored at -20°C until gel electrophoresis was performed.

2.2.5.8 The production of the DNA sequencing ladder

A DNA sequencing ladder was required to run alongside the PCR samples on the gel to identify the exact sequence being protected from the DNase I digest. To do this, the -617 to +90 topo IIα promoter region was amplified using the T7 and biotinylated Sp6 primers with the pGEM-T-617 topo IIα vector as a template. The PCR products were then purified using streptavidin-coated magnetic beads as follows.

20 μL of beads were washed in 1x BW buffer. These beads were then resuspended in 2x BW buffer and an equal volume of PCR products added. After a 15 minute room temperature incubation, the beads were washed with 1x BW buffer. The beads were then incubated in 8 μL 0.1 M NaOH for 10 minutes at room temperature followed by 50 μL 0.1 M NaOH and then washed with 1x BW buffer and TE respectively. Finally the beads were resuspended in 7 μL H_20 for the direct use in sequencing reactions. The sequencing reactions were performed as described in the instructions for the Sequenase™ version 2.0 DNA sequencing kit using primer #2 as the sequencing primer. These sequencing products would be derived from the 3' region of the topo IIα promoter, the region being investigated by the *in vivo* footprinting primers. These reactions were stored at -20°C until gel electrophoresis.

2.2.5.9 Gel electrophoresis

A 0.4 mm thick sequencing gel (8% acrylamide, 7 M urea in 0.1 M TBE) was prepared and pre-run at 75 W for 1 hr until the gel temperature was ~50°C. The DNA pellet (from 2.2.6.7) was resuspended in 2 μL water and 4 μL of formamide loading dye (95% (v/v) formamide, 20 mM EDTA pH 8.0, 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromophenol blue) was added. Prior to loading on the gel, the sample was heated to 95°C for 3 min and then chilled. 3 μL of the DNA sample was loaded onto

the gel. The sequencing reactions were heated for 3 minutes at 74 °C and 3.5 μL were loaded onto the gel which was then electrophoresed at 75 W until the bromophenol blue had run off and xylene cyanol had reached the bottom of the gel.

2.2.5.10 Electroblotting

Approximately 40 cm of the gel above the xylene cyanol band was transferred onto nylon membrane using the Owl Semi-dry Electroblotter System at 12 V, 1.6 amp (max) for 45 minutes. The membrane was briefly dried and the DNA fixed onto the membrane by placing the DNA side of the membrane on an UV transilluminator for 30 seconds.

2.2.5.11 Probe synthesis

To produce the PCR product to be used as a probe, PCR was performed as previously described using the primers R2 and primer #3 with the pGEMT-617 vector as a template. The resulting ~300 bp product was purified using the Qiagen MiniElute™ kit as per manufacturer's instructions and gel quantified. This kit used the salt and pH-dependent nucleic acid binding properties of silica-gel membrane to purify the PCR product. 50 ng of the PCR product was then labeled as per the instructions. Briefly the PCR product was boiled for 5 minutes to denature and quickly placed on ice to prevent renaturation. This product together with 50 μCi $^{32}\alpha$-dCTP was then added to Ready-To-Go DNA Labeling Beads (-dCTP) and left for 15 min at 37°C. The labeling reaction was then purified using a ProbeQuant™ G-50 microcolumn by centrifugation at 3000 rpm for 2 min. The eluted probe was then counted using Cerenkov counting to ensure incorporation was ~25%. The probe was stored at 4°C until use.

2.2.5.12 Hybridisation

The membrane was soaked in 0.1 M TBE and placed inside a hybridisation tube. The membranes were pre-hybridized in hybridisation buffer (0.25 M sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA) for 15 min to 2 hr at 60°C The membrane was

then incubated with all the prepared probe in hybridisation solution overnight at 60°C. The membrane was first washed with prewarmed buffer A (20 mM sodium phosphate pH 7.2, 2.5% SDS, 1 mM EDTA, 0.25% BSA) for 5 minutes and then with buffer B (20 mM sodium phosphate pH 7.2, 1% SDS, 1 mM EDTA), with the buffer changed 5 times over 30 minutes. The membrane was then dried at room temperature and exposed to x-ray film for 1-7 days at -70°C with intensifier screens.

Chapter Three Analysis of Drug-exposed Breast Cancer Cells

3.1 Introduction

The aim of this research was to identify specific promoter region(s) that were involved in the down-regulation of topo IIα gene expression in drug-resistant breast cancer cells (Wosikowski *et al.*, 1997; Matsumoto *et al.*, 1997; Asano *et al.*, 1996b; Zhou *et al.*, 1999c). Previous studies have implicated various promoter elements and transcription factors as mediating such changes including ICB elements, NF-Y and Sp3 (Morgan & Beck, 2001; Wang *et al.*, 1997a; Takano *et al.*, 1999; Kubo *et al.*, 1995; Mo *et al.*, 1997). Although specific elements and cognate transcription factors have been identified as having potential roles in topo IIα regulation in response to drug exposure, it is likely that these mechanisms are cell type-specific. Consequently all promoter elements and their cognate transcription factors should be investigated for a potential role in the regulation of topo IIα expression in drug-resistant breast cancer cells.

In the current research the MDA-MB-231 and MCF-7 breast cancer cell lines were exposed to doxorubicin and the surviving cells isolated. The mechanism of survival was then investigated in these cells, with alterations in the regulation of topo IIα gene expression of particular interest. The method of doxorubicin exposure may be critical to obtain cells which are most similar to the resistant cells seen in clinical drug resistance. As outlined below either pulsed or continuous drug exposure have been used to identify mechanisms of multidrug resistance. The reasons for using the pulsed method in the current research are discussed as follows.

3.2 Generation of doxorubicin 'resistant' breast cancer cell lines

As a treatment regimen, chemotherapy destroys only a portion of dividing cells on single exposure so multiple exposures of chemotherapeutic drugs are required to kill all cancer cells. However, due to the toxicity of the treatment on critical proliferating tissues like bone marrow and the gastrointestinal epithelium, subsequent chemotherapy must be timed to allow recovery of these non-cancerous tissues. Most chemotherapy drugs are therefore given at 3-4 week intervals (McKinnell *et al.*, 1998).

Two main strategies are used for the *in vitro* production of resistant cancer cell lines. The most popular strategy is the long-term exposure of cells to increasing doses of chemotherapeutic drug. The resulting resistant cells can then survive when exposed to constant "non-physiological" drug concentrations (examples include Cowan *et al.*, 1986).

Due to the non-clinical doses and exposure times used, cells produced by long-term drug exposure may only give an indication of the possible resistance mechanisms available to a cell and may not reflect the mechanisms that occur *in vivo*. Most cell lines produced by this method are highly drug-resistant, to a degree two orders of magnitude higher than that normally seen *in vivo*. Resistance of this type can be limited to only a few types of drugs and is sometimes unstable in the absence of the drug - neither of these effects is seen in clinical drug resistance (reviewed in Davey & Davey, 1998).

A second strategy is to mimic the standard chemotherapy regimen, where the cells *in vivo* are exposed to short term pulses of the drug every 3-4 weeks. Cells treated in this manner should be more clinically relevant (Davey & Davey, 1998). For example Yang & Trujillo (1990) produced multidrug-resistant human colon cancer cells by long-term continuous exposure or 1 hour pulses of doxorubicin every 3 weeks. These authors found that the resistant cells grown under continuous drug exposure had altered phenotypic properties including decreased growth rate and colony-forming ability compared to the parental cells and the drug resistance was reversed in the absence of the drug. The "pulsed" resistant cells, however, had a similar phenotype to the parental

cell line and maintained the resistant phenotype in drug-free conditions. Similarly, resistant squamous lung cancer cells produced by pulsed clinical doses of doxorubicin showed stable resistance with broad cross-resistance to a range of drugs (NicAmhlaoibh *et al.*, 1999).

In the study described below two breast cancer cell lines were exposed to pulsed doses of doxorubicin to produce cells with a more "physiological" response to the drug. Research investigating the pharmacokinetics and metabolism of doxorubicin in patients undergoing chemotherapy revealed some variation in the drug plasma concentration after drug administration and the rate of removal from the body. Depending upon the drug dose given, the plasma drug concentrations ranged from 1 to > 5 μM initially after bolus injection, with this dropping to < 1 μM within an hour. A summary of results from the literature are shown in table 3.1. Doxorubicin was used at concentrations within 1-5 μM in this study.

3.3 Experimental design

The initial aim was to use stable cell lines containing deletion constructs of the topo IIα promoter to investigate the drug-induced effects on topo IIα gene regulation. Stable lines were generated and then exposed to pulsed doses of the common chemotherapeutic drug doxorubicin and the changes in topo IIα expression monitored in the surviving cells. It was anticipated that this approach would allow the identification of specific promoter regions that were involved in the regulation of promoter activity.

		Time after drug administration							
Reference	dose of doxorubicin	just after injection	1 hr	6 hr	12 hr	24 hr	48 hr	60 hr	210 hr
Wilkinson & Mawer (1974)	60 mg/m^2 3 minutes	1.7 μM 1.1 μg/mL	-	-	0.3 μM 0.2 μg/mL	0.15 μM 0.1 μg/mL	0.12 μM 0.08 μg/mL	-	-
Piscitelli *et al.* (1993)	45-72 mg/m^2 1 hr	1.7 μM 1.1 μg/mL	0.15 μM 0.1 μg/mL	0.045 μM 0.03 μg/mL	0.03 μM 0.02 μg/mL	-	0.015 μM 0.01 μg/mL	-	-
Greene *et al.* (1983)	75 mg/m^2 15 min	>5 μM 3 μg/mL	0.1 μM 0.06 μg/mL	-	0.09 μM 0.06 μg/mL	0.06 μM 0.04 μg/mL	0.04 μM 0.03 μg/mL	-	-
Muller *et al.* (1993)	35 mg/m^2 1-5 min	2.3 μM 1.5 μg/mL	-	-	-	-	-	0.15 μM 0.1 μg/mL	0.0015 μM 0.001 μg/mL
Brenner *et al.* (1985)	60 mg/m^2 5 min	3 μM 2 μg/mL	-	0.3 μM 0.2 μg/mL	0.08 μM 0.05 μg/mL	0.05 μM 0.03 μg/mL	0.02 μM 0.01 μg/mL	0.01 μM 0.007 μg/mL	-
Creasey *et al.* (1976)	50-60 mg/m^2	1.7 μM 1.1 μg/mL	0.9 μM 0.6 μg/mL	0.3 μM 0.2 μg/mL	-	0.15 μM 0.1 μg/mL	-	-	-
Robert *et al.* (1982)	50 mg/m^2 3 min	3 μM 2 μg/mL	0.2 μM 0.13 μg/mL	0.07 μM 0.05 μg/mL	-	0.03 μM 0.02 μg/mL	-	-	-
Benjamin *et al.* (1974)	60 mg/m^2 1-5 min	1 μM 0.7 μg/mL	-	0.2 μM 0.1 μg/mL	0.15 μM 0.1 μg/mL	0.08 μM 0.05 μg/mL	-	-	-

Table 3.1. Summary of the plasma doxorubicin concentration seen in patients after treatment with doxorubicin. The approximate concentration in μM and μg/mL has been noted.

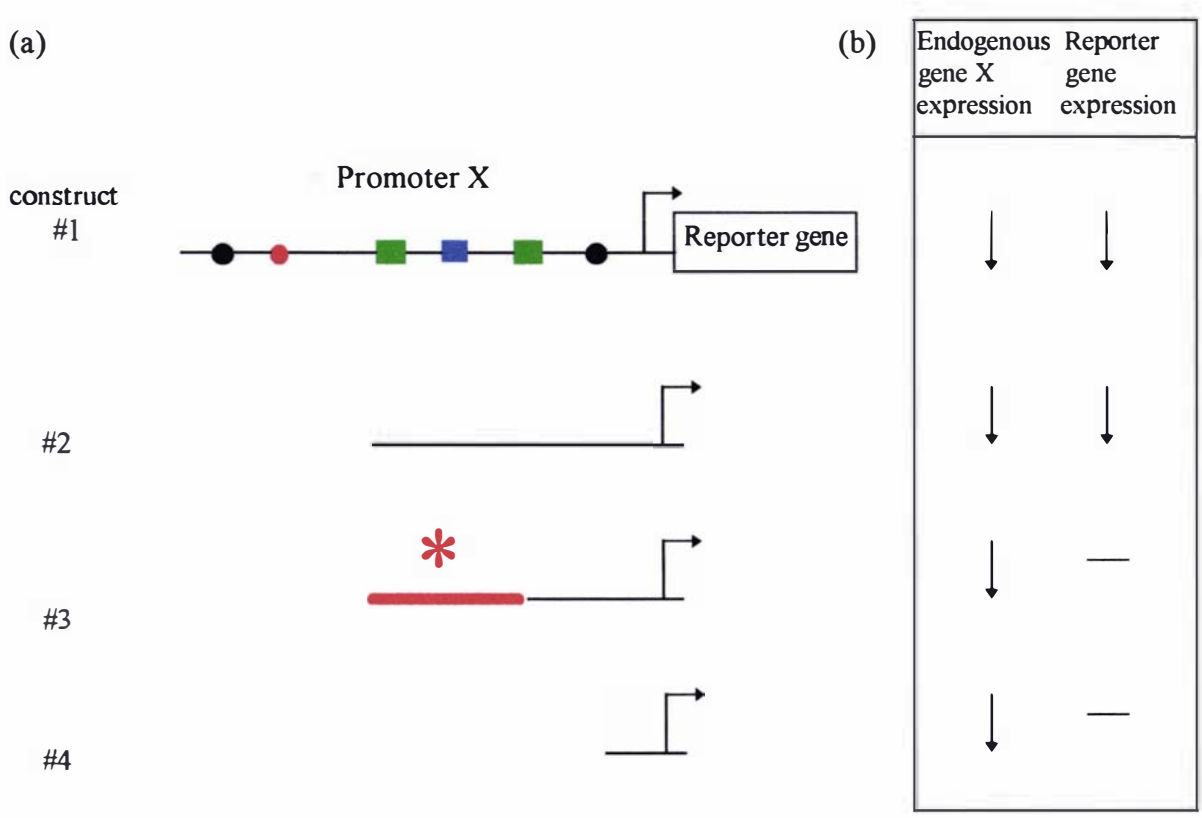


Figure 3.1. The experimental design to identify specific regions involved in transcriptional regulation of promoter X. (a) The different promoter deletion constructs linked to the reporter gene. Different promoter elements were represented by differently coloured circles or squares. (b) The alterations in the levels of endogenous gene X and the reporter gene in the cells that survived drug exposure.

Figure 3.1 outlines the theory behind this experimental system using a hypothetical promoter X. The stable cell lines containing different promoter deletion constructs linked to a reporter gene were treated with the drug. The promoter deletions were designed to include or exclude specific promoter elements which may have roles in the regulation of the promoter (figure 3.1 (a)). The stable cell lines all respond to the drug in the same way and down-regulate the transcription of the endogenous gene X, but show varying regulation of the reporter gene linked to different promoter constructs (figure 3.1 (b)). Cell lines that contain construct #1 and #2 both down-regulate the reporter gene, so the promoter constructs contain the promoter region involved in the down regulation. On the other hand Cell lines containing constructs #3 and #4 do not down-regulate the reporter and thus are lacking the required promoter region. This would indicate that the region present in construct #2 and absent in construct #3 is involved in the down-regulation (labeled by a red star and line).

This experimental system was based on the assumptions that (1) the mechanism of regulation of the topo IIα promoter in breast cancer cells would be similar to that seen in other cell types; (2) the levels of the reporter protein will reflect the transcriptional activity of both the recombinant construct and endogenous topo IIα promoter; (3) the stable cell lines differ only in the topo IIα promoter construct i.e. no other significant changes have occurred during the integration of the reporter vector and subsequent selection and (4) the stable cell lines respond to the drug in the same way.

These assumptions needed to be justified experimentally. To this end, stable cell lines were exposed to doses of doxorubicin to produce individual surviving cell lines that were then analysed for expression of both the reporter gene and the endogenous topo IIα gene.

This Chapter is divided into three distinct sections, the first investigating whether the basal regulation of the topo IIα promoter in the breast cancer cell lines was similar to that in other lines. The second section compares reporter gene expression with endogenous topo IIα gene expression in an attempt to validate the use of reporter system. The final section analyses the expression of topo IIα and the hGH reporter in a range of stable cell lines that survived exposure to doxorubicin.

3.4 Basal regulation of the topo IIα promoter in the breast cancer cell lines

Prior to studying putative changes in the regulation of the human topoisomerase IIα promoter in breast cancer cell lines surviving doxorubicin exposure, the promoter was further studied to define those regions important for basal transcription. To do this, transient transfection experiments using a range of deletion constructs of the topo IIα promoter were carried out.

Promoter constructs corresponding to the topo IIα -1964, -617, -144, -101 and -56 to +90 (tsp = +1) regions, linked to the hGH reporter gene (Appendix 6) were provided by Richard Isaacs (Isaacs *et al.*, 1996).

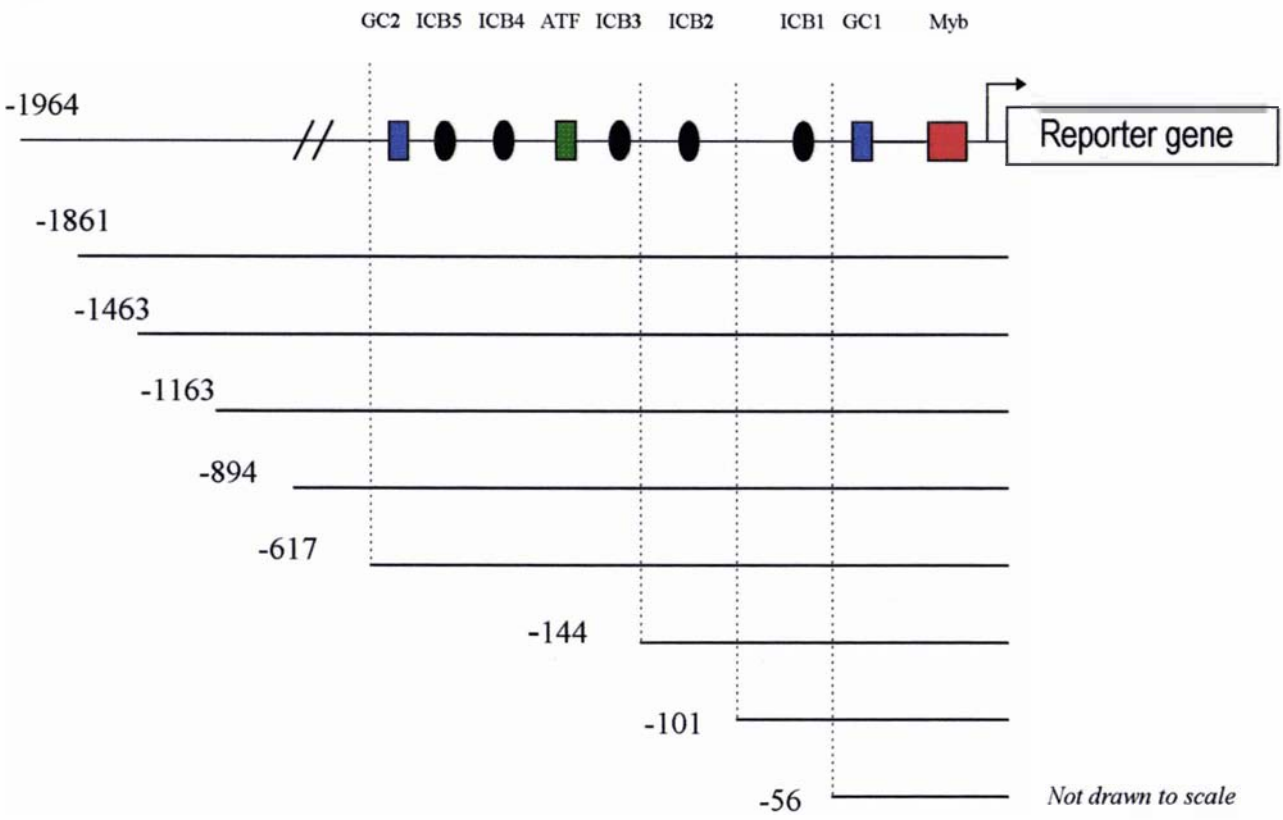


Figure 3.2. The topo IIα promoter constructs used in this research

The three shortest constructs had been designed to investigate the roles of GC1, ICB1 and ICB2 in the regulation of topo IIα transcription. The -617 construct contains the minimal promoter region while the -1964 construct contains the maximum topo IIα regulatory region studied to date (figure 3.2). Additional deletion constructs -1861, -1463, -1163 and -894 were prepared to further define the 5' most region of the topoisomerase IIα promoter.

The reporter constructs were assayed for transcriptional activity using transient transfection of cells in culture followed by analysis of reporter protein activity. A

transient transfection method was used rather than a stable system as high but temporary amounts of the reporter protein can be produced, with a large number of samples able to be analyzed within a short time (Sambrook & Russell, 2001). The firefly luciferase reporter gene system was chosen as it is a sensitive and quick assay and does not require radioactivity or specific antibodies. Cells were co-transfected with a bacterial β-galactosidase expression vector to normalize for variations in cell number, extract preparation and transfection efficiency (Sambrook & Russell, 2001). The pGL2Control vector, which has the luciferase gene controlled by the strong SV40 promoter, was used as a control to check the luciferase assay reagents.

All the pHGH reporter constructs were subcloned into the luciferase reporter vector pGL3B and the calcium phosphate method was used to transfect cells with reporter vectors.

3.4.1 Breast carcinoma cell lines

MCF-7 and MDA-MB-231 breast carcinoma cell lines were chosen for this current study as both had been used in earlier studies investigating the development of drug resistance (examples include Cowan *et al.*, 1986; Schiemann *et al.*, 1998; Wosikowski *et al.*, 1997; Matsumoto *et al.*, 1997; Zhou *et al.*, 1999c). These two cell lines were selected as both were derived from epithelial breast carcinomas, but differ in expression of estrogen receptor and p53. It is not known whether expression of these genes directly affects the development of drug resistance via the regulation of the topo IIα promoter. MCF-7 cells are estrogen receptor-positive with wildtype p53, while MDA-MB-231 cells are estrogen receptor-negative with mutant p53 (Elstner *et al.*, 1995; Xu & Loo, 2001; Bartek *et al.*, 1990).

3.4.2 The sequencing of the topo IIα promoter

The 5' sequence of the topo IIα promoter had not been formally published, so the first step in this study was to completely sequence ~2 kb of the upstream region of the gene. A range of primers was used to sequence both the pHGH1964 construct and the

same region generated by PCR from human genomic DNA. All sequencing was carried out by the Massey University DNA Sequence Analysis Service. Appendix 7 shows the summary of the DNA sequencing analysis.

The sequence from -577 to +119 agreed with the sequence published by Sng *et al.* (1999) (Accession no AJ011741), and with the -562 to +90 sequence from Hochhauser *et al.* (1992). The sequence differs only slightly with the sequence from Isaacs *et al.* (1996) (-617 to +90) where an additional G was observed within the GC2 element in the sequence from this study and in that of Sng *et al.* (1999) (Appendix 7 (b)). The only major difference seen was an additional ~50 bp from the -411 to -363 region which was absent from the GenBank sequence (Accession no. X66794, Appendix 7 (a)). The intronic sequence from +120 to +125 also differs from that published by Sng *et al.* (1999) (Appendix 7 (c)).

Some consistent one base differences were detected between the constructs produced from pHGH1964 (Isaacs *et al.*, 1996) and constructs made using New Zealand-derived genomic DNA as a template. The differences are summarised in Appendix 7 (a). It is not known whether these slight differences represent sequencing errors, a PCR-induced change or are due to natural polymorphisms, but as the differences are single bases changes and did not appear to be in regions thought to be significant for transcription, the constructs were used in functional assays.

A scan for putative transcription factor binding sites in the topo IIα promoter sequence revealed an extremely large number and range of putative binding sites (Appendix 8). This confirmed the necessity of using the deletion constructs in transient transfection experiments to identify specific regions of the promoter involved in the up or down-regulation of basal transcription.

3.4.3 Transient transfection with the -1964, -1861, -1463, -1163, -894, -617,-144, -101, -56 and pGL3B reporter constructs

Conditions for transient transfection were optimized to produce optimal luciferase and β-galactosidase levels. As described in Appendix 9, 3 μg of the pGL3B construct and 2 μg of the pSV-β-gal or pCH110 vector were found to produce optimal levels of reporter activity.

MCF-7 cells were transiently transfected with pGL3B1964, pGL3B1861, pGl3B1463, pGL3B1163, pGL3B894 pGL3B617, pGL3B144, pGL3B101 and pGL3B56 vectors and the relative topo IIα construct promoter activity examined. This allowed the assessment of the importance of specific promoter regions in the -1964 to +90 region to basal topo IIα transcription. Each experiment was performed a minimum of three times and in triplicate within each experiment, to measure the reproducibility of the experiment.

The transient transfection results are summarized in Appendix 10. These results indicate that GC1 and myb have a small but significant effect on basal transcription ($p < 0.01$). The addition of the region including ICB1 (-57 to -101) caused a slight increase in transcription although this was not significantly different to the pGL3B56 value ($p > 0.05$). The addition of the region containing ICB2 (-102 to -144) caused a 4.8-fold significant increase ($p < 0.01$) in promoter activity. The further addition of the region containing ICB3, ATF, ICB4, ICB5 and GC2 also caused a further increase in promoter activity. The addition of the ~ 1.3 kb region 5' to GC2 did not have a significant effect on the maximal topo IIα basal promoter activity, so there was no evidence of a repressive or activating region in the -617 to -1964 region of the topoisomerase IIα promoter in either of two breast cancer cell lines or in HeLa cells.

3.4.4 Summary of basal regulation of topo IIα

Although direct comparisons between the published results and the results from the current experiments were not possible due to differences in cell type, transfection method, reporter vector and promoter construct, the trends can be examined.

The result from the transient transfections using pGL3B1964, 1861, 1463, 1163, 894, 617 and 56 vectors show that there was no evidence for either an activation or repressive region in the -617 to -1964 region of the topo IIα promoter. This result is contradictory to the results reported by Hochhauser *et al.* (1992), where in HeLa cells, the full length promoter (~2 kb) had ~ $\frac{1}{3}$ the activity of the -557 to +90 region. Possible reasons for these differences could be due to differences in the transfection methods, the reporter vector, the reporter assay used, or even differences in the age or history of the HeLa cells.

The results from the experiments with the shorter topo IIα promoter constructs confirm that specific elements can have differing roles in the regulation of basal transcription depending upon the cell type investigated. The transient transfection results suggest that in breast cancer cells, ICB1 has little effect on basal transcription. However in murine fibroblast, human erythroleukemia and human urinary cancer cells (Wang *et al.*, 1997b; Loflin *et al.*, 1996; Furukawa *et al.*, 1998), ICB1 was found to cause a modest increase in promoter activity. This is the same trend as reported for HeLa cells, where ICB1 caused a significant promoter activation (Hochhauser *et al.*, 1992).

The addition of ICB2 caused a significant increase in promoter activity in the breast cancer cell line which was also observed in urinary, murine fibroblast, human leukemia and human epithelial cells (Furukawa *et al.*, 1998; Chen *et al.*, 1999; Morgan and Beck, 2001; Takano *et al.*, 1999). The further increase in transcriptional activity that was seen with the addition of the promoter region 5' to ICB2 was also reflected in these cell lines (Chen *et al.*, 1999; Wang *et al.*, 1997b; Morgan and Beck, 2001; Furukawa *et al.*, 1998).

ICB2 seemed important as a positive inducer of transcription in a majority of the cell lines. GC1 appeared to have a limited effect on basal transcription in HeLa cells (Hochhauser *et al.*, 1992). ICB4 had a limited effect in epithelial, urinary and murine fibroblast cells but caused activation in leukemic and murine embryo fibroblast cells (Furukawa *et al.*, 1998; Chen *et al.*, 1999; Wang *et al.*, 1997b; Morgan and Beck, 2001; Takano *et al.*, 1999). A possibility for these differences could be due to the presence of different activators and repressors in the different cell types or cell lines or to the presence of co-regulatory factors.

These aspects of this study demonstrate that the mechanism for topo IIα promoter regulation in breast cancer lines is likely to be similar to that in other cell lines. While the subsequent research is focused on breast cancer cells and their response to doxorubicin in terms of modulation of topo IIα expression, the results obtained should have relevance for other cell types.

Analysis of these data indicated that the mechanisms governing the basal regulation of the topo IIα promoter are likely to be similar in all cells but the extent of the effect of each element may be cell-type specific depending on the presence of cell-specific transcription factors and co-regulators.

3.5 Verification of the reporter system

The verification of the reporter system was necessary to validate the use of this reporter system prior to the exposure and analysis of drug-exposed cells.

3.5.1 The production of stable cell lines containing the topo IIα promoter deletion constructs

Two topo IIα promoter deletion constructs were used for initial experiments in both breast cancer cell lines. The constructs chosen were the -1964, which is the maximum topo IIα regulatory region studied to date, and the -144 construct which lacks the potentially crucial region upstream of ICB2. The -1964 and -144 human topoisomerase

IIα promoter constructs inserted into the pHGH vector were supplied by Dr. Richard Isaacs (Isaacs *et al.*, 1996) (Appendix 6). Stable lines with either pHGH1964 and pHGH144 were produced by co-transfection with pMAMNeo and maintenance on G418 as described in section 2.2.2.8 to 2.2.2.10. The stable lines produced were labeled MDA1964, MDA144, MCF-7-1964 and MCF-7-144 (the first 3 letters represent the parental cell line, the numbers following represent the topo IIα-hGH reporter construct stably integrated in the cells).

The reporter gene for these experiments needed to reflect the activity of the promoter, be secreted by the cells, be readily detectable, and so allow the monitoring of gene expression without destruction of the cells. Human growth hormone (hGH) met these requirements (Selden *et al.*, 1986).

3.5.2 Investigation of the hGH reporter protein, endogenous topo IIα mRNA and protein in stable cell lines

Endogenous topo IIα gene expression was compared to hGH reporter gene expression to validate the use of this reporter system prior to exposure and analysis of the doxorubicin-exposed stable cell lines.

An hGH ELISA (Enzyme-linked immunosorbent assay) was used to investigate the levels of hGH protein produced by the stable cell lines. The level of hGH secreted was normalized to the number of cells plated (this assumed that all cells were expressing the reporter protein). Immunoblots were used to detect topo IIα protein while real time RT-PCR (reverse transcription polymerase chain reaction) was used to detect topo IIα mRNA levels. To account for variations in these parameters, the levels of topo IIα mRNA and protein were compared to internal controls. These relative values were then compared to the value of the control (unexposed) cells to identify any changes in expression.

Internal controls should be ubiquitously expressed genes expressed in relatively high levels and be unaffected by the experimental treatment being investigated. As no single

control has been proven to be infallible (Bustin, 2000), multiple controls should be used if possible. α-tubulin was an appropriate control for immunoblots as only drugs such as taxol or paclitaxel that target the tubulin protein have been found to produce resistant cells with either mutations or changes in the expression levels of various tubulin isotypes (Minotti *et al.*, 1991; Kavallaris *et al.*, 1997; Haber *et al.*, 1995; Dumontet *et al.*, 1996; Han *et al.*, 2000; Giannakakou *et al.*, 1997; Keates *et al.*, 1981). In addition α-tubulin mRNA has been shown to be unchanged after short-term doxorubicin exposure (Kurabayashi *et al.*, 1993; Kurabayashi *et al.*, 1995; Kunisada *et al.*, 2000) and in doxorubicin-resistant cells (Wang *et al.*, 1999a).

A variety of methods can be used to analyse the levels of specific mRNAs in cells including northern blotting, RNase protection assays and RT-PCR (reviewed in Bustin, 2000). Real time RT-PCR using the Lightcycler™ system was used as it was shown to be highly sensitive and reproducible (section 3.5.2.2). GAPDH (glyceraldehyde 3-phosphate) and 18S rRNA were used as controls of real time RT-PCR. There is no evidence of changes to GAPDH levels in doxorubicin-resistant cells (Pourquier *et al.*, 1998; Daschner *et al.*, 1999) or upon doxorubicin exposure (Kurabayashi *et al.*, 1993; Boucek *et al.*, 1999; Morceau *et al.*, 1996; Jeannesson *et al.*, 1997; Fornari *et al.*, 1996; Fornari *et al.*, 1994). 18S rRNA has been shown to be a reliable internal control under a variety of conditions (Selvey *et al.*, 2001; Schmittgen & Zakrajsek, 2000; Roberts-Thomson *et al.*, 2000; Stürzenbaum & Kille, 2001) and the levels of 18S rRNA were unchanged in rabbit heart tissue after two exposures to doxorubicin (Boucek *et al.*, 1999).

3.5.2.1 Analysis of the reporter gene expression before and after confluence

The expression of the -1964 topo IIα promoter construct has been shown to reflect the expression of endogenous topo IIα in MCF-7 and NIH3T3 cells with decreased expression seen in cells at full confluence (Isaacs *et al.*, 1996). To confirm that this also holds true for the MDA-MB-231 breast cancer cell line, whole cell protein extracts and total cellular RNA were prepared from freely proliferating cells and fully

confluent MDA1964 cells and assayed for topo IIα protein, mRNA and reporter activity.

The level of hGH protein secreted by the cells increased as the cells were proliferating, and plateaued after the cells had reached confluence (figure 3.3). This confirmed that topo IIα gene expression was maximal in the proliferating MDA1964 cells, and a down-regulation of topo IIα promoter construct activity occurred at confluence.

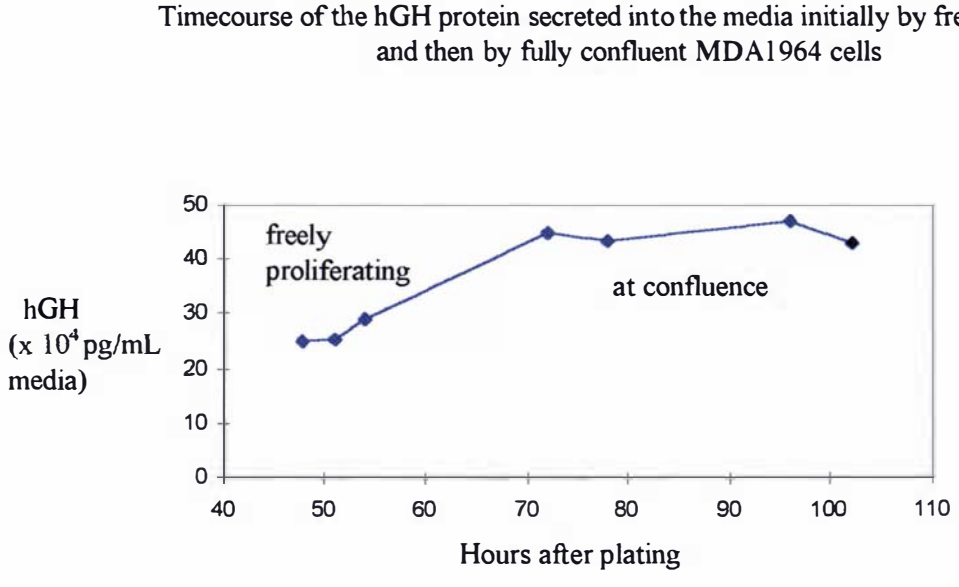
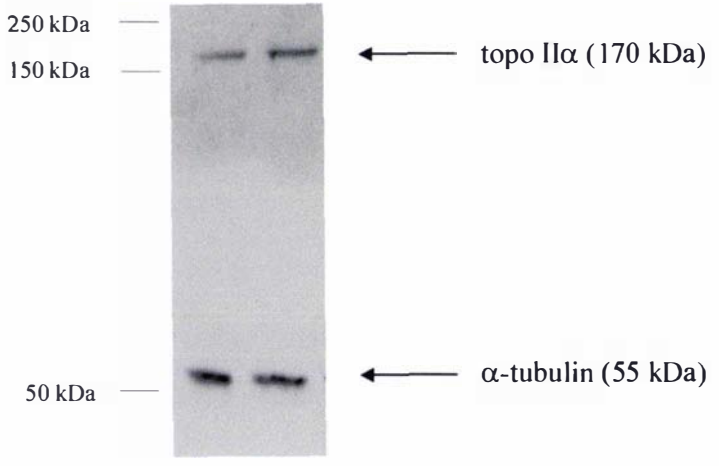


Figure 3.3. hGH protein secreted by the MDA1964 cells before and after 100% confluence. 48 hours after MDA-MB-231 cells were plated, the cells were freely proliferating; at 70 hours the cells had grown to 100% confluence. Samples of the media the cells were grown in were taken at 48, 52, 54, 72, 76, 96 and 102 hours after the cells were plated. The concentration of the hGH protein was determined using the hGH ELISA kit and expressed in pg/mL media.

3.5.2.2 Endogenous topo IIα gene expression in confluent and freely proliferating cells

Whole cell protein extracts were prepared from the cells before and after 100% confluence to monitor the endogenous topo IIα protein levels. MDA1964 cells grown to 100% confluence for 12-24 hours had reduced topo IIα protein levels (52 ± 13%) compared to cells that were freely proliferating (figure 3.4).



Relative topo IIα protein	52 ± 13%	100%

Figure 3.4. Immunoblot of whole cell extracts (Ladias et al., 1992) from freely proliferating and cells that had been 100% confluent for 12-18 hours. 5 μg of whole cell extracts were separated on an 8% SDS-PAGE gel. The proteins were transferred onto positively charged nylon membrane for 90 minutes at 450 mA and incubated in 1% blocking solution in TBST for 2 hours. The membrane was cut horizontally at the 75 kDa marker, with the top half of the membrane was incubated in topo IIα antibody (1:1,000), and the remaining membrane incubated in α-tubulin antibody (1:2,000) overnight at 4℃. Both antibodies were diluted in 0.5% blocking solution in TBS. The membranes were washed over 40 minutes with TBST and incubated in secondary antibodies for 30 minutes (1:10,000 anti goat IgG for topo IIα, 1:5,000 anti mouse IgG for α-tubulin diluted in 0.5% blocking solution). The membrane was then washed over 1 hour with TBST and the proteins detected using a Roche chemiluminescent (POD) detection kit. The Fujifilm Darkbox II using the LAS Pro 1000 program was used to detect the signal. The program Image Gauge was used to quantify protein bands. The value of the topo IIα was calculated relative to the α-tubulin value for the given sample. The proliferating value for the amount of topo IIα protein (relative to α-tubulin) was arbitrarily set at 100%. The mean (± standard deviation) topo IIα protein levels for proliferating and confluent MDA1964 cells were as indicated beneath the figure. At least 3 experiments were performed.

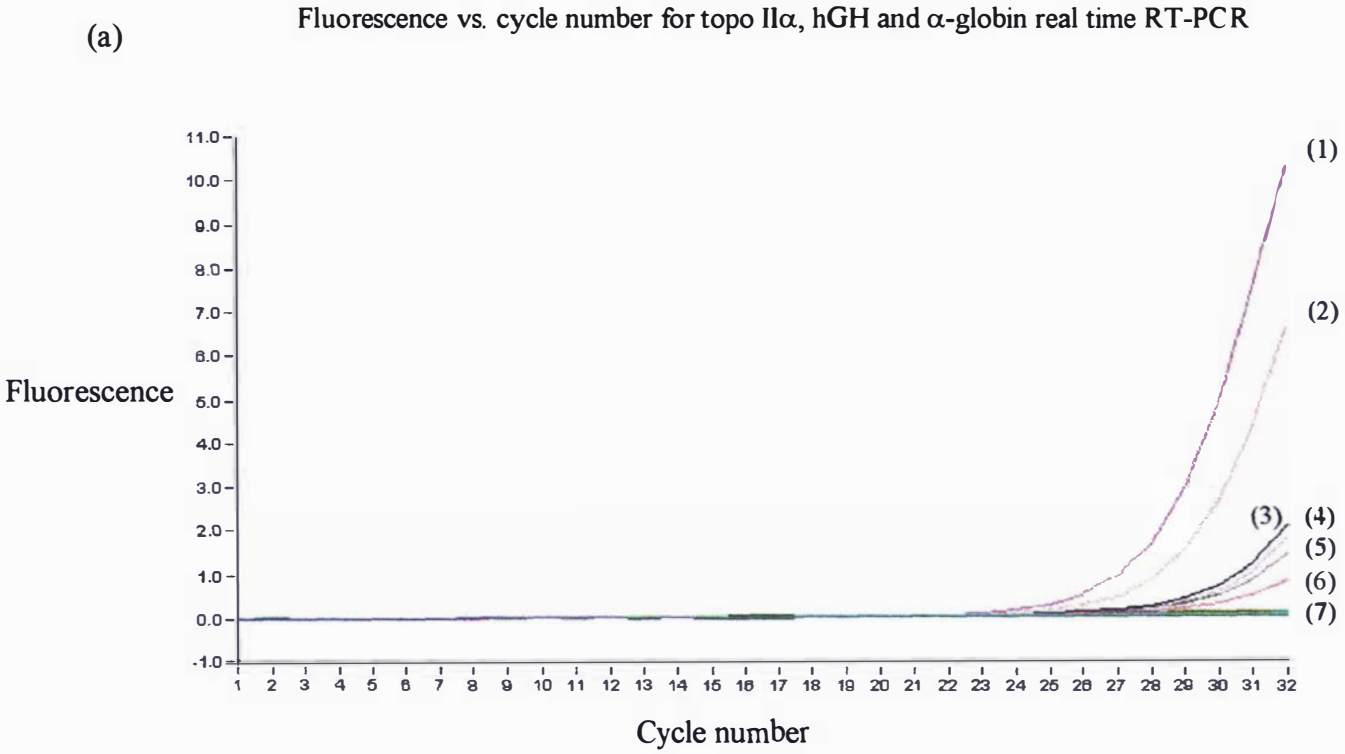
The amount of topo IIα mRNA was examined in freely proliferating and fully confluent cells. As GAPDH mRNA levels have been found to halve in confluent renal carcinoma cells relative to freely proliferating cells (Vilà *et al.*, 2000), an exogenous internal control K562 RNA was included as described in Isaacs *et al.* (1996). K562 cells are known to over-express α-globin mRNA, therefore equal amounts of RNA from freely proliferating and confluent cells were spiked with RNA from K562 cells. The levels of α-globin cDNA were used to account for any differences in the reverse transcription efficiency between the freely proliferating and confluent samples.

Figure 3.5 shows the results of a real time RT-PCR experiment. Table 3.2 summarises the results from at least three experiments. The levels of topo IIα and hGH relative to α-globin were compared in samples prepared from freely proliferating and confluent cells. Compared to freely proliferating cells, a decrease in both topo IIα (23 ± 5%) and hGH (57 ± 12%) mRNA levels were observed in the sample from fully confluent cells. It was noted that the α-globin level was increased in the confluent sample when compared to the proliferating sample, indicating an enhanced reverse transcription efficiency in fully confluent cells.

3.5.2.3 Summary of endogenous topo IIα and reporter gene expression

After the cells had been 100% confluent for at least 12-18 hours, a decrease in both topo IIα mRNA (23%) and protein (52%) was seen. At the same time, the hGH mRNA decreased (57%) while no new hGH protein was secreted by the cells after 100% confluence was reached. It was shown that the decrease in topo IIα mRNA was significantly different to that seen with the hGH reporter ($p < 0.01$). These results may suggest that an element was missing from the -1964 construct that was required for a significant decrease in the topo IIα promoter activity. Another possibility is that differences in the stability of hGH and topo IIα protein or mRNA may cause such a result. These results indicate that the reporter construct may not accurately reflect topo IIα expression.

(a)



(b)

Growth state	Relative topo IIα mRNA levels	Relative topo IIα protein levels	Relative hGH mRNA levels
Freely proliferating	100%	100%	100%
Fully confluent	23 ± 5%	52 ± 13%	57 ± 12%

Figure 3.5. (a) Example of the fluorescence vs. cycle number results of a real time RT-PCR experiment using the reverse transcription reaction derived from either confluent or free RNA spiked with K562 RNA as the template. (1) α-globin, confluent(con) (2) α-globin, freely proliferating (fr)(3) hGH (fr) (4) hGH (con) (5) topo IIα (fr) (6) topo IIα (con). 5 μg RNA plus 1 μg K562 RNA was treated with DNase 1 and then reverse transcribed as described in section 2.2.3. 5 μL of the RT reaction (1:5 dilution) was added to the PCR reaction mix containing 0.5 μM of each primer (Appendix 4), 1x SYBR Green 1 Faststart Master Mix and 3-4 mM $MgCl_2$. To confirm that the product was specific for cDNA, samples minus reverse transcriptase were included (not shown). To confirm the absence of primer dimers and contamination, water only controls (7) were included. Melting curve analysis and diagnostic digests were carried out to confirm the identity of the PCR products. (b) Table 3.2 summarizes the real time RT-PCR results from at least three experiments. The relative values of either topo IIα/α-globin or hGH/α-globin freely proliferating results were arbitrarily set at 100% and the fully confluent results compared to these values. The average (± standard deviation) values are shown. See Appendix 17 to see a typical data set with full analysis.

The difference between the reduction of topo IIα mRNA (23%) and that of hGH mRNA (57%) at confluence may be due to differences in the mRNA stability. hGH has been reported to have a very stable message, with a $t_{½}$ of ~50 hr seen with rat GH mRNA in somatrophic pituitary cells (Yaffe & Samuels, 1984), although this value may be less in cells that do not normally express hGH mRNA. The $t_{½}$ for topo IIα mRNA has been reported to vary depending upon the cell line, with the values ranging from < 2 hr in K562 cells to ~12 hr in epidermoid cancer cell lines (Ritke & Yalowich, 1993; Mo *et al.*, 1997; Withoff *et al.*, 1996b; Asano *et al.*, 1996c; Kubo *et al.*, 1995). There may also be a cell cycle influence on mRNA stability as Goswami *et al.* (1996) found that in HeLa cells, the $t_{½}$ of topo IIα at G_1 was ~30 min, while at S/G_2 the $t_{½}$ is > 4 hr.

The $t_{½}$ for the topo II protein has also been found to vary between cell lines from ~3 hr in primary chicken embryo fibroblast cells to ~25-27 hr in HeLa cells (Heck *et al.*, 1988; Kroll & Rowe, 1991; Kimura *et al.*, 1996; Salema *et al.*, 2001). The topo IIα protein stability has been found to be cell cycle-dependent, with rapid degradation seen from M to G_1 phases (Salema *et al.*, 2001; Heck *et al.*, 1988; Kimura *et al.*, 1996). A longer protein than mRNA stability may explain why the topo IIα mRNA decreased to 23% while the topo IIα protein levels was decreased to only 52% of the freely proliferating levels after the same time.

The stability of hGH protein over the time scale of the experiment was not surprising as the protein is believed to be extremely stable (Selden *et al.*, 1986), and as it is secreted would not be subject to intracellular degradation processes.

The results from these experiments suggested that the activity of the -1964 topo IIα promoter construct integrated into the MDA-MB-231 genome did not accurately reflect the endogenous topo IIα promoter activity and therefore this system may not be suitable for the investigation of the regulation of the topo IIα promoter in drug surviving cells. Nevertheless the regulation of topo IIα promoter reporter constructs in drug surviving cells was investigated.

3.5.3 Analysis of the breast cancer cells treated with doxorubicin

Cells were exposed to drug concentrations of 0.05, 0.5, 5 and 20 μM of doxorubicin for 1 hour and the effect on the cells observed after a week. The two lowest drug concentrations either temporarily inhibited or killed only a limited number of cells while the 20 μM dose killed all cells (results not shown). A dose of 5 μM doxorubicin was used for the subsequent experiments as this dose was close to the maximal *in vivo* drug concentration after bolus injection. This dose killed a majority of cells with individual colonies growing from the small number of cells surviving the treatment.

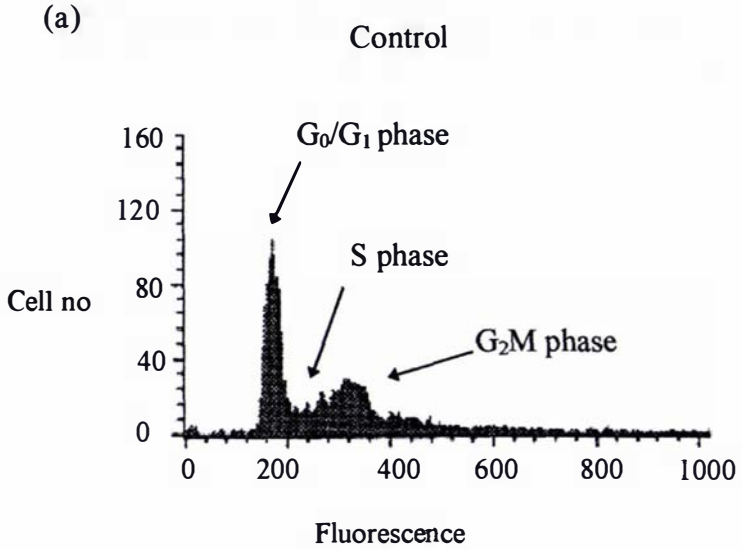
The MDA1964, MDA144, MCF-7-1964 and MCF-7-144 cell lines were exposed to a 1 hour dose of 5 μM doxorubicin and then washed repeatedly to remove the drug. New media was added and the cells returned to the incubator. The media was changed regularly until distinct colonies had grown (normally around 3-4 weeks for MDA-MB-231 cells and 6-7 weeks for MCF-7 cells). Once these colonies were of sufficient size, they were individually transferred to new plates. When the cell lines had produced sufficient numbers, the cells were passaged into two populations. One population was frozen or maintained and the other exposed to a further dose of doxorubicin. The cell lines produced are described in tables 3.4, 3.5 and 3.6.

FACS (fluorescence activated cell sorting) analysis was used to monitor the short term effect of the drug on the cell cycle 24 hours after drug exposure. The 24 hour time period was chosen as this interval has been shown to be sufficient to observe drug-induced alterations to the cell cycle profile (Andres *et al.*, 1998). Analysis of the MDA1964 cells 24 hours after drug exposure showed that the cells were arrested in S and G_2/M phase (figure 3.6). Compared to untreated cells, the amount of cells in S phase had increased from 8% to 13%, the proportion of G_2/M cells had increased from 46% to 68%, while the G_0/G_1 cells had decreased from 46% to 19% (table 3.3). The absence of $<G_0/G_1$ fluorescence indicated that apoptosis had not yet begun.

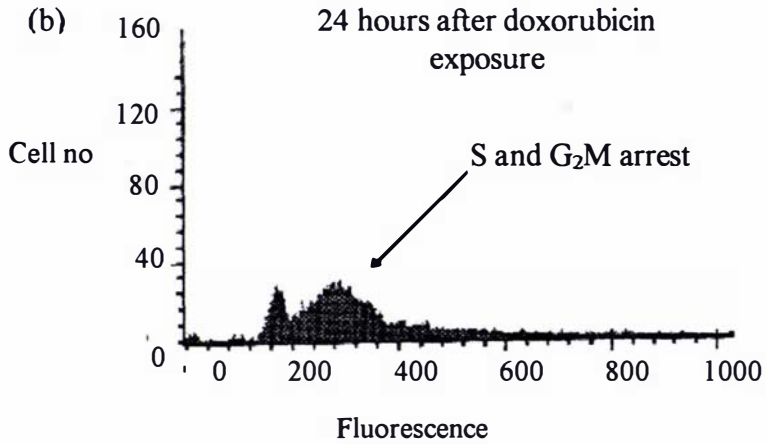
Similar G_2/M arrests have been seen 24 hours after short term doxorubicin exposure of lymphoid (Barlogie *et al.*, 1976), murine erythroleukemic (Zucker *et al.*, 1991), and

CEM cells (Krishan & Frei, 1976). This indicated that the breast cancer cell lines initially responded to doxorubicin in a manner similar to other cell lines.

(a)



(b)



(c)

Cell cycle phase	G_0/G_1	S	G_2/M
(-) drug	46%	8%	46%
(+) drug	19%	13%	68%

Figure 3.6. FACS analysis of MDA1964 cells exposed to doxorubicin. MDA1964 cells were exposed to 5 μM doxorubicin for 1 hour. After 24 hours, the unexposed or exposed cells were harvested, fixed in 70% ethanol in PBS at room temperature for 30 minutes and then left overnight at 4℃. The cells were then pelleted, resuspended in staining solution (100μg/ml RNase, 40 μg/ml propidium iodide in PBS) and incubated at 37℃ for 30 minutes. The cells were then analysed using a Becton Dickinson FACS machine. (a) and (b) show the fluorescence profile of either the control cells or cells exposed to doxorubicin. (c) Table 3.3 shows the % of cells in the G_0/G_1, S and G_2/M phases in the control and doxorubicin-exposed cells.

3.5.3.1 Expression of hGH and topo IIα mRNA and protein in the breast cancer cell lines

The region of the topo IIα promoter which contains the maximal promoter activity (-617 to + 90) was sequenced in both the reporter construct and endogenous gene to ensure that no changes, insertions, deletions or mutations had occurred following drug exposure. Automated sequencing was performed as described in section 2.2.1.9. No changes to the nucleotide sequence were detected (results not shown).

hGH protein, endogenous topo IIα protein and the hGH mRNA were measured in selected cell lines. Up to six of the individual cell lines derived from drug exposure of the MDA1964, MDA144, MCF-7-1964 and MCF-7-144 parental cell lines were selected at random for further analysis. The amount of hGH protein secreted from the cells was determined by ELISA analysis and normalized against the number of cells. This value was compared to the hGH/cell values of control cells with the control arbitrarily set to 100%.

Table 3.4 and 3.5 shows the variation seen between lines derived from the same parental line and exposed to the drug at the same time. The cell lines from MDA1964 all showed an increase of hGH/cell from 400-700%. The hGH mRNA was quantified relative to 18S rRNA. The increase in hGH/cell was reflected by an increase in hGH mRNA in the specific cell lines investigated. The cells from MDA144 showed either little or no change (E1 and F1) or a decreased hGH/cell value (D1). In the cell line D2, the hGH/cell value had increased from ~40% (seen in D1) to ~100%. Therefore the change in hGH/cell was not maintained after a second drug exposure.

The cells from MCF-7-1964 showed small changes in hGH/cell compared to the control values while MCF-7-144 cell lines H1 and H2 showed variations in hGH/cell from 30 to 155%.

(a) MDA1964 cell lines

Individual clones isolated after one drug dose	Initial hGH/cell	Final hGH/cell	hGH mRNA
#1 (cell line B1)	663%	235%	-
#2	475%	-	-
#3 (cell line A1)	713 ± 30%	474 ± 133%	606%
#4	413%	-	-
#5	573%	-	-
#6 (cell line C1)	663%	-	-

Individual clones isolated after exposing A1 to a second drug dose	Initial hGH/cell	Final hGH/cell	hGH mRNA
#3a (cell line A2)	376%	281 ± 142%	271%
#3b	224%	-	-
#3c	167%	-	-
#3d	235%	-	-

(b) MDA144 cell lines

Individual clones isolated after one drug dose	Initial hGH/cell	Final hGH/cell	Clones isolated after a second drug dose	Initial hGH/cell
#1	40%	-	-	-
#2 (cell line E1)	80%	-	-	-
#3 (cell line F1)	100%	-	-	-
#4 (cell line D1)	37 ± 5%	62 ± 22%	#4a (cell line D2)	123 ± 20%
#5	40%	-	-	-
#6	40%	-	-	-

Table 3.4. The initial and final (after freezing) hGH/cell values for individual (a) MDA1964 and (b) MDA144 cell lines. The control (no drug) hGH/cell result was arbitrarily set at 100% and the other hGH/cell values compared to this. The first generation cell lines were clonal cell lines isolated after 1 dose of doxorubicin. The second generation cell lines were isolated after exposing a first generation cell line (either A1 or D1) to a further dose of doxorubicin. The hGH mRNA was calculated relative to 18S rRNA using real time RT-PCR, with the control value arbitrarily set at 100%.

(a) MCF-7-1964 cell lines

Individual clones isolated after one drug dose	Initial hGH/cell	Final hGH/cell	Clones isolated after a second drug dose	Initial hGH/cell
#1	93%	-	-	-
#2	79%	-	-	-
#4 (cell line G1)	113%	137%	#4a (cell line G2)	97%
#5	96%	-	-	-

(b) MCF-7-144 cell lines

Individual clones isolated after one drug dose	Initial hGH/cell	Final hGH/cell	Clones isolated after a second drug dose	Initial hGH/cell	Final hGH/cell
#1	127%	-	-	-	-
#2	82%	-	-	-	-
#3	209%	-	-	-	-
#4 (cell line H1)	155%	76 ± 21%	#4a (cell line H2)	30%	103 ± 40%
#5	245%	-	-	-	-
#6	191%	-	-	-	-

Table 3.5. The initial and final (after freezing) hGH/cell values for individual (a) MCF-7-1964 and (b) MCF-7-144 cell lines. The control (no drug) hGH/cell result was arbitrarily set at 100% and the other hGH/cell values compared to this. The first generation cell lines were clonal cell lines isolated after 1 dose of doxorubicin. The second generation cell lines were isolated after exposing a first generation cell line (either G1 or H1) to a further dose of doxorubicin.

The initial changes were not maintained when specific clones were frozen, thawed and cultured (table 3.4 and 3.5). These results suggested that treatment with doxorubicin could cause alterations to topo IIα reporter construct activity in specific cell lines, but these changes are not stable on freezing or storage.

Four cell lines from the MDA1964 and MDA144 and two from the MCF-7-1964 and MCF-7-144 surviving clones were selected for further analysis. These cell lines are detailed in table 3.6 and have been used in the subsequent research.

Parental cell line	Cell line name	Description
MDA1964	A1	Survived one dose of doxorubicin
	A2	Cell line derived from exposing A1 to a second dose of doxorubicin
	B1	Survived one dose of doxorubicin
	C1	Survived one dose of doxorubicin
MDA144	D1	Survived one dose of doxorubicin
	D2	Cell line derived from exposing D1 to a second dose of doxorubicin
	E1	Survived one dose of doxorubicin
	F1	Survived one dose of doxorubicin
MCF-7-1964	G1	Survived one dose of doxorubicin
	G2	Cell line derived from exposing G1 to a second dose of doxorubicin
MCF-7-144	H1	Survived one dose of doxorubicin
	H2	Cell line derived from exposing H1 to a second dose of doxorubicin

Table 3.6. Description of the cell lines used in the current study. The parental stable cell line, cell line designation and method of drug exposure are detailed. All cell lines were exposed to either a single or double dose of doxorubicin (5μM for1 hour), the cells were then washed and replaced in the incubator. Once individual colonies were observed, random clones were isolated and then amplified to obtain sufficient cells for analysis.

The levels of topo IIα protein (relative to α-tubulin levels) were evaluated in each of these cell lines. Topo IIα mRNA levels (relative to 18S rRNA) were also investigated in specific cell lines. Because of the dramatic increase in hGH expression from the -1964 promoter reporter construct seen in the MDA1964 resistant cell lines (table 3.4), it was expected that the levels of topo IIα expression would also be elevated in these cell lines. This was not the case. The cell lines either showed no change in topo IIα protein levels (B1 and C1) or reduced levels (A1 and A2) compared to the control line. In the cell lines A1 and A2, the decrease in topo IIα protein levels did not correlate with a decrease in topo IIα mRNA levels (figure 3.7 (a)).

A disparity between the hGH and topo IIα results was also seen with the MCF-7-1964 cell lines. Endogenous topo IIα protein was increased in the G1 cell line (256 ± 90%) (figure 3.7 (c)) but the hGH/cell ratio had a value of ~100% compared to the control (table 3.5).

Overall the results of this experiment suggested that the reporter protein levels did not accurately reflect the levels of the topo IIα protein expressed from the endogenous gene in the surviving cell lines.

The cell lines expressing the truncated -144 topo IIα promoter construct may not be expected to reflect endogenous topo IIα levels if this promoter construct was missing promoter elements responsible for the changes in transcriptional regulation. The cells produced from MDA144 had hGH/cell values of 40-100% compared to the control (table 3.4). While decreased relative amounts of topo IIα protein were seen in all 4 cell lines tested (figure 3.7 (b)), there was no correlation of the hGH/cell values with endogenous topo IIα levels, with cell line E1 and F1 having the lowest topo IIα levels and the hGH/cell values close to control levels. When the amounts of topo IIα mRNA was investigated in a specific cell line, a decrease in topo IIα mRNA was seen which correlated with the decrease in topo IIα protein (figure 3.7 (b)).

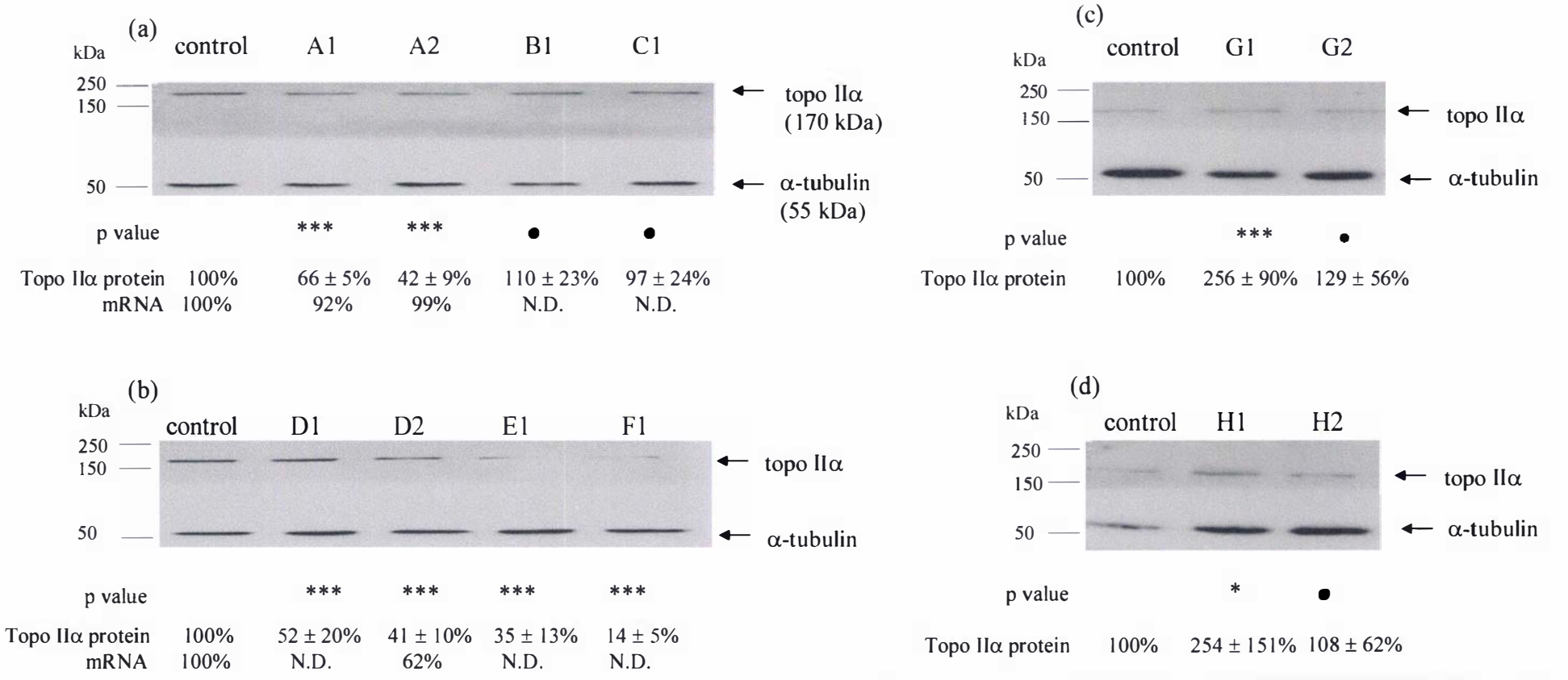


*Figure 3.7. Example of an immunoblot of whole cell extracts (Ladias et al., 1992) from the (a) MDA1964, (b) MDA144, (c) MCF-7-1964 and (d) MCF-7-144 cell lines. 5-10 μg of cell extracts were separated on an 8% SDS-PAGE gel. The proteins were transferred onto positively charged nylon membrane for 90 minutes at 450 mA and incubated in 1% blocking solution in TBST for 2 hours. The membrane was cut horizontally at the 75 kDa marker, with the top half of the membrane incubated in topo IIα antibody (1:1,000), and the remaining membrane incubated in α-tubulin antibody (1:2,000) overnight at 4 ℃. The antibodies were diluted in 0.5% blocking solution. The membranes were washed over 40 minutes with TBST and incubated in secondary antibodies diluted in 0.5% blocking solution for 30 minutes (1:10,000 anti goat IgG for topo IIα, 1:5,000 anti mouse IgG for α-tubulin). The membrane was then washed over 1 hour with TBST and the proteins detected using a Roche chemiluminescent (POD) detection kit. The Fujifilm Darkbox II using the LAS Pro 1000 program were used to detect the signal. The program Image Gauge was used to quantify the protein bands. At least 3 experiments were performed. The value of topo IIα was calculated relative to the α-tubulin value for the given sample. The value of the amount of topo IIα protein (relative to α-tubulin) for the control (no drug) cell line was arbitrarily set at 100%. The Student T-test (2 tailed) was performed to determine the statistical significance of these results compared to the control values and labeled underneath • $p>0.05$ (not significant), * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. The average (± standard deviation) topo IIα protein levels (relative to α-tubulin) and the topo IIα mRNA levels (relative to 18S rRNA) are indicated below the figures. N.D. = not determined.*

A lack of correlation between topo IIα protein levels and hGH/cell was also seen with the MCF-7-144 cell lines. The hGH/cell ratio of H1 was ~100-150% while in H2 cells hGH/cell ratios of ~30-40% were seen (table 3.5) whereas the relative amounts of topo IIα protein were increased in H1 and at control levels in H2 (figure 3.7 (d)).

3.6 Discussion

The objective of this aspect of the research was to use a series of deletion constructs linked to the hGH reporter gene to identify regions involved in the regulation of the topo IIα promoter in drug-surviving cells. Previously it was stated that for the experimental strategy outlined to work, four assumptions were made. The first assumption was that the mechanism of topo IIα regulation was similar to that seen in other cell types. The results from the transient transfections showed that this was likely to be the case. The second assumption was that the reporter protein would reflect the activity of the endogenous and full length introduced topo IIα promoter. While alterations in the levels of the hGH reporter protein were seen in surviving cells containing the full length topo IIα promoter construct, these changes did not correspond to changes in endogenous topo IIα protein levels. The lack of correlation between the hGH reporter protein and endogenous topo IIα protein levels showed that this was not a valid reporter system to monitor endogenous topo IIα protein levels after exposure to doxorubicin in these surviving cell lines. This system may still be valid to use to investigate the regulation of the topo IIα promoter under other physiological conditions, but cannot be used to assess promoter activity after exposure to this chemotherapy drug.

The reason for this disparity is not clear. It is possible that changes occurred in the cells upon drug exposure and that these changes 'uncoupled' the hGH reporter expression from the endogenous topo IIα expression. The site of integration of the promoter constructs may be subject to position effects that respond to drug i.e. near or in a promoter or enhancer that up-regulates specific genes in response to stress, near a DNA region that is susceptible to drug-induced genomic amplification or even a region where changes in methylation status can occur. For example Tada *et al.* (2000) and

Kusaba *et al.* (1999) showed that demethylation and altered chromatin structure of the *MDR1* promoter occurred during drug treatment. Topo II poisons have been proposed to inhibit methylation by altering the conformation of the DNA so that it becomes a poor substrate for DNA cytosine 5'-methyltransferase (Nyce, 1997).

It was also noted that in the MDA1964 cell lines, the topo IIα mRNA levels did not correlate with protein levels in the cell lines analysed, while in the MDA144 cell line investigated, the topo IIα protein did seem to show a correlation. An alternate mechanism may be occurring in these MDA144 cell lines. For example a transcriptional regulation may be occurring where reduced topo IIα protein levels are due to reduced promoter activity. Alternatively, a decrease in mRNA stability may have occurred which caused the decreases in topo IIα protein. Doxorubicin exposure has been previously shown to alter mRNA stability (Morceau *et al.*, 1996; Aries *et al.*, 1996; Jeannesson *et al.*, 1997)

In specific MDA1964 cell lines a post-transcriptional or translational regulation may have caused the decrease in topo IIα protein seen. For example, in the MDA1964 cell lines A1 and A2, the topo IIα mRNA levels were unchanged, while a decrease in topo IIα protein was seen. Changes to the cellular localization or structure of the topo IIα mRNA could result in a decrease in translation of the topo IIα protein, or a decrease in the stability of the topo IIα protein could also cause the decrease in relative amount of topo IIα protein seen.

A number of topo IIα proteins with altered cellular localization have been detected in a range of cell lines and resistant cells. These proteins range in size from 145-170 kDa and most lack the C-terminal region or the nuclear localization sequence and so are predominantly cytoplasmic (Mo & Beck, 1997; Yu *et al.*, 1997; Wessel *et al.*, 1997; Mirski *et al.*, 2000; Harker *et al.*, 1995b). The possibility exists that this kind of resistance mechanism could be occurring in the surviving cell lines. If the topo IIα protein size has not dramatically altered, this kind of mechanism would not be observed in the immunoblot experiments using whole cell extracts. This kind of

mechanism may be detected by using nuclear extracts in immunoblots or by immunocytostaining to visualize the location of the topo IIα protein within the cell.

The third and fourth assumptions were that the different stable cell lines would be unchanged by the integration of the construct and that all stable cell lines would respond to the drug in the same way. This response needed to be consistent in all cells to be able to compare the stable lines and so identify specific regions of the topo IIα promoter involved in its regulation upon drug exposure. The results suggest that this may not be the case. The resistant cell lines derived from the same parental stable cell line showed variation in the extent of the changes in topo IIα protein levels (from no change to a significant decrease) indicating that the cells were responding to the drug in different ways, resulting in cells with differing phenotypes.

Variation in cellular response to drugs has been observed previously. Chen *et al.* (1994), Heenan *et al.* (1997) and Richardson & Siemann (1997).showed that there was variation in the resistant cells produced under identical treatment conditions. Variation was observed in the number of clones produced per plate, the pattern of resistance within these clones or in the maintenance of this resistance under drug-free conditions. These results give support to the hypothesis that the resistance to doxorubicin is due to a spontaneous event rather than due to a pre-existing variant and that each clone is a descendant from a cell which had a single advantageous mutational event.

Another interesting observation was that the hGH/cell values changed after the cell lines were frozen and stored. A possible reason for this could be that even though every attempt was made to produce cell lines derived from single cells, multiple populations of cells were present in each cell line and a different population of cells survived the freezing process in a more viable state and so could become dominant. Another possibility is that the cells were genetically unstable and so upon freezing or multiple cell divisions allows cells with the most stable genetic composition to predominate. This could result in changes to the reporter construct copy number or the DNA environment and so may explain the changes in hGH levels seen over time. A possible mechanism for this could be due to the hypomethylation of chromatin that can

occur during drug treatment. Hypomethylation can lead to chromosomal instability, aneuploidy, mutations, the reactivation of transposons and gene up-regulation (reviewed in Esteller & Herman, 2002).

Levels of the hGH reporter protein did not correlate with endogenous topo IIα and variable responses to doxorubicin were observed. Therefore the experimental strategy of identifying specific regions of the topo IIα promoter involved in the transcriptional regulation in cells that survive drug exposure could not be used. However, the levels of topo IIα protein in the surviving cells differed from that in the control cells. Therefore individual stable cell lines were investigated with the aim of identifying mechanisms that may have caused the changes in the endogenous topo IIα levels. This work is described in Chapter Four. Other mechanisms that may contribute to the drug survival of the cell lines specifically MDR1, MRP1 and Hsp70 were also investigated and the results described in Chapter Five.

Chapter Four Analysis of Transcription Factors in Drug-exposed Breast Cancer Cells

4.1 Introduction

A number of transcription factors have been implicated in the regulation of the topo IIα promoter including NF-Y, Sp1 and Sp3 (Morgan & Beck, 2001; Wang *et al.*, 1997a; Joshi *et al.*, 2000; Magan, 2002; Kubo *et al.*, 1995; Mo *et al.*, 1997). Therefore, surviving breast cancer cell lines were analysed for the relative amounts of these proteins using semi-quantitative immunoblotting.

NF-YA was investigated as DNA binding of the trimer has been correlated with NF-YA levels (Chang & Liu, 1994; Bolognese *et al.*, 1999; Marziali *et al.*, 1999). The levels of protein were investigated rather than mRNA as NF-YA protein has been found to vary under specific conditions indicating post-transcriptional regulation (Bolognese *et al.*, 1999; Marziali *et al.*, 1999). NF-YA exists as long (NF-YAL, 44-45 kDa) and short isoforms (NF-YAS, 41-42 kDa) in mammalian cells (Bolognese *et al.*, 1999; Morgan & Beck, 2001; Chang & Liu, 1994; Ishimaru *et al.*, 1997). A goat polyclonal antibody directed against the C-terminal of human NF-YA was used that would detect both NF-YAL and NF-YAS isoforms. Both isoforms were investigated as there is evidence that the NF-Y trimer containing different NF-YA isoforms may have a differential effect on transcription on specific promoters (Gu *et al.*, 1999; Chang & Liu, 1994).

Total Sp1 protein was detected using a rabbit polyclonal antibody directed towards an internal domain of human Sp1. In the immunoblots, Sp1 can be detected as a ~95 kDa band with a slightly higher molecular weight indistinct band (~105 kDa). The 105 kDa band is thought to be post-translationally modified, either by phosphorylation or glycosylation (Jackson *et al.*, 1990; Jackson *et al.*, 1988).

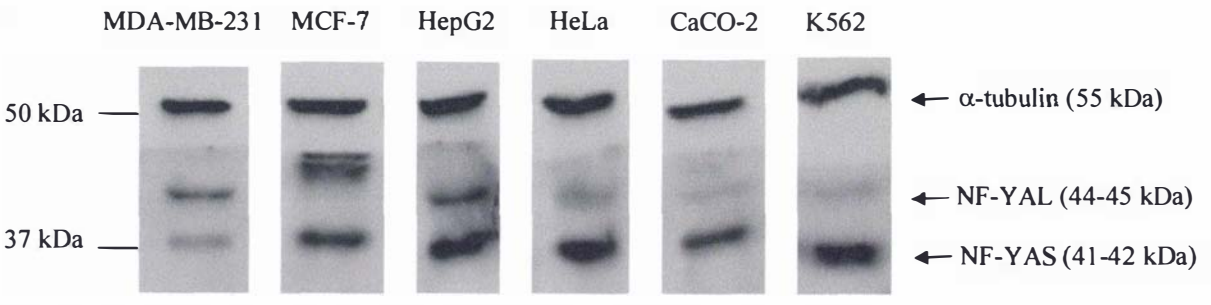
Sp3 exists as three isoforms within the cell, a ~115, 70 and 68 kDa bands (Ammanamanchi & Brattain, 2001a). These isoforms result from differential internal translation initiation, with the shorter isoforms being able to bind DNA, but having little transactivational activity and are thought to act as inhibitors to both Sp1 and Sp3-mediated transactivation (Kennett *et al.*, 1997). Therefore the total Sp3 and the proportion of isoforms were investigated. A rabbit polyclonal antibody directed against a C-terminal epitope of human Sp3 was used which could detect all of the Sp3 isoforms.

Semiquantitative immunoblotting was used to initially investigate the relative amounts and proportion of the NF-YA isoforms in the parental cell lines to identify any significant variations between the two parental breast cell lines and the parental stable cell lines. The relative amounts of Sp1 protein and the relative amounts and proportions of the Sp3 and NF-YA isoforms were then investigated in each of the two parental and surviving cell lines. Cell lines that had been frozen, thawed and maintained without further drug exposure were also used to investigate changes in transcription factors that may have occurred subsequent to freezing and storage. To correct for variations in loading, the amount of each protein was calculated relative to α-tubulin.

4.2 Analysis of long and short NF-YA isoforms, Sp1 and Sp3 in various cancer cell lines

Whole cell extracts were prepared and analysed by immunoblotting as described in section 2.2.4. The immunoreactive proteins were visualised and the bands quantified relative to α-tubulin.

(a)



Total NF-YA relative to α-tubulin	43 ± 5%	109 ± 21%	89 ± 34%	89 ± 25%	81 ± 8%	117%

(b)

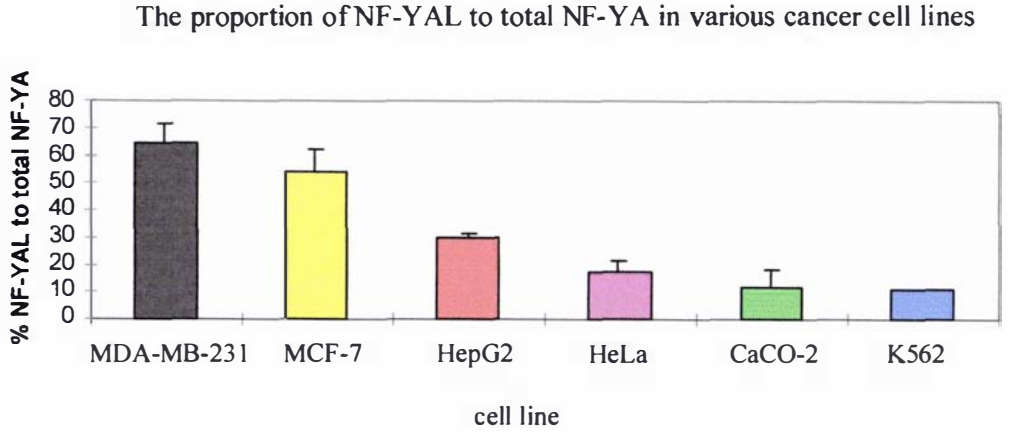


Figure 4.1. (a) Example of an immunoblot of NF-YA isoforms using whole cell extracts (Ladias et al., 1992) from MDA-MB-231, MCF-7, HepG2, HeLa, CaCO-2 and K562 cell lines. 10 μg of cell extracts were separated on an 8% SDS-PAGE gel. The proteins were transferred onto positively charged nylon membrane for 90 minutes at 450 mA and incubated in 1% blocking solution in TBST for 2 hours. The membrane was cut horizontally just below the 50 kDa marker. The bottom half of the membrane was incubated in NF-YA antibody (1:500 in 0.5% blocking solution in TBS), and the remaining membrane incubated in α-tubulin antibody (1:2,000 in 0.5% blocking solution) overnight at 4℃. The membranes were washed over 40 minutes and incubated in secondary antibodies for 30 minutes (1:2,500 anti goat IgG for NF-YA 1:5,000 anti mouse IgG for α-tubulin in 0.5% blocking solution). The membrane was then washed over 1 hour with TBST and the proteins detected using the Roche Chemiluminescence Blotting Substrate (POD) Kit. To detect the signals the Fujifilm Darkbox II using the LAS1000 Pro program was used. The program Image Gauge was used to quantify the protein bands. The average (± standard deviation) value of the total NF-YA (long and short) levels was calculated relative to the α-tubulin value for a given sample and shown as a % below the figure. (b) Graph of the average (± standard deviation) relative amounts of NF-YAL isoform to total NF-YA in the cell lines. The experiments were performed at least two times.

As seen in figure 4.1 (a, b) the proportion of NF-YAL varied greatly between the untreated cell lines. The MDA-MB-231 and MCF-7 breast cancer cells had a substantially higher proportion of the long isoform than the other cell lines tested, while HepG2 (hepatocarcinoma), HeLa (cervical), CaCO-2 (colon carcinoma) and K562 (leukemia) cells contained a majority of the shorter isoform. It was noted that the long isoforms of NF-YA found in MCF-7 cells had a lower mobility than the same isoform in the other cell lines. This could be due to a post-translational modification although such modifications have yet to be identified.

When the total amounts of NF-YA protein were examined relative to α-tubulin, it was seen that the MDA-MB-231 cells produced ~$\frac{1}{2}$ the amount of total NF-YA when compared to the other cell lines (figure 4.1 (a)).

The relative amount of Sp1, Sp3 and Sp3 isoforms within parental cell lines were determined. MCF-7 cells had 2-fold more Sp1 (relative to α-tubulin) than the MDA-MB-231 cells (results not shown). An indistinct slightly higher molecular weight protein was seen above the main Sp1 band in Sp1 immunoblots of MDA-MB-231 cell extracts and most likely represents phosphorylated or glycosylated Sp1. Contrary to what was seen in the MDA-MB-231 cell line, this higher molecular weight band was the predominant isoform observed in Sp1 immunoblots of MCF-7 cell extracts (compare figure 4.3 b, f and 4.5 b, f).

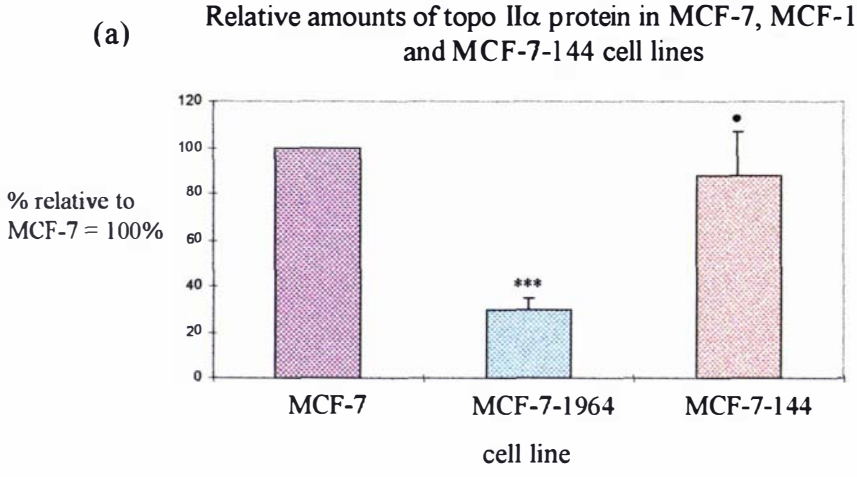
The 115 kDa isoform of Sp3 generally accounted for ~50% of the total Sp3 protein in both MDA-MB-231 and MCF-7 cell extracts with the two smaller isoforms representing approximately equal amounts of the remaining total Sp3 protein (Appendix 12). MCF-7 cell extracts contained over twice the Sp3 levels (relative to α-tubulin) compared to the MDA-MB-231 cell extracts (results not shown), but as MCF-7 cells also had increased relative amounts of Sp1, the overall ratio of Sp1:Sp3 was similar between the cell lines.

4.3 Analysis of transcription factors in parental stable cell lines

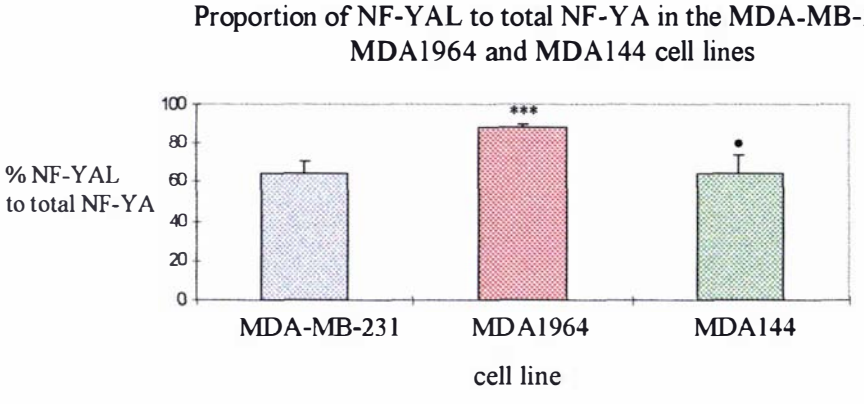
The relative amounts of the topo IIα, Sp1, Sp3 and total NF-YA were similar in the MDA-MB-231, MDA1964 and MDA144 cell lines (results not shown). The MDA1964 cell line had a slightly increased, but statistically significant, proportion of long NF-YA isoform to total NF-YA when compared to MDA-MB-231 and MDA144 cell lines (figure 4.2 (b)).

The MCF-7-144 and MCF-7-1964 cell lines showed some differences to the parental MCF-7 cell line. MCF-7-1964 had a ~1/4 the relative amount of topo IIα protein (figure 4.2 (a)) when compared to the MCF-7 and MCF-7-144 cell lines while the MCF-7-144 cell line had a reduced proportion of NF-YAL isoform when compared to MCF-7 and MCF-7-1964 (figure 4.2 (c)). It is not known how these differences may alter drug response, but confirmed that alterations can occur during the production of stable cell lines.

(a)



(b)



(c)

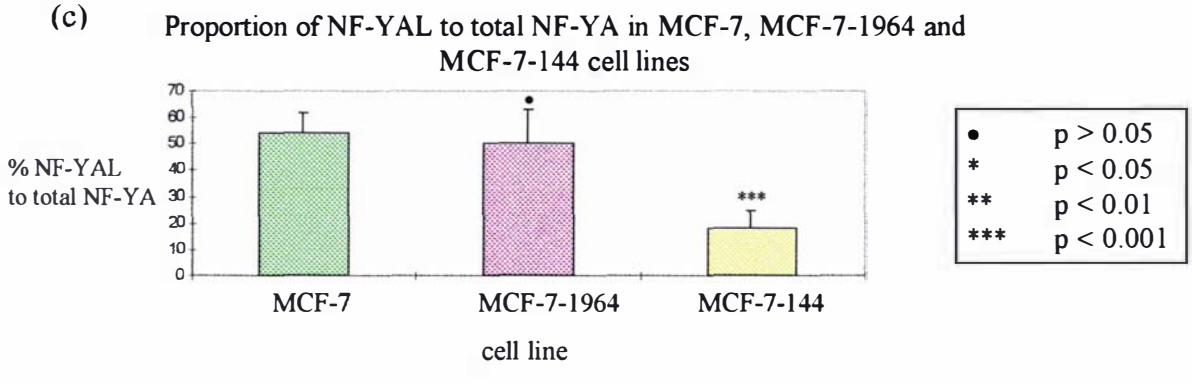


*Figure 4.2. Summary of the immunoblot experiments to determine the relative amounts of (a) topo IIα. The value of the MCF-7 cell line was arbitrarily set at 100%. The relative proportion of NF-YAL to total NF-YA in the (b) MDA-MB-231 and (c) MCF-7 cell lines. The immunoblots were carried out as described in figure 4.1. The average (± standard deviation) values are shown. At least three experiments were performed. The Student T test (2 tailed) was used to determine the statistical significance of these results compared to the parental cell line values using Excel. • $p > 0.05$ (deemed non significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

4.4 Analysis of transcription factors in the drug-exposed cell lines

Whole cell protein extracts were prepared and analysed as described in section 4.2.

4.4.1 The MDA1964 and MDA144 cell lines

The relative amounts of transcription factors analysed in the MDA1964 and MDA144 lines varied (figure 4.3, 4.4). In cell line A1 (MDA1964), a decrease in the relative amount of topo IIα protein was seen without any changes in the relative amounts of Sp1, Sp3 or NF-YA. In cell line A2 (the cells from A1 that survived a second dose of doxorubicin), a further significant decrease ($p < 0.01$) in topo IIα protein was observed with a statistically significant increase in the relative amount of NF-YA. Cell lines B1 (MDA1964) and C1 (MDA1964) did not show changes in the relative amounts of topo IIα protein, but a statistically significant decrease in the total amount of Sp3 protein was seen (figure 4.3, 4.4). The proportions of NF-YA, Sp1 and Sp3 isoforms were unchanged in each of the cell lines tested (results not shown).

All of the MDA144 cell lines had decreased relative amounts of topo IIα when compared to the control. All decreases were statistically significant at the 99.9% level. Cell line D1 exhibited no significant changes in Sp3 or NF-YA and a slight decrease in Sp1, while cell line D2 (the cells from D1 that survived a second dose of doxorubicin), which had a statistically significant increase in the amount of total NF-YA protein showed no further reduction ($p > 0.05$) in the relative amounts of topo IIα protein. Cell line F1 exhibited both a statistically significant decrease in Sp1 protein and increase in NF-YA protein, while in cell line E1 only a statistically significant decrease in Sp1 was observed (figure 4.3, 4.4). The proportions of NF-YA, Sp1 and Sp3 isoforms were unchanged in each of the cell lines tested (Appendix 11).

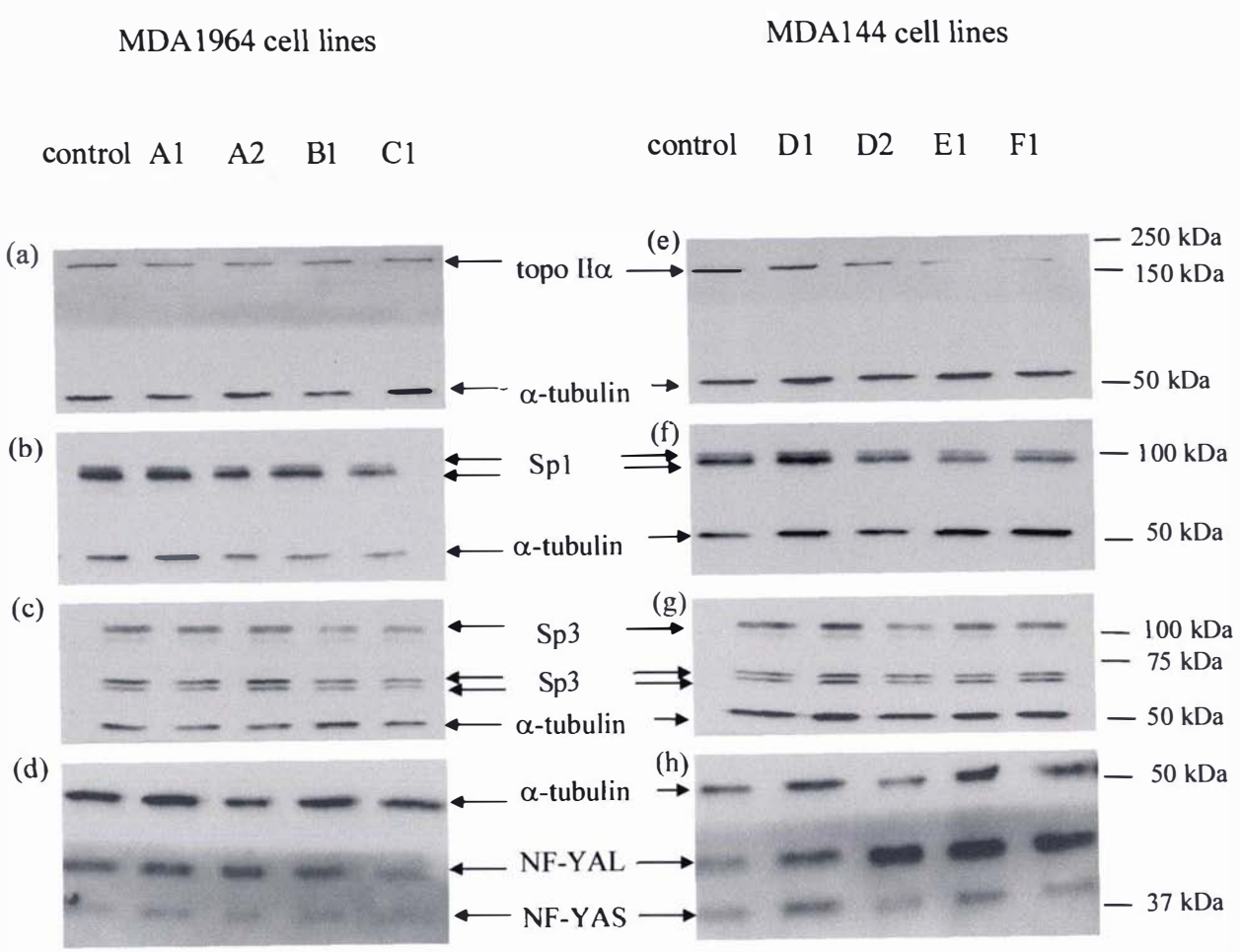
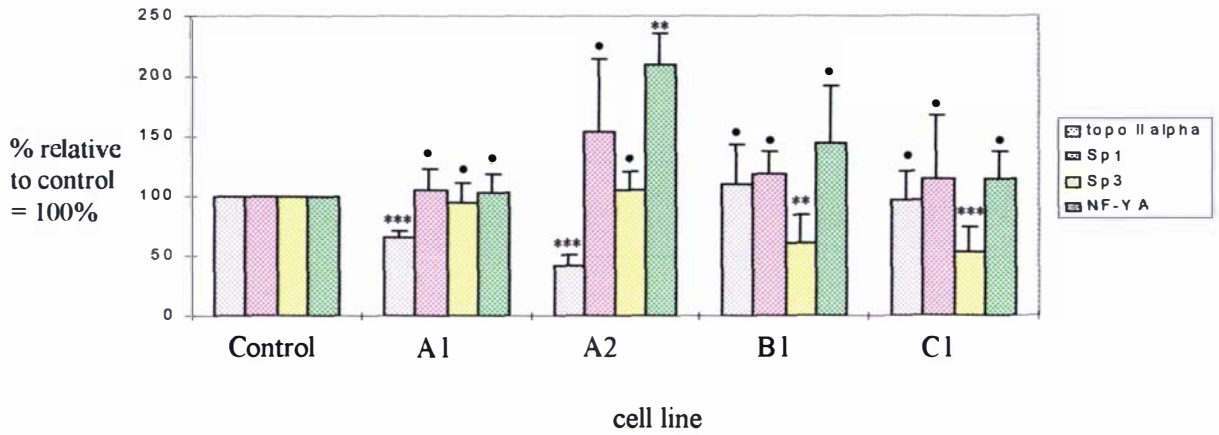


Figure 4.3. Example of an immunoblot of (a),(e) topo IIα, (b),(f) Sp1, (c),(g) Sp3 and (d),(h) NF-YA of whole cell extracts (Ladias et al., 1992) from the MDA1964 and MDA144 cell lines. 5 μg of cell extracts were separated on an 8% SDS-PAGE gel. The proteins were transferred onto positively charged nylon membrane for 90 minutes at 450 mA and incubated in 1% blocking solution in TBST for 2 hours. The membrane was cut horizontally just below the 50 kDa marker, at the ~60 kDa position and at the ~150 kDa marker. The >150 kDa section was incubated in topo IIα antibody (1:1,000), the >60-150 kDa section was incubated in either Sp1 antibody (1:1,000) or Sp3 antibody (1:1,000), the 50-60 kDa section was incubated in α-tubulin antibody (1:2,000) and the <50 kDa section was incubated in NF-YA antibody (1:500). The antibodies were diluted in 0.5% blocking solution in TBS overnight at 4°C. The membranes were washed over 40 minutes with TBST and incubated in secondary antibodies for 30 minutes (1:5,000 anti goat IgG for topo IIα, 1:5,000 anti rabbit IgG for Sp3, 1:10,000 anti rabbit IgG for Sp1, 1:5,000 anti mouse IgG for α-tubulin and 1:2,500 anti goat IgG for NF-YA in 0.5% blocking solution). The membrane was then washed over 1 hour with TBST and the proteins detected using the Roche Chemiluminescence Blotting Substrate (POD) Kit. The Fujifilm Darkbox II using the LAS1000 Pro program was used to detect the signal. The program Image Gauge was used to quantify the protein band signal. The value of the topo IIα, Sp1, Sp3 or NF-YA was calculated relative to the α-tubulin value for the given sample. The relative value for the control sample was arbitrarily set at 100%, with the other values compared to this. At least three experiments were performed.

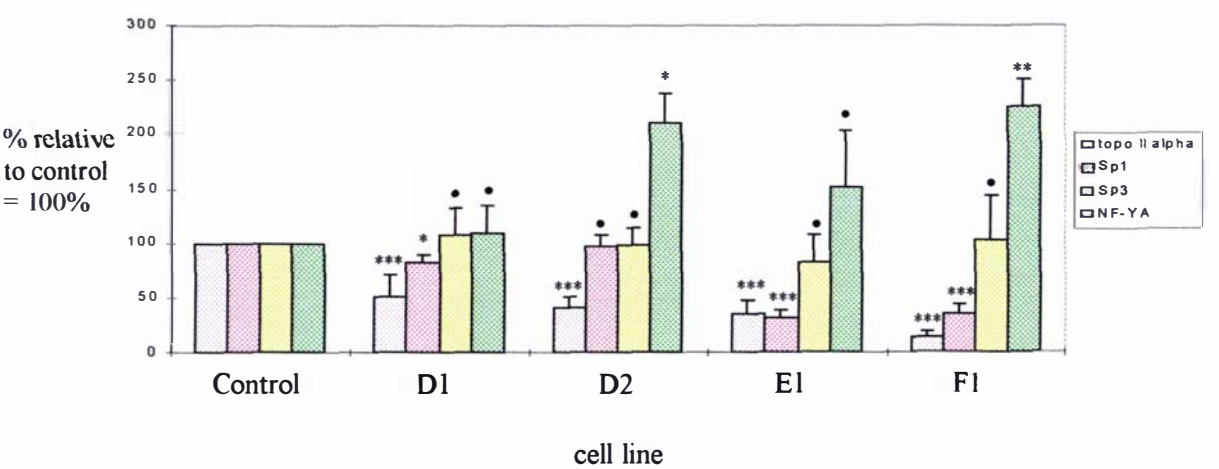
(a)

The relative amounts of topo IIα, Sp1, total Sp3 and total NF-YA protein in the MDA1964 cell lines



(b)

The relative amounts of topo IIα, Sp1, total Sp3 and total NF-YA protein in the MDA144 cell lines



•	$p > 0.05$
*	$p < 0.05$
**	$p < 0.01$
***	$p < 0.001$

*Figure 4.4. Summary of the immunoblot results from the (a) MDA1964 cell lines and (b) MDA144 cell lines. The amount of topo IIα (blue bars), Sp1 (red), Sp3 (yellow) and NF-YA (green) protein was calculated relative to α-tubulin, with the average (± standard deviation) values shown. The value for the control was arbitrarily set at 100%. Immunoblots were performed as described in figure 4.3. At least three experiments were carried out. The Student T test (2 tailed) was performed on the data to determine the statistical significance of these results when compared to the control values using Excel. • $p > 0.05$ (deemed non significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional results are detailed in Appendix 11.*

4.4.2 The MCF-7-1964 and MCF-7-144 cell lines

In contrast to the MDA1964 and 144 cell lines no decreases in topo IIα protein were observed in the MCF-7-1964 and 144 cell lines after drug exposure. Cell line H1 had elevated relative amounts of topo IIα protein ($p < 0.05$) but no significant changes in the amounts of Sp1 or Sp3. A slight reduction in NF-YA was observed ($p < 0.05$) which was probably due to the reduction in the long isoform (figure 4.5, 4.6). The cell line G1 had elevated relative amounts of topo IIα protein and NF-YA protein compared to the control ($p < 0.001$). However cell lines G2 (MCF-7-1964) and H2 (MCF-7-144) showed no significant changes in either topo IIα or any of the transcription factors analysed (figure 4.5, 4.6). The proportions of the 105 kDa band to the total Sp1 and Sp3 isoforms were unchanged in the cell lines tested (Appendix 12).

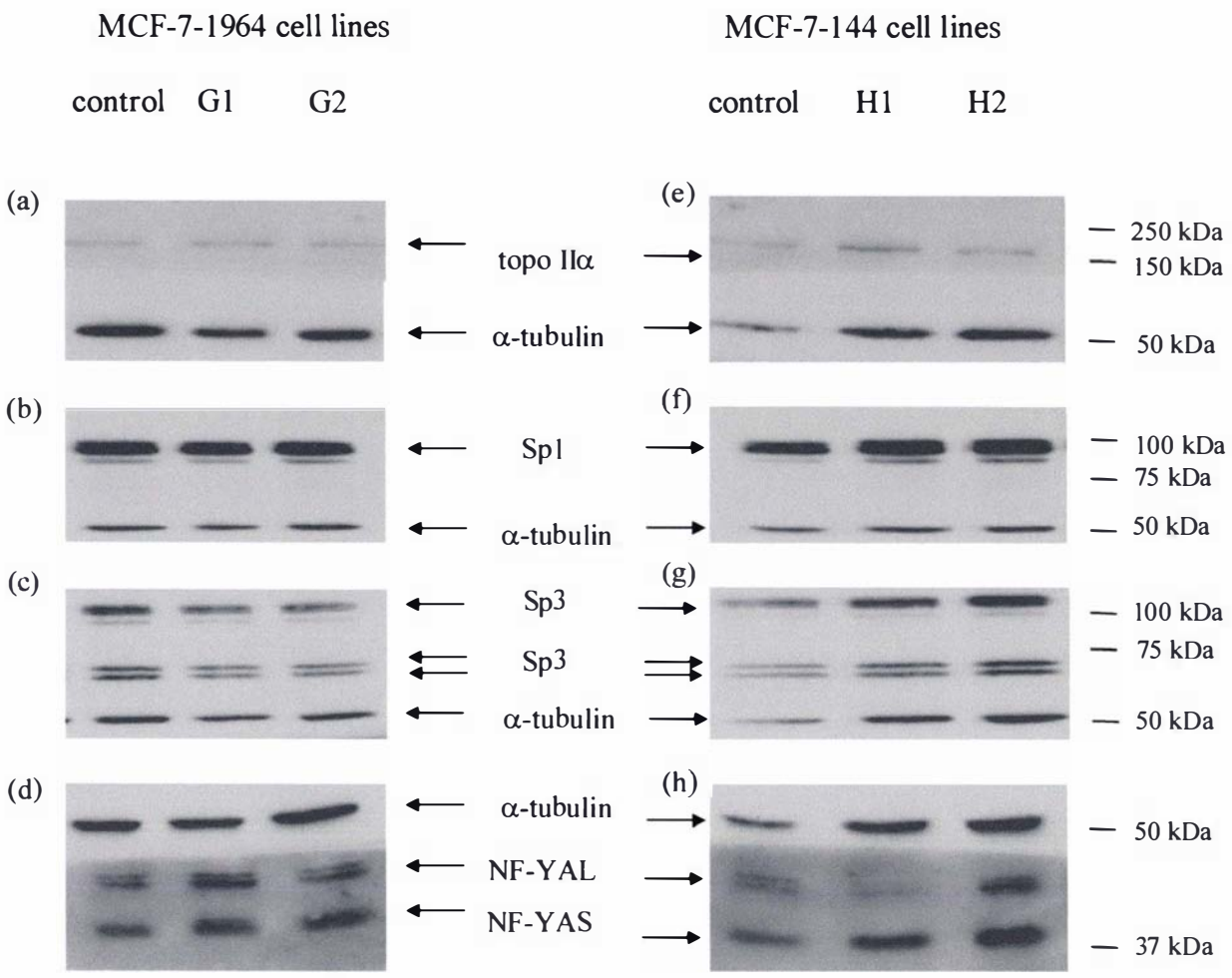
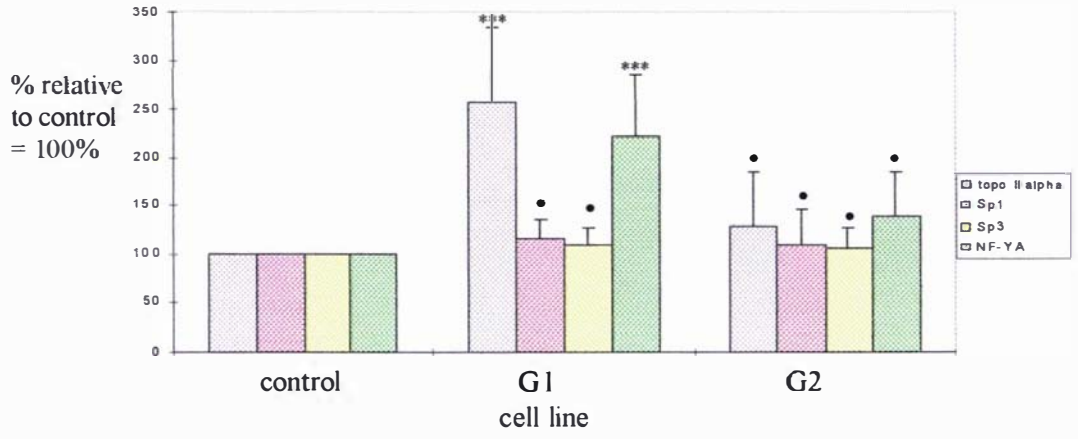
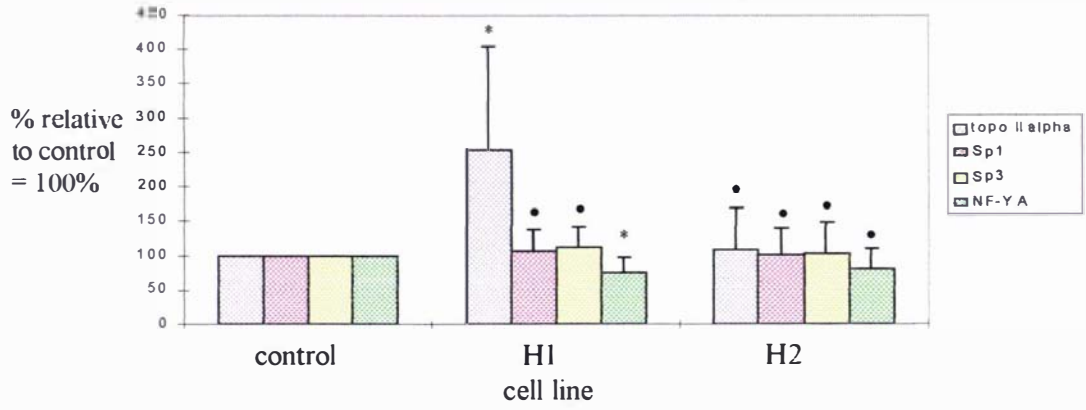


Figure 4.5. Example of an immunoblot of (a),(e) topo IIα, (b),(f) Sp1,(c),(g) Sp3 and (d),(h) NF-YA of whole cell extracts (Ladias et al., 1992) from the MCF-71964 and MCF-7144 cell lines. 10 μg of cell extracts were separated on an 8% SDS-PAGE gel. The proteins were transferred onto positively charged nylon membrane for 90 minutes at 450 mA and incubated in 1% blocking solution in TBST for 2 hours. The membrane was cut horizontally just below the 50 kDa marker, at the ~60 kDa position and at the ~150 kDa marker. The >150 kDa section was incubated in topo IIα antibody (1:1,000), the >60-150 kDa section was incubated in either Sp1 antibody (1:1,000) or Sp3 antibody (1:1,000), the 50-60 kDa section was incubated in α-tubulin antibody (1:2,000) and the <50 kDa section was incubated in NF-YA antibody (1:500). The antibodies were diluted in 0.5% blocking solution in TBS overnight at 4 ℃. The membranes were washed over 40 minutes with TBST and incubated in secondary antibodies for 30 minutes (1:5,000 anti goat IgG for topo IIα, 1:5,000 anti rabbit IgG for Sp3, 1:10,000 anti rabbit IgG for Sp1, 1:5,000 anti mouse IgG for α-tubulin and 1:2,500 anti goat IgG for NF-YA in 0.5% blocking solution). The membrane was then washed over 1 hour and the proteins detected using the Roche Chemiluminescence Blotting Substrate (POD) Kit. To detect the signal, the Fujifilm Darkbox II using the LAS1000 Pro program was used. The program Image Gauge was used to quantify the protein band signal. The value of the topo IIα, Sp1, Sp3 or NF-YA was calculated relative to the α-tubulin value for the given sample. The relative value for the control sample was arbitrarily set at 100%, with the other values compared to this.

(a) The relative amounts of topo IIα, Sp1, total Sp3 and total NF-YA protein in MCF-7-1964 cell lines



(b) The relative amounts of topo IIα, Sp1, total Sp3 and total NF-YA protein in MCF-7-144 cell lines



(c) Proportion of NF-YAL isoform to total NF-YA in the MCF-7-144 cell lines

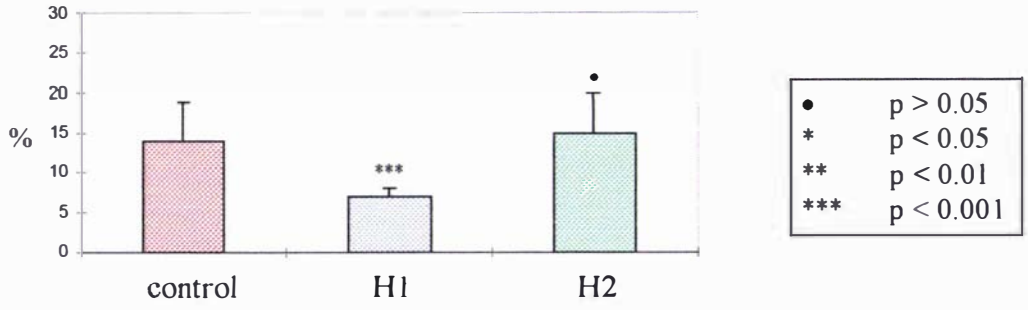


*Figure 4.6. Summary of the immunoblot results of (a) MCF-7-1964 and (b) MCF-7-144 cell lines. The amount of topo IIα (blue bars), Sp1 (red), Sp3 (yellow) and NF-YA (green) protein was calculated relative to α-tubulin for both cell lines. The value for the control was arbitrarily set at 100%, with the average (± standard deviation) values shown (c) Graph of the proportion of NF-YAL to total NF-YA in MCF-7-144 cell lines. Immunoblots were performed as described in figure 4.3 and at least three experiments were performed,. The Student T test (2 tailed) was performed on the data to determine the statistical significance of these results when compared to the control values using Excel. • $p > 0.05$ (deemed non significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional results are detailed in Appendix 12.*

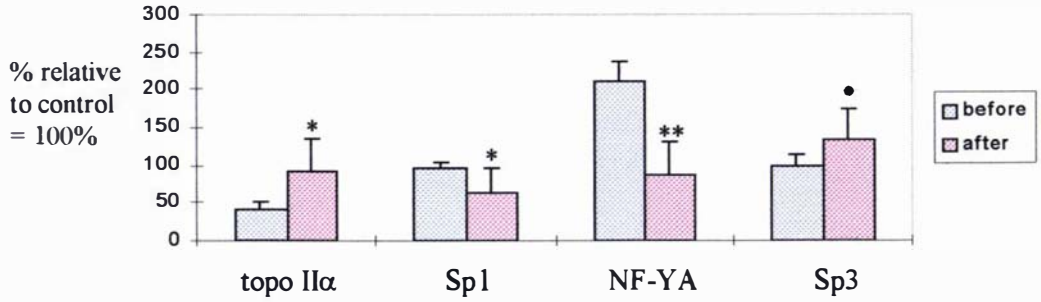
4.4.3 Effect of freezing and thawing on surviving cell lines

Whole cell extracts were prepared and analysed as described in section 4.2 from MDA1964 and 144 cell lines that had been frozen, thawed and maintained without further exposure to doxorubicin.

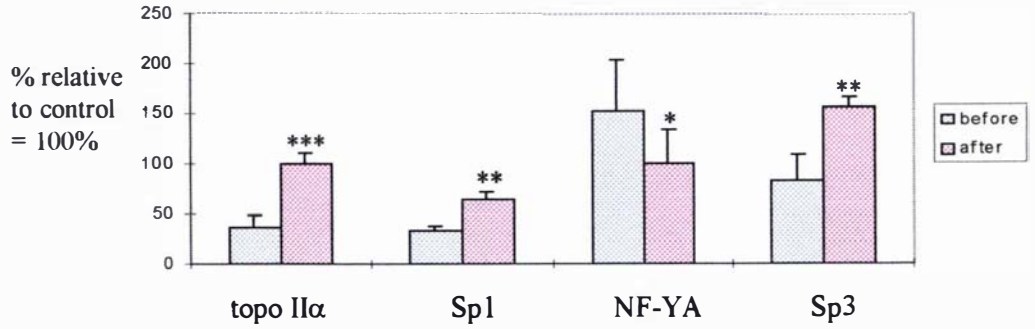
The relative amounts of topo IIα and NF-YA protein had returned to near control levels in cell extracts prepared from cell lines A1 and A2 after freezing and thawing. Inconsistencies were also observed for Sp3. Cell extracts prepared from cell line C1 (which initially demonstrated decreased amounts of Sp3) exhibited control levels of Sp3 while those from cell line B1 had increased amounts of Sp3 (Appendix 13).

A summary of the immunoblotting results for cell lines D2, E1 and F1, all of which initially showed decreased amounts of topo IIα is presented in figure 4.7. The relative amounts of topo IIα returned to control values for all cell lines. The elevated amounts of NF-YA previously observed in cell lines D2 and F1 also returned to control values, and elevated levels of Sp3 were now observed in cell lines E1 and F1. The relative amounts of Sp1 in E1 and F1 remained decreased after freezing, but to a lesser extent (63 ± 9%, 57 ± 15% respectively). The proportions of the 105 kDa band to total Sp1 were unchanged as were the proportion of NF-YA and Sp3 isoforms (data not shown).

(a) Summary of the immunoblotting results from cell line D2 before and after freezing



(b) Summary of the immunoblotting results from cell line E1 before and after freezing



(c) Summary of the immunoblotting results from cell line F1 before and after freezing

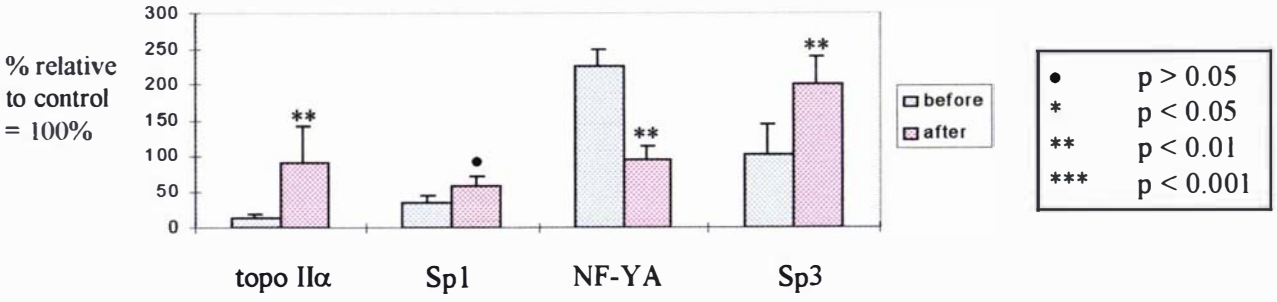


Figure 4.7. Summary of the immunoblot results of topo IIα, Sp1, NF-YA and Sp1 proteins before (blue bars) and after (red) the (a) D2, (b) E1 and (c) F1 cell lines had been frozen. At least three experiments were performed. The amounts of topo IIα, Sp1, Sp3 and NF-YA protein were calculated relative to α-tubulin. The value for the control was arbitrarily set at 100%, with the average (± standard deviation) values shown. Immunoblots were carried out as described in figure 4.3. The Student T test (2 tailed) was performed to determine the statistical significance of the" after freezing" results compared to the "before freezing" results using Microsoft Excel. Full results are detailed in Appendix 13.

4.5 Discussion

The exposure of MDA-MB-231 or MCF-7 cells to 1-2 doses of doxorubicin, resulted in the isolation of individual surviving cell lines. Specific cell lines were amplified and relative amounts of topo IIα, NF-YA, Sp1 and Sp3 protein were investigated.

Of the MDA-MB-231 cell lines that survived doxorubicin exposure, 6/8 cell lines showed decreased relative amounts of topo IIα protein. Of these, 3/8 also had increased amounts of NF-YA protein, with 1/3 of these lines also having decreased amounts of Sp1 protein. The cell line which showed the greatest decrease in the amounts of topo IIα protein had both increased NF-YA and decreased Sp1 protein. Two cell lines (A1, D1) which had decreased topo IIα had no highly significant change in amounts of Sp1, Sp3 or NF-YA, while two cell lines (B1, C1) which had no changes in topo IIα had decreased amounts of Sp3.

Cell line D2 was derived by exposing cell line D1 to a second dose of doxorubicin. Cell line D1 had decreased topo IIα protein with a slight decrease in Sp1 levels. Cell line D2 had significantly similar amounts of topo IIα protein to D1 ($p > 0.05$). The Sp1 levels had returned to wild type amounts and an increase in NF-YA was now observed. Cell line E1 had decreased topo IIα protein with a statistical significant decrease in Sp1 levels while cell line F1 which had the greatest reduction in topo IIα protein showed both an increase in NF-YA protein and a decrease in Sp1 protein. All of these cell lines had a reduction in the amounts of topo IIα protein, which seems to indicate that the decrease in topo IIα protein may provide an adaptive advantage for survival during drug exposure and as such is maintained in cell lines after a second drug exposure.

However in cell lines A1 and A2 a decrease in topo IIα protein was seen without alterations to the amounts of topo IIα steady-state mRNA (section 3.5.3.1). This result could indicate that post-transcriptional controls were involved. In addition an increase in the relative amounts of NF-YA was seen in cell line A2.

With the MCF-7 cell lines, increased topo IIα protein was seen in 2 of the cell lines (G1/H1). In one of these (G1), the relative amount of total NF-YA was increased while in the other cell line (H1), a decrease in the proportion of the NF-YAL isoform

was seen. These changes were not maintained upon a second drug exposure, where both the amounts of topo IIα and NF-YA protein had returned to control levels (G2, H2).

The up-regulation of topo IIα expression has been described in cells resistant to alkylating agents (Pu & Bezwoda, 1999; Pu & Bezwoda, 2000) and platinum derivatives (Minagawa *et al.*, 1997) but only rarely for doxorubicin (Yu *et al.*, 2000) and topo II catalytic inhibitors (Morgan & Beck, 2001). Thielmann & Popanda (1998) showed that two hours after the exposure of fibroblasts to doxorubicin, over-expression of topo IIα protein was seen, but began to decline after six hours. In addition Asano *et al.* (1996a) showed that during induced expression of topo IIα, the increase in topo IIα levels was only transient and after 24 hours had reduced to normal levels. It was proposed that a negative feedback loop occurred with the presence of high exogenous topo IIα decreasing endogenous topo IIα promoter activity.

This result may indicate that the increase in topo IIα protein levels did not provide an adaptive advantage during drug exposure and as such was not maintained after a second drug exposure. The chronic over-expression of topo IIα may be detrimental to cellular viability. McPherson & Goldenberg (1998) showed that cells with excessive amounts of topo IIα showed features of apoptosis and that the production of stable cell lines over-expressing topo IIα was difficult (McPherson *et al.*, 1997). Of the 32 cell lines produced only 2 cell lines had intact topo IIα cDNA, the other cell lines showing rearrangements in the cDNA. A reason for the increased levels of topo IIα protein could be that duplication of the topo IIα gene may have occurred in these cell lines. This duplication event may be unfavorable for survival and as such was lost after a further drug exposure.

In the MDA1964 and MDA144 cell lines that were maintained in the absence of the drug or without further drug exposure, the alterations in topo IIα and specific transcription factors were not maintained. The amounts of topo IIα and NF-YA had returned to control levels in all the cell lines. In the cell lines that had previously had reduced Sp1 protein, the relative amounts of Sp1 levels had approximately doubled. In

4/8 cell lines, the amount of total Sp3 protein had doubled from initial levels. These results suggest that drug exposure produces transient changes in gene expression with the overall phenotype determined by the product of these changes. This result may indicate that decreased topo IIα protein may not be advantageous in the absence of the selective pressure and so is not maintained in these cell lines.

It has been shown that changes to expression seen after drug exposure may not be maintained over time or without further drug exposure. Withoff *et al.* (1996b) showed that in a doxorubicin-resistant small cell lung carcinoma cell line which had decreased topo IIα mRNA and protein after selection, the relative amounts of both had doubled when the cells were incubated for six months without further drug exposure. Similar trends were seen by Budworth *et al.* (1997) and Chen *et al.* (1990) where the expression of MDR1 and BCRP in doxorubicin-resistant MCF-7 cell lines were not maintained in the absence of the drug.

4.5.1 NF-Y and modulation of topo IIα gene expression

The proportions of NF-YAL and NF-YAS were investigated in the parental breast cancer and other cell lines as there is evidence that the different NF-YA isoforms may have a differential effect on transcription on specific promoters (Gu *et al.*, 1999; Chang & Liu, 1994).

The breast cancer cell lines contain a majority of the long isoform, compared to the other cell lines investigated which contained a majority of the short isoform. While MCF-7 and the other cell lines contain similar relative amounts of total NF-YA protein, MDA-MB-231 had substantially less total NF-YA protein. The effect of these differences on the regulation of the topo IIα gene in the two breast cancer cell lines is not known but could account for cell-specific differences in topo IIα regulation.

NF-Y has been implicated in the down-regulation of the topo IIα promoter in drug-resistant cells, where reduced NF-Y binding to ICBs 1-4 has been correlated to down-regulation of topo IIα promoter activity (Wang *et al.*, 1997a; Joshi *et al.*, 2000). But

the results from this current research in MDA144 cell lines implicate NF-Y as a repressor, with increased NF-YA seen with decreased topo IIα protein. This disparity agrees with results from Magan (2002) and Morgan & Beck (2001) where NF-Y was implicated as acting as an activator or repressor of topo IIα transcription depending on promoter context.

NF-YA seems to act as a potential activator in the MCF-7 cell lines. Increased NF-YA protein was seen in cells with increased topo IIα protein (G1), but these changes were not maintained when exposed to a second drug dose (G2). The total amount of NF-YA protein had slightly decreased in cell line H1, and a significant decrease in the amounts of NF-YAL, or increase in the amounts of NF-YAS was seen. The significance of this result is not known. As the two isoforms of NF-YA arise through alternate splicing, this result may indicate that that the splicing mechanism that produces the mRNA for the short isoform has been stimulated in these cells. Another possibility is that the mRNA or protein for the NF-YAS isoform has increased stability (or conversely a decrease in stability of the NF-YAL) in these cell lines. There is no direct evidence for either of these possibilities.

There is evidence that the NF-Y trimer containing the different NF-YA isoforms may have a differential effect on regulating transcription on specific promoters (Gu *et al.*, 1999). The increase in the relative amounts of total NF-YA and the increase in the proportion of NF-YAS were seen in two different cell lines. These events could both result in increased amounts of NF-YAS-containing trimer and if this complex has a transactivating role, increased transcription of the topo IIα gene may occur. After exposing both the cell lines to another drug dose, the increased levels of topo IIα protein, total NF-YA and the enhanced proportion of NF-YAS were no longer observed.

To complicate matters, cell line A2 also had an increase in the relative amounts of NF-YA. If it is assumed that NF-YA can modulate topo IIα transcription, it is not known why topo IIα mRNA levels were unaffected in these cells. One possibility is that the chromatin structure of the promoter is such that increased amounts of NF-YA have no

effect on transcription i.e. the transcription factor cannot bind or bend the DNA. In these cell lines, a disparity between the endogenous and exogenous topo IIα gene expression has already been shown so it may be possible that such a change in chromatin structure has occurred at least to specific regions of the genome. Changes to local chromatin structure and methylation status have also been seen after drug treatment (Tada *et al.*, 2000; Kusaba *et al.*, 1999).

If the topo IIα promoter is in a "non-responsive" chromatin structure then a post-transcriptional mechanism may be an alternative method to reduce the amounts of topo IIα protein within a cell. To cause such a reduction in the amounts of topo IIα protein, either a decrease of the topo IIα mRNA stability (which should be reflected by a decrease in steady-state mRNA) or a decrease in topo IIα protein stability could occur. The degradation of specific proteins may be a common mechanism to develop or maintain drug resistance. Spataro *et al.* (1997) showed that the transient over-expression of a proteosome subunit could confer resistance to doxorubicin while proteasome inhibitors have been found to overcome specific drug resistance (reviewed in Murray & Norbury, 2000).

The mechanism(s) by which the NF-YA can be up-regulated is not yet known. Both post-transcriptional and post-translational mechanisms have been suggested (Bolognese *et al.*, 1999; Marziali *et al.*, 1999; Eggen *et al.*, 2001). Whether drug-induced changes in NF-YA protein result from transcriptional or post-transcriptional mechanisms remain to be investigated.

4.5.2 Alterations in the amount of Sp1 protein

Sp1 has been described as a potential activator of the basal topo IIα promoter activity in transient transfection experiments through binding to GC1 and GC2 (Magan, 2002). The results from the current research also implicate Sp1 as a activator with decreased amounts of Sp1 protein correlated with decreased relative amounts of topo IIα protein.

The mechanism of this down-regulation has yet to be investigated, but both transcriptional and post-translational mechanisms have been implicated in the regulation of Sp1. These include transcriptional down-regulation by MyoD in muscle cells (Viñals *et al.*, 1997) and the up-regulation, as well as increased Sp1 phosphorylation and DNA binding after the exposure of cells to phorbol esters (Noé *et al.*, 2001) and epidermal growth factor (Chupreta *et al.*, 2000). The phosphorylation of Sp1 can have either a positive (Zheng *et al.*, 2000; Rohlff *et al.*, 1997) or negative (Borellini *et al.*, 1990; Leggett *et al.*, 1995; Daniel *et al.*, 1996; Wang *et al.*, 1999c) effect on DNA binding depending on promoter context. Phosphorylation has also been shown to modify Sp1 protein turnover (Mortensen *et al.*, 1997). Glycosylation of Sp1 does not affect DNA binding but may enhance transcriptional activation (Jackson & Tjian, 1988). Post-transcriptional modifications were not investigated in the current study so the relevance of such mechanisms in the drug-induced changes in Sp1 expression cannot be evaluated.

4.5.3 Sp3 and topo IIα regulation

Sp3 has been implicated as a potential repressor of topo IIα transcription in etoposide- and teniposide-resistant human epidermoid cell lines (Kubo *et al.*, 1995) or as an activator in a merbarone-resistant CEM line (Mo *et al.*, 1997). Results from Magan (2002) showed that Sp3 can bind to GC1 and GC2, but further transient experiments have proven difficult due to the Sp3 expression vector affecting the expression of a transfection control (personal communication). However, the trends in these experiments suggest that Sp3 may act as a repressor of topo IIα transcription.

In the initial surviving MDA144 cell lines, no changes in either the total amount of Sp3 or the proportions of the three isoforms was seen. However, a decrease in the relative amounts of Sp3 protein were seen in two MDA1964 cells lines, neither of which showed any changes to the relative amounts of topo IIα protein. Sp3 levels had also increased in cell lines that had been subjected to freezing and thawing. This corresponded to a concomitant increase in topo IIα protein. Little is known about the regulation of expression of Sp3, although 5-aza-cytidine (Ammanamanchi & Brattain,

2001b) and hypoxia (Discher *et al.*, 1998) have been shown to decrease Sp3 protein levels. The relevance of the changes observed in Sp3 levels in the current study in terms of the development of drug resistance were not investigated.

There was no clear trend of the down-regulation of topo IIα in surviving cell lines although changes in transcription factors implicated in topo IIα gene expression were observed. Collectively, these data suggest that down-regulation of topo IIα may be associated with drug resistance in some instances but other mechanisms are also likely to be responsible. These changes were not maintained in the cell lines grown without further exposure to doxorubicin. This suggests that the changes in gene expression that occur upon drug exposure to doxorubicin are transient. The expression profiles of many additional genes will need to be studied before any real conclusions can be drawn about the mechanisms governing drug resistance. A proposed model for the regulation of the topo IIα promoter will be discussed in Chapter Seven.

Changes in the proliferation rate or cell cycle profile may result from drug exposure and may alter expression of proteins like topo IIα. The over-expression of proteins such as MDR1, MRP1 or Hsp70 or the altered expression of topo IIβ may all contribute to the survival of breast cancer cell lines during drug exposure and so were investigated in Chapter Five.

Chapter Five Other Potential Resistance Mechanisms

5.1 Introduction

Drug resistance has been related to several different mechanisms including proliferation rate, chromosome duplication and the over-expression of drug efflux pumps or heat shock protein Hsp70. Therefore selected surviving cell lines were analysed for doubling time as a measure of proliferation rate, and were also subjected to FACS analysis to check for polyploidy and changes in cell cycle progression. MDR1 and MRP1 mRNA and Hsp70 protein content were also analysed. An attempt to quantify topo IIβ mRNA was unsuccessful as were attempts to quantify MDR1, MRP1 and topo IIβ protein using a variety of different immunoblotting conditions.

5.2 The analysis of the doubling time in the surviving cell lines

Cell counts of both MDA1964 and MDA144 selected cell lines were carried out over a period of time as described by Davis (1994). It was not possible to do similar experiments with the MCF-7 cell lines, as the strong aggregation of the cells made cell counts inaccurate. Slight increases in doubling time compared to the control cells were observed (table 5.1) but there was no clear correlation with the levels of topo IIα (Appendix 14).

As cells with unaltered amounts of relative topo IIα protein still had increased doubling times, this may indicate that another mechanism rather than amounts of topo IIα protein was causing the increase in doubling time seen.

(a)

Cell line	Relative topo IIα protein	Doubling time (hr)	Doubling time relative to control	p value
MDA1964 control	100%	32.4 ± 1.6	100%	-
A1	66 ± 5%	44.3 ± 9.0	137%	*
A2	42 ± 9%	36.4 ± 8.7	112%	n.s
B1	110 ± 23%	37.3 ± 1.0	115%	*
C1	97 ± 24%	36.9 ± 4.7	114%	n.s

(b)

Cell line	Relative topo IIα protein	Doubling time (hr)	Doubling time relative to control	p value
MDA144 control	100%	35.5 ± 14.0	100%	-
D1	52 ± 20%	31.4 ± 7.1	88%	n.s
D2	41 ± 10%	41.0 ± 23.5	115%	n.s
F1	14 ± 5%	39.2 ± 8.4	110%	n.s

- *Table 5.1. The doubling time and the relative amounts of the topo IIα protein of the (a) MDA1964 and (b) MDA144 cell lines. The value of topo IIα protein relative to α-tubulin was compared to the control which was arbitrarily set at 100%. The average (± standard deviation) values are shown. The doubling time for MDA-MB-231 is 30.3 ± 1.6 hr. The doubling time experiments were performed as described in section 2.2.3.10. The doubling time of either the MDA1964 or MDA144 control was arbitrarily set at 100%. The Student T-test (2 tailed) was performed to determine the statistical significance of these results compared to the control results. n.s $p > 0.05$, * $p < 0.05$.*

5.3 The analysis of cell cycle profile of selected surviving cell lines

Topoisomerase IIα expression can vary through the cell cycle, with the highest expression during S/G_2/M and lowest during G_0/G_1. Therefore the cell cycle profile of the surviving cells was investigated to ensure that the changes to the relative amounts

of topo IIα protein levels seen were not simply a consequence of increased proportions of cells in G_0/G_1 or due to cells with altered numbers of chromosomes. Fluorescence activated cell sorting (FACS) analysis was used to determine the proportion of cells in the different phases of the cell cycle as previously described in section 2.2.2.13.

While most of the MDA1964, MDA144 and MCF-7-144 cell lines had similar cell cycle profiles to the control cell lines (results not shown), some differences were seen in specific MDA1964 cell lines. Compared to control cells the A1 cell line exhibited a decrease in the proportion of G_0/G_1 cells and increased $>G_2M$ cells. However in the A2 cell line (isolated after exposing A1 cells to a second drug dose), the proportion of cells in each cell cycle phase had returned to near control levels (figure 5.1). These results did not correlate with changes in the relative amount of topo IIα in these cell lines.

5.4 The expression of genes implicated in drug resistance in breast cancer cells

RT-PCR or real time RT-PCR were initially used to detect the expression of MDR1 and MRP1 in the surviving cell lines. Immunoblotting was used in an attempt to detect P-glycoprotein and MRP1 protein. The relative amounts of the inducible Hsp70 protein were also determined in the surviving cell lines, while the detection of topo IIβ mRNA and protein was also attempted by the above methods.

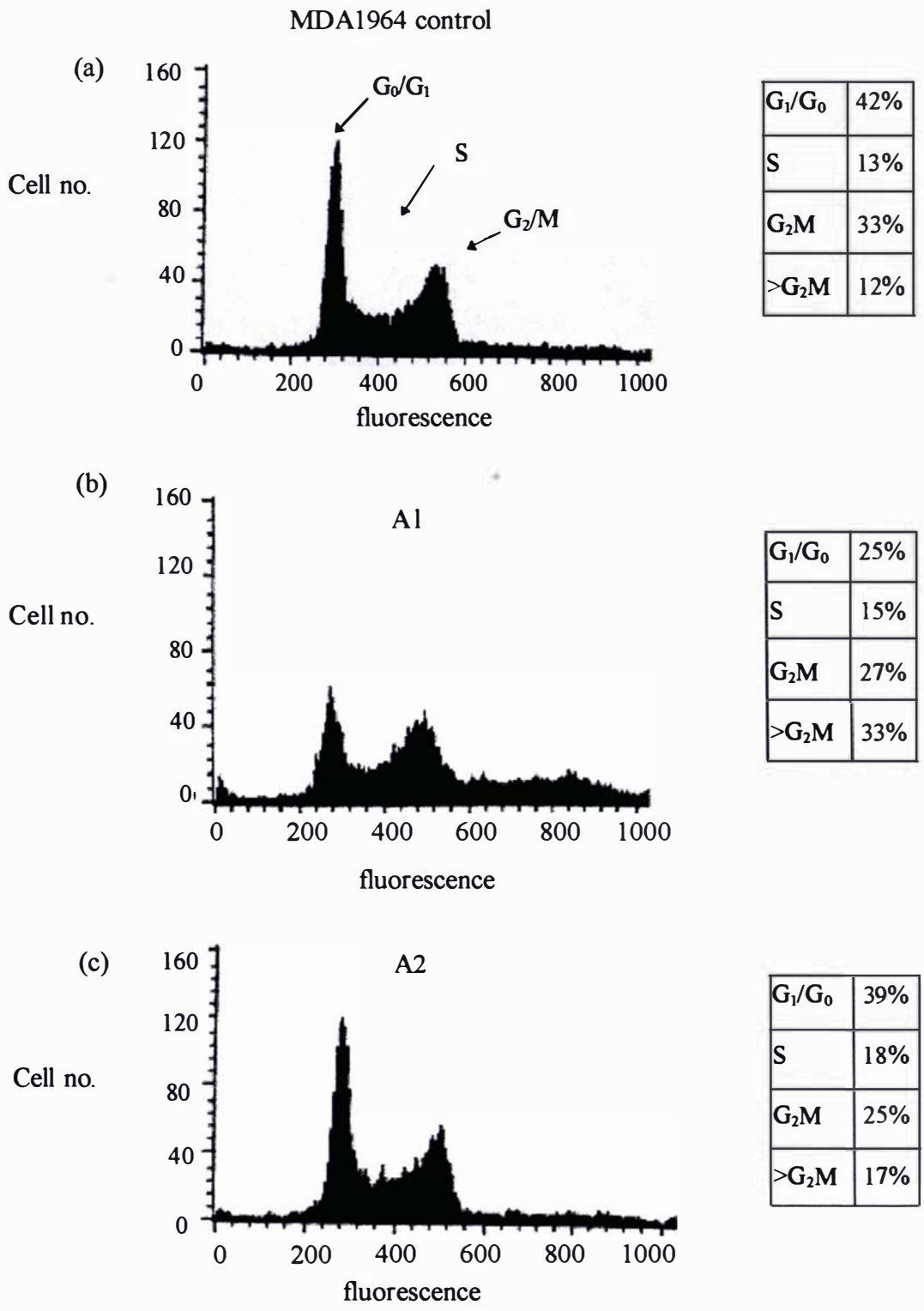


Figure 5.1. FACS analysis of (a) MDA1964 control, (b) A1 and (c) A2 cell lines. The cells were harvested, fixed in 70% ethanol in PBS at room temperature for 30 minutes and then left overnight at 4℃. The cells were then pelleted, resuspended in staining solution (100μg/ml RNase, 40 μg/ml propidium iodide in PBS) and incubated at 37℃ for 30 minutes. The cells were then analysed using a Becton Dickinson FACS machine. The proportion of cells in G_1/G_0, S, G_2M and $>G_2M$ were quantified using the program Image Gauge, calculated relative to the total cells counted and detailed beside each profile.

5.4.1 Alterations in the expression of MDR1 in surviving cell lines

As MDR1 and MDR3 genes share high identity, primers were designed to bind to divergent sequences to distinguish between the two mRNAs. As MDA-MB-231 and MCF-7 cell lines do not normally express MDR1 mRNA (Appendix 15), RT-PCR was used to detect the presence of MDR1 mRNA in the surviving cell lines. RNA from CaCO-2 or HepG2 cells which are known to express MDR1 mRNA (Gutmann *et al.*, 1999; Chan *et al.*, 2000; Jongsma *et al.*, 2000) was used as a positive control (Appendix 15). The detection of GAPDH cDNA was used to confirm that the reverse transcription reaction was successful. Diagnostic digests were used to confirm the identity of all of the products.

The expression of MDR1 mRNA could not be detected in either the MDA1964 (figure 5.2), MDA144, MCF-7-1964 or MCF-7-144 cell lines investigated (results not shown).

Immunoblotting was attempted to confirm the lack of MDR1 expression in the surviving cell lines. While a range of cell extraction and immunoblotting techniques were performed (section 2.2.4) it was not possible to detect P-glycoprotein in whole cell extracts from either the CaCO-2 or HepG2 cell lines. Therefore immunoblot experiments to detect P-glycoprotein could not be performed with the surviving cell lines.

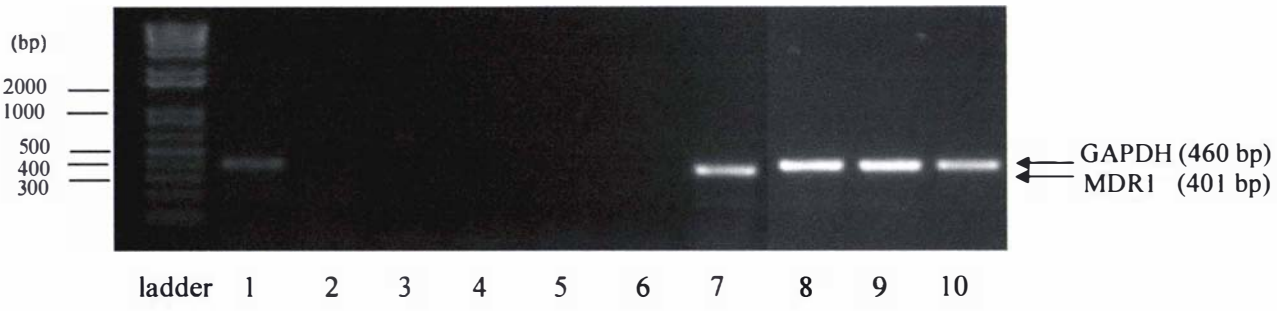


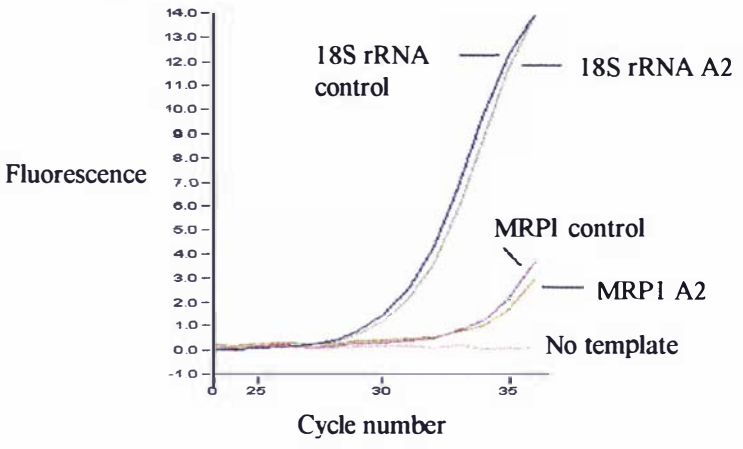
Figure 5.2. RT-PCR to detect MDR1 mRNA in selected MDA1964 cell lines. RNA was extracted from the cell lines and reverse transcription reactions were performed to produce cDNA. PCR was carried out as described in section 2.2.3 using either the MDR1 or GAPDH primers (Appendix 4). CaCO-2 or HepG2 cDNA was used as a positive control for MDR1 primers. GAPDH was used as a control for the RNA loading and the reverse transcriptase reaction. To confirm the products seen were amplified from cDNA and not genomic DNA, RT reactions without reverse transcriptase (no RT) were performed. No template reactions were performed to check for contamination. 5-10 µl of the PCR products were separated on a 1% agarose gel in 1x TAE (80 V for 30 min) and stained with ethidium bromide. (1) and (7) MDR1 positive control, (2) no RT control, (3) MDA1964 control, (4) A1 cell line, (5) A2 cell line, (6) no template, (8), (9) and (10) GAPDH control for the reverse transcription reaction for the control, A1 and A2 cell lines respectively.

5.4.2 Alterations in the expression of MRP1 in the surviving cell lines

The expression of MRP1 was investigated in selected cell lines. As MRP1 mRNA is ubiquitously expressed in MDA-MB-231 and MCF-7 cells (Appendix 16), a more quantitative method was required to detect differences in MRP1 expression. The LightCycler™ real time RT-PCR system was used to detect differences in MRP1 mRNA expression as described in section 2.2.3. The amounts of MRP1 mRNA relative to 18S rRNA were analysed in selected surviving cell lines.

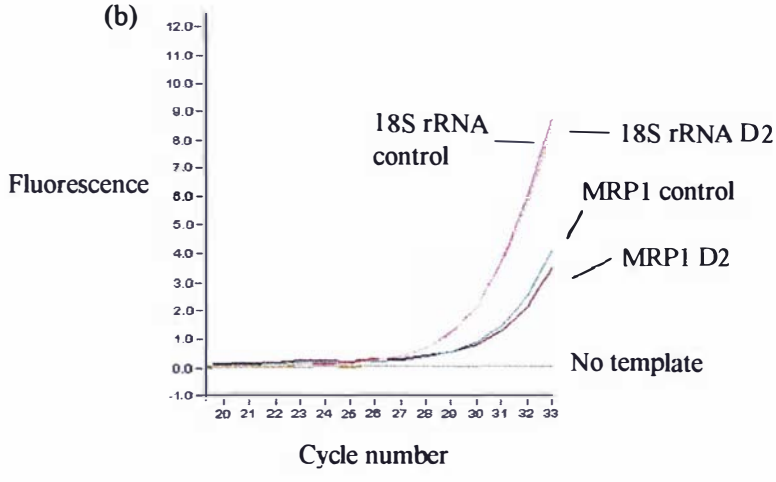
As illustrated in figure 5.3, there was no significant change in MRP1 mRNA levels in the cell lines investigated. As with MDR1, immunoblotting was attempted to confirm the wild-type expression of MRP1 protein in the surviving cell lines, and like the P-gp experiments it was not possible to detect MRP1 protein in any of the cell lines which were known to express MRP1 mRNA including CaCO-2, HepG2 and HeLa cells (Appendix 16). Therefore immunoblot experiments to quantify MRP1 protein could not be performed with the surviving cell lines.

(a)



Cell line	relative amount of MRP1 mRNA
MDA1964 control	100%
A2	100%

(b)



Cell line	relative amount of MRP1 mRNA
MDA144 control	100%
D2	91%

Figure 5.3. Example of the fluorescence vs. cycle number results of a real time RT-PCR investigating MRP1 mRNA relative to 18S rRNA in (a) MDA1964 control and cell line A2 and (b) MDA144 control and cell line D2. 6 μg of the RNA was treated with DNase I and then reverse transcribed as described in section 2.2.3. 5 μL of the RT reaction (1:5 dilution) was added to the PCR reaction mix containing 0.5 μM of each primer (Appendix 4), 1x SYBR Green 1 Faststart Master Mix and 3-4 mM $MgCl_2$. To confirm that the product was specific for cDNA, RT minus samples were included (not shown). To confirm the absence of primer dimers and contamination, a water only control was included. Melting curve analysis and diagnostic digests were carried out to confirm the identity of the PCR products. The amount of MRP1 mRNA was calculated relative to 18S rRNA, with the control value set at 100%.

5.4.3 Alterations to the relative amounts of the Hsp70 protein in the surviving cell lines

The relative amounts of the inducible Hsp70 protein were investigated in the parental and surviving cell lines by immunoblotting. While the parental and stable cell lines MDA-MB-231, MDA1964 and MDA144 had similar levels of Hsp70 protein (data not shown), the MCF-7-144 cell line had significantly decreased amounts of Hsp70 when compared to MCF-7 and MCF-7-1964 (figure 5.4). It is not known if this may affect survival upon drug exposure.

It was noted that Hsp70 was detectable in both MDA1964, MDA144 and MCF-7-1964 and MCF-7-144 control cells. This was not due to the cells being grown in neomycin-containing media as the Hsp70 protein was also detected at similar levels in the parental cell lines (results not shown).

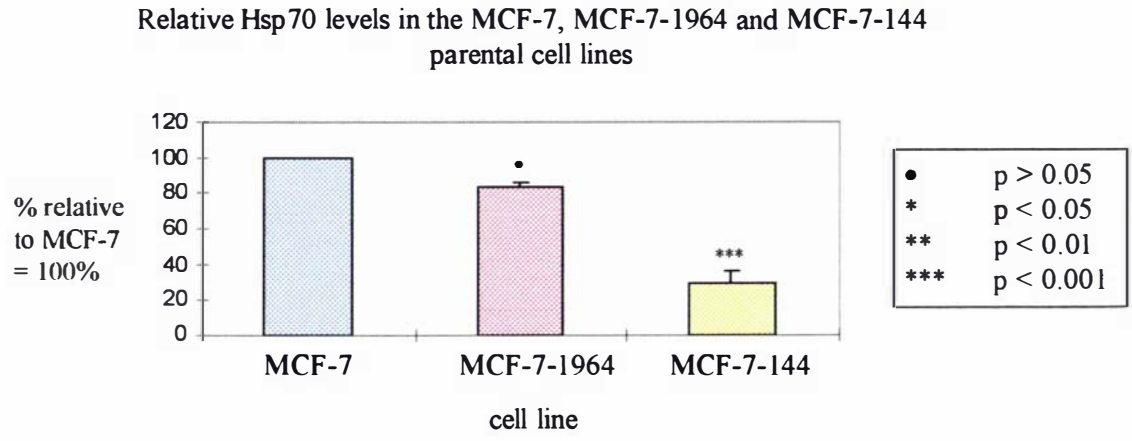


*Figure 5.4. Summary of immunoblot results investigating the amount of Hsp70 protein relative to the α-tubulin in the MCF-7, MCF-7-1964 and MCF-7-144 cell lines. The immunoblots were performed as described in figure 4.3. The amount of Hsp70 protein was calculated relative to α-tubulin. The value for MCF-7 was arbitrarily set at 100 %, with the average (± standard deviation) values shown. The Student T test (2 tailed) was performed to determine the statistical significance of these results compared to the MCF-7 values using Excel. • $p > 0.05$ (deemed non significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

The level of Hsp70 did not vary significantly in the MDA1964 and MCF-7-1964 surviving cell lines (Appendix 11, 12). A decrease in the relative amounts of Hsp70 protein was seen in the E1 and F1 cell lines (figure 5.5 a, b), while an increase in the relative amounts of Hsp70 protein was seen in cell line H1 (202 ± 41%). This increase was lost after a further drug exposure (cell line H2) (figure 5.5 c, d).

5.5 Discussion

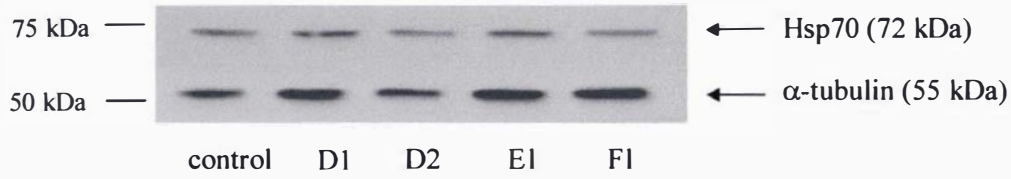
No meaningful relationship between amounts of topo IIα protein, the doubling time or cell cycle profile was seen in the surviving cell lines. As multiple mechanisms of resistance are commonly found in drug-exposed cells, the expression of MDR1, MRP1, Hsp70 were investigated. Expression of MDR1 mRNA was not detected, neither was the over-expression of MRP1 mRNA in the selected cell lines investigated. Attempts made to confirm these results at the protein level through immunoblotting were unsuccessful. While an increase in the relative amounts of the Hsp70 protein was seen in one MCF-7-144 cell line (H1), this up-regulation was not maintained when this cell line was exposed to a second drug dose (H2). Conversely, a decrease in the relative amounts of Hsp70 was seen in several other surviving cell lines (E1 and F1).

The MCF-7-144 parental cell line had significantly lower amounts of Hsp70 protein than MCF-7 and MCF-7-1964. How these alterations may affect the drug response is not known, but this result indicated that in the production of stable cell lines and selection by neomycin, changes can occur to the cells and it cannot be assumed that the stable cell lines are phenotypically the same as the parental lines.

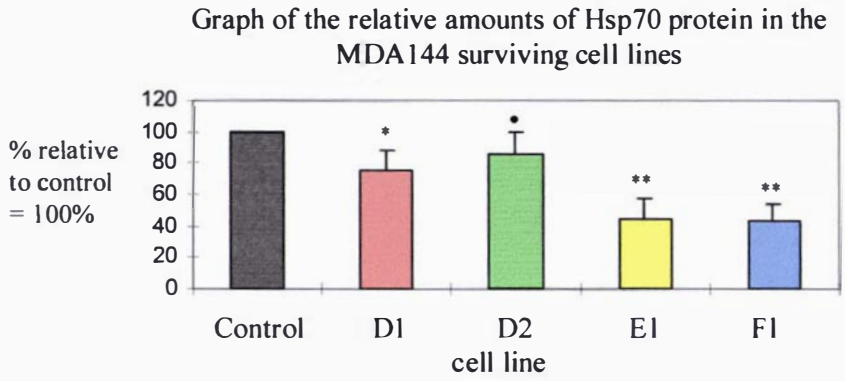
5.5.1 Alterations of doubling time in selected surviving cell lines

The results indicated that the topo IIα protein may not have a significant effect on the proliferation rate in specific cell lines and that other mechanisms are involved in the changes to the proliferation rate seen.

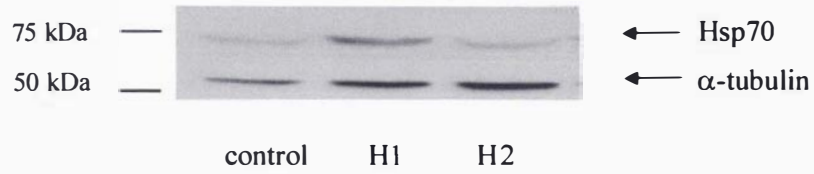
(a)



(b)



(c)



(d)

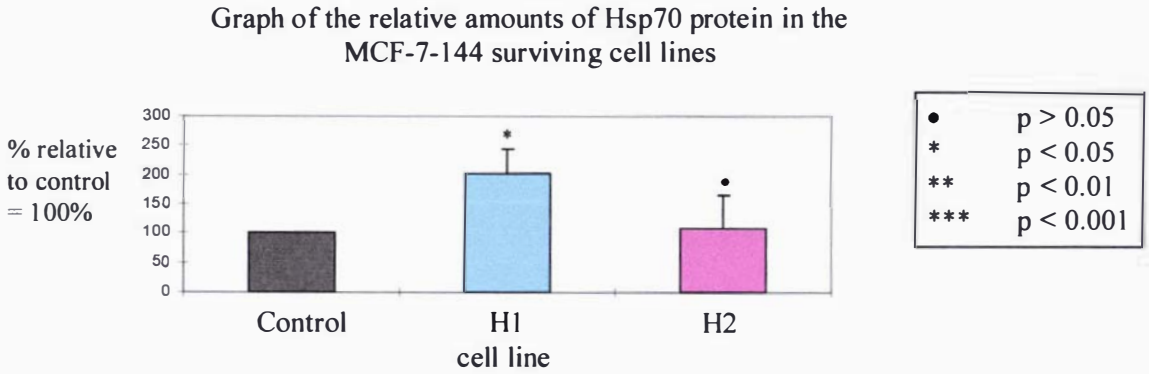


*Figure 5.5. Example of an immunoblot to investigate the amount of Hsp70 protein relative to the α-tubulin in the (a) MDA144 and (c) MCF-7-144 cell lines. The immunoblots were performed as described in figure 4.3. Summary of the immunoblot results of at least three experiments for the (b) MDA144 and (d) MCF-7-144 cell lines. The amount of Hsp70 protein was calculated relative to α-tubulin. The value for the control was arbitrarily set at 100% with the average (± standard deviation) values shown. The Student T test (2 tailed) was performed to determine the statistical significance of these results compared to the control values using Excel. • $p > 0.05$ (deemed non significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

In agreement with this, significant changes to the doubling time were seen with doxorubicin-resistant lung, breast and human stomach adenocarcinoma cell lines and teniposide-resistant (VM-26^R) human leukemic cells without any changes to the topo IIα protein (Son *et al.*, 1998; de Jong *et al.*, 1990; Danks *et al.*, 1988; Lee *et al.*, 1997). Therefore changes in the amount of topo IIα protein do not necessarily result in changes to the doubling time, or vice versa. This differs from the results from Brown *et al.* (1995) where an increase in doubling time from ~25 to ~75 hours was seen in lymphoblastic leukemia cells which had 3 fold less topo IIα protein.

5.5.2 Alterations to the cell cycle profile of selected surviving cell lines

No changes in the cell cycle profiles were observed for the selected MDA144, MCF-7-1964 and MCF-7-144 cell lines investigated. While specific MDA1964 cell lines did show changes to the proportions of the number of cells in each cell cycle phase, these changes did not correlate with the relative amount of topo IIα protein seen in these cells. An increased population of cells with fluorescence (and therefore amount of DNA) greater than G_2M cells was seen in the A1 cell line. This may indicate a population of polyploid cells as seen by Melixetian *et al.* (2000). It has also been shown that the exposure of cells to doxorubicin can cause changes to the chromosome number of specific cells (Aly *et al.*, 1999). Another possibility is that the increased fluorescence is due to a population of cells with a slightly different phenotype. If this phenotype allowed the cells to clump tightly together, then the cells may not be physically separated during the cell sorting and so doublets with increased fluorescence would be seen. The proportion of the $>G_2M$ population had reduced in the A2 cell line. This may indicate that this population was sensitive to the drug and so was eliminated after a further drug exposure. Therefore the changes to the relative amount of topo IIα protein did not correlate to changes in the proportion of cells in various cell cycle stages or to significant changes in the chromosome number of the surviving cells investigated.

5.5.3 The expression of MDR1 and MRP1 in the surviving cell lines

MDR1 mRNA expression was not detected in the surviving cell lines investigated. To confirm these results, immunoblotting was attempted to detect P-glycoprotein. Attempts to optimize the cell extraction and immunoblotting method by using cell extracts from a cell line known to express P-gp were fruitless. Although RT-PCR showed that MDR1 mRNA was detected in CaCO-2 cells, P-gp could not be detected. This could be due to inappropriate extraction or immunoblot conditions, or due to the CaCO-2 cells no longer being able to express P-gp. It also may be possible that the culturing conditions, age etc. of the cell line may have affected the ability to express P-gp. It was shown that while Gutmann *et al.* (1999) and Hamilton *et al.* (2001) could detect P-gp in the CaCO-2 cells, Jacob *et al.* (2001) and Beaumont *et al.* (1998) could not.

As MRP1 mRNA was ubiquitously expressed in all the cell lines investigated, real time RT-PCR was used to determine relative MRP1 mRNA levels. No changes in the relative amounts of MRP1 mRNA were seen in the surviving cell lines investigated. As with MDR1, immunoblotting was attempted to confirm these results and as with the P-gp experiments, MRP1 protein was not detectable in any of the cell lines investigated including CaCO-2 from which MRP1 protein has previously been detected (Gutmann *et al.*, 1999).

In summary, MDR1 and MRP1 did not seem to be involved in the survival of the breast cancer cell lines to doses of doxorubicin. This agreed with research which had shown that MDR1 may not have a role in the development of resistance in breast tumours (Faneyte *et al.*, 2001; Yang *et al.*, 1999; Arnal *et al.*, 2000).

5.5.4 Hsp70 expression in the surviving cell lines

There was no correlation between Hsp70 levels, topo IIα levels and drug survival. The relative amounts of Hsp70 were found to be increased in cell line H1, but this up-regulation was not maintained after a second drug exposure. This may indicate that the

increase in relative amounts of Hsp70 protein did not provide the cells with a selective advantage during drug exposure and so was not maintained. Two cell lines (E1, F1) showed a decrease in the relative amounts of Hsp70 protein. Decreased amounts of Hsp70 protein have been seen upon chronic exposure and during prolonged or daily repeated exposure to electromagnetic fields. This decrease resulted in reduced cytoprotection, but the exact mechanism of this down-regulation is not known (Di Carlo *et al.*, 2002).

5.5.5 Expression of topo IIβ in the surviving cell lines

Unfortunately it was not possible to investigate the role of topo IIβ in the surviving cells. The topo IIβ protein could not be detected using immunoblotting and the quantification of the topo IIβ mRNA was not possible using the real time RT-PCR system.

A reason that topo IIβ protein was not detectable may be due to the amount of topo IIβ protein being too low to be detectable by immunoblots. It has been shown previously that the amount of topo IIα and topo IIβ protein can vary significantly in different cell lines, with topo IIβ protein tending to be found in much lower amounts than topo IIα protein. For example, in HeLa and T47D cells, the topo IIβ amounts were between 7-12% of the topo IIα amounts (Jenkins *et al.*, 1992).

Another possibility is that the topo IIβ protein may be difficult to transfer, although altering immunoblotting conditions had no effect. Other research has shown that topo IIα and topo IIβ proteins can be transferred and detected under the same conditions. so this is unlikely (Herzog *et al.*, 1998; Hashimoto *et al.*, 1995; Harker *et al.*, 1995a; Gao *et al.*, 1999; Feldhoff *et al.*, 1994; Errington *et al.*, 1999; Dereuddre *et al.*, 1997; Canitrot *et al.*, 1998). Another possibility is that the topo IIβ protein may be very susceptible to protease degradation and so may be degraded before immunodetection was possible. It has been shown that topo IIβ protein was much more sensitive to proteolysis when compared to the topo IIα protein (Prosperi *et al.*, 1996; Boege,

1996). Therefore the extraction conditions may need to be optimised to minimize any potential proteolysis.

The topo IIβ cDNA was difficult to amplify under both RT-PCR and real time RT-PCR experimental conditions. The reason for the poor efficiency could be due to low abundance of the mRNA or due to the presence of secondary structure in the topo IIβ mRNA that make it difficult to produce either the cDNA or PCR product. The exact reason for these difficulties could be investigated.

Cell proliferation, cell cycle progression, MDR1, MRP1 or Hsp70 did not seem to have any significant role in survival of cells to doxorubicin. While decreases in topo IIα gene expression may be implicated in specific cell lines, no common mechanism of resistance was apparent.

Chapter Six Development of an *in vivo* footprinting system

6.1 Introduction

The functional significance of changes in specific transcription factors in surviving cells remains to be determined. NF-Y, Sp1 and Sp3 binding sites have been identified on the topo IIα promoter and each has been implicated in the regulation of the topo IIα transcription. Therefore an *in vivo* analysis of the promoter in surviving cells was appropriate. *In vivo* footprinting should identify important regions of the promoter occupied by specific factors. The results of such a study should not only confirm the functional significance of NF-Y, Sp1 and Sp3 in topo IIα transcription but should also identify other binding sites to provide candidates for further study.

An *in vivo* footprinting assay was developed to allow the analysis of the topo IIα promoter *in vivo* in terms of any change in DNA-protein interactions that may occur on drug exposure or on the development of drug resistance.

6.2 Overview of in vivo footprinting

In vivo footprinting using ligation-mediated PCR (LMP) provides a "high resolution, nucleotide-level analysis of chromatin" (Gould, 1998). By this method, DNase I introduces breaks into DNA. The binding of proteins to the DNA protects the bound region from digestion, and the absence of these digestion products is an indication of where these proteins are bound. LMP using gene-specific primers provides a highly sensitive and specific method to detect breaks in a specific DNA region (Carey & Smale, 2000; Gould, 1998). *In vivo* footprinting provides a "snapshot" of the regions of the native promoter structure that interacts with proteins within the nucleus under specific experimental conditions.

The method for *in vivo* footprinting was adapted from that described in Carey & Smale (2000) for the nuclei isolation and DNase I digestion and Gould (1998) for the rest of the protocol.

Nuclei were isolated from cells and incubated with DNase I, an enzyme which causes essentially random double-stranded DNA breaks. As illustrated in figure 6.1, DNase I should digest all DNA that is not protected. A range of different sized DNA fragments should be produced. If each nucleotide was cleaved only once, a range of fragments would be seen (black fragments). As any bound protein will protect against digestion, a protected region should be apparent corresponding to the regions where proteins are bound (red fragments). By running a sequencing ladder corresponding to the entire unprotected region under study, the exact position of the protein binding can be detected.

6.3 Ligation-mediated PCR

Using ligation-mediated *in vivo* footprinting only gene-specific products were amplified and detected. The overall experimental protocol is outlined in figure 6.2. A biotinylated gene-specific primer (#1) was extended with Sequenase™ 2.0 at 48°C to produce blunt-ended products. A double-stranded linker was then ligated to the primer extension product.

These products were then purified using streptavidin-coated magnetic beads. To amplify the ligation products, the longest linker primer, a gene-specific primer #2 and *Taq* polymerase was used. This primer #2 overlapped primer #1 by a few nucleotides. The PCR products were then separated on a 8% acrylamide gel, transferred to nylon membrane by semi-dry electroblotting and the membrane hybridised with a gene-specific probe. This probe was produced by PCR using a primer (#3) that overlapped primer #2 and an appropriate upstream primer.

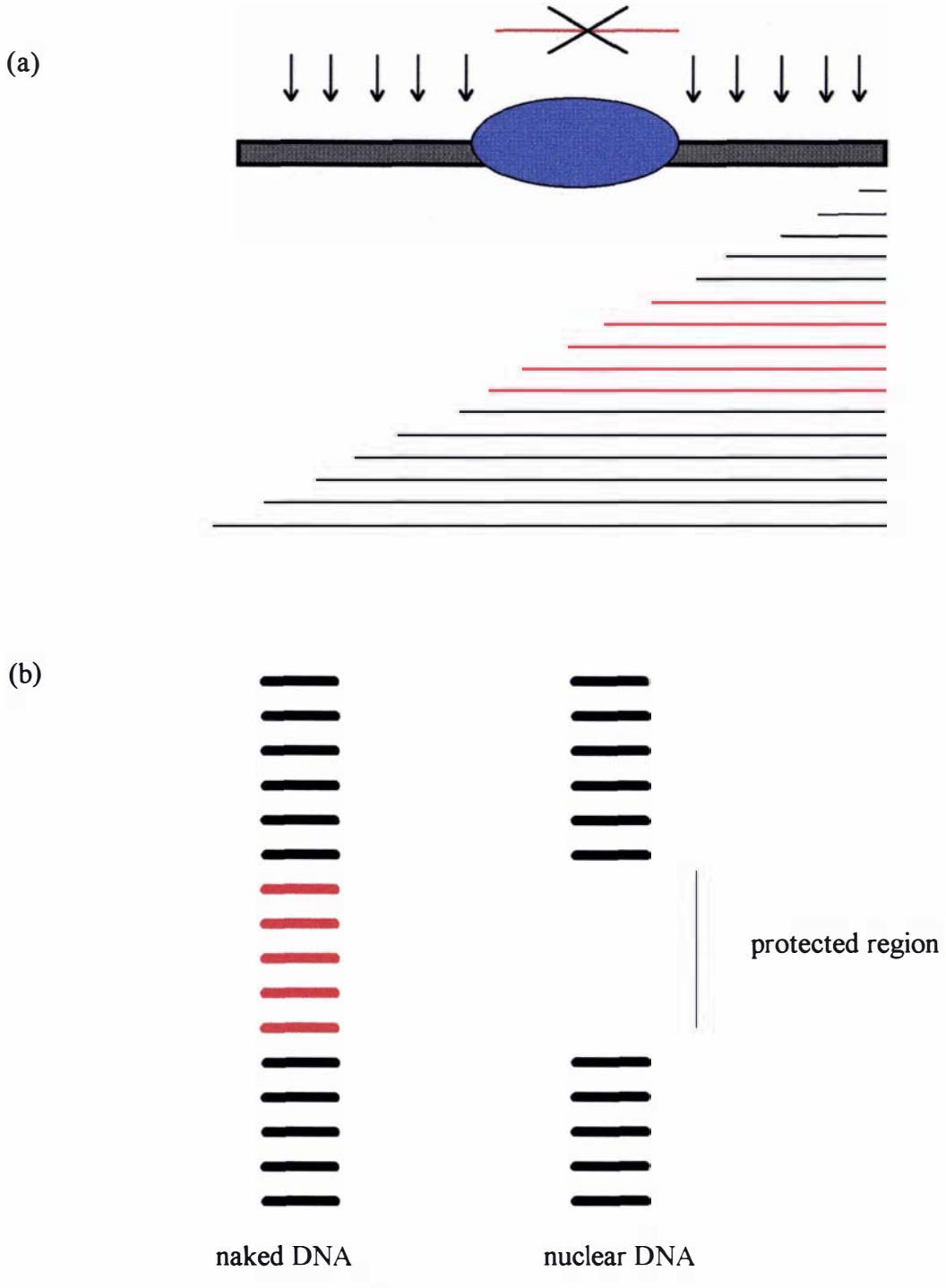
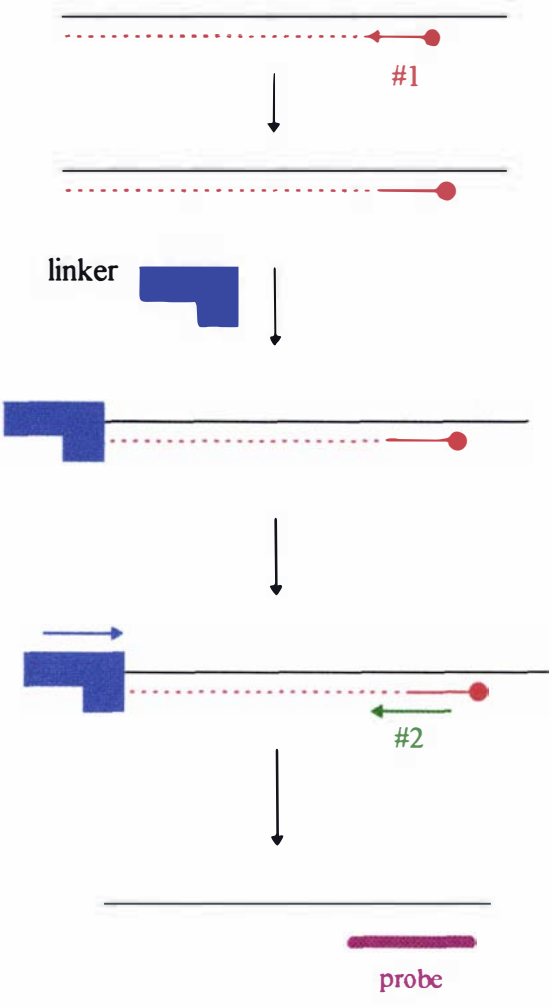


Figure 6.1. (a) Examples of possible DNase I digestion products, black fragments are possible products, red fragments are products that would not be produced due to the bound protein protecting the DNA from digestion. (b) Diagram of the DNase I footprint.



Primer extension with gene-specific biotinylated primer #1 and Sequenase™

The products are blunt-end ligated to the linker

5' GCGGTGACCCGGGAGATCTGAATTC 3'
3'CTAGACTTAAG 5'

The product is purified using streptavidin-coated beads

PCR is performed using the linker primer, nested primer #2 and *Taq* polymerase

PCR products are run on gel, transferred onto nylon membrane and hybridized with labeled probe. The probe used is made from nested primer #3

Figure 6.2. Diagram of the experimental protocol for the primer extension, ligation and PCR amplification.

6.4 Method development : in vivo footprinting

A number of parameters needed to be optimized for the *in vivo* footprinting to be successful. These included the extraction of the nuclei, the conditions for the DNase I digestion and the design of the gene-specific primers.

6.4.1 DNase I digestion

Two alternate methods were available to DNase I digest the native chromatin. The first, outlined in Gould (1998), involved the weakening of the cell membrane by detergents to allow the entry of the DNase I into the cell and subsequently into the nucleus. This method failed to yield substantial amounts of digested DNA possibly due to the DNA being bound up with the cell debris.

The second method (Carey & Smale, 2000), which involved the isolation of the nuclei followed by DNase I digestion proved to be a more reliable method. Due to the limitations of the *in vivo* footprinting method, digestion conditions were required to yield a majority of digestion products between ~300 to 600 bp in size. These were determined by trial and error. Incubation of nuclei with 3 μg of DNase I for 7 minutes produced products between ~200 to <1000 bp. Figure 6.3 shows an example of an alkaline gel showing the single-strand DNA fragments resulting from a range of DNase I incubation times.

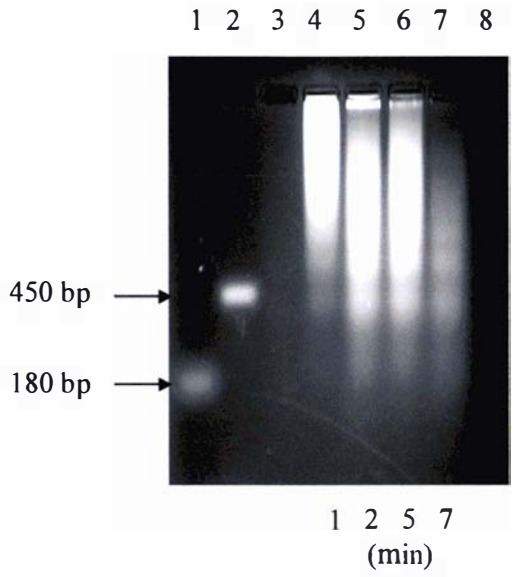


Figure 6.3. DNase I digestion products separated on a 1% agarose alkaline gel. Nuclei were digested with 3 μg DNAse I for 1,2 5 or 7 minutes. As described in section 2.2.5.3, the digests were stopped by the addition of EDTA, SDS and proteinase K. The samples were incubated overnight and the DNA extracted using phenol/chloroform and ethanol precipitation protocols. EDTA was added to the DNA to a final concentration of 10 mM and 6x alkaline loading dye (300 mM NaOH, 6 mM EDTA, 18% Ficoll (type 400), 0.15% bromocresol green, 0.25% xylene cyanol) was then added to the sample. The samples electrophoresed on a 1% agarose alkaline gel in 1x alkaline running buffer (50 mM NaOH, 1 mM EDTA) at 20V overnight. The gel was then neutralized in 1 M Tris-HCl pH 7.6, 1.5 M NaCl, and stained with ethidium bromide. 180 bp and 450 bp PCR products were included as size markers. (1) 180 bp marker, (2) 450 bp marker, (3) and (8) no DNA, (4) 1 minute,(5) 2 minutes, (6) 5 minutes (7) 7 minute DNAse 1 incubation.

6.4.2 The design of topo IIα promoter primers

As the footprinting method has been proposed to give good experimental data up to ~300 bp from the primers used, the initial 3' region of the topo IIα promoter was investigated. The primers chosen are detailed in figure 6.4. The following parameters were used to design the primers as described in Gould (1998); primer #1 had a T_m between 48-56°C to allow the primer extension to occur at 48°C; primer #2 overlapped primer #1 and had a T_m between 53-60°C to facilitate the PCR reaction with the linker primer (T_m 57°C) and primer #3 overlapped primer #2 by a few nucleotides. The primers were nested to enhance the specificity of the reactions.

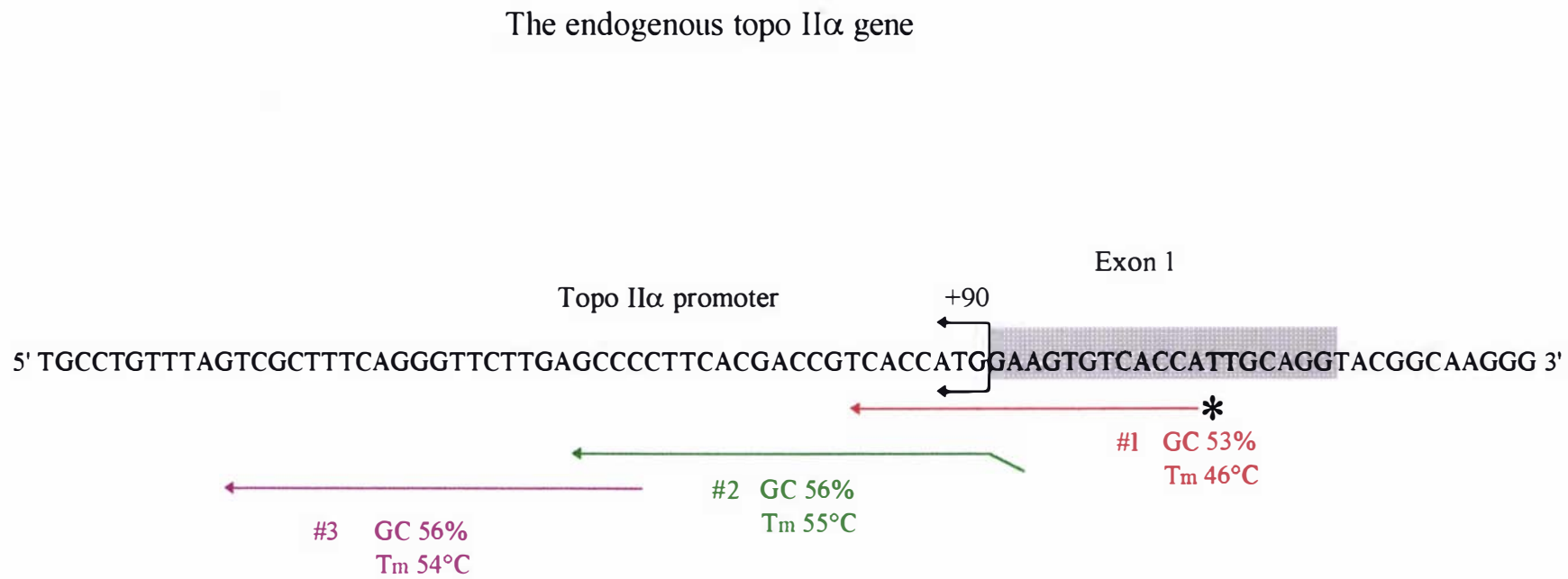


*Figure 6.4. Diagram of the primers used in the in vivo footprinting experiment * biotinylated.*

6.5 In vivo footprinting of the topo IIα promoter shortly after doxorubicin exposure.

Potential alterations to protein binding at the 3' topo IIα promoter region were investigated shortly after the exposure of MDA-MB-231 cells to doxorubicin. MDA-MB-231 cells were exposed to 0 or 5 μM doxorubicin for 1 hour. The cells were then washed and incubated in fresh media. After 6 hours, the nuclei were isolated from the cells and digested by DNase I as described in sections 2.2.5.1 to 2.2.5.3. Primer extension, ligation and PCR reactions were carried out as described in sections 2.2.5.4 to 2.2.5.7. The resulting PCR products were electrophoresed, transferred onto nylon membrane by semidry electroblotting and hybridised with a topo IIα promoter-specific probe. After washing, the membrane was exposed to X-ray film for 2-7 days.

The results from the initial experiment are shown in figure 6.5. This shows that a ladder of products was seen for the control and drug-exposed nuclei. Technical difficulties precluded the determination of the exact sequence that corresponded to this ladder, however a number of differences were observed between the control and drug-exposed nuclei. Compared to the control ladder, potential areas of DNase I protection may be seen in the drug-exposed sample at (a) and (c). This may indicate preferential protein binding at these sites during drug exposure. DNase I hypersensitive sites are frequently seen near protected regions and may reflect conformational changes to the DNA upon protein binding (Carey & Smale, 2000). Potential hypersensitive sites were seen at (b) and (d), both near possible protected regions.

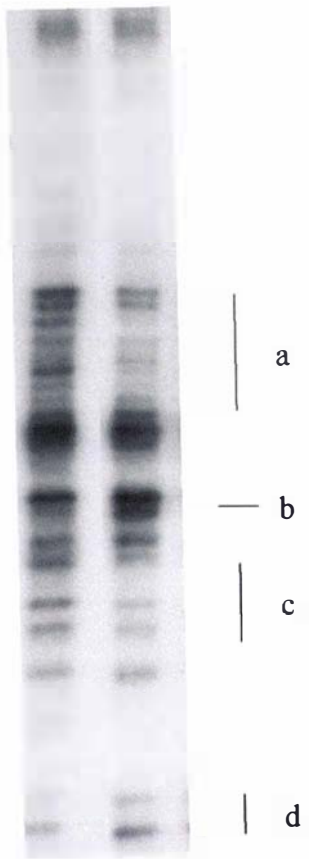


Figure 6.5. MDA-MB-231 cells were exposed to 5 μM doxorubicin for 1 hour, the cells were then washed and incubated in new media for a further 6 hours. Nuclei were then isolated from the cells and digested with 3 μg DNase I for 7 minutes at 37℃. To stop the digestion, the nuclei were incubated overnight with EDTA, SDS and proteinase K. The DNA was then treated with RNase at 37℃ for 2 hours, purified using phenol/chloroform extraction, ethanol precipitated and the pellet resuspended in TE. Primer extension was performed using primer 1 (figure 6.4) and Sequenase™ version 2.0 with the samples heated at 67℃ for 15 min to inactivate the enzyme. The primer extension products were then ligated to the linker and the ligation products were purified using magnetic beads. These products were then used in a PCR reaction using primer #2 and the linker primer. These products were then electrophoresed on an 8% acrylamide 7 M urea gel. This gel was then semidry electroblotted onto nylon membrane, and the membrane hybridised overnight using a topo IIα promoter-specific probe. The membrane was washed with prewarmed buffer A (20 mM sodium phosphate pH 7.2, 2.5% SDS, 1 mM EDTA, 0.25% BSA) for 5 minutes and then with buffer B (20 mM sodium phosphate pH 7.2, 1% SDS, 1 mM EDTA), with the buffer changed 5 times over 30 minutes. The membrane was then dried at room temperature and exposed to X-ray film for 7 days at -70℃ with intensifier screens.

The results from this initial experiment showed that it was possible to use this method to observe alterations to protein binding at the topo IIα promoter after drug exposure. While these results are preliminary, they suggest that alterations to proteins binding to the topo IIα promoter can occur shortly after drug exposure. It will be important to identify both specific proteins and cognate DNA binding sites as well as determine the effect such protein-DNA interactions have on topo IIα transcription. A detailed study of this type should provide important insights into the molecular events that occur upon drug exposure and may ultimately lead to the down-regulation of the topo IIα promoter that results in the drug-resistant phenotype.

With only slight modifications to the footprinting experimental procedure, nucleosomal positioning (with Mnase I), nucleosome remodelling (with restriction endonucleases) and DNA methylation analysis (Carey & Smale, 2000) could also be performed during or after drug exposure, while the use of *in vivo* protein-DNA cross-linking immunoprecipitation experiments could be used to confirm the identity of proteins bound to this promoter region (Carey & Smale, 2000).

Chapter Seven Summary and Discussion

7.1 Experimental summary

The overall aim of the research described in this thesis was to investigate the regulation of the human topoisomerase IIα gene in breast cancer cell lines exposed to doxorubicin, specifically to identify regions of the topo IIα promoter involved in the down-regulation that has been observed in cells after drug exposure. Drug-exposed stable breast cancer cell lines containing various deletion constructs of the topo IIα promoter were analysed for the differential expression of a reporter gene. Immunoblot analysis was used to observe alterations in the amounts of topo IIα protein in the surviving cells, as well as alterations to the amounts of transcription factors NF-Y, Sp1 and Sp3. The maintenance of these alterations was investigated as were the roles of other resistance mechanisms.

The main findings are summarised as follows :

(1) The basal regulation of the topo IIα promoter in MCF-7 and MDA-MB-231 breast cancer cell lines was similar to that reported in other cell lines. Converse to previous reports the addition of the ~ 1.3 kb region 5' to GC2 had little effect on the maximal topo IIα basal promoter activity indicating the absence of repressive or activator elements in this region. No significant differences were observed in basal transcription driven by the deletion constructs in the ER positive, p53 wild type (MCF-7) and the ER negative, mutant p53 (MDA-MB-231) cell lines. It was therefore concluded that the MCF-7 and MDA-MB-231 cell lines would be appropriate for use in studying the regulation of topo IIα after exposure to doxorubicin.

(2) The hGH reporter mRNA and protein did not appear to accurately reflect the expression of the endogenous topo IIα mRNA and protein in the stable cell line containing the -1964 topo IIα hGH reporter construct. These results indicated that the stable cell lines containing different regions of the topo IIα promoter could not be used

to identify the regions involved in the down-regulation of the gene in drug-exposed cells.

(3) A down-regulation of topo IIα expression at the protein level was observed concomitantly with the up-regulation of the NF-YA subunit of the NF-Y trimer in some cell lines derived from the MDA144 stable line. The amounts of Sp1 protein remained unchanged or were reduced. In the selected cell line investigated, the decrease in topo IIα protein was mirrored by a decrease in topo IIα mRNA indicating a likely transcriptional regulation mechanism.

(4) The decrease in topo IIα protein observed in cell lines derived from the MDA1964 stable line was not mirrored by a decrease in topo IIα mRNA indicating that a post-transcriptional mechanism may be involved. A decrease in total Sp3 protein was observed in two other cell lines without any change in the amount of topo IIα protein.

(5) The changes observed in the amounts of topo IIα, Sp1, Sp3 and NF-YA proteins in the MDA1964 and MDA144 stable lines appeared to be transient and not maintained after freezing and thawing or without further drug exposure.

(6) An up-regulation of topo IIα expression at the protein level was observed in the MCF-7 cell lines following a single drug exposure, with either an increase in total NF-YA protein, or a decrease in both total NF-YA and the proportion of the NF-YA long isoform. Upon a further drug exposure, neither the up-regulation of topo IIα nor changes to NF-YA protein were maintained.

(7) No correlation between topo IIα levels and either cell cycle profile or doubling time was observed in the surviving cell lines and there was no evidence of nucleotide changes in the initial ~700 bp of the topo IIα promoter. There was no evidence for an involvement of MDR1 or MRP1 in the survival of the cell lines investigated. Increased amounts of Hsp70 protein were observed in one cell line, but as this was not maintained after a further drug exposure, it was not thought to have a significant role in cell survival.

This study implicates NF-Y and Sp1 as being potential modulators of topo IIα expression in cell lines that survived doxorubicin exposure. This current research in MDA144 cell lines implicate NF-Y as a repressor and Sp1 as an activator, with increased NF-YA and decreased Sp1 protein observed with decreased topo IIα protein. This result appeared contradictory to the results seen with one MCF-7 cell line, where NF-Y may act as an activator and the studies in doxorubicin-resistant hemapoietic cells where reduced NF-Y was correlated to topo IIα down-regulation (Wang *et al.*, 1997a). But these results may reflect cell type-specific expression or regulation of factors involved in the modulation of the topo IIα promoter rather than a different mechanism of promoter regulation. Other than in this study, alterations to Sp3 levels have yet to be detected in doxorubicin-resistant cell lines.

7.2 Model for the regulation of the topo IIα promoter

From what is already known about the regulation of the topo IIα promoter and speculation about the roles of specific transcription factors in drug resistance, a model can be proposed to describe a mechanism of regulation of the topo IIα promoter in drug-exposed cells.

For simplicity only GC1, ICB1 and ICB2 were initially considered in this model. Although not definitively proven it is possible that NF-Y, Sp1 and Sp3 have roles in modulating topo IIα promoter activity. Since NF-Y can perturb, distort or bend DNA or remodel the chromatin in other promoters, including the murine topo IIα promoter (Ronchi *et al.*, 1995; Coustry *et al.*, 2001; Liberati *et al.*, 1998; Korner & Muller, 2000; Li *et al.*, 1998), it is possible that this transcription factor has a similar role at the human topo IIα promoter. Sp1 is normally considered to be an activator while Sp3 can act as a repressor or an activator depending on promoter context (Parnaik, 1999; Suske, 1999; Lania *et al.*, 1997). Interactions between Sp1, Sp3 and NF-Y have also been demonstrated on other promoters (Magan, 2002; Roder *et al.*, 1997; Bigger *et al.*, 1997; Tsai *et al.*, 1999; Liang *et al.*, 2001; Inoue *et al.*, 1999).

Investigation into the basal regulation of the human topo IIα promoter has shown that NF-Y may act as an activator at ICB1 and a repressor at ICB2; Sp1 acts as an activator, and Sp1 and Sp3 can bind to GC1 and GC2; NF-Y bound at ICB1 is likely to interact with Sp1 bound at GC1 (Magan, 2002) and ICB3 and ICB4 can also bind NF-Y but the functional significance of these interactions is unknown. It has been shown that NF-Y is likely to act as a repressor at ICB3 (Morgan & Beck, 2001) and Sp3 has been implicated as a potential repressor or activator of the topo IIα promoter in various resistant cell lines (Kubo *et al.*, 1995; Mo *et al.*, 1997).

Transcriptional regulation can be a complex process, with a number of factors contributing to the overall outcome. Such complex regulation is likely to be the case for the topo IIα promoter. While it may be possible that the alterations to NF-Y, Sp1 and Sp3 in the surviving cell lines may be unrelated to the alterations in topo IIα regulation seen, this seems unlikely given the current understanding of topo IIα promoter regulation. In the model described below for topo IIα regulation, NF-Y acts to modulate the activity of Sp1 and Sp3, with these transcription factors determining whether activation or repression results.

In this model, during basal transcription, NF-Y is bound at ICB1 and Sp1 at GC1 in a tight complex. This interaction between NF-Y and Sp1 may enhance DNA binding and/or transactivation possibly through the direct interaction between these factors and the transcriptional machinery or due to NF-Y-induced perturbations of the chromatin structure. As Sp1 is tightly bound at GC1, Sp3 cannot bind (figure 7.1 (a)).

Upon an increase in NF-Y levels, NF-Y can now bind to the lower affinity binding site ICB2. A possible consequence of this binding may be the disruption of the NF-Y/Sp1 complex downstream of ICB2. The binding of the complex to DNA or the ability to transactivate may be affected and consequently have an inhibitory effect on transcription (figure 7.1 (b)). It has been proposed that NF-Y also acts as a repressor at ICB3 (Morgan & Beck, 2001), and therefore the binding of NF-Y to any sites upstream of ICB1 may have an additional inhibitory effect.

If Sp1 bound at GC1 is required for the high affinity binding of NF-Y to ICB1, then a subsequent reduction in the cellular levels of Sp1 may inhibit the assembly and DNA binding of the NF-Y/Sp1 complex and so further inhibit promoter activity. An enhancement of NF-Y binding to ICB2 may occur. A consequence of the reduced amount or disruption to DNA binding of the NF-Y/Sp1 complex, may be that the putative repressor Sp3 is now be able to compete with Sp1 for binding to GC1 (figure 7.1 (c)). In this model a reduction to the amounts in Sp3 alone would have little effect on promoter activity.

Another possibility is that DNA looping occurs facilitated by Sp1 proteins (Mastrangelo *et al.*, 1991) bound to the two GC elements, although such an interaction has yet to be described for the topo IIα promoter. The modulation of the amounts or activity of Sp1 could cause alterations in the looping and subsequent protein-protein interactions and as such may have an effect upon transcription.

This model may explain the observations in the frozen cell lines that no longer showed decreased amounts of topo IIα protein after being thawed. In those cell lines that had previously shown increased NF-YA and decreased Sp1 protein, the relative amount of NF-YA returned to control levels, while the Sp1 levels had doubled to be approximately half the control levels. In these cell lines, the levels of Sp3 were also increased.

Reduction in the amounts of NF-Y may result in decreased binding of NF-Y to ICB2. This would prevent the disruption of the complex bound at ICB1/GC1 and return topo IIα promoter activity to basal levels. If this occurred, then the additional increase in the amounts of Sp1 protein may result in higher levels of Sp1/NF-Y complex and so would cause the activation of the topo IIα promoter above basal levels. The maintenance of the ratios of activators to repressors could be a mechanism to maintain the required topo IIα levels and prevent over-expression of topo IIα.

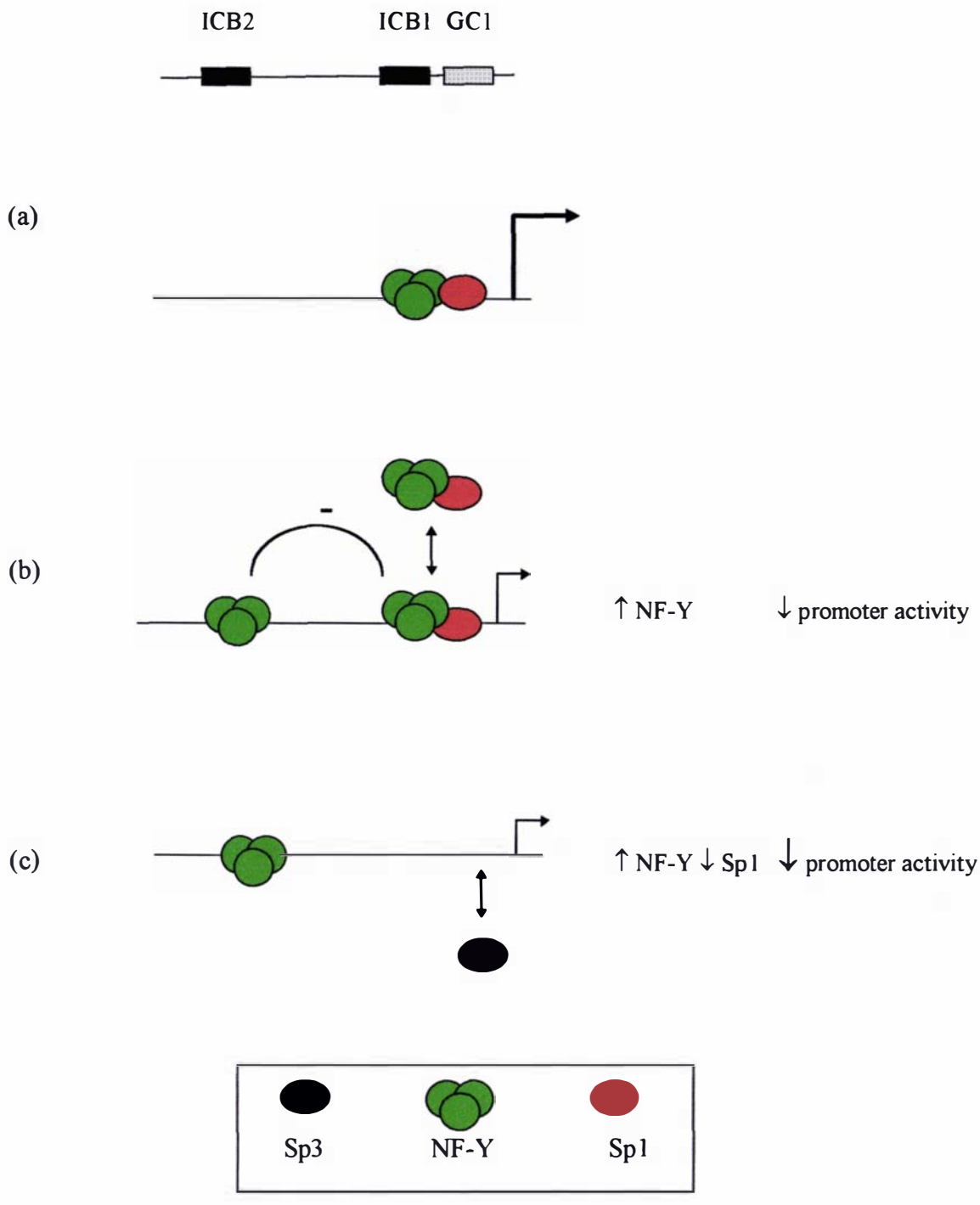


Figure 7.1. Diagram of the proposed interactions occurring at the topo IIα promoter (a) NF-Y is bound to ICB1 and Sp1 is bound at GC1 under basal conditions (b) Upon the increase in amounts of NF-Y, NF-Y now binds to ICB2 causing a disruption of the Sp1/NF-Y complex downstream. This disruption may reduce DNA binding and so allow Sp3 to compete for binding to GC1 (c) The reduction in the amounts of Sp1 would further reduce the Sp1/NF-Y complex binding to GC1/ICB1, further inhibiting promoter activity. Sp3 may now to bind to GC1 without competition.

The cell type- or drug-specific modifications to NF-Y, Sp1 or Sp3 DNA binding or transcriptional activity, either through direct modulation of the factors themselves or the modulation of regulatory factors, may ultimately determine whether activation or repression of the topo IIα promoter activity occurs. This may be the basis of the cell type difference seen. For example if NF-Y was modified in MCF-7 cells to bind to ICB1 but not ICB2, then an enhancement of NF-Y levels could cause promoter activation rather than repression. While such modifications or regulatory factors are as yet unknown, the following summary outlines the potential candidates or regulatory pathways that may have roles in the cell type- or drug-specific regulation of the topo IIα promoter during or after drug exposure.

7.3 Potential role of other transcription factors in the modulation of the topo IIα promoter during or after drug exposure

A number of putative transcription factor binding sites have been identified in the topo IIα promoter sequence. These factors have previously been shown to be involved in doxorubicin resistance or have been modified upon drug exposure and so may have potential roles in the modulation of the topo IIα promoter during or after drug exposure. The analysis of the topo IIα promoter sequence, using the GCG Tfsites database (University of Wisconsin Genetics Computer Group) and the TRANSFAC Version 4.0 database (Wingender *et al.*, 2000), has identified binding sites for such transcription factors in the topo IIα promoter including YB-1, Egr-1, NF-κB, AP-1 and ATF (figure 7.2).

A range of transcription factors has been proposed to be involved in the response to specific anticancer drugs although only limited research has been performed connecting these proteins and drug-induced signaling pathways. The production of reactive oxygen species by doxorubicin metabolism is thought to be a major inducer of the levels or activity of transcription factors like Egr-1 and NF-κB, while the ATF/AP-1 family may have roles in the regulation of genes upon doxorubicin exposure. Table 7.1 shows examples of transcription factors that have been shown to be modulated upon exposure to doxorubicin. Each of these has the potential to interact with the topo IIα

promoter and modulate expression (Bargou *et al.*, 1997; Shibao *et al.*, 1999; Arai *et al.*, 2000; Maestre *et al.*, 2001; Bottero *et al.*, 2001; Daschner *et al.*, 1999; Hai & Hartman, 2001) and are therefore candidates for future research.

7.4 Potential cell type-specific modulators of topo IIα expression between the MDA-MB-231 and MCF-7 cell lines

The MDA-MB-231 and MCF-7 cell lines differ in ER and p53 status. The presence of wild type p53 enhanced doxorubicin sensitivity (Hochhauser *et al.*, 1999; Seth *et al.*, 1997), while estrogen responsiveness enhanced doxorubicin resistance (Teixeira *et al.*, 1995; Zampieri *et al.*, 2002). Both of these mechanisms may modulate resistance or topo IIα promoter activity and may explain why a majority of the MDA-MB-231-derived cell lines had decreased amounts of topo IIα while the MCF-7-derived cell lines did not.

There is evidence that mutant forms of p53 can have roles in the regulation of gene expression even if the protein can no longer bind to DNA. While a p53 mutant protein may not be able to bind to DNA but may still be able to interact with other factors e.g. Sp1 (Torgeman *et al.*, 2001) and Ets (Sampath *et al.*, 2001). In the topo IIα promoter, putative Ets-like binding sites have been identified at -34 (near to GC1) and -467 (between the putative CREB/AP-1/ATF site and GC2) (figure 7.2). The possibility exists that the mutant p53 may have a role in modulating topo IIα transcription by either interacting with Sp1 and/or NF-Y or by directly binding to the Ets protein bound at -34 (if this site is shown to be functional) and then interacting with the nearby Sp1/NF-Y complex. ER has been shown to interact with Sp1 and NF-Y (Saville *et al.*, 2000; Farsetti *et al.*, 2001) and so ER may bind to either or both factors and modulate transcription.

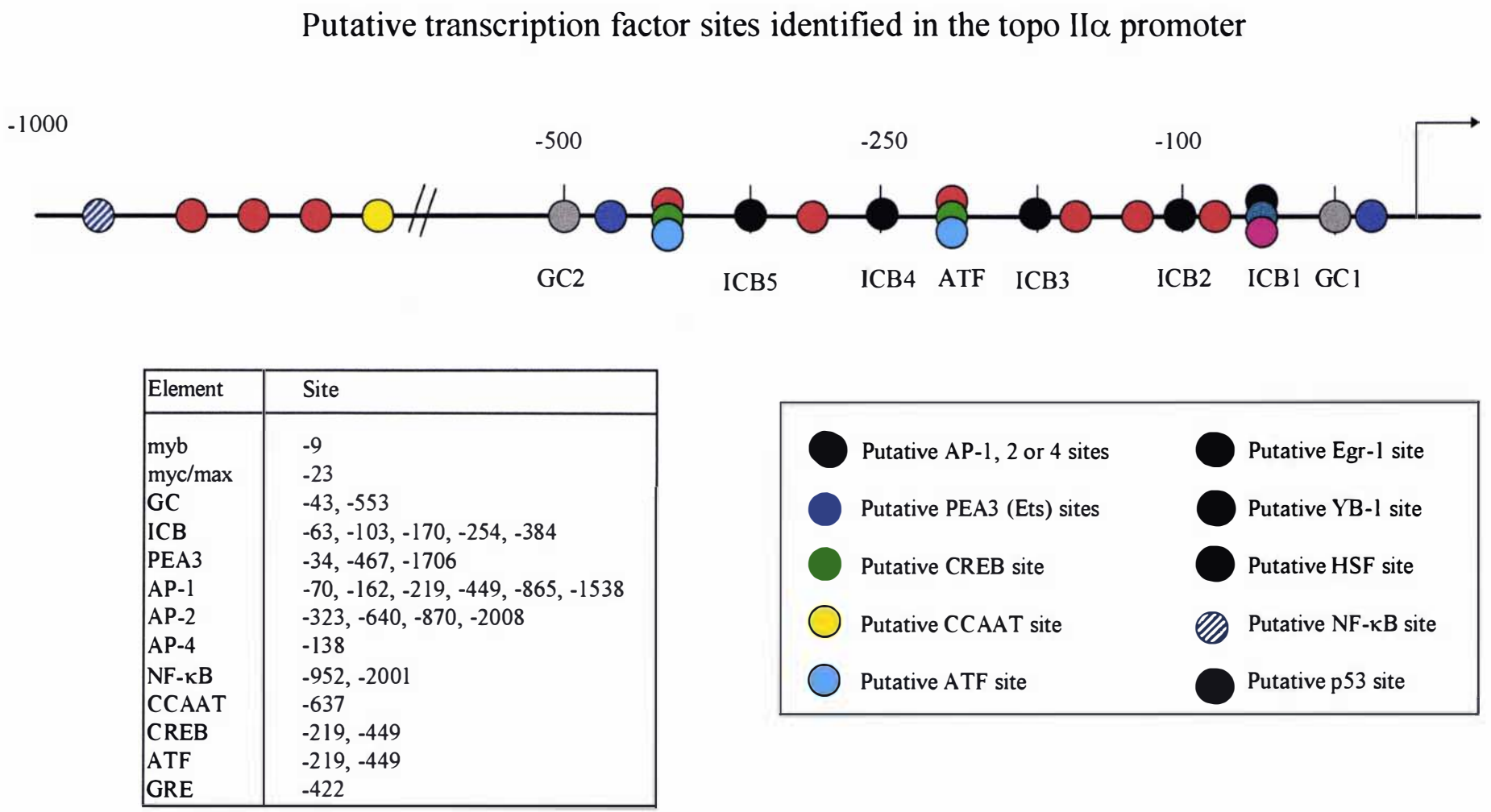


Figure 7.2. Diagram of the putative transcription factor binding sites identified by the GCG Tfsites database (University of Wisconsin Genetics Computer Group) and the TRANSFAC Version 4.0 database (Wingender et al., 2000) in the initial 1000 bp of the human topo IIα promoter.

Transcription factor	Role	Binding sequence (5' → 3')	Upon drug exposure or in resistant cells	Pathway or mediator	Reference
NF-Y	Ubiquitous transcription factor	ICB box	↓ NF-Y binding in dox^R multiple myeloma cells	?	Wang *et al.* (1997a)
Sp1	Ubiquitous transcription factor from Sp family	GC-rich sequence	↑ Sp1 in dox^R HL-60 cells	?	Borellini *et al.* (1990)
Sp3	Ubiquitous transcription factor from Sp family	GC-rich sequence	↓ Sp3 in mer^R CEM cells ↑ Sp3 in VP-16^R and VM-24^R KB cells	? ?	Mo *et al.* (1997) Kubo *et al.* (1995)
YB-1	Induced after environmental stress and cell proliferation	Y box ICB CTGATTGG	Nuclear localization in dox^R MCF-7 cells	?	Bargou *et al.* (1997)
Egr-1	Induced by stimuli that promotes oxidative stress e.g. tissue injury/ mitogens	GCGGGGGCG GAGGGGGCG	(+) dox ↑ Egr-1 levels	p44/p42 MAPK H_2O_2	Gashler & Sukhatme (1995) Nose & Ohba (1996) Saadane *et al.* (2001) Arai *et al.* (2000) Huang *et al.* (1997)
NF-κB	Induced as a stress response	GGGAATTCT	(+dox / +2-AAF) Degradation of IκB repressor, nuclear localization of NF-κB	double-stranded DNA breaks/ membrane interaction PI3K	Maestre *et al.* (2001) Bottero *et al.* (2001) Lin *et al.* (1998) Kuo *et al.* (2002)
ATF	Induced as a stress response	ATF element	(+) dox ↑ ATF3	JNK/SAPK	Hai & Hartman (2001)
AP-1	Mediator of stress response pathways, found as a c-fos/c-jun heterodimer	TGAGTCA	dox^R MCF-7 cells ↑ c-fos ↑ c-jun	?	Daschner *et al.* (1999)

Table 7.1. Examples of transcription factors modulated either after doxorubicin exposure or in doxorubicin-resistant cell lines

In summary, in the cell lines isolated, alterations to Sp1 and/or NF-YA were observed, with differences between the MDA-MB-231 and MCF-7 cell lines. As well as NF-Y and Sp1 or Sp3, the topo IIα promoter contains a number of putative sites for transcription factors known to be modulated after drug exposure and so may be potential candidates for investigation. This current study provides preliminary data indicating potential roles of specific transcription factors in topo IIα expression. A number of experiments need to be performed to confirm and extend the results of this research.

7.5 Signaling pathways activated by doxorubicin

It has been shown that a number of signaling pathways have been activated by doxorubicin, or found to be up-regulated in specific resistant cell lines and which may modulate transcription factors previously mentioned. These include the JNK/SAPK, ERK (p44/42), p38 (Osborn & Chambers, 1996; Herr *et al.*, 1997; Herr *et al.*, 1999; Arai *et al.*, 2000) and PKA pathways (Scala *et al.*, 1995; Glazer & Rohlff, 1994) (figure 7.3).

Increased PKC activity or differential expression of PKC isoforms have been seen in doxorubicin-resistant MCF-7, HL-60 and fibrosarcoma cells (O'Brian *et al.*, 1989; McClean & Hill, 1992; Davies *et al.*, 1996). The PKC-mediated signal transduction pathway was implicated in the nuclear translocation of YB-1, a potential modulator (Koike *et al.*, 1997).

HER2 (neu/Erb-B2) is a 185 kDa transmembrane glycoprotein which has proposed roles in events like cellular differentiation. HER-2 is part of a closely related growth factor receptor family which includes EGFR, HER3 and HER4. The ligand-mediated homo- or heterodimerization of family members results in the phosphorylation of specific receptor tyrosine residues which can act as docking sites for various signaling molecules including Ras (Kaptain *et al.*, 2001; Clynes *et al.*, 1998; Hutchinson & Muller, 2000; Bange *et al.*, 2001).

Examples of signaling pathways activated after doxorubicin exposure or in drug-resistant cell lines

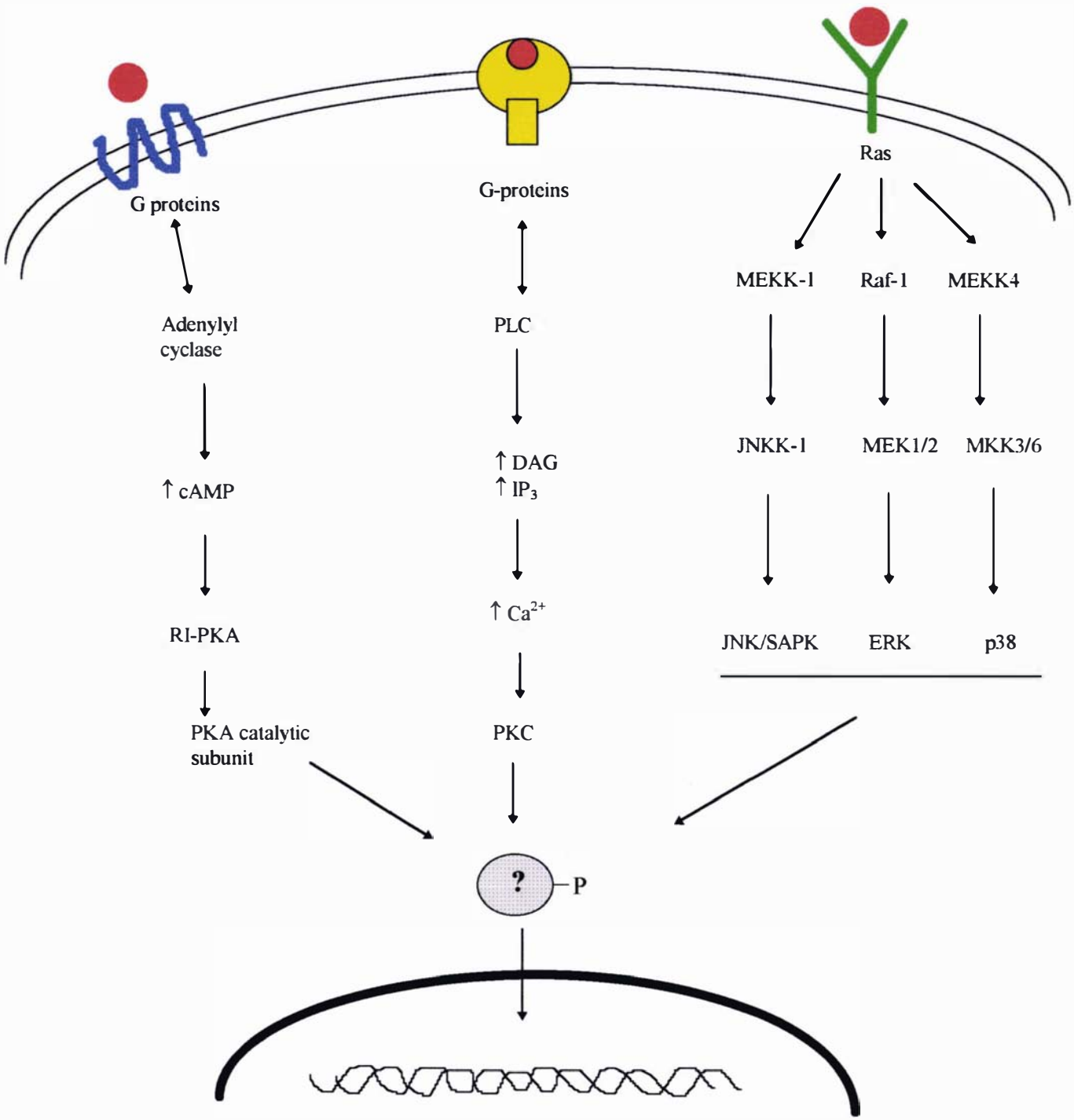


Figure 7.3. Simplified diagram of the MAPK, PKA and PKC-mediated signaling pathways that have proposed roles in drug exposure or drug resistance (adapted from Tamura et al., 2002 ; Kyriakis et al., 2001; English et al., 2002; Dorn et al., 2002).

This family of receptors has been implicated in topo IIα regulation and drug sensitivity. Increased EGFR was seen in doxorubicin-resistant MCF-7 cells and after doxorubicin exposure (Rohlff & Glazer, 1995). While Harris *et al.* (1998), Harris *et al.* (2001) and Lupu *et al.* (1996) showed that the activation or up-regulation of HER2 enhanced topo IIα promoter activity and doxorubicin sensitivity. In agreement with these results, the MEK/ERK and SEK/SAPK pathways have been proposed to mediate the Ras-induced stimulation of the human topo IIα promoter through the putative Ets-like site at -467 (Chen *et al.*, 1999).

A potential connection between these signaling pathways and the transcription factors identified through this current study can be seen. It has been shown that Sp1 can be phosphorylated by a wide variety of signaling pathways (Zheng *et al.*, 2000; Rohlff *et al.*, 1997; Noé *et al.*, 2001; Pal *et al.*, 1998; Liu *et al.*, 2001; Ma *et al.*, 2001; Armstrong *et al.*, 1997; Chupreta *et al.*, 2000).

Phosphorylation has a number of effects on Sp1 including increased protein turnover (Mortensen *et al.*, 1997) and increased or decreased DNA binding (Zheng *et al.*, 2000; Rohlff *et al.*, 1997; Noé *et al.*, 2001; Chupreta *et al.*, 2000; Compe *et al.*, 2001).

Other more general effects of doxorubicin exposure include alterations to DNA methylation status and chromatin structure (Tada *et al.*, 2000; Kusaba *et al.*, 1999); down-regulation of components of RNA polymerase II (Fanciulli *et al.*, 1996; Fanciulli *et al.*, 1998; Bruno *et al.*, 1998) and gene-specific damage (Clary *et al.*, 1998).

7.6 Future directions

The experiments described in this thesis show that the use of stable cell lines containing topo IIα reporter constructs to identify the regions of the promoter involved in regulation after drug exposure was not a valid approach. Instead, investigating endogenous topo IIα regulation should provide meaningful data.

This work and that of others has implicated the transcription factors NF-Y, Sp1 and Sp3 in topo IIα gene expression. A number of experiments could be performed to define the roles of these factors in topo IIα gene regulation during and after drug exposure. The *in vivo* interaction between these proteins and the topo IIα promoter could be investigated. A comparison between the *in vivo* footprints of the topo IIα promoter between the control and drug-exposed cells would confirm the relative importance of specific ICB and GC elements in transcriptional regulation. This method could be used to provide evidence of the proposed model ; if correct the increased binding of proteins to ICB2 and decreased binding to ICB1 and GC1 should be detectable. Differences in the cell type-specific binding of factors to specific promoter elements may be observed, as would changes to binding at other promoter elements. This may identify new candidates for topo IIα gene regulation and provide more definitive information concerning the signaling pathways involved. Chromatin immunoprecipitation assays could also be used to identify the DNA targets of Sp1, Sp3 and NF-Y. Any protein/DNA binding interactions could be confirmed *in vitro* using EMSA or reporter gene assays. It may also be important to assess mRNA and protein turnover.

Alterations to topo IIα catalytic activity may be determined by measuring topo II-mediated unknotting of P4 bacteriophage DNA or the decatenation of kinetoplast DNA from *Trypanosoma* or *Crithidia fasciculata* (Boege, 1996). Topo II-mediated cleavage complexes can be detected through the covalent attachment of the topo II protein to the DNA. The selective binding of topo II-DNA complex to nitrocellulose, or the inability of such complexes to enter a SDS-PAGE gel are the basis of the filter elution and immunoblot depletion assays respectively (Boege, 1996). To detect gene-specific DNA damage, stop-assay PCR can be used. In this method, the inability of promoter-spanning primers to amplify PCR products is used as a measure of DNA damage (Clary *et al.*, 1998).

One significant difficulty of this research was the transient nature of the alterations to both topo IIα and putative transcription factors. The study of drug-exposed cells over shorter time courses may be a more tenable approach. Changes in gene expression

occurring immediately after drug exposure prior to the development of resistance may provide more useful insights into the mechanism involved in drug resistance. This approach may also yield new candidates for study. As the surviving cell lines did not show consistent changes in topo IIα expression, it is extremely likely that alternative mechanisms are involved in the drug resistant phenotype. It will be important to use both genomic and proteomic approaches to ensure both transcriptional and post-transcriptional mechanisms are investigated. Once candidate genes are identified a series of functional assays can be devised to confirm a role in drug resistance.

7.7 Conclusion

The research described in this thesis provides the starting point to investigate the regulation of a gene that may have a crucial effect on the action of many anticancer drugs. An understanding of the regulation of topo IIα gene expression during drug exposure may provide novel targets for pharmaceutical intervention that prevent down-regulation of the gene, or cause the up-regulation of the gene; both of these events should enhance chemosensitivity of cancer cells and drug cytotoxicity. The development of such specific drugs would be highly beneficial in the treatment of cancer and as such is an exciting and worthwhile area of research.

Bibliography

Aas, T., Borresen, A. L., Geisler, S., Smith-Sorensen, B., Johnsen, H., Varhaug, J. E., Akslen, L. A. & Lonning, P. E. (1996). Specific p53 mutations are associated with *de novo* resistance to doxorubicin in breast cancer patients. *Nature Medicine* **2,** 811-814.

Abe, T., Fukamachi, Y., Kanazawa, Y., Furukawa, H., Shimizu, K., Hirano, T., Kasai, H., Kashimura, M. & Higashi, K. (1996). Inhibition of Nucleolar Function and Morphological Change by Adriamycin Associated with Heat Shock Protein 70 Accumulation. *Japanese Journal of Cancer Research* **87,** 945-951.

Adachi, N., Nomoto, M., Kohno, K. & Koyama, H. (2000). Cell-cycle regulation of the DNA topoisomerase IIα promoter is mediated by proximal CCAAT boxes : possible involvement of acetylation. *Gene* **245,** 49-57.

Agoff, S. N., Hou, J., Linzer, D. I. H. & Wu, B. (1993). Regulation of the Human hsp70 Promoter by p53. *Science* **259,** 84-87.

Aly, M. S., Othman, O. E. & El Nahas, S. M. (1999). Specific Numerical Chromosomal Aberrations Induced by Adriamycin. *Environmental and Molecular Mutagenesis* **33,** 161-166.

Ammanamanchi, S. & Brattain, M. G. (2001a). Sp3 Is A Transcriptional Repressor of Transforming Growth Factor-β Receptors. *The Journal of Biological Chemistry* **276,** 3348-3352.

Ammanamanchi, S. & Brattain, M. G. (2001b). 5-AzaC Treatment Enhances Expression of Transforming Growth Factor-β Receptors through Down-regulation of Sp3. *The Journal of Biological Chemistry* **276,** 32854-32859.

Andoh, T. & Ishida, R. (1998). Catalytic inhibitors of DNA topoisomerase II. *Biochimica et Biophysica Acta* **1400,** 155-171.

Andres, J. L., Fan, S., Turkel, G. J., Wang, J.-A., Twu, N.-F., Yuan, R.-Q., Lamszus, K., Goldberg, I. D. & Rosen, E. M. (1998). Regulation of BRCA1 and BRCA2 expression in human breast cancer cells by DNA-damaging agents. *Oncogene* **16,** 2229-2241.

Aoyama, M., Grabowski, D. R., Dubyak, G. R., Constantinou, A. I., Rybicki, L. A., Bukowski, R. M., Ganapathi, M. K., Hickson, I. D. & Ganapathi, R. (1998). Attenuation of drug-stimulated topoisomerase II-DNA cleavable complex formation in wild-type HL-60 cells treated with an intracellular calcium buffer is correlated with decreased cytotoxicity and site-specific hypophosphorylation of topoisomerase IIα. *Biochemical Journal* **336,** 727-733.

Arai, M., Yoguchi, A., Takizawa, T., Yokoyama, T., Kanda, T., Kurabayashi, M. & Nagai, R. (2000). Mechanism of Doxorubicin-Induced Inhibition of Sarcoplasmic Reticulum Ca^{2+}-ATPase Gene Transcription. *Circulation Research* **86,** 8-14.

Aries, A., Trentesaux, C., Ottolenghi, S., Jardillier, J.-C., Jeannesson, P. & Doubeikovski, A. (1996). Activation of Erythroid-Specific Promoters During Anthracycline-Induced Differentiation of K562 Cells. *Blood* **87,** 2885-2890.

Armstrong, S. A., Barry, D. A., Leggett, R. W. & Mueller, C. R. (1997). Casein Kinase II-mediated Phosphorylation of the C Terminus of Sp1 Decreases Its DNA Binding Activity. *The Journal of Biological Chemistry* **272,** 13489-13495.

Arnal, M., Franco, N., Fargeot, P., Riedinger, J.-M., Brunet-Lecomte, P. & Lizard-Nacol, S. (2000). Enhancement of *mdr1* gene expression in normal tissue adjacent to advanced breast cancer. *Breast Cancer Research and Treatment* **61,** 13-20.

Arts, H. J. G., van der Zee, A. G. J., de Jong, S. & de Vries, E. G. E. (2000). Options for modulation of drug resistance in ovarian cancer. *International Journal of Gynecological Cancer* **10,** 47-52.

Asano, T., An, T., Mayes, J., Zwelling, L. A. & Kleinerman, E. S. (1996a). Transfection of Human Topoisomerase IIα into Etoposide-resistant cells : transient increase in sensitivity followed by down-regulation of the endogenous gene. *Biochemical Journal* **319,** 307-313.

Asano, T., An, T., Zwelling, L. A., Takano, H., Fojo, A. T. & Kleinerman, E. S. (1996b). Transfection of a Human Topoisomerase IIα Gene into Etoposide-resistant Human Breast Tumor Cells Sensitizes the Cells to Etoposide. *Oncology Research* **8,** 101-110.

Asano, T., Zwelling, L. A., An, T., McWatters, A., Herzog, C. E., Mayes, J., Loughlin, S. & Kleinerman, E. S. (1996c). Effect of transfection of a *Drosophila* topoisomerase II gene into a human brain tumor cell line intrinsically resistant to etoposide. *British Journal of Cancer* **73,** 1373-1380.

Austin, C. A. & Marsh, K. L. (1998). Eukaryotic DNA topoisomerase II β. *BioEssays* **20,** 215-226.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1991). *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley Intersciences, New York, USA.

Badie, C., Itzhaki, J. E., Sullivan, M. J., Carpenter, A. J. & Porter, A. C. G. (2000). Repression of *CDK1* and Other Genes with CDE and CHR Promoter Elements during DNA Damage-Induced G_2/M Arrest in Human Cells. *Molecular and Cellular Biology* **20,** 2358-2366.

Bakshi, R. P., Galande, S. & Muniyappa, K. (2001). Functional and Regulatory Characteristics of Eukaryotic Type II DNA Topoisomerase. *Critical Reviews in Biochemistry and Molecular Biology* **36,** 1-37.

Baldini, N. (1997). Multidrug resistance - a multiplex phenomenon. *Nature Medicine* **3,** 378-380.

Bange, J., Zwick, E. & Ullrich, A. (2001). Molecular targets for breast cancer therapy and prevention. *Nature Medicine* **7,** 548-552.

Bargou, R. C., Jurchott, K., Wagener, C., Bergmann, S., Metzner, S., Bommert, K., Mapara, M. Y., Winzer, K.-J., Dietel, M., Dorken, B. & Royer, H.-D. (1997). Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic *MDR1* gene expression. *Nature Medicine* **3,** 447-450.

Barlogie, B., Drewinko, B., Johnston, D. A. & Freireich, E. J. (1976). The Effect of Adriamycin on the Cell Cycle Traverse of a Human Lymphoid Cell Line. *Cancer Research* **36,** 1975-1979.

Bartek, J., Iggo, R., Gannon, J. & Lane, D. P. (1990). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* **5,** 893-899.

Barthelmes, H. U., Grue, P., Feineis, S., Straub, T. & Boege, F. (2000). Active DNA Topoisomerase IIα Is A Component of the Salt-stable Centrosome Core. *The Journal of Biological Chemistry* **275,** 38823-38830.

Bassi, L. & Palitti, F. (2000). Anti-topoisomerase drugs as potent inducers of chromosomal aberrations. *Genetics and Molecular Biology* **23,** 1065-1069.

Beaumont, P. O., Moore, M. J., Ahmad, K., Payne, M. M., Lee, C. & Riddick, D. S. (1998). Role of Glutathione *S*-Transferases in the Resistance of Human Colon Cancer Cell Lines to Doxorubicin. *Cancer Research* **58,** 947-955.

Beck, J., Handgretinger, R., Dopfer, R., Klingebiel, T., Niethammer, D. & Gekeler, V. (1995). Expression of mdr1, mrp, topoisomerase IIα/β, and cyclin A in primary or relapsed states of acute lymphoblastic leukaemias. *British Journal of Haematology* **89,** 356-363.

Beck, W. T., Morgan, S. E., Mo, Y. & Bhat, U. G. (1999). Tumor cell resistance to DNA topoisomerase II inhibitors: new developments. *Drug Resistance Updates* **2,** 382-389.

Beketic-Oreskovic, L., Duran, G. E., Chen, G., Dumontet, C. & Sikic, B. I. (1995). Decreased Mutation Rate for Cellular Resistance to Doxorubicin and Suppression of mdr1 Gene Activation by the Cyclosporin PSC 833. *Journal of the National Cancer Institute* **87,** 1593-1602.

Benjamin, R. S., Wiernik, P. H. & Bachur, N. R. (1974). Adriamycin Chemotherapy - Efficacy, Safety, and Pharmacologic Basis of an Intermittent Single High-Dosage Schedule. *Cancer* **33,** 19-27.

Berger, J. M., Gamblin, S. J., Harrison, S. C. & Wang, J. C. (1996). Structure and Mechanism of DNA Topoisomerase II. *Nature* **379,** 225-232.

Bergman, A. M., Giaccone, G., van Moorsel, C. J. A., Mauritz, R., Noordhuis, P., Pinedo, H. M. & Peters, G. J. (2000). Cross-resistance in the 2',2'-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cell line AG6000 to standard and investigational drugs. *European Journal of Cancer* **36,** 1974-1983.

Berthosis, Y., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: Implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences USA* **83,** 2496-2500.

Bigger, C. B., Melnikova, I. N. & Gardner, P. D. (1997). Sp1 and Sp3 Regulate Expression of the Neuronal Nicotinic Acetylcholine Receptor β4 Subunit Gene. *The Journal of Biological Chemistry* **272,** 25976-25982.

Bindall, R. D., Carlson, K. E., Katzenellenbogen, B. S. & Katzenellenbogen, J. A. (1988). Lipophilic Impurities, not Phenolsulfonphthalein, Account for the Estrogenic Activity in Commercial Preparations of Phenol Red. *Journal of Steroid Biochemistry* **31,** 287-293.

Bjergbaek, L., Jensen, S., Westergaard, O. & Andersen, A. H. (1999). Using a Biochemical Approach to Identify the Primary Dimerization Regions in Human DNA Topoisomerase IIα. *The Journal of Biological Chemistry* **274,** 26529-26536.

Boege, F. (1996). Analysis of Eukaryotic DNA Topisomerases and Topoisomerase - Directed Drug Effects. *Journal of Clinical Chemistry and Clinical Biochemistry* **34,** 873-888.

Bolognese, F., Wasner, M., Lange-zu Dohna, C., Gurtner, A., Ronchi, A., Muller, H., Manni, I., Mossner, J., Piaggio, G., Mantovani, R. & Engeland, K. (1999). The cyclin B2 promoter depends on NF-Y, a trimer whose CCAAT-binding activity is cell-cycle regulated. *Oncogene* **18,** 1845-1853.

Borellini, F., Aquino, A., Josephs, S. F. & Glazer, R. I. (1990). Increased Expression and DNA-Binding Activity of Transcription Factor Sp1 in Doxorubicin-Resistant HL-60 Leukemia Cells. *Molecular and Cellular Biology* **10,** 5541-5547.

Borellini, F. & Glazer, R. I. (1993). Induction of Sp1-p53 DNA-binding Heterocomplexes during Granulocyte/Macrophage Colony-stimulating Factor-dependent Proliferation in Human Erythroleukemia Cell Line TF-1. *The Journal of Biological Chemistry* **268,** 7923-7928.

Bottero, V., Busuttil, V., Loubat, A., Magné, N., Fischel, J.-L., Milano, G. & Peyron, J.-F. (2001). Activation of Nuclear Factor κB through the IKK Complex by the Topoisomerase Poisons SN38 and Doxorubicin: A Brake to Apoptosis in HeLa Human Carcinoma Cells. *Cancer Research* **61,** 7785-7791.

Boucek, R. J., Miracle, A., Anderson, M., Engelman, R., Atkinson, J. & Dodd, D. A. (1999). Persistent Effects of Doxorubicin on Cardiac Gene Expression. *Journal of Molecular and Cellular Cardiology* **31,** 1435-1446.

Bourhis, J., Benard, J., Hartmann, O., Boccon-Gibod, L., Lemerle, J. & Riou, G. (1989). Correlation of MDR1 Gene Expression With Chemotherapy in Neuroblastoma. *Journal of the National Cancer Institute* **81,** 1401-1405.

Brandt, T. L., Fraser, D. J., Leal, S., Halandras, P. M., Kroll, A. R. & Kroll, D. J. (1997). c-Myb trans-Activates the Human DNA Topisomerase IIα Gene Promoter. *The Journal of Biological Chemistry* **272,** 6278-6284.

Brenner, D. E., Galloway, S., Cooper, J., Noone, R. & Hande, K. R. (1985). Improved high-performance liquid chromatography assay of doxorubicin : Detection of circulating aglycones in human plasma and comparison with thin-layer chromatography. *Cancer, Chemotherapy and Pharmacology* **14,** 139-145.

Bronstein, I., Fortin, J., Stanley, P. E., Stewart, G. S. A. B. & Kricka, L. J. (1994). Chemiluminescent and Bioluminescent Reporter Gene Assays. *Analytical Biochemistry* **219,** 169-181.

Brown, G. A., McPherson, J. P., Gu, L., Hedley, D. W., Toso, R., Deuchars, K. L., Freedman, M. H. & Goldberg, G. J. (1995). Relationship of DNA Topoisomerase IIα and β Expression to Cytotoxicity of Antineoplastic Agents in Human Acute Lymphoblastic Leukemia Cell Lines. *Cancer Research* **55,** 78-82.

Bruno, T., Leonetti, C., Aloe, S., Iacobini, C., Floridi, A., Di Tondo, U., Punturieri, A. & Fanciulli, M. (1998). Levels of expression of hRPB11, a core subassembly subunit of human RNA polymerase II, affect doxorubicin sensitivity and cellular differentiation. *FEBS Letters* **427,** 241-246.

Budworth, J., Gant, T. W. & Gescher, A. (1997). Co-ordinate loss of protein kinase C and multidrug resistance gene expression in revertant MCF-7/Adr breast carcinoma cells. *British Journal of Cancer* **75,** 1330-1335.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W. & Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *The Journal of Clinical Investigation* **104,** 263-269.

Burden, D. A., Goldsmith, J. & Sullivan, D. M. (1993). Cell-cycle-dependent phosphorylation and activity of Chinese-hamster ovary topoisomerase II. *Biochemical Journal* **293,** 297-304.

Burden, D. A. & Sullivan, D. M. (1994). Phosphorylation of the α- and β-Isoforms of DNA Topoisomerase II Is Qualitatively Different in Interphase and Mitosis in Chinese Hamster Ovary Cells. *Biochemistry* **33,** 14651-14655.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25,** 169-193.

Canitrot, Y., de Murcia, G. & Salles, B. (1998). Decreased expression of topoisomerase IIβ in poly(ADP-ribose) polymerase-deficient cells. *Nucleic Acid Research* **26,** 5134-5138.

Capranico, G., Binaschi, M., Borgnetto, M. E., Zunino, F. & Palumbo, M. (1997). A protein-mediated mechanism for the DNA sequence-specific action of topoisomerase II poisons. *TIPS* **18,** 323-329.

Carey, M. & Smale, S. T. (2000). *Transcriptional Regulation in Eukaryotes : Concepts, Strategies, and Techniques*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Chan, J. Y.-W., Chu, A. C.-Y. & Fung, K.-P. (2000). Inhibition of P-glycoprotein expression and reversal of drug resistance of human hepatoma HepG2 cells by multidrug resistance gene (mdr1) antisense RNA. *Life Sciences* **67,** 2117-2124.

Chang, Z.-F. & Liu, C.-J. (1994). Human Thymidine Kinase CCAAT-binding Protein Is NF-Y, Whose A Subunit Expression Is Serum-dependent in Human IMR-90 Diploid Fibroblasts. *The Journal of Biological Chemistry* **269,** 17893-17898.

Chen, G. K., Duran, G. E., Mangili, A., Beketic-Oreskovic, L. & Sikic, B. I. (2000). *MDR1* activation is the predominant resistance mechanism selected by vinblastine in MES-SA cells. *British Journal of Cancer* **83,** 892-898.

Chen, G., Jaffrezou, J., Fleming, W. H., Duran, G. E. & Sikic, B. I. (1994). Prevalence of Multidrug Resistance Related to Activation of the *mdr*1 Gene in Human Sarcoma Mutants Derived by Single-Step Doxorubicin Selection. *Cancer Research* **54,** 4980-4987.

Chen, G., Templeton, D., Suttle, S. P. & Stacey, D. W. (1999). Ras stimulates DNA topoisomerase IIα through MEK : A link between oncogenic signaling and a therapeutic target. *Oncogene* **18,** 7149-7160.

Chen, Y., Mickley, L. A., Schwartz, A. M., Acton, E. M., Hwang, J. & Fojo, A. T. (1990). Characterization of Adriamycin-resistant Human Breast Cancer Cells Which Display Overexpression of a Novel Resistance-related Membrane Protein. *The Journal of Biological Chemistry* **265,** 10073-10080.

Cho, Y., Gorina, S., Jeffrey, P. D. & Pavletich, N. P. (1994). Crystal Structure of a p53 Tumor Suppressor-DNA Complex: Understanding Tumorigenic Mutations. *Science* **265,** 346-355.

Chupreta, S., Du, M., Todisco, A. & Merchant, J. L. (2000). EGF stimulates gastrin promoter through activation of Sp1 kinase activity. *American Journal of Physiology and Cell Physiology* **278,** C697-C708.

Ciocca, D. R., Fuqua, S. A. W., Lock-Lim, S., Toft, D. O., Welch, W. J. & McGuire, W. (1992). Response of Human Breast Cancer Cells to Heat Shock and Chemotherapeutic Drugs. *Cancer Research* **52,** 3648-3654.

Clary, A., Larrue, A., Pourquier, P. & Robert, J. (1998). Transcriptional down-regulation of c-*myc* expression in an erythroleukemic cell line K562, and its doxorubicin-resistant variant by two topoisomerase II inhibitors, doxorubicin and amsacrine. *Anti-Cancer Drugs* **9,** 245-254.

Clynes, M., Daly, C., NicAmhlaoibh, R., Cronin, D., Elliot, C., O'Connor, R., O'Doherty, T., Connolly, L., Howlett, A. & Scanlon, K. (1998). Recent developments in drug resistance and apoptosis research. *Critical Reviews in Oncology/Hematology* **28,** 181-205.

Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V. & Deeley, R. G. (1992). Overexpression of a Transporter Gene in a Multidrug-Resistant Human Lung Cancer Cell Line. *Science* **258,** 1650-1654.

Cole, S. P. C. & Deeley, R. G. (1998). Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. *BioEssays* **20,** 931-940.

Cole, S. P. C., Sparks, K. E., Fraser, K., Loe, D. W., Grant, C. E., Wilson, G. M. & Deeley, R. G. (1994). Pharmacological Characterization of Multidrug Resistant MRP-transfected Human Tumor Cells. *Cancer Research* **54,** 5902-5910.

Compe, E., De Sousa, G., Francois, K., Roche, R., Rahmani, R., Torresani, J., Raymondjean, M. & Planells, R. (2001). Spot 14 protein interacts and co-operates with chicken ovalbumin upstream promoter-transcription factor 1 in the transcription of the L-type pyruvate kinase gene through a specificity protein 1 (Sp1) binding site. *Biochemical Journal* **358,** 175-183.

Coon, J. C., Marcus, E., Gupta-Burt, S., Seelig, S., Jacobson, K., Chen, S., Renta, V., Fronda, G. & Preisler, H. D. (2002). Amplification and Overexpression of Topoisomerase IIα Predict Response to Anthracycline-based Therapy in Locally Advanced Breast Cancer. *Clinical Cancer Research* **8,** 1061-1067.

Coustry, F., Hu, Q., de Crombrugghe, B. & Maity, S. N. (2001). CBF/NF-Y Functions Both in Nucleosomal Disruption and Transcription Activation of the Chromatin-assembled Topoisomerase IIα Promoter. *The Journal of Biological Chemistry* **276,** 40621-40630.

Covacci, V., Bruzzese, N., Sgambato, A., Ganapathi, R., Cittadini, A. & Wolf, F. I. (2000). Effect of Extracellular Magnesium on Topoisomerase II Activity and

Expression in Human Leukemia HL-60 Cells. *Journal of Cellular Biochemistry* **78,** 325-333.

Cowan, K. H., Batist, G., Tulpule, A., Sinha, B. K. & Myers, C. E. (1986). Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rat. *Proceedings of the National Academy of Sciences USA* **83,** 9328-9332.

Cowell, I. G., Okorokov, A. L., Cutts, S. A., Padget, K., Bell, M., Milner, J. & Austin, C. (2000). Human Topoisomerase IIα and IIβ Interact with the C-terminal Region of p53. *Experimental Cell Research* **255,** 86-94.

Crawford, A. R., Smith, A. J., Hatch, V. C., Oude Elferink, R. P. J., Borst, P. & Crawford, J. M. (1997). Hepatic Secretion of Phospholipid Vesicles in the Mouse Critically Depends on mdr2 or MDR3 P-Glycoprotein Expression. *Journal of Clinical Investigation* **100,** 2562-2567.

Creagh, E. M., Sheehan, D. & Cotter, T. G. (2000). Heat shock proteins - modulators of apoptosis in tumour cells. *Leukemia* **14,** 1161-1173.

Creasey, W. A., McIntosh, L. S., Brescia, T., Odujinrin, O., Aspnes, G. T., Murray, E. & Marsh, J. C. (1976). Clinical Effects and Pharmacokinetics of Different Dosage Schedules of Adriamycin. *Cancer Research* **36,** 216-221.

Currie, R. A. (1998). NF-Y Is Associated with the Histone Acetyltransferases GCN5 and P/CAF. *The Journal of Biological Chemistry* **273,** 1430-1434.

Daniel, S., Zhang, S., DePaoli-Roach, A. A. & Kim, K.-H. (1996). Dephosphorylation of Sp1 by Protein Phosphatase 1 Is Involved in the Glucose-mediated Activation of the Acetyl-CoA Carboxylase Gene. *The Journal of Biological Chemistry* **271,** 14692-14697.

Danks, M. K., Schmidt, C. A., Cirtain, M. C., Suttle, D. P. & Beck, W. T. (1988). Altered Catalytic Activity of and DNA Cleavage by DNA Topoisomerase II from Human Leukemic Cells Selected for Resistance to VM-26. *Biochemistry* **27,** 8861-8869.

Daschner, P. J., Ciolino, H. P., Plouzek, C. A. & Yeh, G. C. (1999). Increased AP-1 activity in drug resistant human breast cancer MCF-7 cells. *Breast Cancer Research and Treatment* **53,** 229-240.

Davey, R. & Davey, M. (1998). The extended-MDR phenotype. *Cytotechnology* **27,** 237-247.

Davies, R., Budworth, J., Riley, J., Snowden, R., Gescher, A. & Gant, T. W. (1996). Regulation of P-glycoprotein *1* and *2* gene expression and protein activity in two MCF-7/Dox cell line subclones. *British Journal of Cancer* **73,** 307-315.

Davis, B. J. (1964). Disc Electrophoresis - II Method and Application To Human Serum Proteins. *Annals of the New York Academy of Sciences* **121,** 404-427.

Davis, J. M. (1994). *Basic Cell Culture : A Practical Approach*. IRL Press at Oxford University Press, Oxford.

de Jong, M. C., Slootstra, J. W., Scheffer, G. L., Schroeijers, A. B., Puijk, W. C., Dinkelberg, R., Kool, M., Broxterman, H. J., Meloen, R. H. & Scheper, R. J. (2001). Peptide Transport by the Multidrug Resistance Protein MRP1. *Cancer Research* **61,** 2552-2557.

de Jong, S., Zijlstra, J. G., de Vries, E. G. E. & Mulder, N. H. (1990). Reduced DNA Topoisomerase II Activity and Drug-induced DNA Cleavage Activity in an Adriamycin-resistant Human Small Cell Lung Carcinoma Cell Line. *Cancer Research* **50,** 304-309.

Deffie, A. M., Batra, J. K. & Goldenberg, G. J. (1989). Direct Correlation between DNA Topoisomerase II Activity and Cytotoxicity in Adriamycin-sensitive and -resistant P388 Leukemia Cell Lines. *Cancer Research* **49,** 58-62.

Den Boer, M. M., Pieters, R. & Veerman, A. J. P. (1998). Mechanisms of cellular anthracycline resistance in childhood acute leukemia. *Leukemia* **12,** 1657-1670.

Dereuddre, S., Delaporte, C. & Jacquemin-Sablon, A. (1997). Role of Topoisomerase II β in the Resistance of 9-OH-ellipticine-resistant Chinese Hamster Fibroblasts to Topoisomerase II Inhibitors. *Cancer Research* **57,** 4301-4308.

Dereuddre, S., Frey, S., Delaporte, C. & Jacquemin-Sablon, A. (1995). Cloning and characterization of full-length cDNAs coding for the DNA topoisomerase II β from Chinese hamster lung cells sensitive and resistant to 9-OH-ellipticine. *Biochimica et Biophysica Acta* **1264,** 178-182.

DeVore, R. F., Corbett, R. H. & Osheroff, N. (1992). Phosphorylation of Topoisomerase II by Casein Kinase II and Protein Kinase C : Effects on Enzyme-mediated DNA Cleavage/Religation and Sensitivity to the Antineoplastic Drugs Etoposide and 4'-(9-Acridinylamino) methane-sulfon-*m*-anisidide. *Cancer Research* **52,** 2156-2161.

Di Carlo, A., White, N., Guo, F., Garrett, P. & Litovitz, T. (2002). Chronic Electromagnetic Field Exposure Decreases HSP70 Levels and Lowers Cytoprotection. *Journal of Cellular Biochemistry* **84,** 447-454.

Di Leo, A., Gancberg, D., Larismont, D., Tanner, M., Jarvinen, T., Rouas, G., Dolci, S., Leroy, J.-Y., Paesmans, M., Isola, J. & Piccari, M. J. (2002). HER-2 Amplification and Topoisomerase IIα Gene Aberrations as Predictive Markers in Node-positive Breast Cancer Patients Randomly Treated Either with an Anthracycline-based Therapy or with Cyclophosphamide, Methotrexate, and 5-Fluorouracil. *Clinical Cancer Research* **8,** 1107-1116.

Diah, S. K., Smitherman, P. K., Aldridge, J., Volk, E. L., Scheider, E., Townsend, A. J. & Morrow, C. S. (2001). Resistance to Mitoxantrone in Multidrug-resistant MCF7 Breast Cancer Cells: Evaluation of Mitoxantrone Transport and the Role of Multidrug Resistance Protein Family Proteins. *Cancer Research* **61,** 5461-5467.

Ding, H., Benotmane, A. M., Suske, G., Collen, D. & Belayew, A. (1999). Functional Interactions between Sp1 and Sp3 and the Helicase-like Transcription Factor Mediate Basal Expression from the Human Plasminogen Activator Inhibitor-1 Gene. *The Journal of Biological Chemistry* **274,** 19573-19580.

Dingemans, A. C., Pinedo, H. M. & Giaccone, G. (1998). Clinical resistance to topoisomerase-targeted drugs. *Biochimica et Biophysica Acta* **1400,** 275-288.

Discher, D. J., Bishopric, N. H., Wu, X., Peterson, C. A. & Webster, K. A. (1998). Hypoxia Regulates β-Enolase and Pyruvate Kinase-M Promoters by Modulating Sp1/Sp3 Binding to a Conserved GC Element. *The Journal of Biological Chemistry* **273,** 26087-26093.

Dorn, A., Bollekens, J., Staub, A., Benoist, C. & Mathis, D. (1987). A Multiplicity of CCAAT Box-Binding Proteins. *Cell* **50,** 863-872.

Downes, C. S., Mullinger, A. M. & Johnson, R. T. (1991). Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. *Proceedings of the National Academy of Sciences USA* **88,** 8895-8899.

Drake, F. H., Hofmann, G. A., Bartus, H. F., Mattern, M. R., Crooke, S. T. & Mirabelli, K. (1989). Biochemical and Pharmalogical Properties of p170 and p180 Forms of Topoisomerase II. *Biochemistry* **28,** 8154-8160.

Dumontet, C., Duran, G. E., Steger, K. A., Beketic-Oreskovic, L. & Sikic, B. I. (1996). Resistance Mechanisms in Human Sarcoma Mutants Derived by Single-Step Exposure to Paclitaxel (Taxol). *Cancer Research* **56,** 1091-1097.

Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. S. & Liu, L. F. (1985). Topoisomerase II is a Structural Component of Mitotic Chromosome Scaffolds. *Journal of Cell Biology* **100,** 1706-1715.

Eder, J. P. J., Chan, V. T.-W., Niemierko, E., Teicher, B. A. & Schnipper, L. E. (1993). Conditional Expression of Wild-type Topoisomerase II Complements a Mutant Enzyme in Mammalian Cells. *The Journal of Biological Chemistry* **268,** 13844-13849.

Eggen, B. J. L., Benus, G. F. J. D., Folkertsma, S., Jonk, L. J. & Kruijer, W. (2001). TAK1 activation of the mouse JunB promoter is mediated through a CCAAT box and NF-Y. *FEBS Letters* **506,** 267-271.

Elstner, E., Linker-Israeli, M., Said, J., Umiel, T., de Vos, S., Shintaku, I. P., Heber, D., Binderup, L., Uskokovic, M. & Koeffler, H. P. (1995). 20-epi-Vitamin D_3

Analogues: A Novel Class of Potent Inhibitors of Proliferation and Inducers of Differentiation of Human Breast Cancer Cell Lines. *Cancer Research* **55,** 2822-2830.

Emili, A., Greenblatt, J. & Ingles, C. J. (1994). Species-Specific Interaction of the Glutamine-Rich Activation Domains of Sp1 with the TATA Box-Binding Protein. *Molecular and Cellular Biology* **14,** 1582-1593.

English, J. M. & Cobb, M. H. (2002). Pharmacological inhibitors of MAPK pathways. *Trends in Pharmacological Sciences* **23,** 40-45.

Epsztejn, S., Glickstein, H., Picard, V., Slotki, I. N., Breuer, W., Beaumont, C. & Cabantchik, Z. I. (1999). H-ferritin subunit overexpression in erythroid cells reduces the oxidative stress response and induces multidrug resistance properties. *Blood* **94,** 3593-3603.

Errington, F., Willmore, E., Tilby, M. J., Li, L., Li, G., Li, W., Baguley, B. C. & Austin, C.A. (1999). Murine Transgenic Cells Lacking DNA Topoisomerase IIβ Are Resistant to Acridines and Mitoxantrone : Analysis of Cytotoxicity and Cleavable Complex Formation. *Molecular Pharmacology* **56,** 1309-1316.

Escargueil, A. E., Plisov, S. Y., Filhol, O., Cochet, C. & Larsen, A. K. (2000). Mitotic Phosphorylation of DNA Topoisomerase II α by Protein Kinase CK2 Creates the MPM-2 Phosphoepitope on Ser-1469. *The Journal of Biological Chemistry* **275,** 34710-34718.

Esteller, M. & Herman, J. G. (2002). Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *Journal of Pathology* **196,** 1-7.

Estey, E., Adlakha, R. C., Hittelman, W. N. & Zwelling, L. A. (1987). Cell Cycle Stage Dependent Variations in Drug-Induced Topoisomerase II Mediated DNA Cleavage and Cytotoxicity. *Biochemistry* **26,** 4338-4344.

Evers, R., Zaman, G. J. R., van Deemter, L., Jansen, H., Calafat, J., Oomen, L. C. J. M., Oude Elferink, R. P. J., Borst, P. & Schinkel, A. H. (1996). Basolateral Localization and Export Activity of the Human Multidrug Resistance-associated Protein in Polarized Pig Kidney Cells. *Journal of Clinical Investigation* **97,** 1211-1218.

Falck, J., Jensen, P. B. & Sehested, M. (1999). Evidence for Repressional Role of a Inverted CCAAT Box in Cell Cycle-dependent Transcription of the Human DNA Topoisomerase IIα Gene. *The Journal of Biological Chemistry* **274,** 18753-18758.

Faneyte, I. F., Kristel, P. M. P. & van de Vijver, M. J. (2001). Determining *MDR1*/P-Glycoprotein Expression in Breast Cancer. *International Journal of Cancer* **93,** 114-122.

Galvagni, F., Capo, S. & Oliviero, S. (2001). Sp1 and Sp3 Physically Interact and Co-operate with GABP for the Activation of the Utrophin Promoter. *Journal of Molecular Biology* **306,** 985-996.

Gao, H., Huang, K.-C., Yamasaki, E. F., Chan, K. K., Chohan, L. & Snapka, R. M. (1999). XK469, a selective topoisomerase IIβ poison. *Proceedings of the National Academy of Sciences USA* **96,** 12168-12173.

Gashler, A. & Sukhatme, V. P. (1995). Early Growth Response Protein 1 (Egr-1): Prototype of a Zinc-finger Family of Transcription Factors. *Progress in Nucleic Acid Research and Molecular Biology* **50,** 191-224.

Giannakakou, P., Sackett, D. L., Kang, Y.-K., Zhan, Z., Buters, J. T. M., Fojo, T. & Poruchynsky, M. S. (1997). Paclitaxel-resistant Human Ovarian Cancer Cells Have Mutant β-Tubulins That Exhibit Impaired Paclitaxel-driven Polymerization. *The Journal of Biological Chemistry* **272,** 17118-17125.

Giocanti, N., Hennequin, C., Balosso, J., Mahler, M. & Favaudon, V. (1993). DNA Repair and Cell Cycle Interactions in Radiation Sensitization by the Topoisomerase II Poison Etoposide. *Cancer Research* **53,** 2105-2111.

Glazer, R. I. & Rohlff, C. (1994). Transcriptional regulation of multidrug resistance in breast cancer. *Breast Cancer Research and Treatment* **31,** 263-271.

Gnjatic, S., Cai, Z., Viguier, M., Chouaib, S., Guillet, J.-G. & Choppin, J. (1998). Accumulation of the p53 Protein Allows Recognition by Human CTL of a Wild-Type p53 Epitope Presented by Breast Carcinomas and Melanomas. *The Journal of Immunology* **160,** 328-333.

Goldenberg, G. J., Wang, H. & Blair, G. W. (1986). Resistance to Adriamycin: Relationship of Cytotoxicity to Drug Uptake and DNA Single- and Double-Strand Breakage in Cloned Cell Lines of Adriamycin-sensitive and -resistant P388 Leukemia. *Cancer Research* **46,** 2978-2983.

Goswami, P. C., Roti Roti, J. L. & Hunt, C. R. (1996). The Cell Cycle-Coupled Expression of Topoisomerase IIα during S Phase is Regulated by mRNA Stability and Is Disrupted by Heat Shock or Ionizing Radiation. *Molecular and Cellular Biology* **16,** 1500-1508.

Goswami, P. C., Sheren, J., Albee, L. D., Parsian, A., Sim, J. E., Ridnour, L. A., Higashikubo, R., Gius, D., Hunt, C. R. & Spitz, D. R. (2000). Cell Cycle-coupled Variation in Topoisomerase IIα mRNA Is Regulated by the 3'-Untranslated Region. *The Journal of Biological Chemistry* **275,** 38384-38392.

Gould, H. (1998). *Chromatin - A Practical Approach*. Oxford University Press, New York.

Gould, S. J. & Subramani, S. (1988). Firefly Luciferase as a Tool in Molecular and Cell Biology. *Analytical Biochemistry* **175,** 5-13.

Grabowski, D. R., Holmes, K. A., Aoyama, M., Ye, Y., Rybicki, L. A., Bukowski, R. M., Ganapathi, M. K., Hickson, I. D. & Ganapathi, R. (1999). Altered Drug Interaction and Regulation of Topoisomerase II β : Potential Mechanisms Governing Sensitivity of HL-60 Cells to Amsacrine and Etoposide. *Molecular Pharmacology* **56,** 1340-1345.

Grant, C. E., Hipfner, D. R., Valdimarrson, G., Almquist, K. C., Cole, S. P. C. & Deeley, G. (1994). Overexpression of Multidrug Resistance-associated protein (MRP) Increases Resistance to Natural Product Drugs. *Cancer Research* **54,** 357-361.

Greene, R. F., Collins, J. M., Jenkins, J. F., Speyer, J. L. & Myers, C. E. (1983). Plasma Pharmacokinetics of Adriamycin and Adriamycinol : Implications for the Design of *in Vitro* Experiments and Treatment Protocols. *Cancer Research* **43,** 3417-3421.

Gu, Z., Kuntz-Simon, G., Rommelaere, J. & Cornelis, J. (1999). Oncogenic Transformation-Dependent Expression of a Transcription Factor NF-Y Subunit. *Molecular Carcinogenesis* **24,** 294-299.

Gutmann, H., Fricker, G., Torok, M., Michael, S., Beglinger, C. & Drewe, J. (1999). Evidence for Different ABC-Transporters in Caco-2 Cells Modulating Drug Uptake. *Pharmaceutical Research* **16,** 402-407.

Haber, M., Burkhart, C. A., Regl, D. L., Madafiglio, J., Norris, M. D. & Band Horwitz, S. (1995). Altered Expression of Mβ2, the Class II β-Tubulin Isotype, in a Murine J774.2 Cell Line with a High Level of Taxol Resistance. *The Journal of Biological Chemistry* **270,** 31269-31275.

Hai, T. & Hartman, M. G. (2001). The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors : activating transcription factors proteins and homeostasis. *Gene* **273,** 1-11.

Hamilton, K. O., Backstrom, G., Yazdanian, M. A. & Audus, K. L. (2001). P-Glycoprotein Efflux Pump Expression and Activity in Calu-3 Cells. *Journal of Pharmaceutical Sciences* **90,** 647-658.

Han, E. K.-H., Gehrke, L., Tahir, S. K., Credo, R. B., Cherian, S. P., Sham, H., Rosenberg, S. H. & Ng, S.-C. (2000). Modulation of drug resistance by α-tubulin in paclitaxel-resistant human lung cancer cell lines. *European Journal of Cancer* **36,** 1565-1571.

Hansen, R. K., Parra, I., Lemieux, P., Oesterreich, S., Hilsenbeck, S. G. & Fuqua, S. A. (1999). Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. *Breast Cancer Research and Treatment* **56,** 187-196.

Harker, W. G., Slade, D. L., Parr, R. L., Feldhoff, P. W., Sullivan, D. M. & Holguin, M. (1995a). Alterations in the Topoisomerase IIα Gene, Messenger RNA, and Subcellular Protein Distribution as well as Reduced Expression of the DNA Topoisomerase IIβ Enzyme in a Mitoxantrone-resistant HL-60 Human Leukemia Cell Line. *Cancer Research* **55,** 1707-1716.

Harker, W. G., Slade, D. L., Parr, R. L. & Holgun, M. H. (1995b). Selective Use of an Alternative Stop Codon and Polyadenylation Signal within Intron Sequences Leads to a Truncated Topoisomerase II α Messenger RNA and Protein in Human HL-60 Leukemia Cells Selected for Resistance to Mitoxantrone. *Cancer Research* **55,** 4962-4971.

Harris, L. N., Yang, L., Liotcheva, V., Pauli, S., Iglehart, J. D., Colvin, O. M. & Hsieh, T. (2001). Induction of Topoisomerase II Activity after ErbB2 Activation Is Associated with a Differential Response to Breast Cancer Chemotherapy. *Clinical Cancer Research* **7,** 1497-1504.

Harris, L. N., Yang, L., Tang, C., Yang, D. & Lupu, R. (1998). Induction of Sensitivity to Doxorubicin and Etoposide by Transfection of MCF-7 Breast Cancer Cells with Heregulin β-2. *Clinical Cancer Research* **4,** 1005-1012.

Hashimoto, S., Chatterjee, S., Ranjit, G. B., Bao, C., Ford, J., Ganapathi, R., Berger, S. J. & Berger, N. A. (1995). Drastic Reduction of Topoisomerase IIα Associated with Major Acquired Resistance to Topoisomerase II Active Agents but Minor Perturbations of Cell Growth. *Oncology Research* **7,** 407-416.

Hazelhurst, L. A., Foley, N. F., Gleason-Guzman, M. C., Hacker, M. P., Cress, A. F., Greenberger, L. W., de Jong, M. C. & Dalton, W. S. (1999). Multiple Mechanisms Confer Drug Resistance to Mitoxantrone in the Human 8226 Myeloma Cell Line. *Cancer Research* **59,** 1021-1028.

He, M., Rennie, P. S., Dragowska, V., Nelson, C. C. & Jia, W. (2002). A mutant P53 can activate apoptosis through a mechanism distinct from those induced by wild type P53. *FEBS Letters* **517,** 151-154.

Heck, M. M. S., Hittelman, W. N. & Earnshaw, W. C. (1988). Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proceedings of the National Academy of Sciences USA* **85,** 1086-1090.

Heenan, M., O'Driscoll, L., Cleary, I., Conolly, L. & Clynes, M. (1997). Isolation From a Human MDR Lung Cell Line of Multiple Clonal Subpopulations which Exhibit Significantly Different Drug Resistance. *International Journal of Cancer* **71,** 907-915.

Herbomel, P., Bourachot, B. & Yaniv, M. (1984). Two Distinct Enhancers with Different Cell Specificities Coexist in the Regulatory Region of Polyoma. *Cell* **39,** 653-662.

Herr, I., Wilhelm, D., Böhler, T., Angel, P. & Debatin, K.-M. (1997). Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *The EMBO Journal* **16,** 6200-6208.

Herr, I., Wilhelm, D., Böhler, T., Angel, P. & Debatin, K.-M. (1999). JNK/SAPK Activity Is Not Sufficient For Anticancer Therapy-Induced Apoptosis Involving CD95-L, TRAIL and TNF-α. *International Journal of Cancer* **80,** 417-424.

Herzog, C. E., Holmes, K. A., Tuschong, L. M., Ganapathi, R. & Zwelling, L. A. (1998). Absence of Topoisomerase IIβ in an Amsacrine-resistant Human Leukemia Cell Line with Mutant Topoisomerase IIα. *Cancer Research* **58,** 5298-5300.

Herzog, C. E. & Zwelling, L. A. (1997). Evaluation of a Potential Regulatory Role for Inverted CCAAT Boxes in the Human Topoisomerase IIα Promoter. *Biochemical and Biophysical Research Communications* **232,** 608-612.

Hill, K. A. & Sommer, S. S. (2002). p53 as a Mutagen Test in Breast Cancer. *Environmental and Molecular Mutagenesis* **39,** 216-227.

Hochhauser, D., Stanway, C. A., Harris, A. L. & Hickson, I. D. (1992). Cloning and Characterization of the 5'-Flanking region of the Human Topoisomerase IIα Gene. *The Journal of Biological Chemistry* **267,** 18961-18965.

Hochhauser, D., Valkov, N. I., Gump, J. A., Wei, I., O'Hare, C., Hartley, J., Fan, J., Bertino, J. R., Banerjee, D. & Sullivan, D. M. (1999). Effects of Wild-Type p53 Expression on the Quantity and Activity of Topoisomerase IIα and β in Various Human Cancer Cell Lines. *Journal of Cellular Biochemistry* **75,** 245-257.

Holmes, D. S. & Quigley, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114,** 193-197.

Holzmayer, T. A., Hilsenbeck, S., Von Hoff, D. D. & Roninson, I. B. (1992). Clinical Correlates of MDR1 (P-glycoprotein) Gene Expression in Ovarian and Small-Cell Lung Carcinomas. *Journal of the National Cancer Institute* **84,** 1486-1491.

Hooijberg, J. H., Broxterman, H. J., Kool, M., Assaraf, Y. G., Peters, G. J., Noordhuis, P., Scheper, R. J., Borst, P., Pinedo, H. M. & Jansen, G. (1999). Antifolate Resistance Mediated by the Multidrug Resistance Proteins MRP1 and MRP2. *Cancer Research* **59,** 2532-2535.

Hopfner, R., Mousli, M., Jeltsch, J., Voulgaris, A., Lutz, Y., Marin, C., Bellocq, J., Oudet, P. & Bronner, C. (2000). ICBP90, a Novel Human CCAAT Binding Protein, Involved in the Regulation of Topoisomerase IIα Expression. *Cancer Research* **60,** 121-128.

Hortobagyi, G. N. (1997). Anthracyclines in the Treatment of Cancer. *Drugs* **54,** 1-7.

Houlbrook, S., Harris, A. L., Carmichael, J. & Stratford, I. J. (1996). Relationship between Topoisomerase II Levels and Resistance to Topoisomerase II Inhibitors in Lung Cancer Cell Lines. *Anticancer Research* **16,** 1603-1610.

Hsiang, Y., Wu, H. & Liu, L. F. (1988). Proliferation-dependent Regulation of DNA Topoisomerase II in Cultured Human Cells. *Cancer Research* **48,** 3230-3235.

Huang, R.-P., Fan, Y., Ni, Z., Mercola, D. & Adamson, E. D. (1997). Reciprocal Modulation Between Sp1 and Egr-1. *Journal of Cellular Biochemistry* **66,** 489-499.

Huber, S. A. (1992). Heat-Shock Protein Induction in Adriamycin and Picornavirus-Infected Cardiocytes. *Laboratory Investigation* **67,** 218-224.

Hutchinson, J. N. & Muller, W. J. (2000). Transgenic mouse models of human breast cancer. *Oncogene* **19,** 6130-6137.

Inoue, T., Kamiyama, J. & Sakai, T. (1999). Sp1 and NF-Y Synergistically Mediate the Effect of Vitamin D_3 in the $p27^{Kip1}$ Gene Promoter That Lacks Vitamin D Response Elements. *The Journal of Biological Chemistry* **274,** 32309-32317.

Inoue, H., Nojima, H. & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96,** 23-28.

Isaacs, R. J., Davies, S. L., Sandri, M. I., Redwood, C., Wells, R. J. & Hickson, I. D. (1998). Physiological regulation of eukaryotic topoisomerase II. *Biochimica et Biophysica Acta* **1400,** 121-137.

Isaacs, R. J., Davies, S. L., Wells, N. J. & Harris, A. L. (1995). Topoisomerases II α and β as therapy targets in breast cancer. *Anti-Cancer Drugs* **6,** 195-211.

Isaacs, R. J., Harris, A. L. & Hickson, I. D. (1996). Regulation of the Human Topoisomerase IIα Gene Promoter in Confluence-arrested Cells. *The Journal of Biological Chemistry* **271,** 16741-16747.

Ishida, R., Sato, M., Narita, T., Utsumi, K. R., Nishimoto, T., Morita, T., Nagata, H. & Andoh, T. (1994). Inhibition of DNA Topoisomerase II by ICRF-193 Induces Polyploidization by Uncoupling Chromosome Dynamics from Other Cell Cycle Events. *The Journal of Cell Biology* **126,** 1341-1351.

Ishimaru, F., Mari, B. & Shipp, M. A. (1997). The Type 2 CD10/Neutral Endopeptidase Promoter: Functional Characterization and Tissue-Specific Regulation by CBF/NF-Y Isoforms. *Blood* **89,** 4136-4145.

Jackson, S. P., MacDonald, J. J., Lees-Miller, S. & Tjian, R. (1990). GC Box Binding Induces Phosphorylation of Sp1 by a DNA-Dependent Protein Kinase. *Cell* **63,** 155-165.

Jackson, S. P. & Tjian, R. (1988). O-Glycosylation of Eukaryotic Transcription Factors: Implications for Mechanisms of Transcriptional Regulation. *Cell* **55,** 125-133.

Jacob, S., Aguado, M., Fallik, D. & Praz, F. (2001). The Role of the DNA Mismatch Repair System in the Cytotoxicity of the Topoisomerase Inhibitors Camptothecin and Etoposide to Human Colorectal Cancer Cells. *Cancer Research* **61,** 6555-6562.

Jaffrezou, J., Chen, G., Duran, G. E., Kuhl, J. & Sikic, B. I. (1994). Mutation Rates and Mechanisms of Resistance to Etoposide Determined From Fluctuation Analysis. *Journal of the National Cancer Institute* **86,** 1152-1158.

Järvinen, T. A. H. & Liu, E. T. (2000). Effects of *HER-2/neu* on chemosensitivity of tumor cells. *Drug Resistance Updates* **3,** 319-324.

Järvinen, T. A. H., Tanner, M., Barlund, M., Borg, A. & Isola, J. (1999). Characterization of Topoisomerase IIα Gene Amplification and Deletion in Breast Cancer. *Genes, Chromosomes and Cancer* **26,** 142-150.

Järvinen, T. A. H., Tanner, M., Rantanen, V., Barlund, M., Borg, A., Grenman, S. & Isola, J. (2000). Amplification and Deletion of Topoisomerase IIα Associate with ErbB-2 Amplification and Affect Sensitivity to Topoisomerase II Inhibitor Doxorubicin in Breast Cancer. *American Journal of Pathology* **156,** 839-847.

Jeannesson, P., Lahlil, R., Chenais, B., Devy, L., Gillet, R., Aries, A., Morceau, F. & Trentesaux, C. (1997). Anthracyclines as Tumor Cell Differentiating Agents: Effects on the Regulation of Erythroid Gene Expression. *Leukemia and Lymphoma* **26,** 575-587.

Jedlitschky, G., Leier, I., Buchholz, U., Barnouin, K., Kurz, G. & Keppler, D. (1996). Transport of Glutathione, Glucuronate and Sulfate Conjugates by the *MRP* Gene-encoded Conjugate Export Pump. *Cancer Research* **56,** 988-994.

Jenkins, J. R., Ayton, P., Jones, T., Davies, S. L., Simmons, D. L., Harris, A. L., Sheer, D. & Hickman, I. D. (1992). Isolation of cDNA clones encoding the β isozyme of human DNA topoisomerase II and localization of the gene to chromosome 3p24. *Nucleic Acid Research* **20,** 5587-5592.

Jin, S. & Scotto, K. W. (1998). Transcriptional Regulation of the *MDR1* Gene by Histone Acetyltransferase and Deacetylase Is Mediated by NF-Y. *Molecular and Cellular Biology* **18,** 4377-4384.

Jongsma, A. P. M., Riethorst, A., Lankelma, J., Dekker, H. & Westerhoff, H. V. (2000). Evaluating limited specificity of drug pumps. *European Journal of Biochemistry* **267,** 5369-5377.

Joshi, A. A., Wang, Q. & Suttle, D. P. (2000). p53 Modulation of DNA Topoisomerase IIα Gene Expression. *Cell Biochemistry and Biophysics* **33,** 209-216.

Kaptain, S., Tan, L. K. & Chen, B. (2001). Her-2/*neu* and Breast Cancer. *Diagnostic Molecular Pathology* **10,** 139-152.

Kaufmann, S. H., Gore, S. D., Miller, C. B., Jones, R. J., Zwelling, L. A., Schneider, E., Burke, P. J. & Karp, J. E. (1998). Topoisomerase II and the Response to Antileukemic Therapy. *Leukemia and Lymphoma* **29,** 217-237.

Kaufmann, S. H., McLaughlin, S. J., Kastan, M. B., Liu, L. F., Karp, J. E. & Burke, P. J. (1991). Topoisomerase II Levels during Granulocytic Maturation *in Vitro* and *in Vivo*. *Cancer Research* **51,** 3534-3543.

Kavallaris, M., Kuo, D. Y.-S., Burkhart, C. A., Regi, D. L., Norris, M. D., Haber, M. & Band Horwitz, S. (1997). Taxol-resistant Epithelial Ovarian Tumors Are Associated with Altered Expression of Specific β-Tubulin Isotypes. *Journal of Clinical Investigation* **100,** 1282-1293.

Keates, R. A. B., Sarangi, F. & Ling, V. (1981). Structural and functional alterations in microtubule protein from Chinese hamster ovary cell mutants. *Proceedings of the National Academy of Sciences USA* **78,** 5638-5642.

Kellner, U., Hutchinson, L., Seidel, A., Lage, H., Danks, M. K., Dietel, M. & Kaufmann, S. H. (1997). Decreased Drug Accumulation in a Mitoxantrone-Resistant Gastric Carcinoma Cell Line in the Absence of P-Glycoprotein. *International Journal of Cancer* **71,** 817-824.

Kennett, S. B., Moorefield, K. S. & Horowitz, J. M. (2002). Sp3 Represses Gene Expression via the Titration of Promoter-specific Transcription Factors. *The Journal of Biological Chemistry* **277,** 9780-9789.

Kennett, S. B., Udvadia, A. J. & Horowitz, J. M. (1997). Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acid Research* **25,** 3110-3117.

Kerr, D. J. (1996). Mechanisms of cytotoxic drug resistance in breast cancer. *The Breast* **5,** 293-297.

Khelifa, T., Rene, B., Le Mee, S., Lambert, B., Saucier, J., Markovits, J., Jacquemin-Sablon, H. & Jacquemin-Sablon, A. (1999). Transfection of 9-Hydroxyellipticine-resistant Chinese Hamster Fibroblasts with Human Topoisomerase IIα cDNA : Selective Restoration of the Sensitivity to DNA Religation Inhibitors. *Cancer Research* **59,** 4927-4936.

Kim, W., Kakehi, Y., Kinoshita, H., Arao, S., Fukumoto, M. & Yoshida, O. (1996). Expression Patterns of Multidrug-resistance (MDR1), Multidrug Resistance-associated Protein (MRP), Glutathione-S-Transterase-π (GST- π) and DNA Topoisomerase II (Topo II) Genes in Renal Cell Carcinomas and Normal Kidney. *Journal of Urology* **156,** 506-511.

Kimura, K., Nozaki, N., Enomoto, T., Tanaka, M. & Kikuchi, A. (1996). Analysis of M Phase-specific Phosphorylation of DNA Topoisomerase II. *The Journal of Biological Chemistry* **271,** 21439-21445.

Kimura, K., Saijo, M., Ui, M. & Enomoto, T. (1994). Growth State- and Cell Cycle-dependent Fluctuation in the Expression of Two Forms of DNA Topoisomerase II and Possible Specific Modification of the Higher Molecular Weight Form in the M Phase. *The Journal of Biological Chemistry* **269,** 1173-1176.

Koike, K., Uchiumi, T., Ohga, T., Toh, S., Wada, M., Kohno, K. & Kuwano, M. (1997). Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Letters* **417,** 390-394.

Korner, K. & Muller, R. (2000). *In Vivo* Structure of the Cell Cycle-regulated Human *cdc25C* Promoter. *The Journal of Biological Chemistry* **275,** 18676-18681.

Koutsodontis, G., Tentes, I., Papakosta, P., Moustakas, A. & Kardassis, D. (2001). Sp1 Plays a Critical Role in the Transcriptional Activation of the Human Cyclin-dependent Kinase Inhibitor p21 *WAF1/Cip1* Gene by the p53 Tumor Suppressor Protein. *The Journal of Biological Chemistry* **276,** 29116-29125.

Krishan, A. & Frei, E. I. (1976). Effect of Adriamycin on the Cell Cycle Traverse and Kinetics of Cultured Human Lymphoblasts. *Cancer Research* **36,** 143-150.

Krishna, R. & Mayer, L. D. (2000). Multidrug resistance (MDR) in cancer - Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *European Journal of Pharmaceutical Sciences* **11,** 265-283.

Kroll, D. J. & Rowe, T. C. (1991). Phosphorylation of DNA Topoisomerase II in a Human Tumor Cell Line. *The Journal of Biological Chemistry* **266,** 7957-7961.

Kruh, G. D., Chan, A., Myers, K., Gaughan, K., Miki, T. & Aaronson, S. A. (1994). Expression Complementary DNA Library Transfer Establishes *mrp* as a Multidrug Resistance Gene. *Cancer Research* **54,** 1649-1652.

Kubo, T., Kohno, K., Ohga, T., Taniguchi, K., Kawanami, K., Wada, M. & Kuwano, M. (1995). DNA Topoisomerase IIα Gene Expression under Transcriptional Control in Etoposide/Teniposide-resistant Human Cancer Cells. *Cancer Research* **55,** 3860-3864.

Kubo, A., Yoshikawa, A., Hirashima, T., Masuda, N., Takada, M., Takahara, J., Fukuoka, M. & Nakagawa, K. (1996). Point Mutations of the Topoisomerase IIα Gene in Patients with Small Cell Lung Cancer Treated with Etoposide. *Cancer Research* **56,** 1232-1236.

Kunisada, K., Negoro, S., Tone, E., Funamoto, M., Osugi, T., Yamada, S., Okabe, M., Kishimoto, T. & Yamauchi-Takihara, K. (2000). Signal transducer and activator

of transcription 3 in the heart transduces not only a hypertrophic signal but a protective signal against doxorubicin-induced cardiomyopathy. *Proceedings of the National Academy of Sciences USA* **97,** 315-319.

Kuo, M. T., Liu, Z., Wei, Y., Lin-Lee, Y.-C., Tatebe, S., Mills, G. B. & Unate, H. (2002). Induction of human *MDR1* gene expression by 2-acetylaminofluorene is mediated by effectors of the phosphoinositide 3-kinase pathway that activate NF-κB signaling. *Oncogene* **21,** 1945-1954.

Kurabayashi, M., Dutta, S., Jeyaseelan, R. & Kedes, L. (1995). Doxorubicin-Induced Id2A Gene Transcription Is Targeted at an Activating Transcription Factor/Cyclic AMP Response Element Motif through Novel Mechanisms Involving Protein Kinases Distinct from Protein Kinase C and Protein Kinase A. *Molecular and Cellular Biology* **15,** 6386-6397.

Kurabayashi, M., Jeyaseelan, R. & Kedes, L. (1993). Antineoplastic Agent Doxorubicin Inhibits Myogenic Differentiation of C2 Myoblasts. *The Journal of Biological Chemistry* **268,** 5524-5529.

Kusaba, H., Nakayama, M., Harada, T., Nomoto, M., Kohno, K., Kuwano, M. & Wada, M. (1999). Association of 5' CpG demethylation and altered chromatin structure in the promoter region with transcriptional activation of the multidrug resistance 1 gene in human cancer cells. *European Journal of Biochemistry* **262,** 924-932.

Kusumoto, H., Rodgers, Q. E., Boege, F., Raimondi, S. C. & Beck, W. T. (1996). Characterization of Novel Human Leukemic Cell Lines Selected for Resistance to Merbarone, a Catalytic Inhibitor of DNA Topoisomerase II. *Cancer Research* **56,** 2573-2583.

Kyriakis, J. M. & Avruch, J. (2001). Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation. *Physiological Reviews* **81,** 807-869.

Ladias, J. A. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V. & Cladaras, C. (1992). Transcriptional Regulation of Human Apolipoprotein Genes ApoB, ApoCIII, and ApoAII by Members of the Steroid Hormone Receptor Superfamily HNF-4, ARP-1, EAR-2, and EAR-3. *The Journal of Biological Chemistry* **267,** 15849-15860.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227,** 680-685.

Lage, H., Helmbach, H., Dietel, M. & Schadendorf, D. (2000). Modulation of DNA topoisomerase II activity and expression in melanoma cells with acquired drug resistance. *British Journal of Cancer* **82,** 488-491.

Lang, A. J., Mirski, S. E. L., Cummings, H. J., Yu, Q., Gerlach, J. H. & Cole, S. P. C. (1998). Structural Organization of the Human *TOP2A* and *TOP2B* genes. *Gene* **221,** 255-266.

Lania, L., Majello, B. & De Luca, P. (1997). Transcriptional Regulation by the Sp Family Proteins. *International Journal of Biochemistry and Cell Biology* **29,** 1313-1323.

Larsen, A. K. & Skladanowski, A. (1998). Cellular resistance to topoisomerase-targeted drugs : from drug uptake to cell death. *Biochimica et Biophysica Acta* **1400,** 257-274.

Latchman, D. (1995). *Gene Regulation*. Chapman & Hall, London.

Le Mee, S., Chaminade, F., Delaporte, C., Markovits, J., Saucier, J.-M. & Jacquemin-Sablon, A. (2000). Cellular Resistance to the Antitumor DNA Topoisomerase II Inhibitor S16020-2: Importance of the *N*-[2(Dimethylamino)ethyl]carbamoyl Side Chain. *Molecular Pharmacology* **58,** 709-718.

Lee, J. S., Scala, S., Matsumoto, Y., Dickstein, B., Robey, R., Zhan, Z., Altenberg, G. & Bates, S. E. (1997). Reduced Drug Accumulation and Multidrug Resistance in Human Breast Cancer Cells Without Associated P-Glycoprotein or MRP Overexpression. *Journal of Cellular Biochemistry* **65,** 513-526.

Leggett, R. W., Armstrong, S. A., Barry, D. & Mueller, C. R. (1995). Sp1 Is Phosphorylated and Its DNA Binding Activity Down-regulated Upon Terminal Differentiation of the Liver. *The Journal of Biological Chemistry* **270,** 25879-25884.

Lehmann, U., Glockner, S., Kleeberger, W., Feist, H., von Wasielewski, R. & Kreipe, H. (2000). Detection of Gene Amplification in Archival Breast Cancer Specimens by Laser-Assisted Microdissection and Quantitative Real-Time Polymerase Chain Reaction. *American Journal of Pathology* **156,** 1855-1864.

Leier, I., Jedlitschky, G., Buchholz, U., Cole, S. P. C., Deeley, R. G. & Keppler, D. (1994). The *MRP* Gene Encodes an ATP-dependent Export Pump for Leukotriene C_4 and Structurally Related Conjugates. *The Journal of Biological Chemistry* **269,** 27807-27810.

Li, Q., Herrler, M., Landsberger, N., Kaludov, N., Ogryzko, V. V., Nakatani, Y. & Wolffe, A. P. (1998). *Xenopus* NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the *Xenopus* hsp70 promoter *in vivo*. *The EMBO Journal* **17,** 6300-6315.

Li, X., Hooft van Huisjduijnen, R., Mantovani, R., Benoist, C. & Mathis, D. (1992). Intron-Exon Organization of the NF-Y Genes. *The Journal of Biological Chemistry* **267,** 8984-8990.

Liang, F., Schaufele, F. & Gardner, D. G. (2001). Functional Interaction of NF-Y and Sp1 Is Required for Type A Natriuretic Peptide Receptor Gene Transcription. *The Journal of Biological Chemistry* **276,** 1516-1522.

Liberati, C., Ronchi, A., Lievens, P., Ottolenghi, S. & Mantovani, R. (1998). NF-Y Organizes the γ-Globin CCAAT Boxes Region. *The Journal of Biological Chemistry* **273,** 16880-16889.

Lim, K., Lee, J., Yun, K., Son, M., Park, J., Yoon, W. & Hwang, B. (1998). Reduced Level of ATF is Correlated with Transcriptional Repression of DNA Topoisomerase II α Gene During TPA-induced Differentiation of HL-60 Cells. *Biochemistry and Molecular Biology International* **46,** 35-42.

Lin, Z. P., Boller, Y. C., Amer, S. M., Russell, R. L., Pacelli, K. A., Patierno, S. R. & Kennedy, K. A. (1998). Prevention of Brefeldin A-induced Resistance to Teniposide by the Proteasome Inhibitor MG-132 : Involvement of NF-κB Activation in Drug Resistance. *Cancer Research* **58,** 3059-3065.

Liu, A., Prenger, M. S., Norton, D. D., Mei, L., Kusiak, J. W. & Bai, G. (2001). Nerve Growth Factor Uses Ras/ERK and Phosphatidylinositol 3-Kinase Cascades to Up-regulate the *N*-Methyl-D-aspartate Receptor 1 Promoter. *The Journal of Biological Chemistry* **276,** 45372-45379.

Locke, V. L., Davey, R. A. & Davey, M. W. (1999). Altered drug sensitivity in response to idarubicin treatment in K562 human leukaemia cells. *British Journal of Haematology* **106,** 86-91.

Loe, D. W., Deely, R. G. & Cole, S. P. C. (1998). Characterization of Vincristine Transport by the M_r 190,000 Multidrug Resistance Protein (MRP) : Evidence for Cotransport with Reduced Glutathione. *Cancer Research* **58,** 5130-5136.

Loflin, P. T., Altschuler, E., Hochhauser, D., Hickson, I. D. & Zwelling, L. A. (1996). Phorbol Ester-Induced Down-Regulation of Topoisomerase IIα mRNA in a Human Erythroleukemia Cell Line. *Biochemical Pharmacology* **52,** 1065-1072.

Lohri, A., Reuter, J., Gudat, F. & Herrman, R. (1997). Topoisomerase IIα mRNA and Tumour Cell Proliferation in non-Hodgkin's Lymphoma. *Journal of Clinical Pathology* **50,** 22-26.

Lotfi, K., Månsson, E., Chandra, J., Wang, Y., Xu, D., Knaust, E., Spasokoukotskaja, T., Liliemark, E., Eriksson, S. & Albertioni, F. (2001). Pharmacological basis for cladribine resistance in a human acute T lymphoblastic leukemia cell line selected for resistance to etoposide. *British Journal of Haematology* **113,** 339-346.

Lupu, R., Cardillo, M., Cho, C., Harris, L., Hijazi, M., Perez, C., Rosenberg, K., Yang, D. & Tang, C. (1996). The significance of *heregulin* in breast cancer tumor progression and drug resistance. *Breast Cancer Research and Treatment* **38,** 57-66.

Ma, W., Lim, W., Gee, K., Aucoin, S., Nandan, D., Kozlowski, M., Diaz-Mitoma, F. & Kumar, A. (2001). The p38 Mitogen-activated Kinase Pathway Regulates the Human Interleukin-10 Promoter via the Activation of Sp1 Transcription Factor in Lipopolysaccharide-stimulated Human Macrophages. *The Journal of Biological Chemistry* **276,** 13664-13674.

Maestre, N., Tritton, T. R., Laurent, G. & Jaffrézou, J.-P. (2001). Cell Surface-directed Interaction of Anthracyclines Leads to Cytotoxicity and Nuclear Factor κB Activation but not Apoptosis Signaling. *Cancer Research* **61,** 2558-2561.

Magan, N. (2002). "Basal Transcription of Human Topoisomerase II". MSc thesis. Massey University, Palmerston North, New Zealand.

Maity, S. N. & de Crombrugghe, B. (1998). Role of the CCAAT-binding protein CBF/NF-Y in transcription. *Trends in Biological Sciences* **23,** 174-178.

Mantovani, R. (1999). The molecular biology of the CCAAT-binding factor NF-Y. *Gene* **239,** 15-27.

Marchetti, F., Bishop, J. B., Lowe, X., Generoso, W. M., Hozier, J. & Wyrobek, A. J. (2001). Etoposide induces heritable chromosomal aberrations and aneuploidy during male meiosis in the mouse. *Proceedings of the National Academy of Sciences USA* **98,** 3952-3957.

Marie, J.-P., Zittoun, R. & Sikic, B. I. (1991). Multidrug Resistance (*mdr*1) Gene Expression in Adult Acute Leukemias: Correlations With Treatment Outcome and In Vitro Drug Sensitivity. *Blood* **78,** 586-592.

Marx, G., Zhou, H., Graves, D. E. & Osheroff, N. (1997). Covalent Attachment of Ethidium to DNA Results in Enhanced Topoisomerase II-Mediated DNA Cleavage. *Biochemistry* **36,** 15884-15891.

Marziali, G., Perrotti, E., Ilari, R., Coccia, E. M., Mantovani, R., Testa, U. & Battistini, A. (1999). The Activity of the CCAAT-box Binding Factor NF-Y Is Modulated Through the Regulated Expression of Its A Subunit During Monocyte to Macrophage Differentiation: Regulation of Tissue-Specific Genes Through a Ubiquitous Transcription Factor. *Blood* **93,** 519-526.

Mastrangelo, I. A., Courey, A. J., Wall, J. S., Jackson, S. P. & Hough, P. V. C. (1991). DNA looping and Sp1 multimer links: A mechanism for transcriptional synergism and enhancement. *Proceedings of the National Academy of Sciences USA* **88,** 5670-5674.

Matsumoto, Y., Kunishio, K. & Nagao, S. (1999). Increased phosphorylation of DNA topoisomerase II in etoposide resistant mutants of human glioma cell line. *Journal of Neuro-Oncology* **45,** 37-46.

Matsumoto, Y., Takano, H. & Fojo, T. (1997). Cellular Adaptation to Drug Exposure : Evolution of the Drug-resistant Phenotype. *Cancer Research* **57,** 5086-5092.

Matuoka, K. & Chen, K. Y. (1999). Nuclear Factor (NF-Y) and Cellular Senescence. *Experimental Cell Research* **253,** 365-371.

McClean, S. & Hill, B. T. (1992). An overview of membrane, cytosolic and nuclear proteins associated with the expression of resistance to multiple drugs in vitro. *Biochimica et Biophysica Acta* **1114,** 107-127.

McKinnell, R. G., Parchment, R. E., Peratoni, A. O. & Pierce, G. B. (1998). *The Biological Basis of Cancer*. Cambridge University Press, Cambridge, UK.

McPherson, J. P., Deffie, A. M., Jones, N. R., Brown, G. A., Deuchars, K. L. & Goldenberg, G. J. (1997). Selective Sensitization of Adriamycin-Resistant P388 Murine Leukemia Cells to Antineoplastic Agents Following Transfection with Human DNA Topoisomerase IIα. *Anticancer Research* **17,** 4243-4252.

McPherson, J. P. A. & Goldenberg, G. J. (1998). Induction of Apoptosis by Deregulated Expression of DNA Topoisomerase IIα. *Cancer Research* **58,** 4519-4524.

Mechetner, E., Kyshtoobayeva, A., Zonis, S., Kim, H., Stroup, R., Garcia, R., Parker, R.J. & Fruehauf, J. P. (1998). Levels of Multidrug Resistance (MDR1) P-Glycoprotein Expression by Human Breast Cancer Correlate with *in Vitro* Resistance to Taxol and Doxorubicin. *Clinical Cancer Research* **4,** 389-398.

Melixetian, M. B., Beryozkina, E. V., Pavlenko, M. A. & Grinchuk, T. M. (2000). Altered expression of DNA-topoisomerase IIα is associated with increased rate of spontaneous polyploidization in etoposide resistant K562 cells. *Leukemia Research* **24,** 831-837.

Melnikova, I. N. & Gardner, P. D. (2001). The Signal Transduction Pathway Underlying Ion Channel Gene Regulation by Sp1-c-Jun Interactions. *The Journal of Biological Chemistry* **276,** 19040-19045.

Meschini, S., Calcabrini, A., Monti, E., del Bufalo, D., Stringaro, A., Dolfini, E. & Arancia, G. (2000). Intracellular P-Glycoprotein Expression is Associated with the Intrinsic Multidrug Resistance Phenotype in Human Colon Adenocarcinoma Cells. *International Journal of Cancer* **87,** 615-628.

Minagawa, Y., Kigawa, J., Irie, T., Kanamori, Y., Itamochi, H., Cheng, K. & Terakawa, N. (1997). Enhanced Topoisomerase I Activity and Increased Topoisomerase II α Content in Cisplatin-resistant Cancer Cell Lines. *Japanese Journal of Cancer Research* **88,** 1218-1223.

Minotti, A. M., Barlow, S. B. & Cabral, F. (1991). Resistance to Antimitotic Drugs in Chinese Hamster Ovary Cells Correlates with Changes in the Level of Polymerized Tubulin. *The Journal of Biological Chemistry* **266,** 3987-3994.

Mirski, S. E. L., Sparks, K. E., Yu, Q., Lang, A. J., Jain, N., Campling, B. G. & Cole, S. P. C. (2000). A Truncated Cytoplasmic Topoisomerase IIα in a Drug-resistant Lung Cancer Cell Line is Encoded by a *TOP2A* Allele with a Partial Deletion of Exon 34. *International Journal of Cancer* **85,** 534-539.

Mo, Y. & Beck, W. T. (1997). Heterogeneous Expression of DNA Topoisomerase IIα Isoforms in Tumor Cell Lines. *Oncology Research* **9,** 193-204.

Mo, Y., Wang, Q. & Beck, W. T. (1997). Down-regulation of Topoisomerase IIα in CEM Cells Selected for Merbarone Resistance Is Associated with Reduced Expression of Sp3. *Cancer Research* **57,** 5004-5008.

Modrak, D. E., Draper, M. P. & Levy, S. B. (1997). Emergence of Different Mechanisms of Resistance in the Evolution of Multidrug Resistance in Murine Erythroleukemia Cell Lines. *Biochemical Pharmacology* **54,** 1297-1306.

Montgomery, R. B., Guzman, J., O'Rouke, D. M. & Stahl, W. L. (2000). Expression of oncogenic epidermal growth factor receptor family kinases induces paclitaxel resistance and alters beta-tubulin isotype expression. *The Journal of Biological Chemistry* **275,** 17358-17363.

Morceau, F., Chénais, B., Gillet, R., Jardillier, J.-C., Jeannesson, P. & Trentesaux, C. (1996). Transcriptional and Posttranscriptional Regulation of Erythroid Gene Expression in Anthracycline-induced Differentiation of Human Erythroleukemic Cells. *Cell Growth and Differentiation* **7,** 1023-1029.

Morgan, S. E. & Beck, W. T. (2001). Role of an Inverted CCAAT Element in Human Topoisomerase IIα Gene Expression in ICRF-187-Sensitive and -Resistant CEM Leukemic Cells. *Molecular Pharmacology* **59,** 203-211.

Morgan, S. E., Cadena, R. S., Raimondi, S. C. & Beck, W. T. (2000). Selection of Human Leukemic CEM Cells for Resistance to the DNA Topoisomerase II Catalytic Inhibitor ICRF-187 Results in Increased Levels of Topoisomerase IIα and Altered G_2/M Checkpoint and Apoptotic Responses. *Molecular Pharmacology* **57,** 296-307.

Mortensen, E. R., Marks, P. A., Shiotani, A. & Merchant, J. L. (1997). Epidermal Growth Factor and Okadaic Acid Stimulate Sp1 Proteolysis. *The Journal of Biological Chemistry* **272,** 16540-16547.

Motta, M. C., Caretti, G., Badaracco, G. F. & Mantovani, R. (1999). Interactions of the CCAAT-binding Trimer NF-Y with Nucleosomes. *The Journal of Biological Chemistry* **274,** 1326-1333.

Muller, C., Chatelut, E., Gualano, V., De Forni, M., Huguet, F., Attal, M., Canal, P. & Laurent, G. (1993). Cellular pharmacokinetics of doxorubicin in patients with chronic lymphocytic leukemia : comparison of bolus administration and continuous infusion. *Cancer Chemotherapy and Pharmacology* **32,** 379-384.

Murray, R. Z. & Norbury, C. (2000). Proteasome inhibitors as anti-cancer agents. *Anti-Cancer Drugs* **11,** 407-417.

Naito, S., Yokomizo, A. & Koga, H. (1999). Mechanisms of drug resistance in chemotherapy for urogenital carcinoma. *International Journal of Urology* **6,** 427-439.

Nakajima, T., Ohi, N., Arai, T., Nozaki, N., Kikuchi, A. & Oda, K. (1995). Adenovirus E1A-induced apoptosis elicits a steep decrease in the topoisomerase IIα level during the latent phase. *Oncogene* **10,** 651-662.

NicAmhlaoibh, R., Heenan, M., Cleary, I., Touhey, S., O'Loughlin, C., Daly, C., Nunez, G., Scanlon, K. J. & Clynes, M. (1999). Altered Expression of mRNAs for Apoptosis-Modulating Proteins in a Low Level Multidrug Resistant Variant of a Human Lung Carcinoma Cell Line that also Expresses *mdr1* mRNA. *International Journal of Cancer* **82,** 368-376.

Nielsen, D., Eriksen, J., Maare, C., Litman, T., Kjaersgaard, E., Plesner, T., Friche, E. & Skovsgaard, T. (2000a). Characterisation of Non-P-Glycoprotein Multidrug-Resistant Ehrlich Ascites Tumour Cells Selected for Resistance to Mitoxantrone. *Biochemical Pharmacology* **60,** 363-370.

Nielsen, D., Maare, C., Eriksen, J., Litman, T., Friche, E. & Skovsgaard, T. (2000b). Characterisation of Multidrug-Resistant Ehrlich Ascites Tumour Cells Selected *In Vivo* for Resistance to Etoposide. *Biochemical Pharmacology* **60,** 353-361.

Noé, V., Alemany, C., Nicolás, M. & Ciudad, C. J. (2001). Sp1 involvement in the 4β-phorbol 12-myristate 13-acetate (TPA)-mediated increase in resistance to methotrexate in Chinese hamster ovary cells. *European Journal of Biochemistry* **268,** 3163-3173.

Nose, K. & Ohba, M. (1996). Functional activation of the *egr*-1 (early growth response-1) gene by hydrogen peroxide. *Biochemical Journal* **316,** 381-383.

Nyce, J. W. (1997). Drug-induced DNA hypermethylation: A potential mediator of acquired drug resistance during cancer chemotherapy. *Mutation Research* **386,** 153-161.

O'Brian, C. A., Fan, D., Ward, N. E., Seid, C. & Fidler, I. J. (1989). Level of protein kinase C activity correlates directly with resistance to adriamycin in murine fibrosarcoma cells. *FEBS Letters* **246,** 78-82.

O'Connor, P. M., Jackman, J., Bae, I., Myers, T. G., Fan, S., Mutoh, M., Scudiero, D. A., Monks, A., Sausville, E. A., Weinstein, J. N., Friend, S., Fornace, A. J. J. & Kohn, K. W. (1997). Characterization of the *p53* Tumor Suppressor Pathway in Cell Lines of the National Cancer Institute Anticancer Drug Screen and Correlations with the Growth-Inhibitory Potency of 123 Anticancer Agents. *Cancer Research* **57,** 4285-4300.

Oesterreich, S., Weng, C., Qui, M., Hilsenbeck, S. G., Osborne, C. K. & Fuqua, S. A. W. (1993). The Small Heat Shock Protein hsp27 Is Correlated with Growth and Drug Resistance in Human Breast Cancer Cell Lines. *Cancer Research* **53,** 4443-4448.

Ornstein, L. (1964). Disc Electrophoresis I - Background and Theory. *Annals of the New York Academy of Sciences* **121,** 321-349.

Osborn, M. T. & Chambers, T. C. (1996). Role of the Stress-activated/c-Jun NH_2-terminal Protein Kinase Pathway in the Cellular Response to Adriamycin and Other Chemotherapeutic Drugs. *The Journal of Biological Chemistry* **271,** 30950-30955.

Owen, G. I., Richer, J. K., Tung, L., Takimoto, G. & Horwitz, K. B. (1998). Progesterone Regulates Transcription of the $p21^{WAF1}$ Cyclin-dependent Kinase Inhibitor Gene through Sp1 and CBP/p300. *The Journal of Biological Chemistry* **273,** 10696-10701.

Pal, S., Claffey, K. P., Cohen, H. T. & Mukhopadhyay, D. (1998). Activation of Sp1-mediated Vascular Permeability Factor/Vascular Endothelial Growth Factor Transcription Requires Specific Interaction with Protein Kinase Cζ. *The Journal of Biological Chemistry* **273,** 26277-26280.

Parnaik, V. K. (1999). New insights into the multiple functions of Sp1, a ubiquitous transcription factor. *Current Science* **76,** 166-172.

Paul, S., Breuninger, L. M., Tew, K. D., Shen, H. & Kruh, G. D. (1996). ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specificity transporter. *Proceedings of the National Academy of Sciences USA* **93,** 6928-6934.

Petruti-Mot, A. S. & Earnshaw, W. C. (2000). Two differentially spliced forms of *topoisomerase IIα* and β mRNAs are conserved between birds and humans. *Gene* **258,** 183-192.

Philipsen, S. & Suske, G. (1999). A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acid Research* **27,** 2991-3000.

Piscitelli, S. C., Rodvold, K. A., Rushing, D. A. & Tewksbury, D. A. (1993). Pharmacokinetics and pharmacodynamics of doxorubicin in patients with small cell lung cancer. *Clinical Pharmacology and Therapeutics* **53,** 555-561.

Pope, B. & Kent, H. M. (1996). High efficiency 5 minute transformation of *Escherichia coli*. *Nucleic Acid Research* **24,** 536-537.

Pourquier, P., Montaudon, D., Huet, S., Larrue, A., Clary, A. & Robert, J. (1998). Doxorubicin-Induced Alterations of c-*myc* and c-*jun* Gene Expression in Rat Glioblastoma Cells : Role of c-*jun* in Drug Resistance and Cell Death. *Biochemical Pharmacology* **55,** 1963-1971.

Promega pGL3B Luciferase Reporter Vector Technical Manual. (1994).

Prosperi, E., Negri, C., Bottiroli, G. & Astaldi Ricotti, G. C. B. (1996). Immunocytometric determination of the α and β isoforms of human DNA topoisomerase II: influence of different fixatives. *Analytical Cellular Pathology* **10,** 137-148.

Pu, Q. Q. & Bezwoda, W. R. (1999). Induction of Alkylator (Melphalan) Resistance in HL60 Cells Is Accompanied by Increased Levels of Topoisomerase II Expression and Function. *Molecular Pharmacology* **56,** 147-153.

Pu, Q. Q. & Bezwoda, W. R. (2000). Alkylator Resistance in Human B Lymphoid Cell Lines: (2) Increased Levels of Topoisomerase II Expression and Function in a Melphalan-Resistant B-CLL Cell Line. *Anticancer Research* **20,** 2571-2580.

Qui, J., Catapano, C. V. & Fernandes, D. J. (1996). Formation of Topoisomerase IIα Complexes with Nascent DNA Is Related to VM-26-Induced Cytotoxicity. *Biochemistry* **35,** 16354-16360.

Ramachandran, C. & Melnick, S. J. (1999). Multidrug Resistance in Human Tumors - Molecular Diagnosis and Clinical Significance. *Molecular Diagnosis* **4,** 81-94.

Rattner, J. B., Hendzel, M. J., Furbee, C. S., Muller, M. T. & Bazett-Jones, D. P. (1996). Topoisomerase IIα Is Associated with the Mammalian Centromere in a Cell Cycle- and Species-specific Manner and Is Required for Proper Centromere/Kinetochore Structure. *Journal of Cell Biology* **134,** 1097-1107.

Richardson, D. S. & Johnson, S. A. (1997). Anthracyclines in haematology : preclinical studies, toxicity and delivery systems. *Blood Reviews* **11,** 201-223.

Richardson, M. E. & Siemann, D. W. (1997). Tumor Cell Heterogeneity : Impact on Mechanisms of Therapeutic Drug Resistance. *International Journal of Radiation Oncology Biology Physics* **39,** 789-793.

Ritke, M. K., Allan, W. P., Fattman, C., Gunduz, N. N. & Yalowich, J. C. (1994). Reduced Phosphorylation of Topoisomerase II in Etopside-Resistant Human Leukemia K562 Cells. *Molecular Pharmacology* **46,** 58-66.

Ritke, M. K. & Yalowich, J. C. (1993). Altered gene expression in human leukemia K562 cells selected for resistance to etoposide. *Biochemical Pharmacology* **46,** 2007-2020.

Robert, J., Illiadis, A., Hoerni, B., Cano, J., Durand, M. & Lagarde, C. (1982). Pharmacokinetics of Adriamycin in Patients with Breast Cancer : Correlation between Pharmacokinetic Parameters and Clinical Short-term Response. *European Journal of Cancer and Clinical Oncology* **18,** 739-745.

Robert, J. & Larsen, A. K. (1998). Drug resistance to topoisomerase II inhibitors. *Biochimie* **80,** 247-254.

Roberts-Thomson, S. J., Holman, N. A., May, F. J., Lee, W.-J. & Monteith, G. R. (2000). Development of a real-time RT-PCR assay for plasma membrane calcium ATPase isoform 1 (PMCA1) mRNA levels in a human breast epithelial cell line. *Journal of Pharmacological and Toxicological Methods* **44,** 513-517.

Roca, J., Berger, J. M., Harrison, S. C. & Wang, J. C. (1996). DNA Transport by a type II topoisomerase : Direct evidence for a two-gate Mechanism. *Proceedings of the National Academy of Sciences USA* **93,** 4057-4062.

Roca, J. & Wang, J. C. (1992). The Capture of a DNA Double Helix by an ATP-Dependent Protein Clamp : A Key Step in DNA Transport by Type II DNA Topoisomerases. *Cell* **71,** 833-842.

Roca, J. & Wang, J. C. (1994). DNA Transport by a Type II DNA Topoisomerase : Evidence in Favor of a Two-gate Mechanism. *Cell* **77,** 609-616.

Roder, K., Wolf, S. S., Beck, K. & Schweizer, M. (1997). Cooperative Binding of NF-Y And Sp1 at the DNase I-hypersensitive Site, Fatty Acid Synthase Insulin-responsive Element 1, Located at -500 in the Rat Fatty Acid Synthase Promoter. *The Journal of Biological Chemistry* **272,** 21616-21624.

Rohlff, C., Ahmad, S., Borellini, F., Lei, J. & Glazer, R. I. (1997). Modulation of Transcription Factor Sp1 by cAMP-dependent Protein Kinase. *The Journal of Biological Chemistry* **272,** 21137-21141.

Rohlff, C. & Glazer, R. I. (1995). Regulation of Multidrug Resistance Through the cAMP and EGF Signalling Pathways. *Cellular Signalling* **7,** 431-443.

Ronchi, A., Bellorini, M., Mongelli, N. & Mantovani, R. (1995). CCAAT-box Binding Protein NF-Y (CBF, CPI) recognizes the minor groove and distorts DNA. *Nucleic Acids Research* **23,** 4565-4572.

Roninson, I. B., Chin, J. E., Choi, K., Gros, P., Housman, D. E., Fojo, A., Shen, D.-W., Gottesman, M. M. & Pastan, I. (1986). Isolation of human *mdr* DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proceedings of the National Academy of Sciences USA* **83,** 4538-4542.

Rzepecki, R. & Fisher, P. A. (2000). During both interphase and mitosis, DNA topoisomerase II interacts with DNA as well as RNA through the protein's C-terminal domain. *Journal of Cell Science* **113,** 1635-1647.

Saadane, N., Yue, P., Alpert, L., Mitmaker, B., Kirby, G. M. & Chalifour, L. E. (2001). Diminished molecular response to doxorubicin and loss of cardioprotective effect of dexrazoxane in *Egr-1* deficient female mice. *Canadian Journal of Physiology and Pharmacology* **79,** 533-544.

Saitoh, N., Goldberg, I. & Earnshaw, W. C. (1995). The SMC proteins and the coming of age of the chromosome scaffold hypothesis. *BioEssays* **17,** 759-766.

Saleem, A., Ibrahim, N., Patel, M., Li, X., Gupta, E., Mendoza, J., Pantazis, P. & Rubin, E. H. (1997). Mechanisms of Resistance in a Human Cell Line Exposed to Sequential Topoisomerase Poisoning. *Cancer Research* **57,** 5100-5106.

Salema, L., Lam, V., McPherson, J. P. & Goldenberg, G. J. (2001). Role of proteasomal degradation in the cell cycle-dependent regulation of DNA topoisomerase IIα expression. *Biochemical Pharmacology* **61,** 795-802.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning - A Laboratory Manual*, Third edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sampath, J., Sun, D., Kidd, V. J., Grenet, J., Gandhi, A., Shapiro, L. H., Wang, Q., Zambetti, G. P. & Schuetz, J. D. (2001). Mutant p53 Cooperates with ETS and Selectively Up-regulates Human MDR1 Not MRP1. *The Journal of Biological Chemistry* **276,** 39359-39367.

Sandri, M. I., Isaacs, R. J., Ongkeko, W. M., Harris, A. L., Hickson, J. D., Broggini, M. & Vikhanskaya, F. (1996). p53 regulates the minimal promoter of the human topoisomerase IIα gene. *Nucleic Acid Research* **24,** 4464-4470.

Sarto, C., Binz, P.-A. & Mocarelli, P. (2000). Heat shock proteins in human cancer. *Electrophoresis* **21,** 1218-1226.

Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J.-Å. & Safe, S. (2000). Ligand-, Cell-, and Estrogen Receptor Subtype (α/β)-dependent Activation at GC-rich (Sp1) Promoter Elements. *The Journal of Biological Chemistry* **275,** 5379-5387.

Scala, S., Budillon, A., Zhan, Z., Cho-Chung, Y. S., Jefferson, J., Tsokos, M. & Bates, S. E. (1995). Downregulation of *mdr*-1 Expression by 8-Cl-cAMP in Multidrug Resistant MCF-7 Human Breast Cancer Cells. *The Journal of Clinical Investigation* **96,** 1026-1034.

Schiemann, S., Schwirzke, M., Brunner, N. & Weidle, U. H. (1998). Molecular analysis of two mammary carcinoma cell lines at the transcriptional level as a model system for progression of breast cancer. *Clinical and Experimental Metastasis* **16,** 129-139.

Schinkel, A. H., Roelofs, M. E. M. & Borst, P. (1991). Characterization of the Human *MDR*3 P-Glycoprotein and Its Recognition by P-Glycoprotein-specific Monoclonal Antibodies. *Cancer Research* **51,** 2628-2635.

Schmittgen, T. D. & Zakrajsek, B. A. (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods* **46,** 69-81.

Segars, J. H. & Driggers, P. H. (2002). Estrogen action and cytoplasmic signaling cascades. Part 1: membrane-associated signaling complexes. *Trends in Endocrinology and Metabolism* **13,** 349-354.

Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M. & Moore, D. D. (1986). Human Growth Hormone as a Reporter Gene in Regulation Studies Employing Transient Gene Expression. *Molecular and Cellular Biology* **6,** 3173-3179.

Selvey, S., Thompson, E. W., Matthaei, K., Lea, R. A., Irving, M. G. & Griffiths, L. R. (2001). β-Actin - an unsuitable internal control for RT-PCR. *Molecular and Cellular Probes* **15,** 307-311.

Seth, P., Katayose, D., Li, Z., Kim, M., Wersto, R., Craig, C., Shanmugam, N., Ohri, E., Mudahar, B., Rakkar, A. N. S., Kodali, P. & Cowan, K. (1997). A recombinant adenovirus expressing wild type p53 induces apoptosis in drug-resistant human breast cancer cells : A gene therapy approach for drug-resistant cancers. *Cancer Gene Therapy* **4,** 383-390.

Shibao, K., Takano, H., Nakayama, Y., Okazaki, K., Nagata, N., Izumi, H., Uchiumi, T., Kuwano, M., Kohno, K. & Itoh, H. (1999). Enhanced Coexpression of YB-1 and DNA Topoisomerase IIα Genes in Human Colorectal Carcinomas. *International Journal of Cancer* **83,** 732-737.

Sigal, A. & Rotter, V. (2000). Oncogenic Mutations of the p53 Tumor Suppressor: The Demons of the Guardian of the Genome. *Cancer Research* **60,** 6788-6793.

Slapak, C. A., Daniel, J. C. & Levy, S. B. (1990). Sequential Emergence of Distinct Resistance Phenotypes in Murine Erythroleukemia Cells under Adriamycin Selection: Decreased Anthracycline Uptake Precedes Increased P-Glycoprotein Expression. *Cancer Research* **50,** 7895-7901.

Slapak, C. A., Fracasso, P. M., Martell, R. L., Toppmeyer, D. L., Lecerf, J. & Levy, S. B. (1994). Overexpression of the Multidrug Resistance-associated Protein (MRP) Gene in Vincristine but not Doxorubicin-selected Multidrug-resistant Murine Erythroleukemia Cells. *Cancer Research* **54,** 5607-5613.

Smit, J. J. M., Schinkel, A. H., Mol, C. A. A. M., Majoor, D., Mooi, W. J., Jongsma, A. M., Lincke, C. R. & Borst, P. (1994). Tissue Distribution of the Human MDR3 P-Glycoprotein. *Laboratory Investigation* **71,** 638-649.

Smith, A. J., van Helvoort, A., van Meer, G., Szabo, K., Welker, E., Szakacs, G., Varadi, A., Sarkadi, B. & Borst, P. (2000). MDR3 P-glycoprotein, a Phosphatidylcholine Translocase, Transports Several Cytotoxic Drugs and Directly Interacts with Drugs as Judged by Interference with Nucleotide Trapping. *The Journal of Biological Chemistry* **275,** 23530-23539.

Sng, J., Heaton, V. J., Bell, M., Maini, P., Austin, C. A. & Fisher, L. M. (1999). Molecular cloning and characterization of the human topoisomerase IIα and IIβ genes

: evidence for isoform evolution through gene duplication. *Biochimica et Biophysica Acta* **1444,** 395-406.

Somasundaram, K. & El-Deiry, W. S. (2000). Tumor Suppressor p53 : Regulation and Function. *Frontiers in BioScience* **5,** D424-D437.

Son, Y. S., Suh, J. M., Ahn, S. H., Kim, J. C., Yi, J. Y., Hur, K. C., Hong, W., Muller, M. T. & Chung, I. K. (1998). Reduced activity of topoisomerase II in an Adriamycin-resistant human stomach-adenocarcinoma cell line. *Cancer Chemotherapy and Pharmacology* **41,** 353-360.

Spataro, V., Toda, T., Craig, R., Seeger, M., Dubiel, W., Harris, A. L. & Norbury, C. (1997). Resistance to Diverse Drugs and Ultraviolet Light Conferred by Overexpression of a Novel Human 26 S Proteasome Subunit. *The Journal of Biological Chemistry* **272,** 30470-30475.

Stoner, M., Wang, F., Wormke, M., Nguyen, T., Samudio, I., Vyhlidal, C., Marme, D., Finkenzeller, G. & Safe, S. (2000). Inhibition of Vascular Endothelial Growth Factor Expression in HEC1A Endometrial Cancer Cells through Interactions of Estrogen Receptor α and Sp3 Proteins. *The Journal of Biological Chemistry* **275,** 22769-22779.

Stürzenbaum, S. R. & Kille, P. (2001). Control genes in quantitative molecular biological techniques: the variability of invariance. *Comparative Biochemistry and Physiology Part B* **130,** 281-289.

Su, W., Jackson, S., Tjian, R. & Echols, H. (1991). DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1. *Genes and Development* **5,** 820-826.

Sugimoto, K., Yamada, K., Egashira, M., Yazaki, Y., Hirai, H., Kikuchi, A. & Oshima, K. (1998). Temporal and Spatial Distribution of DNA Topoisomerase II Alters During Proliferation, Differentiation and Apoptosis in HL-60 Cells. *Blood* **91,** 1407-1417.

Suske, G. (1999). The Sp-family of transcription factors. *Gene* **238,** 291-300.

Tada, Y., Wada, M., Kuroiwa, K., Kinugawa, N., Harada, T., Nagayama, J., Nakagawa, M., Naito, S. & Kuwano, M. (2000). *MDR1* Gene Overexpression and Altered Degree of Methylation at the Promoter Region in Bladder Cancer during Chemotherapeutic Treatment. *Clinical Cancer Research* **6,** 4618-4627.

Takano, H., Ise, T., Nomoto, M., Kato, K., Murakami, T., Ohmori, H., Imamura, T., Nagatani, G., Okamoto, T., Ohta, R., Furukawa, M., Shibao, K., Izumi, H., Kuwano, M. & Kohno, K. (1999). Structural and functional analysis of the control region of the human DNA topoisomerase IIα gene in drug-resistant cells. *Anti-Cancer Drug Design* **14,** 87-92.

Takano, H., Kohno, K., Ono, M., Uchida, Y. & Kuwano, M. (1991). Increased Phosphorylation of DNA Topoisomerase II in Etoposide-resistant Mutants of Human Cancer KB Cells. *Cancer Research* **51,** 3951-3957.

Tamura, S., Hanada, M., Ohnishi, M., Katsura, K., Sasaki, M. & Kobayashi, T. (2002). Regulation of stress-activated protein kinase signaling pathways by protein phosphatases. *European Journal of Biochemistry* **269,** 1060-1066.

Tan, B., Piwnica-Worms, D. & Ratner, L. (2000). Multidrug resistance transporters and modulation. *Current Opinion in Oncology* **12,** 450-458.

Tanikawa, J., Ichikawa-Iwata, E., Kanei-Ishii, C. & Ishii, S. (2001). Regulation of c-Myb Activity by Tumor Suppressor p53. *Blood Cells, Molecules, and Diseases* **27,** 479-482.

Teixeira, C., Reed, J. C. & Pratti, M. A. C. (1995). Estrogen Promotes Chemotherapeutic Drug Resistance by a Mechanism Involving *Bcl-2* Proto-Oncogene Expression in Human Breast Cancer Cells. *Cancer Research* **55,** 3902-3907.

Thielmann, H. W. & Popanda, O. (1998). Doxorubicin and γ rays increase the level of DNA topoisomerase IIα in nuclei of normal and xeroderma pigmentosum fibroblasts. *Journal of Cancer Research and Clinical Oncology* **124,** 355-366.

Thompson, D. A. & Weigel, R. J. (1998). Characterization of a gene that is inversely correlated with estrogen receptor expression (ICERE-1) in breast carcinomas. *European Journal of Biochemistry* **252,** 169-177.

Tolner, B., Hartley, J. A. & Hochhauser, D. (2001). Transcriptional Regulation of Topoisomerase IIα at Confluence and Pharmacological Modulation of Expression by *bis*-Benzimidazole Drugs. *Molecular Pharmacology* **59,** 699-706.
Topcu, Z. (2001). DNA topoisomerases as targets for anticancer drugs. *Journal of Clinical Pharmacy and Therapeutics* **26,** 405-416.

Torgeman, A., Mor-Vaknin, N., Zelin, E., Ben-Aroya, Z., Löchelt, M., Flügel, R. M. & Aboud, M. (2001). Sp1-p53 Heterocomplex Mediates Activation of HTLV-1 Long Terminal Repeat by 12-*O*-Tetradecanoylphorbol-13-acetate That Is Antagonized by Protein Kinase C. *Virology* **281,** 10-20.

Towatari, M., Adachi, K., Marunouchi, T. & Saito, H. (1998). Evidence for a critical role of DNA topoisomerase II α in drug sensitivity revealed by inducible antisense RNA in a human leukaemia cell line. *British Journal of Haematology* **101,** 548-551.

Trock, B. J., Leonessa, F. & Clarke, R. (1997). Multidrug Resistance in Breast Cancer : a Meta-analysis of MDR1/gp170 Expression and Its Possible Functional Significance. *Journal of the National Cancer Institute* **89,** 917-931.

Tsai, C., Lee, C., Chien, C., Kuo, S. & Chang, Y. (1999). Additive Effect of Sp1 and Sp3 in Regulation of the ED-L1E Promoter of the EBV LMP 1 Gene in Human Epithelial Cells. *Virology* **261,** 288-294.

Tsutsui, K., Tsutsui, K., Okada, S., Watanabe, M., Shohmori, T., Seki, S. & Inoue, Y. (1993). Molecular Cloning of Partial cDNAs for Rat DNA Topoisomerase II Isoforms and Their Differential Expression in Brain Development. *The Journal of Biological Chemistry* **268,** 19076-19083.

van der Bliek, A. M., Baas, F., Ten Houte de Lange, T., Kooiman, P. M., van der Velde-Koerts, T. & Borst, P. (1987). The human *mdr3* gene encodes a novel P-glycoprotein homologue and gives rise to alternatively spliced mRNAs in liver. *The EMBO Journal* **6,** 3325-3331.

van der Bliek, A. M., Kooiman, P. M., Schneider, C. & Borst, P. (1988). Sequence of *mdr*3 cDNA encoding a human P-glycoprotein. *Gene* **71,** 401-411.

van der Zee, A. G. J., Hollema, H. H., De Bruijn, H. W. A., Willemse, P. H. B., Boonstra, H., Mulder, N. H., Aalders, J. G. & de Vries, E. G. E. (1995). Cell Biological Markers of Drug Resistance in Ovarian Carcinoma. *Gynecologic Oncology* **58,** 165-178.

van Helvoort, A., Smith, A. J., Sprong, H., Frtizsche, I., Schinkel, A. H., Borst, P. & van Meer, G. (1996). MDR1 P-Glycoprotein Is a Lipid Translocase of Broad Specificity, While MDR3 P-Glycoprotein Specifically Translocates Phosphatidylcholine. *Cell* **87,** 507-517.

van Slooten, H.-J., van de Vijver, M. J., Borresen, A.-L., Eyfjord, J. E., Valgardsdottir, R., Scherneck, S., Nesland, J. M., Devilee, P., Cornelisse, C. J. & van Dierendonck, J. H. (1999). Mutations in Exons 5-8 of the *p53* Gene, Independent of Their Type and Location, are Associated with Increased Apoptosis and Mitosis in Invasive Breast Carcinoma. *Journal of Pathology* **189,** 504-513.

Vargas-Roig, L. M., Gago, F. E., Tello, O., Aznar, J. C. & Ciocca, D. R. (1998). Heat Shock Protein Expression and Drug Resistance in Breast Cancer Patients Treated with Induction Chemotherapy. *International Journal of Cancer* **79,** 468-475.

Vassetzky, Y. S., Alghisi, G. & Gasser, S. M. (1995). Topoisomerase II mutations and resistance to anti-tumor drugs. *BioEssays* **17,** 767-774.

Vilà, M. R., Nicolás, A., Morote, J., de Torres, I. & Meseguer, A. (2000). Increased Glyceraldehyde-3-Phosphate Dehydrogenase Expression in Renal Cell Carcinoma Identified by RNA-Based, Arbitrarily Primed Polymerase Chain Reaction. *Cancer* **89,** 152-164.

Viñals, F., Fandos, C., Santalucia, T., Ferré, J., Testar, X., Palacin, M. & Zorzano, A. (1997). Myogenesis and MyoD Down-regulate Sp1. *The Journal of Biological Chemistry* **272,** 12913-12921.

Volm, M. (1998). Multidrug Resistance and its Reversal. *Anticancer Research* **18,** 2905-2918.

Wang, H., Jiang, Z., Wong, Y. W., Dalton, W. S., Futscher, B. W. & Chan, V. T. (1997a). Decreased CP-1 (NF-Y) Activity Results in Transcriptional Down-Regulation of Topoisomerase IIα in a Doxorubicin-Resistant Variant of Human Multiple Myeloma RPMI 8226. *Biochemical and Biophysical Research Communications* **237,** 217-224.

Wang, J. C. (1996). DNA Topoisomerases. *Annual Review of Biochemistry* **65,** 635-692.

Wang, K., Ramji, S., Bhathena, A., Lee, C. & Riddick, D. S. (1999a). Glutathione *S*-transferases in wild-type and doxorubicin-resistant MCF-7 human breast cancer cell lines. *Xenobiotica* **29,** 155-170.

Wang, Q., Zambetti, G. P. & Suttle, D. P. (1997b). Inhibition of DNA Topoisomerase IIα Gene Expression by the p53 Tumor Suppressor. *Molecular and Cellular Biology* **17,** 389-397.

Wang, W., Dong, L., Saville, B. & Safe, S. (1999b). Transcriptional Activation of E2F1 Gene Expression by 17β -Estradiol in MCF-7 Cells Is Regulated by NF-Y-Sp1/Estrogen Receptor Interactions. *Molecular Endocrinology* **13,** 1373-1387.

Wang, Z., Zhang, Y., Lu, J., Sun, S. & Ravid, K. (1999c). Mpl Ligand Enhances the Transcription of the Cyclin D3 Gene: A Potential Role for Sp1 Transcription Factor. *Blood* **93,** 4208-4221.

Watt, P. M. & Hickson, I. D. (1994). Structure and function of type II DNA topoisomerases. *Biochemical Journal* **303,** 681-695.

Webb, C. D., Latham, M. D., Lack, R. B. & Sullivan, D. M. (1991). Attenuated Topisomerase II Content Directly Correlates with a Low Level of Drug Resistance in a Chinese Hamster Ovary Cell Line. *Cancer Research* **51,** 6543-6549.

Wells, N. J., Fry, A. M., Guano, F., Norbury, C. & Hickson, I. D. (1995). Cell Cycle Phase-specific Phosphorylation of Human Topoisomerase IIα. *The Journal of Biological Chemistry* **270,** 28357-28363.

Welshon, W. V., Wolf, M. F., Murphy, C. S. & Jordan, V. G. (1988). Estrogenic activity of phenol red. *Molecular and Cellular Endocrinology* **57,** 169-178.

Wessel, I., Jensen, L. H., Jensen, P. B., Falck, J., Rose, A., Roerth, M., Nitiss, J. L. & Sehested, M. (1999). Human Small Cell Lung Cancer NYH Cells Selected for Resistance to the Bisdioxopiperazine Topoisomerase II Catalytic Inhibitor ICRF-187 Demonstrate a Functional R162Q Mutation in the Walker A Consensus ATP Binding Domain of the α Isoform. *Cancer Research* **59,** 3442-3450.

Wessel, I., Jensen, P. B., Falck, J., Mirski, S. E. L., Cole, S. P. C. & Sehested, M. (1997). Loss of Amino Acid ^{1490}Lys-Ser-Lys^{1492} in the COOH-Terminal Region of Topoisomerase IIα in Human Small Cell Lung Cancer Cells Selected for Resistance to Etoposide Results in an Extranuclear Enzyme Localization. *Cancer Research* **57,** 4451-4454.

Wilkinson, P. M. & Mawer, G. E. (1974). The Persistence of Adriamycin in Man and Rat. *British Journal of Clinical Pharmacology* **1,** 241-247.

Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Prüß, M., Reuter, I. & Schacherer, F. (2000). TRANSFAC: an integrated system for gene expression regulation. *Nucleic Acid Research* **28,** 316-319.

Withoff, S., de Jong, S., de Vries, E. G. E. & Mulder, N. H. (1996a). Human DNA Topoisomerase II : Biochemistry and Role in Chemotherapy Resistance. *Anticancer Research* **16,** 1867-1880.

Withoff, S., Keith, W. N., Knol, A. J., Coutts, J. C., Hoare, S. F., Mulder, N. H. & de Vries, E. G. E. (1996b). Selection of a subpopulation with fewer DNA topoisomerase IIα gene copies in a doxorubicin-resistant cell line panel. *British Journal of Cancer* **74,** 502-507.

Withoff, S., Smit, E. F., Meersma, G., J,, van Den Berg, A., Timmer-Bosscha, H., Kok, K., Postmus, P. E., Mulder, N. H., De Vries, E. G. E. & Buys, C. H. C. M. (1994). Quantitation of DNA Topoisomerase IIα Messenger Ribonucleic Acid Levels in a Small Cell Lung Cancer Cell Line and Two Drug Resistant Sublines Using a Polymerase Chain Reaction-Aided Transcript Titration Assay. *Laboratory Investigation* **71,** 61-66.

Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K. & Drake, F. H. (1991). Proliferation-and Cell Cycle-dependent Differences in Expression of the 170 Kilodalton and 180 Kilodalton Forms of Topoisomerase II in NIH-373 Cells. *Cell Growth and Differentiation* **2,** 209-214.

Wosikowski, K., Schuurhuis, D., Kops, G. J. P. L., Saceda, M. & Bates, S. E. (1997). Altered Gene Expression in Drug-resistant Human Breast Cancer Cells. *Clinical Cancer Research* **3,** 2405-2414.

Wurzer, G., Herceg, Z. & Wesierska-Gadek, J. (2000). Increased Resistance to Anticancer Therapy of Mouse Cells Lacking the Poly(ADP-ribose) Polymerase Attributable to Up-Regulation of the Multidrug Resistance Gene Product P-Glycoprotein. *Cancer Research* **60,** 4238-4244.

Xu, J. & Loo, G. (2001). Different Effects of Genistein on Molecular Markers Related to Apoptosis in Two Phenotypically Dissimilar Breast Cancer Cell Lines. *Journal of Cellular Biochemistry* **82,** 78-88.

Yaffe, B. M. & Samuels, H. H. (1984). Hormonal Regulation of the Growth Hormone Gene. *The Journal of Biological Chemistry* **259,** 6284-6291.

Yamada, K., Tanaka, T., Miyamoto, K. & Noguchi, T. (2000). Sp Family Members and Nuclear Factor-Y Cooperatively Stimulate Transcription from the Rat Pyruvate Kinase M Gene Distal Promoter Region via Their Direct Interactions. *The Journal of Biological Chemistry* **275,** 18129-18137.

Yang, L. & Trujillo, J. M. (1990). Biological Characterization of Multidrug-resistant Human Colon Carcinoma Sublines Induced/Selected by Two Methods. *Cancer Research* **50,** 3218-3225.

Yang, X., Uziely, B., Groshen, S., Lukas, J., Israel, V., Russell, C., Dunnington, G., Formenti, S., Muggia, F. & Press, M. F. (1999). *MDR1* Gene Expression in Primary and Advanced Breast Cancer. *Laboratory Investigation* **79,** 271-280.

Yoon, J. H., Kim, J. K., Rha, G. B., Oh, M., Park, S., Seong, R. H., Hong, S. H. & Park, D. (1999). Sp1 mediates cell proliferation-dependent regulation of rat DNA topoisomerase IIα gene promoter. *Biochemical Journal* **344,** 367-374.

Yu, D., Ma, C. & Chang, S. (2000). Establishment and characterization of renal cell carcinoma cell lines with multidrug resistance. *Urology Research* **28,** 86-92.

Yu, Q., Mirski, S. E. L., Sparks, K. E. & Cole, S. P. C. (1997). Two COOH-Terminal Truncated Cytoplasmic Forms of Topoisomerase IIα in a VP-16-Selected Lung Cancer Cell Line Result from Partial Gene Deletion and Alternative Splicing. *Biochemistry* **36,** 5868-5877.

Zaman, G. J. R., Flens, M. J., van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J. & Borst, P. (1994). The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proceedings of the National Academy of Sciences USA* **91,** 8822-8826.

Zaman, G. J. R., Versantvoort, C. H. M., Smit, J. J. M., Eijdems, E. W. H. M., de Haas, M., Smith, A. J., Broxterman, H. J., Mulder, N. H., de Vries, F. G. E., Baas, F. & Borst, P. (1993). Analysis of the Expression of *MRP*, the Gene for a New Putative Transmembrane Drug Transporter in Human Multidrug Resistant Lung Cancer Cell Lines. *Cancer Research* **53,** 1747-1750.

Zampieri, L., Bianchi, P., Ruff, P. & Arbuthnot, P. (2002). Differential Modulation by Estradiol of P-Glycoprotein Drug Resistance Protein Expression in Cultured MCF7 and T47D Breast Cancer Cells. *Anticancer Research* **22,** 2253-2260.

Zhang, C. C., Yang, J., Bash-Babula, J., White, E., Murphy, M., Levine, A. J. & Hait, W.N. (1999). DNA Damage Increases Sensitivity to *Vinca* Alkaloids and Decreases Sensitivity to Taxanes through p53-dependent Repression of Microtubule-associated Protein 4. *Cancer Research* **59,** 3663-3670.

Zhang, H., D'Arpa, P. & Liu, L. F. (1990). A Model for Tumor Cell Killing by Topoisomerase Poisons. *Cancer Cells* **2,** 23-27.

Zheng, X.-L., Matsubara, S., Diao, C., Hollenberg, M. D. & Wong, N. C. W. (2000). Activation of Apoplipoprotein A1 Gene Expression by Protein Kinase A and Kinase C through Transcription Factor, Sp1. *The Journal of Biological Chemistry* **275,** 31747-31754.

Zhou, R., Vitols, S., Gruber, A., Lilemark, J., Wang, Y. & Lilemark, E. (1999a). Etoposide-induced DNA strand breaks in relation to p-glycoprotein and topoisomerase II protein expression in leukaemic cells from patients with AML and CLL. *British Journal of Haematology* **105,** 420-427.

Zhou, R., Wang, Y., Gruber, A., Larsson, R., Castanos-Velez, E. & Liliemark, E. (1999b). Topoisomerase II-mediated alterations of K562 drug resistant sublines. *Medical Oncology* **16,** 191-198.

Zhou, R. H., Wang, P., Zou, Y., Jackson-Cook, C. K. & Povirk, L. F. (1997). A Precise Interchromosomal Reciprocal Exchange between Hot Spots for Cleavable Complex Formation by Topoisomerase II in Amsacrine-treated Chinese Hamster Ovary Cells. *Cancer Research* **57,** 4699-4702.

Zhou, Z., Zwelling, L. A., Kawakami, Y., An, T., Kobayashi, K., Herzog, C. & Kleinerman, E. S. (1999c). Adenovirus-mediated Human *Topoisomerase IIα* Gene Transfer Increases the Sensitivity of Etoposide-resistant Human Breast Cancer Cells. *Cancer Research* **59,** 4618-4624.

Zucker, R. M., Adams, D. J., Bair, K. W. & Elstein, K. H. (1991). Polyploidy Induction as a Consequence of Topoisomerase Inhibition. *Biochemical Pharmacology* **42,** 2199-2208.

Zwelling, L. A., Mayes, J., Hinds, M., Chan, D., Altschuler, E., Carroll, B., Parker, E., Deisseroth, K., Radcliffe, A., Seligman, M., Li, L. & Farquar, D. (1991). Cross-Resistance of an Amsacrine-Resistant Human Leukemia Line to Topoisomerase II Reactive DNA Intercalating Agents. Evidence for Two Topoisomerase II Directed Drug Actions. *Biochemistry* **30,** 4048-4055.

Appendix 1. Literature review of the basal transcription of the topo IIα promoter in different cell lines

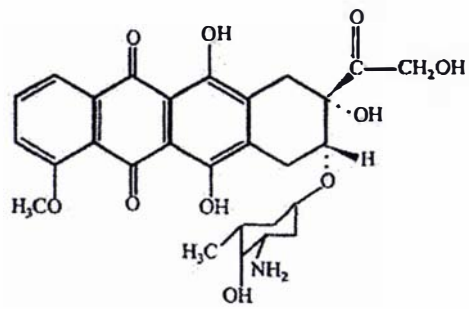
Promoter elements contained	This study MCF-7 breast cancer cell line	Hochhauser *et al.* (1992) HeLa (human cervical)	NIH3T3 Chen *et al.* (1999) (murine fibroblast)	Wang *et al.* (1997b) BALB/c p53 -/- (murine embryo fibroblast)	Loflin *et al.* (1996) K562 (human erythro-leukemia)	Furukawa *et al.* (1998) T24 (human transitional urinary cancer)	Morgan and Beck (2001) CEM (human leukemia)	Takano *et al.* (1999) KB (human epithelial)
5' GC2	106% (-1964)	35% (-2400) 72% (-1200)	-	-	75% (-2400) 34% (-1200)	-	-	-
GC2 → (-562)	100% (-617)	-	-	-	-	-	-	-
ICB5 → (-385)	-	100% (-557)	100% (-557)	100% (-557)	100% (-557)	-	100% (-557)	-
ICB4 → (-259)	-	97% (-295)	133% (-382)	85% (-382)	282% (-295)	100% (-295)	63% (-382)	100% (-295)
ATF → (-226)	-	-	123% (-252)	55% (-252)	-	-	18% (-252)	-
ICB3 → (-175)	-	-	150% (-182)	31% (-182)	-	91% (-197)	36% (-182)	100% (-197)
ICB2 → (-108)	17% (-144)	-	50% (-162) 48% (-122)	6.3% (-162) 3.4% (-142) 2.5% (-122)	-	40% (-154)	21% (-162)	85% (-154)
ICB1 → (-68)	3.6% (-101)	58% (-90)	-	2% (-90)	11.7% (-90)	9% (-94)	2% (-90)	25% (-74)
GC1 (-51)	1.5% (-56)	-	-	-	-	-	-	-
myc/max → (-29)	-	4.8% (-32)	8.3% (-32)	0.4% (-32)	16% (-32)	-	-	-
Myb → (-16)	-	-	-	-	-	1.6% (-20)	-	10% (-20)
	-	4.9% (-2)	-	-	1.5% (-2)	-	-	-

Appendix 1. Summary of the results from published transient transfection experiments. In most cases the value of the -617 or -557 construct was arbitrarily valued at 100% with the rest of the values compared to this. The 5' end of the construct is stated in the () relative to the tsp. The first column states the promoter elements contained within the construct i.e. ICB1→ contains ICB1, GC1, myc/max and the Myb element, with the exact position of the element in promoter.

Appendix 2. Table of the mechanisms of drug action and resistance mechanisms

Drug	Class	Drug Mechanisms	Major *In vitro* resistance mechanism
Doxorubicin (dox) (Adriamycin) Others anthracyclines include Idarubicin (ida) Daunorubicin (dau)	Anthracyclines	Topo II poison DNA intercalation Production of free radicals Induction of apoptosis Membrane modulation Inhibition of DNA polymerase, RNA polymerase, DNA helicase	Topo II MDR1 MRP1 GST/GSH
Mitoxantrone (mito)	Anthracenedione	Topo II intercalative poison Induce apoptosis	Topo II, MDR1, MRP1 GSH
9-OH-ellipticine	Ellipticines	Topo II intercalative poison	Topo II
mAMSA (amsacrine)	Aminoacridines	Topo II intercalative poison	Topo II
Etoposide (VP-16) Teniposide (VM-26)	Epipodophyllotoxins	Topo II poison - non-intercalator Induce apoptosis	Topo II, MDR1, MRP1 GST
ICRF8/159/187	Bisdioxopiperazines	Topo II catalytic inhibitor	Topo II
Merbarone (mer)	Barbiturate derivative	Topo II catalytic inhibitor	Topo II
Paclitaxel (pac) (Taxol)	Taxanes	promotes microtubule (MT) assembly/ inhibits MT disassembly Induce apoptosis	MDR1 altered α/β tubulin structure, change in tubulin levels or polymerization
Melphalan (mel)	Alkylating agent	DNA crosslinks	GSH, DNA repair metallothionein
Cisplatin (cis)	Platinum derivative	DNA inter/intracalator Production of free radicals	GSH metallothioein DNA repair enzymes Thymidylate synthase

(adapted from (Naito et al., 1999 ; Kerr, 1996 ; van der Zee et al., 1995 ; Volm, 1998 ; Arts et al., 2000 ; Andoh & Ishida, 1998).



The structure of doxorubicin from Austin *et al.* (1998).

Appendix 3. Examples of drug resistant cell lines which have shown changes in the amounts of topo IIα or topo IIβ

Topo II poisons (intercalators) - Anthracyclines

cell type	drug	topo IIα	topo IIβ	reference
MES-SA sarcoma	dox	↓ (R/P)	↔ (R/P)	Beketic-Oreskovic *et al.* (1995)
MCF-7	"	↓ (R)	n/a	Wosikowski *et al.* (1997)
small cell lung cancer	"	↓ (R/P)	n/a	Withoff *et al.* (1994)
stomach adenocarcinoma	"	↓ (P)	↔ (P)	Son *et al.* (1998)
renal	"	↑ (R)	n/a	Yu *et al.* (2000)
multiple myeloma	"	↓ (R)	n/a	Wang *et al.* (1997a)
murine erythroleukemic	"	↓ (R/P)	↓ (R/P)	Modrak *et al.* (1997)
K562	ida	↓ (P)	↓ (P)	Zhou *et al.* (1999b)
"	"	↓ (P)	n/a	Locke *et al.* (1999)

Topo II poisons - other intercalactors

HL-60	m-AMSA	n/a	↓ (R/P)	Herzog *et al.* (1998)
myeloma RPMI 8226	mito	↓ (P)	↓ (P)	Hazelhurst *et al.* (1999)
K562	"	↓ (P)	↓ (P)	Zhou *et al.* (1999b)
HL-60	"	↓ (R)	↓ (R)	Harker *et al.* (1995)
Gastric	"	↓ (P)	n/a	Kellner *et al.* (1997)
EAT cells	"	↓ (P)	↓ (P)	Nielsen *et al.* (2000b)
Chinese hamster lung (CHL)	9-OH-ellipticine	↓(R/P)	↓ (R/P)	Khelifa *et al.* (1999)
Chinese hamster ovary (CHO)	"	n/a	↓ (P)	Dereuddre *et al.* (1995) Dereuddre *et al.* (1997)

Alkylating agents

HL-60	mel	↑ (P)	n/a	Pu & Bezwoda (1999)
lymphocytic cells	"	↑ (P)	↑ (P)	Pu & Bezwoda (2000)

Topo II poisons (non intercalators) - Epipodophyllotoxins

CHL	VP-16	↓ (P)	↔ (P)	Hashimoto *et al.* (1995)
CEM	"	↓ (P)	↓ (P)	Lotfi *et al.* (2001)
KB epidermoid	"	↓ (R)	n/a	Kubo *et al.* (1995)
MES-SA sarcoma	"	↓ (R)	↓ (R)	Jaffrezou *et al.* (1994)
melanoma	"	↓ (R/P)	↓ (R/P)	Lage *et al.* (2000)
MDA-MB-231	"	↓ (R)	n/a	Matsumoto *et al.* (1997) ; Asano *et al.* (1996b) ; Zhou *et al.* (1999b)
EAT cells	"	↓ (P)	↓ (P)	Nielsen *et al.* (2000a)
Human glioma	"	↓ (R/P)	n/a	Matsumoto *et al.* (1999)
K562	"	↓ (R)	↔ (R)	Melixetian *et al.* (2000)
U-937 monoblastic leukemia	"	↓ (R/P)	↔ (R/P)	Saleem *et al.* (1997)

Topo II catalytic inhibitors

CEM	mer	↓ (P)	↑ (P)	Kusumoto *et al.* (1996)
"	"	↓ (R)	n/a	Mo *et al.* (1997)
CEM leukemic	ICRF-187	↑ (R/P)	n/a	Morgan *et al.* (2000)
small cell lung cancer	"	↑ (P)	↔ (P)	Wessel *et al.* (1999)
CHL cells	topo II inhibitor	↔ (R/P)	↓ (R/P)	Le Mee *et al.*, 2000)

Other drugs

cervical	cis	↑ (P)	n/a	Minagawa *et al.* (1997)
ovarian	"	↑ (P)	n/a	Minagawa *et al.* (1997)
"	gemcitabine	↓ (R)	n/a	Bergman *et al.* (2000)

Appendix 3. Examples of the changes to topo IIα or topo IIβ mRNA (R) or protein (P) in a range of drug-resistant cell lines. ↔ no change, ↑ increase ↓ decrease dox = doxorubicin, ida = idarubicin, mito = mitoxantrone, mel = melphalan, VP-16 = etoposide, mer = merbarone, cis = cisplatin.

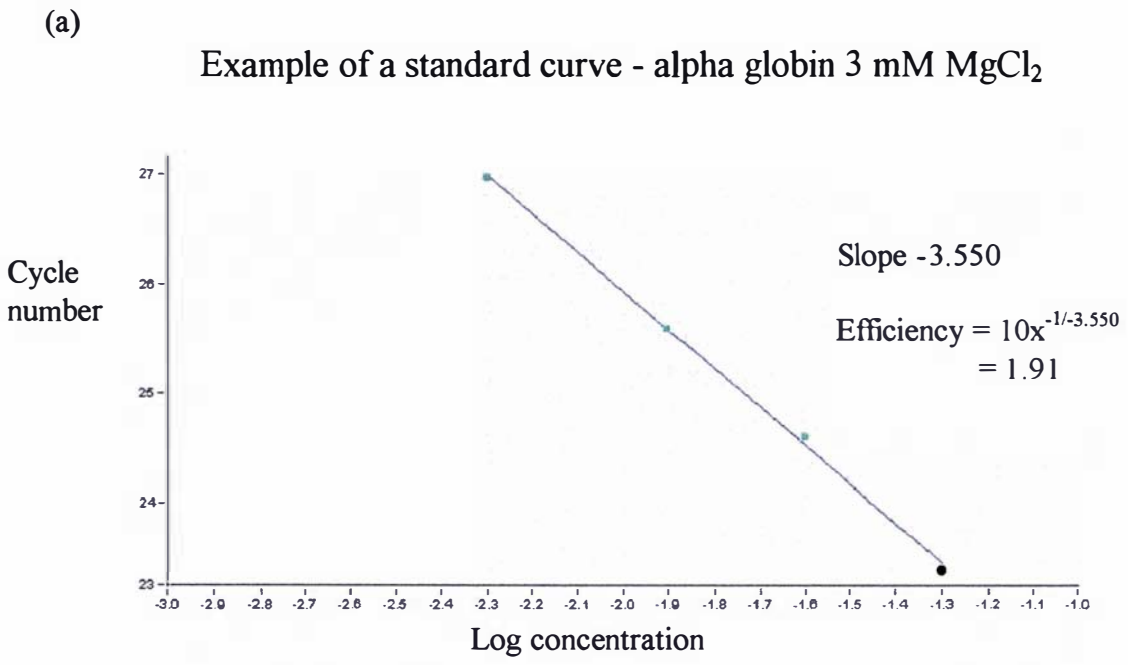
Appendix 4. Summary of the primers used in RT-PCR and real time RT-PCR

mRNA	Primer	Position	PCR product size.	Accession no	Diagnostic digest (approx. size of products)	PCR Efficiency ($MgCl_2$)	Tm (%GC)	Reference
topo IIα *	sense 5' GTAGCAATAATCTAAACCTCT 3' anti 5' GGTTGTAGAATTAAGAATAGC 3'	nt 2367-2377 nt 2775-2797	430 bp	J04088	*Dde* I 330 + 100 bp	1.98 (4 mM)	85.5°C (42%)	Lohri *et al.* (1997)
hGH	sense 5' TTTGGCCTGCTCTGCCTG 3' anti 5' GTTTGGATGCCTTCCTCTAGGTC 3'	nt 37-54 nt 423-446	409 bp	E00141	*Pvu* II 110 + 290 bp	1.95 (4 mM) 1.81 (3 mM)	90.0°C (54%)	-
GAPDH	s 5' CGGGAAGCTTGTGATCAATGG 3' a 5' GGCAGTGATGGCATGGACTG 3'	nt 252-272 nt 590-609	357 bp	M33197	*Nco* I 120 + 230 bp	1.80 (3 mM)	90.0°C (54%)	Beck *et al.* (1995)
18S rRNA	for 5' CGAAGACGATCAGATACCG 3' rev 5' GGCATCACAGACCTGTT 3'	nt 1047-1066 nt 1487-1503	456 bp	X03205	*Dde* I 290 +110 +50 bp	2.04 (3 mM) 1.87 (4 mM)	90.0°C (55%)	-
α-globin	sense 5' GACCTGCACGCGCACAAGCTTC 3' anti 5' CGCAGGGGTGAACTCGGCGG 3'	nt 294-317 nt 382-399	105 bp	V00493	*Dde* I 50 + 55 bp	1.97 (4 mM)	91.0°C (65%)	-
topo IIβ	for 5' AGCGTAGGCTACATGG 3' rev 5' CAGGCTCTACACGTTG 3'	nt 2069-2084 nt 2548-2563	495 bp	X68060	*Dde* I 100 + 400 bp	1.46 (3 mM)	85.0°C (40%)	
MRP1	sense 5' ATCAAGACCGCTGTCATTGG 3' anti 5' GAGCAAGGATGACTTGCAGG 3'	nt 1379-1398 nt 1538-1559	181 bp	LO5628	*Dde* I 110 + 70 bp	2.04 (3 mM)	89.0°C (53%)	Kim *et al.* (1996)
MDR1	sense 5' CTGGTGTTTGGAGAAATGACAG 3' anti 5' CCCAGTGAAAAATGTTGCCATTGAC 3'	nt 632-653 nt 1009-1033	400 bp	G187468	*Pvu* II 210 + 190 bp	-	-	Futscher *et al.* (1993)
MDR3	sense 5' GCTGTTTTCCACCACAATTG 3' anti 5' CTGTGACCACCGTGTAAAC 3'	nt 1473-1494 nt 2101-2120	647 bp	M23234	*Hinc* II 260 + 390 bp	-	-	-
GAPDH	sense 5' GTCATGGGTGTGAACCATGAG 3' anti 5' GACAACTTCAGTCTCCTCTGGT 3'	nt 455-475 nt 903-924	469 bp	M33197	*Xba* I 350 + 120 bp	-	-	-

*Appendix 4. Table of the primers used in the RT-PCR and real time RT-PCR reactions in this current study. The MRP1 primers were used in both reactions, the topo IIα, 18S rRNA, α-globin, GAPDH (s/a) and topo IIβ were used for real time RT-PCR reactions, while MDR1, MDR3 and GAPDH (sense/anti) were used for RT-PCR reactions. * these primers were designed to avoid the alternate splice sites between exon 8 - 11 (Petruti-Mot et al., 2000).*

Appendix 5. An example of a PCR "standard curve" used to determine the PCR efficiency of the PCR reaction in a real time RT-PCR experiment

(a)

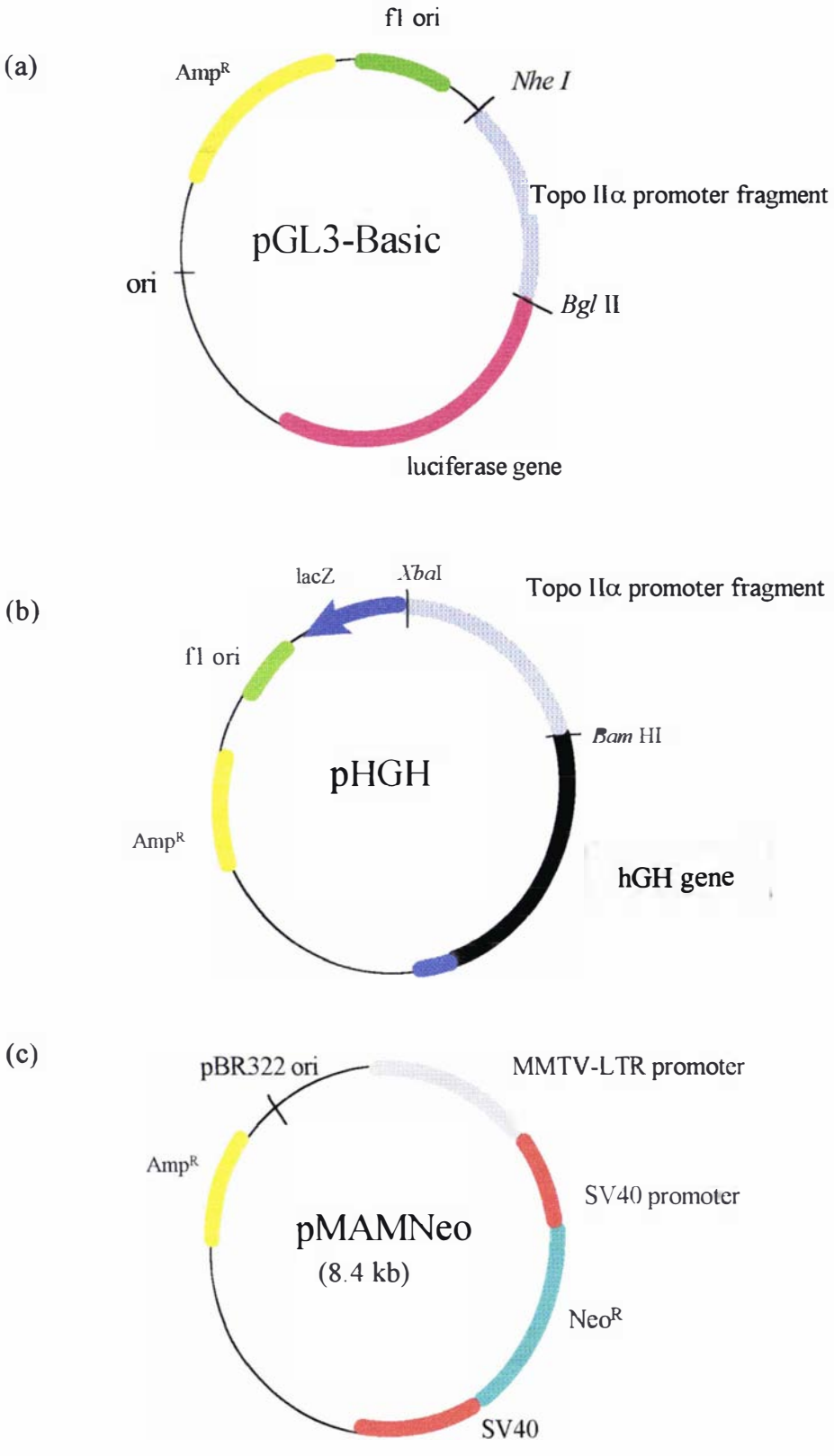


(b)

cell line	18S rRNA cross-over cycle no	18S rRNA "concentration"	topo IIα (T) cycle no	topo IIα "concentration"	ratio (T/18s)
standard curve control	-	-	29.73	0.05*	-
MDA144 control	27.93	0.175	25.88	0.723	4.14 (100%)
D2	27.91	0.177	26.54	0.456	2.58 (62%)

Appendix 5. (a) The graph of log concentration vs. cycle number for the alpha globin primer set (b) Example of data from a real time RT-PCR experiment. The standard curve control was arbitrarily valued at 0.05 (the dilution factor of the sample and the "value" from the standard curve).*

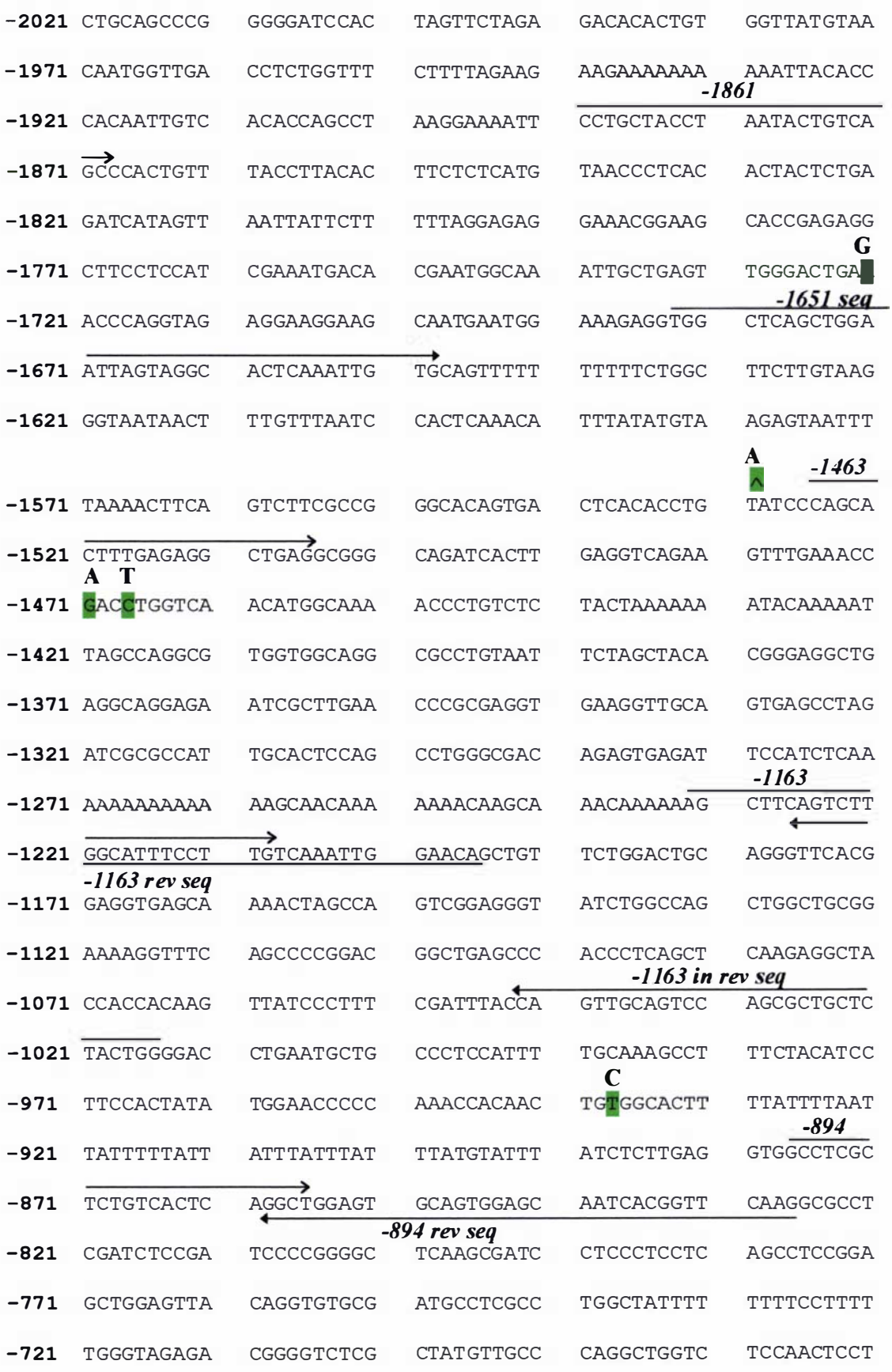
Appendix 6. Diagram of the vectors used in this study

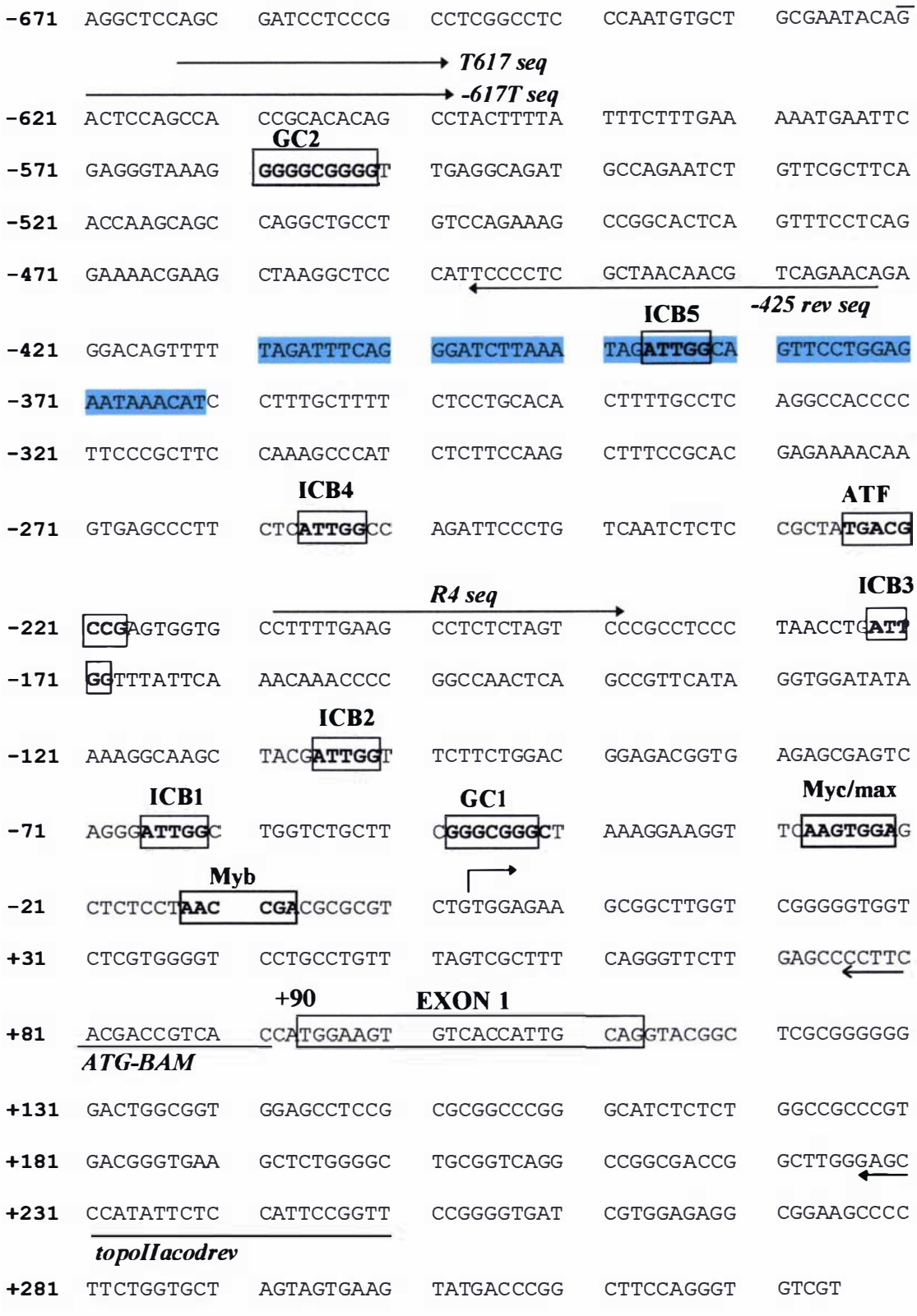


Appendix 6. Diagrams of (a) pGL3Basic, (b) pHGH and (c) pMAMNeo

Appendix 7. The sequence of the human topo IIα promoter

(a)




-671 AGGCTCCAGC GATCCTCCCG CCTCGGCCTC CCAATGTGCT GCGAATACAG
T617 seq
-617T seq
-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
-425 rev seq
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
R4 seq
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
EXON 1
+81 ACGACCGTCA CCATGGAAGT GTCACCATTG CAGGTACGGC TCGCGGGGGG
ATG-BAM
+131 GACTGGCGGT GGAGCCTCCG CGCGGCCCGG GCATCTCTCT GGCCGCCCGT
+181 GACGGGTGAA GCTCTGGGGC TGCGGTCAGG CCGGCGACCG GCTTGGGAGC
+231 CCATATTCTC CATTCCGGTT CCGGGGTGAT CGTGGAGAGG CGGAAGCCCC
topoIIacodrev
+281 TTCTGGTGCT AGTAGTGAAG TATGACCCGG CTTCCAGGGT GTCGT

(b)

This study	Isaacs *et al.* (1996)
GC2 TAAA**G**GGGGCGGGGT	GC2 TAAAGGGGCGGGGT

(c)

This study	Sng *et al.* (1999)
(+117) GGC**TCGCGG**GGGGGACT	(+117) GGC**AA**GGGGGACT

Appendix 7. (a) Summary of the human topo IIα promoter as sequenced from genomic DNA in this study. The primers for both sequencing (seq) and cloning are labeled. The cloning primers had a Xba I site added to the 5' end while the ATG-BAM had a 5' Bam HI site added. The position of the differences between the sequence derived in this study and that of Hochhauser, University of Oxford, unpublished, are highlighted in green. The different nucleotides are shown in bold. The sequence missing from the GenBank sequence X66794 is highlighted in blue. Differences between the sequence from this study and (b) Isaacs et al. (1996) (c) Sng et al. (1999).

Appendix 8. Putative transcription factors binding sites in the human topo IIα promoter

Summary of some of the results of the identification of putative transcription factor binding sites found in the human topo IIα promoter using the GCG Tfsites database (University of Wisconsin Genetics Computer Group) and the TRANSFAC Version 4.0 database (Wingender *et al.*, 2000). (+) in the sense strand, (-) in the antisense strand.

Factor	Position
AHRARNT	-1422(+)
AP1	-1966(+), -1919(-), -1880(-), -1758(+) -1546(+), -1545(+/-),-1493(-), -1470(-), -1215(-), -873(-), -868(+/-), -437(-), -247(+/-), -228(+), -169(+),-79(-), +80(-)
AP2	-2017(+/-), -1302(-), -879(-), -812(+), -647(-), -330(-)
AP3	-1984(+), -951(-)
AP4	-1742(-), -1681(+/-), -1528(+),-1200(+/-), -1135(+/-), -1104(-), -1088(+), -1032(-), -775(-), -638(-), -145(+)
ATF	-439(-), -229(+), +78(-)
BARBIE	-1695(+), -604(-), -569(+)
BRN2	-1820(+), -1751(+), -918(-), -402(+)
CAAT	-1751(-), -1698(-), -1607(+), -1159(+), -525(+), -390(-), -260(-), -176(-), -109(-), -69(-)
CDPCR3HD	-2010(-), -1326(+), -824(+), -393(+)
C/EBP B	-1982(+), -1905(+), -1865(-), -1773(-) -1717(+), -1633(+),-1129(+), -997(+), -985(-), -974(-), -850(+), -483(-), -478(+), -341(-), -293(-), -45(+)
CETS1P54	-1896(-), -1677(+), -1382(+), -1108(+), -781(-), -768(+), -475(+), -389(-), -324(-), -98(+), +34(-)
CHOP	-1317(-)
C-MYB	-1347(+), -1051(+), -949(-), -426(+), -391(+), -19(-)
CP2	-1325(-), +13(-)
CREBP1CJUN	-1492(-), -436(-)
CREBP1	-1979(-)
CREB	-1492(-), -438(-), -436(-), -230(+), -228(+), -226(+), +79(-), +81(-)
CREL	-1899(-), -1786(-), -1222(+), -960(-), -386(+)
DELTAEF1	-1899(+), -1864(+), -1720(-), -1690(-), -1540(+), -1493(-), -1474(+), -1348(-), -1173(-), -1017(+), -886(-), -763(-), -183(+), -135(-)
E2F	-1125(-)
E2	-846(+)
E47	-1542(-), -1203(+/-), -1202(+), -766(+), -765(+), -550(+)
E4BP4	-1981(+/-), -1848(+)
ER	-1975(+), -1469(-)

Factor	Position
Ets-1	-1772(-)
FREAC2	-1869(-), -1614(-), -375(+)
FREAC4	-1869(-)
FREAC7	-923(-), -916(-), -912(-), -908(-), -900(-), -375(+)
GATA1	-1536(-), -1147(-), -1065(-), -1063(-), -897(-), -895(-), -132(+), -131(+)
GATA2	-1824(+), -1063(-), -895(-)
GATA3	-1824(+/-), -1063(-), -895(-), -412(+), -403(+)
GATA	-1533(-), -1148(-), -1066(-), -898(-), -128(+)
GC	-563(+), -195(-)
GFI1	-1370(+), -1296(-), -1062(-), -849(+), -544(+), -421 (-), -401(-), -262(-), -247(+), -187(-), -120(-), -80(-)
GKLF	-1961(-), -1057(-), -743(-), -572(+), -567(+), -566(+), -469(+), -362(-), -325(-), -324(-), -306(-), -265(-), -246(-)
GRE	-429(+/-)
HFH1	-1868(+), -915(+), -372(-)
HFH2	-1938(-), -1937(-), -1811(+), -1649(+), -1613(+), -1600(-), -1437(-), -1273(-), -1272(-), -1271(-), -1270(-), -1269(-), -1262(-), -1254(-), -1246(-), -1242(-), -924(+), -915(+), -911(+),-596(+), -372(-)
HFH3	-1939(-), -1938(-), -1868(+), -1649(+), -1648(+), -1601(-), -1438(-), -1432(-), -1274(-),-1273(-), -1272(-), -1271(-),-1270(-), 1269(-), -1259(-), -1255(-), -1247(-), -1243(-)
HFH3	-924(+),-922(+), -915(+), -911(+), -907(+), -899(+), -740(+), -596(+), -373(-), -167(-)
HFH8	-1868(+), -1613(+), -1255(-),-915(+), -913(+), -907(+), -899(+), -373(-), +42(+)
HLF	-1980(+/-), -1847(-)
HNF1	-1815(+)
HNF3B	-1939(-), -1938(-), -1879(+), -1813(+), -1651(+), -1601(-), -1438(-), -1432(-), -1274(-), -1273(-), -1272(-), -1271(-),-1270(-), -1269(-), -1255(-), -1247(-), -1243(-), -926(+), -917(+), -913(+), -909(+), -901(+), -742(+), -598(+), -373(-), -282(-), -167(-),+40(+)
IK1	-453(-), -325(-), -252(-)
IK2	-2014(+), -1734(+), -1533(-), -1384(+), -1062(-), -1020(+), -815(-), -794(-), -660(-), -647(-), -477(+), -458(-), -452(-), -406(+), -324(-), -251(-), -196(-), -189(-), -74(+)
IRF1	-1056(-), -732(-)
IRF2	-1056(-)
LMO2COM	-1682(+/-), -1540(-), -1534(-), -1499(-), -1201(+/-), -1145(-), -1137(+/-), -1063(-), -1046(+), -948 (-), -895(-), -764(+), -549(+), -129(+), -33(+)
LYF1	-1801(+), -645(-), -187(-)
MYCMAX	-1499(+), -1070(+)
MYOD	-1681(+/-), -1540(-), -1539(+), -1201(+/-), -1200(+/-), -1137(+), -1136(+/-), -1045(-), -947(+), -764(+), -763(-)

Factor	Position
MYOD	-548(-)
MZF1	-2014(+), -1020(+), -811(-), -448(-)
NF1	-1750(+), -1638(+), -1411(+), -1225(+), -1147(-), -1141(+), -626(-), -552(-), -389(+), -340(-), -259(+), -161(-), -68(+)
NFAT	-1944(+), -1903(+), -1796(+),-1699(+), -1220(-), -1127(+), -733(-), -589(+), -483(-),-476(+), -293(-)
NRF2	-1685(-), -1376(+), -324 (-)
NFκB50	-2011(+)
NFκB65	-1222(+)
NFκB	-2011(+), -1475(-), -960(-)
NFY	-1211(-), -1208(-), -1157(+), -646(+), -644(+), -394(-), -391(-), -264(-), -261(-), -180(-), -178(+), -177(-), -113(-), -110(-), -73(-), -70(-)
NKX25	-1854(-), -1814(+), -1812(-), -1523(-),-1496(-), -936(-), -927(+), -925(-), -346(-), -275(+),-31(+)
NMYC	-1386(-), -287(-), +26(+)
NRF2	-1789(+)
OCT1	-1751(-), -1595(+), -1589(-), -1292(+), 932(+), -601(+), -133(-)
PADS	-1501(-), -952(-), +23(+)
PEA1	-1544(-)
PEA3	-1900(+), -1807(-), -1793(+), -1771(+), -1711(+), -1707(+), -1217(+), -1039(+), -730(+), -480(+), -473(+), -39 (+)
RFX1	-1985(+), -1339(-), -1212(+), -699(-)
RORA1	-1965(-), -1496(+)
RREB1	-955(+)
S8	-1937(+), -1818(+), -1816(-), -1679(+), -1623(-), -1581(-), -1431(+), -1399(-), -931(+), -929(-), -922(+)
SOX5	-1975(+), -1923(+), -1919(-), -1657(-)
SP1	-1097(-), -563(+), -194(-), -153(+)
SREBP1	-1876(+), -1499(+)
SRY	-1976(+), -1920(-), -1658(-), -1615(-), -1433(+), 1260(+), -1252(+), -1244(+), -441(+), -279(+),-164(+)
STAT	-1631(+)
T3R	-1495(+)
TAL1ALPHA4E7	-1203(+/-)
TAL1BETA47	-1203(+)
TAL1BETAITF2	-1203(+/-)
TAXCREB	-1182(+)
TCF11	-1973(-), -1914(+), -1875(+), -1847(+), -1820(+), -1765(-), -1553(-), -1465(+), -1210(+), -868(+), -587(-), -432(+), -242(+), -235(-), -74(+)
TH1E47	-1966(+), -1644(+), -1198(+), -1147(+), -544(-), -257(-)
TST1	-1676(+), -1401(-), -931(-), -600(-), -580(+)

Factor	Position
USF	-1538(+), -1498(+), -1497(+), -1385(+), -1384(-), -762(-), -285(-), -226(-), -275(-), -222(-), -31(-), +27(-),+30(+)
VBP	-1980(+/-), -1847(-), -1589(-)
VMYB	-1792(+), -1651(-), -1044(-), -947(+), -402(-), -385(-), -142(-), -141(-), -17(+)
XFD1	-1869(-), -916(-), -912(-), -908(-), -900(-)
XFD2	-1869(-), -923(-), -916(-) -912(-), -908(-), -373(+)
ZID	+62(-)

Appendix 9. The optimization results for the transient transfection experiments

(a) MCF-7

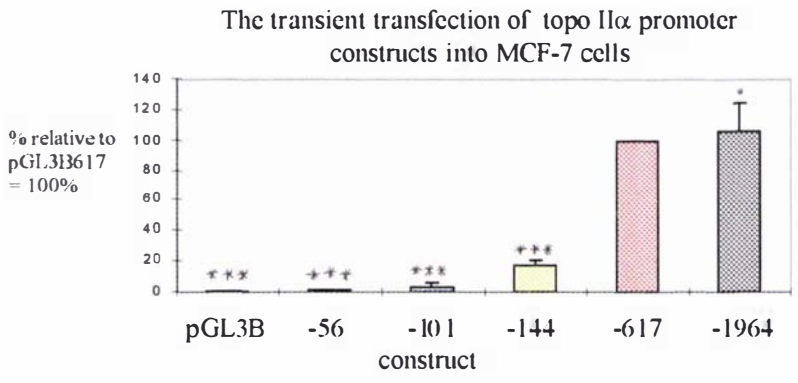
Incubation time		6 hr		18 hr	
pGL3B617	pCH110	Luciferase (relative light units)	β-gal (A_{405})	Luciferase (relative light units)	β-gal (A_{405})
1 μg	1 μg	~6	~0.2	~2.5	~0.20
2 μg	1 μg	~6	~0.2	~2.0	~0.15
3 μg	1 μg	~10	~0.2	~3.0	~0.15
3 μg	2 μg	~11	~0.3	~2.0	~0.20

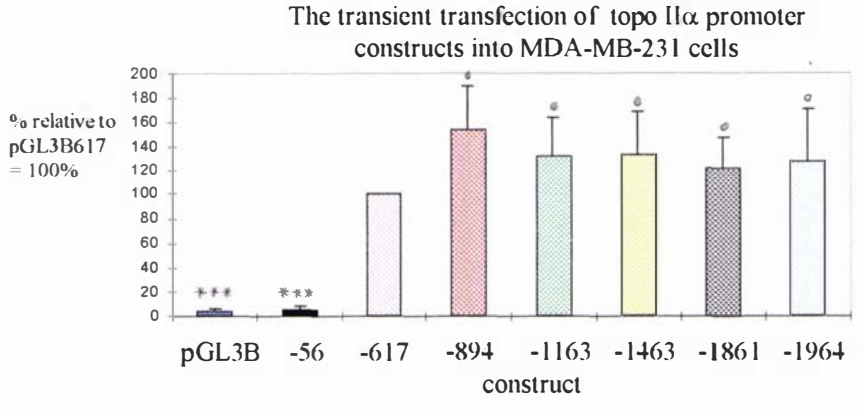
(b) MDA-MB-231

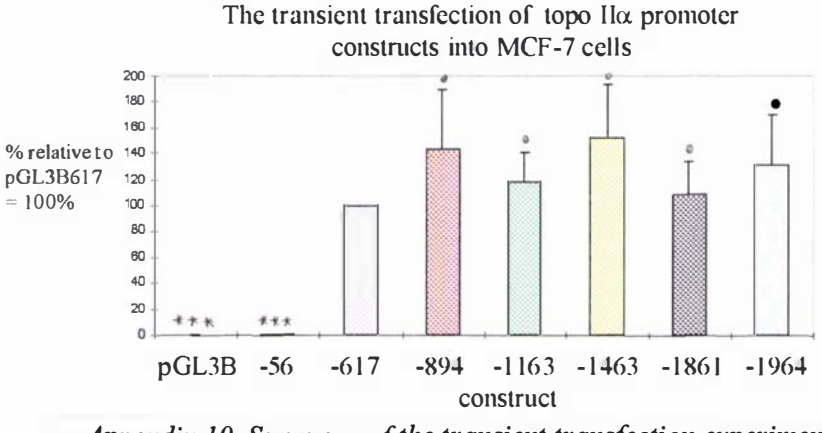
Incubation time		6 hr		18 hr	
pGL3B617	pCH110	Luciferase (relative light units)	β-gal (A_{405})	Luciferase (relative light units)	β-gal (A_{405})
1 μg	1 μg	~0.3	~0.1	~0.03	~0.1
2 μg	1 μg	~0.5	~0.1	~0.08	~0.1
3 μg	1 μg	~2	~0.1	~0.10	~0.1
3 μg	2 μg	~1	~0.15	~0.15	~0.1

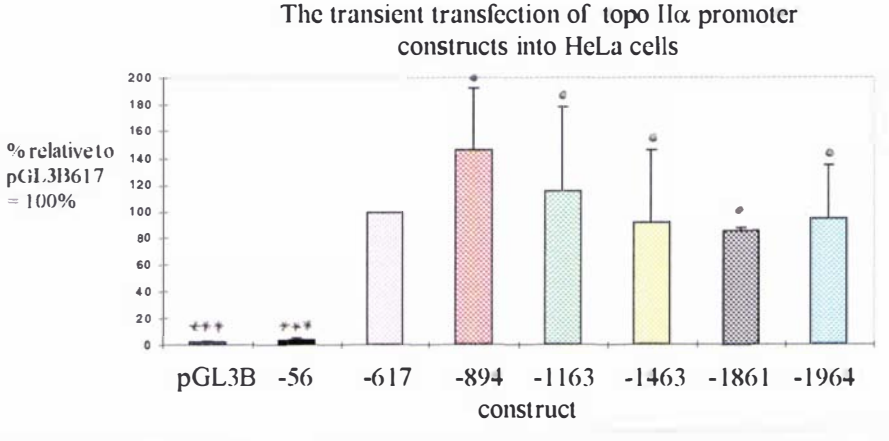
Appendix 9. The results from (a) MCF-7 and (b) MDA-MB-231 cells transiently transfected with pGL3B617. In each experiment either 1, 2 or 3 μg of pGL3B617 was co-transfected with 1 or 2 μg pCH110. After 6 or 18 hours the cells were washed and incubated for a further 18 hours. The cells were then harvested and the luciferase and β-galactosidase levels determined.

Appendix 10. Summary of the results of transient transfection experiments investigating the activity of different topo IIα promoter constructs in the breast cancer cell lines



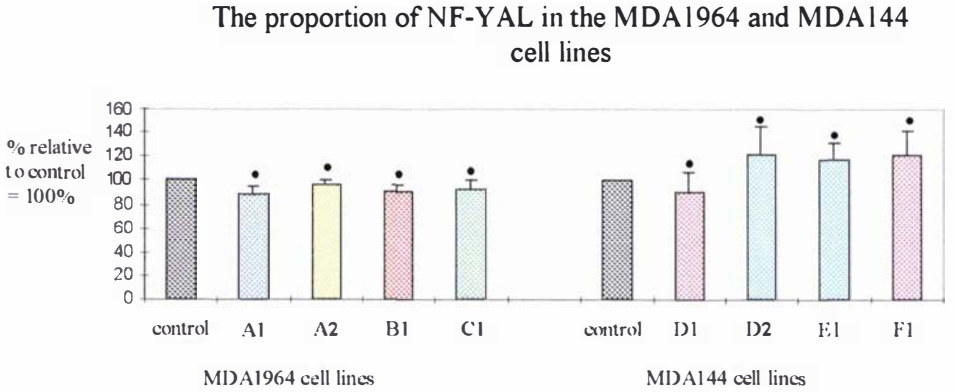


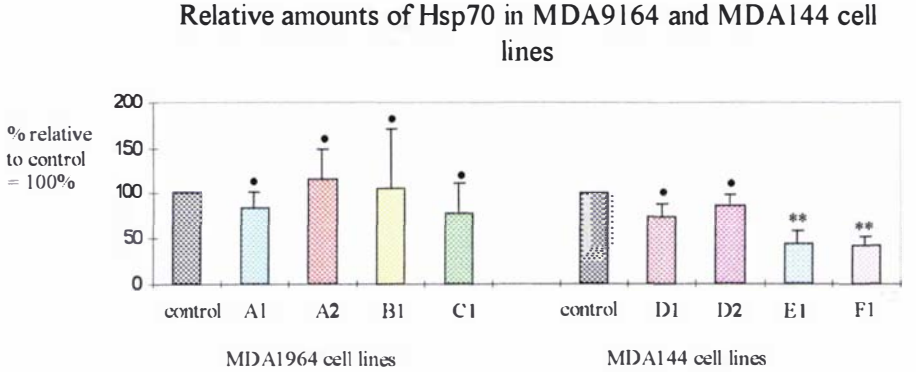


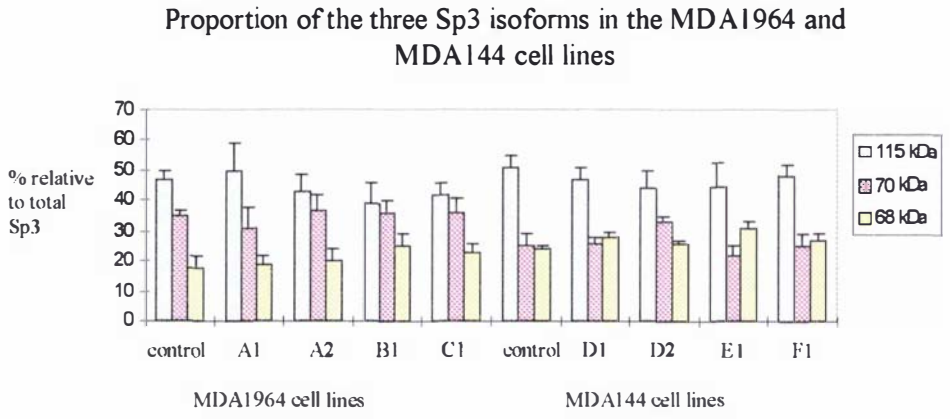


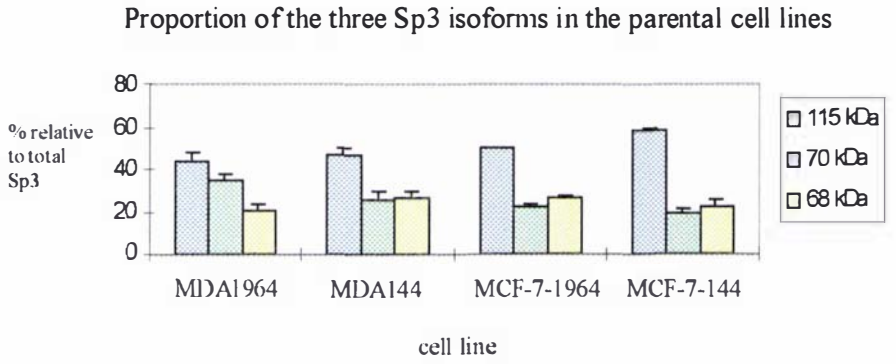
*Appendix 10. Summary of the transient transfection experiments of topo IIα promoter constructs using MDA-MB-231, MCF-7 and HeLa cell lines. In each experiment 3 μg of each luciferase reporter construct was co-transfected with 2 μg β-galactosidase reporter vector into each cell line. After 24 hours the cells were harvested and the luciferase and β-galactosidase levels determined. The luciferase levels were normalized to the β-galactosidase levels and the pGL3B617 result was arbitrarily set at 100% and the rest of the results compared to this. The average (± standard deviation) data are shown. Each experiment was performed at least three times and in triplicates within each experiment. The Student T test (2 tailed) was performed to determine the statistical significance of these results compared to that of the pGL3B617 construct *** $p < 0.005$, • $p > 0.05$.*

Appendix 11. Additional immunoblot results for the MDA1964 and MDA144 cell lines



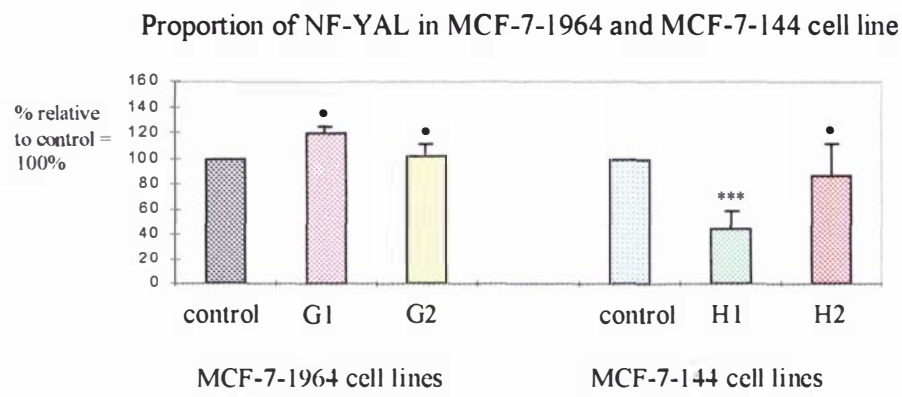


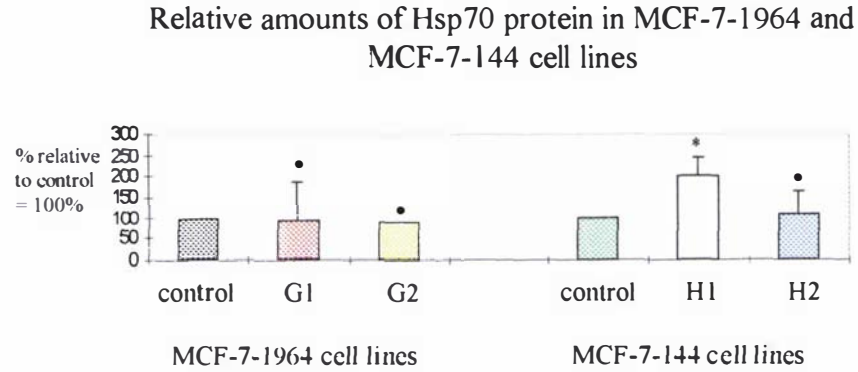


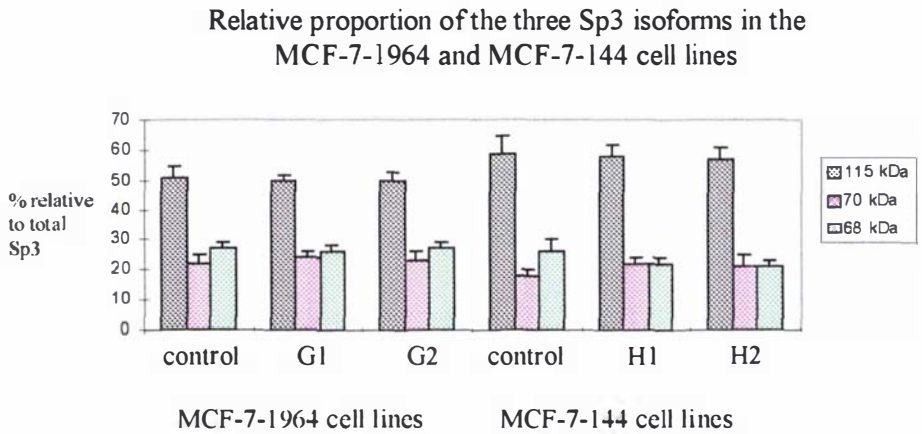


*Appendix 11. Summary of the immunoblot results using whole cell extracts from MDA1964 and MDA144 cell lines. The immunoblots were carried out as described in figure 4.3. At least three experiments were performed. The p values were calculated by the Student T-test (two-tailed) using Microsoft Excel. · $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Appendix 12. Additional immunoblot results for the MCF-7-1964 and MCF-7-144 cell lines

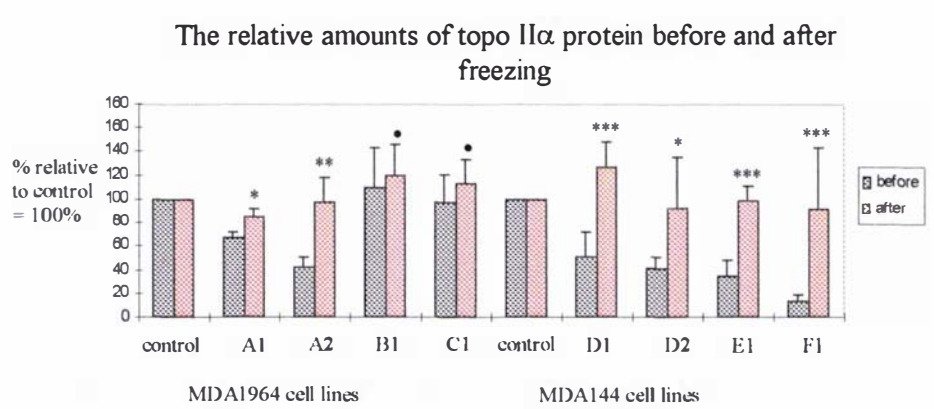


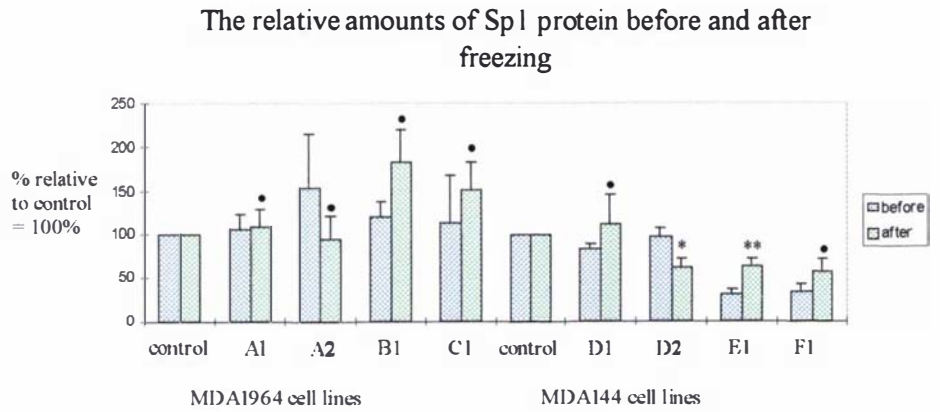


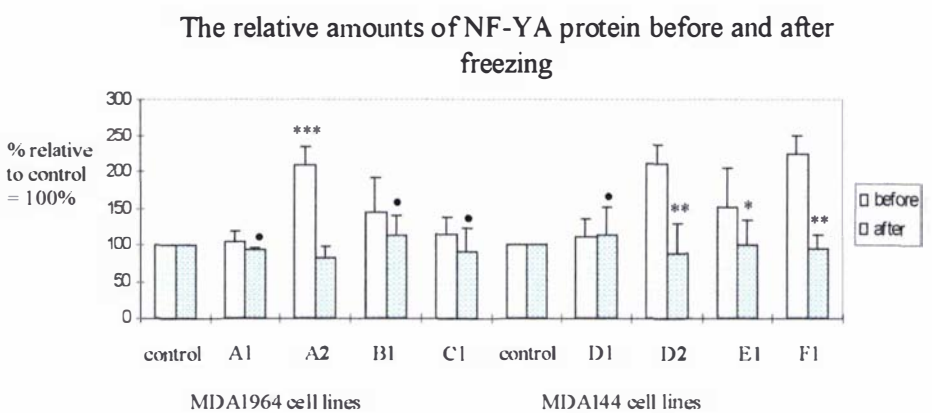


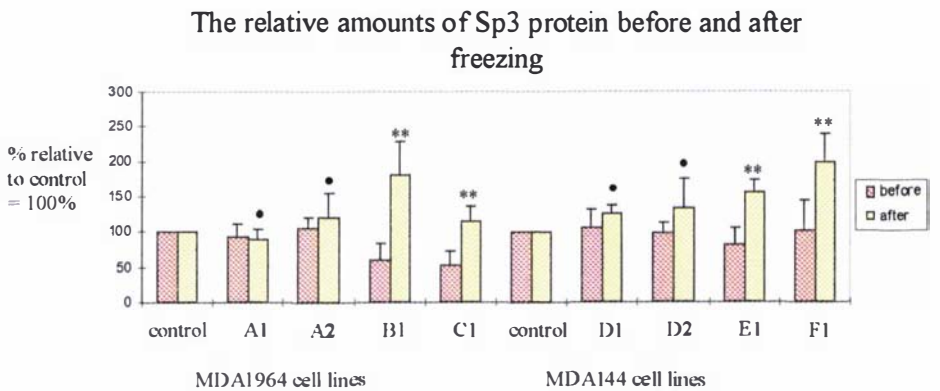
*Appendix 12. Summary of the immunoblot results using whole cell extracts from MCF-7-1964 and MCF-7-144 cell lines. The immunoblots were carried out as described in figure 4.3. At least three experiments were performed. The p values were calculated by the Student T-test (two-tailed) using Microsoft Excel. · $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Appendix 13. Comparison of the "before" and "after" freezing immunoblot results for the MDA1964 and MDA144 cell lines





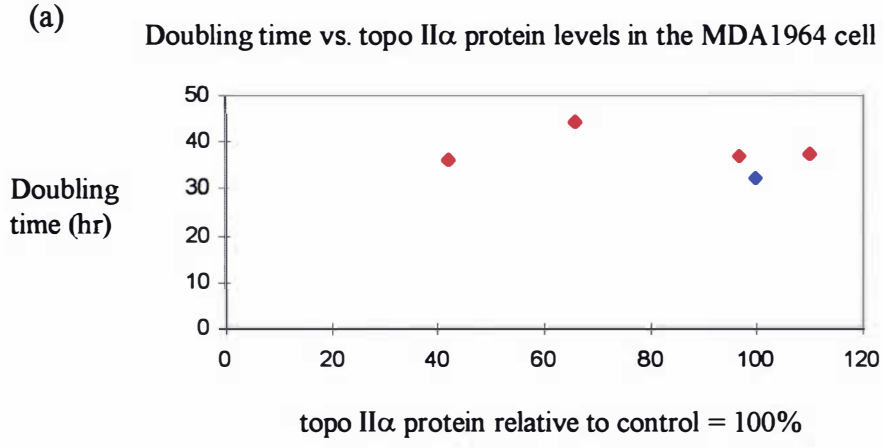




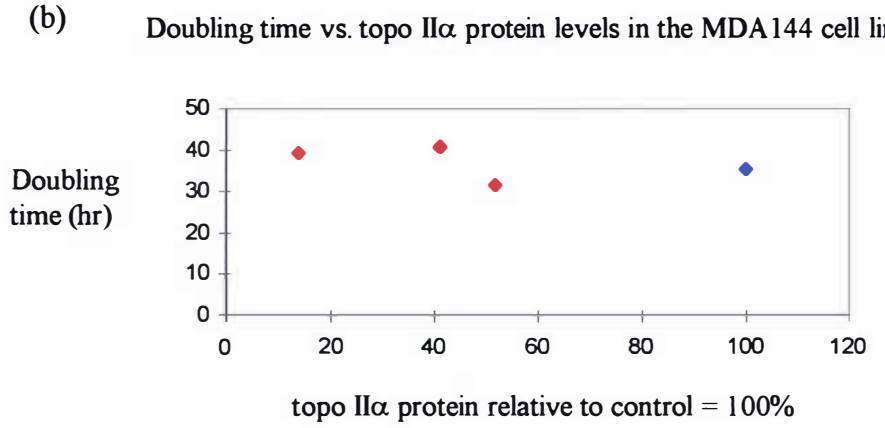
*Appendix 13. Summary of the immunoblot results using whole cell extracts from MDA1964 and MDA144 cell lines before and after freezing. The immunoblots were carried out as described in figure 4.3. At least three experiments were performed. The p values were calculated by the Student T-test (two-tailed) using Microsoft Excel · $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Appendix 14. Correlation between the doubling time and the relative amounts of topo IIα protein for the MDA1964 and MDA144 cell lines.

(a)



(b)

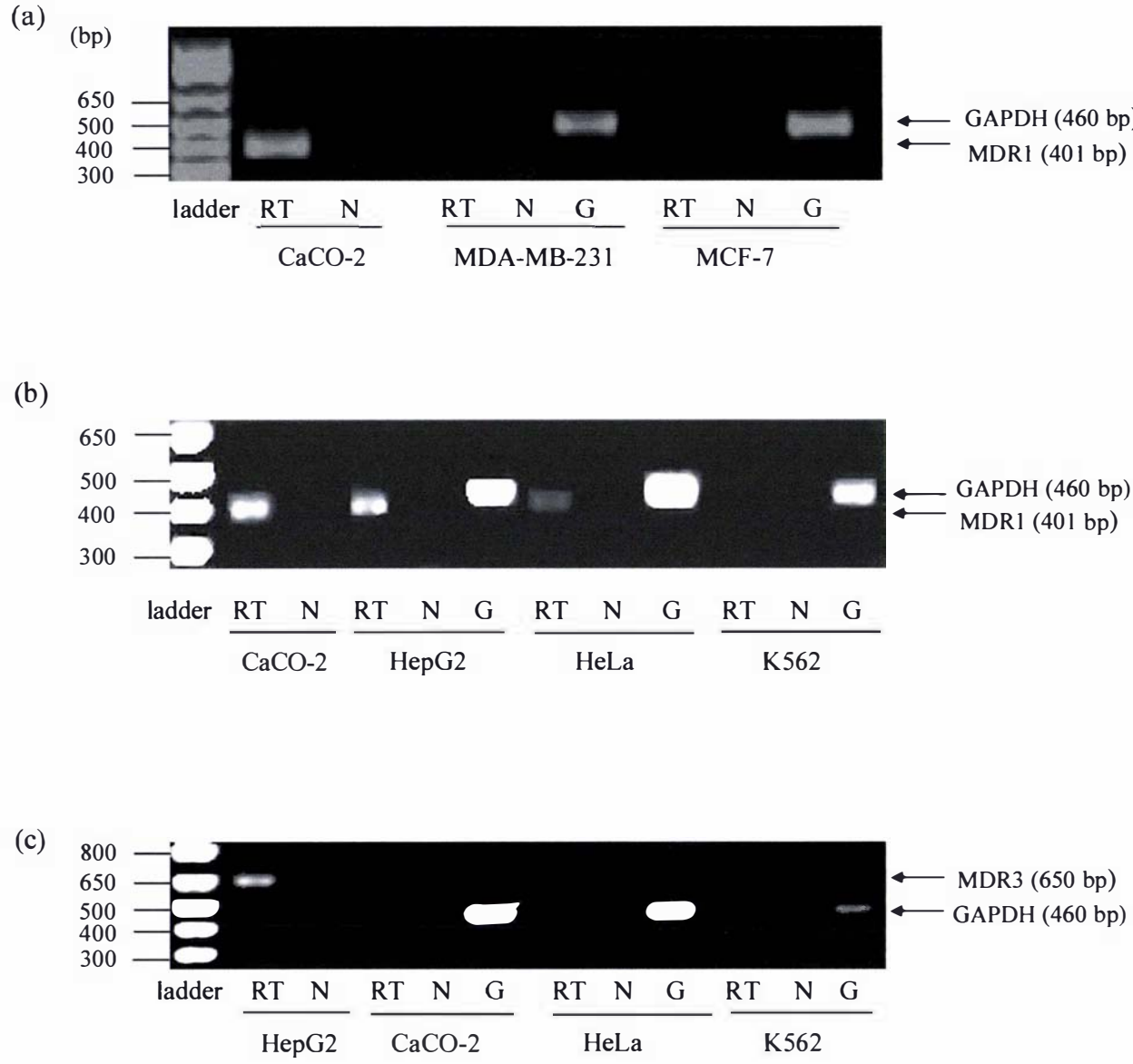


(c)

Cell line	Correlation
MDA1964	-0.353
MDA144	-0.436

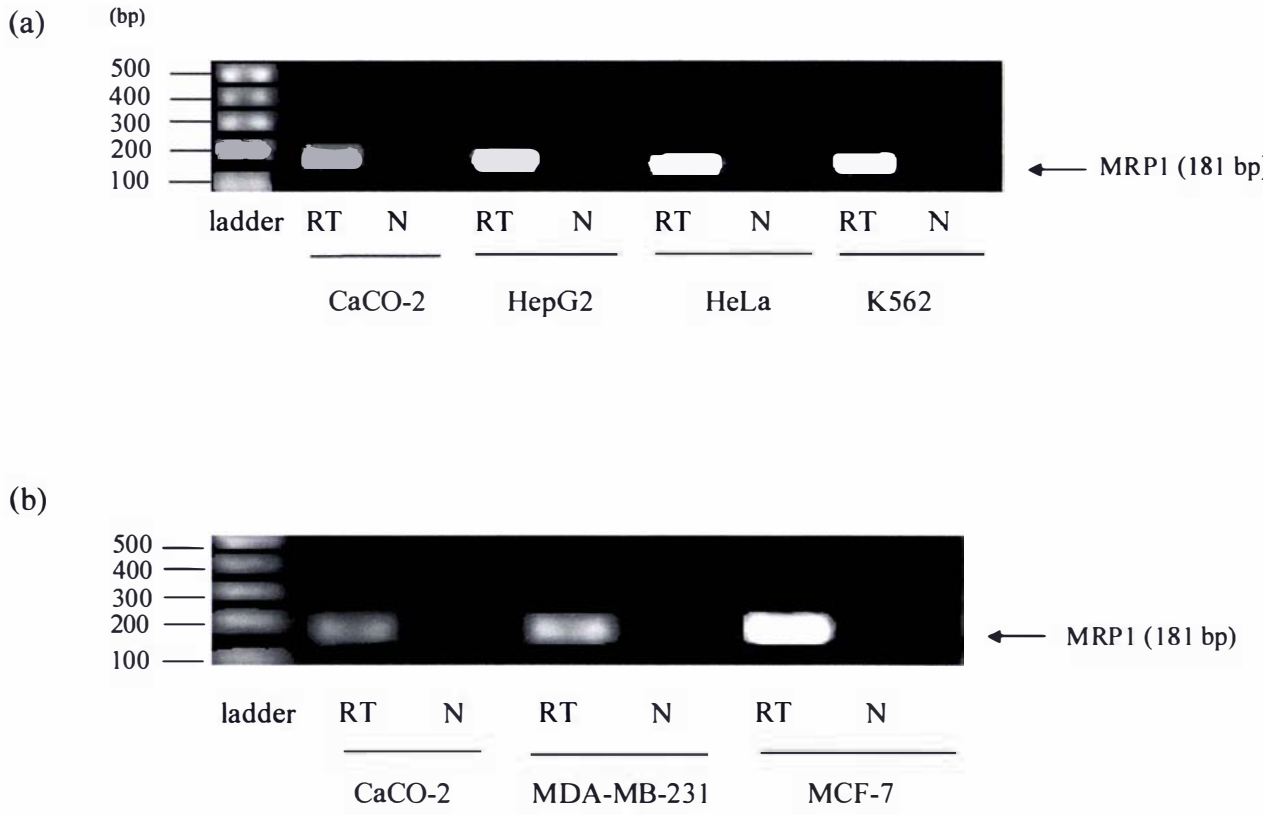
Appendix 14. Scatter graph of doubling time vs. topo IIα protein levels for the (a) MDA1964 and (b) MDA144 cell lines. The control cell line is indicated by a blue dot. (c) The results of correlation analysis for MDA1964 and MDA144 cell lines using Microsoft Excel. A correlation of -1.00 indicates a significant result.

Appendix 15. The expression of MDR1 and MDR3 mRNA in various cancer cell lines



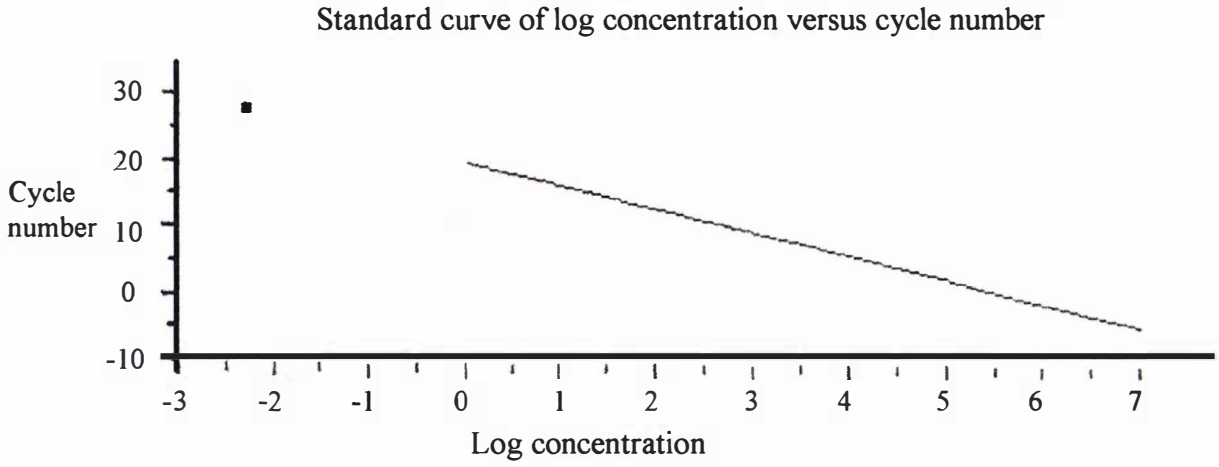
Appendix 15. RT-PCR to detect (a) (b) MDR1 and (c) MDR3 mRNA in MDA-MB-231, MCF-7, CaCO-2, HepG2, HeLa or K562 cell lines. CaCO-2 cDNA was used as a positive control for MDR1, while HepG2 cDNA was used as a positive control for MDR3. GAPDH (G) was used as a control for the RNA loading and the reverse transcriptase reaction (RT). To confirm that the products seen were amplified from cDNA and not genomic DNA, RT reactions where the reverse transcriptase was omitted (N) were performed. A no template control was performed to check for contamination. The RT-PCR was performed as described in section 2.2.3. 5-10 µl of the PCR products were separated on a 1% agarose gel in 1x TAE (80 V for 30 min) and stained with ethidium bromide.

Appendix 16. The expression of MRP1 mRNA in various cancer cell lines



Appendix 16. RT-PCR to detect MRP1 mRNA in (a) CaCO-2, HepG2, HeLa and K562 and (b) MDA-MB-231, MCF-7 and CaCO-2 cell lines. CaCO-2 cDNA was used as a positive control for MRP1. To confirm that the products seen were amplified from cDNA and not genomic DNA, RT reactions where the reverse transcriptase was omitted (N) were performed. No template reactions were performed to check for contamination. The RT-PCR was performed as described in section 2.2.3. 5-10 μl of the PCR products were separated on a 1% agarose gel in 1x TAE (80 V for 30 min) and stained with ethidium bromide.

Appendix 17. Example of a real time RT-PCR experiment to determine the levels of topo IIα and hGH mRNA in confluent and freely proliferating cells



Example of the results from a real time RT-PCR experiment to determine the levels of topo IIα and hGH mRNA in confluent and freely proliferating cells. RNA was extracted from MDA-MB-231 cells that were either freely proliferating or grown to confluence. 5 μg RNA plus 1μg K562 RNA was treated with DNAse I and then reverse transcribed as described in section 2.2.3. 5 μL of the RT reaction (1:5 dilution) was added to the PCR reaction mix containing 0.5 μM of each primer (Appendix 4), 1x SYBR Green 1 Faststart Master Mix and 3-4 mM $MgCl_2$. The "concentration" of the standard was arbitrarily set at $5x10^{-3}$ (the dilution factor used for this sample). To account for variations between experiments, the cross over cycle number of the standard was used to correctly position the standard curve (above). The "concentration" of the other PCR products was determined from the standard curve of cycle number versus log concentration of the standard (hGH cDNA). The "concentration" of the topo IIα mRNA and hGH mRNA was calculated relative to α-globin mRNA. The mRNA "concentrations" of the freely proliferating samples were arbitrarily set at 100% and the mRNA "concentrations" of the confluent cells compared to this (below).

	Cross over cycle	"concentration"	"concentration" relative to α-globin	% relative to the free value
Standard	27.57	5.000E-03	-	-
topo IIα freely proliferating	26.84	8.087E-03	5.72	100%
hGH freely proliferating	27.65	4.726 E-03	33.50	100%
α-globin freely proliferating	24.94	2.828 E-03	1.00	-
topo IIα confluent	28.26	3.165 E-03	1.20	21%
hGH confluent	27.46	5.370 E-03	20.38	61%
α-globin confluent	23.99	5.270 E-03	1.00	-