A yeast model of Bloom's syndrome.

Thesis

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A Yeast Model of Bloom's Syndrome

Thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Bloom's syndrome (BS) is a rare inherited disorder associated with growth retardation, immunodeficiency and a predisposition to cancers of all types. At a cellular level BS is associated with marked chromosomal instability, as manifest by interchanges between homologous chromosomes and sister chromatids. BS arises as a consequence of defects in a DNA helicase, BLM, which is a member of the RecQ family of helicases. Defects in other RecQ family helicases, derived from prokaryotic and lower eukaryotic cells, also result in a loss of genomic integrity. This suggests that this group of helicase subfamily share a degree of functional conservation.

In this thesis, the validity of using the budding yeast, *S.cerevisiae,* as a model system for BS has been assessed. In this organism, the structural homologue of the BLM protein is Sgs1p. Deletion of Sgs1p is associated with mitotic hyperrecombination and defective chromosome segregation. Deletion of *SGS1* also suppresses the slow growth and hyperrecombination phenotype of *top3* mutants, which are defective in topoisomerase III. Ectopic expression of a wild type *SGS1* gene in *sgs1Δtop3Δ* mutants reinduced the slow growth defect, and this effect was reproduced when the human *BLM* gene was expressed. This result suggested firstly, that the yeast and human proteins share functional similarities and secondly, that the interaction between the RecQ family helicases and topoisomerase III is highly conserved. On this basis, the remainder of this thesis focussed upon the role of topoisomerase III.

Deletion of the *S.cerevisiae TOP3* gene leads to a slow growth phenotype accompanied by an accumulation of cells with a late S/G2 content of DNA. *top3Δ* mutants exhibit a *RAD24/RAD9*-dependent delay in the G2 phase, suggesting a role for topoisomerase III in the resolution of abnormal DNA structures/damage arising during S-phase. Consistent with this notion, *top3Δ* strains are defective in the intra-S-phase checkpoint that slows the rate of S-phase progression following exposure to DNA damaging agents and are sensitive to killing by a variety of DNA damaging agents, including ultra-violet light, γ-rays and the alkylating agent MMS. This S-phase checkpoint defect was not associated with a failure to induce expression of DNA damage response genes. Consistent with an S-phase specific role for topoisomerase III, expression of the *TOP3* mRNA is activated in the late G1 phase, and DNA damage checkpoints operating outside of S-phase are unaffected by deletion of *TOP3*. All of the phenotypic consequences of loss of topoisomerase III function were suppressed by deletion of *SGS1*. These data implicate topoisomerase III and, by inference, Sgs1p in a checkpoint role in response to DNA damage arising during S-phase. A model to explain the role of these proteins during DNA replication is proposed.

CONTENTS

To my parents

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The last three years have felt very much like a non-ending obstacle course: one obstacle is negotiated only for another to block the way. It has without doubt been the most challenging and difficult period of my medical career. I hope that this time spent in laboratory research has taught me two things: first, the ability to critically appraise medical or scientific research and second, a real appreciation of just how hard it is to be successful in pursuing a scientific career. I would like to thank all those people who have helped me during this time.

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ABBREVIATIONS

ATP	adenosine triphosphate
ATM	ataxia telangiectasia mutated
BLM	Bloom's syndrome mutated
bp	base pairs
cdc	cell division cycle
cDNA	complementary DNA
CHEF	clamped homogeneous electric fields
DNA	deoxyribonucleic acid
DSB	double strand break
dsDNA	double-stranded DNA
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FACs	fluorescence-activated cell sorting
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HU	hydroxyurea
kb	kilobase pairs
kDa	kilodaltons
mRNA	messenger RNA
MMS	methyl methane sulfonate
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SCE	sister chromatid exchange
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulphate

ssDNA	single-stranded DNA
TBE	tris/borate/EDTA buffer
Tris	tris (hydroxymethyl)-aminomethane
ts	temperature sensitive
v/v	volume for volume
w/v	weight for volume
WRN	Werner's syndrome mutated

Chapter 1

Introduction

1.1 Introduction

The loss of genomic integrity is an invariant property of tumour cells. The study of inherited cancer-prone disorders has shed considerable light upon the means by which human cells maintain genomic integrity. Interpretation of the accumulating data in this field has been facilitated by comparative analyses in prokaryotic and eukaryotic model systems. These studies have demonstrated, for the most part, a remarkable degree of functional conservation of the individual players and pathways involved in the maintenance of genome stability in all organisms.

In the last decade, the cloning of genes defective in the disorders ataxia telangiectasia, xeroderma pigmentosum and hereditary non-polyposis colon cancer has generated important information regarding the role of cell cycle checkpoints, nucleotide excision repair and mismatch repair, respectively, in preventing genome instability and suppressing cancer (Shiloh, 1997; Wood, 1997; Jiricny, 1996). The prior dissection of many aspects of these processes in model systems was such that, at least in the cases cited above, putative functions could be assigned to many of the cloned human genes almost as soon as their protein coding sequences were available.

In this thesis, a yeast model has been employed in order to understand in greater detail, the basis for the loss of genomic integrity associated with the cancer-prone disorder, Bloom's syndrome (BS). This disorder arises as a consequence of a defect in a DNA helicase, which has structural similarity to a family of helicases found in prokaryotes and lower eukaryotes, the so-called RecQ family (Ellis et al., 1995). Defects in another RecQ family helicase are also found in another inherited disease, the progeria-like syndrome Werner's syndrome (WS) (Yu et al., 1996). To date, studies in prokaryotic (Hanada et al., 1997; Harman and Kowalczykowski, 1998) and yeast model systems (Watt et al., 1996; Stewart et al., 1997) suggest that the fundamental defect in both BS and WS is one of recombinational repair.

In the following section, a brief overview of recombinational repair will be presented, together with an analysis of how specific defects in this mode of repair, can result in other disorders associated with predisposition toward cancer.

1.2 The 'recombinational repair' disorders

1.2.1 Recombination- principles

Genetic recombination is the molecular process by which new combinations of genetic material are generated (reviewed in Friedberg et al., 1995). Recombination functions to generate genetic diversity either through conjugational recombination in prokaryotes or meiosis in eukaryotes. It is essential for repair of damaged DNA, particularly of double strand breaks (DSBs). DSBs can arise as a result not only of damage (caused by ionising irradiation or free radicals) but also as intermediates in 'scheduled' DNA rearrangements (mating type switching in yeast or V(D)J recombination in mammalian cells). A failure to resolve such lesions can have catastrophic consequences to the cell, since even a single unrepaired DSB can be a lethal event (Frankenberg-Schwager and Frankenberg, 1990). Recombination interfaces or overlaps with nearly all aspects of DNA metabolism, a fact that until recently has been underplayed in the literature. Thus, one difficulty in assessing the various pathways involved is that a single protein can affect a multitude of processes, some in which its function is an absolute requirement and others where it is not essential but can still affect the outcome.

Four broad classes of recombination event have been proposed (Heyer and Kohli, 1994); (1) homologous recombination, which depends upon extensive sequence homology and can be defined according to the locations of the sequences combining; (2) site-specific recombination, including V(D)J rearrangements in lymphoid cells, where recombination occurs at specific sequences, sharing little or no homology; (3) transposition, in which DNA sequences are moved from one site in the genome to another in a random fashion; and (4) illegitimate recombination, in which neither significant sequence homology nor specific locations are involved, and which can result in insertions, deletions and other DNA rearrangements. Two basic mechanisms of recombination are homologous recombination involving strand exchange, and non-homologous recombination which involves 'end ining' of DNA. These are described briefly below, together with emerging evidence to suggest that defects in these two processes in humans can pre-dispose to the development of cancer.

1.2.2 Homologous recombination

The primary mechanism for the formation of heteroduplex DNA in homologous recombination involves strand exchange, and this is likely to be applicable to both

prokaryotes and eukaryotes (reviewed in Eggleston and West, 1996). This type of recombination comprises several steps: (1) processing of a duplex molecule to form the substrate for strand exchange (that is, linear DNA with a single-stranded tail); (2) alignment of the homologous parental molecules; (3) strand invasion and the formation of a 4-stranded structure, known as a Holliday junction; (4) migration of the Holliday junction to extend the region of heteroduplex DNA; and (5) resolution of the interlinked molecules to give recombinant progeny. A diagrammatic representation of this basic process, together with the key players involved at each stage in prokaryotes is shown in Figure 1.1. It seems likely that the fundamental features of this mode of recombination are conserved in eukaryotes. In *E.coli,* three distinct pathways of homologous recombination can be defined at a genetic level: the RecBCD pathway, the RecF pathway and the RecE pathway (Dunderdale and West, 1994; West, 1994). Although previous studies, skewed toward an analysis of conjugal recombination, suggested that the RecBCD pathway was the primary pathway for homologous recombination in this organism, a more recent consensus is that the RecF pathway is just as important to the cell and plays a key role in post-replication gap repair (as discussed in section 1.3). In eukaryotes, homologous recombination that does not involve strand exchange and which involves proteins that participate in nucleotide excision repair ('single strand annealing') has been described (Haber, 1992).

1.2.3 RecA and RecA homologues

Central to each of the recombination pathways in *E.coli* is the RecA protein, which catalyses homologous pairing and strand exchange (Kowalczykowski and Eggleston, 1994; Roca and Cox, 1997). This protein binds single-stranded DNA (ssDNA) with high affinity and stoichiometry of one RecA monomer per three nucleotides, to form helical nucleoprotein filaments in which the DNA is extended and underwound (Stasiak and Egelman, 1988). The RecA-bound filaments provide the structural framework for a homology search which involves the pairing with duplex DNA (Howard-Flanders et al., 1984). In this structure, two homologous DNA molecules are brought in close proximity and ATP-dependent strand exchange occurs with a 5'-3' polarity relative to the ssDNA (Cox and Lehman, 1981).

The budding yeast *RAD51* gene, a member of the *RAD52* epistasis group, encodes a eukaryotic homologue of RecA (Aboussekhra et al., 1992; Basile et al., 1992; Shinohara

et al., 1992). Like RecA, yeast Rad51 protein forms helical nucleoprotein filaments (Ogawa et al., 1993) and promotes homologous pairing and strand transfer reactions (Sung, 1994), although the polarity of strand transfer is opposite, being 3'-5' (Sung and Robersson, 1995). Transcription of the *RAD51* gene is upregulated at the G1/S cell cycle transition and also following exposure to DNA-damaging agents (Basile et al., 1992). Deletion of *RAD51*, results in sensitivity to ionising radiation and to methyl methane sulfonate (MMS), and is associated with defects in meiosis or mating-type switching (Petes et al., 1991). Levels of spontaneous and radiation-induced mitotic recombination are reduced significantly in *rad51* mutants. Similar phenotypes are associated with defects in other *RAD52*-epistasis group genes, including *RAD52*, *RAD54* and *RAD57* (Petes et al., 1991).

Other homologues of RecA have been identified in both the mouse (Morita et al., 1993) and humans (Shinohara et al., 1993). Electron microscopic visualisation of human Rad51-DNA complexes shows that it forms RecA-like nucleoprotein filaments (Benson et al., 1994) and *in vitro* studies confirm that it catalyses ATP-dependent homologous pairing and strand transfer reactions (Baumann et al., 1996), albeit with an opposite polarity of strand transfer to the prokaryotic protein (Baumann and West, 1997). Mammalian Rad51 protein is upregulated at the G1/S cell cycle phase transition (Yamamoto et al., 1996a) and is expressed at high levels in gonadal and lymphoid tissues (Shinohara et al., 1993). This latter finding is consistent with a role in meiosis and V(D)J recombination. A role in meiosis is further suggested by the identification of human Rad51 protein in discrete foci on spermatocytes during meiotic prophase I when chromosomes undergo synapsis (Barlow et al., 1997).

In mouse somatic cells, Rad51 is induced following treatment with DNA damaging agents (Haaf et al., 1995). This role of RecA-like proteins in mediating double strand break repair is emphasised by the recent finding that the genes *XRCC2* and *XRCC3*, which complement the radiation-sensitivity of rodent cell mutants, both encode proteins with significant homology to RecA (Liu et al., 1998). In contrast to RecA or *S.cerevisiae* Rad51, mammalian Rad51 appears to be essential. Targeted disruption of murine *RAD51* gene leads to an embryonic lethal phenotype and it has not been possible to generate *RAD51-/-* ES cells (Lim and Hasty, 1996; Tsuzuki et al., 1996). This suggests that *RAD51* may be required for cell viability in mammalian cells, perhaps through essential roles in cell proliferation and/or genomic maintenance. In this context, the interactions

identified between human Rad51 and Brca1 and Brca2, both of which are defective in familial breast/ovarian cancer (reviewed in Zhang et al., 1998), may provide clues as to the nature of these essential functions.

1.2.4 Brca1 and Brca2 - a role in recombination?

About 1 in 20 cases of breast cancer in women results from a hereditary predisposition to the disease (Stratton and Wooster, 1996). Two breast cancer susceptibility genes, *BRCA1* and *BRCA2* have been cloned, which appear to be responsible for the majority of inherited breast cancer, but very few sporadic cases (Stratton, 1996). Women who inherit a defective *BRCA* gene have an 85% risk of breast cancer by the age 70 (Easton, 1997). *BRCA* genes act as tumour suppressors as the wild type gene is lost in the tumours of heterozygous carriers. Individuals who carry the defective genes are also at risk of other cancers, including ovary, prostate and pancreas.

Several lines of evidence have suggested a role for Brca1 and Brca2 in recombinational repair of DNA damage. First, both proteins associate with Rad51 protein. Brca1 and Rad51 proteins co-immunoprecipitate and co-localise in discrete nuclear foci of mitotic cells. In human meiotic cells, Brca1 and Rad51 proteins are associated with the unsynapsed elements of the synaptonemal complex (Scully et al., 1997a). Brca2 and Rad51 interact in a 2-hybrid assay and their tissue expression pattern is similar (Sharan et al., 1997). Second, Rad51 and Brca1/2 show co-ordinate cell cycle regulation (Rajan et al., 1996; Yamamoto et al., 1996a). Third, as with Rad51, both proteins appear to be involved in the cellular response to ionising irradiation (Scully et al., 1997a and 1997b; Thomas et al., 1997). Rad51/Brca1 nuclear foci disperse upon DNA damage, concomitant with phosphorylation of the Brca1 protein. Disruption of *BRCA2*, results in embryos that are hypersensitive to ionising irradiation, as observed in *RAD51* null embryos and arrest at a similar stage in embryonic development (Lim and Hasty, 1996; Tsuzuki et al., 1996; Scully et al., 1997a). Indeed certain cancer cell lines derived from *BRCA2*-defective individuals show marked sensitivity to ionising irradiation (Abbott et al., 1998).

A small number of recent studies have assessed the phenotype of cells homozygous for a defective murine *BRCA2* gene, encoding a truncated form of the protein. These homozygotes show gross growth retardation, the development of thymic lymphomas, and cells derived from them show sensitivity to DNA damaging agents (Connor et al.,

1997; Friedman et al., 1998; Patel et al., 1998). Most strikingly, such cells also demonstrate numerous spontaneous chromosomal anomalies, including chromatid breaks and the formation of triradials and quadriradials (Patel et al., 1998), features observed in *RAD51*-deficient embryos (Lim and Hasty, 1996).

1.2.5 Non-homologous recombination

Chromosomal breaks may be repaired by a process that requires little or no homology (reviewed in Chu, 1997; Tsukamoto and Ikeda, 1998). Non-homologous end joining (NHEJ) may be site-specific (as for V(D)J recombination) or non-specific (as for illegitimate recombinational repair of DSBs induced by DNA damaging agents). Both site-specific and non-specific NHEJ are likely to share similarities in their basic mechanism and in this regard may employ a similar protein machinery. In vertebrates, NHEJ appears to be the primary mode of repair for ionising irradiation-induced DSBs, whereas in budding yeast homologous recombination is the most prominent mode of repair (Friedberg et al., 1995). Thus, in the latter organism, studies of NHEJ usually require the disablement of *RAD52*-dependent homologous recombination functions.

The three major components necessary for NHEJ that appear to be conserved in all eukaryotes, are the Ku proteins, DNA ligase IV and the Rad50/Mre11 complex. A diagrammatic representation of a model for the steps involved in NHEJ is presented in Figure 1.2. The Ku proteins (Ku70 and Ku80) bind to the ends of DSBs and promote their alignment and/or their ligation (Dvir et al., 1993; Gottlieb and Jackson, 1993; Boulton and Jackson 1996a and 1996b). They may also act to bind other components of the NHEJ machinery including, in vertebrates, the serine/threonine protein kinase DNA-PK catalytic subunit (Hartley et al., 1995). In *S.cerevisiae*, loss of Ku70 or 80, results in an overall reduction in the frequency of joining of linearised plasmids with cohesive ends, and in those plasmids that are recovered, the joining is error-prone (Boulton and Jackson, 1996a and 1996b; Milne et al., 1996). Loss of either of the Ku proteins in budding yeast results in an increase in radiation sensitivity, but only when *RAD52*-dependent homologous recombination is disabled (Boulton and Jackson, 1996a and b; Siede et al., 1996c). A number of ionising irradiation-sensitive rodent cell lines are complemented by expression of human genes encoding Ku proteins (including *XRCC5* and *XRCC6*), and these cell lines are also defective in V(D)J recombination (Tsukamoto and Ikeda, 1998).

A second component of the NHEJ pathway are DNA ligases. These enzymes act to catalyse the joining of nicks in duplex DNA and the ends of DSBs during many aspects of DNA metabolism (Tomkinson and Levin, 1997). In *S.cerevisiae*, DNA ligase IV functions in Ku-dependent NHEJ, presumably at the final ligation step (Schar et al., 1997; Teo and Jackson, 1997; Wilson et al., 1997). In humans, it is less clear which of the four DNA ligases has an important role in NHEJ, since they can all promote end-joining of DNA *in vitro* (Ramsden and Gellert, 1998). The finding that human DNA ligase IV also interacts with Xrcc4, another participant in mammalian NHEJ, make it the most likely candidate (Critchlow et al., 1997; Grawunder et al., 1997). This is further suggested by the recent finding that *S.cerevisiae* DNA ligase IV interacts with an Xrcc4 homologue which is also defective in NHEJ (Herrmann et al., 1998).

The Rad50 complex also appears to participate in the same NHEJ pathway although the precise biochemical functions of this complex are not well understood. In *S.cerevisiae*, this complex consists of Rad50p, Mre11p and Xrs2p, and 2-hybrid analyses indicate that the three proteins physically interact (Alani et al., 1990; Malone et al., 1990; Ajimura et al., 1993; Johzuka and Ogawa, 1995). Mutants defective in these proteins are defective in meiosis but remain proficient in mitotic homologous recombination. These proteins participate in the Ku-dependent repair of DSBs and consequently, rejoining efficiencies of linearised plasmids is reduced in the respective mutants (Scheistl et al., 1994; Milne et al., 1996; Tsukamoto et al., 1997; Boulton and Jackson, 1998). This defect in NHEJ presumably underlies the sensitivity of the mutants to ionising irradiation. Human Mre11 and Rad50 homologues have also been identified and, as in yeast, appear to act together as a complex (Petrini et al., 1995; Dolganov et al., 1996). A possible human Xrs2 homologue, p95, has also been identified (see discussion below). The role of this complex in mediating NHEJ repair is supported by the finding that human Mre11 and Rad50 co-localise in nuclear foci following treatment with agents that induce DNA DSBs (Maser et al., 1997). Mre11 associates with DSBs shortly following irradiation (Nelms et al., 1998). Attempts to generate null *MRE11-/-* mutant ES cells show that *MRE11* is essential for viability (Xiao and Weaver, 1997).

1.2.6 Human disorders with potential defects in non-homologous recombination.

Defects in NHEJ might result in the loss of genomic stability and the development of cancer. This is suggested by the finding that human Mre11/Rad50 proteins act together

in a complex with another protein, p95, which is defective in Nijmegen Breakage syndrome (NBS). The protein p95 shows modest homology in the N-terminal region to budding yeast Xrs2 (Carney et al., 1998; Varon et al., 1998). NBS is a rare autosomal recessive disorder characterised by microcephaly, immunodeficiency and an increased risk of haematological malignancy (van der Burgt et al., 1996). At a cellular level, NBS shows increased sensitivity to ionising irradiation, as well as increased levels of spontaneous and induced levels of chromosomal fragility (Shiloh, 1997). Cells fail to induce p53 at the G1/S checkpoint and show the phenomenon of radio-resistant DNA synthesis, due to lack of an S-phase checkpoint that normally slows the rate of DNA replication when cells are damaged (Jongmans et al., 1997; Sullivan et al., 1997). In common with Rad50 and Mre11, p95 accumulates with similar distribution in cell nuclei following irradiation (Carney et al., 1998).

Many researchers have been struck by the similar clinical and cellular phenotypes of NBS and another inherited human disorder associated with genomic instability, ataxia telangiectasia (AT) (Shiloh, 1997; Stumm et al., 1997). AT is an autosomal recessive disorder characterised by progressive cerebellar degeneration, telangiectasia, immunodeficiency and a predisposition to haematological malignancy. *ATM*, the gene mutated in AT encodes a 350 kDa protein with significant homology to the signal transduction molecule, phosphatidyl inositol (PI)-3 kinase and to the checkpoint proteins Mec1 in budding yeast and Rad3, in fission yeast (Savitsky et al., 1995). At a cellular level NBS and AT are almost indistinguishable, in that they both show chromosomal instability, loss of checkpoints and sensitivity to ionising irradiation. Chromosomal instability in NBS cells is more marked, however (Shiloh, 1997). Fusion of AT and NBS cells fails to complement the chromosome instability, suggesting that p95 and ATM may act in the same pathway (Stumm et al., 1997). Furthermore, the formation of Rad50/Mre11 foci in AT cells post-irradiation is diminished (Maser et al., 1997) and studies of joining proficiency of linearised plasmids suggest a defect in NHEJ (Runger et al., 1992).

One explanation for the pleiotropic effects associated with loss of ATM is that this protein acts to sense damage to cellular macromolecules and activates signal transduction pathways that induce DNA repair, cell cycle checkpoints or other pathways involved in limiting the effects of oxidative stress (reviewed in Shiloh, 1997; Taylor, 1998). The p95 protein may form part of this signal transduction pathway leading to DNA repair and

activation of the cell cycle checkpoints. A key question to be answered is to what degree, if any, do either of these proteins participate in DNA repair. It is clear that not all the phenotypes of AT cells can be explained in terms of checkpoint deficiency. Thus, AT cells are sensitive to ionising irradiation even when cells are not cycling (reviewed in Jeggo et al., 1998). Furthermore, under non-cycling conditions the rejoining of DSBs is defective in AT cells. Whether AT or NBS cells are defective in NHEJ or in a distinctive repair pathway remains to be determined.

Another disorder associated with chromosomal instability is Fanconi's anaemia (FA). This disease is associated with progressive pancytopenia, growth retardation, skeletal malformations and predisposition to leukaemia (Auerbach et al., 1989). The most consistent cellular feature in FA is an increase in spontaneous chromosomal breakage, amplified following exposure to cross-linking agents (Liu et al., 1994; Friedberg et al., 1995). At a molecular level, the main finding is one of deletional mutation (Papadopoulo et al., 1990; Bredberg et al., 1995). FA is an autosomal recessive disorder with 8 complementation groups (Buchwald, 1995; Joenje et al., 1997). The genes for two of these groups, *FAA* (Lo Ten Foe et al., 1996; The FA/BC consortium, 1996) and *FAC* (Strathdee et al., 1992) have been cloned. Neither of the encoded proteins shares homology with other known proteins or contain any recognised motifs, suggesting that they act in a novel pathway unique to vertebrates. The FAA and FAC proteins form a complex that translocates to the nucleus (Kupfer et al., 1997) and both proteins are expressed in tissues with rapidly dividing cells (Krasnoshtein and Buchwald, 1996). The sensitivity of FA cells to DNA cross-linking agents has suggested that such cells may be defective in recombinational repair, since in prokaryotes and eukaryotes this mode of repair is invoked following treatment with the same agents (reviewed in Digweed and Sperling, 1996). Although no defect in homologous recombinational repair has been described (Thyagarajan and Campbell, 1997), other studies have implicated defects in NHEJ. Although the overall ligation frequency of linearised plasmids is the same as control cells, this occurs at the expense of an increase in deletions or other complex mutations (Runger et al., 1993; Escarceller et al., 1997 and 1998). No general defect in NHEJ can be proposed, however, since FA cells are not sensitive to ionising irradiation (Friedberg et al., 1995).

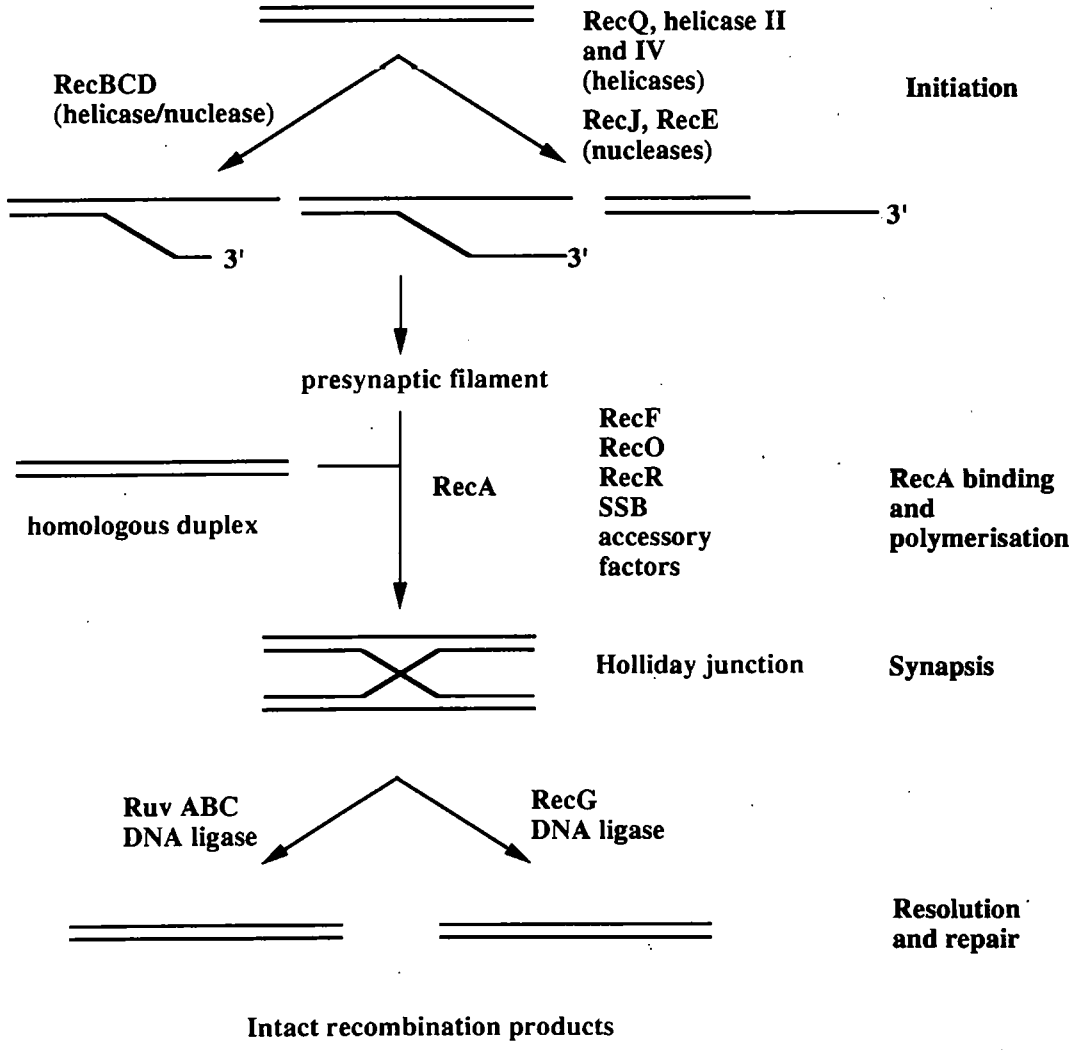


Figure 1.1: Homologous recombination in *E. coli*. RecBCD, RecJ, RecQ, and RecE proteins use their helicase and/or nuclease functions to generate regions of ssDNA that are recognised by RecA. Assembly of the RecA filament may be facilitated by RecF, RecO, RecR and SSB proteins. RecA forms helical nucleoprotein filaments and promotes interactions with other duplex DNA molecules, leading to homologous pairing and strand exchange. This recombination intermediate in which 2 DNA molecules are connected by a crossover, is referred to as a Holliday junction. Resolution of this structure by the action of RuvABC or RecG, results in the formation of nicked duplex products that are repaired by DNA ligase.

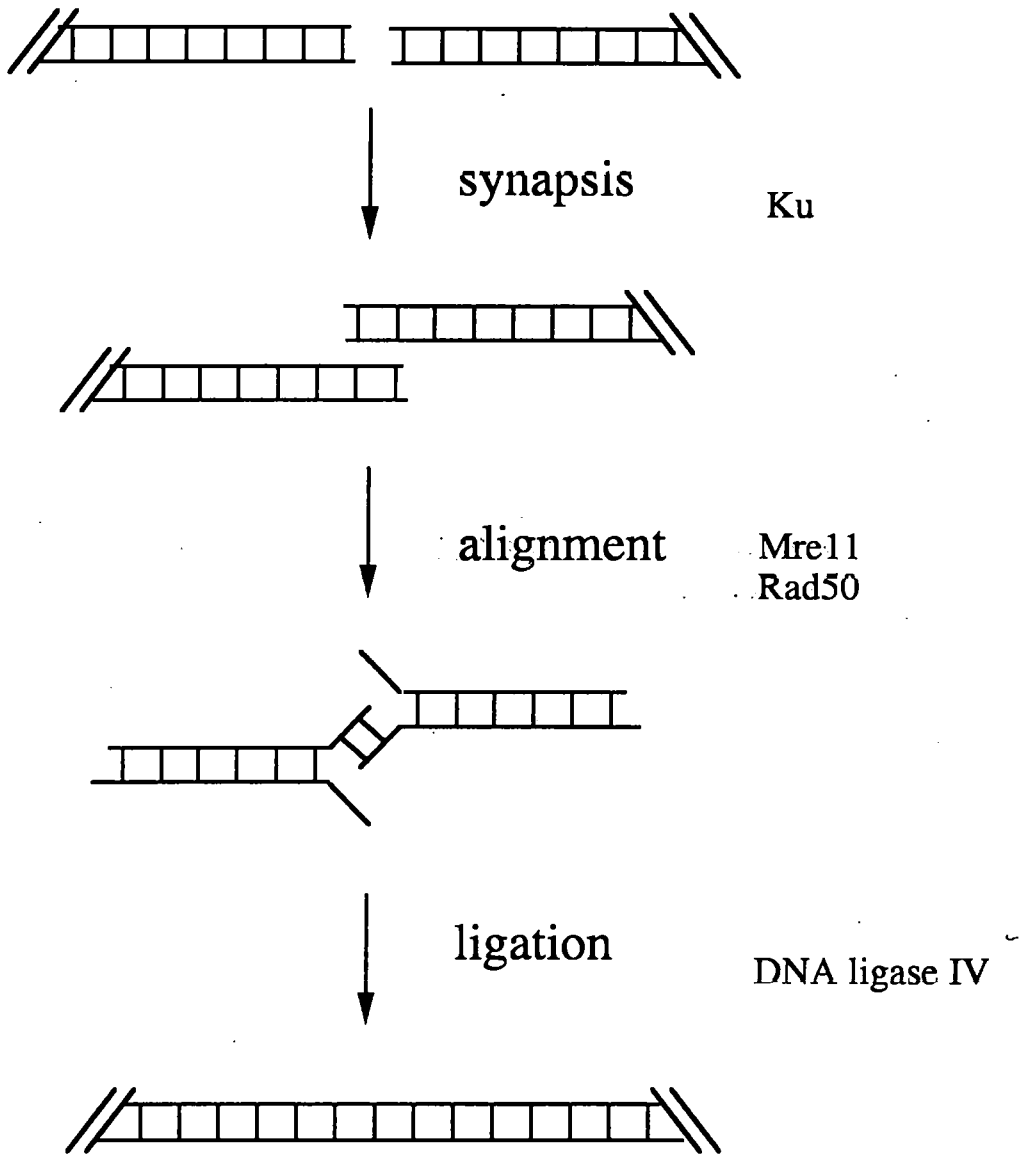


Figure 1.2: A model for DNA intermediates in non homologous end joining Non-homologous end joining (NHEJ) proceeds through a series of steps including synapsis of DNA ends and alignment by micro-homology pairing. Microhomology alignment could occur by unwinding of the DNA ends (as shown), or alternatively by strand invasion/displacement, or by exonuclease digestion of one of the strands on each end. Gap filling and ligation then complete the repair of the double strand break. Proteins thought to participate in NHEJ and the stage at which they act are indicated (see text for details).

1.3 Human disorders associated with defects in RecQ family helicases.

So far, an attempt has been made to show how defects in highly conserved recombination pathways may result in genome instability and cancer-predisposition. In the following sections, two putative recombinational repair disorders, Bloom's syndrome (BS) and Werner's syndrome (WS) will be discussed in the context of information derived from prokaryotic and eukaryotic model systems. Both these inherited disorders result from defects in similar proteins that show a high degree homology to RecQ, a participant in the RecF pathway of recombination in *E.coli* (Nakayama et al., 1984: Ellis et al., 1995; Yu et al., 1996).

1.3.1 Bloom's syndrome: clinical phenotype

In 1954, a New York dermatologist, David Bloom, described the association of 'congenital telangiectatic erythema resembling lupus erythematosus' in association with short stature in children (Bloom, 1954). Over the next decade, individuals with a similar constellation of features were studied and entered into what we now know as the 'Bloom's Registry'. By 1965, the predisposition to malignancy and its likely, autosomal recessive inheritance had been noted (German et al., 1965; German, 1969). Since that time, and until 1991, any individual with Bloom's syndrome was eligible for entry to the Bloom's register, allowing the prospective follow-up of a cohort of individuals and the accumulation of considerable clinical and experimental data. By 1991, the total number of individuals accrued to this database was 168 (German, 1997). The development of the Registry was the key determinant in the eventual identification of the *BLM* gene, defects in which are responsible for this disorder.

The incidence of BS is extremely low in the general population, but occurs in about 1 in 58,000 Ashkenazi Jews (German, 1979). No definitive data exists concerning any anomalies or cancer risk in heterozygous relatives of affected individuals. The main clinical features of Bloom's syndrome are growth retardation, a sun-sensitive facial erythema, immunodeficiency and a marked predisposition to malignancy of all types (German, 1993). At birth, individuals are noted to have a proportionately small body size and few attain full adult height. This is associated with a characteristic appearance which comprises dolichocephaly (long, thin cranium), malar and mandibular hypoplasia. The

predominant skin lesion is a photo-sensitive rash affecting the face, dorsa of the hands and forearms. This is sometimes accompanied by patchy areas of both hyper- and hypo-pigmentation (German, 1995). Patients present with repeated infections, primarily bacterial infections affecting the respiratory tract and this a major cause of mortality in affected individuals. All men with BS are infertile (small testes associated with a complete failure of spermatogenesis), whilst women with BS are subfertile. Other features reported in association with BS include persistent vomiting and diarrhoea in infancy, diabetes mellitus, a high-pitched voice, subnormal intelligence and a number of minor anatomical anomalies.

The feature which is of most interest to the scientific community is the proneness to cancer in BS individuals. By 1996, a total of 100 cancers (arising in 71 persons) had been reported amongst the 168 individuals in the BS Registry (German, 1997). The mean age of death in BS is 23.8 years and the majority of deaths are due to malignancy. More than 1 in 4 BS individuals who develop a malignancy go on to develop one or more further primaries. In general, the spectrum of cancers mirrors that of the general population although with a vastly increased incidence. In the first 2 decades of life, leukaemias and lymphomas predominate, and in the 3rd and 4th decades of life epithelial cancers become more common. The overall picture is of an increased risk, a normal spectrum of cancers and an age distribution which is skewed markedly to a younger age range, as compared to normal individuals. No significant differences in terms of cancer grade, cytogenetics or treatment responses are described. No excess treatment-related radiosensitivity has been reported.

1.3.2 Werner's syndrome: clinical phenotype

WS was first identified in 1904 by Otto Werner in his doctoral thesis 'Cataract in combination with scleroderma' (Werner, 1904). He reported 4 siblings with similar clinical findings: short stature; senile appearance; greying of the hair occurring in the 2nd decade of life; cataracts occurring in the 3rd decade; scleroderma-like skin changes; and ovarian failure. Further characterisation of the disorder by Oppenheimer and Kugel in 1934, Thannhausser in 1945 and by Epstein et al. in 1966 identified WS as a disorder associated with growth retardation, some features of accelerated ageing, cancer-predisposition and chromosomal instability.

The inheritance of WS is autosomal recessive, and the frequency of homozygotes ranges from 1 to 25 per 10^6, with increases in incidence reported in Japan and Sardinia (reviewed in Thweatt and Goldstein, 1993). No definitive data exist in relation to the phenotype associated with heterozygotes. The first recognised and consistent feature of WS is a failure of the adolescent growth spurt, such that adults are short in stature. Skin anomalies are prominent and include scleroderma-like atrophic changes affecting the face or distal extremities and ulceration affecting the legs. Both females and males are subfertile. Features considered representative of progeria include premature onset of cataracts, greying of the hair, coronary and peripheral vascular disease, diabetes mellitus and osteoporosis. The mean age of death is 47 years, and the most common cause is ischaemic heart disease (Epstein, 1966). Despite these features, WS cannot be really be classified as a disorder of premature ageing since there is no proportional advancement in time of all manifestations of ageing and secondly, no proportional advancement of all diseases or all causes of death. In particular, the absence of Alzeimher's disease suggests that the assignment of WS as an ageing disorder is imprecise. Furthermore, in contrast to normal ageing, there is a propensity to rare, non-epithelial cancers. A comprehensive review of 124 case reports of cancer in WS in the world literature, reveals a ratio of epithelial: non-epithelial cancers of 1:1, as compared to the usual 10:1 (Goto et al., 1996). Prominent amongst the non-epithelial tumours are soft tissue sarcomas, osteosarcomas, myeloid disorders and benign meningiomas. As in BS, no consistent differences in the grade of cancer, cytogenetics or treatment responses are reported. No excess treatment-related radiosensitivity has been described. A comparison of the respective clinical phenotypes of WS and BS is shown in Table 1.1.

Table 1.1: Comparison of the clinical features of Bloom's and Werner's syndromes

feature	Bloom's syndrome	Werner's syndrome
onset	prenatal	post -adolescent
features of ageing	not prominent	yes
skin	facial erythema	scleroderma-like
immunodeficiency	yes	no
fertility	males infertile, females subfertile	subfertility
mean age at death (major cause)	24 years (cancer)	47 years (cardiac disease)
cancer	all types	non-epithelial

1.3.3 Cellular phenotypes associated with Bloom's and Werner's syndromes

Cells derived from individuals with both BS and WS grow slowly, with a reduced rate of transit through S-phase (Hand and German, 1975; Gianelli et al., 1977; Ockey and Saffhill, 1986; Fujikawa-Yamamoto et al., 1987; Salk, 1982; Giesler et al., 1997). Newly synthesised DNA strands elongate more slowly in both cell types and this is associated with the presence of abnormal replication intermediates in BS cells (Lonn et al., 1990) and a decreased frequency of initiation events in WS cells (Hanaoka et al., 1985). WS cells also demonstrate premature replicative senescence in culture (Martin et al., 1970; Salk et al., 1981: Salk, 1982; Tollefsbol and Cohen, 1984), although this has not been studied in BS cells. In contrast to cells from other disorders associated with genome instability, both BS and WS cells are not particularly sensitive to DNA damaging agents

(reviewed in Friedberg et al., 1995). Although there are a small number of reports suggesting sensitivity to UV or cross-linking agents in cells derived from affected individuals, these are the exception rather than the rule.

A gross level of spontaneous chromosomal instability is evident in both diseases. A dramatic representation of this is the large numbers of epithelial cells from BS individuals containing micronuclei, which are produced when aberrant chromosomes or chromosome fragments lag at anaphase and are surrounded at telophase by their own nuclear membrane (Rosin and German, 1985). At a cytogenetic level the nature of the genomic instability in the two disorders is distinctive.

BS stands out from other chromosome instability syndromes because of its unique tendency for increased exchanges between homologous chromosome segments. This is manifested as an increase in sister chromatid exchanges (SCEs) or interchanges between homologous chromosomes (quadriradials and telomere associations), both of which occur spontaneously and which are amplified following exposure to various DNA-damaging agents (Kuhn and Therman, 1986). The ten fold increase in the number of SCEs as compared to wild type cells when BS cells are incubated in the presence of bromodeoxyuridine is specific to this disorder. Single chromosome breaks and chromatid translocations between nonhomologous chromosomes are also increased. In WS, cytogenetic abnormalities include deletions, reciprocal translocations and inversions, an appearance termed 'variegated translocation mosaicism' (Salk et al., 1985). A wide spectrum of break points is found in the WS karyotype, with specific clusters affecting 1q12, 5q12 and 6cen. WS cells do not demonstrate an elevated rate of SCEs.

The most elegant evidence that the cytological changes in BS described above reflect an increase in somatic recombination, has been the study of affected individuals who are heterozygous for the MN blood group (Kyoizumi et al., 1989; Langlois et al., 1989). Glycophorin A (GPA) is a cell surface erythrocyte protein which occurs in two forms, M and N, and is the product of codominantly expressed alleles. Using a flow cytometric approach to assess the cell surface expression of the M and N antigens, loss of heterozygosity was detected at a rate 50-fold greater than normal individuals, such that about 1 in 1000 erythroid cells in BS individuals were the progeny of cells that had been altered by a recombinational event between the GPA alleles. Hemizygous or partial loss variants were also markedly elevated (50-80 fold) in BS individuals. Although, this hemizygous variant could arise as a result of chromosomal breakage, comparisons with

the frequencies of these events with other situations in which chromosome breaks are observed (for instance, in individuals exposed to ionising irradiation), suggest that additional mechanisms resulting in this genetic variant are involved in BS individuals (Langlois et al., 1987).

At a molecular level, no consistent representation of the events that lead to mutation in BS and WS individuals has yet emerged. In contrast to hereditary non-polyposis colonic cancer which is associated with a mismatch DNA repair defect, there is no increase in microsatellite instability in either disorder (Foucault et al., 1996; Brooks-Wilson et al., 1997). A marked increase in rates of mutation at the *HPRT* locus in BS and WS individuals appears to arise as a consequence of large deletions (Warren et al., 1981; Vijayalaxmi et al., 1983; Fukuchi et al., 1989; Tachibana et al., 1996) . In an attempt to characterise the mechanism of this 'hyper-mutability' in more detail, plasmid products derived from normal, BS and WS cells following transfection of linearised plasmid DNA have been assessed. For BS cells, the efficiency of plasmid re-joining was reduced 1.3-3 fold, and the spontaneous frequency of the mutations in the derived products was up to 21-fold greater than normal controls. The type of mutations identified included deletions, insertions, point mutations or complex rearrangements at joining sites (Runger and Kraemer, 1989). In contrast, in WS the efficiency of plasmid rejoining is normal and although this ligation is error-prone as compared to wild type cells, the complexity of the mutations (deletions) was less marked than in BS (Runger et al., 1994).

No explanation currently exists to explain the immunodeficiency associated with BS. Abnormalities identified include low immunoglobulin levels, reduced *in vitro* proliferative responses to mitogen stimulation, depressed natural killer activity, impairment of B-cell differentiation and reduced helper T-cell function (Hutteroth et al., 1975; Weemaes et al., 1979; Taniguchi et al., 1982; Ueno et al., 1985). However, in studies to date no abnormalities in either signal or coding joint formation of V(D)J recombination have been identified (Hsieh et al., 1993). Furthermore, BLM does not appear to be involved in the process of immunoglobulin hypermutation (Sack et al., 1998).

1.3.4 The identification of *BLM* and *WRN* genes.

The process that led up to the identification of the *BLM* gene involved at its ultimate stage, a completely novel strategy and is thus described in some detail. At an early

stage, BS was believed to be a single gene disorder as evidenced by the absence of complementation in fusion experiments between cell lines derived from BS individuals of different ethnic backgrounds (Weksberg et al., 1988). The chromosomal location was identified by complementation analysis following microcell-mediated transfer of human chromosomes into a transformed BS fibroblast cell line, which showed that chromosome 15 was able to correct the elevated SCE rate (McDaniel and Schultz, 1992). Analysis of affected persons whose parents are cousins (and thus, who are likely to have inherited an identical defective gene) allowed genetic mapping to a band 15q26.1 and tight linkage between the proto-oncogene *FES* and the putative *BLM* gene (German et al., 1994). Further linkage analysis indicated that *BLM* was just proximal to *FES* and to *D15S127* (a CA repeat locus) and could be assigned to an interval no greater than 2cM (Ellis et al., 1994).

Although it is likely that this standard positional cloning method approach would have allowed the eventual identification of *BLM*, a novel method that exploited an observation made nearly twenty years earlier was used. It had been noted that in certain individuals with BS, a small number of cells could be isolated with normal rates of SCE formation (German et al., 1977). This high SCE/low SCE mosaicism was identified only in individuals whose parents were not known to share a common ancestor. In multiple low SCE cells studied, polymorphic loci distal to *BLM* on 15q had become homozygous, whereas polymorphic loci proximal to *BLM* remained heterozygous. These observations, supported the hypothesis that low SCE cells arose through recombination between different *blm* alleles in persons who had inherited paternally and maternally derived *blm* alleles mutated at different sites (Ellis et al., 1995). This type of recombinational event in a precursor stem cell could thus give rise to a cell whose progeny had a functional wild type gene and phenotypically a low SCE rate (Figure 1.3). Consistent with this model, certain low SCE cell lines demonstrated a reduction to homozygosity at loci distal but not proximal to the *BLM* locus. These observations and hypothesis led to a cloning method referred to as somatic crossover point (SCP) mapping (Ellis et al., 1995). In this strategy the aim was to identify the most proximal polymorphic locus that was constitutionally heterozygous but that had been reduced to homozygosity in the low SCE cells, and to identify the most distal polymorphic locus that remained constitutionally heterozygous in them. *BLM* would then be in the short interval defined by the reduced (distal) and unreduced (proximal) heterozygous markers. A schematic diagram of this SCP approach

is shown in Figure 1.3. The candidate gene identified in this way was proven to be the disease-related gene, by a number of methods including single-stranded conformational polymorphism analysis of sequences generated by RT-PCR from patients and control RNA, which permitted identification of mutations in 10 individuals with BS (Ellis et al., 1995). The *BLM* gene was predicted to encode a 1417 amino acid protein with significant homology to the RecQ family helicases.

Within a few months of this discovery, positional cloning identified the gene defective in WS. The *WRN* gene, located to 8p12, was also found to encode a RecQ family helicase (Yu et al., 1996). Thus within a very short period of time, two distinct human disorders associated with gross chromosomal instability were found to result from defects in proteins from the same DNA helicase family.

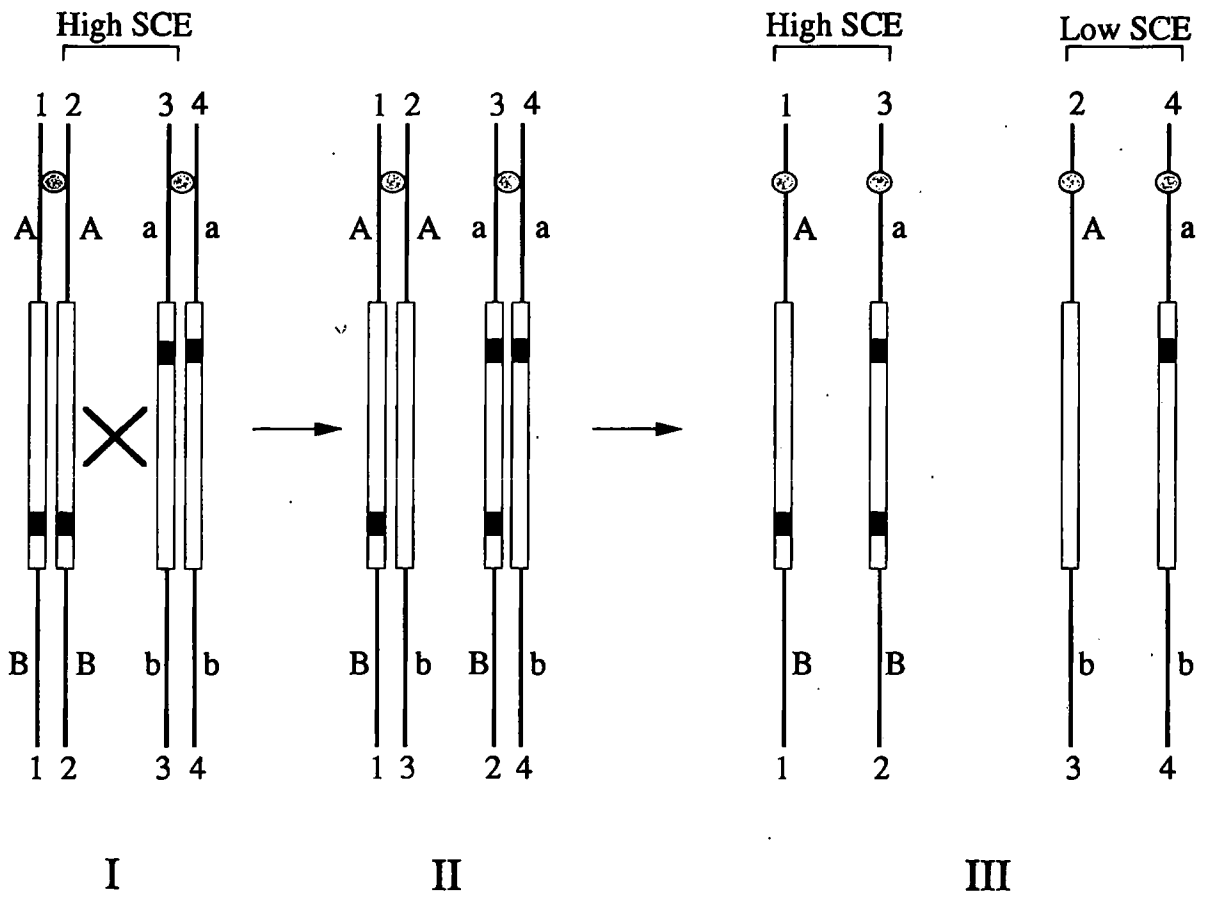


Figure 1.3: Intragenic somatic recombination in Bloom's syndrome. The high frequency of recombination in BS can generate a wild type *BLM* allele in compound heterozygotes, accounting for the minor population of lymphocytes with normal levels of sister chromatid exchange (SCE) observed in these individuals. **I** Each of 2 mutations in *BLM* (the yellow rectangle), represented by the blue and red boxes, is at a different site in the gene. *A/a* and *B/b* are polymorphic microsatellite markers, located respectively proximal to and distal to the *BLM* locus. The centromere is represented by a grey circle. **II** Following homologous interchange between chromatids 2-2 and 3-3 at a point between the sites of mutation within *BLM*, a wild type gene is generated on chromatid 2-3, that corrects to normal the high SCE phenotype of BS cells. The distal marker *b* becomes associated with the wild type gene on chromatid 2-3. **III** By segregational events at mitosis, 2 pairs of daughter molecules are possible (only one of the possible pairs are shown). If chromosomes 2-3 and 4-4 co-segregate to the same daughter cell (on the right), the distal marker becomes homozygous *b/b* and the proximal marker remains heteozygous *A/a*. This cell will have a low SCE phenotype.

1.4 The RecQ subfamily of DNA helicases

1.4.1 DNA helicases

DNA helicases catalyse the disruption of hydrogen bonds that hold two strands of a DNA duplex together (Matson et al., 1994; Lohman and Bjornson, 1996). This NTP-driven process generates single-stranded DNA intermediates or templates for DNA repair, replication, recombination and transcription. The majority of helicases contain 7 helicase 'signature' motifs. The first (Ia) and third motifs (II), corresponding to Walker boxes A and B respectively, bind to ATP. Motifs Ib and III-VI are highly conserved and specific to helicases, although not all proteins with these motifs necessarily demonstrate helicase activity *in vitro* (Gorbalenya and Koonin, 1993). Their biological importance is emphasised by the finding of large numbers of helicases in most organisms whose genomes have been extensively studied. In budding yeast, there may be as many as 41 helicases (Ellis, 1997).

DNA helicases can be categorised biochemically by their substrate preference, direction of unwinding, processivity and their interaction with other proteins or co-factors. For example, some helicases act on blunt-ended duplexes, whereas others require ssDNA overhangs or fork-like structures. Using end-labelled substrates it is possible to ascertain the direction of helicase translocation through the duplex. Mechanistically, there are two broad groups of helicases (Matson et al., 1994): those requiring a 3' flanking strand of ssDNA and those requiring a 5' flanking strand (known as 3'-5' and 5'-3' polarity helicases respectively).

On the basis of sequence homology, there are five classes of helicase (Gorbalenya and Koonin, 1993). Most 3'-5' helicases are members of superfamilies I or II. These two families contain proteins which contain the seven conserved helicase motifs and a number of these proteins are thought to act as dimers. Superfamily III represents helicases derived from DNA or RNA viruses, while superfamilies IV and V contain 5'-3' hexameric helicases related either to the *E.coli* replicative helicase DnaB or the bacterial transcription terminator, Rho. The RecQ helicases form a subfamily of superfamily II.

Sequence analysis of helicases in superfamilies I and II indicate that the greatest variation is seen in regions between motifs Ia and II, and between IV and V, and at the N- or C-termini (Bird et al., 1998). This is consistent with a modular structure, in which insertions at the positions described above, could modify helicase activity. The structure

of a small number of helicases have been resolved and demonstrate significant similarity in their pattern of folding, although the relative positions of the individual subdomains are different (Subramanya et al., 1996; Korolev et al., 1997). The nucleotide binding pocket is formed by several of the motifs conserved in superfamilies I and II, and is situated at the bottom of a cleft that runs between two domains.

A common feature in most superfamily I and II helicases, is the internal repeat of two RecA-like subdomains, suggesting that these domains may be the minimal structure necessary for helicase activity (Bird et al., 1998). This homology is likely to represent significant overlap in the mechanism of action for helicase and RecA proteins. Thus RecA destabilises a DNA duplex to enable invasion of a homologous strand, whereas helicases catalyse only the first step of this reaction. Both sets of proteins couple the binding and hydrolysis of nucleotides to conformational changes that in turn alter the affinity of enzymes for different forms of DNA. Two models have been proposed to explain the molecular mechanism that results in unwinding. The 'inchworm" model suggests that the helicase molecule translocates through the DNA in discrete steps coupled to the hydrolysis of ATP (Yarranton and Geftner, 1979). In this scenario, the helicase could conceivably act as a monomer. The 'active rolling' model proposes that helicases act by an alternation in the affinity of the enzyme for ss- or dsDNA that is controlled by the binding and subsequent hydrolysis of ATP (Wong and Lohman, 1992). This second model requires that the helicase acts as an oligomer and second, that a helicase subunit can bind ss- and dsDNA, but not both simultaneously.

1.4.2 The RecQ family

Since the isolation of *E. coli* RecQ in 1984 (Nakayama et al., 1984) there has been a steady increase in the identification of related family members, either through the direct cloning of genes defective in BS and WS, or more recently through searching the rapidly expanding genome databases. In the most recent estimate, there are at least 16 members of the RecQ family (see legend to Figure 1.4 for accession numbers). In general, it appears that the number of RecQ family helicases expressed in each organism is directly related to the complexity of that organism, such that prokaryotes and unicellular eukaryotes possess only a single RecQ helicase, whereas *C. elegans* possesses at least four, and *H. sapiens* has at least five. Attempts to classify members of this family on the basis of overall size, direct sequence comparison or phyllogenetic analyses have not

proven particularly useful to date. The ultimate aim should, of course, be to categorise individual members of this family on the basis of function.

In Figure 1.4, an alignment of 6 key members of the RecQ family is shown. Each protein is characterised by a central domain of ~600 amino acids that contains the 7 so-called helicase signature motifs, including a putative ATP-binding sequence (Walker A box) in motif I, and a DExH-box in motif II. Amongst all of the proteins in this family, strong sequence similarity is restricted to this central domain both within and adjacent to the 7 helicase motifs. The 4 larger proteins, Sgs1p, Rqh1p, WRN and BLM, also share an area of extended sequence homology C-terminal to the helicase domain. Although there is little primary sequence homology outside of these regions, the N- and C-terminal regions of the larger proteins appear to perform several roles. The N-terminal domain in each case includes blocks of sequence comprising multiple acidic amino acids that might be involved in protein and/or DNA interactions. Motifs homologous to the nuclease domains in RNaseD and certain proof-reading DNA polymerases have been identified in the N-terminal domain of WRN (Mushegian et al., 1997), suggesting that WRN protein may possess nuclease activity. The N-terminal domain of Sgs1p has been shown to be important for binding to both topoisomerase III (Gangloff et al., 1994) and topoisomerase II (Watt et al., 1995)(see below). A nuclear localisation signal (NLS) sequence has been identified in the C-terminal domain of both WRN (a group of basic amino acids at residues 1370-1375, Matsumoto et al., 1997) and BLM (a bipartite NLS between residues 1334-1349, Kaneko et al., 1997) (Figure 1.4).

To date, the spectrum of mutations identified in WS individuals are 4 nonsense (codons, R369X, R888X, Q1164X, R1304X), 1 frameshift (nucleotide, 1167delA), 3 exon skipping (exons 20, 26 and 32) and one large deletion (>15kb). It is of interest that many of the *WRN* gene mutations identified in WS individuals (5 of the 9 mutations described) are predicted not to disrupt the helicase domain, but to truncate the protein resulting in loss of the NLS sequence (Yu et al., 1996; Goto et al., 1997; Yu et al., 1997). In the most recent analyses of cell lines derived from 35 non-Ashkenazi Jewish individuals with BS (Ellis et al., 1995; Dr. N. Ellis, personal communication), the spectrum of mutations identified were 5 missense (codons, Q672R, G891E, C901Y, C1055S, G1036T), 8 nonsense (codons, S186X, K272X, R364X, S595X, Q645X, Q700X, Q752X, R899X), 6 frameshift (nucleotides, 1610insA, 2042insG, 2281delATCTGAinsTAGATTC, 2996delC, 32223delT, 3333delT), 2 exon-skipping

(exon2, exon6) and 1 large ~750bp deletion (identified by Southern). Within this non-Ashkenazi Jew group, 8 unrelated individuals had the same mutation, evidence for small founder effects. A single frameshift mutation (2281delATCTGAinsTAGATTC) accounts for the majority of *BLM* mutations within the Ashkenazi Jewish group of individuals with BS. A screen of 1491 anonymous, unrelated Ashkenazi Jewish individuals with no known family history of BS found that the carrier rate was 1 in 107 for this mutation (Straughen et al., 1998; Dr. N. Ellis, personal communication). The majority of the mutations described above for BS-affected individuals either severely truncate the BLM protein or cause amino acid substitutions in residues within or adjacent to the helicase motifs and are predicted therefore to eliminate helicase function (Ellis et al., 1997). The significance of the difference in mutational spectra between *BLM* and *WRN*, and how this might relate to the different cellular phenotypes of WS or BS cells is not yet clear.

In an attempt to assign specific functions to each of the different RecQ family members, 3 general strategies have been deployed: (1) an assessment of the cellular phenotype associated with the absence of that protein; (2) a genetic approach to establish functional relationships with replication/repair/recombination pathways; (3) a biochemical assessment of the enzymatic activities of a purified family member. Summaries of the findings for each of these approaches (Nakayama et al., 1984; Gangloff et al., 1994; Watt et al., 1995; Hanada et al., 1997; Watt et al., 1996; Sinclair and Guarente, 1997; Stewart et al., 1997; Murray et al., 1997; Kuhn and Therman, 1986; Runger and Kraemer, 1989; Lonn et al., 1990; Giannelli et al., 1997; Friedberg et al., 1995; Hoehn et al., 1975; Fukuchi et al., 1989; Hanaoka et al., 1985; Okada et al., 1998; Thweatt and Goldstein, 1993; Umezu and Nakayama, 1993; Harman and Kowalczykowski, 1998; Bennett et al., 1998; Karow et al., 1997; Gray et al., 1997; Suzuki et al., 1997; Shen et al., 1998) are shown in Tables 1.2 and 1.3. In comparing the various properties of each enzyme, it is important to make the following qualification. In each of the 5 cases where mutants exist, loss of a RecQ family helicase does not compromise cell viability, and therefore it is likely that other proteins can partially substitute or compensate for the loss of a RecQ helicase. This functional redundancy, which must in some cases involve enzymes with no structural similarity to RecQ helicases, has the potential to at least partially mask or distort the 'true' phenotypic effects of loss of a RecQ helicase.

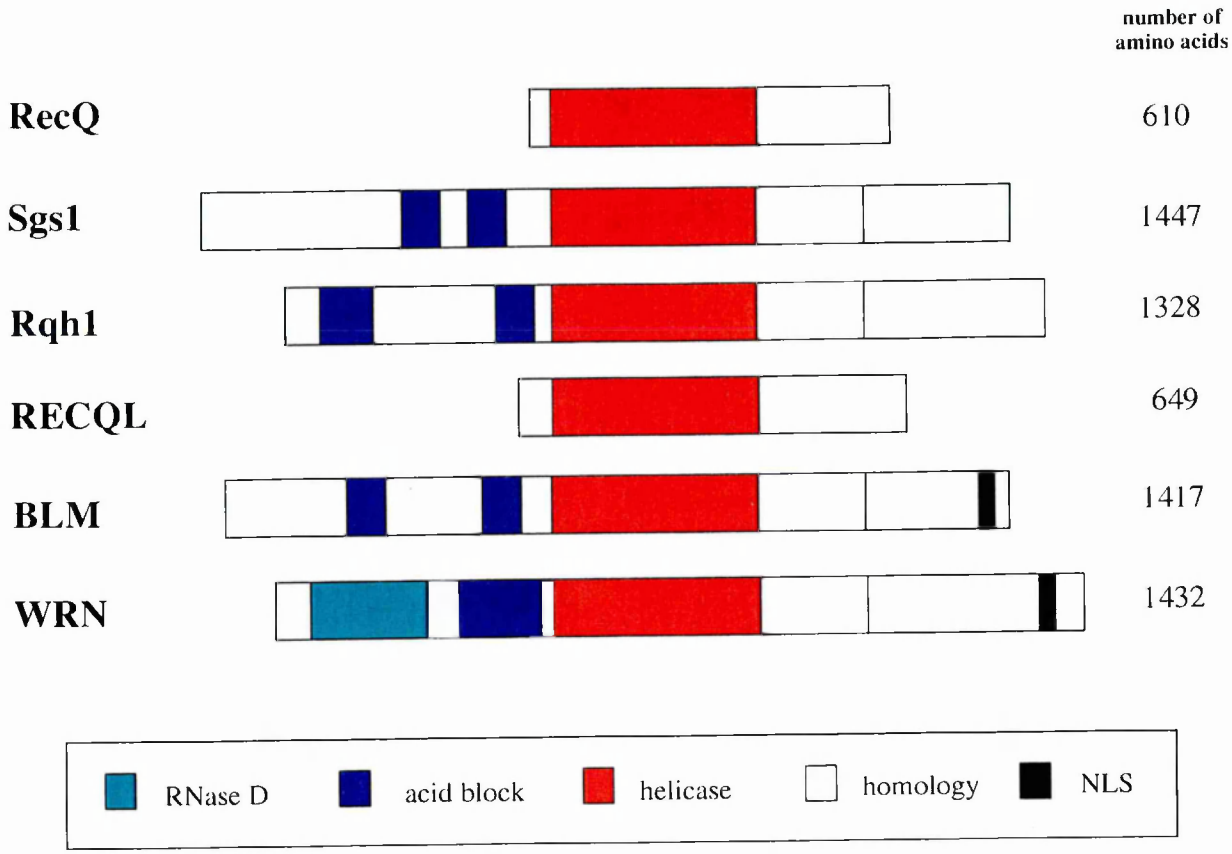


Figure 1.4: Schematic representation of the structures of six members of the RecQ helicase family. The proteins shown are *E. coli* RecQ, *S. cerevisiae* Sgs1, *S. pombe* Rqh1, and *H. sapiens* RECQL, BLM and WRN, as indicated on the left. The size of each protein is shown on the right. Regions corresponding to RNase D motifs, blocks of acidic amino acids, helicase domains, extended regions of homology outside of the core helicase domain, and nuclear localisation sequences (NLS) are indicated and shown in the key below. Accession numbers for these and other RecQ family members are as follows: RecQ (A35776); Sgs1 (L07870); Rqh1 (Z54354); uncharacterized members in *C. elegans* (Z38112, U00052, U29097); and *H. sapiens* RECQL (L36140), BLM (U39817), WRN (L76937) and 2 other uncharacterized members (H16879, Z15298).

	recQ	*SGS1*	*rqh1*	*BLM*	*WRN*
NATURE OF GENOMIC INSTABILITY	elevated illegitimate recombination	• hyper-recombination • extrachromosomal rDNA circles	increase in recombination induced by HU	• SCEs • telomere association, quadriradials • insertions, deletions, loss of heterozygosity	• variegated translocation mosaicism • deletions
REPLICATION ABNORMALITIES	role in RecF pathway	moderate sensitivity to HU	sensitive to HU	• retarded replication fork progression • abnormal replication intermediates	• abnormal replication intermediates • sensitivity to S-phase specific agents
CELLULAR RESPONSES TO DNA DAMAGE	UV sensitive (*recBCsbcA/BC*)	moderate sensitivity to UV	sensitive to UV	not sensitive to UV	not sensitive to UV
FEATURES OF AGEING	?	• reduced life span, nucleolar fragmentation • no telomere shortening	?	?	• reduced life span • shortened telomeres
CHROMOSOME LOSS/NON-DISJUNCTION	?	meiotic/mitotic chromosome non-disjunction	HU-stimulated chromosome loss	subfertility	subfertility

TABLE 1.2: Phenotypes of mutants lacking a RecQ family helicase

	RecQ	Sgs1	Rqh1	BLM	WRN
SIZE	610 aa	1447 aa	1328 aa	1417 aa	1432 aa
POLARITY OF HELICASE	3'-5' helicase	3'-5' helicase	not known	3'-5' helicase	3'-5' helicase
ABILITY TO INITIATE HOMOLOGOUS PAIRING	Yes	not known	not known	not known	not known
NUCLEIC ACID SUBSTRATES	• DNA duplex • blunt -ended • forked • 3 or 4 way junctions	• DNA duplex • forked • DNA/RNA hybrids	not known	• DNA duplex • forked • synthetic cruciforms • G4 DNA	• DNA duplex • DNA/RNA hybrid
PROTEIN INTERACTIONS	not known	Topoisomerases II and III	not known	not known	not known

TABLE 1.3: Properties of RecQ family helicase proteins

1.4.3 Are RecQ helicases involved in 'replication fork repair'?

A significant amount of evidence, derived mainly from studies on bacterial and yeast model systems, suggests that RecQ family helicases may act upon DNA structures that arise during the DNA synthesis phase (Tables 1.2 and 1.3). DNA replication is potentially hazardous to the integrity of cellular DNA for numerous reasons. For example, accumulating evidence indicates that replication fork progression is not always smooth but may be interrupted, for example, by the presence of lesions in the template (reviewed in Bierne and Michel, 1994; Friedberg et al., 1995; Kuzminov, 1995). This can have the effect either that a pre-existing single strand break in the DNA template is converted to a far more cytotoxic double strand break, or that the replication fork will stall and subsequently collapse. Alternative mechanisms by which this collapse could be precipitated include the collision of a replication fork either with a converging protein complex (a replication or transcription complex), or with an abnormal DNA structure (e.g. a cruciform). Collapse and disassembly of the fork may result in the formation of cytotoxic DNA strand breaks. Failure to resolve such lesions and to successfully re-initiate replication would result in the loss of genetic information and the likely loss of cell viability. DNA structures that might exist at a stalled fork, such as single strand gaps in the lagging strand and the free 3' end of the leading strand, are also likely to be highly recombinogenic.

Cells have developed a number of strategies for dealing with the deleterious consequences of replication fork breakdown (for recent reviews, see Bierne and Michel, 1994; Kuzminov, 1995). Lesions blocking replication fork progression can either be repaired directly, or be by-passed through the activity of a DNA polymerase equipped to catalyse translesion synthesis. Alternatively, extensive studies in prokaryotes and, more recently, in eukaryotes (Zhou and Rothstein, 1997; Merrill and Holm, 1998), suggest the involvement of genetic recombination functions in the 'repair' of DNA structural abnormalities arising during DNA replication. For example, single-stranded lesions that are converted to DNA DSBs at the replication fork could be repaired by a mechanism involving homologous recombination with the intact sister-chromatid. A second possible pathway for recombination-mediated replication fork repair is via post-replication gap filling (Kogoma, 1997; Cox, 1997). In this, replication re-initiates downstream of the site of fork blockage, leaving single-stranded gaps opposite the original lesion, which are subsequently filled by homologous recombination (Figure 1.5).

In prokaryotes, post-replication gap filling is dependent upon the RecF pathway for genetic recombination. Indeed, a number of studies have shown functional connections between DNA replication and the RecF pathway. Thus, for example, the *recF* and *recR* genes are found within operons encoding DNA synthesis genes (Flower and McHenry, 1991). Certain forms of aberrant replication, including plasmid linear-multimer formation or rifampicin-resistant plasmid replication, have been shown to be RecF-dependent (Kusano et al., 1989; Magee and Kogoma, 1990). Moreover, loss of RecF pathway genes results in resistance to thymine starvation, a process that is also thought to result in abnormal replication (Nakayama et al., 1984). To explain these connections, Courcelle et al. (1997) have suggested that the replisome disengages from the template at a DNA lesion, and that RecF pathway proteins are required to subsequently 'reassemble' a replisome complex at the site of the replication fork following removal of the lesion by specific DNA repair enzymes. The precise means by which this re-initiation of replication at non-origin sites occurs is not clear, but seems to involve PriA-mediated priming of new DNA synthesis from sites of D-loops in the template. This would clearly permit replication to be re-initiated at the precise position where it faltered, an essential factor if loss or gain of genetic information is to be prevented.

Although *recQ* is a member of the RecF pathway of recombination in *E.coli*, its precise function in relation to the processes described above has yet to be established. However, a recent detailed biochemical assessment of the catalytic activities of the RecQ protein provides firm evidence that RecQ, in association with RecA and SSB, can initiate homologous DNA pairing (Harman and Kowalczykowski, 1998), a finding consistent with much of the genetic data derived from studies of *recQ* mutants. In addition, RecQ exhibits a substrate 'promiscuity' in that it can unwind with similar efficiency a broad range of DNA substrates representative of structures generated during replication or recombination, including duplexes with blunt ends or 3' and 5' overhangs, 3- or 4-way DNA junctions and forked DNA structures (Harman and Kowalczykowski, 1998). Thus, the likely ability of RecQ to initiate recombination from internal nicks or small gaps (an ability not shared by the RecBCD enzyme, the major initiator of recombination in *E.coli*) make it an attractive candidate for dealing with structures generated at a stalled or collapsed replication forks. Consistent with this, in experimental systems designed to reproduce the effect of replication fork stalling *in vivo* (using plasmids carrying specific *E.coli* replication termination sites) an increase in the frequency of deletions arising via

illegitimate recombination has been observed (Bierne et al., 1997). Structurally similar deletions arise at an elevated frequency in *recQ* mutants (Hanada et al., 1997). It seems likely that the existence of a mechanism to resolve aberrant structures generated during the process of DNA replication is important in all cells, including those of higher eukaryotes.

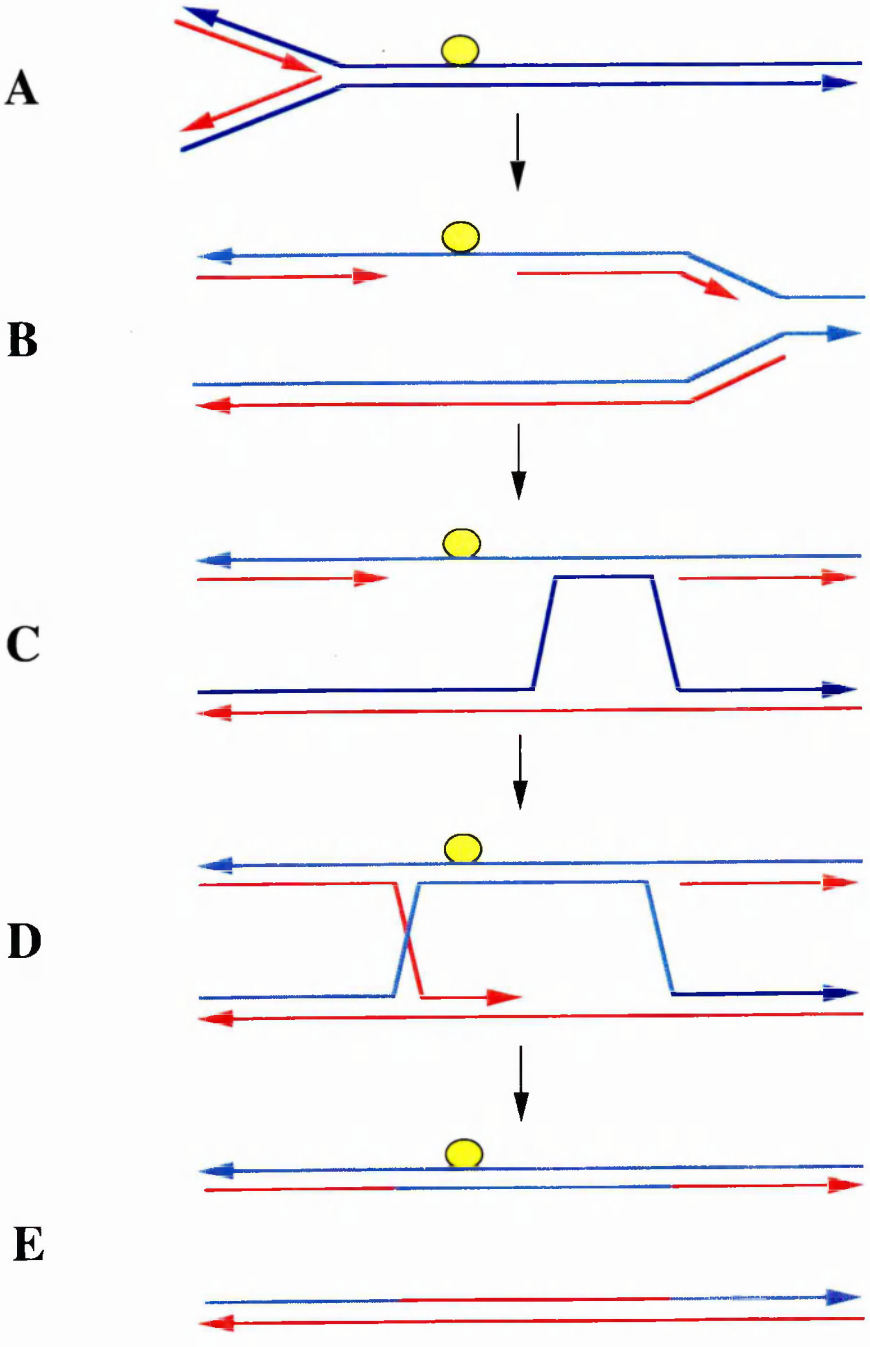


Figure 1.5: Post-replication gap repair. (A) A replication fork approaches a lesion (e.g. a pyrimidine dimer) on the template, leading to replication fork arrest and disassembly. (B) Replication reinitiates downstream of the blockade, leaving a gap opposite the lesion. (C) RecA (or one of its eukaryotic homologues) polymerises on single-stranded DNA at the gap, and promotes homologous pairing and strand exchange with the intact strand of the daughter duplex, forming a D-loop. (D) Extended strand exhange allows the 3' end of the newly synthesised DNA to invade this structure, leading to the formation of a Holliday junction. The gap is then filled in by DNA synthesis. (E) Resolution of the Holliday junction and ligation completes the reaction. Removal of the lesion can then be catalyzed by the appropriate DNA repair pathway (i.e. nucleotide excision repair). Blue lines represent template DNA and red lines represent newly synthesised DNA.

1.4.4 Evidence for replication abnormalities in the absence of RecQ family helicases in eukaryotes

There is emerging evidence from studies in yeasts that replication does not proceed normally in absence of a RecQ helicase. When fission yeast *rqh1* mutants are exposed to the DNA synthesis inhibitor, hydroxyurea (HU) they lose viability and exhibit a 'cut' ('cell untimely torn') phenotype in which the septum often bisects an undivided nucleus (Stewart et al., 1997). These mutant cells show a normal cell cycle checkpoint response to the initial insult, in that they show cell elongation, but no cell division. However *rqh1* mutants lose viability and initiate 'cuts' when the cells attempt to resume DNA replication following the removal of the HU. Thus, fission yeast cells are defective in the 'recovery' from S-phase arrest in the absence of a functional $rqh1^+$ gene. Genetic studies also indicate that $rqh1^+$ is necessary for viability in certain mutants showing defects in the elongation step of DNA synthesis (Murray et al., 1997). This implies that if replication fork progression is inhibited, Rqh1p is necessary in some way to facilitate the process of re-initiating DNA replication. The marked increase in levels of chromosome loss and recombination following exposure of *rqh1* mutants to HU (Stewart et al., 1997) is consistent with a failure to resolve lesions and/or aberrant structures arising at the sites of stalled replication forks.

Budding yeast mutants lacking *SGS1*, the homologue of $rqh1^+$, were reported to be sensitive to HU while this thesis was in preparation (Yamagata et al., 1998). Furthermore, in the absence of HU, *sgs1* mutants show elevated rates both of mitotic recombination (both inter- and intra-chromosomal between repetitive sequences) and mitotic chromosome nondisjunction (Watt et al, 1995 and 1996). Sgs1p also acts to suppress deletions arising through illegitimate recombination, in a similar manner to RecQ in *E.coli* (Yamagata et al., 1998). Taken together, these findings suggest that the RecQ-family helicases in prokaryotes and lower eukaryotes share similar fundamental functions.

It seems probable that the putative defects in replication described above, observed in the absence of RecQ helicase function in yeasts, are also evident in human cells. As stated earlier studies of fibroblasts derived from patients with WS and BS showed either retarded replication fork progression or the accumulation of abnormal replication intermediates (Giannelli et al., 1977; Hanaoka et al., 1985; Lonn et al., 1990). BS cells also show a marked elevation in the rate of sister-chromatid exchanges which arise in S-

phase when cells are exposed to bromo-deoxyuridine (Kuhn and Therman, 1986). The finding that WS fibroblasts (Okada et al., 1998) show specific sensitivity to S-phase specific agents, such as the topoisomerase I inhibitor campothecin, suggests that this helicase functions primarily during the period in which the cell replicates its DNA. The recent finding that the formation of 'replication foci' in *Xenopus*, is dependent upon FFA-1 which shows a high degree of homology to the WRN protein, provides more direct evidence of a role in DNA replication (Yan et al., 1998).

1.4.5 Defects in RecQ helicases are associated with a reduction in life span.

Individuals with WS show many of the features of normal ageing, but display them several decades earlier in life than do unaffected individuals (Epstein et al., 1966). At this stage it is not clear how deficiency in a RecQ helicase leads to premature replicative senescence. Studies utilising *sgs1* mutants of budding yeast have suggested the possible role in the ageing process of a failure to suppress instability in DNA repeat sequences, particularly in the tandemly duplicated rDNA locus (Sinclair and Guarente, 1997). Guarente and colleagues have shown that there is a more rapid accumulation of extra-chromosomal rDNA circles (ERCs) in *sgs1* mutants than in wild-type cells, and that this chromosomal instability is associated with a 60% reduction in life span. This abnormality in controlling life span in *sgs1* mutants is associated with other features found in 'old' yeast cells, including sterility, nucleolar fragmentation and relocalisation of Sir proteins to the nucleolus. Immunolocalisation of Sgs1p demonstrates that the protein is concentrated in the nucleolus, where it is proposed to act in the suppression of ERC formation. The number of ERCs in aged yeast cells was estimated to be between 500-1000 copies per cell and it has been hypothesised that the build up of ERCs directly results in the loss of viability by titrating out essential proteins necessary for replication or transcription. In this context, it is noteworthy that premature introduction of rDNA circles containing a weak replication origin into wild type cells, markedly reduces life span. Repeat sequence instability in *sgs1* mutants is partially *RAD52*-dependent (Watt et al., 1996), which implicates recombination functions in ERC formation. However, the nature of any putative recombinogenic structures arising at the rDNA locus are as yet not known. Since it is likely that ERC formation is *RAD52*-dependent, another key question to be addressed is whether deletion of *RAD52* in an *sgs1* mutant improves life span.

It is not clear whether a similar process can account for the replicative senescence in mammalian cells. Although the human WRN protein immunolocalises to the nucleolus, the murine homologue does not (Markinak et al., 1998), and there is no evidence that rDNA copy number increases in human cells undergoing replicative senescence.

1.5 Functional relationships between topoisomerases and RecQ family helicases

Topoisomerases catalyse the interconversion of topological isomers of DNA and are required for the resolution of torsional stress in the DNA and for the unlinking of topologically-intertwined molecules (Wang, 1996). For an excellent discussion of the potential roles of interactions between topoisomerases and helicases, which is beyond the scope of this introduction the reader is referred to Duguet (1997).

Interactions have been identified between the RecQ family helicases and topoisomerases in both human and budding yeast cells. For example, a reduction in the cellular level of topoisomerase IIα levels has been documented in cells derived from individuals with BS (Foucault et al., 1997). In budding yeast, deletion of *SGS1* results in a synergistic growth defect in combination with a *top1* mutation and a chromosome segregation defect that is epistatic to that of a *top2-4* conditional mutation (Watt et al., 1995; Lu et al., 1996). Moreover, both topoisomerase II and III are reported to interact physically with Sgs1 protein (Gangloff et al., 1994; Watt et al., 1995). However, perhaps the most compelling evidence for a functional link between Sgs1p and a topoisomerase is that deletion of *SGS1* strongly suppresses the slow growth and hyper-recombination phenotype of *top3* mutants (Gangloff et al., 1994). By understanding the function of topoisomerse III, it is reasonable to suppose that it will be possible to determine in greater detail the function of Sgs1p and perhaps other protein members of the RecQ family.
In the following sections an overview of the topoisomerases and their biological functions will be presented together with a more detailed description of the topoisomerase III family. Current hypotheses regarding how this group of enzymes might act in concert with the RecQ family helicases will also be detailed.

1.5.1 Topology-definitions

Topoisomerases are enzymes that catalyse the inter-conversion of topological isomers of DNA (Bates and Maxwell, 1993; Watt and Hickson, 1994; Wang, 1996). Topological isomers are those DNA molecules which differ only in their *linking number*, which is the number of times two strands cross each other when the DNA molecule is projected onto a plane. This is due to a combination of the degree of *twist* (the extent of coiling of the two DNA strands about the axis of the DNA helix) and *writhe* (the coiling of the helical axis in space). Within supercoiled DNA, further configurations can arise including *catenation*, which is the linking of two or DNA more molecules in which at least one strand of each duplex is in the form of a closed ring; and *knotting* which is the irreducible entanglement of a single DNA molecule.

Topoisomerases can be classified into two broad groups on the basis of their catalytic action (Wang, 1996). Type I topoisomerases produce transient single strand breaks, within a DNA duplex allowing the other DNA strand of the helix to pass through the break with subsequent religation of the primary strand. Type II topoisomerases introduce transient double strand breaks, enabling the passage of an intact DNA duplex through the break before it reseals. According to these abilities, type I topoisomerases can alter the linking number in steps of one, whereas type II topoisomerases alter the linking number in steps of two.

1.5.2 Topoisomerases-classification

A table summarising the features of the best studied topoisomerases is presented in Table 1.4 (Bates and Maxwell, 1993; Watt and Hickson, 1994; Wang, 1996). There are two groups of class I enzymes, type IA enzymes which are found in both prokaryotes and eukaryotes, and type IB topoisomerases found exclusively in eukaryotes. Type IA topoisomerases act as monomers, form a covalent link with the 5' phosphoryl end of cut DNA and with one exception are ATP-independent. This family consists of the topoisomerase III subfamily, *E.coli* topoisomerase I and 'reverse gyrase' subfamily. This latter group of enzymes are found in hyper-thermophilic archaebacteria and catalyse the ATP-dependent introduction of positive supercoils into DNA (see section 1.5.8 for a further discussion of their function).

Type IB topoisomerases also act as monomers and are ATP-independent, but differ from the type IA enzymes in that they bind from a covalent link to the 3' phosphoryl end of cut DNA. All eukaryotic type I topoisomerases are members of this group. These

enzymes can relax both negative and positive supercoils and act primarily in replication and in transcription, where they act to limit excessive supercoiling which arises as proteins translocate through a DNA duplex.

Type II topoisomerases are ATP-dependent, function as dimers and form a covalent link with the 5' phosphoryl end of cut DNA. This group includes bacterial gyrase and topoisomerase IV, and all of the eukaryotic type II topoisomerases. These enzymes act either in controlling the level of supercoiling or as decatenases. This latter function is of most importance during the final stages of replication.

Table 1.4 Summary of the classification and functions of the topoisomerases

Class	name	organism	function
IA	Topoisomerase I	*E.coli*	relaxes negative supercoils
	Topoisomerase III	*E.coli*	genome stability/plasmid segregation
	Topoisomerase III	*S.cerevisiae*	genome stability
	Topoisomerase IIIα	Human	not known
	Topoisomerase IIIβ	Human	not known
	reverse gyrase	thermophilic bacteria	induces positive supercoiling
IB	Topoisomerase I	*S.cerevisiae*	relaxes negative and positive supercoils
	Topoisomerase I	Human	relaxes negative and positive supercoils
II	DNA gyrase	*E.coli*	induces negative supercoils
	Topoisomerase IV	*E.coli*	chromosome segregation
	Topoisomerase II	*S.cerevisiae*	chromosome segregation
	Topoisomerase IIα	Human	not known
	Topoisomerase IIβ	Human	not known

1.5.3 Biological functions of topoisomerases: an overview

Although at one level the topoisomerases have simple biochemical activities, their ability to influence nearly all aspects of DNA metabolism, make an assessment of their cellular functions more difficult. In the following brief overview the role of topoisomerases in two key areas will be discussed: their role in DNA replication and their involvement in maintaining genome stability. For an extensive review of the functions of the topoisomerases, see Wang, 1996 (and references therein).

Topoisomerases have important roles in both the initiation and elongation steps of DNA replication. In both cases, this function is dependent upon the ability of certain topoisomerases to modulate the level of DNA supercoiling. DNA supercoiling is a property of almost all DNA in the form of closed circular DNA. In eukaryotes, linear

DNA is fixed at multiple sites to the nuclear matrix, and the domains between such attachment sites behave as closed circular molecules, since their ends are in fixed orientation relative to one another (Bates and Maxwell, 1993). Such closed circular molecules or closed domains are almost invariably supercoiled.

The role of topoisomerases in supercoiling homeostasis is relevant to the initiation step of DNA replication, since the binding of sequence specific proteins to 'origins of replication' (a necessary antecedent to the assembly of a replication complex), is stimulated by negative supercoiling of the DNA. For example, the binding of the *E.coli* initiation protein DnaA to *oriC*, the origin of replication, is more stable if *oriC* is located on negatively supercoiled DNA (Fuller and Kornberg, 1983). In lower eukaryotes, the efficiency of the autonomously replicating sequence (ARS) to act as replication origins is also dependent upon the degree of supercoiling (Umek and Kowalski, 1988).

DNA replication involves the translocation of a replisome through the DNA duplex. This tracking process has the potential to generate positive supercoils ahead of the complex (Liu and Wang, 1987). Constraints upon the movement of the ends of the DNA molecule through anchoring to other proteins (Cook, 1991) or the viscosity of the nuclear milieu, also result in the formation of positive supercoils, since they cannot diffuse across the template. Topoisomerases can therefore act as 'swivelases' which relax positive supercoils and allow DNA chain elongation. In prokaryotes, this effect is achieved primarily by DNA gyrase which introduces negative supercoils to counteract the formation of positive supercoils. In eukaryotes either topoisomerase I or II activity is sufficient to fulfil this function. Loss of topoisomerase I in budding yeast results in a retardation of nascent chain elongation (Kim and Wang, 1989) and topoisomerase I is reported to preferentially associate with the replication fork (Wang and Liu, 1990).

At the terminal stages of DNA replication as two replication forks converge, the unwinding of the parental DNA strands between the two forks may precede their replication into two daughter helices. The product of replication may then be a pair of catenated DNA rings in the case of closed circular DNA, or catenated topological domains in the case of linear replicons (reviewed in Wang, 1996). In principle, catenated DNA molecules may be resolved by either type I or type II topoisomerases, but in the case of type I molecules, the reaction requires a nick, or single strand gap in at least one of the substrate DNAs. In prokaryotes, decatenation is achieved by topoisomerase IV and to a lesser extent, by gyrase and topoisomerase III (Hiasa et al., 1994; Hiasa and Marians,

1994; Zechiedrich and Cozzarelli, 1995). In eukaryotes, there is substantial evidence also that type II topoisomerases are the predominant effectors of decatenation. In *S.cerevisiae*, shift of the conditional mutant *top2-4*, results in cellular progression through anaphase with entangled chromatids, leading to chromosome loss or breakage (Holm et al., 1985, 1989; Spell and Holm, 1994). This appears to affect primarily large chromosomes which were found to break in a 200kb region near the centromere, but smaller intertwined chromosomes appear to resolve without breakage, presumably through unwinding of the intertwinings off the ends of the chromosome arms. The role of topoisomerase II in chromosome segregation in higher eukaryotes has been confirmed by a large number of studies (reviewed in Wang, 1996). Thus, in mammalian cells, immunodepletion of topoisomerase II (Yang et al., 1987) or its inactivation by drugs (Roca et al., 1994) leads to inhibition of decatenation and chromosome separation.

Evidence supporting the role of topoisomerases in maintaining genome stability is mainly derived from studies in *S.cerevisiae*. Inactivation of any of the known three topoisomerases in this organism results in marked increases in mitotic homologous recombination. This is manifested primarily in loss of rDNA stability for *top1* and *top2* conditional mutants (Christman et al., 1989; Kim and Wang, 1989), whereas all repetitive sequences are affected by loss of topoisomerase III (Wallis et al., 1989; Gangloff et al., 1994; Kim et al., 1995) (see 1.5.5). In cells of a *top1Δ top2ts* double mutant grown at the restrictive temperature, over half of the rDNA genes are present in the form of extrachromosomal rings (Kim and Wang, 1989) . Expression of either *TOP1* or *TOP2* in this mutant promotes re-integration of the rings back into the rDNA locus.

A number of mechanisms have been proposed to explain the loss of genomic stability when topoisomerases are inactivated (reviewed in Wang et al., 1990; Duguet, 1997). The first is that hypernegative supercoiling, by promoting the inadvertent pairing of DNA molecules, may itself stimulate recombination, particularly in heavily transcribed regions such as rDNA. This seems unlikely as the hyper-recombination phenotype is observed in *top1* or *top2* single mutants in which hyper-negative supercoiling is not evident. A second explanation is that topoisomerases may be involved directly in recombination, perhaps by acting to resolve plectonemic recombinational junctions, a process that is similar mechanistically to the separation of interwound parental strands during replication.

Illegitimate recombination can be affected both negatively and positively by topoisomerase activity. Thus, on the one hand *E.coli* topoisomerase I appears to be necessary for illegitimate recombination events involving the formation of deletions that occur following perturbations of DNA replication (Zu and Scheistl, 1996; Bierne et al., 1997). On the other hand, inactivation of *E.coli* topoisomerase III results in an increase in the spontaneous rates of deletion formation (Schofield et al., 1992). Treatment of eukaryotic cells with topoisomerase inhibitors, which act to stabilise covalent intermediates between DNA and the topoisomerases, results in chromosomal rearrangements, deletions, sister chromatid exchanges, quadriradials and an increase in integration events (Anderson and Berger, 1994). At a molecular level, there is good evidence that some of these events arise secondarily to non-homologous recombination (Bae et al., 1988 and 1991; Bodley et al., 1993; Aratani et al., 1996). However, since topoisomerase-inhibiting drugs also induce DNA damage, interpreting their effects and extrapolating them to topoisomerase function is difficult.

1.5.4 The topoisomerase III family

An alignment of the key members of this family is shown in Figure 1.6. Most of the information concerning this family of topoisomerases is derived from the bacterial or yeast enzymes. Where tested, these enzymes possess similar biochemical activities *in vitro*, although few direct comparisons have been made (DiGate and Marians, 1988; Hiasa and Marians, 1994; Zhang et al., 1995 and 1996; Kim and Wang, 1995; Hanai et al., 1996). However, it seems likely that their *in vivo* functions will differ at least in some respects, since in higher eukaryotes these enzymes are essential, whereas in prokaryotes and in *S.cerevisiae* these enzymes are not necessary for viability. At the time of writing, two new members to this family have been identified. An *S.pombe* sequence with a high degree of homology to budding yeast and mammalian topoisomerase III enzymes has recently become available, although the phenotype of the corresponding null mutant has not been reported. In humans, a putative *TOP3β* , predicted to encode an 753 residue protein, has also recently been identified in the immunoglobulin λ locus at 22q.11-12 (Kawasaki et al., 1997). The region of homology to other members of the topoisomerase III family extends throughout the first ~600 amino acids. Like human topoisomerase IIIα, the predicted protein has a C-terminal domain but it shares no significant motifs or homologies to other proteins.

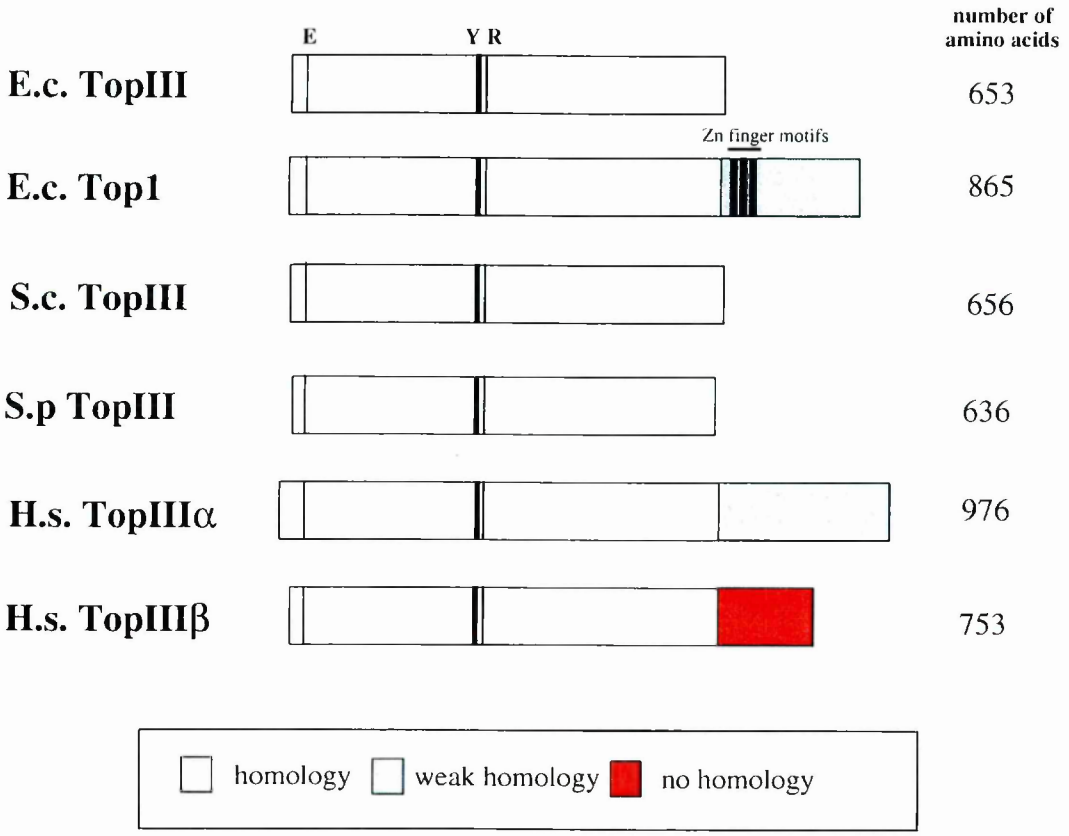


Figure 1.6: Schematic representation of the structures of members of the topoisomerase III family. The proteins shown are *E.coli* topoisomerase III and topoisomerase I (the latter shown for comparison), and topoisomerase III enzymes of *S.cerevisiae*, *S.pombe* and *H.sapiens*, as indicated on the left. The size of each protein is shown on the right. Regions corresponding to areas of homology, weak homology or no homology are as indicated in the key below the diagram. Three black vertical bars in *E.coli* topoisomerase I indicate the positions of tetracysteine motifs that may be involved in DNA binding. Alanine substitution at the highly conserved residues E (glutamine-9) and R (argininine-321), both of which are in close proximity to the active site tyrosine (Y-319), results in the loss of DNA relaxation activity of *E.coli* topoisomerase I.

A comparison of the various properties of the topoisomerase III family is shown below.

Table 1.5 A comparison of bacterial, yeast and human topoisomerase III enzymes

	E. coli	*S.cerevisiae*	*H.sapiens* *
size	653 aa	656 aa	976 aa
DNA binding	ssDNA	ssDNA	NT†
relaxation of supercoils	relaxes negative supercoils	relaxes negative supercoils (weak)	relaxes negative supercoils (weak)
decatenation	yes	NT	NT
replication	supports nascent chain elongation	NT	NT
effect of gene deletion on genome stability	deletional mutations	instability of repetitive sequences	not known

* human topoisomerase IIIα

† NT - not tested

1.5.5 *E.coli* topoisomerase III

E. coli topoisomerase III, although structurally similar to *E.coli* topoisomerase I, is biochemically and functionally distinct. It was originally purified as a superhelical DNA relaxation activity from cells containing a deletion of the gene (*topA*) encoding topoisomerase I (Srivenugopal et al., 1984). However, unlike topoisomerase I, topoisomerase III mediated relaxation of negatively supercoiled DNA is only detectable under extreme conditions such as high temperature, low salt and low Mg^{2+} (DiGate and Marians, 1988). In contrast, decatenation of paired plasmids or late stage replication intermediates is proficient under standard conditions (Hiasa and Marians, 1994; Hiasa et al., 1994). *E. coli* topoisomerase III is also able to support *in vitro* nascent DNA chain elongation, whereas topoisomerase I is inhibitory in this regard (Hiasa and Marians, 1994). The finding that topoisomerase III can support chain elongation is of some interest, since it was originally thought to be dependent upon the ability of an enzyme to act as a 'swivelase', removing positive supercoils as they build up ahead of a replication fork. Two hypotheses have been put forward to explain this apparent paradox (Hiasa and Marians, 1994; Wang, 1996). One potential explanation is that topoisomerase III could catalyse the passage of one parental strand through another by acting on a single stranded region near the replication fork, thus reducing the degree of positive supercoiling. An alternative hypothesis is that positive supercoils can equilibrate across the DNA template, taking the form of catenanes behind the fork and positive coils ahead of the fork. Topoisomerase III could act on catenanes behind the replication fork and ensure its progression. Since topoisomerase III is partially redundant with gyrase and topoisomerase IV with respect to its ability to support *in vitro* replication (Hiasa and Marians, 1994), it is probable that this does not represent the major function of this enzyme. Consistent with this, the only cellular phenotype associated with loss of topoisomerase III activity is an excessive rate of spontaneous deletion formation between regions involving short homologies (Schofield et al., 1992) and it is not clear how this relates to any of the reported biochemical properties of this enzyme.

Although not necessary for viability, the recent finding that a large number of conjugative plasmids isolated from Gram positive and negative bacteria encode polypeptides with extensive sequence similarity to topoisomerase III (Li et al., 1997) is indicative of the biological importance of this topoisomerase subfamily. One of these, TraE protein derived from the plasmid RP4, exhibits biochemical activity identical to that

of topoisomerase III. Based upon the observations described above, one possibility is that this group of plasmid-derived topoisomerase III-like proteins act to resolve plasmid DNA replication intermediates either during vegetative replication or in conjugative DNA transfer.

In view of the extensive sequence similarity between the type IA topoisomerases it is thought likely that they share common mechanisms of action at a biochemical level. At the time of writing, the crystal structure of none of the topoisomerase III enzymes has been determined. However, a 67 kDa fragment of *E.coli* topoisomerase I (comprising the N-terminal 596 residues) has been crystallised and its structure solved (Lima et al., 1994). The polypeptide folds into four distinct domains: domains I and IV form the 'base' of the fragment and domains III and II the 'lid'; a pair of long strands connect the 'lid' to the 'base', and the four domains and the connecting strands enclose a hole with an average diameter of 30 Å. The active site nucleophile Tyr-319 is located in domain III, at the junction between this domain and domains I an IV. This strategic location suggests that the DNA strand containing the phosphotyrosyl bond is bound to the enzyme across domain III and the base comprising I and IV. In order to provide room for the binding of a DNA strand, the lid (domains III and II) and the base (I and IV) must move away relative to each other from their positions, using the connecting strands as hinges. A diagrammatic representation of the proposed mode of action for type IA topoisomerases is presented in Figure 1.7. More recently, site-directed mutagenesis of residues conserved in all type IA topoisomerases has been reported (Chen and Wang, 1998). Alanine substitution at Glu-9 or Arg-321 (both of which are in close proximity to the active site tyrosine Y319) , both resulted in a loss of DNA relaxation activity in *E.coli* topoisomerase I.

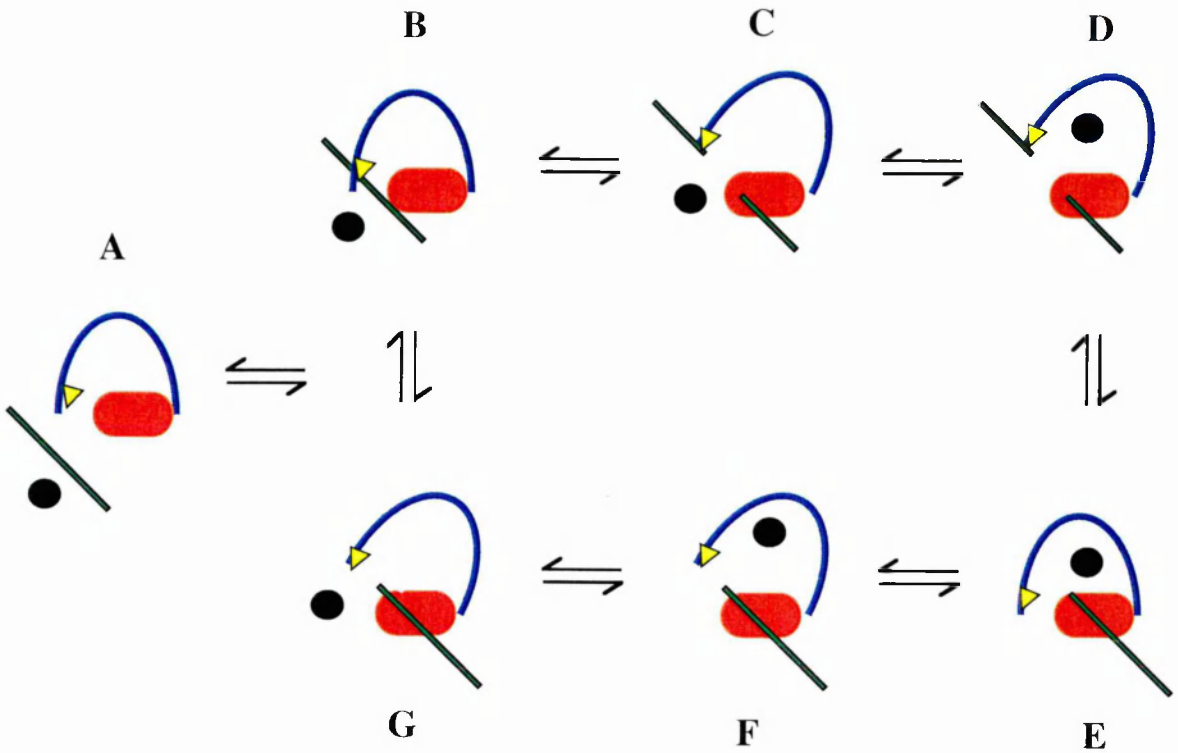


Figure 1.7: Model for type IA topoisomerase strand passage reaction (Lima et al., 1994). Intermediate steps for a strand passage reaction resulting in the catenation/decatenation of double strand rings where one ring has a pre-existing single-stranded region. Type IA topoisomerases can cleave only ssDNA. In this reaction, the single stranded region is cleaved and the duplex portion of a different molecule is passed through the break. In the diagram shown, the 'passing' strand (P) can be either be internal or external (P_{in} or P_{ex}) to the enzyme's central cavity, and is represented by the closed black circle. The single-stranded gated strand (G) can be either opened or closed (G_O or G_C) and is represented by the green tube. The enzyme (E) can also be either an open or closed state (E_O or E_C) and consists of a base (red rectangle) and a lid (blue arc). No directionality for this mechanism is known and all intermediates could proceed in either direction as indicated by the double arrows. **A** ($P_{ex} + E_CG_C$). In this state neither the passing or gated strands are associated with the protein. The gated strand and the enzyme are in a closed state. **B** ($P_{ex} + E_CG_C$). The gated strand interacts covalently with the enzyme (both in a closed state) and the passing strand is external to the enzyme. **C** ($P_{ex} + E_OG_O$). The passing element is external to the protein and both the gated strand and the protein are open. Tyr-319 (yellow triangle) is shown covalently attached to the 5'-phosphoryl end of the gated strand. The 3'-OH end of the gated strand interacts non-covalently with the base of the enzyme. **D** ($P_{in} + E_OG_O$). The passing strand passes through the break in the gated strand and is now internal to the enzyme. **E** ($P_{in} + E_CG_C$). The passing strand is internal to the enzyme, but both the enzyme and the gated strand are closed. The gated strand interacts non-covalently with the base of the enzyme. **F** ($P_{in} + E_OG_C$). The passing strand can exit from the protein's central cavity as it is now in the open state. **G** ($P_{ex} + E_OG_C$). The enzyme remains open and interacts non-covalently with the gated strand, whilst the passing strand exits the enzyme.

1.5.6 *S.cerevisiae* topoisomerase III

The first eukaryotic type IA topoisomerase identified was budding yeast topoisomerase III (Wallis et al., 1989). *S.cerevisiae top3* mutants show defective cell cycle progression such that cells appear to delay with a late S/G2 DNA morphology (Gangloff et al., 1994), which has been interpreted as evidence for a role in late replication as described below (Section 1.5.8). Diploids homozygous for *top3* are unable to sporulate suggesting a role in meiosis, although at what stage during meiosis *top3* cells are defective has not been defined. Like its prokaryotic counterpart, loss of yeast topoisomerase III activity results in a loss of genomic stability (Wallis et al., 1989; Bailis et al., 1992; Gangloff et al., 1994). Mutations in the *TOP3* gene result in an increase in the frequency of *RAD52*-dependent loss of a suppressor tRNA marker flanked by δ sequences (short, repetitive sequences present in the yeast transposon Ty). The hyper-recombination phenotype in *top3* mutants is distinct from that observed in *top1* and *top2ts* mutants, which affects stability only of the highly repetitive rDNA (Christman et al., 1989; Kim and Wang, 1989). In contrast, *top3* mutants show increased recombination affecting all repetitive sequences studied, including δ sequences, rDNA and unlinked homologous *SAM* genes. In wild type cells, recombination at these loci occurs at a low frequency. It appears, however that in *top3* mutants, not only is the overall frequency altered but that the mechanism of recombination is distinct. Thus, recombination in *top3* mutants between the unlinked homologous *SAM1* and *SAM2* genes was partially dependent upon the nucleotide excision repair gene, *RAD1*. Since Rad1p possesses exonuclease activity, it was proposed by the authors of this study, that this protein processes DNA lesions generated in the absence of topoisomerase III and facilitates their repair by *RAD52*-dependent recombination.

Biochemically, the yeast topoisomerase III enzyme resembles *E.coli* topoisomerase III, in that it partially relaxes negatively, but not positively supercoiled DNA , binds with strong preference to ssDNA and binds to sequences very similar to that of the *E.coli* enzyme (Kim and Wang, 1995). *In vivo* experiments, in which the other two yeast topoisomerases, I and II, are inactivated confirm that topoisomerase III has only weak activity in relation to the relaxation of negative supercoils (Wang, 1996). It appears unlikely therefore that a failure to modulate the degree of supercoiling in *top3* mutants underlies their hyper-recombination phenotype.

1.5.7 Mammalian topoisomerase III enzymes

Human *TOP3α,* encoding a 976 residue protein, is a single copy gene located on chromosome 17p11.2-12 (Hanai et al., 1996). This protein shows 44% identity and 61% similarity with yeast topoisomerase III over the first ~ 600 amino acids. However, in addition, the human enzyme has a C-terminal segment of over 300 amino acids with no counterpart in the yeast enzyme and which contains no significant motifs, but possesses weak homology with the C-terminal domain of *E.coli* topoisomerase I. At a biochemical level, human topoisomerase IIIα can partially relax highly negatively supercoiled DNA, although information regarding sequence recognition or the ability to support *in vitro* replication are as yet not available. *TOP3α* mRNA is constitutively expressed in multiple normal tissues (Fritz et al., 1997).

Little is known, as yet, about the biological functions of mammalian topoisomerase IIIα *in vivo.* A murine *TOP3α* has been identified which is highly expressed at the mRNA level in the testis (Seki et al., 1998). Targeted disruption of murine *TOP3α* has recently been reported (Li and Wang, 1998). Heterozygous *top3α*$^{+/-}$ mutant mice resembled phenotypically *top3α*$^{+/+}$ litter mates. No viable *top3α-/-* progeny were obtained. Examination of embryos or blastocysts cultured *in vitro,* indicated that implantation of *top3*$^{-/-}$ embryos and the induction of decidualisation in the uterus could occur, but that viability was compromised at an early stage of development. This suggests that mammalian topoisomerase III performs an essential role, perhaps through a role in cell proliferation or genomic maintenance.

Indirect evidence regarding the functions of human topoisomerase III are derived from studies designed to identify genes able to complement the cellular AT phenotype. Expression of a truncated (amino acids 141-976) topoisomerase IIIα was able to partially correct the radiosensitivity, hyper-recombination and radio-resistant DNA synthesis phenotypes of an AT cell line (Meyn et al., 1993; Fritz et al., 1997). The effect was apparently related to a dominant negative phenomenon, since this effect was reproduced by an antisense construct, but not by expression of the full length gene. The interpretation of this finding is fraught with difficulty since there are a large number of unrelated genes which are able to complement the AT phenotype. Furthermore, it is difficult to distinguish between dominant negative effects that relate to the true functions of an overexpressed gene and artefactual effects of overexpression. Another potential clue to the role of topoisomerase III is that the *TOP3α* gene is consistently deleted in

individuals with Smith-Magenis syndrome (SMS) in which multiple congenital abnormalities/mental retardation are associated with an interstitial 4Mb deletion of chromosome 17p11.2 (Elsea et al., 1998). However, since the deletion is extremely large and likely to involve many other genes, and since the other *TOP3α* gene still remains intact, it seems extremely unlikely that partial loss of topoisomerase III activity has any role in the pathogenesis of this disorder.

1.5.8 Models to explain the interaction between Sgs1p and topoisomerase III

A number of hypotheses have been put forward to explain the functional basis for the interaction between Sgs1p and topoisomerase III. As discussed above, budding yeast topoisomerase III is a type IA topoisomerase, as are the hyperthermophilic 'reverse gyrases'. Many workers in the topoisomerase field have been attracted by the potential mechanistic similarity of an Sgs1/Top3 complex to the reverse gyrase enzyme found in hyperthermophilic archaebacteria. Reverse gyrase is composed of both a helicase-like domain and a type IA topoisomerase domain either in the same polypeptide or in the form of a heterodimeric complex (Confalonieri et al., 1993; Duguet, 1997). Movement of the helicase domain through the DNA duplex produces 2 waves of supercoiling, positive in front of and negative behind the translocating complex. Selective relaxation of the negatively supercoiled regions by topoisomerase III would, as a consequence, generate net positive supercoiling. In thermophiles, such an effect might act to prevent denaturation of DNA during growth at elevated temperatures. However, attempts to generate positive supercoiling activity *in vitro* using a recombinant Sgs1p fragment in the presence of topoisomerase III have so far proven unsuccessful (Bennet et al., 1998).

There are many alternative suggestions for the functional role of an association between a RecQ helicase and a topoisomerase. For example, a direct role for a helicase in conjunction with a topoisomerase in recombination has been suggested previously by others (Wang et al., 1990: Wang, 1996). Thus, depending upon the site of its activity, the helicase/topoisomerase complex could act either to disrupt inadvertently paired DNA strands, or to promote branch migration. Moreover, such a complex could act either as a 'reformatase' to eliminate abnormal DNA structures, such as triple helices, or act to rapidly reform a plectonemically-wound double helix during branch migration of recombination junctions.

An alternative and appealing possibility is that the RecQ helicase/topoisomerase complex acts to facilitate catalysis of the late stages of replication, as has been proposed previously (Gangloff et al., 1994; Watt et al., 1995). As two replication forks converge, a build up of positive supercoils and/or steric hindrance between the advancing replisomes could inhibit the final stages of DNA replication. Moreover, the priming of new lagging strand synthesis might not be possible under these circumstances. It has been proposed that Sgs1p might unwind the unreplicated region between the advancing forks. This would have the effect of introducing intertwinings between the daughter DNA molecules, which would have to be resolved by the decatenation activity of a topoisomerase prior to anaphase. If replication of the partially single-stranded DNA molecules were completed prior to decatenation, then topoisomerase II (which uniquely can introduce double-strand breaks into DNA) would be the only enzyme capable of unlinking the resulting duplex catenanes (Watt et al., 1995). Alternatively, topoisomerase III, which is able efficiently to decatenate DNA molecules that contain single-stranded regions, could act on the DNA prior to the 'filling-in' of the small single-stranded gaps left at the sites of the converging forks (Gangloff et al., 1994). This might explain why Sgs1p interacts with both topoisomerases II and III. Failure to resolve abnormal DNA structures that arise at the terminal stages of DNA replication could lead to DNA breaks and hence to aberrant recombination, as is observed in mutants deficient in either RecQ helicases or topoisomerases.

1.6 Conclusions

One form of genome instability particularly associated with cancer is chromosomal rearrangement. Rearrangement of DNA that results in amplification, deletion or altered expression of oncogenes or tumour suppressor genes occurs at a greatly increased rate in cancer cells, and may accelerate disease progression. Accordingly, defects in recombinational repair are likely to contribute to both tumour formation and progression. A better understanding of how this repair process contributes to the maintenance of genome stability in normal cells will hopefully lead ultimately to better treatments for cancers in which this process is defective.

In prokaryotes, the RecF pathway appears to have a key role in the recombinational repair of stalled replication forks. The only evidence that a similar pathway might exist

in eukaryotes is the finding of homologues to RecQ helicase, an *E.coli* protein, which is aligned to the bacterial RecF pathway. The more recent finding that defects in RecQ family helicases in humans result in a predisposition to cancer underlines their biological significance. The interactions between the RecQ family helicases and the topoisomerases may provide important clues to how these proteins, acting in concert, influence genome stability.

In the following chapters, an attempt will be made to address to what degree the human BLM protein and the lower eukaryotic Sgs1p share not only structural similarity, but also functional similarity. A primary aim of this initial work was to assess the validity or otherwise for using yeast as a model system for BS. The results of these experiments led directly to a study of the role of topoisomerase III in *S.cerevisiae,* in relation to the effect of this enzyme upon the cell cycle and the cellular response to DNA damage. In the final part of this thesis a model will be presented which uses information derived from this work, to explain the cellular roles of topoisomerase III and Sgs1p.

Chapter 2

Materials and Methods

2.1 Chemicals

Unless otherwise stated, all chemicals used in this work were purchased from the Sigma Chemical Company. Bacterial and yeast media were supplied by ICRF Central Services, Clare Hall. Radioisotopes were provided by Amersham UK, Ltd.

2.2 Enzymes

Unless otherwise stated, all enzymes in this work were purchased from Boehringer Mannheim. Zymolyase-20T was obtained from AMS Biotechnology (Europe) Ltd. and Glusulase was purchased from DuPont.

2.3 Preparation and manipulation of DNA

2.3.1 Preparation of plasmid DNA

Plasmid DNA was prepared from *E.coli* saturated cultures using the alkaline lysis method as described by Sambrook et al. (1989). Alternatively, both small and large scale preparations of plasmid DNA were obtained using Promega 'Wizard' mini- or maxi-prep kits (which employ a silica-based resin to bind DNA) according to the manufacturer's instructions.

DNA to be sequenced was prepared by a modified alkaline lysis/PEG precipitation procedure. A single bacterial colony was inoculated into 4.5ml Terrific Broth [1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 17mM KH_2PO_4, 72mM K_2HPO_4] and grown with aeration at 37°C to saturation. Cells were then pelleted and resuspended in 200µl of GTE buffer (50mM glucose, 25mM Tris-HCl, pH8.0, 10 mM EDTA, pH8.0). Lysis was achieved by the addition of 300µl of freshly prepared 0.2M NaOH/1% (w/v)SDS and then the contents mixed gently until the solution cleared. After incubation on ice for 5 minutes, the solution was neutralised by the addition of 300µl 3M potassium acetate, pH4.8, followed by mixing and a further incubation on ice. Cellular debris was removed by centrifugation in an Eppendorf 5415 C bench top micro-centrifuge at 14,000rpm for 5 minutes and the supernatant transferred to a clean tube. RNase (DNase-free) was the added to a final concentration of 20µg/ml and the solution incubated at 37°C for 20 minutes. Chloroform extraction was then performed twice (each time by adding an equivalent volume of chloroform), before

precipitation of the DNA by adding an equal volume of isopropanol. Precipitated DNA was pelleted by centrifugation at 14,000rpm for 10 minutes and was then washed once with 500µl of 70% ethanol. The pellet was then dissolved in 32µl of ddH_2O and the DNA re-precipitated by the addition first of 8µl 4M NaCl, and then adding 40µl of autoclaved PEG solution [13% (w/v) PEG 8000]. The sample was then mixed and incubated on ice for 20 minutes, before pelleting the DNA by spinning at 14,000rpm at 4°C. The supernatant was removed and the pellet washed with 500µl of 70% ethanol. The pellet was then resuspended in 20µl ddH_2O and stored at -20°C.

DNA prepared for transfection was purified by ultracentrifugation on caesium chloride gradients. DNA generated by the alkaline lysis method (derived from 200-400ml volumes of saturated bacterial culture) was resuspended in 4.1ml TE containing 40µg/ml ethidium bromide and 4.8g caesium chloride. Samples were then pre-cleared by the centrifugation at 2000rpm in a Beckman GPR centrifuge for 10 minutes. The supernatant was then loaded into Beckman ultracentrifugation tubes and ultracentrifuged at 80,000rpm for 3 hours at 20°C using a Vti80 rotor in a Beckman L8-M centrifuge. Using a 20G needle, the plasmid band was aspirated. All the above steps were then repeated to allow a second centrifugation under the same conditions. Following this, ethidium was extracted repeatedly with an equal volume of iso-amyl alcohol and the solution then diluted in 3 volumes of TE (10mM Tris-HCl, pH8.0, 1mM EDTA). 3 volumes of ethanol were then added and the sample was left at -80°C for 15 minutes to precipitate the DNA. Samples were then centrifuged at 10,000rpm and the precipitated DNA washed once in 70% ethanol, before resuspending in 100µl TE.

In all cases, DNA quality was assessed by measuring the OD_{260} and by running the products of the appropriate restriction digest on a 0.8-1.2% agarose gel.

2.3.2 Preparation of yeast genomic DNA

A single yeast colony was inoculated into 5-10 ml liquid YPD (see section 2.10) and grown to saturation. The cells were harvested by centrifugation at 3000rpm in a Beckman GPR centrifuge, room temperature for 5 minutes and then resuspended in 0.5ml solution A [1.2M sorbitol, 200mM Tris-HCl, pH7.5, 20mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 100µg/ml zymolyase 20-T] and cells allowed to spheroplast at 37°C for 20 minutes. Spheroplasts were pelleted by spinning for 2 minutes at 14,000rpm using a bench top microcentrifuge.

In order to lyse the cells and remove RNA and protein, the cell pellet was gently resuspended in 50µl 1M sorbitol and then 0.5 ml solution B [50mM Tris-HCl, pH7.5, 100mM NaCl, 100mM EDTA, 0.5% (w/v) SDS, 0.4mg/ml Proteinase K, 0.1mg/ml RNase]. Following incubation at 65°C overnight, the preparation was gently extracted twice with phenol/chloroform [1:1 (v/v), pH 8.0] and the DNA precipitated by adding 1ml ethanol. After gentle inversion, the DNA was pelleted by centrifugation at 14,000rpm in a bench top microcentrifuge for 10 minutes. The DNA was washed with 1ml 70% ethanol, dried and then resuspended in 200µl TE buffer.

2.3.3 Preparation of intact yeast chromosomal DNA

Intact yeast chromosomal DNA samples were prepared from log phase or arrested cells, as described previously (Louis and Haber, 1990). Briefly, 1 x 10^7 cells derived from log phase or arrested cultures were pelleted and then washed in 1ml cold 50mM EDTA. Following centrifugation at 14,000rpm in a bench top microcentrifuge the pellet was resuspended in 200µl of 50mM EDTA and left on ice. For each sample, 100µl SCE/zymolyase solution [1M sorbitol, 0.1M sodium citrate, 0.05M EDTA, 5% (v/v) 2-mercaptoethanol and 3mg/ml zymolyase-20T] and 0.5 ml low melting point agarose solution [1% (w/v) low melting point agarose, 0.45M EDTA, 0.1M Tris-HCl, pH8.0] were added simultaneously. The cells were gently mixed and then placed into an agarose plug mould. After setting, the plugs were put into clean 1.5ml eppendorf tubes and overlaid with 0.5ml of 0.45M EDTA, 0.1M Tris-HCl, pH8.0, 5% (v/v) 2-mercaptoethanol. After incubation at 37°C for at least 4 hours, the above solution was removed and the plugs overlaid with 0.5ml of 1% (v/v) sarkosyl, 1mg/ml proteinase K, 0.1 mg/ml RNase, 0.4M EDTA and incubated at 37°C overnight. The plugs were then washed with cold 0.5M EDTA and then stored under 0.45M EDTA, 0.1M Tris-HCl, pH8.0 at 4°C.

2.3.4 Polymerase chain reaction (PCR)

All polymerase chain reactions involving the generation of products used for subsequent cloning employed Expand™ High Fidelity PCR System (Boehringer Mannheim) according to the manufacturers instructions. This system is composed of thermostable Taq and Pwo polymerases. The latter enzyme possesses 3'-5' exonuclease activity which is reported to result in a higher fidelity of DNA synthesis than Taq alone

(Barnes, 1994). In a standard reaction, 50-100 ng of template DNA and 20pmoles of each primer were used for a 50µl reaction. For each reaction, the optimal $MgCl_2$ concentration, annealing temperature, extension times and number of cycles was determined. Negative controls for PCR were reactions without template DNA. In all cases where DNA for cloning was being generated, the minimum number of cycles that generated a product visible on agarose gel electrophoresis was used. In order to generate a *BLM* product, pooled PCR products derived from 5-10 individual reactions were used in order to reduce the number of cycles required. All oligonucleotides were generated by the ICRF Oligonucleotide Synthesis Service, Clare Hall and supplied deprotected and dried down. The side products of synthesis were removed by ethanol precipitation.

To generate a full length *SGS1* cDNA the 5' primer 66436 (5'-GAGGA**GGATCC**AGGATTATGGTGACGAAGCCGTCACA-3') and 3' primer 66435 (5'-ATAAGAAT**GCGGCCGC**TTACATCACTTTCTTCCTCTGTAGTG-3') were used. The 5' primer included a *BamH1* site (bold) and a yeast Kozak consensus (underlined) and the 3' primer included a *Not1* site (bold). Template DNA was cosmid 9646 (kindly donated by Dr. Ed Louis, Institute of Molecular Medicine, Oxford). Conditions for PCR were as follows; 1st step, 93°C (2minutes) for 1 cycle; 2nd step, 55°C (1 minute), 68°C (7 minutes) and 93°C (30 seconds) repeated for 5 cycles; and final step, 55°C (1 minute) followed by 68°C (15 minutes) for 1 cycle.

A full length *BLM* cDNA was generated in 2 sections, a 5' 1.4 kb (Z) and a 3' 2.9kb (X/Y) with an overlapping unique *EcoR1* site. PCR(Z) was generated using the 5' primer F1 (5'-AGAGGA**CTCGAG**AGGATTATGGCTGCTGTTCCTCA-3') and 3' primer R2 (5'-TCCTAGGGTGGTAGTCAGTAAACA-3'). The 5' primer included a *Xho1* site (bold) and a yeast Kozak consensus (underlined). Template DNA was a human B-cell cDNA library (kindly donated by Dr. D. Simmons, Institute of Molecular Medicine, Oxford). Conditions for PCR(Z) were as follows: 1st step, 93°C (2 minutes) for 1 cycle; 2nd step, 55°C (1 minute), 68°C (3 minutes) and 93°C (30 seconds) repeated for 32 cycles; and final step, 55°C (1 minute) followed by 68°C (10 minutes) for 1 cycle.

PCR (X/Y) was generated using the 5' primer F3 (5'-ATA CAG GCC TGA TTC ACT -3') and the 3' primer R1 (5'-CTC GAG CAT TGA GAT TCG GTT GTT ATG AGA ATG-3'), and using the same template DNA. Conditions for PCR (X/Y) were as follows: 1st step, 93°C (2 minutes) for 1 cycle; 2nd step, 55°C (1 minute), 68°C (5

minutes) and 93°C (30 seconds) repeated for 18 cycles; and final step, 55°C (1 minute) followed by 68°C (15 minutes) for 1 cycle.

To generate a full length *TOP3* cDNA the 5' primer 84735 (5'-GAGGA**GGATCC**ACAATAATGAAAGTGCTATGTGTCGC-3') and the 3' primer 84734(5'-ATAAGAAT**GCGGCCGC**TTACATGGATGCCTTGACAC-3') were used. The 5' primer incorporated a *BamH1* site (bold) and the 3' primer incorporated a *Not1* site (bold). Template DNA was pRK500 (kindly donated by J.Wang) and the polymerase used was Taq. Conditions for PCR were as follows: 1st step, 94°C (2 minutes) for 1 cycle; 2nd step, 55°C (1 minute), 72°C (5 minutes) and 94°C (30 seconds) repeated for 7 cycles; and final step, 55°C (1 minute) followed by 72°C (5 minutes) for 1 cycle.

2.3.5 Restriction digest

DNA was digested with the appropriate restriction enzyme according to the manufacturers instructions assuming that 1 μg of DNA is digested by 1 unit of enzyme at 37°C (or other temperature depending on the enzyme utilised). DNA fragments generated by PCR were usually digested overnight, while other restriction digests were incubated for 1-4 hours.

2.3.6 Ligations

Standard ligation protocols were used in the generation of constructs detailed in Section 2.3.7 (Sambrook et al., 1989). Ligations were carried out using an insert: vector ratio of 10:1. DNA following treatment with restriction enzymes or PCR products were separated by agarose gel electrophoresis and/or purified using a GeneClean kit (BIO 101 Inc.) according to the manufacturers instructions. Insert and vector DNA was then combined in a total volume of 10μl containing 20mM Tris-HCl, pH7.6, 5mM $MgCl_2$, 10mM DTT, 50mg/ml BSA, 1mM ATP (0.5mM for blunt end ligations) and 1 unit T4 DNA ligase. Ligations were allowed to proceed for 1 hour at room temperature. Ligations of insert and the pCR-Script vector (Stratagene) were carried out according to the manufacturer's instructions. Controls, included in each ligation series, were: uncut vector, to confirm transformation efficiency; cut vector plus DNA ligase, to detect religation frequency; and cut vector alone, to confirm complete cutting and background religation. Ligation transformants were screened by colony PCR and subsequently by restriction digests of 'miniprep' DNA. For colony PCR, 5μl of saturated solution derived

from individual ligation transformants was added to a 50µl PCR reaction. The conditions for PCR were as above except that the initial denaturation of the 1st step was 94°C for 4 minutes, and in the 2nd step, only 15 cycles were performed.

2.3.7 Plasmids

A list of plasmids used in this study for expression in yeast or mammalian cells is shown in Tables 2.1-2.3.

pJH626.T is a derivative of the pJH626 expression vector (Sikorski and Hieter, 1989) in which the promoter for *S. cerevisiae* triose phosphate isomerase (a glycolytic enzyme with constitutive levels of expression) has been inserted between the *EcoR1-HindIII* sites within the multiple cloning site region. This construct was generated by Dr. Charles Redwood (Institute of Molecular Medicine, Oxford).

A cDNA encoding the full length open reading frame of *SGS1* was generated by PCR as described above and cloned into the pCR-Script SK(+) vector (Stratagene). The cDNA was then inserted between the *BamH1-Not1* sites of pYES2 (to give pRKC2), pJH626.T (to give pRKC3) and pCDNA3 (to give pRKC4). The same cDNA was cloned between the *Xho1-Not1* sites of pREP3X to generate pRKC5.

Pooled 1.4 kb 5' (Z) and 2.9 kb 3' (X/Y) *BLM* cDNA PCR products were cloned into pCR-Script and plasmids derived from several of the resulting transformants sequenced. To generate pYES2, pJH626.T and pCDNA3 derivatives containing *BLM* , the strategy is set out in Section 3.1 was performed. This involved a 4-way ligation employing restriction digest products designed to exclude PCR-induced errors. The resulting constructs were pRKC15 (pYES2-*BLM*), pRKC16 (pJH626.T-*BLM*) and pRKC17 (pCDNA3-*BLM*). To generate pRKC18 (pREP3X-*BLM*) a 3-way ligation was performed in which a *Xho1-AvrII* 5' fragment of *BLM* (derived from a pCR-Script plasmid containing the 5' 1.4 kb *BLM* pcr product) and an *AvrII-Not1* 3' fragment of *BLM* (derived from pRKC15) were inserted into the *Xho1-Not1* sites of pREP3X. A description of the modifications of pRKC15, to generate histidine-tagged BLM have been described previously (Karow et al., 1997). To confirm the *BLM* sequence, constructs were sequenced using ABI^{TM} Dye Terminator Cycle Sequencing (Perkin Elmer Corporation).

The full length *TOP3* cDNA was cloned into pT7.Blue (Invitrogen) to generate pRKC19. Construction of pRKC20, used for the one-step gene replacement of *TOP3* is described in Section 2.14.

Table 2.1 Mammalian expression vectors

name	comment
pCDNA3	*G418*^{R}, CMV promoter (Invitrogen)
pRKC4	pCDNA3, *SGS1* cloned between *BamH1-Not1* sites
pRKC17	pCDNA3, *BLM* cloned between *BamH1-Not1* sites

Table 2.2 *S. cerevisiae* expression vectors

name	comment
pYES2	*URA3*, *GAL1* promoter, multicopy (Invitrogen)
pJH626.T	*URA3*, *TPI* promoter, single copy
pSE556	*URA3*, *GAL1* promoter, single copy (Navas et al., 1996).
pJA98	pSE556 incorporating *RAD53*
pNKY2070	*LEU2*, *PHO5* promoter, multicopy (Kim and Wang, 1992)
pRK500	pNKY2070 incorporating *TOP3*
pYCP1F16	*TRP1*, *GAL1* promoter, multicopy (Foreman and Davis, 1994)
pRAD53HA	pYCP1F6 incorporating HA-tagged *RAD53* (de la Torre-Ruiz et al., 1998)
pRS426	*URA3*, multicopy (Sugimoto et al., 1997)
pDM198	pRS426 incorporating *TEL1*
pRKC2	pYES2, *SGS1* cloned between *BamH1-Not1* sites
pRKC3	pJH626.T, *SGS1* cloned between *BamH1-Not1* sites
pRKC15	pYES2, *BLM* cloned between *BamH1-Not1* sites
pRKC16	pJH626.T, *BLM* cloned between *BamH1-Not1* sites

Table 2.3 *S. pombe* expression vectors

name	comment
pREP3X	*LEU2*, *NMT1* promoter
pRKC5	pREP3X, *SGS1* cloned between *Xho1-Not1* sites
pRKC18	pREP3X, *BLM* cloned between *Xho1-Not1* sites

2.4 Preparation of RNA

The method employed the Qiagen 'RNeasy' kit which involves selective RNA binding (species > 200kb) to a silica gel. A number of modifications were made to the manufacturers instructions in order to increase the yield of RNA. 1 x 10^8 yeast cells were pelleted and frozen immediately on dry ice before storage at -80°C. Each pellet was allowed to thaw on ice, then resuspended in 100µl lysis buffer RLT and an equivalent volume of sterile, glass beads (diameter 0.2mm) added. The sample was then agitated in an homogeniser (Hybaid) for 45 seconds, before adding 500µl RLT buffer. All subsequent steps were performed at room temperature. To remove cellular debris, the sample was centrifuged at 14,000rpm for 2 minutes in an Eppendorf bench top microcentrifuge. The supernatant was then transferred to a new eppendorf containing 500µl 70% ethanol and mixed. 500µl of this mixture was then added to an RNeasy spin column which was then spun for 15 seconds at 14,000rpm and the flow through discarded. This step was then repeated for the remaining mixture. 700µl of wash buffer RW1 was then spun through the column as previously. Two washes with 500µl of buffer RPE were then performed. After the final wash, the column was spun again for 2 minutes. The column was then placed in a new 1.5ml collection tube and 25µl RNase-free H_2O added directly to the column membrane. The column was left at room temperature for 10 minutes, before spinning for 2 minutes at 14,000rpm. This step was then repeated. The final concentration was then determined by measuring the OD_{260} and samples were stored at -80°C. When working with RNA, precautions were taken to avoid RNase contamination. All solutions were made up with DEPC-treated H_2O. Plastic disposable pipettes, freshly opened micropipette tips and eppendorfs were used. Electrophoresis apparatus and circulation pump tubing were all cleaned with 0.2% (v/v) Absolve (DuPont) before rinsing thoroughly in ddH_2O. All working surfaces were cleaned with methanol.

2.5 Preparation of protein extracts

Total cellular yeast protein extracts were prepared using a TCA extraction technique (Fioani et al. 1994). Briefly, 1 x 10^8 cells were pelleted and resuspended in 50µl 20% (v/v) trichloroacetic acid (TCA) and an equal volume of 0.2mm glass beads added. After

vortexing for 3 minutes, the supernatant was then transferred to a new eppendorf. The remaining cell debris was resuspended in 5% (v/v) TCA and again the supernatant transferred. Precipitated protein was pelleted by spinning at 14,000rpm in a bench top centrifuge for 3 minutes. The supernatant was then removed and discarded and the protein pellet resuspended in 100µl 1 x SDS loading buffer [60mM Tris-HCl, pH6.8, 1% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 1% (v/v) 2-mercaptoethanol]. The pH of the protein sample was corrected by the addition of 2.5µl 3M Tris-HCl, pH8.5 and then the samples boiled for 3 minutes. Protein concentrations were determined using the Bradford reaction (Sambrook et al., 1989), which was calibrated using a standard curve for human albumin.

Total protein from GM8505 Bloom's fibroblasts was prepared by collecting 1 x 10^6 cells and resuspending the pellet in 200µl 1 x SDS loading buffer. Samples were boiled for 5 minutes and the DNA sheared by passing the sample repeatedly through a 20G needle.

2.6 Electrophoretic techniques

2.6.1 Agarose gel electrophoresis

DNA samples were electrophoresed through 0.8-1.2% agarose gels in TAE buffer (40mM Tris-HCl, pH8.5, 2mM EDTA) to which 0.5µg/ml ethidium bromide was added and using a horizontal gel apparatus (Pharmacia). DNA samples containing 1/5 volume DNA sample buffer [30% (w/v) sucrose, 100mM EDTA, 0.01% (w/v) bromophenol blue] were electrophoresed at 75mA until the dye had reached the desired distance. DNA was visualised by exposure to UV light from a high intensity, long range wavelength transmitter (Fotodyne). Photographs of gels were taken using an MP-4 Polaroid camera following the manufacturer's instructions. When DNA bands needed to be cut from gels, this was performed using a disposable scalpel under low intensity UV light.

RNA samples were electrophoresed through a 1.2% agarose gel in 20mM phosphate buffer (7mM Na_2HPO_4, 13 mM NaH_2PO_4, pH6.6) using a horizontal gel apparatus (BioRad) with continuous buffer circulation. RNA samples made up to a total volume of 5µl were mixed with 7.3µl glyoxylation buffer [10% (v/v) deionised glyoxal, 75% (v/v) deionised DMSO, 0.01M phosphate buffer, pH 6.6) and 4µl RNA loading buffer [50%

(v/v) glycerol, 0.01M phosphate buffer, pH 6.6, 0.4% (w/v) bromophenol blue]. Samples were then electrophoresed at 4V/cm for 4-6 hours.

2.6.2 Pulse field gel electrophoresis

Samples representing approximately 1 x 10^7 yeast cells were applied to a 1% agarose gel slab and chromosomes were separated using a Biorad contour-clamped homogenous electric field (CHEF) apparatus (CHEF-DRII). Separation was achieved in 24 hours at 200V with initial and final switching times being 60 and 90 seconds, respectively. The running buffer used was 0.5 x TBE (44.5mM Tris, 44.5mM boric acid, 1mM EDTA), which was maintained at a temperature of 11°C by rapid recirculation while running the gel at an ambient temperature of 4°C. The DNA was then transferred to nylon membranes and chromosome IV was detected with an *HO* gene probe, using standard protocols.

2.6.3 Polyacrylamide electrophoresis of proteins

This was performed according to standard protocols (Sambrook et al., 1989). Protein samples were heated at 95°C for 3 minutes in 1 X SDS loading buffer [60mM Tris-HCl, pH6.8, 1% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 1% (v/v) 2-mercaptoethanol]. SDS polyacrylamide gels were prepared using BioRad Mini-Protean vertical gel electrophoresis apparatus. The resolving gel (6.5-12% acrylamide, ratio to bis-acrylamide 37.5:1 to 150:1) was prepared in 375 mM Tris-HCl, pH8.8, 0.1% (w/v) SDS. The stacking gel (4% acrylamide, ratio to bisacrylamide 37.5:1) was made up in 125 mM Tris-HCl, pH6.8, 0.1% (w/v) SDS. Running buffer was 190mM glycine, 25mM Tris, 0.1% (w/v) SDS. Proteins were visualised by staining gels with 0.1% (w/v) Coomassie blue, 50% (v/v) methanol, 7% (v/v) glacial acetic acid.

For analysis of Rad53, protein extracts were separated by SDS-PAGE using 6.5% acrylamide gels prepared using an 80:1 acrylamide:bisacrylamide ratio. For analysis of BLM protein, proteins were separated using a 10% gel with a 37.5:1 acrylamide:bis-acrylamide ratio.

2.7 Southern protocols

Following the separation of DNA by electrophoresis, the agarose gel was exposed to UV light for 2-3 minutes, and immersed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 minutes. The gel was then rinsed in ddH_2O and immersed in neutralising solution (1M ammonium acetate, 0.02M NaOH) for 60 minutes. DNA was transferred overnight to Hybond N nylon membranes by capillary diffusion in 20 x SSC (3M NaCl, 0.3M sodium citrate). The DNA was then cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene).

Probes for Southern blotting were randomly labelled with $\alpha^{32}P$ dCTP using 'Ready-to-go' labelling beads (Pharmacia-Biotech), according to the manufacturers instructions. The *HO* cDNA was kindly provided by Dr. E. Louis, Institute of Molecular Medicine, Oxford.

Hybridisation was performed using a formamide-based buffer (Sambrook et al., 1989). Blots were pre-hybridised in a Hybaid oven at 42°C for 6 hours in pre-hybridisation buffer [50% deionised formamide, 10% (w/v) dextran sulphate, 0.2% (w/v) BSA, 0.2% (w/v) polyvinyl-pyrrolidine(MW 40,000), 0.2% (w/v) Ficoll (MW 400,000), 0.1% (w/v) sodium pyrophosphate, 1% (w/v) SDS, 50mM Tris-HCl, pH7.5 and 100µg/ml denatured salmon sperm DNA). Heat denatured probe was then added and the hybridisation allowed to continue overnight. Non-hybridised probe was removed by washing the membrane 3 times in low stringency buffer [2x SSC, 0.1% (w/v) SDS] at room temperature, followed by 2 washes in high stringency buffer [0.1 x SSC, 0.1% (w/v) SDS] at 65°C. X-ray films (BioMax, Kodak) were exposed to cling film-wrapped membranes for signal detection.

2.8 Northern protocols

Following the separation of RNA by electrophoresis, transfer of RNA to Hybond N nylon membranes was achieved by capillary diffusion overnight in 40 mM phosphate buffer (14mM Na_2HPO_4, 26mM NaH_2PO_4, pH6.6). RNA was cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene).

RNR1, *RAD51*, *RAD16* and *ACT1* DNA probes were kindly donated by Dr. Noel Lowndes, ICRF. A 1.9kb *TOP3* DNA probe was generated using the strategy outlined in section 2.3.4. Random labelling of probes was performed as above. Hybridisation was

performed as in section 2.7. Northern blots were quantitated (Phosphoimager; Molecular Dynamics Ltd.) and the values were normalised to an *ACT1* loading control.

2.9 Western protocols

Western transfer was performed according to the method of Towbin et al. (1979). Proteins were transferred to Hybond C super nitrocellulose membrane (Amersham) by electroblotting using a Trans-blot cell (BioRad). Transfer was performed at 60V for 2 hours at 4°C in transfer buffer [48mM Tris, 39mM glycine, 20% (v/v) methanol, 0.037% (w/v) SDS].

A rabbit polyclonal serum to Rad53p (NLO16; de la Torre-Ruiz et al., 1998) was used at a final dilution of 1:10,000 in 1% fat free milk in PBS containing 0.02% Tween-20, with a primary incubation period of 12 hours. For BLM detection, α-BLM (a rabbit polyclonal raised against the N-terminal domain of BLM, kindly donated by Prof. S. Jackson, Cambridge University) was used at a concentration of 1:1000. Horseradish peroxidase-linked secondary antibody (Sigma) was used at 1:10,000 with a 60 minute incubation period.

Chemiluminescent detection, which relies upon the oxidation of luminol by Horseradish Peroxidase was performed using an ECL kit (Amersham), according to the manufacturer's instructions.

2.10 Media for yeast studies

S.cerevisiae strains were grown at 30°C on YPD or the appropriate synthetic complete (SC), or dropout (SD), in the latter case omitting one or more nutritional supplements. YPD is 1% yeast extract, 2% bacto-peptone, 2% dextrose, 2.5% bacto-agar and 0.005% adenine hemisulphate (all w/v) and was supplied by the ICRF, Clare Hall. SC/SD media is (w/v) 0.67% yeast nitrogen base (without amino acids), 0.5% ammonium sulphate, 2% glucose, 2% bacto-agar plus the appropriate amount of complete or drop out mixture (BIO 101). Liquid YPD or SD media was prepared as above but without agar. In experiments designed to identify the effect of regulated expression of genes cloned downstream of the *GAL1* promoter, the carbon source was substituted as appropriate. 5-Fluoro-orotic acid (FOA) media is (w/v) 0.67% yeast nitrogen base, 2%

glucose, 2% bacto-agar, 0.1% FOA (added after autoclaving when media had cooled to 50°C), the appropriate quantity of uracil drop out media and 50mg/l uracil. Hydroxyurea or methyl methane sulphonate containing media were made up as above except with the addition of drug (just prior to pouring in the case of solid media).

S.pombe minimal EMM minimal media (Moreno et al., 1991) was supplied by the ICRF, Clare Hall. The appropriate nutrients were added according to the genotype of the strain being grown. Phloxin B was added at a final concentration of 10μg/ml in viability experiments.

2.11 Human cell culture

Human SV40-transformed fibroblast MRC V, human cervical carcinoma HeLa S3 and SV40-transformed BS-derived GM8505 cells were grown as monolayers in E4 media, supplemented with 15% fetal bovine serum, 3mM glutamine and antibiotics (penicillin 100 units/ml, streptomycin 100 mg/ml and nystatin 50 units/ml). Cells were grown in Petri dishes at 37°C in a humidified atmosphere containing 5% CO_2. For passaging, cell monolayers were detached using 0.02% (w/v) EDTA in PBSA. All cells were tested for mycoplasma contamination and found to be negative.

2.12 *E.coli* bacterial strains

The following strain was used for transformations and for both small and large scale preparations of plasmid DNA:

DH10β: F-, *end*A1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *thi*-1, λ-, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA)U169, ϕ80d*lac*ZΔM15

2.13 Yeast strains

2.13.1 *S.cerevisiae* strains

All experiments were performed in the YP1 strain background except where stated. The following strains were used:

YP1 background:

RKC1a, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 top3::LEU2*

JMK22d, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 sgs1::LYS2*

RKC1c, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 sgs1::LYS2 top3::LEU2*

RKC 1d, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101*

JMK90, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 ku70:: G418^R*

JMK25 6B, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 rad51::LEU2*

M83, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 rad52::LEU2*

M89, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 rad54::LEU2*

JMK91, *MATa leu2Δ his4-R ura3-52 lys2 ade2-101 rad59:: G418^R*

6048b, MATα *leu2Δ HIS4 trp1-H ura3-52 lys2 ade2-101 srs2::LEU2*

Y55 background:

RKC40, *MATa his4-C leu2-R met13-4 cyh2^R lys2-d ura3-1 ade1-1 trp1-H TRP5 CAN1 sgs1::LEU2*

RKC41, *MATα HIS4 leu2-R met13-2 cyh2^R lys2-d ura3-1 ade1-1 TRP1 trp5-1 can1^R sgs1::LEU2*

A364a background:

RKC 31a, *MATa ura3 his3 trp1 leu2*

RKC 31b, *MATa ura3 his3 trp1 le*u2 *top3::G418^R*

RKC 36d, *MATa ura3 his3 trp1 le*u2 *top3::G418^R rad9::HIS3*

DLY 195, *MATa ura3 his3 trp1 leu2 rad9::HIS3* (Lydall and Weinert 1995)

RKC36e, *MATa ura3 his3 trp1 leu2 rad24::URA3*

RKC36f, *MATa ura3 his3 trp1 leu2 rad24::URA3 top3::G418^R*

RKC36g, *MATa ura3 his3 trp1 leu2 rad9::HIS3 rad24::URA3 top3::G418^R*

RKC37e, *MATa ura3 his3 trp1 leu2 rad17::URA3*

RKC37f, *MATa ura3 his3 trp1 leu2 rad17::URA3 top3::G418^R*

RKC37g, *MATa ura3 his3 trp1 leu2 rad17::URA3 rad9::HIS3 top3::G418^R*

RKC12d, *MATa ura3 his3 trp1 leu2 sgs1::URA3*

DLY264, *MATa ura3 his3 trp1 leu2 mec2-1::URA3* (Paulovich and Hartwell, 1995)

DLY 285, *MATa ura3 his3 trp1 leu2 mec1-1:: HIS3* (Paulovich and Hartwell, 1995)

CG378 background:

RKC32/33a, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52*

RKC34/35c, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 top3::G418^R*

CG378-R1, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 rfa2-1* (Santocanale et al., 1995)

CG378-R2, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 rfa2-2* (Santocanale et al., 1995)

CY379, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 pri-M4* (Marini et al., 1997)

RKC34d, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 rfa2-1 top3::G418^R*

RKC35d, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 rfa2-2 top3::G418^R*

RKC39d, *MATa ade5 can1 leu2-3,112 trp1-289 ura3-52 pri-M4 top3::G418^R*

2.13.2 *S.pombe* strains

910a, *leu1-32 ura4-D18 ade6-706*

910b, *leu1-32 ura4-D18 ade6-706 hus2.22*

2.14 Yeast strain construction

In both the A364a and the CG378 strains, *TOP3* was disrupted using pRKC20. In this plasmid the full length open reading frame of *TOP3* incorporating 5'*BamH1* and 3'*Not1* sites was cloned into pT7. Blue (Invitrogen), and the coding sequence was disrupted by insertion of the KanMx:4 module (Wach et al., 1994) between the *Hpa1* and *BstX1* sites (Figure 2.1). This disruption deletes the coding sequence for 289 amino acids including the active site tyrosine. The presence of stop codons in the KanMx4 module in all 3 reading frames precludes the possibility that a Top3-KanMx fusion protein could be synthesised *in vivo*. The product of a *BamH1* and *Not1* digest was used for the transformation, and the resulting transformants were analysed by PCR using primers flanking the *TOP3* gene and within KanMx4 module (Figure 2.1 and 2.2). Primers corresponding to the diagram in Figure 2.1 were:

A(5'-GTCGGATCATACAGGCATGT-3');

B (5'-CATCCTATGGAACTGCCTCGG-3');

C (5'TTCAGAAACAACTCTGGCGCA-3');

D (5'-TAGTCGCGCGTGAATATGCTA-3').

In the YP1 background *TOP3* was disrupted using *Not I*-digested plasmid pWJ258, kindly provided by Dr R. Rothstein. This disruption contains the *LEU2* gene. Transformants were checked by Southern analysis and by PCR using primers flanking the

TOP3 gene and within *LEU2*. The phenotype of *top3Δ* was also confirmed genetically in all backgrounds by the ability of an *sgs1Δ* mutation to rescue the slow growth of the *top3Δ* strains (Gangloff et al., 1994). [1]

SGS1, RAD17 and *RAD24* were deleted in the A364a background by Dr. J Kearsey (Institute of Molecular Medicine, Oxford) using a PCR based strategy (Wach et al., 1994) in which the entire open reading frames of the respective genes were disrupted by the KanMx.4 module. In each case deletions were checked by PCR as outlined above and by their respective phenotypes. In order to change the markers for these knockouts, linear DNA incorporating the entire open reading frame of *URA3* flanked by 45bp of sequence homologous to the 5' and 3' ends of the $G418^R$ was used to transform the various strains. The 5'primer,

(5'-ATC CCC GGG TTA ATT AAG GCG CGC CAG ATC TGT TTA GCT TGC CTC GCT TTT CAA TTC AAT TCA TC-3') and the 3'primer, (5'-GTA TAG CGA CCA GCA TTC ACA TAC GAT TGA CGC ATG ATA TTA CTT AAA TCA TTA CGA CCG AGA T-3') were used to generate this construct using the plasmid pFEP10 (a plasmid containing the *URA3* gene, kindly donated by Dr. E. Louis, Institute of Molecular Medicine, Oxford), as template DNA. Conditions for PCR were as follows: 1st step, 94°C (2 minutes) for 1 cycle; 2nd step, 54°C (1 minute), 72°C (90 seconds) and 94°C (30 seconds) repeated for 7 cycles; and final step, 54°C (1 minute) followed by 72°C (5 minutes) for 1 cycle. The PCR product was used to transform the relevant yeast strains and correct placement of the markers was checked genetically by screening Ura+ transformants for a G418 sensitive phenotype.

The following strains were generated by Dr. J. Kearsey, Institute of Molecular Medicine: JMK22d, JMK25 6B, JMK90, JMK91. The following strains were obtained from Dr. E. Louis: M83, M89, 6048b. Other strains were obtained from the authors indicated. Strains 910a and 910b were obtained from Dr. A Carr (MRC Cell Mutation Unit, Sussex)

[1]Note that the *TOP3* deletion used in the A364a and CG378 strains was not complete. Dominant negative effects relating to the expression of a truncated N-terminal domain have not been excluded. This could be achieved by assessment of the *top3Δ* phenotype in a heterozygous background.

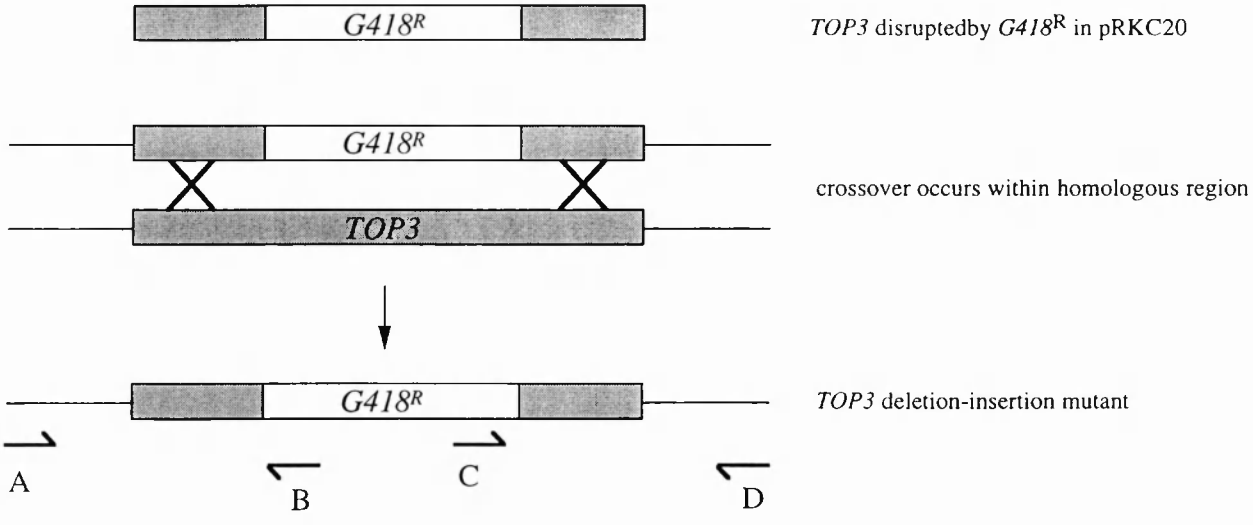


Figure 2.1: One-step gene disruption. A null allele of the *TOP3* gene was generated by deleting the coding sequence for 289 amino acids (including the active site tyrosine) and insertion of the KanMx:4 module. Strains were transformed with this construct and G418$^+$ transformants were selected. The wild type *TOP3* gene was replaced with *top3Δ::G418*^{R} by a double crossover. Primers A and D correspond to flanking sequences 5' and 3' to the *TOP3* gene, whereas primers B and C correspond to internal sequences of the KanMx:4 module.

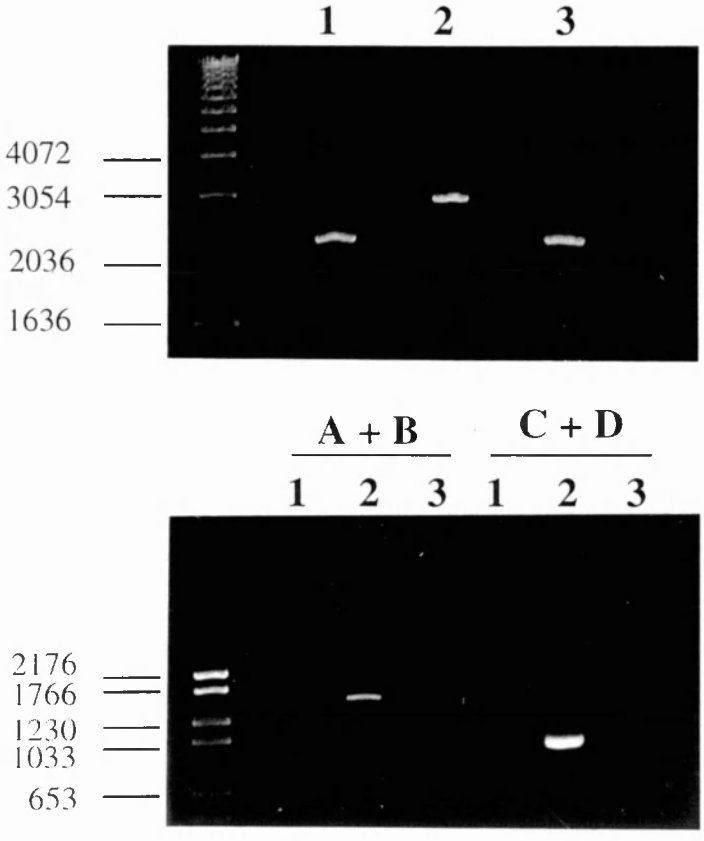


Figure 2.2: Confirmation of correct disruption of the *TOP3* gene. In the A364a and CG378 strains, *TOP3* was disrupted using pRKC20. Wild type diploid G418$^+$ transformants, were allowed to sporulate and genomic DNA prepared from the resulting putative wild type and *top3Δ::G418*^{R} segregants. In the above panels, results for 3 genotypes are shown: (1) wild type; (2) *top3Δ::G418*^{R}; and (3) *rad10Δ::G418*^{R}. Molecular weight markers are shown on the left. In the top panel, PCR reactions using primers A and D (see Figure 2.1) for each of the 3 genotypes are shown. Disruption of the *TOP3* gene results in a 3.0 kb product, whereas an undisrupted gene results in a 2.3 kb product. In the lower panels, PCR reactions using primers A and B (left) and C and D (right) are shown. Disruption of the *TOP3* gene results in a 1.4 kb product for primers A and B, and a 0.9 kb product for primers C and D. No products are expected where the *TOP3* gene remains intact.

2.15 Tetrad analysis

During meiosis and sporulation in *S.cerevisiae,* genes on different chromosomes assort independently, and linked genes can be separated by recombination. Therefore meiosis and sporulation is a relatively straightforward way to create strains with different genotypes. For diploid construction, cells from each haploid isogenic parent with opposite mating types were mixed on the surface of a YPD plate and the mating allowed to proceed overnight at 30°C. The mating mixture was then replica plated to drop-out or minimal media to select for auxotrophic complementation. Alternatively, the mating mixture was plated out for single colonies and diploids selected by their inability to mate with either *Mata* or *Matα* trains.

Diploids constructed in this way were patched onto YPD plates and then after growth for 24 hours at 30°C, were replica plated to a sporulation medium [2% (w/v) potassium acetate, 0.22% (w/v) yeast extract, 0.05% (w/v) glucose, 2.5% (w/v) agar and 0.09% complete amino acid mixture, pH7.0] or minimal sporulation medium [2% (w/v) potassium acetate, 2.5% (w/v) agar and the addition of those amino acids for which the strain is auxotrophic]. The sporulation plates were incubated for between 5-7 days at 25°C. Tetrads were suspended in 100µl sterile dd$H_2$0 to which 0.5µl glusulase had been added and was left at room temperature for 60 minutes. Dissection was performed using an Axioskop (Zeiss) dissecting microscope. Dissected tetrads were grown for 3-4 days at 30°C or at the appropriate temperature for conditional strains.

2.16 Transformation and Transfection procedures

2.16.1 Yeast transformation

S. cerevisiae cells were grown to mid log phase in YPD (OD_{600} 0.4-0.6) and 50 ml of cell culture were harvested by centrifugation at 3000rpm in a Beckman GPR centrifuge, room temperature for 5 minutes. The cells were washed once in sterile ddH_2O and then washed again in 15 ml TE, pH 7.5, 0.1M lithium acetate. The cells were then suspended in 0.5ml TE, pH 7.5, 0.1M lithium acetate. For the transformation, 200µl of the above cell suspension was added to 5µl of 10mg/ml sheared salmon sperm DNA, and 1-5µg of transforming DNA. 1ml of PEG solution [35% (w/v) PEG 4000, 0.1M lithium acetate, TE, pH7.5 was added, mixed well and incubated with shaking at 30°C for

30 minutes and then heat shocked at 42°C for 15 minutes. The cells were then pelleted by microcentrifugation, resuspended in sterile ddH_2O and plated upon the appropriate selective plates. Transformants appeared generally after 3 days incubation at 30°C. Controls with no transforming DNA were run in parallel.

For transformation of *S.pombe* cells were grown to mid log phase in minimal media (OD_{600} 0.3-0.5), cells from 50 ml harvested by centrifugation as above, and then washed once in cold sterile ddH_2O and then washed again in cold in 1M sorbitol. Cells were then resuspended in 200µl cold 1M sorbitol. For the transformation, 40µl of the above cell suspension was added to 100ng of transforming DNA in a cuvette and left on ice for 5 minutes. The cells were transformed with a Gene Pulser apparatus (Bio-Rad) set at 1.5kV, 25µF and 200Ω parallel resistance. Cells were then plated out on the appropriate selective media. Transformants appeared generally after 5 days incubation at 30°C. Controls with no transforming DNA were run in parallel.

2.16.2 Preparation of competent *E.coli*

Cells were prepared according to the method of Sambrook et al. (1989). 400ml of Luria broth (LB) was inoculated with 1ml of saturated culture derived from a single *E.coli* colony and incubated at 37°C until mid log phase (OD_{600} 0.4-0.5). The culture was then chilled on ice for 15 minutes and then centrifuged at 4000rpm in a Beckman GPR centrifuge, 4°C for 10 minutes. The pellet was then resuspended in 300ml of cold, sterile ddH_20 and spun as previously. This step was repeated using 100ml of cold ddH_20 for resuspension, and then repeated again using 40 ml of ice cold 10% glycerol. Following a further centrifugation, the pellet was resuspended in 1ml 10% glycerol, aliquoted, snap frozen and then stored at -80°C. To assess the competency of the cells, 1-10 ng of plasmid DNA were used in a standard transformation reaction.

2.16.3 Transformation of plasmid DNA into *E.coli*

For electroporation of DNA into *E.coli* , the method was that described by Sambrook et al. (1989). Plasmid DNA (~10ng or 0.5 µl of a ligation reaction) was added to a cuvette (on ice) containing 50 µl of electrocompetent cells. The cells were transformed with a Gene Pulser apparatus (Bio-Rad) set at 2.5kV, 25µF and 200Ω parallel resistance. Immediately following transformation, 1ml of LB was added to the cuvette and the

sample incubated at 37°C for 30 minutes, before spreading onto agar plates containing the appropriate antibiotic.

2.16.4 Transfection of human cells

Human cells were transfected either by electroporation or by DOTAP (Boehringer Mannheim). For electroporation, cell monolayers grown in 15cm Petri dishes to 50% confluence were detached by treatment with trypsin and then washed twice in 25ml ice-cold phosphate buffered sucrose (272mM sucrose, 7mM potassium phosphate buffer pH7.4, 1mM $MgCl_2$). Cells were then resuspended in the same buffer at 0.4 x 10^7 cells/ml and 800µl aliquots incubated on ice in the presence of 10µg transfecting DNA in an electroporation cuvette. Samples were then electroporated using a Gene Pulser apparatus (BioRad) using 150-350kV, 25-125µF and 200Ω. Following electroporation the samples were left on ice for an additional 10 minutes, before being plated in 15 cm Petri dishes in E4/15% foetal bovine serum. After 24h 600µg/ml G418 was added. Transfectants appeared generally at around 14 days.

DOTAP is a liposome formulation of the cationic lipid DOTAP. Mixing of DOTAP with DNA results in the formation of stable complexes. These complexes adhere to the cell surface, fuse with the cell membrane and release the DNA into the cytoplasm. For DOTAP transfection, cells were grown to 40-45% confluence in 15cm Petri dishes in E4/15% foetal bovine serum. 15µg transfecting DNA in 10µl TE was made up to a total volume of 210µl with 170µl 20mM HEPES buffer, pH7.4 and 45µl DOTAP transfection reagent. The mixture was then left at room temperature for 15 minutes, before addition to the Petri dish. Cells were then managed as described above.

2.17 Mitotic heteroallelic recombination

A freshly constructed diploid strain (RKC40 x RKC41) homozygous for *sgs1Δ*, but heteroallelic for 2 recessive *met13* alleles was transformed with pJH626.T, pRKC3 and pRKC16 and transformants were selected on SD Ura- plates. Individual colonies from each set of transformants were then diluted in 100 µl sterile ddH_2O and dilutions placed on SD -Met Ura and titred on SD-Ura plates. After 3 days the number of methionine prototrophs were scored and the frequency determined for each colony.

Fluctuation analyses were performed as described by Lea and Coulson (1949) using the method of the median. The rate of heteroallelic recombination per cell per generation was calculated using the following formula: rate=0.4343(median frequency) divided by ln(number cfu/colony). This analysis was repeated 4 times with 11-19 independent colonies resuspended for each test of a given strain.

2.18 Flow Cytometric analyses

Cells were grown in YPD media, collected by centrifugation and resuspended in 70% ethanol. Cells were then washed in 50 mM sodium citrate, pH7.0, and resuspended in the same buffer containing 0.25 mg/ml RNase A. The cells were sonicated and then incubated at 50°C for 60 minutes. Proteinase K was added to a final concentration of 1 mg/ml and the cells were incubated at the same temperature for a further 60 minutes. The cells were then allowed to cool and propidium iodide was added to a concentration of 8 μg/ml. Samples were analysed using a Becton Dickinson FACScan machine incorporating LYSIS2 software. We confirmed that the peak shifting seen in flow cytometric analyses was a reflection of chromosomal DNA synthesis. The shift in the flow cytometric histograms from the G1 to the G2/M position was inhibited by α–factor, which induces a G1 arrest, and by HU, which inhibits DNA synthesis.

2.19 Cell cycle blockade and irradiation procedures

All experiments were performed in YPD. For cell cycle blockade experiments, cultures were grown to early log phase (OD 0.3-0.4). G1 arrest was induced by adding α–factor to a final concentration of 20 μg/ml, and G2/M arrest was achieved by adding MBC to a final concentration of 200 μg/ml and DMSO to 1 %. After the appropriate time intervals, cells were checked microscopically and by flow cytometry to confirm the appropriate arrest phenotype. The method for UV-irradiation (254nm) has been described previously (Aboussekhra et al., 1996). Cell cycle delay during S-phase was analysed as described by Paulovich and Hartwell (1995).

2.20 Survival curves

(i) **HU** A final concentration of 0.2 M HU was added to early log phase cells in YPD at 30°C. Aliquots of cells were removed at timed intervals, washed once, sonicated briefly and dilutions at different concentrations were spread onto YPD agar plates. Colonies were counted after 72 hours growth at 30°C and the % survival determined, relative to the viable colony count prior to addition of HU.

(ii) **MMS** Cells were grown as above, before addition of MMS to concentrations of 0.005 -0.03% for 60 minutes. The MMS was then inactivated by the addition of sodium thiosulphate to 5% and the % survival determined as above.

(iii) **UV irradiation** Cells were grown to log phase, diluted at different concentrations in dH_20 and spread on YPD agar. Plates were irradiated at the indicated doses with 254 nm UV light and after 72 hours growth at 30°C surviving colonies were counted. The % survival was compared to a non-irradiated control.

(iv) **γ-irradiation** Cells were grown to log phase and then irradiated in suspension in 15ml centrifuge tubes using a Gammacell 1000 ^{137}Cs γ-irradiator (Atomic Energy Corporation, Canada) at a dose rate of 3Gy/minute at room temperature. Dilutions at different concentrations were spread onto YPD plates and colonies counted after 72 hours growth at 30°C. The % survival was determined relative to non-irradiated controls.

2.21 Analysis of cell morphology

Cells were fixed with 4% formaldehyde and microtubules were visualised as reported previously (Kilmartin and Adams, 1984). The anti-tubulin antibody was the mouse monoclonal TAT-1 used at 1.500 (kindly provided by Prof. K. Gull, University of Manchester), raised against *Trypanosoma brucei* α-tubulin (Woods et al., 1989) which cross-reacts with *S.cerevisiae* α-tubulin. The secondary antibody (1:250) was Cy3-conjugated rabbit anti-mouse IgG used at 1.250 (Sigma). Cellular DNA was stained using 1μg/ml DAPI. To determine and categorise cell morphologies, at least 200 cells were analysed for each sample. All samples were evaluated single blind.

2.22 Sister Chromatid Exchanges

Metaphase spreads were prepared from cells propagated through 2 rounds of DNA synthesis in the presence of 10μg/ml 5-bromodeoxyuridine. Chromosomes were stained

with Hoechst 33258 and UV photolysis achieved by exposure to both long- and short-wave UV light for 20 minutes at 50°C. Chromosomes were stained with Giemsa and exchanges scored single blind on a Zeiss Axiophot microscope. The SCE frequency was expressed as the number of individual strand exchanges per chromosome. Quantification of the frequency of SCE/chromosome was performed by P. North, Institute of Molecular Medicine, Oxford.

2.23 Statistical methods

Unless otherwise stated, statistical comparisons were performed using a chi-squared analysis (two-tailed). For mitotic heteroallelic recombination, statistical comparisons between different strains within an isogenic series were made by rank order (Wierdl et al., 1996). The frequencies for each colony of the 2 strains being compared were converted to rates using the median method equation described above and ranked together (equal numbers of colonies were used). A chi-squared analysis was performed to test whether one strain had significantly more colonies ranked in the top half of the rate values than the other strain. If the 2 strains had equal rates, then the expected of colonies in the upper half of the rank order for a given strain is 50% of the colonies. A chi-squared value (one-tailed) of >3.85 ($p<0.05$) indicates a significant deviation from the expected 50% and therefore a significant difference in rate between the strains.

Chapter 3

Assessment of the ability of *BLM* to complement *S.cerevisiae sgs1Δ* mutants.

3.1 Introduction

If a human and yeast protein share not only structural, but also functional similarity, there are several advantages in using yeast as a model system for dissecting in detail the properties of the human protein. First, the entire genome of *S.cerevisiae* has now been sequenced (Goffeau et al., 1996). This is relevant to the study of BS, since in humans at least five (and quite possibly more) RecQ-family helicases exist, and this has the potential to mask or distort the effect of loss of function mutations in *BLM*. In budding yeast there is only one RecQ family helicase and thus the effect of loss of function mutations can be more readily assessed. Second, yeast cells grow rapidly with an average generation time of around 2 hours, significantly quicker than cultured mammalian cells. Indeed, both transformed and untransformed BS cell lines are exceptionally difficult to work with, since they demonstrate significantly prolonged generation times combined with a reduced plating efficiency. Third, yeast cells can be manipulated either as diploids or haploids and thus, in the latter case the effects of a recessive mutation(s) can be easily assessed. This contrasts with the difficulty in eliminating a wild type gene in mammalian cells. In yeast, genes can be easily deleted, mutated and reintroduced, overexpressed or tagged.

The experimental advantages of budding yeast are therefore being increasingly exploited to determine the function of genes mutated in human diseases. One example of this approach has been the study of the human *ALD* gene which is mutated in the neurodegenerative disease, adrenoleukodystrophy, a disorder characterised by defective β-oxidation of saturated very long chain amino acids which results in extensive neuronal demyelination (Mosser et al., 1993). The *ALD* gene encodes an ABC transporter localised in the peroxisomal membrane. A structural homologue of this protein, Pat1p, has been identified in *S.cerevisiae* and has been demonstrated to be involved in the import of activated long-chain fatty acids into peroxisomes (Hettema et al., 1996). Using a reversal of this strategy, identification of yeast genes has, on occasions, led directly to the discovery of human genes defective in human disease. For instance, the simple sequence repeat instability observed in yeast *msh2* mutants (Strand et al., 1993) was found to resemble microsatellite instability in human hereditary non-polyposis colon cancer, and this led directly to the cloning of human *MSH2* (Fishel et al., 1993) More

recently, information derived from studies of yeast *bub1* mutants, which are defective in the spindle assembly checkpoint, accelerated the finding that in certain colorectal cancer cell lines showing high rates of aneuploidy, the human *BUB1* was mutated (Hoyt et al., 1991; Cahill et al., 1998).

It is now possible to use yeast to screen for drugs with potential effectiveness against human diseases (reviewed in Hartwell et al., 1997). For, example, yeast mutants defective in highly conserved DNA damage response pathways (such as mismatch repair) can be used as models of the human cancer cells, in order to screen directly for drugs or drug targets. In this way it may be possible to identify drugs with a therapeutic advantage by identifying agents that are more lethal to the mutant than to the wild type yeast cell. Many mutations in human cancers are loss of function mutations and thus, the proteins they encode do not themselves constitute a drug target. One approach that could circumvent this problem is 'synthetic lethal' screening which allows the identification of second site mutations that by themselves are not lethal, but in combination with the primary defect cause lethality. The gene products of these genes may constitute putative secondary drug targets, since their inactivation might be anticipated to kill cells with the primary defect. A more intricate approach appropriate to drug screening might involve the generation of hybrid yeast/human biochemical pathways. For example, in one study a yeast membrane bound chimeric yeast/mammalian Gα protein was used to couple the signal from a rat somatostatin receptor to a yeast reporter system (Price et al., 1995).

A significant number of human disease associated genes show significant sequence similarity to genes identified in the yeast *Saccharomyces cerevisiae*. In the most recent analysis, using the BLAST algorithm (Basic Local Alignment Search Tool), 74 out of 250 human disease-associated genes match budding yeast genes (Altschul et al., 1990; Foury, 1997). A subgroup of these genes match an *S.cerevisiae* protein at least as significantly (BLAST $p< e^{-40}$) as the human *NF1* (defective in neurofibromatosis type 1) and the yeast *IRA2* gene (which encodes an inhibitory regulatory protein). This cut-off is used since, in this particular example, ectopic expression of the catalytic domain of human NF1 complemented the yeast *ira2* mutant. The finding of heterologous complementation combined with biochemical evidence of similar activity (Xu et al., 1990), indicates that these proteins share significant functional properties.

The *BLM* gene product closely matches the proteins encoded by *S.cerevisiae*, *SGS1*, (BLAST $p< e^{-105}$) and in *S.pombe rqh1* (BLAST $p< e^{-143}$). A key question to be

addressed therefore is to what degree the respective proteins share functional similarity. An initial experimental approach to this problem is to assess the ability of one gene to complement the cellular phenotype associated with defects in the other gene. This experimental strategy is outlined below.

3.2 Results

3.2.1 Cloning *BLM*

A cDNA encoding the full length open reading frame of *BLM* was generated by the PCR using DNA from a human B-cell cDNA library as template. The strategy adopted is set out in Figure 3.1. The *BLM* cDNA was generated in 2 sections, a 5' 1.4kb PCR product incorporating a *BamH1* restriction site and yeast Kozak consensus sequence and a 3' 2.9kb fragment incorporating a *Not1* restriction site. In order to reduce the frequency of polymerase-induced errors a proof-reading enzyme was used and the conditions optimised to allow the fewest number of PCR cycles to generate a product. These products were then sequenced in order to screen for changes from the published nucleotide sequence. Two independent 2.9kb products with mutations predicted to alter the open reading frame were subsequently used: product (X) which had an A to G substitution at position 2482, which would have resulted in an alanine to threonine amino acid substitution at codon 827; and product (Y) which had an A to G substitution at position 4207, which would have resulted in a lysine to glutamic acid amino acid substitution at codon 1402 (see Figure 3.1). In order to circumvent these mutations, a 5' *EcoR1 -HindIII* fragment of product (Y) and a 3' *HindIII-Not1*fragment of product (X) were used in the subsequent ligations.

The predicted amino acid sequence of the protein encoded by this cDNA was shown to be identical to the BLM sequence published by Ellis et al. (1995), although the nucleotide sequence contained 5 silent changes: T to C (position 621); A to C (position 981); T to C (position 2286); G to A (position 3102); and C to A (position 3531). Four of these five changes, with the exception of the T to C change at position 2286, are likely to represent polymorphisms, as the same changes were identified in independently generated cDNAs. Examination of codon usage frequencies in different species indicated that the remaining silent mutation was unlikely to have any effect upon expression levels in either yeast or human cells. This cDNA was cloned into mammalian

(pCDNA3), *S.pombe* (pREP.3X) and *S.cerevisiae* (pYES2 and pJH626.T) expression vectors.

In order to obtain recombinant BLM for protein purification, two modifications to the full length *BLM* cDNA were made in work performed in conjunction with J. Karow, Institute of Molecular Medicine. First, a hexahistidine affinity tag was engineered at the 3' end of the coding sequence to permit the expressed protein both to be detected by means of specific anti-histidine tag antibodies and to be purified on nickel-chelate resin. Second, the first five codons of the yeast *TOP2* gene were placed immediately upstream of the complete *BLM* open reading frame. In previous studies it was shown that the presence of these yeast Top2p-derived amino acids improved the yield of expressed recombinant human proteins. The modified *BLM* cDNA was cloned downstream of the *GAL1* promoter in the vector pYES2. The biochemical properties of the BLM protein derived from this construct have been published (Karow et al., 1997).

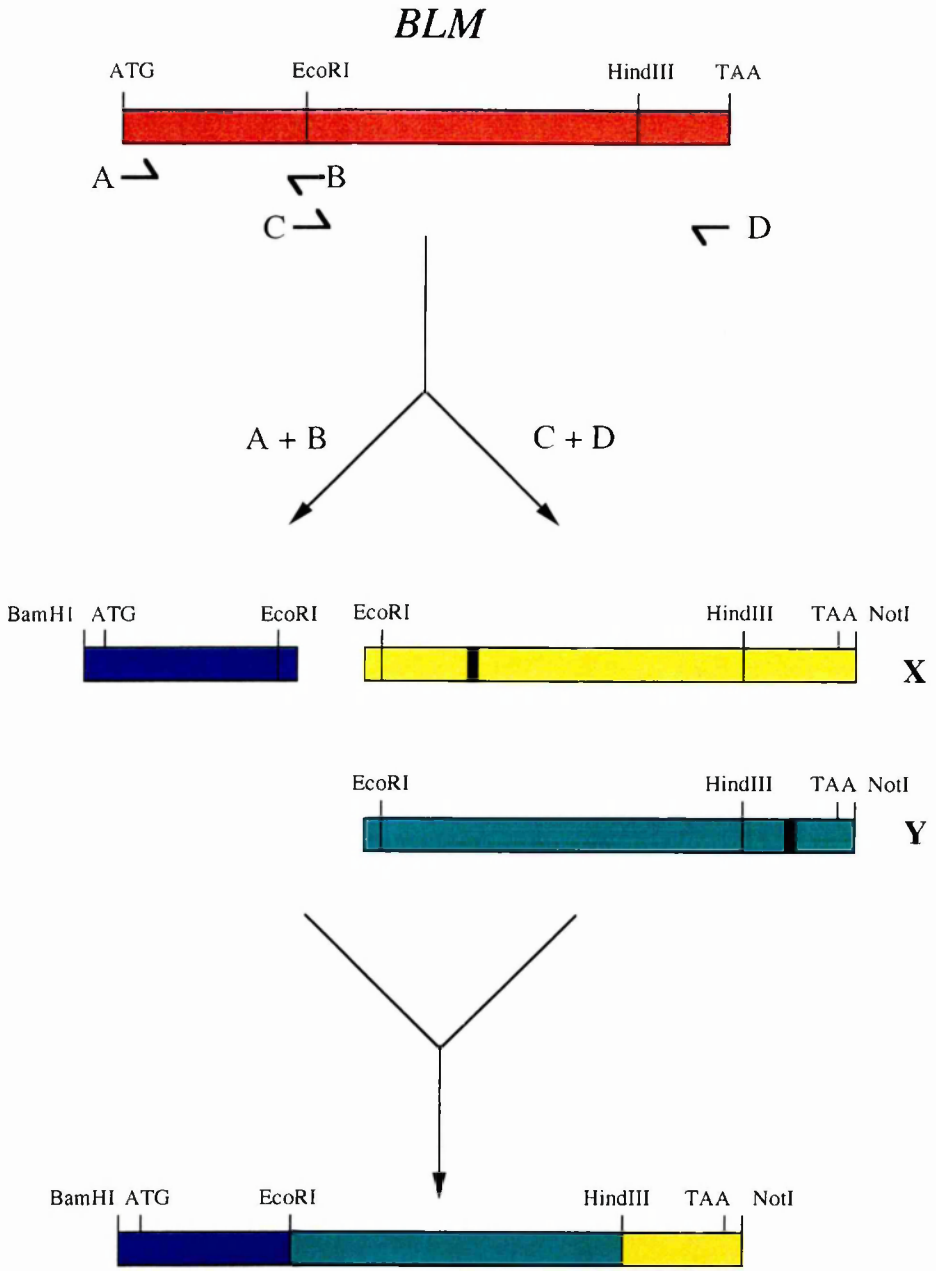


Figure: 3.1 Cloning *BLM*. The *BLM* cDNA was generated in 2 sections, a 5' 1.4kb PCR product incorporating a *BamH1* restriction site and yeast Kozak consensus sequence and a 3' 2.9kb fragment incorporating a *Not1* restriction site. Products were sequenced in order to screen for changes from the published nucleotide sequence. Two independent 2.9kb products with mutations predicted to alter the open reading frame were subsequently used: product (X) which had an A to G substitution at position 2482; and product (Y) which had an A to G substitution at position 4207. Mutations are represented by thick black bars. In order to circumvent these mutations, a 5' *EcoR1 -HindIII* fragment of product (Y) and a 3' *HindIII-Not1* fragment of product (X) were used in the subsequent ligations.

3.2.2 Complementation of the slow growth and elevated sister chromatid exchange phenotype of GM8505 Bloom's fibroblasts by *BLM*.

Recombinant BLM protein derived from the above construct demonstrated DNA-dependent ATPase activity and ATP- and Mg^{2+}-dependent helicase activity with a 3'-5' polarity for unwinding duplex DNA (Karow et al. 1997), features consistent with other members of the RecQ helicase family (Umezu and Nakayama, 1993; Gray et al., 1997; Suzuki et al., 1997; Bennett et al., 1998). Prior to heterologous complementation studies in yeast cells defective in RecQ homologues, confirmation that the *BLM* cDNA was functional and could complement a Bloom's syndrome fibroblast cell line was sought. The GM8505 cell line is an SV40-transformed fibroblast line derived from a patient with BS. This cell line demonstrates all the cardinal features of the BS cellular phenotype, including slow growth, poor plating efficiency and elevated rate of sister chromatid exchange in the presence of bromodeoxyuridine (McDaniel and Schultz, 1992). This latter phenotype was shown previously to be corrected by microcell-mediated transfer of chromosome 15 which contains the *BLM* locus at 15q26.1 (McDaniel and Schultz, 1992).

The unmodified *BLM* cDNA was positioned downstream of the CMV promoter in the vector pCDNA3 which also contains a $G418^R$ reporter, to give pRKC17. GM8505 cells were transfected with either pRKC17 or vector as a control. Selection for permanent transfectants was performed using G418 selection at 0.6 mg/ml. 2-3 weeks following transfection, single colonies were picked and propagated as independent cultures. Comparison of growth rates of 3 independent pRKC17 or vector transfectants demonstrated a mean 3-fold increase in the growth rate of GM8505 cells transfected with the *BLM* cDNA (Figure 3.2). Transfection with the *BLM* cDNA also corrected the poor plating efficiency of the parental GM8505 cell line, as indicated by comparison of cell number at the first time point in Figure 3.2, in which an initial drop is observed in the vector transfectants. This effect upon plating efficiency was confirmed by staining colonies 7 days after a known number of cells were plated (Figure 3.3). Metaphase spreads from pooled *BLM* or vector GM8505 transfectants were then examined for sister chromatid exchanges in work performed by P. North, Institute of Molecular Medicine. The *BLM* transfectants showed a partial reduction in SCE frequency (0.72 SCE/chromosome, n=2816 in *BLM* transfectants, vs. 1.14 SCE/chromosome, n=2698 for the vector controls, $p<0.0001$). These results compare with correction of the elevated

SCE phenotype in the same cell line from 2.12 SCE/chromosome in non-complemented cells to 0.50 SCE/chromosome in cells following minicell transfer of chromosome 15, which contains the *BLM* locus (McDaniel and Schultz, 1992). To confirm that the complementation of slow growth, poor plating efficiency and elevated SCE frequency was as a consequence of expression of the full length BLM protein, detection of the protein was tested by immunoblot analysis of extracts prepared from pRKC17 and vector transfectants in work performed by P. North, Institute of Molecular Medicine. Expression of the full length BLM protein was observed for 3 independent *BLM* clones, but not in the control transfectants (Figure 3.4). The GM8505 cell line is homozygous for a truncation mutation in *BLM* and no BLM protein can be detected (Dr. N. Ellis, Laboratory of Human Genetics, New York Blood Center, personal communication).

In a set of parallel experiments, an attempt was made to transfect GM8505 cells with *SGS1* cDNA which encodes the budding yeast structural homologue of the BLM protein. The full length *SGS1* cDNA was cloned into pCDNA3 to give pRKC4. No transfectants were obtained. In further experiments, alternative transfection methods including electroporation, differing quantities of transfecting DNA and other control fibroblast cell lines were adopted. In all experiments the vector control was transfected with high efficiency, but no *SGS1* transformants were obtainable. Transformation of pRKC4 into *E.coli* conferred resistance to low dose kanamycin confirming that the $G418^R$ marker in this construct was intact. Attempts at transient expression of *SGS1* were not performed.

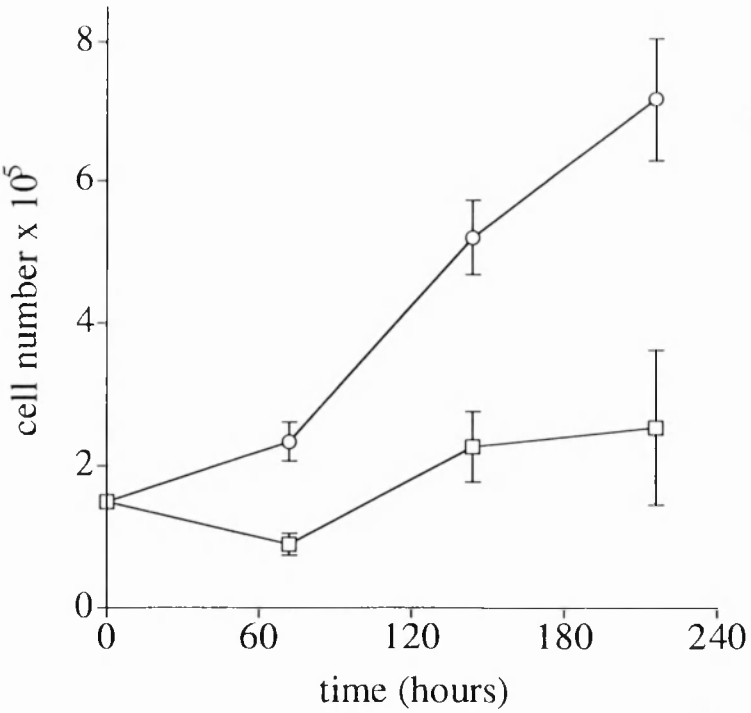


Figure 3.2: Complementation of slow growth rate of GM8505 cells by transfection with *BLM*. Graph showing cell number (±SEM) against time, of 3 independent GM8505 clones, transfected with pRKC17 (*BLM*) or vector control. 1.5 x 10^5 cells derived from log phase cultures of *BLM* (○) and control (□) transfectants, were grown under identical conditions. Note the initial fall in cell number in the control transfectants. Estimated mean generation times were ~80 hours for the *BLM* transfectants and ~240 hours for control transfectants.

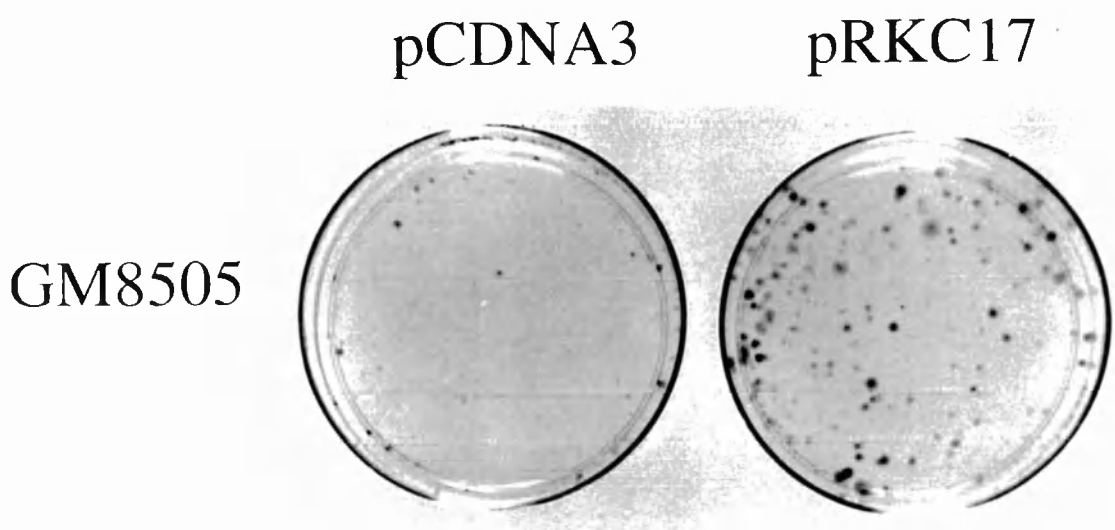


Figure 3.3: Complementation of poor plating efficiency of GM8505 cells by transfection with *BLM*. 5 x 10^4 cells derived from log phase cultures of pRKC17 (*BLM*) transfectants and pCDNA3 (vector) controls were plated into 15cm petri dishes and grown for 7 days, before staining of adherent colonies with 400µg/ml crystal violet.

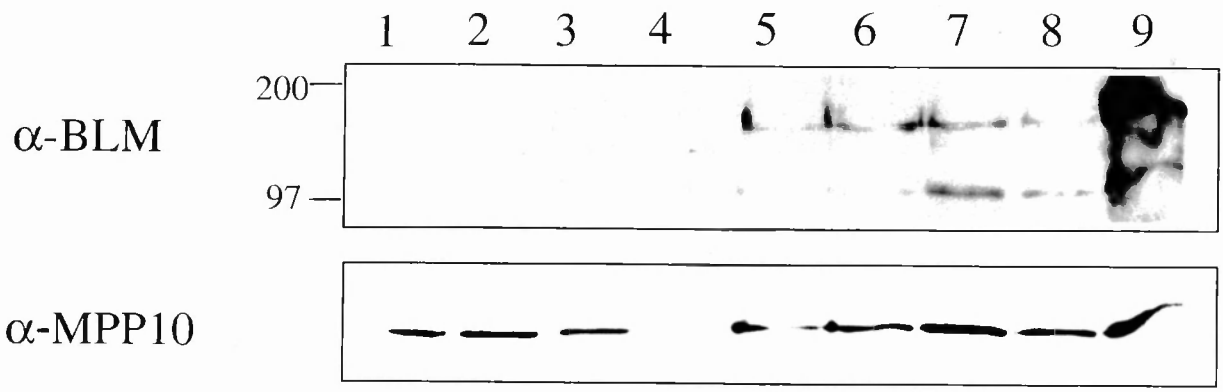


Figure 3.4: Expression of full length BLM protein in GM8505 cells transfected with pRKC17. Protein extracts derived from GM8505 (G) cells were separated by SDS-PAGE, transferred to nylon and immunoblotted with an α-BLM (upper panel) or α-MPP10 (lower panel) antibody. Lanes 1-3 represent control transfectants and lanes 5-8 represent *BLM* transfectants. Lane 9 is a HeLa (H) positive control. MPP10 is a nuclear protein and was run as a loading control. Lanes 1-9 are as follows: (1) G, vector clone 1; (2) G, vector clone 2; (3) G, vector clone 3; (4) empty lane; (5) G, pRKC17 pooled clones; (6) G, pRKC17 clone 1; (7) G, pRKC17 clone 2; (8) G, pRKC17 clone 3; (9) HeLa positive control. BLM protein runs as a band ~170 kDa with a second band visible at ~100 kDa. This presumably represents a breakdown product of BLM as it is not observed in the GM8505 vector controls.

3.2.3 Assessment of ability of *BLM* to complement fission yeast *hus2.22* mutants.

Fission yeast Rqh1p is a structural homologue of the BLM protein. *rqh1* mutants are defective in the recovery from an S-phase arrest induced either by HU or by DNA damage (Stewart et al., 1997). Mutants demonstrate a loss of viability and a 'cut' (cell untimely torn) phenotype when replication is re-initiated following arrest. In this phenotype a septum bisects an undivided nucleus. The *hus2.22* mutation (allelic to *rqh1*+) introduces a stop codon at amino acid position 790, eliminating the remaining 580 amino acids which contain the last two conserved helicase motifs. The phenotype of *hus2.22* mutants is identical to an *rqh1* null mutant.

In order to assess whether *BLM* could complement the phenotype of the *hus2.22* mutant, the full length cDNA was cloned downstream of the thiamine repressible *NMT-1* promoter in the *S.pombe* vector, pREP3X to give pRKC18. In this construct, gene expression is switched off in the presence of thiamine and switched on in its absence. pRKC18 and a vector control were transformed into wild type and *hus2.22* isogenic mutants. Plates containing ~400 independent transformants were then replica plated to 4 different types of selective media containing phloxin B: HU + thiamine; HU and no thiamine; no HU + thiamine; no HU and no thiamine. In the presence of HU, untransformed *hus2.22* mutants grow poorly and colonies are a deep red colour, resulting from the failure of cells to extrude the phloxin B pigment consequent upon a loss of viability. In contrast, wild type cells in the presence of HU form larger colonies which are pink in colour. No differences were observed between *hus2.22 BLM* and vector transformants in the presence of HU with or without thiamine. This suggests that *BLM* is unable to complement the HU- sensitive phenotype of *hus2.22* mutants.

To confirm this in more detail, log phase cultures of *BLM* or vector *hus2.22* transformants were grown either in the presence or absence of thiamine, exposed to HU or mock treated for 4 hours, and then released into media without HU, but again either in the presence or absence of thiamine. The end points determined were the percentage of 'cuts' and viability. Results from 2 independent experiments are shown in Figure 3.5. No reduction in the HU-induced 'cut' phenotype or loss of viability of *hus2.22* cells was evident under conditions in which full length BLM was expressed (Figure 3.6). In experiments performed in parallel, *hus2.22* cells transformed with pRKC5 (a derivative of pREP3x in which the *SGS1* cDNA is cloned downstream of the *NMT-1*promoter), also failed to show complementation of the HU-sensitive phenotype. Isogenic wild type

controls did not demonstrate a 'cut' phenotype or loss of viability in the presence of HU. Sgs1 protein expression, however, was not confirmed. No positive control was run in these experiments, because at the time they were performed only a partial sequence of *rqh1*$^+$ was available.

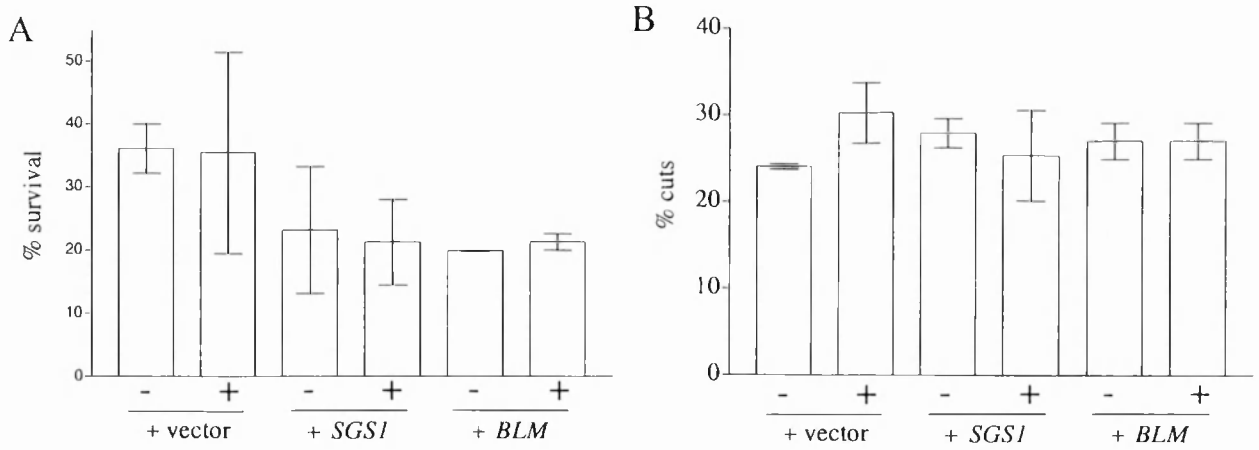


Figure 3.5: Failure of *BLM* to complement the phenotype of *S.pombe hus2.22* mutants. *hus2.22* mutants transformed with pRKC18 (*BLM*) , pRKC5 (*SGS1*) or vector were grown in selective media, either under inducing (+) or repressed (-) conditions at 30°C. At t=0 cells were treated with 10mM HU or mock treated for 4 hours, and then released into fresh media either in the presence or absence of thiamine. After a further 4 hours, the % survival (A) in relation to the t=0 value, and % cells corresponding to a 'cut' phenotype (B) were determined. The % cells with a 'cut' phenotype was determined by scoring 1000 DAPI-stained cells single blind. The means (+ ranges) of 2 independent experiments are shown. In the absence of HU, survival was 100% and the % cuts were < 1%, for each set of transformants.

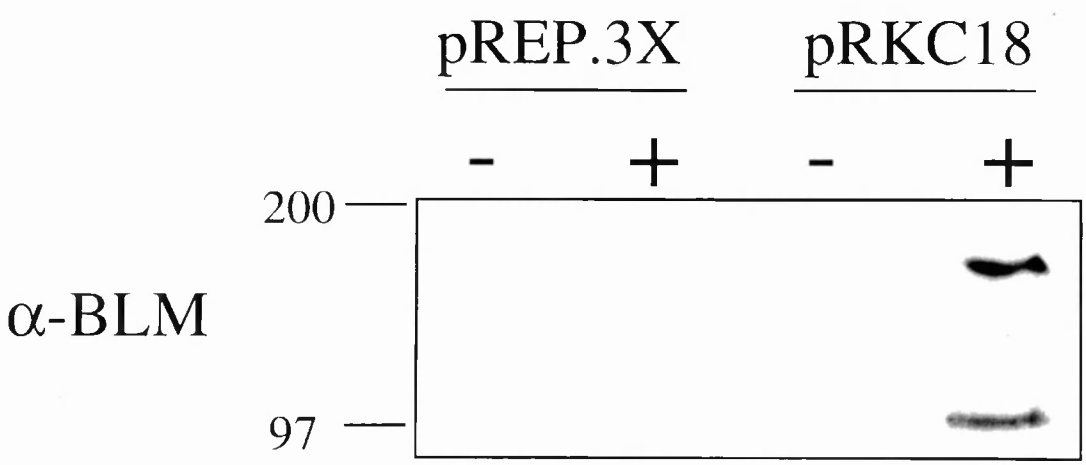


Figure 3.6: Expression of full length BLM protein in *hus2.22* mutants transformed with pRKC18 Protein extracts derived from pRKC18 (*BLM*) 0r pREP.3X (vector) control *hus2.22* transformants were grown under inducing (+) or repressed (-) conditions at 30°C for 22 hours, under the same conditions of the experiment shown in Figure 3.5. As previously, α-BLM identified a 170 kDa band and a 100 kDa band, under inducing conditions.

3.2.4 Assessment of the ability of *BLM* to complement budding yeast *sgs1* mutants.

A number of potential end-points in assessing the ability of *BLM* to complement budding yeast *sgs1* mutants were available and these are described below.

3.2.4a Loss of budding yeast Sgs1p in association with loss of another DNA helicase, Srs2, results in a synergistic growth defect.

SGS1 is a non-essential gene, which suggests either that it serves a non-essential function or that other genes can compensate for its loss (Gangloff et al., 1994; Watt et al., 1995). In *E.coli* , deletion of *recQ* in a *recBC sbcB(C)* background (which activates the RecF recombination pathway) is also compatible with viability (Mendonca et al., 1995). However, loss of both RecQ and another helicase, UvrD (Helicase II) in this genetic background is not compatible with survival (Mendonca et al., 1995). UvrD, like RecQ possesses 3'-5' helicase activity and is thought to initiate recombination events in the RecF pathway (Matson, 1986). Potential explanations for this lethality are that UvrD and RecQ are involved in alternate pathways essential for cell viability or that they act in the same pathway which is knocked out synergistically by the loss of both proteins.

In budding yeast, the helicase Srs2p shows a high degree of homology to UvrD and, like its putative counterpart in *E.coli* possesses 3'-5' helicase activity (Aboussekhra et al., 1989; Rong and Klein, 1993). Mutants defective in Srs2p demonstrate a hyper-recombination phenotype (Rong et al., 1991) and sensitivity to DNA damaging agents (Aboussekhra et al., 1989). The precise mechanism by which Srs2p helicase mediates the cellular response to DNA damage has, however, not been resolved.

Diploids heterozygous for *sgs1Δ::LYS2* and *srs2Δ::LEU2* were constructed (JMK22d x 6048b), allowed to sporulate and the resulting tetrads dissected. Combination of *S.cerevisiae sgs1Δ* and *srs2Δ* mutations resulted in a marked synergistic growth defect This synergistic growth effect was complemented by ectopic expression of a wild type *SGS1* cDNA from a single copy *URA3*-marked plasmid (pRKC3) and could be re-induced by 'chasing out' the plasmid by the growth of transformants on FOA-containing plates (Figure 3.7, Table 3.1). In Table 3.1, the results show genotypes of segregants scored blind for colony size prior to confirmation of markers. Microcolonies were scored as non-viable and not counted. Complementation by *SGS1* was restricted to only half the potential *sgs1Δsrs2Δ* segregants, since the single copy plasmid, pRKC3, only segregates

to approximately half of the spores during meiosis. Each of the 15 double mutants which were viable contained the *URA3*-marked plasmid. The same effect is reproduced in other genetic backgrounds (Dr. J. Kearsey, Institute of Molecular Medicine, personal communication).

3.2.4.b *BLM* fails to complement the synergistic growth defect of *sgs1Δ srs2Δ* double mutants.

This genetic interaction provided a potential means by which to test for the ability of *BLM* to complement an *sgs1Δ*-related phenotype. A diploid heterozygous for *sgs1Δ* and *srs2Δ* was transformed with pRKC16 (a *URA3* -marked single copy plasmid, in which the *BLM* cDNA was cloned downstream of the promoter for the constitutive glycolytic enzyme, triose phosphate isomerase). The resulting transformants were allowed to sporulate and the tetrads were then dissected. The resulting genotypes are shown in Table 3.1. Segregants were scored blind for colony size prior to confirmation of markers. Microcolonies were scored as non-viable. pRKC16 failed to complement the synergistic growth defect of *sgs1Δ srs2Δ* double mutants. Overexpression of *BLM* under inducing conditions from pRKC15 (a multicopy plasmid in which the *BLM* cDNA is positioned downstream of the *GAL1* promoter) also failed to complement in this system (data not shown). As previously, expression of full length BLM was confirmed by immunoblot.

Table 3.1 Genotypes of segregants derived from diploid JMK22d x 6048b

genotype	**+ vector** n=32 tetrads	**+ *SGS1* (pRKC3)** n=39 tetrads	**+ *BLM* (pRKC16)** n=50 tetrads
SGS1 SRS2	29	32	35
sgs1Δ SRS2	25	34	41
SGS1 srs2Δ	26	34	46
sgs1Δsrs2Δ	2	15	2

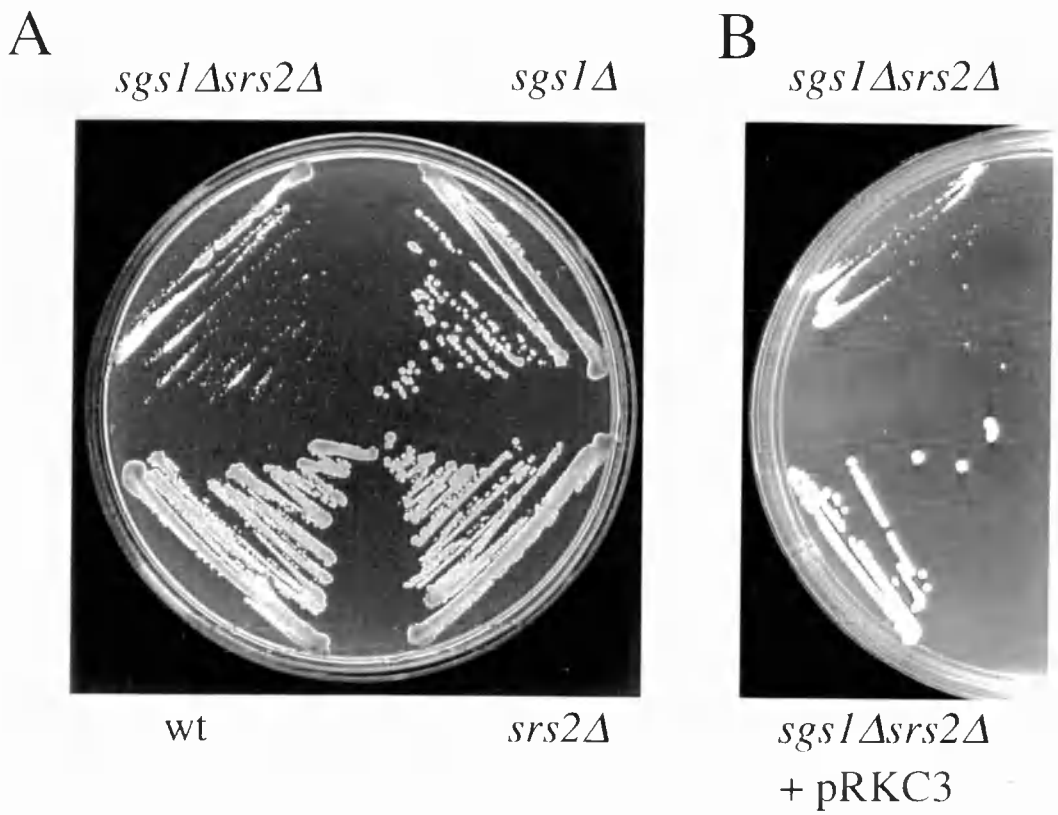


Figure 3.7: *sgs1Δsrs2Δ* demonstrate a synergistic growth defect. Diploids heterozygous for *sgs1Δ::LYS2* and *srs2Δ::LEU2* were constructed (JMK22d x 6048b), allowed to sporulate and the resulting tetrads dissected. (A) A YPD plate showing colony sizes associated with each of the four resulting genotypes, after growth at 30°C for 3 days, is shown. (B) Transformation of the above diploid with a single copy *URA3*-marked pRKC3 (*SGS1*) before sporulation, was able to reverse the slow growth phenotype of the resulting *sgs1Δsrs2Δ* double mutants. Chasing out pRKC3 on FOA (upper quarter of plate), reinduced the synergistic growth defect.

3.2.4c Assessment of the effect of *BLM* upon the hyper-recombination phenotype of *sgs1Δ* mutants

Cells derived from individuals with BS show hyper-recombination (reviewed in German, 1993). This is also the case for *sgs1Δ* cells, manifested as an increase in the frequency of interchromosomal homologous recombination, intrachromosomal excision recombination and ectopic recombination (Watt et al., 1996). In this latter study, the largest difference between *sgs1Δ* and wild type cells was a 13-fold difference in the rate of mitotic recombination between *met13-4* and *met13-2* heteroalleles in diploid cells. In order to test the ability of *BLM* to complement this phenotype, a diploid homozygous for *sgs1Δ* and heterozygous for the two *met13* alleles was constructed, and transformed with pRKC16 (*BLM*), pRKC3 (*SGS1*) and empty vector as a negative control. The rate of recombination is determined by scoring the frequency of methionine prototrophy, which could arise as a result of recombination (involving either crossover or gene conversion) or by back mutation. The overall strategy for this experiment is shown in Figure 3.8a.

To exclude the possibility that methionine prototrophy resulted from reversion of the *met13* allele, the rates for methionine prototroph formation of wild type and *sgs1Δ* haploid strains containing the *met13-4* recessive allele were determined. For 2 independent estimations of the rate of reversion mutation at *met13*, the wild type rate was 3.3×10^{-9} /cell/generation and the *sgs1Δ* rate was 3.0×10^{-9}/cell/generation. Thus, the rate of reversion mutation for this recessive allele is not elevated in *sgs1Δ* haploid mutants. Assuming that the rate of reversion mutation is similar for a *met13-2* recessive allele, any differences observed between wild type and *sgs1Δ* diploid strains is likely to reflect differences in recombination rather than reversion mutation rates.

Four independent experiments (each involving 11 to 19 independent transformants for each plasmid) were performed to estimate the rate of heteroallelic recombination between *met13-4* and *met13-2* and the results are shown in Figure 3.8b. Ectopic expression of *SGS1* as a positive control, resulted in over a five fold reduction in the recombination rate as compared to the vector control. A smaller but consistent reduction was observed for the *BLM* transformants. This difference did not, however, reach statistical significance and time limitations did not permit additional experiments to confirm or refute this reduction.

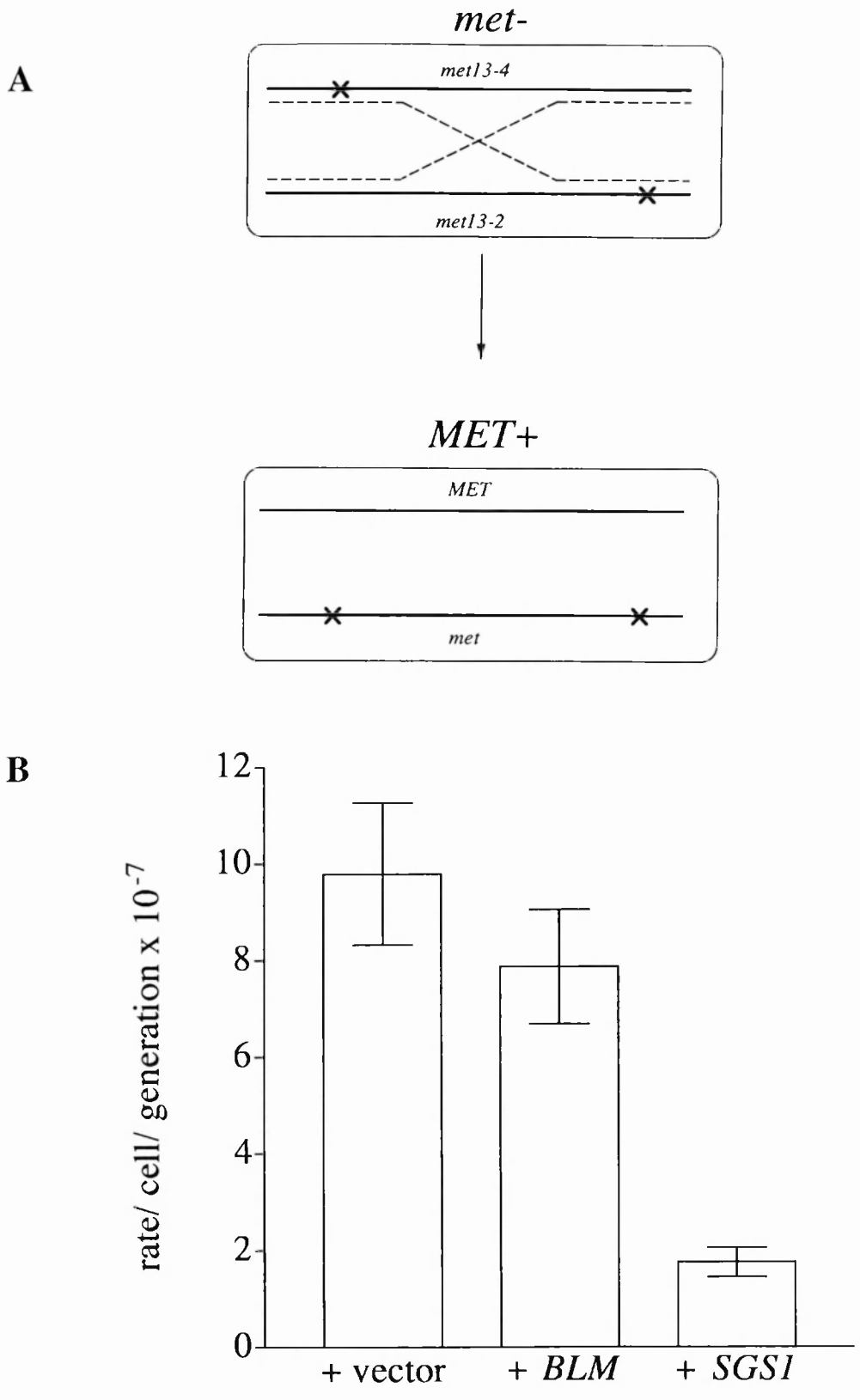


Figure 3.8: Effect of *BLM* on the rate of heteroallelic recombination in *sgs1Δ* mutants. Freshly constructed diploids (RKC40 x RKC41), homozygous for *sgs1Δ* and heterozygous for recessive *met13* alleles, were transformed with pRKC16 (*BLM*), pRKC3 (*SGS1*) or pJH626.T (vector) control. (A) Recombination between *met13-2* and *met13-4* alleles (either by crossover or gene conversion) can generate a wild type *MET13* gene and restore methionine prototrophy. The black crosses represent different mutations in the *MET13* gene. (B) . In each experiment 11-19 independent Ura+ transformants were then resuspended in sterile ddH_2O and plated on SD Ura- Met- plates and titred on SD Ura- plates. Median rates of recombination were then determined and the means (±SEM) of 4 independent experiments are shown.

3.2.4d Expression of both *SGS1* and *BLM* results in the re-induction of slow growth in an *sgs1Δ top3Δ* double mutant.

top3Δ mutants grow slowly, are defective in sporulation and demonstrate elevated rates of mitotic recombination. These phenotypes are suppressed by additional mutations in the *SGS1* gene (Gangloff et al., 1994). Ectopic expression of *SGS1* in an *sgs1Δ top3Δ* double mutant would therefore be expected to result in the re-induction of the slow growth phenotype. Evidence for heterologous complementation would also be obtained if this effect was reproduced by ectopic expression of *BLM*. To test this, wild type and isogenic *sgs1Δ top3Δ* double mutants were transformed with pRKC2 (*SGS1*) and pRKC15 (*BLM*) in which the respective genes are cloned downstream of the *GAL1* promoter, and empty vector as a control. Ten independent transformants from each were then spread on selective plates containing either glucose (repressed conditions) or galactose/raffinose (inducing conditions). Wild type *SGS1* or *BLM* transformants showed no differences from vector controls under inducing or repressed conditions. In contrast, *SGS1* or *BLM sgs1Δ top3Δ* transformants showed a consistent reduction in colony growth, as compared to the vector controls (Figure 3.9). This was confirmed by estimation of generation times (derived from serial determinations of cell number during log phase growth) of the respective transformants under inducing and non-inducing conditions (Table 3.2). Under inducing conditions, *SGS1* transformants demonstrated a nearly 4-fold increase in generation time as compared to vector controls, whereas *BLM* transformants showed a 1.5 fold increase.

Table 3.2 Generation times (±SEM) of *sgs1Δ top3Δ* transformants grown in SD -Ura media.

condition	+ vector	+ *SGS1* (pRKC2)	+ BLM (pRKC15)
2% glucose, n=5	2.80 ± 0.01	3.26± 0.07	2.89± 0.02
2% galactose/ 1% raffinose, n=9	3.62± 0.18	13.69± 0.57	5.55± 0.25

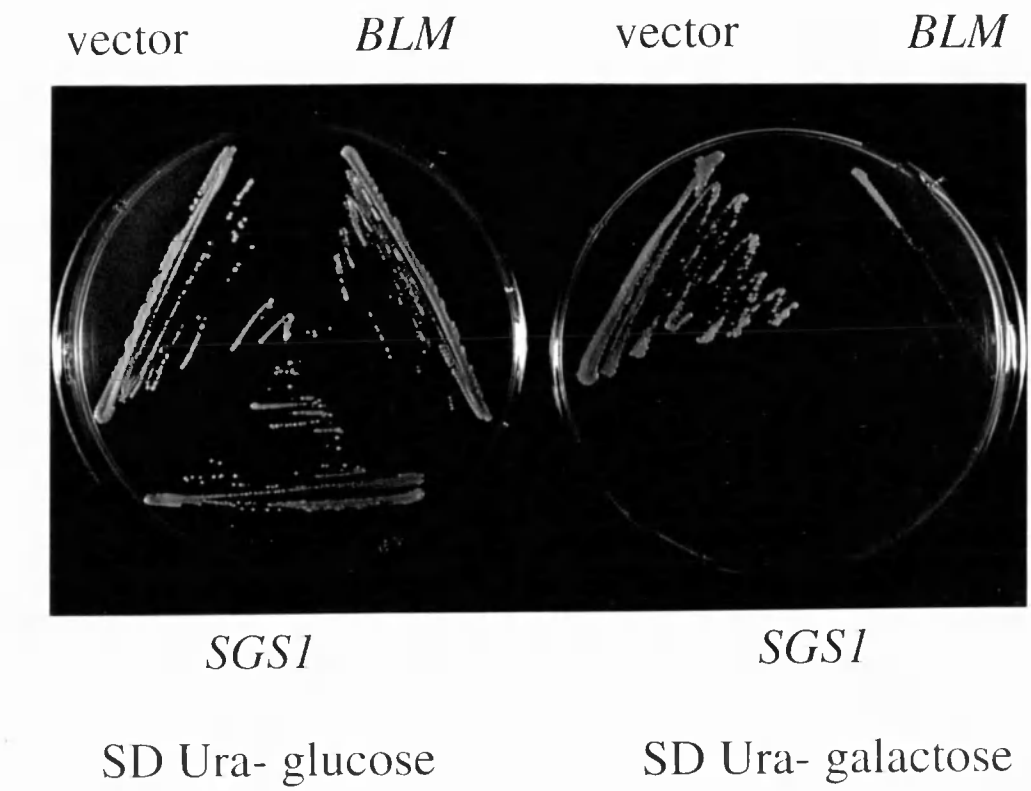


Figure 3.9: Effect of *BLM* upon growth of *sgs1Δtop3Δ* cells. Wild type and *sgs1Δtop3Δ* cells were transformed with pRKC15 (*BLM*), pRKC2 (*SGS1*) and pYES2 (vector) control. In each case the respective genes were cloned downstream of the *GAL1* promoter. Independent transformants were plated onto selective media containing either 2% glucose or 2% galacose/1% raffinose and grown for 3 days at 30°C. Plates showing *sgs1Δtop3Δ* transformants are shown. No effect of expression of *SGS1* or *BLM* was observed in wild type cells. Expression of full length BLM protein under inducing conditions was confirmed as previously by immunoblot.

3.3 Discussion

3.3.1 Complementation of the BS cellular phenotype

The potential advantage of introducing a wild type gene into a cell line containing a 'knockout' mutation is that one can assess the phenotypes of cells that are otherwise isogenic. Results presented in this chapter demonstrate the partial correction of the slow growth, poor plating efficiency and elevated SCE rate of an SV40-transformed BS fibroblast cell line following transfection with the wild type *BLM* cDNA under the regulation of the CMV promoter. The complementation of the BS cellular phenotype combined with the demonstration of DNA-dependent ATPase and helicase activities of the recombinant protein (Karow et al., 1997), have paved the way for combined studies in which the effects of *BLM* mutations upon helicase function *in vitro* can be compared with their effect upon the phenotype of BS or normal cell lines. Other studies ongoing include the evaluation of the response of *BLM* and vector transfectants to a variety of DNA damaging drugs or agents which inhibit DNA synthesis. In the longer term, regulatable expression of a wild type or mutated *BLM* gene, may provide a more powerful tool for dissecting the roles of the protein.

The failure to obtain complete correction of the SCE phenotype, in comparison with previous studies employing the minicell transfer of chromosome 15 into the same cell line (McDaniel and Schultz, 1992), probably reflect differences in the level and timing of mRNA expression. Similar results, confirming partial correction of the SCE phenotype in BS cells have recently been reported by others (Giesler et al., 1997). In this study, the authors reported that *BLM* transfection also resulted in an increase in the rate of S-phase transit as assessed by pulse labelling with bromodeoxyuridine.

The failure to obtain any permanent *SGS1* transfectants in either BS or normal cell lines, despite efficient control transfections performed in parallel was unexpected. Although this might represent a 'toxic' effect of Sgs1p, perhaps secondary to unregulated helicase activity, it was anticipated that a proportion of permanent transfectants (with low or minimal levels of *SGS1* expression), might be viable. One way of addressing this might be to perform transient transfections or to use constructs that allow regulatable expression of *SGS1*.

3.3.2 Synergy between *sgs1Δ* and *srs2Δ*

The synergistic effects upon growth observed in *sgs1Δsrs2Δ* double mutants could result either from an additive negative effect of the two mutations upon a single essential biological pathway, or from the inactivation of 2 different but functionally overlapping pathways. In both scenarios, and in the context of the similar biochemical activities of the respective proteins, it seems reasonable to postulate that both Sgs1p and Srs2p act upon the same group of DNA structures and/or lesions.

A clue to the nature of these putative lesions might be extrapolated from the effects of different DNA damaging agents upon *srs2* mutants. In *S.cerevisiae,* the cellular response to DNA damage is controlled by a set of genes which define three pathways (Game, 1993; Lawrence, 1994; Friedberg et al., 1995). Mutants defective in the *RAD3* excision repair group are primarily sensitive to UV irradiation which induces the formation of pyrimidine dimers and other photoproducts. *RAD52* epistasis group mutants are defective in recombinational repair of double strand breaks and are sensitive to ionising irradiation, but less so to UV. The third set of genes form the more heterogeneous and complex *RAD6* group and are sensitive to the lethal effects of both UV and γ-rays. The *RAD6* group appears to comprise 2 independent pathways, a major pathway mediating error-free post-replication repair, involving Polδp, PCNAp and Mms2p (Torres-Ramos et al., 1996; Giot et al., 1997; McDonald et al., 1997), and a secondary error-prone pathway, involving Rev3p, Rev7p and Rev1p (Lawrence, 1994). It should be noted here that the mechanism for post-replication repair in eukaryotes is not known and its similarity or otherwise to post-replication repair in prokaryotes is not established. Double mutants deficient in both the *RAD6* and *RAD52* pathways demonstrate synergistic interactions (reviewed in Friedberg et al., 1995), suggesting that these two pathways are partly able to substitute for each other, and therefore share some common substrates.

Further evidence for this partial overlap derives from study of *srs2* mutants which are sensitive to both UV and ionising irradiation, and which have complex relationships with both the *RAD6* and *RAD52* epistasis groups. Mutation or loss of Srs2p activity suppresses the radiation sensitivity of *rad6* mutants (Aboussekhra et al., 1989), an effect which is dependent upon the presence of an intact *RAD52* pathway (Scheistl et al., 1990). This led to the proposal that Srs2p plays some role in assigning DNA lesions or repair intermediates down a *RAD6*-dependent post-replication repair pathway, and that in its absence lesions are channelled into recombination repair pathways. In this sense, Srs2p

can be thought of as having 'anti-recombinogenic' activity. This is also illustrated by the fact *srs2* mutants demonstrate a mitotic hyper-recombination phenotype (Rong et al., 1991) and by the finding that overexpression of *SRS2* in a wild type background confers sensitivity to ionising irradiation (Kaytor et al., 1995). Furthermore, *srs2* mutations suppress the radiation sensitivity of partially defective mutants in the *RAD52*-dependent pathway (Milne et al., 1995). Counter to these anti-recombinogenic effects, Srs2p protein is reported to participate directly in recombinational repair of DSBs since *srs2* mutants are defective in gene conversion involving long non-homologous 3' ends (Paques and Haber, 1997). This apparent contradiction mirrors findings for the Srs2 homologue in *E.coli*, UvrD. For example, biochemical studies show that, depending on the assay conditions, UvrD can either promote or inhibit RecA-mediated branch migration (Morel et al., 1993). Similarly, *uvrD* mutants not only stimulate recombination (Washburn and Kushner, 1991), but also have a positive role in the recombination pathway (Horii and Clark, 1973).

A number of hypotheses have been put forward to explain the phenotypes of *srs2* mutants (Friedberg et al., 1995; Chanet et al., 1996; Paques and Haber, 1997) . First, Srs2p could act to prevent inappropriate recombination during processes such as DNA replication which generate potential recombinogenic structures such as ssDNA gaps. Second, Srs2p could act directly to initiate repair of such structures by post-replication repair. Third, Srs2p could have a more direct role in recombination and depending upon the context, could either limit or promote the extension of recombination structures.

What does this information about Srs2p tell us about Sgs1p? One potential explanation is that Sgs1p is involved primarily in recombinational repair and thus, in the absence of both Sgs1p and Srs2p, there is a failure to resolve structures either by post-replication repair or by recombination. The exact nature of these putative structures, the process which generates them and the roles of either of these two proteins in resolving them remain to be determined.

3.3.3 *BLM* partially complements the *sgs1Δ* phenotype

Failure to obtain heterologous complementation could occur for a number of reasons. First, although proteins from distinct organisms may be structurally similar, they may in fact not share functional similarity. For example, BLASTp data suggest that the closest yeast neighbour of the human CFTR protein, defective in cystic fibrosis (Riordan et al.,

1989), is Ycf1p (Szczypka et al., 1994). However, the closest human neighbour to YCf1p is not CFTR, but another ABC transporter, MRP which is associated with multidrug resistance (Ze-Sheng et al., 1996). Both MRP and YCf1p act as glutathione S-conjugate pumps and the MRP gene complements *ycf1* mutants. A second reason for failure to observe complementation, is that although two proteins may be functionally similar, the pathways in which they act or proteins with which they interact may be dissimilar. Third, the timing and levels of expression of many genes may be critical for their function and this may be difficult to achieve experimentally.

Expression of *BLM* in fission yeast failed to complement the effects of loss of Rqh1p activity. Other potential end points for assessing complementation, not published at the time these experiments were performed, would include the effect of *BLM* upon the rates of recombination or chromosomal loss induced by HU in *rqh1* mutants. However, similar failure to obtain complementation by *BLM*, has been found by others (Dr. E. Stewart, Department of Genetics, Harvard Medical School, personal communication).

In budding yeast, there is no equivalent of the 'cut' phenotype seen in fission yeast and as demonstrated in Chapter 4, in the strain background employed for most experiments, *sgs1Δ* mutants do not show significant sensitivity to HU. *BLM* expression did not correct the synergistic slow growth defect of the *sgs1Δsrs2Δ* double mutant.

There was, however, evidence of partial complementation by *BLM* for a subset of the phenotypes associated with *sgs1Δ* mutation. Deletion of *SGS1* results in a marked suppression of the slow growth and mitotic hyper-recombination phenotypes of a *top3Δ* mutant. Inducible ectopic expression of wild type *SGS1* in an *sgs1Δtop3Δ* background, but not in a wild type background, results in the re-induction of slow growth. Similarly, inducible expression of *BLM* in an *sgs1Δtop3Δ* background, but not in a wild type background also induces a slow growth phenotype although not to the same extent as for *SGS1*. This partial complementation suggests not only that BLM and Sgs1p share functional properties, but also that the genetics between topoisomerase III and RecQ family helicases may be highly conserved. While this thesis was in preparation, the ability of *BLM* to complement this subset of *sgs1Δ* phenotypes has been reported by others using strains in which *BLM*, under the control of the *GAPDH* promoter, was integrated into the *SGS1* locus. Furthermore, in this study *BLM* was found to partially complement both the elevated rate of illegitimate recombination and homologous recombination (between direct repeats) in *sgs1Δ* mutants. It seems likely, therefore, that

the small but consistent reduction in the rate of heteroallelic recombination in BLM *sgs1Δ* transformants, measured in this study, does in fact correspond to a partial complementation effect.

Chapter 4

An evaluation of the cell cycle defect in *S.cerevisiae top3Δ* mutants

4.1 Introduction

DNA topoisomerases catalyse the interconversion of topological isomers of DNA and are required for the resolution of torsional stress in DNA and for the unlinking of topologically-intertwined molecules. Budding yeast expresses three topoisomerases, designated topoisomerases I, II and III, all of which are highly conserved in mammalian cells. The role of topoisomerase III in DNA metabolism has remained enigmatic, partly because this enzyme possesses only a very weak DNA relaxation activity on negatively supercoiled DNA and is thought, therefore, unlikely to participate in the maintenance of DNA supercoiling homeostasis (Kim and Wang, 1992; Wang, 1996). It has been suggested previously that this enzyme performs a non-essential role in the segregation of newly-replicated DNA, although there is no direct evidence to support this proposal (Wang, 1996). The bacterial topoisomerase III enzyme shows very similar catalytic properties *in vitro* to that of its eukaryotic counterparts and has been implicated in the unlinking of DNA strands during DNA replication to permit nascent chain elongation and the separation of daughter molecules (Hiasa and Marians, 1994). The slow growth phenotype of *S.cerevisiae top3Δ* mutants is associated with the accumulation of cells in late S/G2 phase of the cell cycle. Thus, one of the starting points in assessing the function of topoisomerase III was to address this defect in the cell cycle.

Cell duplication and division require an orderly and regulated transit through the cell cycle (reviewed in Murray and Hunt, 1993). The eukaryotic cell cycle consists of four phases: **S** phase, when DNA replication takes place; **M** phase, or mitosis, where chromosome segregation occurs; and the gap phases **G1** (before DNA replication) and **G2** (before mitosis). Progression through the cell cycle can easily be evaluated in *S.cerevisiae* since this organism divides by budding (Figure 4.1). Early in the cell cycle a small bud forms which increases progressively in size and eventually separates from the mother cell. Bud formation occurs just after START (see below) and is therefore a useful marker for this restriction point. In contrast to higher eukaryotes, the nuclear envelope does not break down during mitosis and microtubules are nucleated by spindle pole bodies rather than centrosomes. Another key difference is the formation of a pre-mitotic spindle during S-phase, the timing of which corresponds to the period of nuclear

migration, when the nucleus approaches the canal between the mother cell and the bud (reviewed in Futcher, 1993).

Although the cell cycle can be viewed as representing discrete phases as described above, a more modern view is that cell cycle transitions are more complex involving regulation at a number of levels. These encompass changes in cyclin dependent kinase activity which in turn is regulated by proteolysis (reviewed in Nasmyth, 1996; King et al., 1996; Osmani and Ye, 1997), and alterations at the level of the chromosome such that they are either competent or refractory for DNA replication (reviewed in Diffley, 1996; Stillman, 1996). The integration of these activities are essential in order to ensure first, the correct order of events, second, the completion of certain steps (such as DNA replication) prior to the initiation of others (such as mitosis) and third, to prevent the inappropriate repetition of processes that have already been completed.

Superimposed upon this regulatory network are a number of surveillance mechanisms which monitor the completion of individual processes and act either positively or negatively upon subsequent steps. A subset of these mechanisms can be regarded as 'checkpoints' which act to block cell cycle transitions. These are generally defined in terms of the response of cells to 'extrinsic' effects such as DNA damage or inhibition of DNA replication either in replication-defective mutants or following exposure to HU.

In *S.cerevisiae* the G2/M checkpoint induced by DNA damage is thought to be dependent upon proteins that act to sense abnormal DNA structures (for example Rad9p or Rad24p), 'signal transducers', (Mec1p, a homologue of the protein ATM, and Rad53p) and 'effectors' of the cell cycle arrest (Pds1p) (Weinert and Hartwell, 1988; Weinert et al., 1994; Allen et al., 1994; Yamamoto et al., 1996b). Damaged cycling cells arrest with a large budded phenotype and a short spindle, suggesting an arrest in a G2 or metaphase state. In budding yeast, the arrest of cells at this point is mediated by Pds1p, which is required for preventing anaphase when DNA or spindles are damaged. Thus, in this organism the critical event postponed by the DNA damage checkpoint is sister chromatid separation. During the normal cell cycle Pds1p is targeted for degradation by a large protein complex, the anaphase promoting complex (APC), which promotes the ubiquitination of a number of proteins, including cyclins and Pds1p, that contain 'destruction boxes' near their N-termini (Cohen-Fix et al., 1996; Osmani and Ye, 1997). Degradation of Pds1p, releases another protein, Esp1p, which in turn appears to disrupt

cohesive forces between sister chromatids, allowing their separation (Ciosk et al., 1998). In the absence of Pds1p, cells exposed to DNA damage, progress inappropriately through anaphase and lose viability rapidly. In contrast to *S. cerevisiae*, other eukaryotes employ different strategies for inducing a block in cell cycle progression following DNA damage. In fission yeast, cells delay the onset of mitosis by inhibiting the activation of $p34^{cdc2}$ kinase. The onset of mitosis requires the activation and dephosphorylation of a tyrosine residue (Y15) of $p34^{cdc2}$ kinase (reviewed in Kitazono and Matsumoto, 1998). Experiments designed to induce inappropriate dephosphorylation of Y15 of cdc2p, also result in inappropriate progression though mitosis following DNA damage (Enoch and Nurse, 1990; Lundgren et al., 1991). A similar mechanism resulting in a G2 arrest is operational in higher eukaryotes, since expression of a $p34^{cdc2}$ in which 2 major phosphorylation sites have been mutated, results in a shorter delay in the onset of mitosis following damage, than in controls (Jin et al., 1996; Blasina et al., 1997).

Another checkpoint maintains the dependence of mitosis on the prior completion of DNA replication. There are two broad classes of yeast mutants that are defective in this checkpoint. The first class comprises mutants in the *S.pombe rad/hus* (Al-Khodairy and Carr, 1992; Jimenez et al., 1992; Al-Khodairy et al., 1994) and in the *S.cerevisiae MEC1/RAD53* genes (Weinert et al., 1994). Instead of demonstrating a cell cycle arrest following inhibition of DNA replication, these mutants proceed inappropriately through mitosis. These mutants do not appear to be directly defective in replication or mitosis. This group of mutants also show a loss of checkpoint responses following DNA damage and thus, it is likely that the cellular response to DNA damage and replication block share, at least in some respects, a common framework. A second class of mutations are defective in the initiation of DNA replication and include the *S.pombe cdc18, cut5/rad4* and *cdt1* genes (Kelly et al., 1993; Saka et al., 1994; Hofmann and Beach, 1994) and the *S.cerevisiae CDC6* genes (Piatti et al., 1995). These mutants are unable to synthesise DNA and are therefore not viable. However, instead of undergoing a cell cycle arrest like many other mutants defective in DNA replication, this class of mutants progress through mitosis with a haploid content of DNA. A single hypothesis which seeks to explain the phenotypes associated with the two distinct classes of mutant has been proposed (Humphrey and Enoch, 1995). It has been suggested that assembled replication complexes activate the checkpoint pathway, thus preventing mitosis while replication is ongoing. Thus, in the first class of mutants the surveillance mechanism which monitors

this signal is impaired, and in the second class of mutants, no replication complexes are assembled and no inhibitory signal is generated. Consistent with this model is the finding that mutations in certain replication factors can also be associated with a failure to induce the appropriate cell cycle delay following DNA damage or inhibition of DNA replication (Navas et al., 1995; Sugimoto et al., 1997).

In the following sections, an attempt is made to identify the point in the *S.cerevisiae* cell cycle where topoisomerase III acts. Three approaches were adopted. In the first, an attempt was made to clarify the nature of the 'arrest' phenotype observed in *top3Δ* mutants. Second, the effects of perturbing DNA replication in the absence of topoisomerase III were examined. Finally, the cell cycle regulation of *TOP3* mRNA expression was determined.

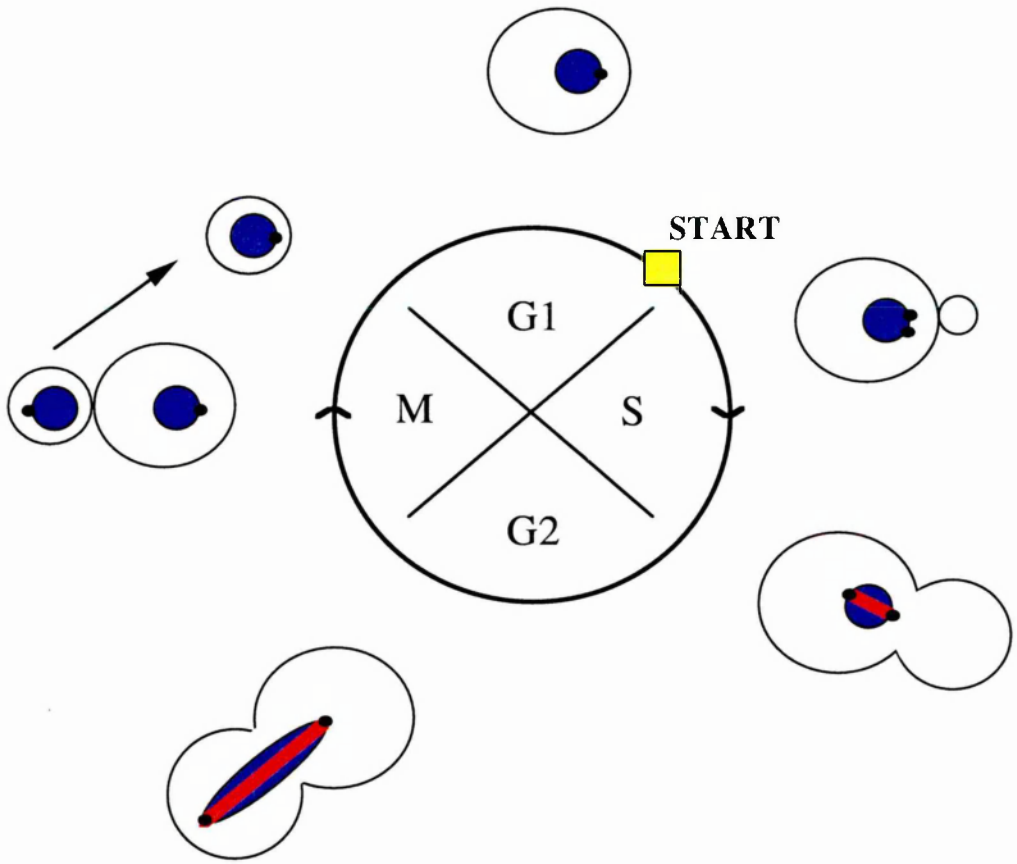


Figure 4.1: Schematic representation of the organisation of the cell cycle of *S.cerevisiae*. Nuclei are shown in blue, spindle pole bodies are represented by black circles and spindles by red bars. See text for details.

4.2 Results

4.2.1 *top3Δ* cells demonstrate a checkpoint-dependent cell cycle delay at G2/M

The cell cycle transit time of *S. cerevisiae top3Δ* mutants is approximately double that of isogenic wild-type strains (Wallis et al., 1989). Previous microscopic studies of asynchronous *top3Δ* cultures revealed an accumulation of large budded cells (~70% of the total population) containing a single nucleus close to the neck of the mother cell (Gangloff et al., 1994), suggesting a delay in the cell cycle in late S/G2. In order to characterise this arrest phenotype in more detail, flow cytometric analyses were performed of wild type and *top3Δ* cells at timed intervals following release from a cell cycle arrest in G1 induced by the α-factor mating pheromone. It should be noted that because *top3Δ* cells delay in late S/G2, it was not possible to obtain a completely synchronous G1 population of mutant cells with α-factor within the time course of the experiment. Figure 4.2 shows that following release from an α-factor arrest, *top3Δ* cells progressed through S-phase at a rate similar to that of wild-type cells, but then delayed in the cell cycle at a point where they had a 2C content of DNA. Consistent with previous studies (Gangloff et al., 1994), therefore, the extended cell cycle time in *top3Δ* strains is at least in part accounted for by this late S/G2 delay. *top3Δ* cells showed a very broad cell size distribution (as measured by estimates of forward light scatter), which can affect the apparent DNA content of cells when measured by flow cytometry (Paulovich and Hartwell, 1995). In order to overcome this problem, contour plots of the flow cytometry data were employed because they reflect more accurately the DNA content of a synchronous population, and in all subsequent experiments such plots were evaluated alongside the more conventional histograms. Analysis of cell size using contour plots (Figure 4.3) indicated that *top3Δ* cells showed a marked and progressive increase in size during the extended delay at late S/G2. It should be noted that *top3Δ* cells emerged from an α-factor arrest about 10-20 minutes earlier than did wild type cells, as assessed microscopically by the appearance of small budded cells (data not shown). This possibly reflects the larger size of the G1-arrested mutant cells, which would influence the rate at which cells pass the cell size restriction point before START (Pringle and Hartwell, 1981). Moreover, *top3Δ* mutants showed the consistent presence of cells with a <1C content of DNA, suggesting an inherent abnormality in chromosome transmission (Figure 4.2).

It is not possible using flow cytometry to distinguish between late S-phase and G2/M cells and it is a possibility, therefore, that a residual level of non-replicated DNA exists in *top3Δ* cells that is sufficient to induce a late S-phase delay. To determine whether the late S/G2 arrested cells had completed DNA replication, intact chromosomes of log phase cells were separated by pulse field gel electrophoresis (Figure 4.4). In this assay, only fully replicated DNA enters the gel, whereas incompletely replicated DNA is retarded in the wells (Hennessy et al. 1991). As controls, wild type cells were treated with HU (blocks in S phase), α-factor (blocks in G1) or MBC (blocks in M). As expected, DNA from HU-treated cells failed to enter the gel, whereas the majority of the fully replicated DNA in α-factor- or MBC-treated cells entered the gel. In common with the findings for wild type cells, the majority of DNA from log phase *top3Δ* cells entered the gel (70.0% ± SEM 2.9 of total DNA for wild type cells vs. 82.3% ± 2.4 for *top3Δ* cells). This latter figure was consistently greater than the % of both large budded cells and cells with a 2C DNA content. Thus, *top3Δ* cells complete bulk DNA replication and arrest at a point in the cell cycle after completion of DNA replication, but prior to chromosome segregation.

The progressive increase in cell size when *top3Δ* mutants attain a 2C content of DNA would be consistent with activation of the G2/M DNA damage checkpoint. To analyse this, the *RAD24* and *RAD9* DNA damage checkpoint genes were deleted in a *top3Δ* background and the cell cycle distribution of the resulting double mutants was determined. As shown in Figure 4.5, asynchronous cells from the *rad9Δ top3Δ* and *rad24Δtop3Δ* double mutants showed a marked reduction in the proportion of cells with a 2C content of DNA. Thus the percentage of cells gated for a 2C content of DNA using the contour plots shown were 61.6% (*top3Δ*), 40.9% (*top3Δrad24Δ*) and 40.3% (*top3Δrad9Δ*). Analysis of contour plots indicated that the *rad24Δtop3Δ* and *rad9Δtop3Δ* cells with a 2C DNA content were of a more uniform size than was observed for a *top3Δ* single mutant population, suggesting that the double mutant cells were progressing more rapidly through G2/M. This suggests that the cell cycle delay in *top3Δ* cells is dependent upon *RAD24* and *RAD9* and, by inference, the G2/M checkpoint. Synchronisation experiments confirmed that deletion of *RAD24* in a *top3Δ* background had the effect of shortening the G2/M phase and not of causing an additional delay in G1, which could have had a similar effect on the appearance of flow cytometric profiles (Figure 4.6). Thus, at 40 minutes following release from an α-factor arrest a significant proportion of

the *top3Δrad24Δ* cells have progressed into the next cell cycle, whereas the single *top3Δ* cells continue to be held up at G2/M. We have not excluded the possibility that deletion of *RAD9* in a *top3Δ* background causes an additional problem in the G1 phase of the cell cycle. These results were confirmed in 2 independent strain backgrounds.

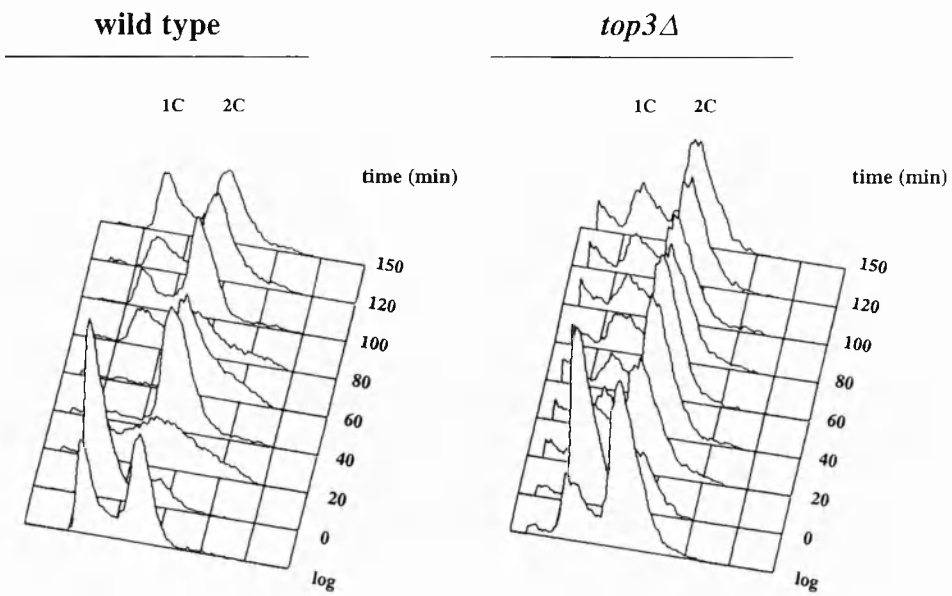


Figure 4.2: Cell cycle in *top3Δ* mutants. Log phase cultures of wild type (RKC1d) and *top3Δ* (RKC1a) cells were arrested in G1 with α–factor for 150 minutes, washed twice and then released into fresh, warmed medium. Flow cytometric analysis was performed at timed intervals. Peaks representing cells with a 1C and 2C content of DNA are indicated. Note the presence of a population of cells with a DNA content less than 1C in the *top3Δ* samples.

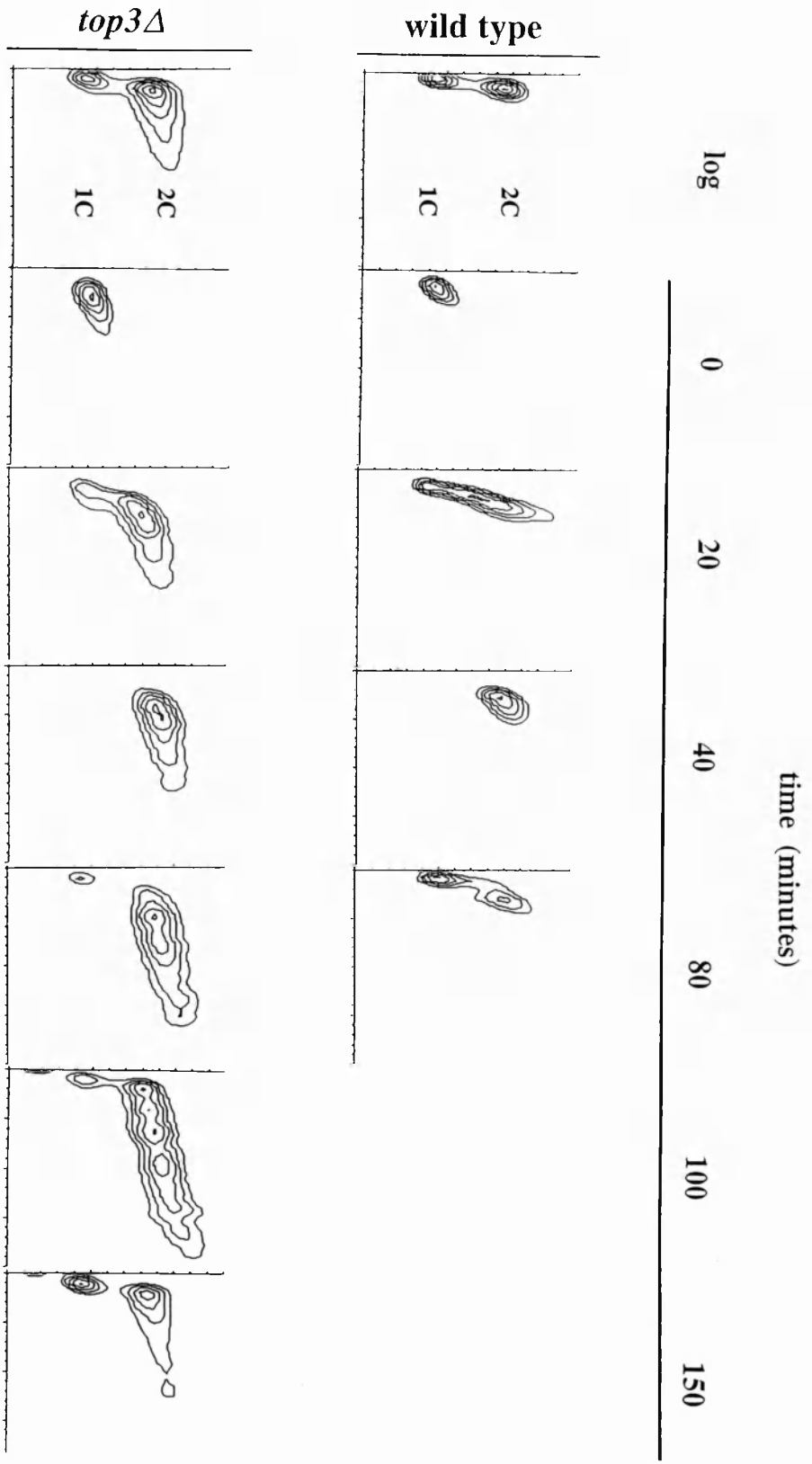


Figure 4.3: Cell cycle changes in cell size and DNA content in *top3Δ* mutants. Flow cytometric plots from the same experiment shown in Figure 4.2, in the form of contour plots with DNA content (y-axis) against forward light scatter (cell size; x-axis). Peak thresholds were set at 15%.

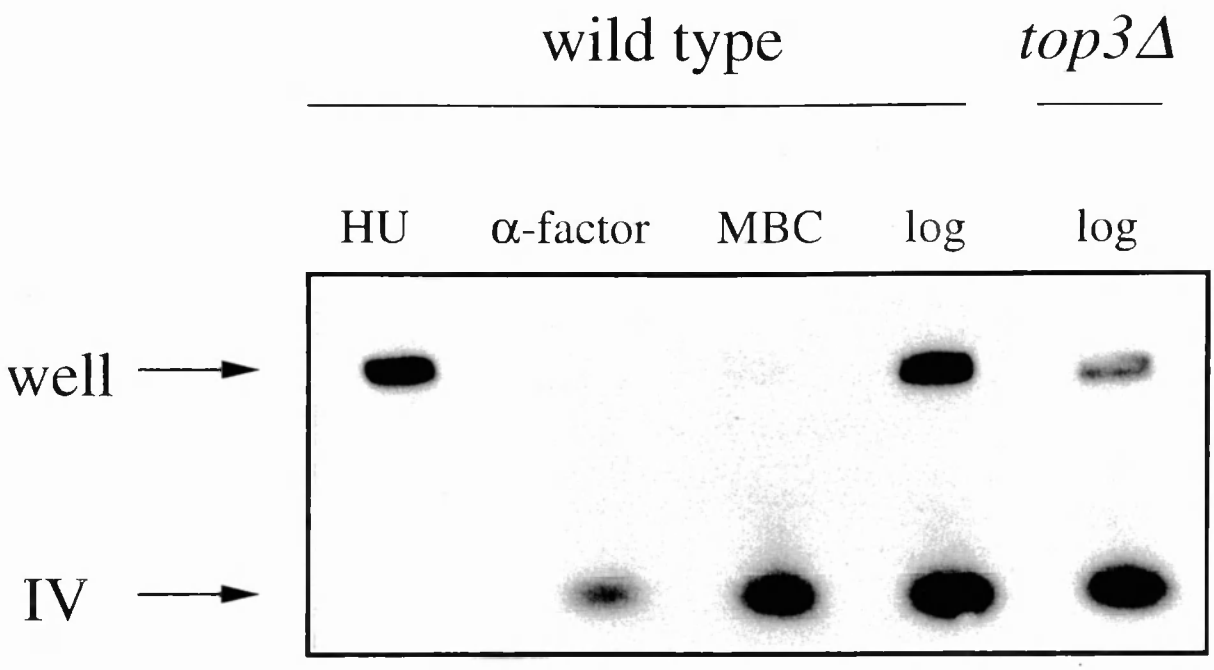


Figure 4.4: Bulk DNA replication is completed in *top3Δ* mutants. Intact chromosomal DNA from log phase cultures of wild-type and *top3Δ* strains, or of wild-type cells arrested with HU, α–factor or MBC, was separated by pulse field gel electrophoresis. After transfer to a nylon membrane, the DNA was probed with the *HO* gene, which reveals chromosome IV. The positions of the wells and of chromosome IV which had migrated into the gel are indicated.

Ethidium bromide staining of the gel (not shown) demonstrated no differences in the staining of larger and smaller chromosomes.

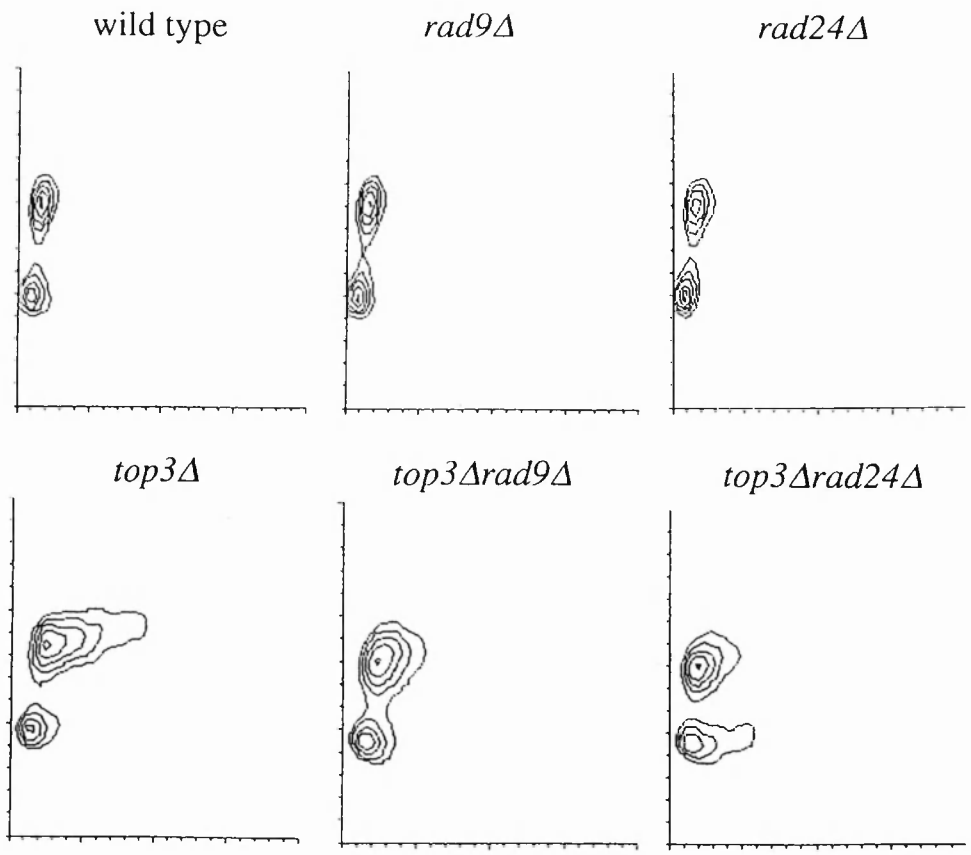


Figure 4.5: *top3Δ* cells demonstrate a *RAD9-/RAD24*-dependent cell cycle delay at G2/M. Flow cytometric contour plots from log phase cultures of RKC 31a (wild-type), RKC31b (*top3Δ),* DLY195 (*rad9Δ*), RKC36e (*rad24Δ*), RKC36f (*top3Δrad24Δ*) and RKC36d (*top3Δrad9Δ* strains).

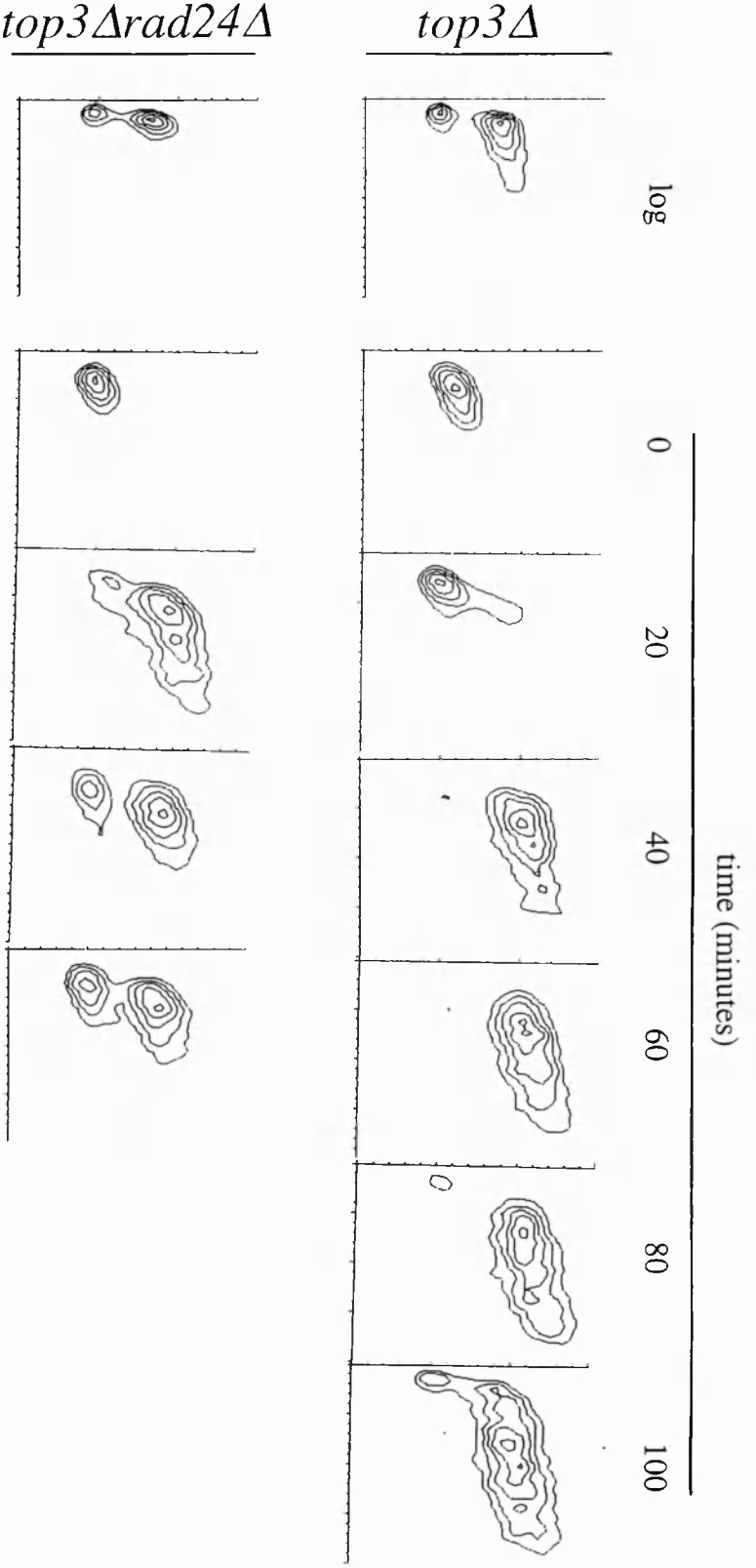


Figure 4.6: Deletion of *RAD24* in *top3Δ* cells shortens the period of delay at G2/M. Log phase cultures of *top3Δ* and *top3Δrad24Δ* cells were arrested in G1 with α-factor and then released into fresh media. Flow cytometric contour plots at timed intervals post-release are shown.

4.2.2 The effect of *SGS1* upon the viability of *top3Δ* mutants

One explanation for the cell cycle studies described above, is that in the absence of topoisomerase III, abnormal DNA structures are generated during replication, that induce a checkpoint-mediated delay at G2/M. Although, this accounts in part for the slow growth phenotype of *top3Δ* mutants, another factor is the poor viability of *top3Δ* cells such that they have a plating efficiency of only ~25% (Figure 4.7). Deletion of *SGS1* in a *top3Δ* background results not only in a reversion to near wild type generation times (Gangloff et al., 1994), but also a marked improvement in cell viability. One possible interpretation of this is that in the absence of topoisomerase III, Sgs1p is involved in generating DNA structures that are potentially cytotoxic during S-phase. If this is the case, then overexpression of Sgs1p might be expected to exert a 'toxic' effect only during DNA replication. On this basis, we tested the effect of Sgs1p overexpression ($Sgs1^{op}$) at different phases of the cell cycle in *sgs1Δtop3Δ* cells. In preliminary results derived from 2 independent experiments, log phase cultures of *sgs1Δtop3Δ* cells containing either pRKC2 (*SGS1* cloned downstream of *GAL1* in a multicopy plasmid) or vector control were arrested either in G1 with α-factor, or in M-phase with MBC. G1 arrested cells were then washed twice and either maintained in G1 with α-factor or released into media without α-factor. M-phase arrested cells were washed twice and then released again into media containing MBC. For each of these conditions, the cells were split into 2 aliquots containing either inducing conditions (galactose/raffinose) or repressed conditions (glucose). As shown in Figure 4.8, a marked reduction in cell viability was noted in those cells released from G1 into S-phase under inducing conditions. In contrast, holding cells in G1 or in M-phase fully or partially protected cells from loss of viability under inducing conditions. Protein expression was not determined in these experiments.

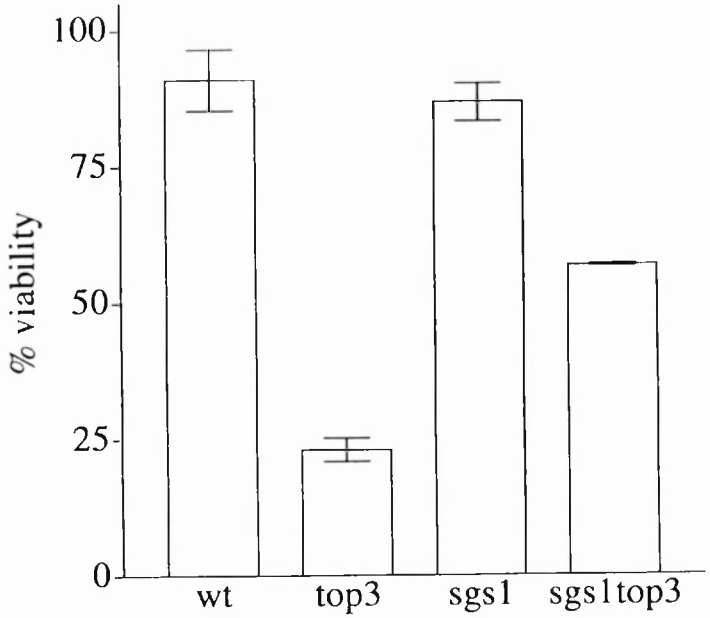


Figure 4.7: Poor viability of *top3Δ* mutants is suppressed by deletion of *SGS1*. Wild type and *sgs1Δ*, *top3Δ* and *sgs1Δtop3Δ* isogenic mutants were grown to log phase and a known number of cells plated onto YPD plates. The number of colony forming units for each strain were counted after 3 days growth at 30°C and the percentage viability calculated. The mean viability (±SEM) of 3-6 independent evaluations are shown.

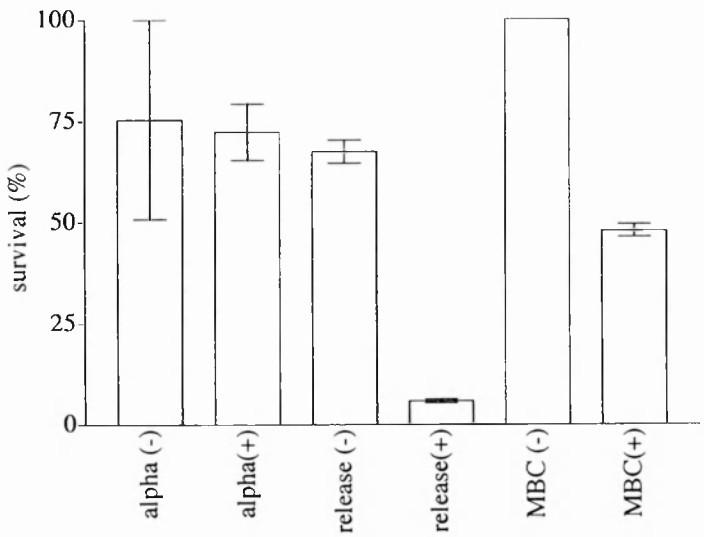


Figure 4.8: *SGS1* overexpression induces a cell-cycle specific loss of viability. *sgs1Δtop3Δ* cells transformed with pRKC2 (*SGS1*) or pYES2 (vector control) were grown to log phase in selective media containing 2% glucose and then arrested in G1with α-factor or in M-phase with MBC for 2 hours. At t=0, G1-arrested cells were then washed twice and then either maintained in G1 with α-factor (alpha) or released into media without α-factor (release) for 2 hours. At t=0, M-phase arrested cells were washed twice and then released into media containing MBC for 2 hours. For each condition, cells were split into 2 aliquots containing 2% glucose (-) or 2% galactose/1% raffinose (+). Mean (+ ranges) % survival in relation to t=0, from 2 independent experiments are shown. All values were corrected for vector control values. G1 and M-phase arrests were confirmed microscopically for the duration of the experiment. Expression of Sgs1p was not confirmed in these experiments.

4.2.3 *top3Δ* mutants lose viability rapidly when DNA replication is blocked by HU

There is substantial evidence supporting a role for topoisomerases during replication, acting both as swivelases and decatenases (Watt and Hickson, 1994; Wang, 1996). Furthermore, prokaryotic topoisomerase III possesses the ability to unlink DNA strands during replication in an *in vitro* system (Hiasa and Marians, 1994). One possible interpretation of the extended delay at G2/M in the absence of functional topoisomerase III is that abnormal DNA structures are generated during the process of DNA replication. This is in keeping with the similarities in flow cytometric profiles between *top3Δ* mutants and those of a number of replication-defective mutants, including *cdc17*, *cdc9* and *cdc44* (Weinert and Hartwell, 1993). In order to study this in more detail, we evaluated the effects on cell survival of inhibiting DNA synthesis with HU. Wild-type and *top3Δ* cells were released from an α-factor-induced G1 arrest into medium containing HU and % survival was quantified at timed intervals thereafter. Figure 4.9 shows that in a *top3Δ* cell population loss of survival occurred rapidly at a dose of HU (200mM) that was non-toxic to wild-type cells, such that following a 2 hour exposure 75% of *top3Δ* cells had either lost viability or were committed to die. Release from α–factor arrest into medium lacking HU was not associated with any loss of survival. Significant loss of viability occurred at a point when *top3Δ* cells were still in early S-phase, as judged by analysis of their DNA content using flow cytometry (see Figure 4.13 below and confirmed using contour plots; data not shown). HU sensitivity of *top3Δ* mutants was confirmed in 3 independent strain backgrounds.

Expression of a wild-type *TOP3* gene in a *top3Δ* mutant fully suppressed both the slow growth phenotype in drug-free medium, and the sensitivity to HU (Figure 4.10). Previous work has shown that deletion of *SGS1* in a *top3Δ* genetic background suppresses the slow growth and mitotic hyper-recombination phenotype characteristic of these cells, and Sgs1p has been shown to bind to topoisomerase III (Gangloff et al., 1994). As shown in Figure 4.11, deletion of *SGS1* also almost completely corrected the HU sensitivity of *top3Δ*cells. Indeed, *top3Δ* mutants could not be propagated on YPD plates containing as little as 2.5mM HU, whereas *sgs1Δtop3Δ* double mutants formed colonies in the presence of 100 mM HU (data not shown). This genetic interaction between *top3* and *sgs1* was confirmed in 2 independent strain backgrounds. This suggests that in the absence of topoisomerase III, Sgs1p activity is maybe in some way dysregulated and that this contributes to the HU sensitive phenotype. To test this, the

effect of Sgs1^{op} in a wild type background was evaluated. In the preliminary experiment shown in Figure 4.12, Sgs1^{op} had a small effect upon the viability of wild type cells in the presence of HU. Expression of Sgs1p in relation to the various conditions for this experiment was not determined.

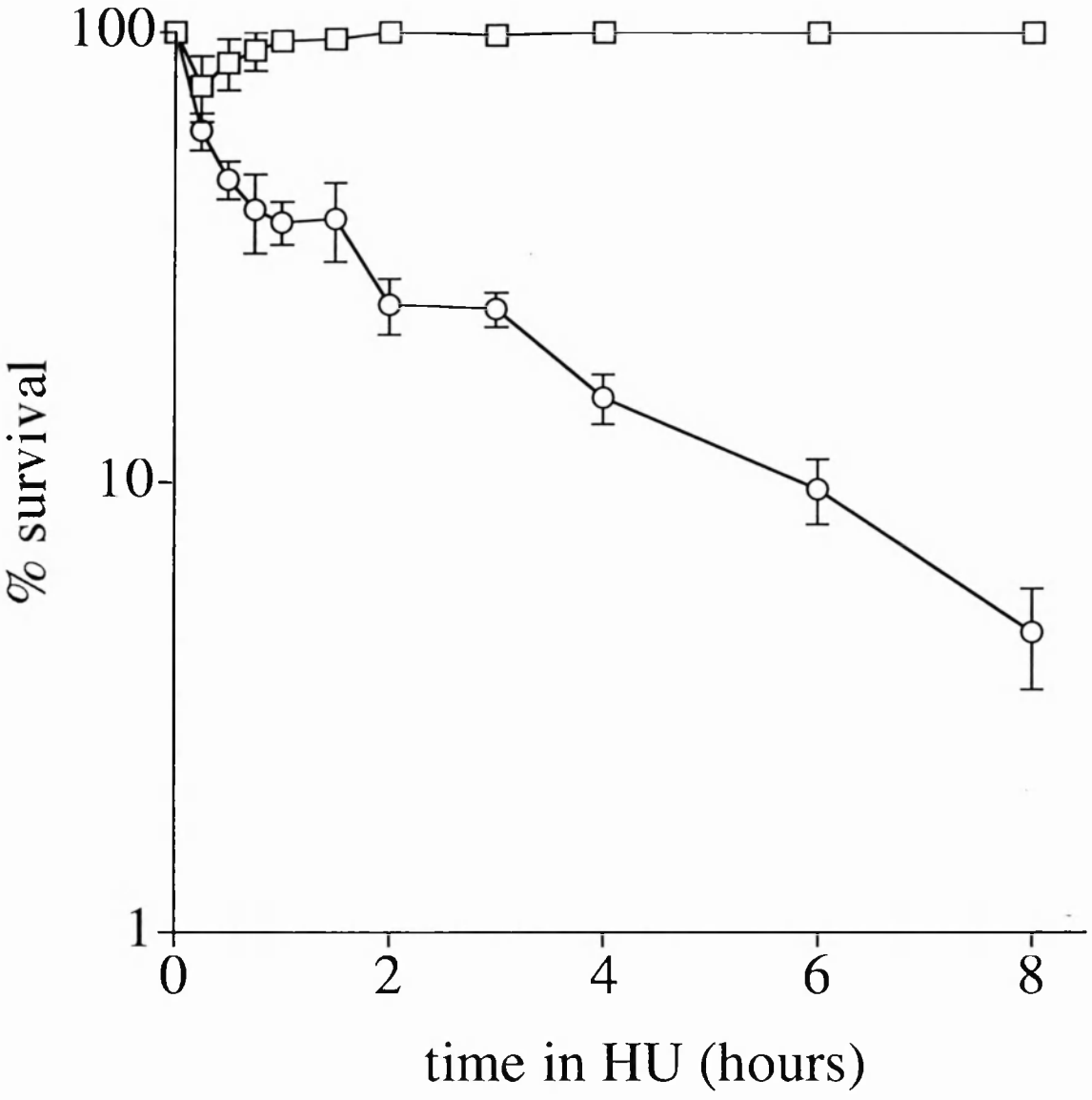


Figure 4.9: *top3Δ* cells lose viability when DNA replication is inhibited Log phase cultures of wild type (□) and *top3* (○) cells were arrested in G1 and released into medium containing 0.2M HU. The mean % survival (±SEM) determined at timed intervals in 3 independent experiments is shown

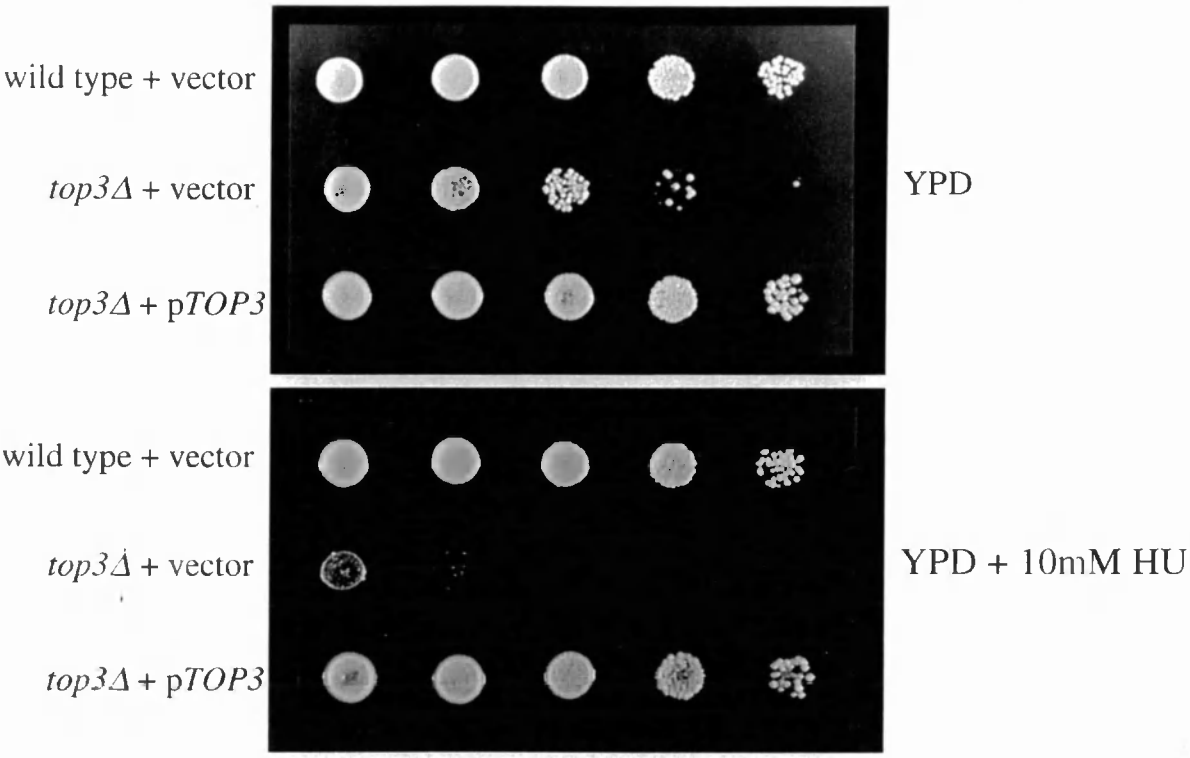


Figure 4.10: Complementation of the HU sensitivity of *top3Δ* mutants by ectopic expression of wild type *TOP3*. Log dilutions of saturated cultures of *top3Δ* cells transformed with pRK500, which encodes wild type *TOP3*, or a vector-only control were plated onto YPD (upper panel) or YPD containing 10mM HU (lower panel).

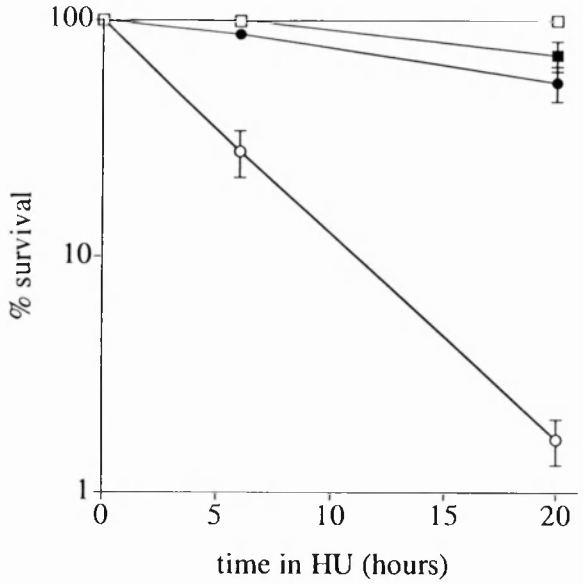


Figure 4.11: Deletion of *SGS1* suppresses the HU sensitivity of *top3Δ* mutants. Early log phase cultures in minimal media of wild-type (□), and isogenic *sgs1* (■), *sgs1 top3* (●) and *top3* (○) mutants were exposed to 0.2M HU at the times indicated and % survival was determined as compared to t=0. Data represent means (±SEM) from 4 independent experiments.

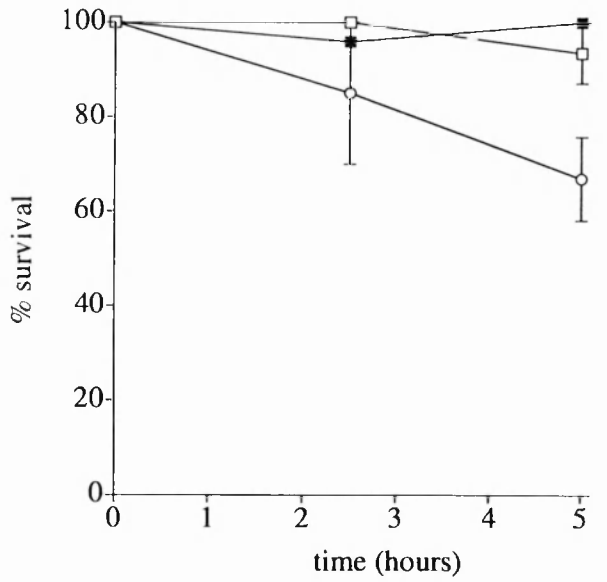


Figure 4.12: *SGS1* overexpression confers HU sensitivity in wild type cells. Wild type cells transformed with pRKC2 (*SGS1*) or pYES2 (vector control) were grown to log phase in selective media containing 2% glucose. At t=0, cells were washed twice and resuspended in selective media containing 2% glucose plus 0.2M HU (□), 2% galactose/1% raffinose plus 0.2M HU (○) and 2% galactose/1% raffinose with no HU (■). At timed intervals %survival in relation to t=0 was determined. The means (±SEM) of 5 experiments are shown for pRKC2 transformants are shown. In all cases and under each condition, % survival in vector controls was 100%. The % survival in cells released into media containing 2% glucose alone was 100% in each of the experiments. Note that the y-axis is linear. Expression of Sgs1p was not confirmed in these experiments.

4.2.4 *top3Δ* cells do not demonstrate an 'arrest' phenotype following exposure to HU

When DNA replication is blocked in wild-type budding yeast with HU, cells arrest with a uniform phenotype of a large-budded morphology containing a short, bar-like spindle and an undivided nucleus close to the bud neck. This arrest phenotype is dependent upon an intact checkpoint apparatus involving 'sensors' (*POL2, RFC5, DPB11*), 'signal transducers' (*MEC1, RAD53*) and as yet undefined 'effectors' of the cell cycle arrest (Lydall and Weinert, 1996). The rapid kinetics of death in HU-treated *top3Δ* cells is similar to that observed previously in checkpoint-deficient mutants. These checkpoint mutants fail to show the uniform arrest phenotype seen in HU-treated wild type cells, and appear to progress through mitosis, as manifested by the presence of long anaphase-like spindles, with a near haploid content of DNA (Allen et al., 1994; Weinert et al., 1994; Navas et al., 1995; Araki et al., 1995; Sugimoto et al., 1997).

To investigate this aspect in *top3Δ* strains, wild type and *top3Δ* cell cultures were arrested in G1 with α factor and then released, one half into media containing HU and the other half into drug-free medium. Nuclear and cellular morphologies were then compared by DAPI/spindle staining at the 2 hour time point when both wild-type and *top3Δ* cells had an S-phase content of DNA (Figure 4.13). The most striking feature of HU-treated *top3Δ* cells was the apparent lack of the normal cell cycle arrest phenotype (despite evidence that replication was inhibited by HU) and a concomitant increase in the % of abnormal cells (Figures 4.14-16). Examples of abnormal nuclear morphologies in HU-treated *top3Δ* cells included DNA that was fragmented, positioned entirely within the bud, or completely absent (despite the presence of mitochondrial DNA staining). These qualitative observations were confirmed quantitatively by scoring the percentage of abnormal cells in the presence or absence of HU (Figure 4.17). Microtubule staining of *top3Δ* cells also demonstrated several abnormalities, including, loop- or spoke-shaped spindles, unusually thick microtubule or inappropriately long spindles. Thus, following a 2 hour exposure to HU, 97% of wild type cells had arrested with a short spindle. In contrast, 23% of *top3Δ* cells had a partially elongated spindle.

The rapid loss of viability in the presence of HU, in association with marked abnormalities of nuclear and spindle morphology could suggest that the coupling of subsequent cell cycle events to the completion of DNA replication is dependent upon functional topoisomerase III. One potential explanation for the loss of viability in HU-treated *top3Δ* cells is that they are entering a near haploid mitosis. However, if mitosis

was inhibited by co-treating *top3Δ* cells with HU and the microtubule poison, MBC, there was no significant suppression of the loss of viability (Figure 4.18). Thus, commitment to death in HU-treated *top3Δ* cells does not appear to require either cytokinesis or progression beyond the arrest point in M-phase induced by MBC, as has been reported recently for hypomorphic mutants of *RAD53* or *MEC1* (Desany et al., 1998).

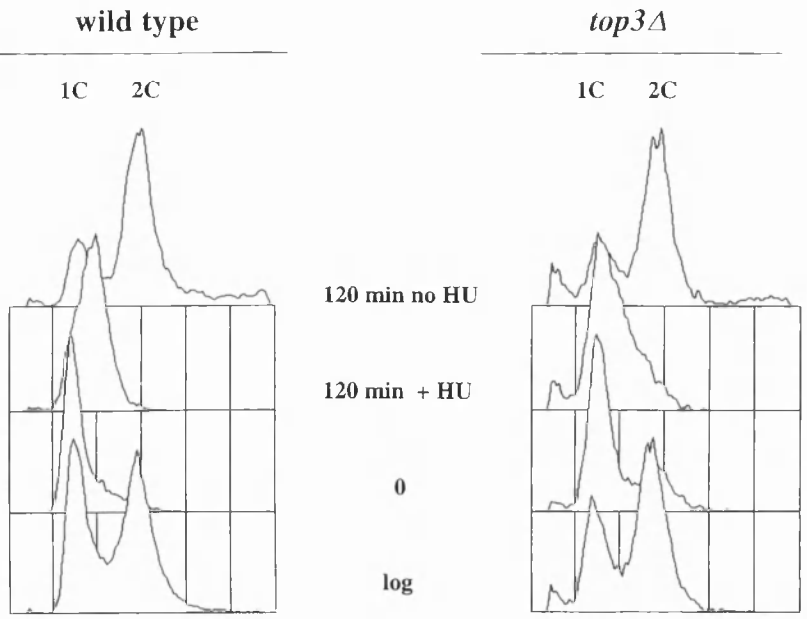


Figure 4.13: DNA content of wild type and *top3Δ* cells treated with HU. Flow cytometry histograms of wild type and *top3Δ* early log phase cultures that were arrested in G1 with α–factor and then released into medium containing either 0.2M HU or no drug as a control.

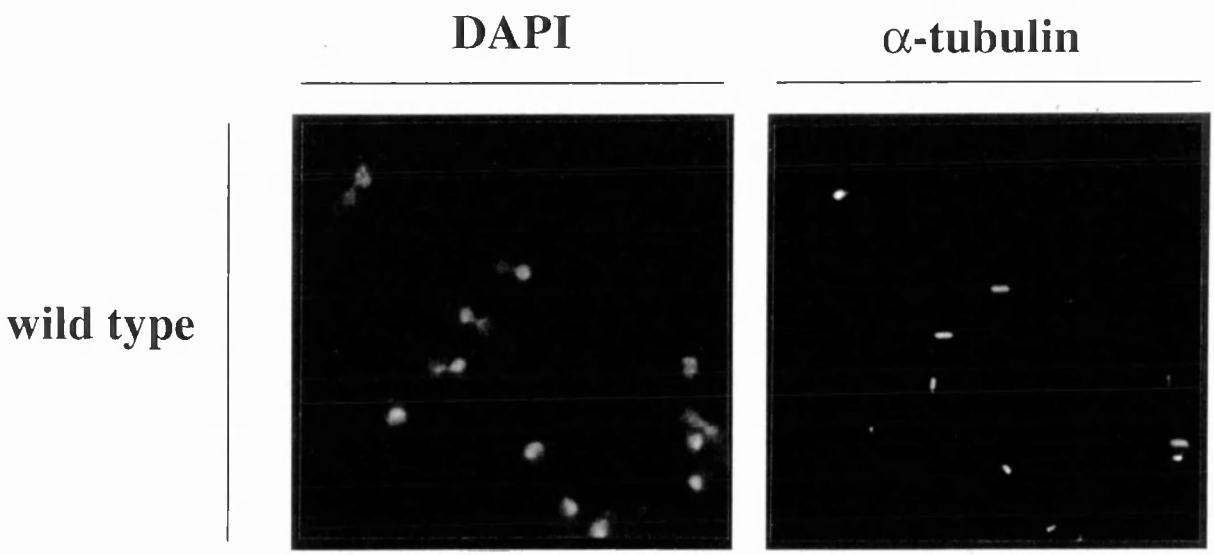


Figure 4.14: Arrest phenotype observed in wild type cells following exposure to HU. Same experiment as in Figure 4.9. Representative photomicrographs of DAPI/tubulin staining of wild-type cells exposed to HU for 2 hours.

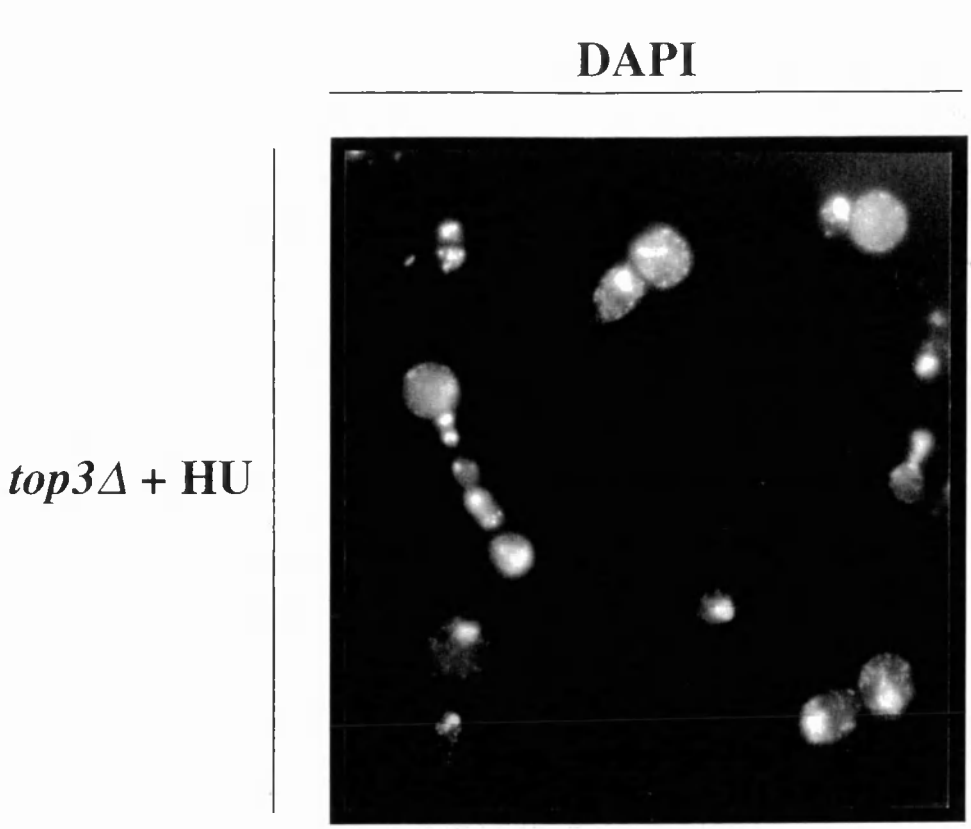


Figure 4.15: Nuclear morphology of *top3Δ* cells exposed to HU. Same experiment as in Figure 4.14. DAPI staining of *top3Δ* cells exposed to HU for 2 hours.

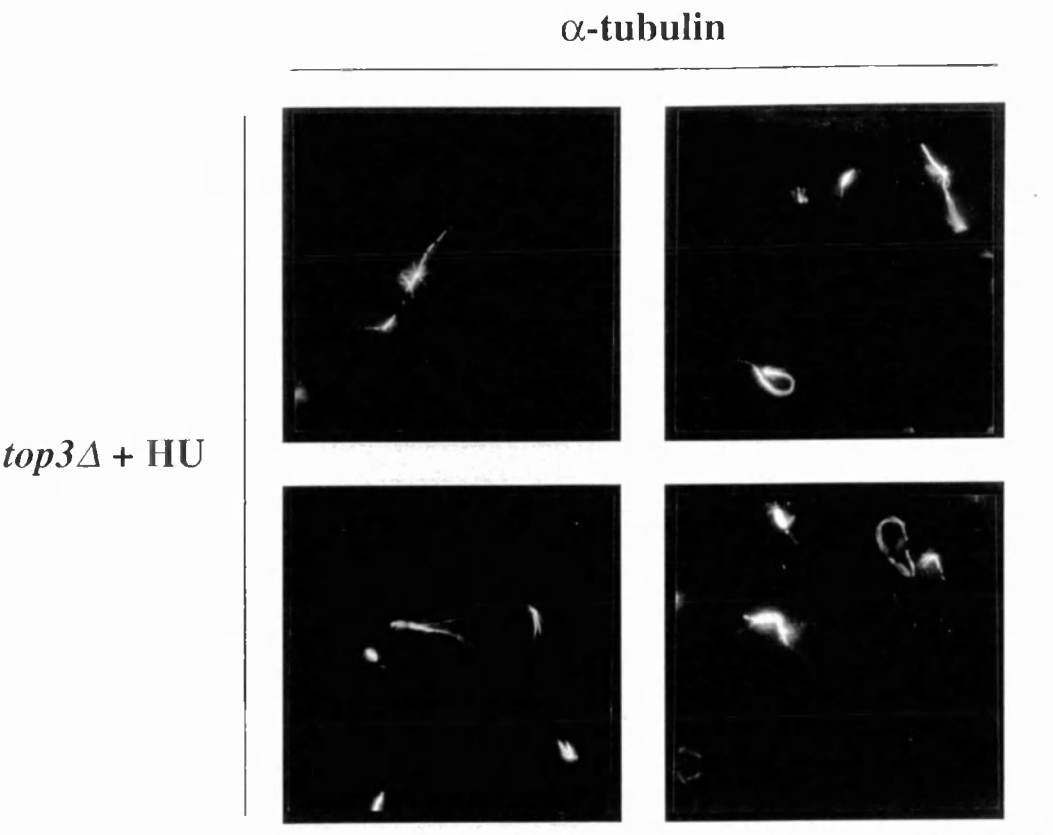


Figure 4.16: Spindle morphology of *top3Δ* cells exposed to HU. Same experiment as in Figure 4.15. Spindle staining of *top3Δ* cells exposed to HU for 2 hours demonstrating the following abnormalities: elongated spindles (top left), loop-pattern (top right), radial pattern (bottom left) and thickened, irregular microtubule array (bottom right).

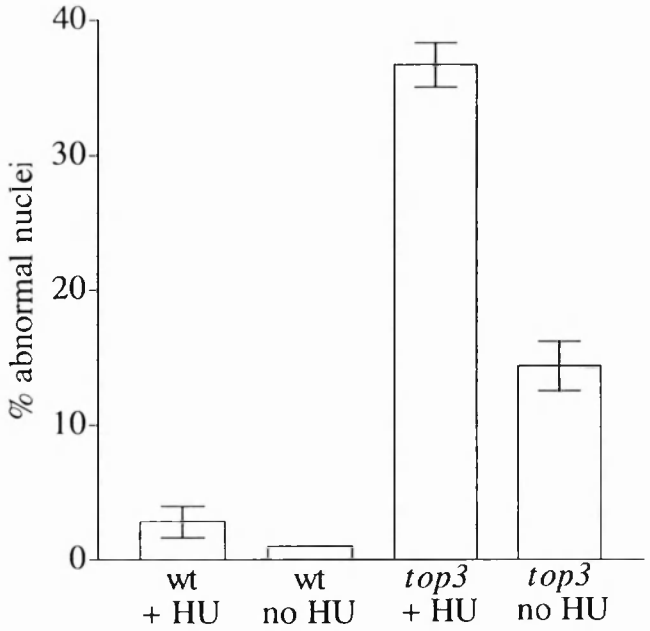


Figure 4.17: Abnormalities in nuclear DNA staining in wild type and *top3Δ* cells following exposure to HU. Abnormalities in nuclear DNA staining were quantified in wild-type and *top3Δ* cells following exposure to 0.2M HU or mock treatment for 2 hours, by scoring at least 200 cells. All assessments were performed single blind and the mean percentage (±SEM) of 3 independent experiments are shown.

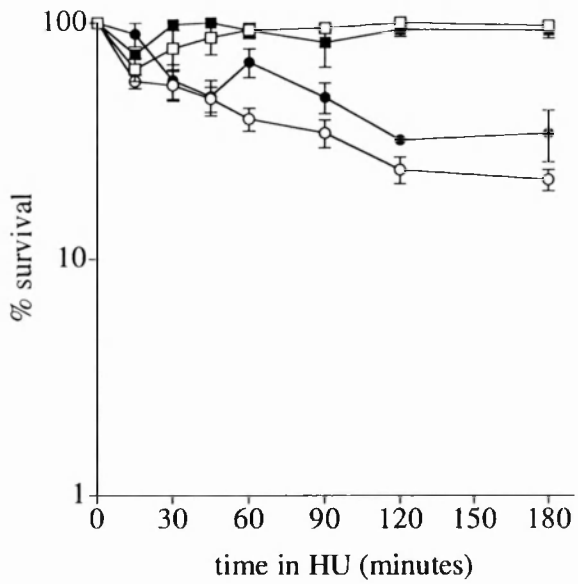


Figure 4.18: Inhibition of mitosis does not reverse the HU sensitivity of *top3Δ* mutants Log phase wild type (□, ■) and *top3Δ* (○, ●) cells were arrested in G1 with α–factor, and then released at t=0 into media containing either 0.2M HU (open symbols) or 0.2M HU plus 200µg/ml MBC (closed symbols). The mean % survival in relation to t=0 (±SEM) from three independent experiments is shown. Values for HU/MBC treated cells were adjusted according to values obtained when cells were released in parallel into media containing MBC alone. In this arm of the experiment, arrest at M-phase was maintained for at least 4 hours. Neither wild type nor *top3Δ* cells showed any significant sensitivity to MBC.

4.2.5 Expression of the *TOP3* gene is cell cycle regulated

Thus far, the data implicate topoisomerase III in an S-phase-specific role in response to DNA perturbations. Analysis of the pattern of *TOP3* gene expression was therefore evaluated in order to provide further evidence for a cell cycle phase-specific role. For this, wild-type cells were arrested in G1 with α-factor and then released into fresh medium. RNA samples were prepared at timed intervals for Northern blot analysis, and cell cycle position was assessed in parallel by analysis of both budding index and DNA content using flow cytometry. In the representative experiment shown in Figure 4.19, synchrony was maintained until the middle of the second cycle. The level of *TOP3* mRNA peaked 20 minutes after release from α-factor arrest at a level 11-fold higher than that in the arrested cells. This time point coincided with onset in the decline of G1 cells, but was prior to the appearance of budded cells. In the second cycle, the peak in *TOP3* mRNA levels was coincident with the rise in the proportion of G1 cells. The estimated size of the *TOP3* transcript was ~2.5 kb. This pattern of transcription was confirmed in a second strain background, and the 2.5kb transcript was undetectable in RNA derived from *top3Δ* cells (data not shown). Thus, it appears that *TOP3* transcripts are low in early G1, appear abruptly around START and decline during late S/G2.

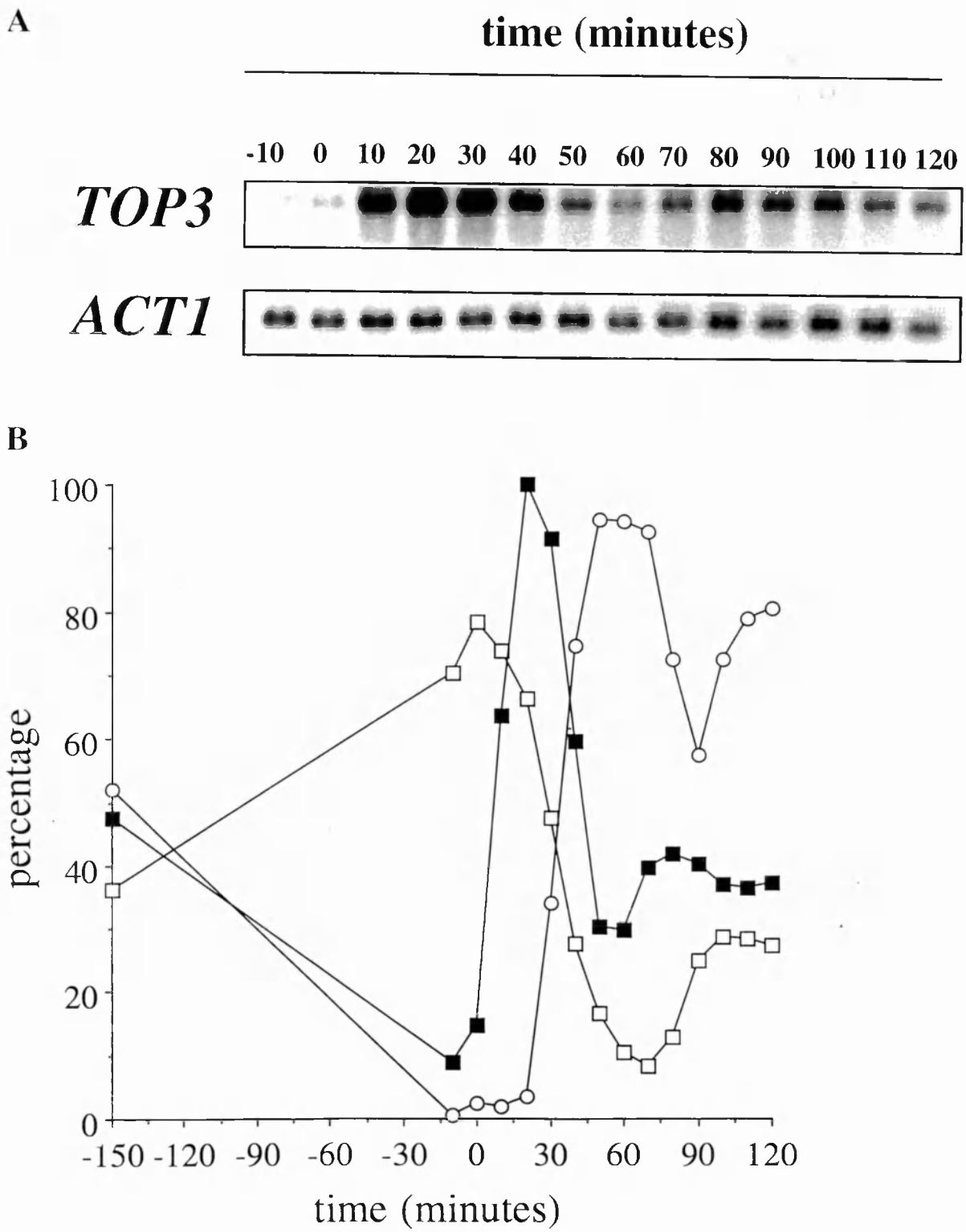


Figure 4.19: Cell cycle regulation of *TOP3* mRNA. Wild type cells (RKC1d) were grown to early log phase, arrested in G1 as described in Figure 6 and then released into fresh warmed medium. Cells were removed at timed intervals for RNA extraction, measurement of budding index and flow cytometric analysis of DNA content. (A) Levels of the *TOP3* and *ACT1* transcripts at timed intervals prior to and following release from G1 arrest. (B) The percentage of budded cells (○) and cells gated with a G1 content of DNA (□) plotted relative to *TOP3* transcript levels (■), quantified from the data in panel (A). The maximal transcript level is given an arbitrary value of 100%. Note that cells lose synchrony at about the mid-point of the 2nd cycle.

4.3 Discussion

4.3.1 *top3Δ* strains are defective in cell cycle progression in the absence of DNA damaging agents or HU

Previous work has shown that the slower doubling time of *top3Δ* strains is a result of an accumulation of cells in the late S/G2 phase of the cell cycle. I have shown here that these cells have completed bulk DNA replication and are arrested at the G2/M DNA damage checkpoint. The most economical explanation for these findings is that some form of abnormal DNA structure and/or DNA lesion is generated during DNA replication and that while this is not sufficient to prevent completion of bulk DNA synthesis, it nevertheless is recognised by the G2/M DNA damage checkpoint machinery as abnormal.

A major challenge is now to identify the abnormal DNA structures that might occur during progression through S-phase in these mutants. It is known that aberrant replicative structures resembling recombination intermediates or late Cairns-type structures can be observed on 2-dimensional gels following drug-mediated inactivation of topoisomerase I or II in budding yeast (Levac and Moss, 1996). However, DNA samples derived from asynchronous cultures of wild-type and *top3Δ* strains exhibit no consistent differences on 2-dimensional gels, even if replication is perturbed by either DNA damage (with 0.01% MMS) or 50mM HU (Dr. J. Kearsey, Institute of Molecular Medicine, personal communication). I conclude that any putative abnormal replication intermediates that might arise in *top3Δ* strains either fall outside the group of structures detectable by this method, or are accumulated at levels below the detection limit.

Preliminary evidence suggests that Sgs1p is implicated in generating these putative abnormal structures. Thus, in an *sgs1Δtop3Δ* mutant, ectopic expression of Sgs1p appears to be particularly deleterious in cells progressing though S-phase. Relative protection was afforded to cells overexpressing Sgs1p, but which were held either in G1 or in M-phase. I have not excluded the possibility that cells lose viability at other points in the cell cycle i.e. in G2 at a point prior to the M-phase block induced by MBC. Despite the obvious qualifications regarding the difficulty in interpreting the deleterious effects of overexpressing proteins, it will be of some interest to assess the effect of Sgs1^{op} upon replicative intermediates detected in 2D gels, since this might give some insight into the nature of abnormal DNA structures present in *top3Δ* mutants.

It has been proposed that topoisomerase III in conjunction with Sgs1p has a role in the terminal stages of DNA replication (Gangloff et al., 1994; Watt et al., 1995). As 2 replication forks converge, a build up of positive supercoils between the two advancing replisomes could inhibit the final stages of DNA replication. It has been suggested that Sgs1p might unwind the unreplicated region between the advancing forks, which would have the effect of introducing intertwinings between the daughter DNA molecules. This would then require the action of topoisomerase II or III to decatenate the daughter molecules prior to anaphase. Three findings suggest that this model does not fully explain the role of topoisomerase III. First, the cell cycle regulation of the *TOP3* transcript suggests that topoisomerase III protein is required at a much earlier stage in the cell cycle. Second, the sensitivity of *top3Δ* mutants to the effect of HU which blocks cells at early S-phase, suggest that its role in replication is likely to be earlier than that predicted by the model. Third, in data not shown, overexpression of topoisomerase II, which might be expected to suppress any defect in decatenation of daughter molecules at the terminal stages of replication, had no effect upon the growth rate or viability of *top3Δ* mutants.

4.3.2 *top3Δ* strains are hypersensitive to HU

Hydroxyurea is an S-phase specific toxin that acts through inhibition of ribonucleotide reductase (reviewed in Elledge et al., 1993). Wild type yeast cells treated with HU arrest in S-phase for approximately 2 hours, but then adapt to the presence of HU by inducing expression of ribonucleotide reductase subunits, permitting continued DNA synthesis. The HU-sensitive mutants of budding and fission yeast that have been identified to date fall into 2 distinct classes depending upon the kinetics of cell death induced by this agent. One class of mutants, which includes those defective in *POL2, RFC5, MEC1* and *RAD53* in *S. cerevisiae*, fail to arrest adequately in the presence of HU and progress into an aberrant mitosis with unreplicated DNA (Allen et al., 1994; Weinert et al., 1994; Navas et al., 1995; Sugimoto et al., 1997). By definition, this class are defective in the S/M checkpoint that monitors whether DNA replication is complete before committing the cells to a mitosis, and these mutants show rapid death kinetics when treated with HU. To date, mutations of this class in *S. cerevisiae* have all been shown to reside in essential genes. Recent evidence suggests that the loss of viability in *rad53* or *mec1* mutants following transient inhibition of DNA synthesis is not the result

of inappropriate anaphase, but rather a failure to complete chromosomal replication (Desany et al., 1998). The second class of HU-sensitive mutants, exemplified by *rqh1* mutants of fission yeast, arrest normally in the presence of HU, but lose viability during the subsequent 'recovery' period when cell cycle progression is re-initiated after a period of adaptation (Stewart et al., 1997; Davey et al., 1998).

One interpretation of the data presented in this study is that *top3* strains are representative of the first class of HU-sensitive mutants in that they lose viability rapidly upon exposure to HU and do not show the normal S/M checkpoint arrest in S-phase. This interpretation is subject to the following two qualifications. First, the viability of *top3Δ* mutants is low and thus, it is difficult to interpret whether the morphological abnormalities observed are occurring in cells already committed to die prior to exposure to HU or cells which only lose viability in the presence of HU. Second, *top3Δ* cells, in the absence of HU, demonstrate a delay in G2/M transition and thus, the kinetics of spindle elongation following release from a G1 arrest are different from wild type cells. In this context, it was not possible to demonstrate a progressive and inappropriate increase in the percentage of cells with anaphase-like spindles in *top3Δ* cells exposed to HU (data not shown). In conclusion, *top3Δ* cells exposed to HU, show rapid loss of viability associated with nuclear fragmentation and abnormal spindle morphology. It is not possible however, to state definitively whether these effects relate to loss of the S/M checkpoint or as a consequence of a defect in some other process, such as DNA repair.

top3Δ mutants are genuinely hypersensitive to HU in that they cannot be propagated on solid medium containing as little as 2.5 mM HU. To my knowledge, based both upon published reports and upon my own studies of various S/M checkpoint-deficient mutants, *top3Δ* strains are substantially more sensitive to HU than any of the previously described checkpoint-deficient mutants of budding yeast. Since many of the previously described HU-sensitive mutants, including *rad53* and *mec1*, are apparently completely defective in the S/M checkpoint, it is difficult to reconcile the extreme sensitivity of *top3Δ* mutants simply with loss of this checkpoint. I would suggest that *top3Δ* mutants are defective in a process such as the processing of DNA lesions in preparation for their repair, that acts to maintain viability during or following exposure to HU. Consistent with this, loss of recombinational repair functions is also associated with extreme sensitivity to HU (Allen et al., 1994). Transient inhibition of DNA synthesis in *rad53* and *mec1* mutants leads to

a subsequent failure to complete chromosomal replication as assessed by pulse field gel electrophoresis (Desany et al., 1998). It will be important to test this in *top3Δ* mutants.

4.3.3 *TOP3* is a putative new member of the SCB box-containing family of genes

The proposal that topoisomerase III has a role in S-phase is supported by the data showing that *TOP3* transcript levels are cell cycle regulated, arising in G1 and declining in late S/G2. A number of genes are transcribed exclusively in the late G1 phase or at the G1/S boundary, including the G1 cyclins and certain genes required for DNA synthesis (reviewed in Schwob and Nasmyth, 1996). These late G1-activated genes can be classified into 2 groups on the basis of *cis*-acting sequences found within their promoter sequences. The first group of genes includes the DNA metabolism genes (e.g. *RFA1-3*, *POL1-3*, *DBF4*) and the *CLB5* and *CLB6* cyclin genes, and the promoter region of these genes contains an element similar to the Mlu1 cell cycle box (MCB element). The second group of genes, including *CLN1*, *CLN2* and *HCS26,* contain a promoter motif termed the SCB element, which acts as a late G1-specific upstream activating sequence and binds the Swi4/Swi6 complex (Ogas et al, 1991). Following S-phase, transcription of both of these groups of genes is down-regulated. In this context, I have identified a potential SCB element in the 5' flanking region of *TOP3* (CGCGAAA, at positions -130 to -124 from the ATG start codon), suggesting that *TOP3* is a new member of the group of genes regulated by the Swi4/Swi6 complex. While this finding would be consistent with the G1 activation of *TOP3* gene expression, it should be noted that the minimal promoter region and positions of any potential transcription start sites in the *TOP3* gene have yet to be characterised.

Chapter 5

The role of *S.cerevisiae* topoisomerase III in mediating checkpoint responses to DNA damage and replication blockade.

5.1 Introduction

Cells respond to DNA damage or to an inhibition of DNA replication by delaying cell cycle progression in order to resolve the resulting abnormal DNA structures. These cell cycle 'checkpoint' pathways are highly conserved in different eukaryotic species and act to preserve both genome integrity and cell viability (reviewed by Hartwell and Weinert, 1989; Carr, 1995; Paulovich et al., 1997). A major function of checkpoint pathways is to ensure that cells do not enter mitosis with non-replicated DNA, or enter S-phase or mitosis with a damaged genome. The first indication these pathways were under genetic control came from the observation that certain mutants of the budding yeast *S. cerevisiae* could be identified that failed to delay mitosis even when chromosomal damage remained unrepaired (Weinert and Hartwell, 1988). It is now clear from the results of many studies that checkpoint pathways are dependent upon the actions of numerous gene products (reviewed by Paulovich et al., 1997).

The genetic and biochemical composition of a particular checkpoint pathway is determined, at least in part, by the point in the cell cycle at which the perturbation of DNA structure/function occurs (Figure 5.1). Entry into S-phase is inhibited if cells incur DNA damage in G1, such as through UV irradiation, and this G1/S DNA damage checkpoint is dependent upon the *RAD9, RAD17, RAD24, MEC1, MEC3, DDC1* and *RAD53* (also called *MEC2/SAD1/SPK1*) genes in *S. cerevisiae* (Siede et al., 1993; Allen et al., 1994; Siede et al., 1994; Longhese et al., 1996; Siede et al., 1996a and 1996b; Longhese et al., 1997). These genes, in conjunction with *PDS1,* are also required for the G2/M checkpoint, which acts to inhibit entry into anaphase when DNA damage has not been repaired (Weinert et al., 1994; Yamamoto et al., 1996). Two additional checkpoints have been identified in budding yeast that function either to slow the rate of progression through S-phase in the presence of DNA damage, the intra-S checkpoint (Paulovich and Hartwell, 1995), or to inhibit entry into anaphase when DNA replication is blocked in response to inhibition of nucleotide biosynthesis by hydroxyurea (HU), the S/M checkpoint (Weinert et al., 1994). A common mechanism underlying both of these S-phase checkpoints is suggested by recent studies, indicating that inhibition of DNA synthesis or DNA damage, leads to a *MEC1/RAD53* -dependent inhibition of late replication origin firing (Santocanale and Diffley, 1998; Shirahige et al., 1998). The

majority of the G1/S and G2/M checkpoint genes play a minor or no role, at least in budding yeast, in the intra-S and S/M checkpoints (Paulovich et al., 1997a and 1997b), with the notable exception of *MEC1* and *RAD53*, which are both essential genes and are required for checkpoint pathways that operate in all phases of the cell cycle (Allen et al., 1994; Weinert et al., 1994; Siede et al., 1996b). In addition to the 'dedicated' checkpoint genes that play a role in S-phase, there are certain gene products that function in S-phase as part of the DNA replication machinery, whilst additionally performing some, as yet unidentified, role in checkpoint surveillance of the genome. These include Polε (DNA polymerase ε) and Dbp11p (Polε-interacting factor), which act in the S/M checkpoint (Navas et al., 1995; Araki et al., 1995), and Pri1p (the catalytic subunit of DNA primase), which acts in the intra-S checkpoint (Marini et al., 1997).

In addition to subdividing checkpoints on the basis of the phase of the cell cycle in which they act, it is possible to categorise individual checkpoint proteins on the basis of whether they function in a DNA damage/abnormal structure 'sensory' role, as 'signal transducers', or as 'targets' of the signalling cascade (Lydall and Weinert, 1996). Thus, loss of a DNA damage sensor, such as Rad9p, Rad24p or Polε, causes a failure to transduce the signal required for arrest of the cell cycle (Navas et al., 1995; Kiser and Weinert, 1996). Part of the signal transduction cascade that is absent in this class of mutants is the *MEC1*-dependent phosphorylation of Rad53p (Navas et al., 1996; Sun et al., 1996; de la Torre-Ruiz et al., 1998). As a result of this defect in signalling, other features of the checkpoint response are absent, including transcriptional upregulation of DNA damage response genes such as *RNR1*, *RAD51* and *RAD16* (de la Torre-Ruiz et al., 1998). Loss of factors acting downstream of the *MEC1*-dependent signalling cascade would not be expected to show deficiency in Rad53p phosphorylation, but instead might be blocked at a subsequent step in the checkpoint pathway, such as prior to induction of cell cycle arrest. Although considerable progress has been made in identifying checkpoint sensors and signal transducers, our knowledge of the targets of these factors, particularly those acting in S-phase, is still very limited.

In the previous chapter, I demonstrated that *top3Δ* mutants were HU-sensitive, although there is no definitive evidence for an associated loss of the S/M checkpoint. In the following sections, an attempt is made to assess the integrity of other cell cycle checkpoints in *top3Δ* mutants and to assess the relationship of topoisomerase III with components of the checkpoint apparatus.

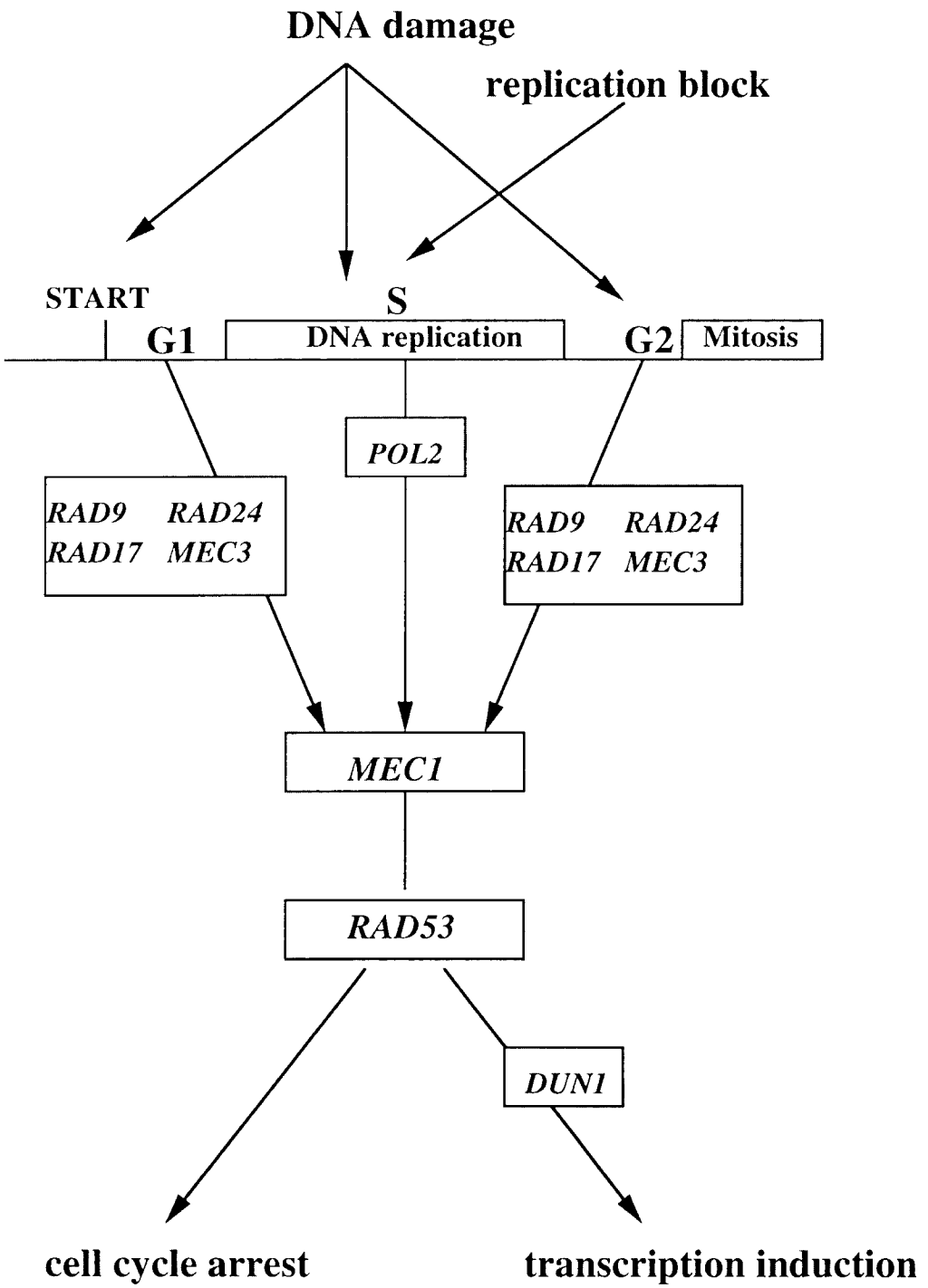


Figure 5.1: Model of cell cycle checkpoint responses in *S.cerevisiae* See text for details (Navas et al., 1996)

5.2 Results

5.2.1 *top3Δ* cells are sensitive to DNA damaging agents

A number of checkpoint mutants are defective in the arrest of the cell cycle in response not only to inhibition of DNA replication, but also to DNA damage, probably reflecting some overlap in the cellular checkpoint response to a variety of different abnormal DNA structures (Lydall and Weinert, 1996). The sensitivity or otherwise of *top3Δ* mutants to DNA damaging agents was therefore tested. *top3Δ* cells were found to be sensitive to the DNA damaging agents MMS (Figure 5.2) and UV-light (Figure 5.3) and to a lesser extent (approximately 2.5-fold) to ionising radiation (Figure 5.4). These results were confirmed in 3 independent strain backgrounds. As was the case for HU-treatment, the sensitivity of *top3Δ* strains to DNA damaging agents was substantially suppressed by deletion of *SGS1* or by ectopic expression of a wild type *TOP3* gene (Figure 5.5).

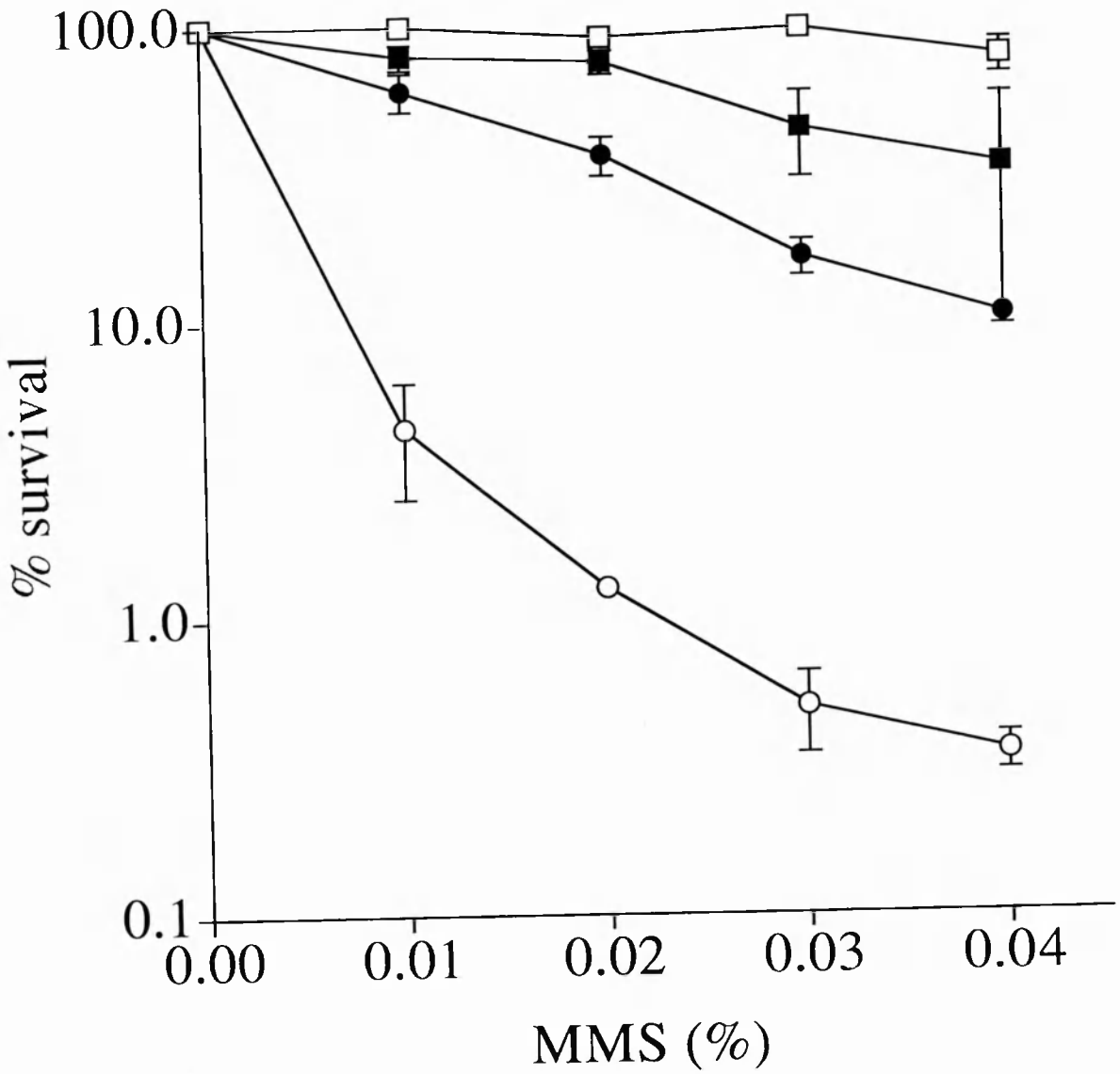


Figure 5.2: Sensitivity of *top3Δ* cells to MMS. Strains RKC1d (wild type, □) JMK22d (*sgs1*, ■), RKC1c (*sgs1top3*, ●) and RKC1a (*top3*, ○) were grown to early log phase in YPD and then at t=0 were exposed to MMS at the concentrations indicated for 60 minutes and % survival determined in relation to t=0. The means (±SEM) of 3 independent experiments are shown.

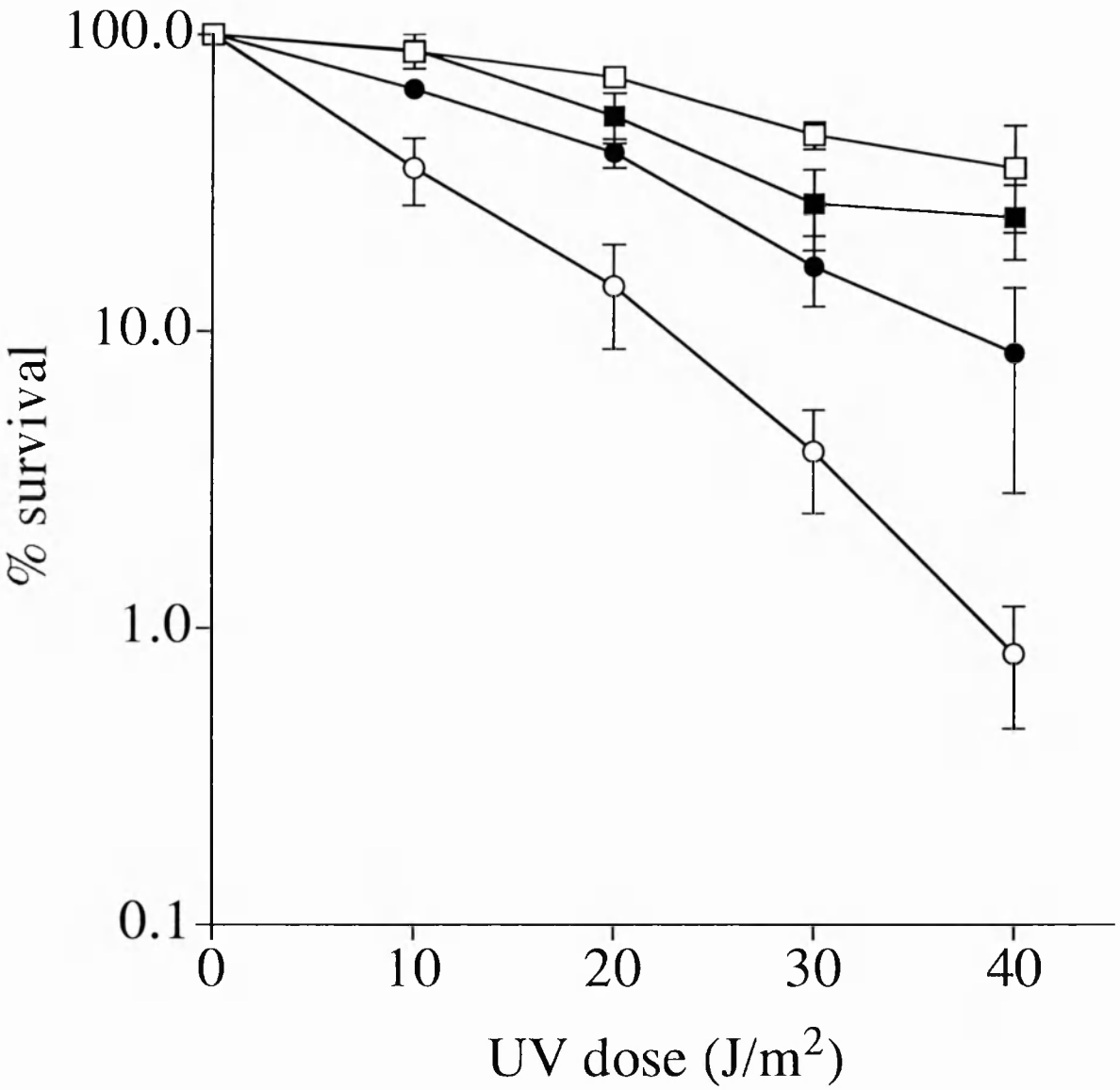


Figure 5.3: Sensitivity of *top3Δ* cells to UV. Strains RKC1d (wild type, □) JMK22d (*sgs1*, ■), RKC1c (*sgs1top3*, ●) and RKC1a (*top3*, ○) were grown to early log phase in YPD and exposed to UV at the doses indicated. % survival was determined in relation to a non-irradiated control. The means (±SEM) of 3 independent experiments are shown.

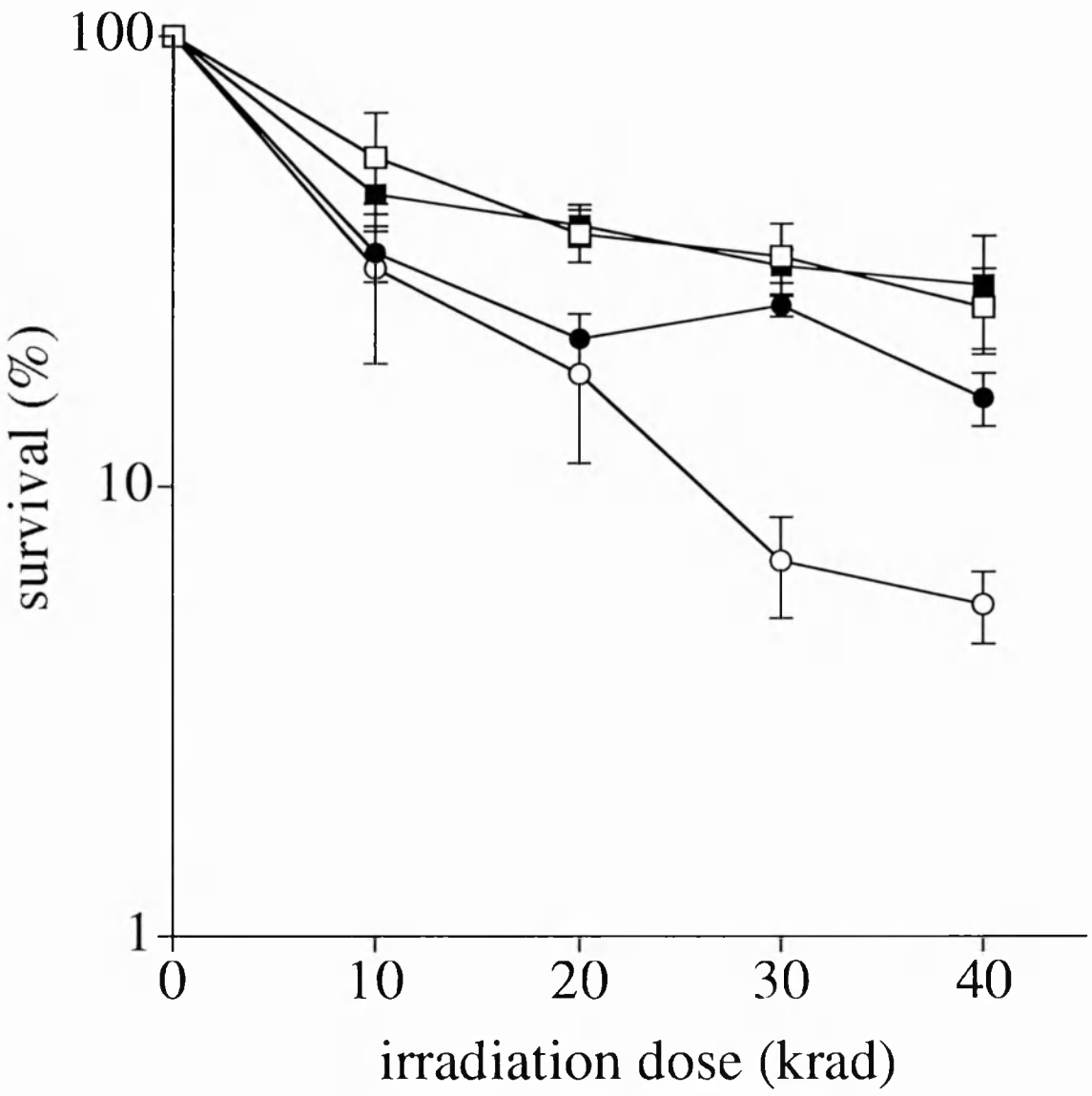


Figure 5.4: Sensitivity of *top3Δ* cells to γ–rays. Strains RKC1d (wild type, □) JMK22d (*sgs1*, ■), RKC1c (*sgs1top3*, ●) and RKC1a (*top3*, ○) were grown to early log phase in YPD and exposed to γ–rays at the doses indicated. Survival was determined in relation to a non-irradiated control. The means (±SEM) of 3 independent experiments are shown.

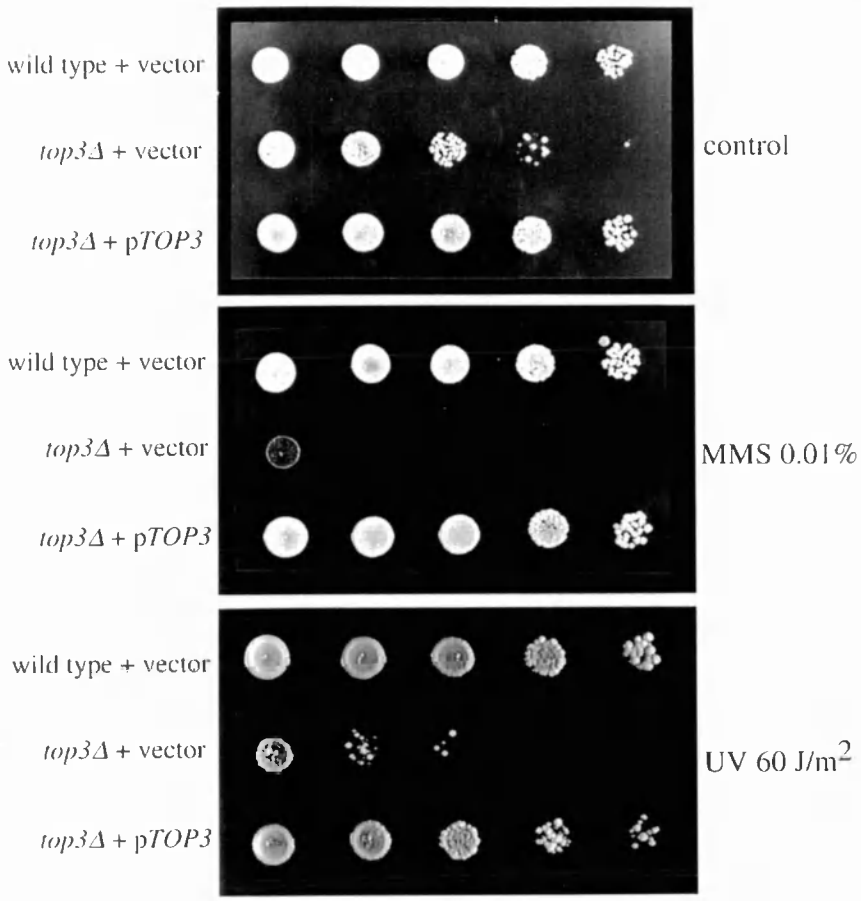


Figure 5.5: Complementation of the MMS and UV sensitivity of *top3Δ* mutants by ectopic expression of wild type *TOP3*. Log dilutions of saturated cultures of *top3Δ* cells transformed with pRK500, which encodes wild type *TOP3*, or a vector-only control were plated onto YPD (upper panel) or YPD containing 0.01% MMS (middle panel) or YPD, followed by UV irradiation at 60J/m^2 (lower panel)

5.2.2 *top3Δ* cells are defective in the intra-S checkpoint, but not in the G1/S or G2/M DNA damage checkpoints

The intra-S checkpoint acts to slow the rate of DNA synthesis when DNA is damaged during S-phase and is dependent upon the products of a number of genes (Paulovich and Hartwell, 1995). The data in Figure 5.6 shows that when *top3Δ* cells were released from an α-factor-induced G1 arrest into medium containing 0.03% MMS, most cells achieved a 2C content of DNA after approximately 60-90 minutes, whereas wild-type cells similarly treated progressed through S-phase much more slowly, such that at 240 minutes the majority of cells still had a < 2C content of DNA[1]. Consistent with a failure to invoke the intra-S checkpoint, the viability of *top3Δ* cells at the 60 minute time point was only <0.2% compared with 86.5% for wild-type cells. In the absence of MMS, *top3Δ* cells entered S-phase more rapidly than wild-type cells when the α-factor was removed (see earlier discussion in section 3.2.1) and the majority of cells have a 2C content of DNA at the 60 minute time point. In contrast, wild type cells attain a 2C content of DNA at 90 minute time point. This difference however cannot account for the dramatic disparity in the rates of S-phase progression in the presence of MMS. It should be noted that there is a partial delay in the rate of S-phase progression in *top3Δ* mutants in the presence of MMS and this is observed in other mutants defective in this checkpoint (Paulovich and Hartwell, 1995; Sugimoto et al., 1997; Paulovich et al., 1997b).

In contrast to the above results, cell cycle checkpoint responses to DNA damage occurring outside of S-phase were apparently unaffected by deletion of *TOP3*. Figure 5.7 shows that when α-factor arrested *top3Δ* cells were UV-irradiated and then released into fresh medium, they showed a marked delay in the rate of progression through the G1/S phase transition, as compared to non-irradiated controls. Indeed, the extent of the delay in *top3Δ* mutants appeared to be somewhat greater than that apparent in irradiated wild-type cells. Thus, while *top3* mutants are proficient in the G1/S checkpoint, it is possible that topoisomerase III plays some role in G1, as supported by the finding that only partial protection was afforded to MMS-treated *top3Δ* mutants by maintaining them in G1 with α-factor (Figure 5.8).

The data presented in chapter 3 indicate that *top3Δ* cells delay at a Rad9p/Rad24p-mediated G2/M checkpoint in the absence of exogenously-added DNA damaging agents. When cells arrested in G2/M with MBC were then UV-irradiated, the extent of subsequent delay in the cell cycle following removal of MBC was comparable in wild-

type and *top3Δ* cells (Figure 5.9), confirming that the G2/M DNA damage checkpoint is intact in *top3Δ* mutants.

Budding yeast cells arrest in mitosis in response to spindle depolymerisation (Clayton et a., 1979). Mutants defective in this spindle-assembly checkpoint, such as *mad1* or *bub1*, continue through mitosis without a spindle in the presence of microtubule poisons and die rapidly (Li and Murray, 1991; Hoyt et al., 1991). However, *top3Δ* cells were not sensitive to MBC, indicating that this checkpoint is also intact (Figure 5.10).

[1]Note that the possibility that the changes in DNA content of *top3Δ* mutants may reflect changes in the content of mitochondrial DNA. This could be excluded by assessing the effect of *TOP3* deletion in strains containing no mitochondrial DNA.

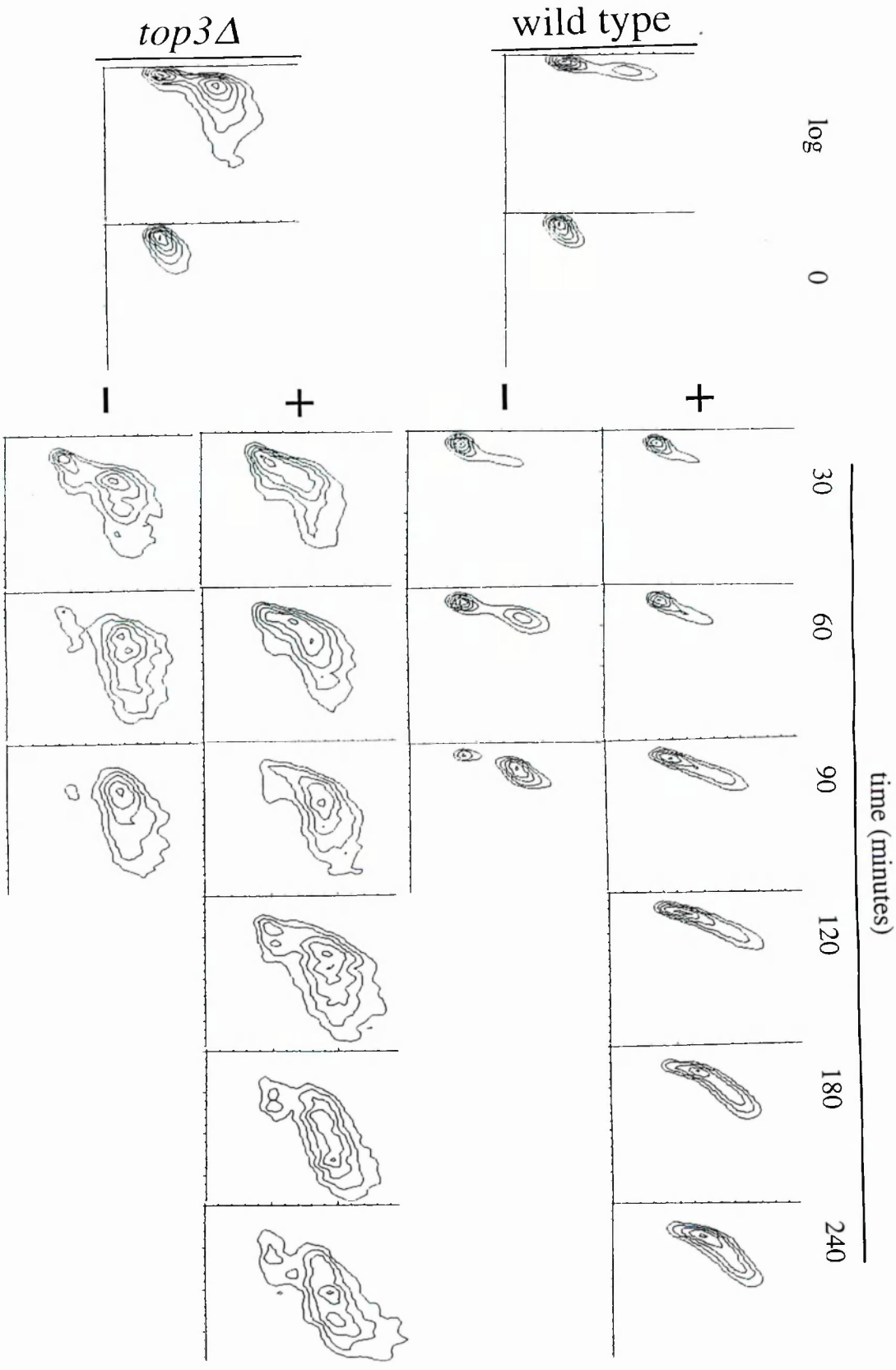


Figure 5.6: *top3Δ* cells are defective in the intra-S checkpoint. To assess the intra-S checkpoint, wild type and *top3Δ* cells were grown in YPD to early log phase and then arrested in G1 with α-factor for 150 minutes. Cells were then washed twice with fresh warmed media and released into media containing either 0.03% MMS (+) or no drug (-) as a control. DNA content was assessed by flow cytometry and contour plots with DNA content (y-axis) against cell size (x-axis) are shown. Thresholds were set at 15%. Similar results were obtained in each of 4 experiments performed.

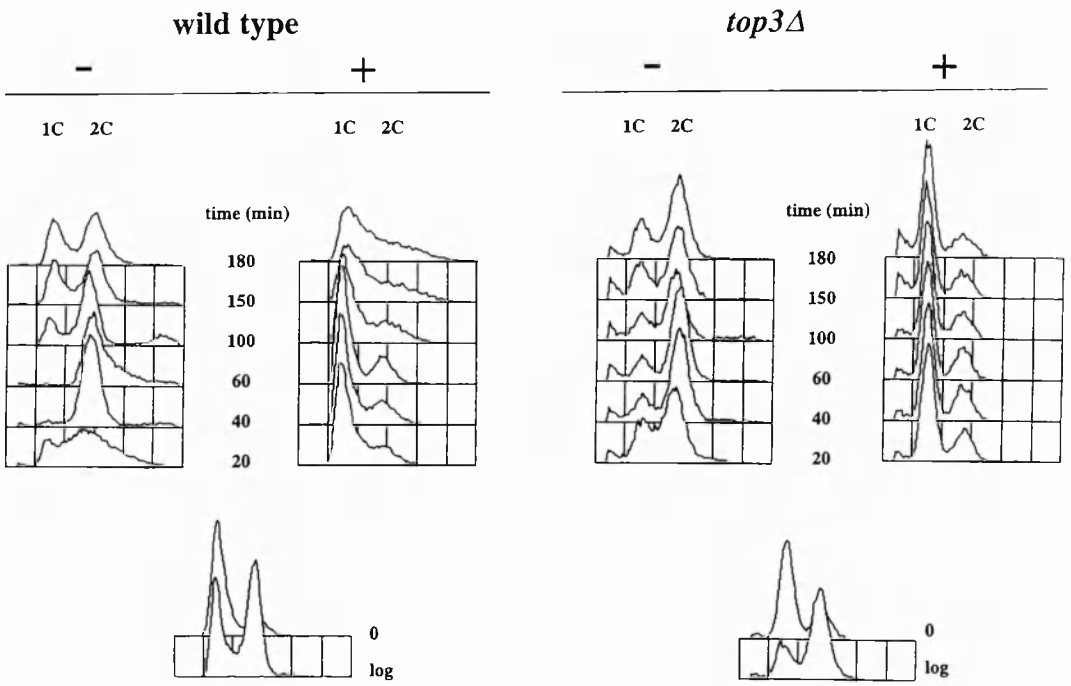


Figure 5.7: *top3Δ* cells are proficient in the G1/S checkpoint. Wild type and *top3Δ* cells were grown in YPD to early log phase and then arrested in G1 with α-factor for 150 minutes, and then irradiated with 80 J/m^2 UV light (+) or mock treated (-), before washing twice and releasing into fresh medium. Similar results were obtained from 4 independent experiments.

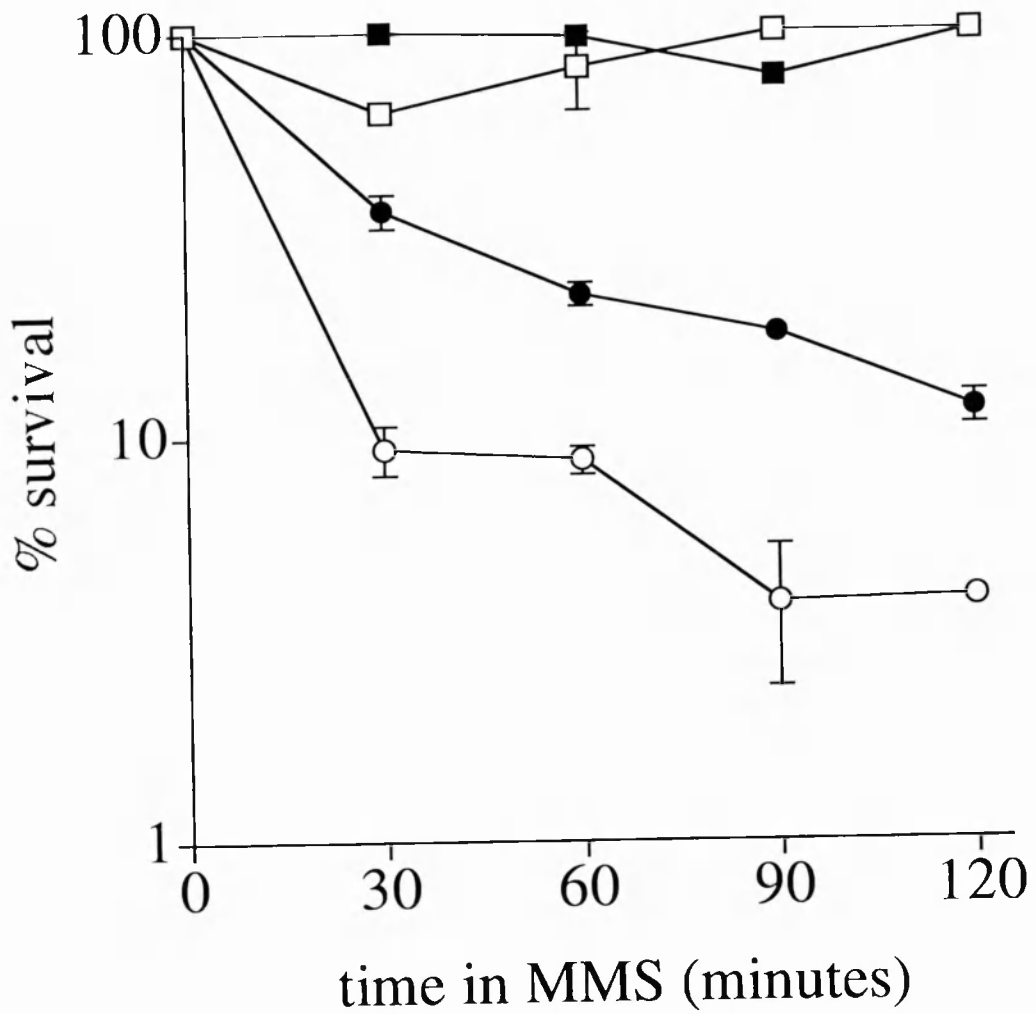


Figure 5.8: Effect of exposure to MMS in cells held in G1 with α-factor. Wild type (□, ■) and *top3Δ* (○, ●) cells were arrested in G1 as above and then released into YPD containing either 0.005% MMS alone (open symbols) or 0.005% MMS plus 20μg/ml α-factor (closed symbols) and % survival was determined at the time points indicated. The effectiveness of the α-factor block in each case was assessed microscopically and by flow cytometry. The means (±SEM) of 3 independent experiments are shown.

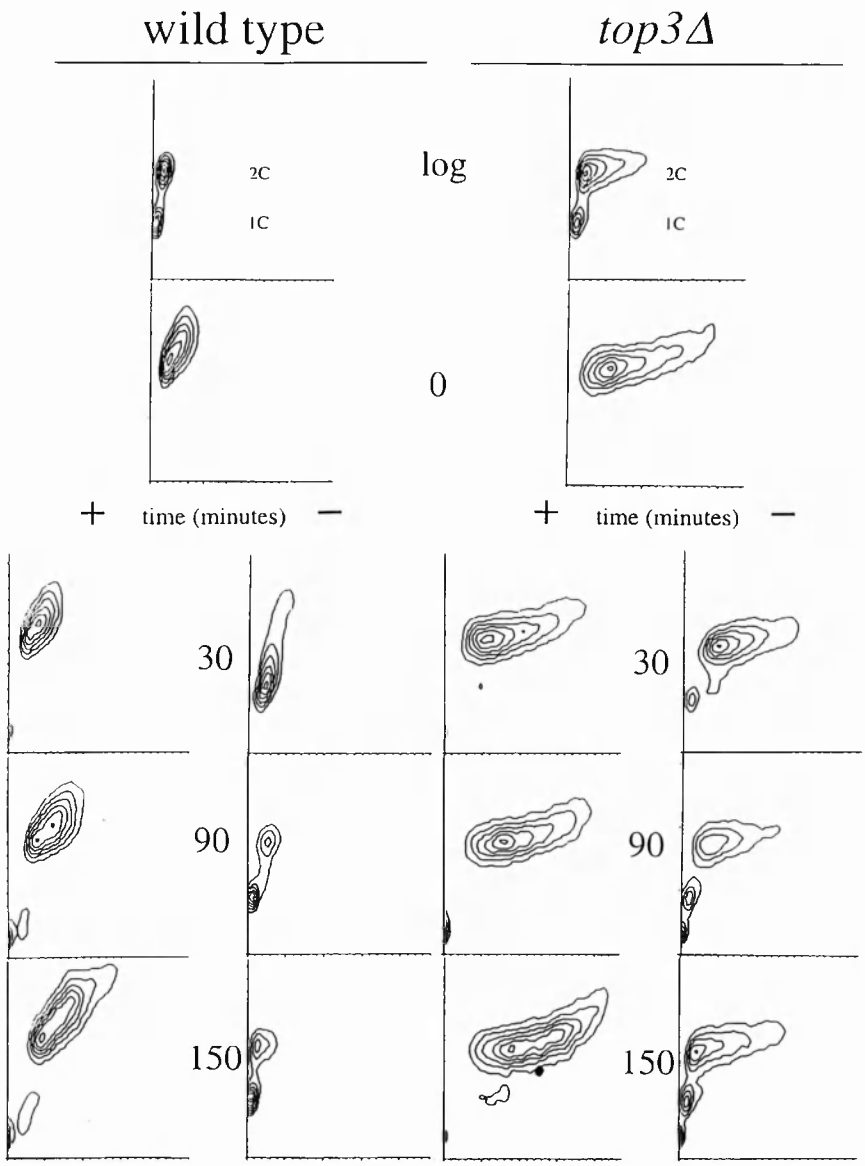


Figure 5.9: *top3Δ* cells are proficient in the G2/M DNA damage checkpoint. Wild type and *top3Δ* cells were grown in YPD to early log phase and arrested in G2/M with MBC 200µg/ml for 2 hours, and then irradiated with 80 J/m^2 UV light (+) or mock treated (-), before washing twice and releasing into fresh medium. Similar results were obtained in 2 independent experiments.

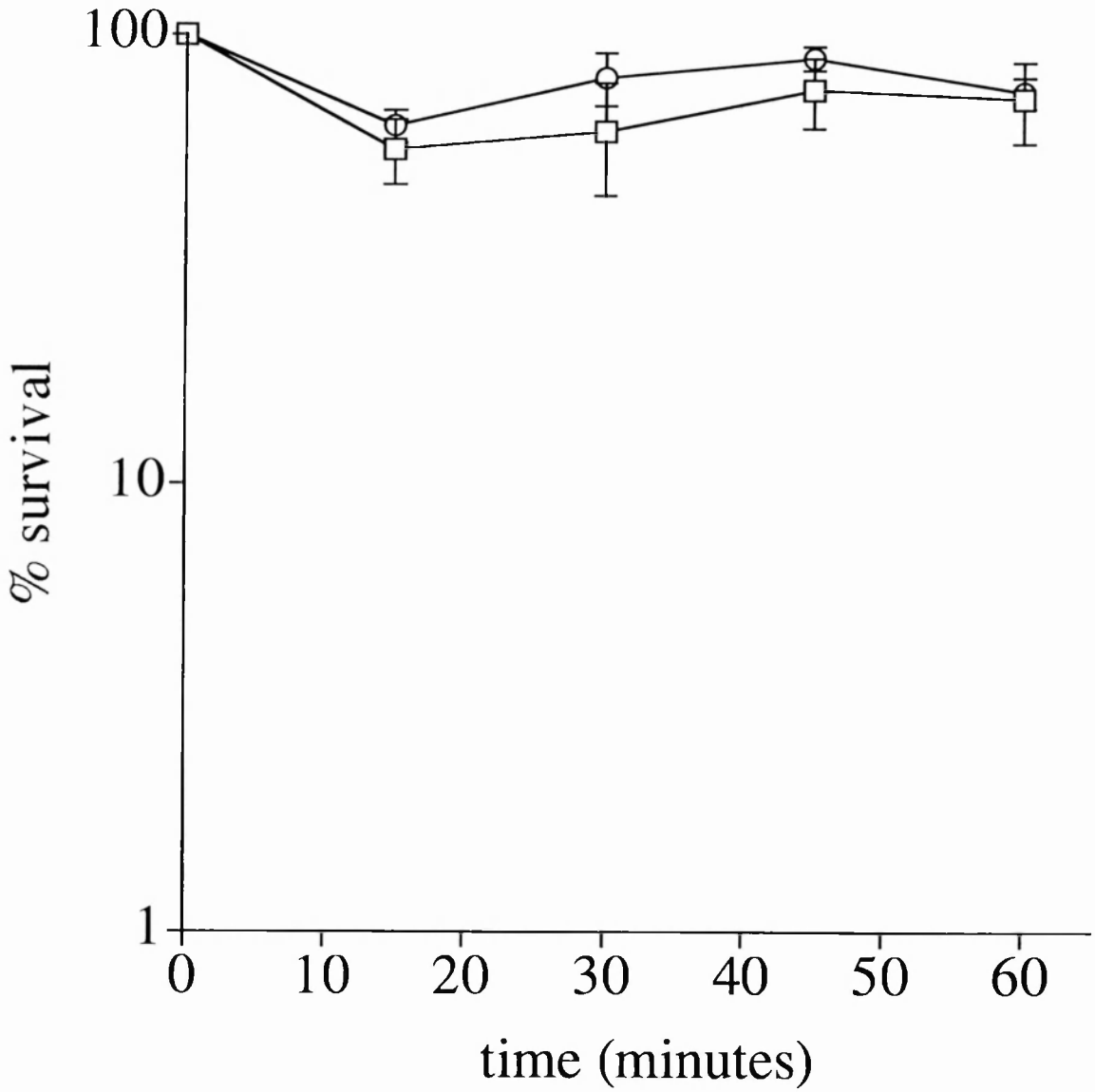


Figure 5.10: Sensitivity of wild type and *top3Δ* cells to the spindle poison, MBC. Log phase wild type (□) and *top3Δ* (○) cells were exposed at t=0 to MBC at 200μg/ml and the % survival in relation to t=0 determined at the times indicated. The means (±SEM) of 3 independent experiments are shown. Note that a positive control was not run in this experiment.

5.2.3 Rad53p phosphorylation and transcriptional upregulation of DNA damage response genes occurs in *top3Δ* cells

Checkpoint proteins can be categorised on the basis of whether they function as 'sensors' of abnormal DNA structure, 'signal transducers', or 'targets' of the signalling apparatus (Lydall and Weinert, 1996). Loss of sensory function might be expected to result in an absence of all downstream responses following DNA damage or replication inhibition, including a failure both to phosphorylate Rad53p and to show transcriptional upregulation of DNA damage response genes. In contrast, loss of a target function would be predicted to result in the variable loss of transcriptional induction or cell cycle arrest (depending upon the point at which the protein acts), but would show intact phosphorylation of Rad53p.

In order to position topoisomerase III function in relation to this broad outline of the checkpoint pathway, an assessment was made of Rad53 phosphorylation and transcriptional induction of DNA damage response genes in wild-type and *top3Δ* cells following release from a G1 arrest into medium containing HU or MMS. Figure 5.11 shows that phosphorylation of Rad53p (as indicated by the appearance of a slower migrating species on SDS-PAGE) occurred in *top3Δ* cells following DNA damage or inhibition of DNA replication. Furthermore, even in the absence of drug treatment, extracts from *top3Δ* cells showed a small fraction of Rad53p that migrated as a lower mobility form (indicated by the arrow marked with an asterisk in Figure 5.11). This aberrant phosphorylation in the absence of extrinsic damage suggests that abnormal structures exist in *top3Δ* cells that result in a degree of constitutive activation of the checkpoint pathway. Quantification of the immunoreactive Rad53 protein suggested that there was a clear attenuation in the extent of Rad53 phosphorylation following HU or MMS treatment in *top3Δ* cells as compared to wild-type cells. Thus, for *top3Δ* cells the maximal % of total Rad53p that existed in the phosphorylated form was 59.4% after HU treatment (91.4% for wild type cells), and 54.7% after MMS treatment (87.9% for wild type). Furthermore, the proportion of Rad53p that was fully phosphorylated following HU or MMS treatment in *top3Δ* cells was substantially less than in wild type cells.

Since transcriptional responses to DNA damage/replication block are at least partially dependent upon an intact Rad53-dependent pathway (Allen et al., 1994), we examined whether transcriptional upregulation of genes, including *RNR1, RAD51*, and *RAD16* occurred in *top3Δ* mutants. In the presence of either HU or MMS, the *RNR1*

transcriptional responses was broadly similar (Figures 5.12 and 5.13). In both wild type and *top3Δ* cells the *RAD51* transcript is upregulated following HU/MMS although this is less easy to interpret since the baseline level of transcription in G1 was 2.5 fold higher in *top3Δ* cells. *RAD16* transcript levels were evaluated since this transcript is not cell cycle regulated. In wild type cells MMS exposure results in a 2.5 fold induction in *RAD16* transcript levels although HU exposure has no effect. In untreated *top3Δ* cells the *RAD16* transcript levels increase 2-fold following release from the G1 arrest and there appears to be no significant induction over and above this following DNA damage or replication block. This data, together with the finding of aberrant Rad53 phosphorylation in untreated cells, would be consistent with the hypothesis that *top3Δ* cells accumulate DNA lesions, resulting in *RAD16* expression through the Rad53p-dependent pathway, which is proficient in these cells. This hypothesis is not supported, however, by the finding of attenuated levels of Rad53p phosphorylation following DNA damage or HU-treatment.

A number of checkpoint mutants defective in sensory (*pol2, rfc5*) or signal transduction (*mec1*) functions show partial rescue of their respective phenotypes by over-expression of either Rad53p or Tel1p, since these proteins probably function downstream of Mec1p (Navas et al., 1996; Sanchez et al., 1996; Sugimoto et al., 1997). However, overexpression of neither *RAD53* (Figure 5.14) nor *TEL1* (not shown) had any significant effect on the HU or DNA damage sensitivity of *top3Δ* cells.

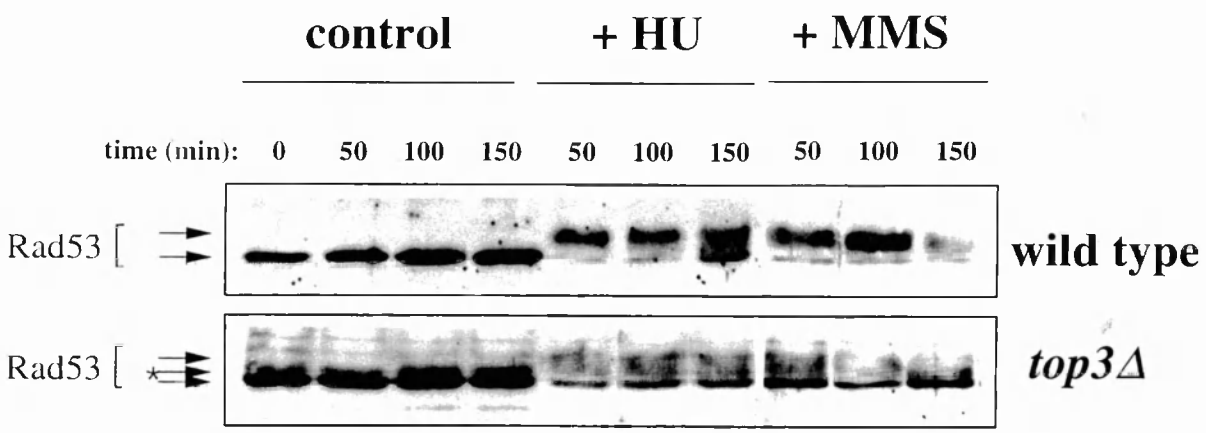


Figure 5.11: **Rad53 phosphorylation in *top3Δ* mutants.** Wild type and *top3Δ* cells were grown in YPD to early log phase and then arrested in G1 with α-factor for 150 minutes. Cells were then released into YPD (control), or into YPD containing either 0.2M HU or 0.03% MMS. Protein extracts derived from cells removed at timed intervals were then separated by SDS-PAGE, transferred to nylon and immunoblotted with an α-Rad53 antibody. A representative Western blot for extracts from wild-type (upper panel) and *top3Δ* (lower panel) cells is shown. Arrows on the left label the positions of non-phosphorylated (lowermost arrow in each panel) and phosphorylated (uppermost arrow) forms of Rad53. The arrow marked with an asterisk defines the position of the additional, lower mobility form of Rad53 seen in *top3Δ* cells in the absence of drug treatments.

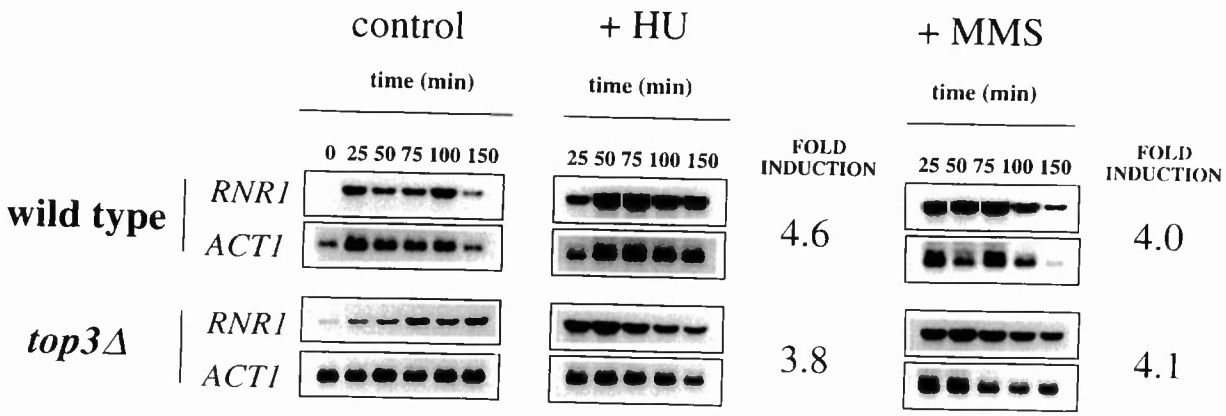


Figure 5.12: **Transcriptional induction following DNA damage or inhibition of replication in *top3Δ* cells.** Wild type and *top3Δ* cells were grown in YPD to early log phase and then arrested in G1 as described in Figure 5.11. Cells were then released into YPD (control), or into YPD containing either 0.2M HU or 0.03% MMS. Cells were removed at timed intervals for RNA extraction and representative Northern blots hybridised with either an *RNR1* or an *ACT1* control probe are shown. Fold induction of the *RNR1* transcript (as indicated to the right of each data set) in the presence of either HU or MMS was determined by dividing the maximal transcript level observed in each case by the transcript level seen in the absence of drug treatment (corrected in all cases for *ACT1* mRNA levels).

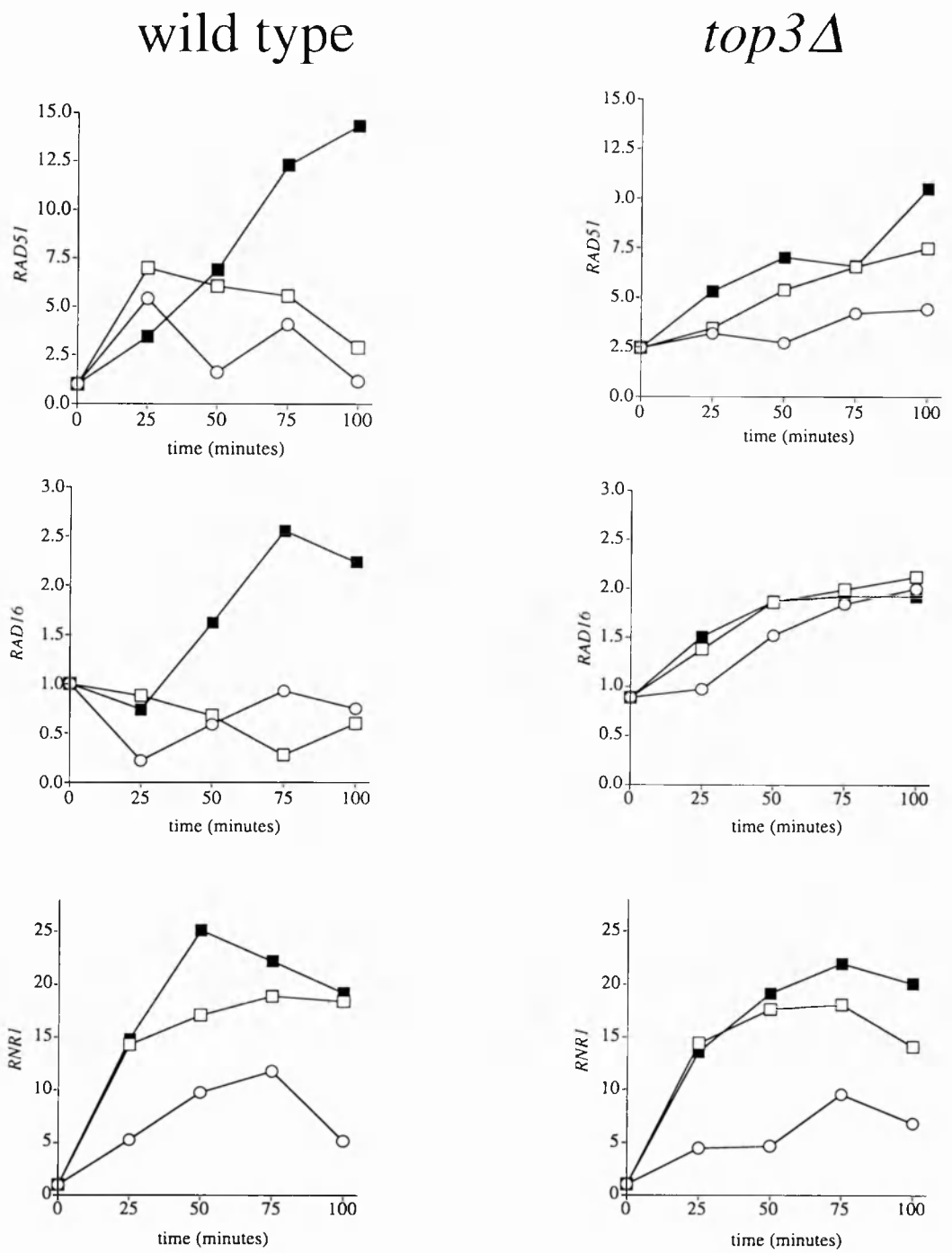


Figure 5.13: Transcriptional induction following DNA damage or inhibition of replication in *top3Δ* cells. Wild type and *top3Δ* cells were grown in YPD to early log phase and then arrested in G1 as described in Figure 5.11. Cells were then released into YPD (control), or into YPD containing either 0.2M HU or 0.03% MMS. Cells were removed at timed intervals for RNA extraction and Northern blots hybridised with *RNR1, RAD51, RAD16* or *ACT1*. Transcript levels were corrected in all cases in relation to the loading control. Graphs showing the fold increase for each of the transcripts following treatment with 0.2M HU (□), 0.03% MMS (■) or mock treatment (○) are shown. In each case the fold increase was determined in relation to the wild type G1 value. Wild type responses are shown on the **left** and *top3Δ* responses on the **right**.

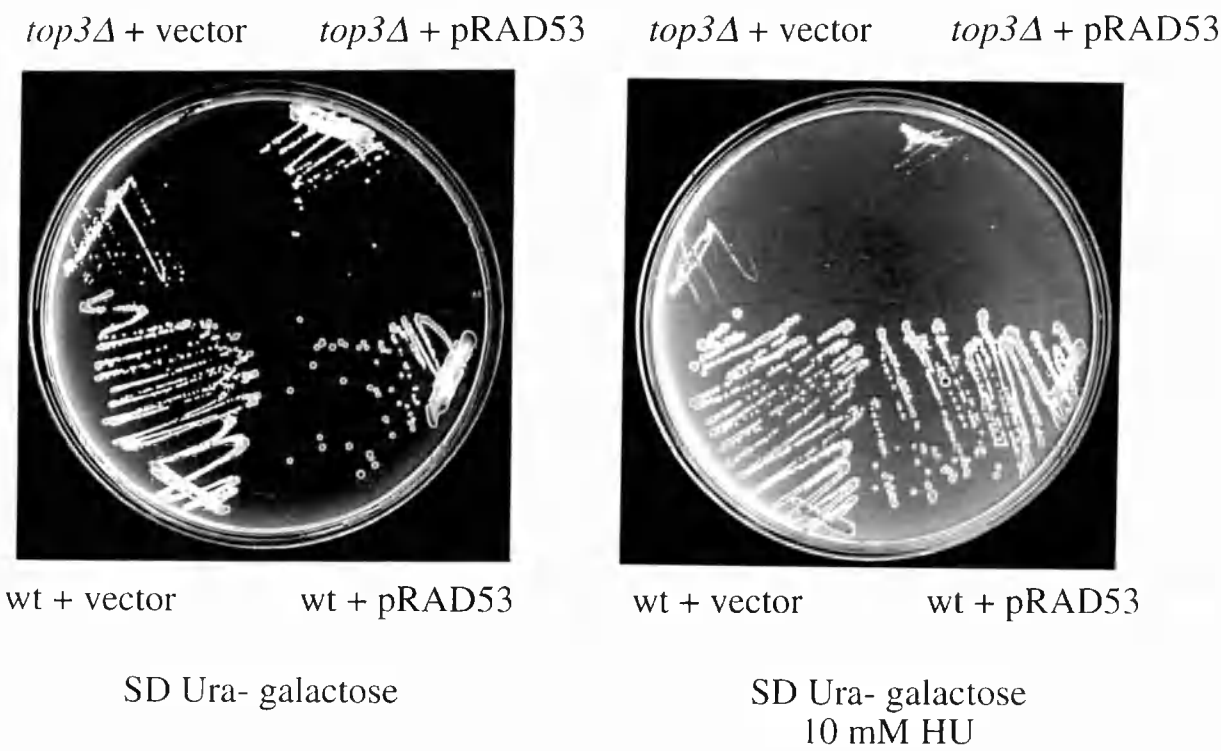


Figure 5.14 : Rad53p overexpression does not reverse the HU sensitivity of *top3Δ* cells Wild type and *top3Δ* cells were transformed with single copy pRAD53 (in which *RAD53* is cloned downstream of the *GAL1* promoter) or vector control, and the resulting transformants spread onto selective plates containing either 2% glucose or 2% galactose/1% raffinose, with and without 10mM HU. Plates showing colony growth in the presence or absence of HU under inducing conditions are shown. Similar negative results were obtained when cells were exposed to MMS or UV. Similar negative results were obtained following overexpression of *RAD53*, also under the contol of the *GAL1* promoter, from a multicopy plasmid. Overexpression of Rad53p was confirmed by immunoblot.

5.2.4 Effect of DNA damage or inhibition of replication upon *TOP3* mRNA expression

As described above, both prokaryotic and eukaryotic cells upregulate the transcription of DNA repair genes following DNA damage or inhibition of DNA replication (Friedberg et al., 1995; Kiser and Weinert, 1996). In order to assess whether *TOP3* transcript levels are similarly upregulated, wild type cells were arrested in G1 and then released into YPD containing HU, MMS or no drug control. RNA samples were prepared at timed intervals for Northern blot analysis. There was an 3-fold increase in the level of *TOP3* mRNA when cells were exposed to MMS, but no significant increases following exposure to HU and UV (Figure 5.15). Controls showed increases in *RNR1* expression following exposure to both agents.

In budding yeast a number of genes whose transcription is upregulated by DNA damage have so-called 'damage response elements' (DREs) in their 5' regions (Sancar, 1985; Siede and Friedberg, 1992; Zheng et al., 1993; Kiser and Weinert, 1996). A search for potential DREs in the 5' sequence upstream of the ORF for *TOP3* revealed potential DREs at positions (relative to the translation initiation codon) -46 to -64 (similarity to an element in the *PHR1* promoter) and -817 to -831 (similar to the *DRE1* element in the *RAD2* promoter and another closely related element in the *RAD53* promoter). An alignment of these putative DREs is shown in Figure 5.16.

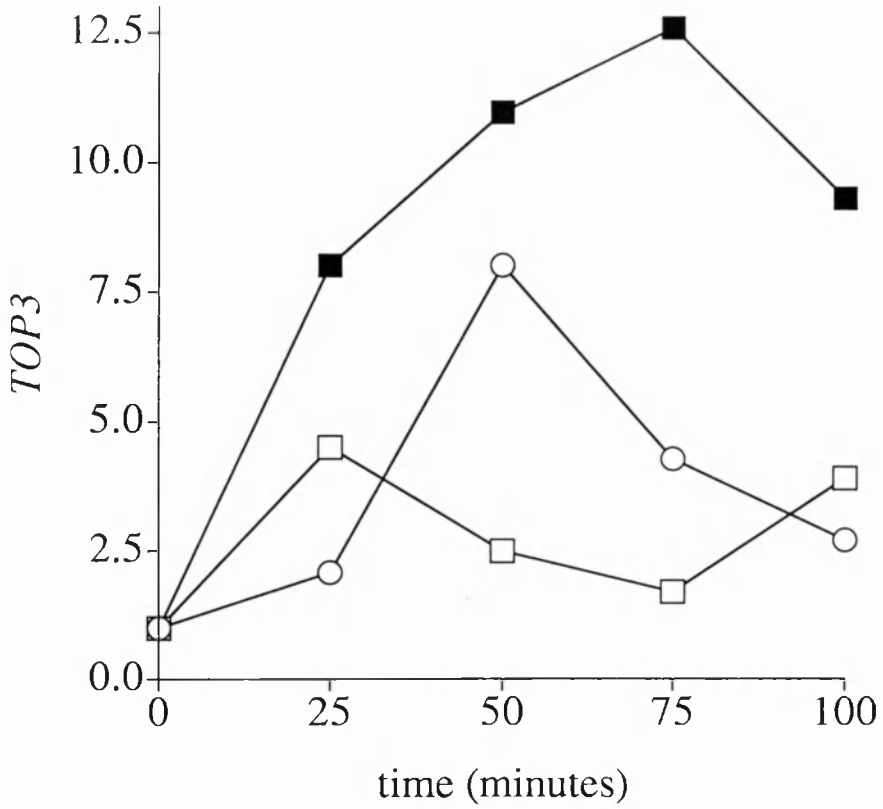


Figure 5.15: Transcriptional induction of *TOP3* following DNA damage or inhibition of replication. Wild type cells were grown in YPD to early log phase, arrested in G1 and then released into YPD containing (1) 0.2M HU, (2) 0.03% MMS or (3) no drug (control). Cells were removed at timed intervals for RNA extraction and a Northern blot hybridised with either a *TOP3* or an *ACT1* probe. Transcript levels were corrected in all cases in relation to the loading control. Graph showing the fold increase for the *TOP3* transcript following treatment with 0.2M HU (□), 0.03% MMS (■) or mock treatment (○) is shown. In each case the fold increase was determined in relation to the G1 value.

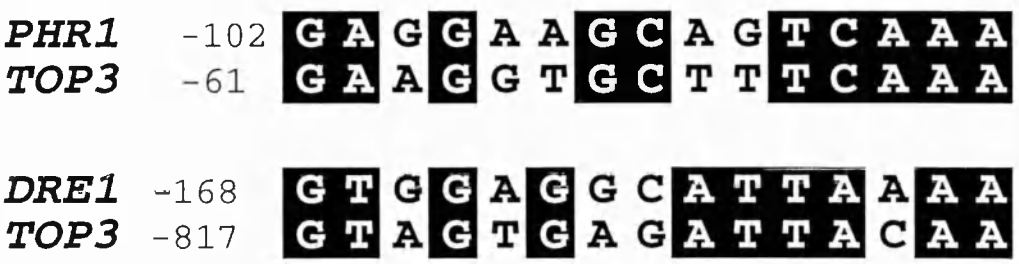


Figure 5.16: Potential damage response elements in the 5' region upstream of the *TOP3* translation initiation codon. Two possible sites with sequence similarity to previously identified damage response elements, *PHR1* and *DRE1*, are shown. Figures to the left correspond to the position of the first nucleotide of each element in relation to the translation initiation codon of the respective genes. The nucleotide sequence with similarity to *PHR1* identified in the *TOP3* 5' region is in reverse orientation to the reported element.

5.2.5 Genetic interactions between *top3* and other checkpoint-deficient mutants

The *MEC1* and *RAD53* genes encode essential proteins involved in the signal transduction pathway that is activated during S-phase by inhibition of DNA replication, and during all phases of the cell cycle by DNA damage (Allen et al., 1994: Weinert et al., 1994; Siede et al., 1996b). Combination of a *top3Δ* mutation in the A364a strain with conditional alleles of *MEC1* (*mec1-1*) and *RAD53* (*mec2-1*) resulted in synthetic lethality (either microcolonies that could not be propagated or no visible colony) after sporulation of the appropriate heterozygous diploids at the permissive temperature (Tables 5.1).

In an effort to characterise genetic interactions between *top3Δ* and other mutations that disable potential targets of the Mec1/Rad53 signal transduction pathway, the effect of combining deletion of *TOP3* with conditional mutations in *RFA2*, which encodes the 34 kDa subunit of single-stranded DNA binding protein, and *PRI1*, which encodes the large subunit of DNA primase, were examined. Rfa2p has roles in both DNA replication and DNA repair, and is one of the very few established 'targets' for the checkpoint kinase cascade operating in S-phase. In budding yeast, Rfa2p is phosphorylated in response to DNA replication block or DNA damage, and this phosphorylation is dependent upon *MEC1,* but not *RAD53* (Brush et al., 1996). Combination of *top3Δ* with either of two independent *rfa2* alleles (*rfa2-1* and *rfa2-2*) resulted in synthetic lethality (Tables 5.1 and 5.2, Figures 5.17 and 5.18), either at the permissive temperature for *top3Δ rfa2-1* double mutants (25^{o}C, n=20 tetrads dissected) or at the semi-permissive temperature for *top3Δ rfa2-2* double mutants (30^{o}C, n=20). Similarly, combination of a *top3Δ* mutation with the *pri1-M4* mutation, which itself leads to a defect in S-phase checkpoint responses to DNA damage (Marini et al., 1997), resulted in synthetic lethality at the permissive temperature for the *pri1-M4* strain (25^{o}C, n=20).

Table 5.1

Genotypes of segregants following sporulation of diploid heterozygous for *x*[1] and *top3Δ*[2]

genotype	*mec1-1* n=35	*mec2-1* n=25	*rfa2-1* n=20	*pri-M4* n=20
TOP3 X	32	19	17	26
TOP3 x	14	27	21	12
top3 X	36	13	23	10
top3 x	0	0	0	0

[1] where *x* is the recessive allele indicated at the top of each column and where *X* is the respective wild type allele·

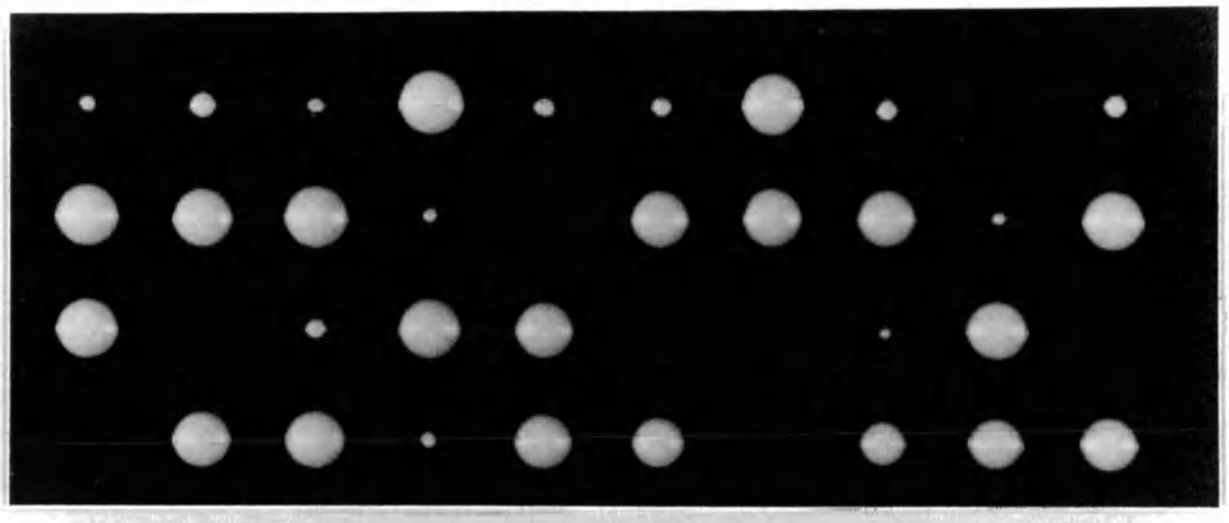
[2] all germinations were performed at 25°C·

Table 5.2

Survival of segregants of diploid heterozygous for *rfa2-2* and *top3Δ* at different temperatures[1]

genotype	**25°C**, n=20	**30°C**, n=20
TOP3 RFA2	18	18
TOP3 rfa2-2	16	16
top3 RFA2	17	17
top3 rfa2-2	19	0

[1] segregants were germinated at 25°C and then spread onto 2 plates, and then allowed to grow at either 25°C or 30°C for 3 days.



3	3	3	2	3	3	1	3	-	3
1	1	2	3	-	2	1	2	3	2
2	-	3	2	2	-	-	3	2	-
-	2	2	3	1	1	-	2	1	1

Figure 5.17: Synthetic lethality of *top3Δ* and *rfa2-1* mutations. CG378 diploids heterozygous for *top3Δ::G418*^{R} and *rfa2-1* were constructed, allowed to sporulate and tetrads dissected onto YPD plates. Spores were then allowed to germinate at 25^{o}C for 3 days. Ten tetrads, each arranged vertically, are shown. Genotypes shown in the table below are as follows: (1)*TOP3 RFA2*; (2) *TOP3 rfa2-1* ; (3)*top3 RFA2*; and (4) *top3 rfa2-1*. No segregants with a type (4) genotype were recovered. In each case, where there is no macroscopic colony, between 10-50 cells were evident on microscopic examination, confirming that germination had taken place, but that subsequent vegetative growth was defective. Genotypes were assigned by screening segregants for the ability to grow in the presence of G418 or at 36^{o}C. Since, both *top3Δ* and *rfa2-1* cells are unable to grow at 36^{o}C, G418-resistant segregants were mated to wild type cells and the resulting diploids dissected. Cells derived from the inital cross were assigned a *top3 RFA2* genotype if no segregants from the second cross that were both G418-sensitive and temperature-sensitive were recovered.

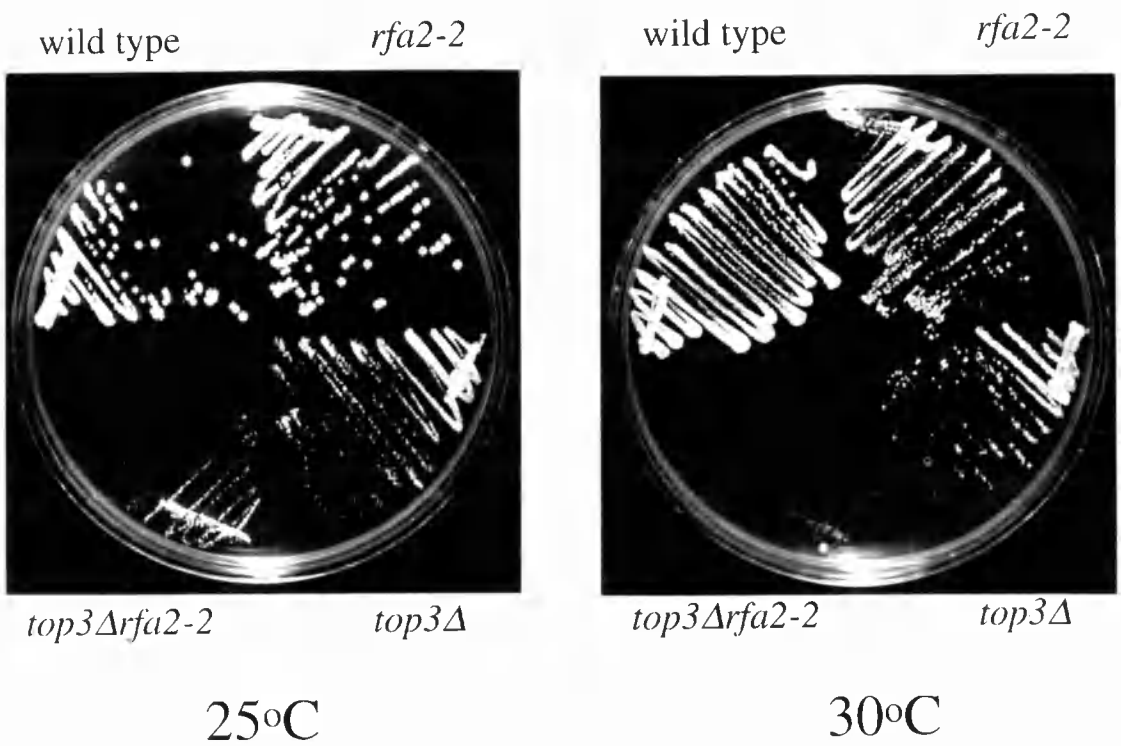


Figure 5.18: Synthetic lethality of *top3Δ* and *rfa2-2* mutations. CG378 wild type, *rfa2-2*, *top3Δ* and *top3Δ rfa2-2* cells were spread on YPD plates and incubated at the permissive (25°C) or semi-permissive (30°C) temperature for 3 days.

5.2.6 Genetic interactions between *top3* and mutations affecting checkpoints outside S-phase.

In the absence of extrinsic damage or inhibitors of replication, synchronous *top3* cells demonstrate a *RAD24/RAD9*-dependent delay at the G2/M checkpoint. Bulk DNA replication as assessed by pulse field gel electrophoresis of intact chromosomal DNA is complete. One explanation put forward in Chapter 4 is that during DNA replication abnormal structures or lesions are generated in *top3* cells that are not sufficient to prevent the completion of bulk DNA synthesis, but nevertheless are recognised by the G2/M damage checkpoint machinery as abnormal.

One prediction of this hypothesis is that loss of the G2/M checkpoint would have deleterious consequences upon the viability of *top3* cells. In order to address this, a series of double mutants were constructed in which the G2/M checkpoint genes *RAD9*, *RAD17* and *RAD24* were deleted in a *top3Δ* background (A364a strain). Unexpectedly, loss of the G2/M checkpoint in a *top3Δ* mutant did not reduce viability significantly (Figure 5.19). Since deletion of *RAD9* or *RAD24* singly, does not completely abolish the G2/M checkpoint (Aboussekhra et al., 1996), a triple mutant in which both these genes were deleted in a *top3Δ* background was constructed. Deletion of both these genes abolishes the G2/M checkpoint almost completely (Aboussekhra et al., 1996; de la Torre-Ruiz et al., 1998). Viability in this triple mutant was not compromised as compared to the *top3Δ* single mutant. This suggests that any DNA lesions generated prior to the induction of the checkpoint-mediated delay in the cell cycle are not repaired to any great extent during the delay period.

Another unexpected finding was that deletion of either *RAD17* or *RAD24*, but not *RAD9*, resulted in an increase in viability of *top3Δ* cells. *RAD9* forms part a distinct epistasis group from the other G2/M checkpoint genes including *RAD17, RAD24, MEC3* and *DDC1*. Members of the latter group, as opposed to *RAD9,* appear to have a role in a meiotic checkpoint (Lydall et al., 1996). Furthermore, it has been suggested that whereas Rad17p and Rad24p promote exonuclease activity in response to certain damage, this activity is antagonised by Rad9p. Since loss of *RAD9* or *RAD17/24* in a *top3Δ* background resulted in equivalent reductions in the cell cycle delay at G2/M, it seems likely that this difference reflects differences in the activities of Rad9p and Rad24p that are independent of their checkpoint role.

Consistent with this, assessment of survival following UV irradiation suggested that *TOP3* had an epistatic relationship with *RAD17/24* but a synergistic relationship with *RAD9* (Figures 5.20, 5.21 and 5.22). The classical interpretation of epistasis data in DNA repair is that mutations which when combined do not lead to increased radiation sensitivity compared to the most sensitive single mutant act in the same pathway. Mutations which do increase radiation sensitivity when combined act in different pathways. In the latter case, if the effect is synergistic it is likely that the respective gene products compete for the same subset of lesions.

In summary, loss of the G2/M checkpoint in a *top3Δ* does not compromise viability. Loss of Rad24p/17p activity actually improves overall viability of a *top3Δ* mutant and the respective mutants show epistatic relationships in relation to DNA damage. It should be noted that the effect of *RAD24* deletion upon *top3Δ* viability was specific to the A364a background and was not reproduced in 2 other strain backgrounds tested. In no background tested was there any significant additional loss of viability following deletion of *RAD9* or *RAD24* in a *top3Δ* background.

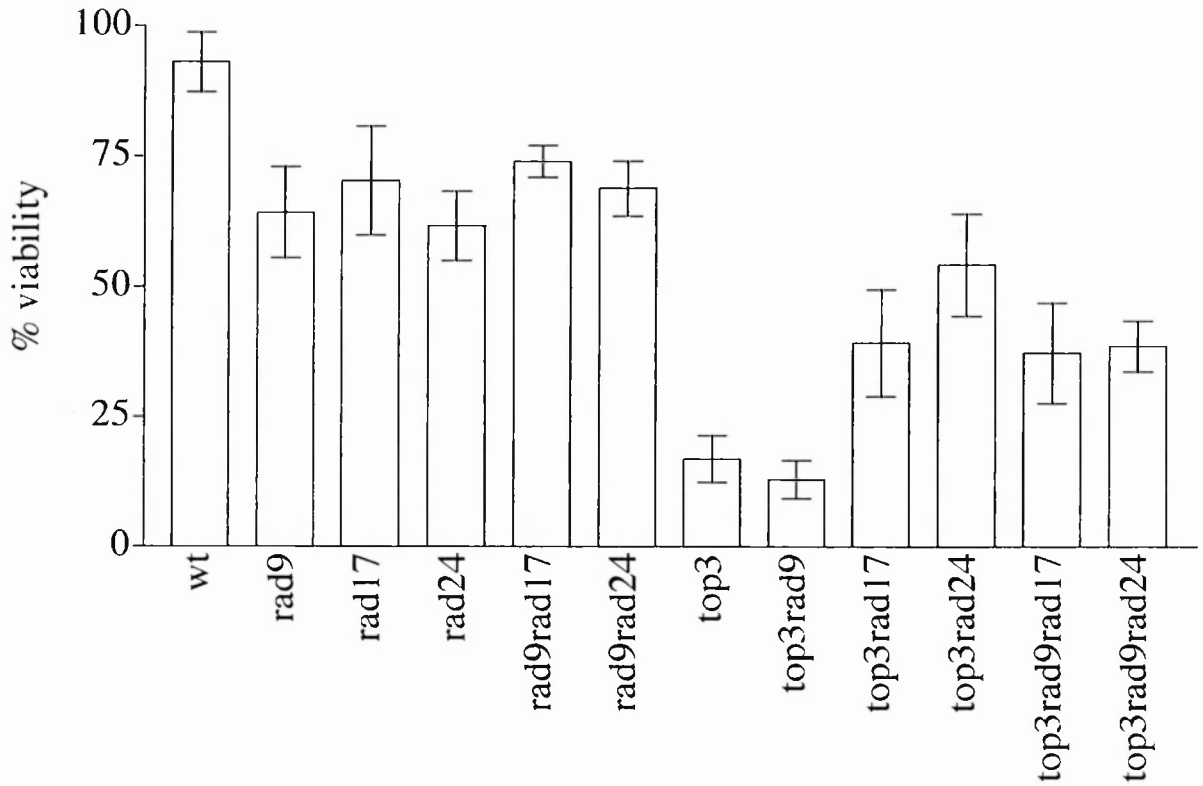


Figure 5.19: Viability of *top3Δ* cells following loss of *RAD9* and *RAD24* genes. Cells derived from log phase cultures of isogenic mutants in the A364a background were counted and a known number of cells plated onto YPD. The number of colony forming units was determined after 3 days growth at 30°C. The means (±SEM) of 3-6 independent experiments are shown.

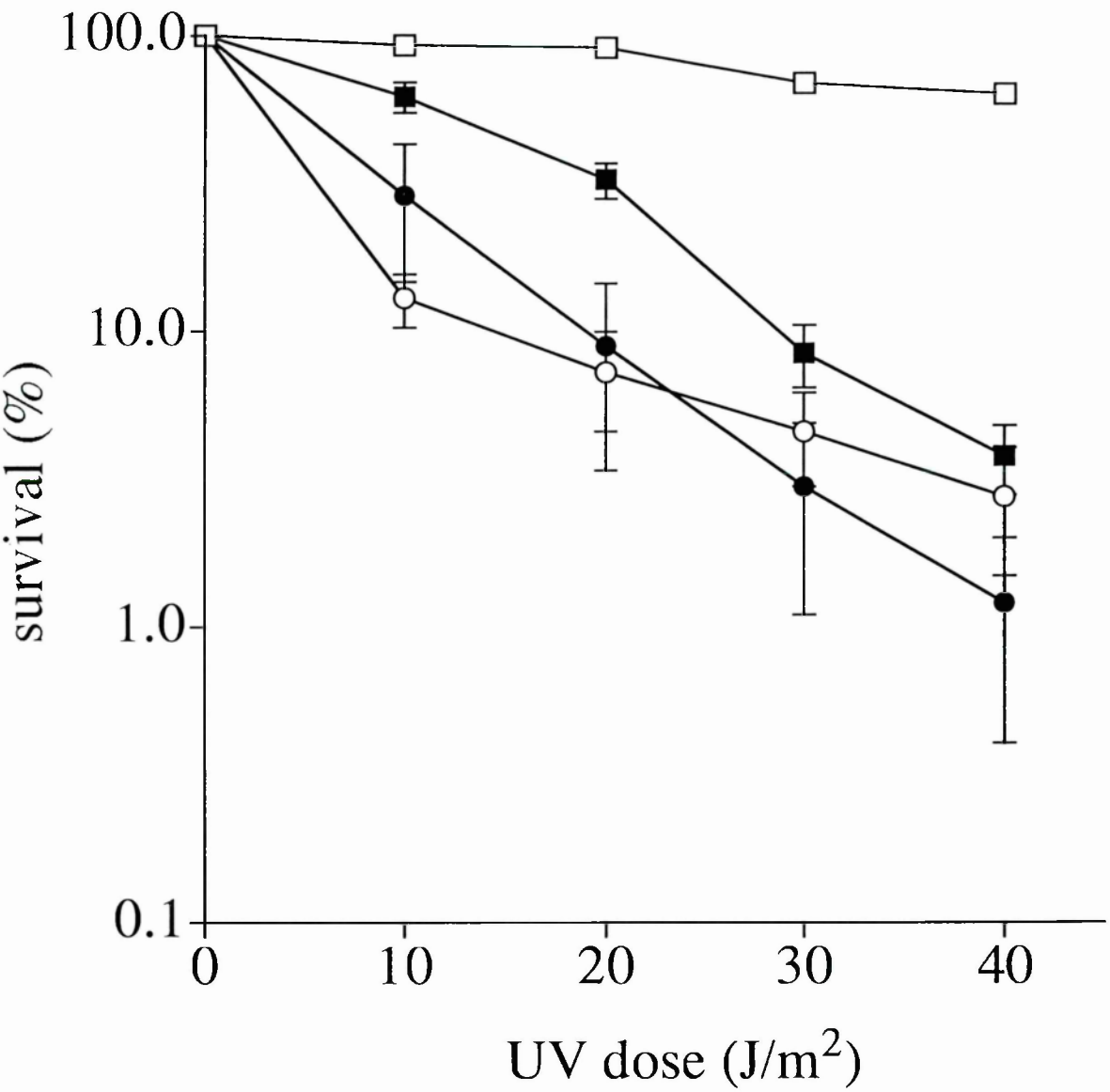


Figure 5.20: Effect of *RAD24* upon UV sensitivity of *top3Δ* mutants. Log phase cultures of A364a wild-type (□), *top3Δ* (○), *rad24Δ* (■) and *top3Δrad24Δ* (●) strains were plated onto YPD, exposed to UV, and % survival determined in relation to non-irradiated controls. Mean (±SEM) % survival from 4 independent experiments are shown.

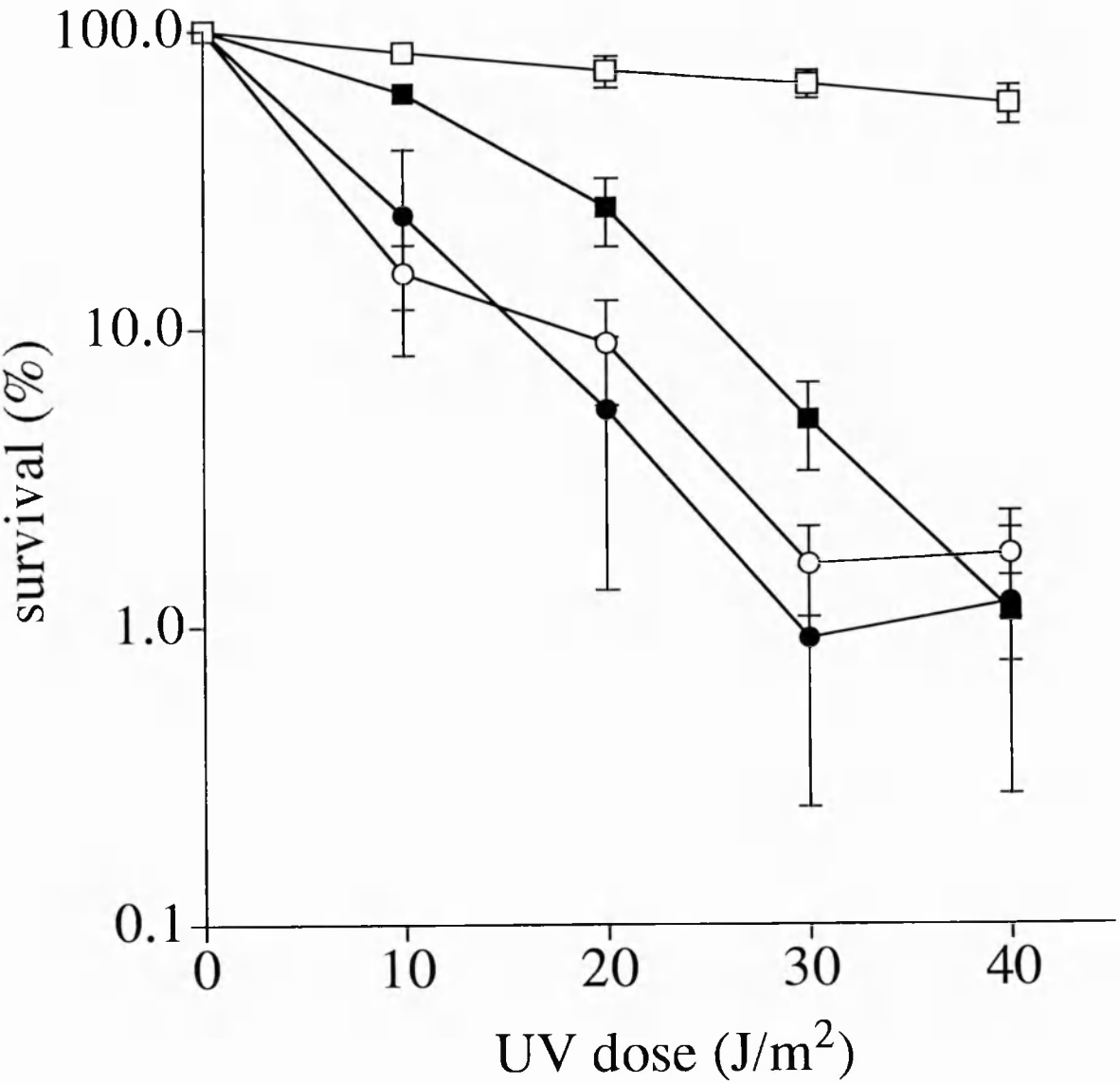


Figure 5.21: Effect of *RAD17* upon UV sensitivity of *top3Δ* mutants. Log phase cultures of A364a wild-type (□), *top3Δ* (○), *rad17Δ* (■) and *top3Δrad17Δ* (●) strains were plated onto YPD, exposed to UV and % survival determined in relation to non-irradiated controls. Mean (±SEM) % survival from 4 independent experiments are shown.

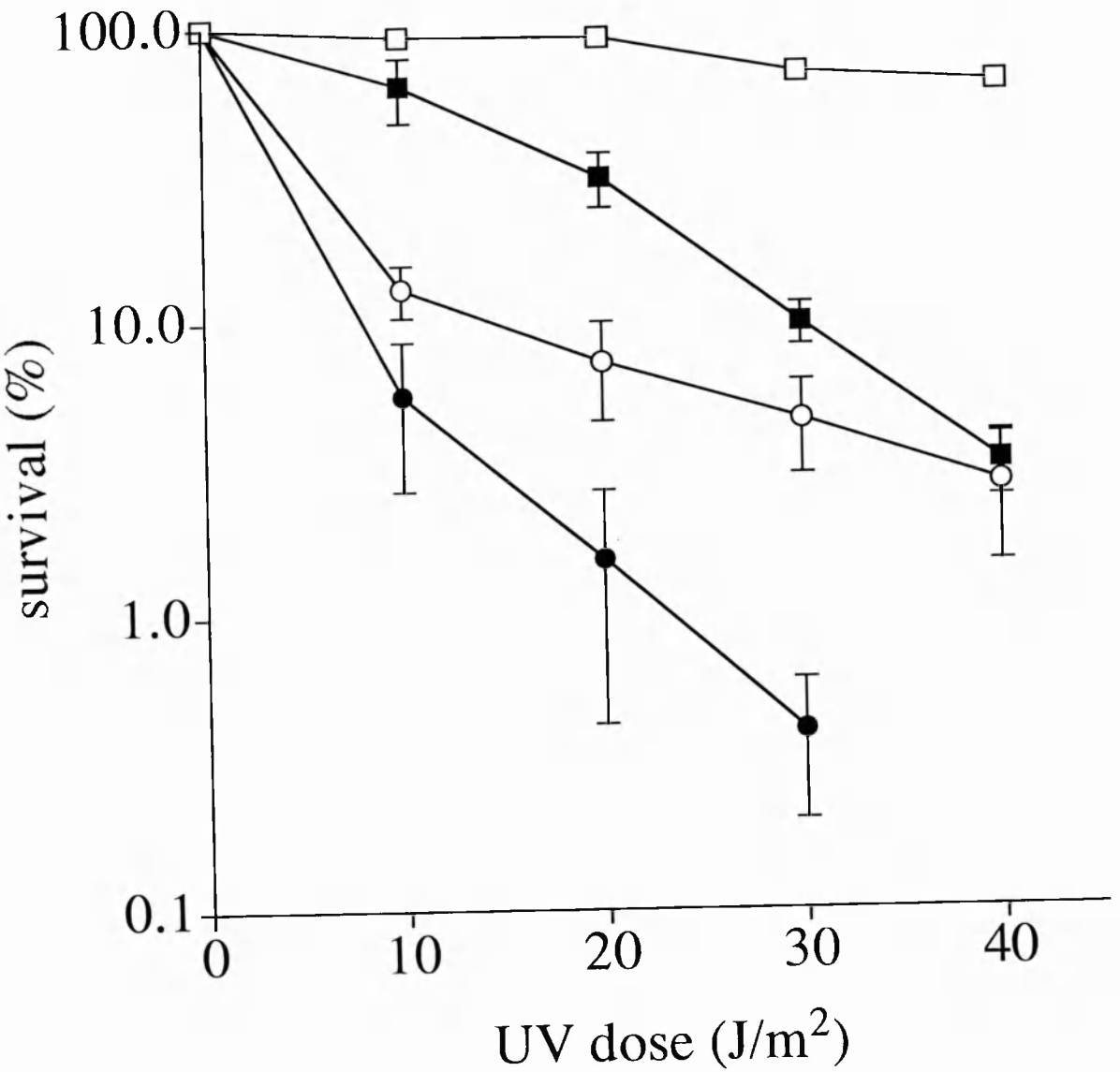


Figure 5.22: Effect of *RAD9* upon UV sensitivity of *top3Δ* cells. Log phase cultures of A364a wild-type (□), *top3Δ* (○), *rad9Δ* (■) and *top3Δrad9Δ* (●) strains were plated onto YPD, exposed to UV and % survival determined in relation to non-irradiated controls. Mean (±SEM) % survival from 4 independent experiments are shown.

5.2.7 Genetic interactions between *top3* mutants and genes which influence recombinational repair.

Loss of each of the 3 topoisomerases in budding yeast results in mitotic hyper-recombination (Christman et al., 1989; Kim and Wang, 1989; Wallis et al., 1989; Gangloff et al., 1994; Kim et al., 1995). In *top3* mutants this is characterised by *RAD52*-dependent recombination between repetitive sequences, including the rDNA (Wallis et al., 1989; Gangloff et al., 1994). A potential role in recombinational repair is suggested by the sensitivity of *top3* mutants to ionising irradiation. The above results also suggested a link to recombination, since *rad24* mutants show an epistatic relationship not only to *top3* mutants but also to *rad52* in relation to sensitivity to UV irradiation (Eckardt-Schupp et al., 1997). It has been proposed that Rad24p is involved in the formation of single stranded substrates for the recombinational repair machinery (Lydall and Weinert, 1995).

Loss of any of the genes of the *RAD52* epistasis group in a *top3Δ* background (YP1 strain) resulted in an improvement in viability as compared to the *top3Δ* single mutant (Figure 5.23). This compared with little effect upon viability following loss of *KU70*, a gene with a role in the non-homologous end-joining of double strand breaks. Comparison of the various isogenic mutants suggests that TOP3 showed an epistatic relationship to *RAD52* in relation to their sensitivity to ionising irradiation (Figure 5.24), although this latter result should be interpreted cautiously since *top3Δ* mutants are only mildly sensitive to ionising irradiation. The effect of *RAD52* deletion upon *top3Δ* viability was reproducible in another independent strain background.

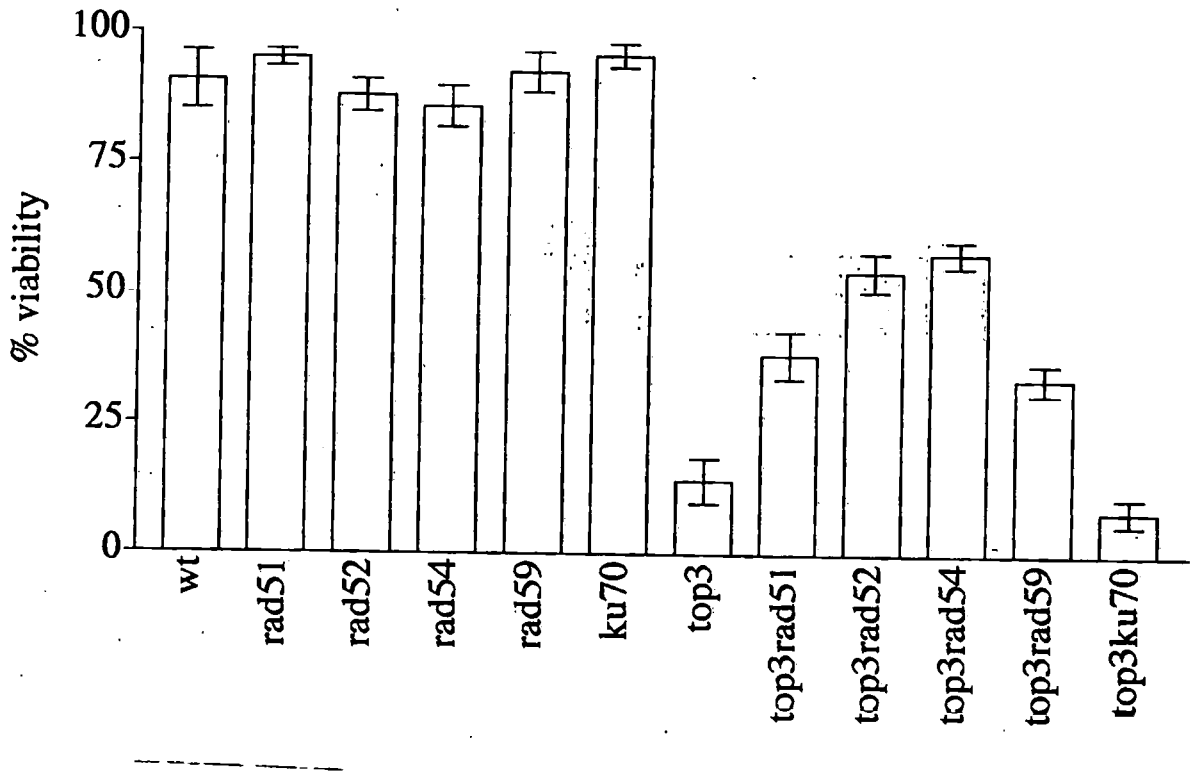


Figure 5.23: Viability of *top3Δ* cells following loss of genes involved in recombinational repair. Cells derived from log phase cultures of isogenic mutants in the YP1 background were counted and a known number of cells plated onto YPD. The number of colony forming units was determined after 3 days growth at 30°C. The means (±SEM) of 3-6 independent experiments are shown.

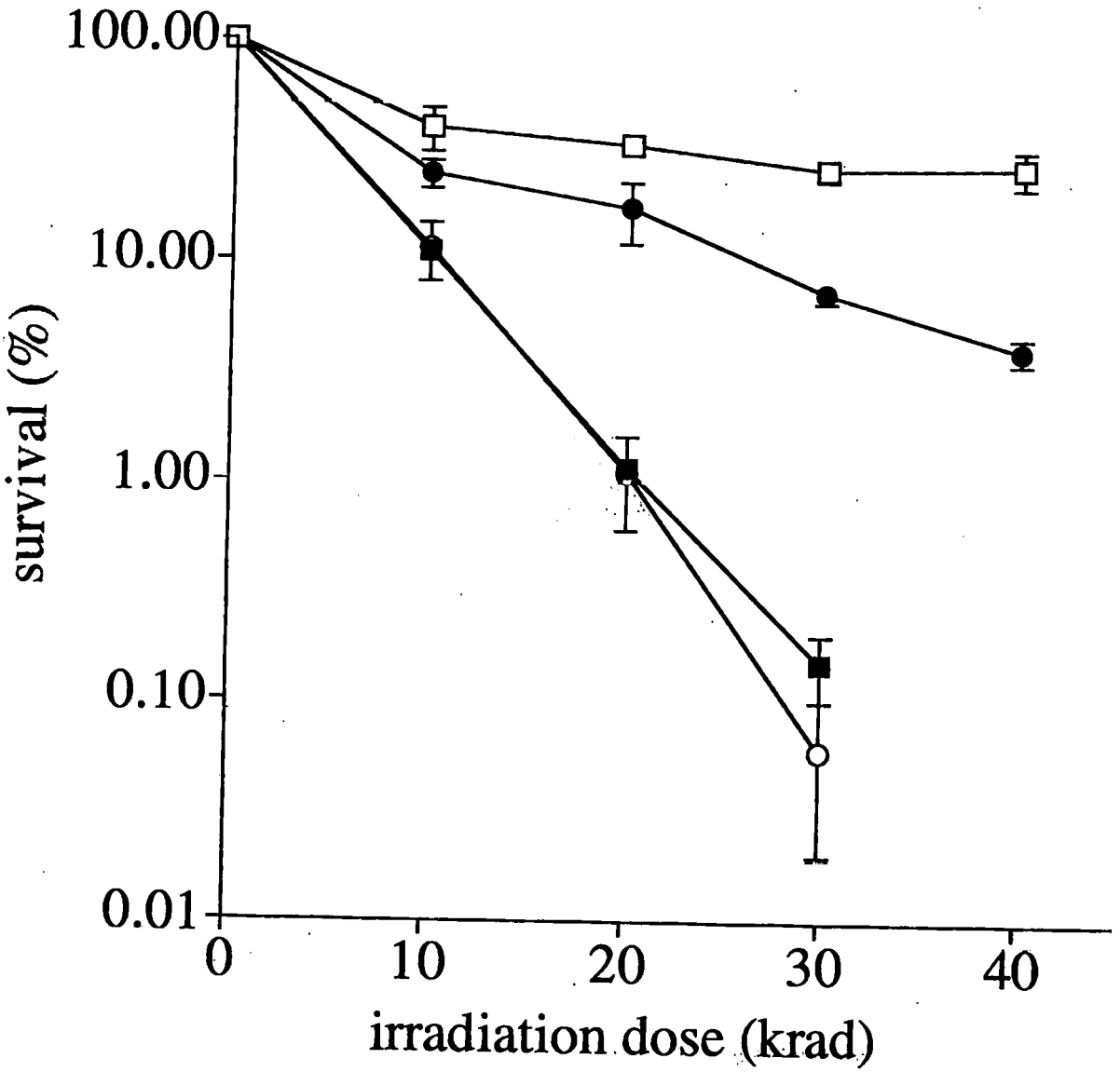


Figure 5.24: Effect of *RAD52* upon γ–ray sensitivity of *top3Δ* cells. Log phase cultures of YP1 wild-type (□), *top3Δ* (○), *rad52Δ* (■)and *top3Δrad52Δ* (●) strains were exposed to γ-rays at the indicated doses and % survival determined in relation to non-irradiated controls. Means (±SEM) of 3 independent experiments are shown.

5.3 Discussion

5.3.1 The role of topoisomerase III in S-phase checkpoint responses

In the previous chapter, evidence was presented demonstrating that untreated *top3Δ* cells delay at G2/M. In the presence of HU *top3Δ* cells lose viability rapidly and this is associated with the finding of cells with fragmented nuclei. Although this latter finding would be consistent with the loss of the S/M checkpoint, it is difficult to interpret morphological changes in cells that already have disturbed cell cycle kinetics. In this chapter, we have shown that *top3* cells are defective in the intra-S checkpoint but other checkpoints outside S-phase are intact.

While there are many examples of mutants that show abnormal responses within S-phase to inhibition of DNA replication or DNA damage, it would appear that no previously described mutant exhibits the same range of phenotypic characteristics as those of a *top3Δ* mutant. Amongst the previously described mutants that lack both the intra-S and the S/M checkpoints are *mec1-1*, *mec2-1* (an allele of *RAD53*) and *rfc5-1* (defective in a subunit of Replication Factor C), all of which are conditional mutations in essential genes (Allen et al., 1994; Weinert et al., 1994; Paulovich and Hartwell, 1995; Sugimoto et al., 1997). However, *mec1-1* and *mec2-1* mutants are defective in all checkpoint responses, including those operating outside of S-phase, and *rfc5-1* mutants are defective in transcriptional induction of DNA damage response genes and phosphorylation of Rad53p in response to DNA damage or replication blockade. To my knowledge, only one mutant has been identified to date that shows intact transcriptional responses to DNA damage, but nevertheless fails to inhibit progression through S-phase in the presence of DNA damage. This is the partially dominant, conditional mutation (*pri-M4*) in the *PRI1* gene, encoding the catalytic subunit of DNA primase (Marini et al., 1997). However, this mutation does not confer HU sensitivity, unlike deletion of *TOP3*. Thus, deletion of *TOP3* is distinctive in that it neither affects checkpoints operating outside of S-phase, nor the ability to upregulate transcription of DNA damage response genes (at least in the case of *RNR1*).

It has not been possible to determine whether topoisomerase III acts upstream of Rad53p in a 'sensing' role or whether it acts downstream as an effector of cell cycle delay. Against topoisomerase III acting downstream is the reduction in the degree of Rad53p phosphorylation following DNA damage or replication block. However, the failure to

observe any effect of overexpressing either *RAD53* or *TEL1* upon either HU or MMS sensitivity of *top3Δ* cells is in contrast to the reported effects of overexpression in *pol2* (Navas et al., 1996) or *rfc-5* (Sugimoto et al., 1997) mutants. Although transcriptional responses appear to be intact, such upregulation is not necessarily dependent upon Rad53p (de la Torre-Ruiz et al., 1998). Thus, at present, two models can be envisaged. In the first topoisomerase III acts upstream of Rad53p as a sensor of abnormal structures. In this case the integrity of the transcriptional responses is dependent upon redundancy in this sensory mechanism. In the second model, topoisomerase III acts downstream of Rad53p as an effector of the cell cycle delay. In this scenario, the attenuation in the degree of Rad53p phosphorylation reflects the loss of viability of *top3Δ* cells in the presence of HU or MMS. This is discussed in more detail in Chapter 6.

5.3.2 *top3Δ* strains are sensitive to several classes of DNA damaging agents

Mutations in a wide variety of genes encoding DNA repair enzymes or checkpoint proteins confer sensitivity to DNA damaging agents (Friedberg et al., 1995). Thus, for example, mutation of nucleotide excision repair genes leads to sensitivity to UV light, while mutation of recombinational repair genes confers sensitivity to ionising radiation and MMS, but not to UV light. *top3Δ* mutants are unusual in being sensitive to all three of these classes of agent, indicating that the protein probably does not play a dedicated role in any single pathway for the repair of specific DNA lesions, but instead operates more generally in cellular responses to DNA damage. To my knowledge, this represents the first evidence in eukaryotes that a topoisomerase can protect cells from the cytotoxic effects of DNA damaging agents. In one report, mutation of prokaryotic topoisomerase I was associated with UV sensitivity and this has been interpreted as evidence that proteins involved in nucleotide excision repair are influenced by the superhelical density of DNA (Sternglanz et al., 1993; Pedrini and Ciarrochi, 1983). Since yeast topoisomerase III does not appear to play any significant role in supercoiling homeostasis (Wang, 1996), this hypothesis is unlikely to apply to *top3Δ* mutants.

The upregulation of the *TOP3* transcript following treatment of wild type cells with MMS, but not HU, may indicate that it has some role in the direct processing of DNA lesions resulting from this agent. This result should be viewed with some caution, however, since the *TOP3* transcript is cell cycle regulated and treatment with MMS perturbs the cell cycle. Experiments that assess *TOP3* transcript and topoisomerase III

protein levels following MMS-exposure of cells held at different points in the cell cycle will need to be performed. The role of the putative DREs identified in the 5' region upstream of the translation initiation codon of the *TOP3* gene has also not been determined. Transcription initiation sites and the stability or otherwise of the topoisomerase III protein are, however, not known. The significance of these sites, together with the role of a putative SCB box (described in Section 4.3.3), will need to be evaluated by assessing the effect of mutation of the various elements.

5.3.3 The relationship of *TOP3* to other genes that mediate S-phase checkpoint responses

Given the intact nature of DNA damage checkpoint responses occurring in G1 and G2, but a failure to invoke the intra-S DNA damage checkpoint, it would appear that DNA damage arising during S-phase presents the most serious threat to *top3Δ* mutants. Consistent with this is the finding that combination of *top3Δ* with mutants that are defective in S-phase checkpoint responses (*mec1-1, mec2-1, pri-M4*)) or in targets of this response (*rfa2-1, rfa2-2*), resulted in a synthetic lethal phenotype. In contrast, combination of *top3Δ* with deletions affecting genes which influence specifically G1 and G2/M checkpoints, did not result in a lethal effect. The difficulty in interpreting this information is that each of the S-phase checkpoint genes, with the exception of *TOP3*, is an essential gene. Loss of an essential function in cells which are already compromised does not necessarily imply that the *top3Δ* mutation and the other S-phase checkpoint mutations act synergistically to knock out an essential checkpoint pathway. Thus, the lethality of combined of *top3Δ* and *pri-M4* mutations may simply reflect a failure to replicate DNA. However, in data not shown, *top3Δ* mutations when combined with a hypomorphic mutation (*pol2-12*) in another essential S-phase specific gene, *POL2* , was not associated with synthetic lethality. Although these latter mutants are reported to be defective in the S/M checkpoint (Navas et al., 1995), I was not able to reproduce this in the A364a background.

I have not excluded the possibility that abnormal DNA structures are generated outside of S-phase that require the action of topoisomerase III. The protracted delay of *top3Δ* cells in the G1 phase following UV-irradiation, the failure to completely reverse the MMS sensitivity of these cells by maintaining them in G1 during drug exposure, and the presence of a minor fraction of phosphorylated Rad53p in undamaged G1-arrested

cells, is consistent with a possible role for topoisomerase III in the G1 phase of the cell cycle. In the absence of DNA damage, however, the rate of progression through the G1/S transition is not prolonged. The nature of any role for topoisomerase III in G1 remains to be determined.

5.3.4 Identification of other potential suppressors of the *top3Δ* phenotype

Genetic suppression of mutants defective in a cellular process can occur in two ways (Murray and Hunt, 1993). Mutational suppression results from a compensatory mutational change that re-establishes function. Dosage suppression infers that an increased concentration of an interacting component might stabilise a complex or promote a reaction by over-riding any block. We have identified two groups of genes, which when deleted, suppress the loss of viability of *top3Δ* mutants. The first includes members of the *RAD24* epistasis group and the second, members of the *RAD52* epistasis group.

The effect of Rad24p or Rad17p appear to be independent of their role in the G2 checkpoint, since deletion of Rad9p does not suppress the low viability of *top3Δ* mutants. In this context, there is some evidence that Rad24p and Rad17p have roles in DNA repair. For example, Rad24p shows sequence similarity to subunits of replication factor C (RFC) (Griffiths et al., 1995). RFC is a five-subunit protein complex that has roles in both DNA replication and repair (Waga and Stillman, 1994; Aboussekhra et al., 1995; McAlear et al., 1996). The similarity of Rad24p to these subunits resides in domains II, III and VIII, and suggests that Rad24p has some RFC-type functions. Like certain *rfc* mutants, *rad24Δ* mutants show a moderate degree of HU sensitivity (Lydall and Weinert, 1997). Furthermore, overexpression of Rad24p strongly reduces the growth rate of yeast strains defective in DNA repair/replication proteins including RFC1p, DNA polα and DNA polδ. More indirect evidence of a role in DNA repair derives from studies that suggest that Rad17p has significant homology with human RAD1 which possesses 3' to 5' exonuclease activity (Parker et al., 1998). Taken together, these results suggest that in the absence of topoisomerase III, Rad17p and Rad24p possess DNA processing functions that are deleterious to cell viability. This effect was only observed in the A364a background and not 2 other backgrounds tested, a finding in keeping with those of others studying the effects of Rad24p overexpression (Lydall and Weinert, 1997). The reason for this is not known. In no background, however, did loss of the

RAD24 or *RAD9* genes confer any significant loss of viability. This might indicate that a substantial fraction of the DNA lesions that led to the induction of the G2/M checkpoint are either irreparable, at least in G2/M, or can be tolerated in subsequent cell cycles.

Deletion of genes in the *RAD52* epistasis group conferred a similar improvement in viability in *top3Δ* mutants as that following deletion of *RAD24*. Thus, recombinational processing of abnormal DNA structures present in the absence of topoisomerase III are damaging to the cell. Furthermore, in work performed while this thesis was in preparation, deletion of *RAD52* has been found to suppress the HU and UV sensitivity of *top3Δ* mutants (Dr. Jonathan Kearsey, Institute of Molecular Medicine, personal communication). As in the case of *RAD24*, there are two general explanations for this suppression. First, topoisomerase III could be involved in the resolution of intermediates generated by Rad52p. Second, in the absence of topoisomerase III DNA lesions might be generated in which are then processed inappropriately by the recombinational repair apparatus.

Chapter 6

Conclusions

6.1 Summary of key findings

The overall aim of this thesis was to improve our understanding of how defects in RecQ helicases in humans can lead to gross genomic stability, that in turn can lead to cancer predisposition or features of premature replicative senescence. The structural similarity of human BLM and *S.cerevisiae* Sgs1p, and the potential similarity of the cellular phenotypes when either of these proteins are defective, alerted us to the use of yeast as a model system (German, 1993; Watt et al., 1996). In the first instance, heterologous complementation studies were performed, which showed that *BLM* could complement a subset of the *sgs1Δ* phenotypes. *SGS1* deletion suppresses the slow growth phenotype of mutants defective in topoisomerase III. Ectopic expression of wild type *SGS1* in an *sgs1Δtop3Δ* mutants re-induces the slow growth phenotype, an effect that is reproduced by ectopic expression of *BLM*. This suggested that the interaction between the RecQ family helicases and this topoisomerase is highly conserved. Thus, the remaining part of this study focused upon the functional role of topoisomerase III using yeast as the model system.

Loss of topoisomerase III results in a cell cycle delay at the G2/M phase of the cell cycle and this delay is dependent upon the DNA damage checkpoint genes *RAD24/RAD9*. This suggested a potential role for topoisomerase III in the resolution of abnormal DNA structures/damage arising during S-phase. Consistent with this concept, *top3Δ* mutants are defective in the intra-S checkpoint that slows the rate of S-phase progression following exposure to DNA damaging agents. Furthermore in the presence of the DNA replication inhibitor hydroxyurea, *top3Δ* mutants demonstrate a rapid loss of viability associated with fragmented nuclei and abnormal nuclear spindles. Whether this latter finding truly represents loss of the S/M checkpoint is not clear since it has proven difficult to interpret changes in spindle elongation in cells that even in the absence of HU show a defective cell cycle. DNA damage checkpoints operating outside S-phase were shown to be unaffected by deletion of *TOP3*. Consistent with this S-phase specific role, expression of the *TOP3* mRNA is activated in late G1 and the *TOP3* gene contains a putative SCB motif in the 5' region upstream of the translation initiation codon. Most of the phenotypic consequences of loss of topoisomerase III are partially suppressed by deletion of *SGS1*, although the effect of *SGS1* deletion in a *top3Δ* background upon the intra-S or S/M checkpoint was not examined.

6.2 Topoisomerase III- a role in checkpoints or repair?

Loss of topoisomerase III confers specific loss of S-phase checkpoints. A number of findings, however, suggest that a primary function of topoisomerase III is to mediate repair of DNA lesions. First, assuming the S/M checkpoint is defective in *top3Δ* mutants, these cells were exquisitely sensitive to HU and to an extent that was greater than other mutants defective in the S/M checkpoint. Second, although the G1/S checkpoint is proficient in *top3Δ* mutants a role in DNA repair at this phase of the cell cycle cannot be excluded. This is suggested by the extended delay following irradiation in G1 phase as compared to wild type cells, the failure to rescue MMS sensitivity of *top3Δ* cells by holding them in G1 with α-factor, and the aberrant phosphorylation of Rad53p in α-factor arrested cells. The potential upregulation of the *TOP3* transcript following exposure of wild type cells to MMS is also consistent with a role in repair. Finally, the genetic interactions between *top3Δ* and *rad24* or *rad52,* are suggestive of functional relationships between topoisomerase III and proteins with DNA repair/processing functions.

There is increasing evidence, both in prokaryotes and eukaryotes, that cellular responses to DNA damage are dependent upon the partial processing of damage to intermediate structures. In bacteria, the 'SOS' pathway mediates the response to DNA damage that results in increased DNA repair and cell survival (Little and Mount, 1982; Friedberg et al., 1995). However, initial damage, such as DSBs is insufficient to activate this pathway, since partial processing of the DSB by RecBCD to generate ssDNA is required before RecA can bind and mediate downstream events including cell cycle alterations and DNA repair (reviewed in Sassanfar and Roberts, 1990).

In budding yeast, there are three lines of evidence that this type of processing is found in eukaryotes. First, UV-induced pyrimidine dimers cannot themselves trigger a checkpoint response (Siede et al., 1994; Weinert, 1997), but instead require the action of excision repair proteins or photolyase. Second, checkpoint mediated arrest during meiosis (during which DSBs are normally degraded to generate 3' single strands) is also postulated to correlate with the presence of single stranded DNA (Weinert, 1997). In one mutant, *rad50S*, DSBs generated during meiosis are not degraded, and cells do not arrest (Alani et al., 1990). A third example provides evidence that checkpoint proteins themselves may be involved in the initial processing of DNA damage. Thus, in *cdc13*

mutants which arrest at G2 at the restrictive temperature, a lesion in dsDNA is converted to ssDNA, an effect which is dependent upon Rad17p/Rad24p (Lydall and Weinert, 1995).

6.3 How does loss of topoisomerase III activity result in loss of checkpoint proficiency?

How can the above findings be incorporated into a model for the action of Sgs1/Top3 during replication? When cells are exposed to any insult that results in replication fork disruption, the cell is likely to require the integrated response of proteins acting not only in cell cycle checkpoint processes, but also in DNA replication, recombination and repair pathways. As discussed above, a common factor in mediating such responses is the partial processing of DNA to single-stranded DNA intermediates. Since both helicases and topoisomerases can generate and process such substrates, one attractive possibility is that the Sgs1/Top3 complex is central to the 'networking' of the various pathways.

In Figure 6.1 a schematic model for the role of Sgs1p/Top3p is shown. I propose that following inhibition of replication or DNA damage, replication forks stall and that Sgs1p/Top3p is involved in the early processing of the resulting abnormal DNA structures. Sgs1/Top3 could serve two functions in this process, which are not mutually exclusive: first, the preparation of lesions for repair by the *RAD52*-dependent recombination machinery, and second, the generation of DNA structures that lead to activation of the checkpoint, thus preventing the firing of new replicons. Murray et al. (1997) have suggested a similar role for fission yeast $rqh1^+$ in preparing DNA lesions at blocked replication forks for the recombinational repair machinery. This model would also be consistent with the proposed role of the RecQ protein, the *E.coli* homologue of Sgs1p, which in concert with RecA can initiate homologous recombination and disrupt joint molecules formed by aberrant recombination (Harmon et al., 1998). In further support of this general concept are the observations that recombination intermediates (Holliday junctions) can be detected in yeast during S-phase and that perturbation of replication leads to an elevation in their frequency (Zhou et al., 1997).

Deletion of *SGS1* results in suppression of the HU, MMS and UV sensitive phenotype of *top3Δ* mutants. It has not been established, however, whether or not *sgs1Δtop3Δ* double mutants are checkpoint proficient. If the double mutant is checkpoint

proficient, then one explanation could be that the phenotype of *top3Δ* mutants relates primarily to dysregulation of Sgs1p activity. Such dysregulated activity could interfere (either directly or indirectly) with the checkpoint machinery. Alternatively, deletion of *SGS1* could allow the activation of a parallel and partially redundant pathway that could lead to the activation of the checkpoint. If the *sgs1Δtop3Δ* double mutant remained checkpoint defective, this would suggest that the sensitivity of *top3Δ* mutants to inhibition of replication or DNA damage, relates primarily to a repair rather than a checkpoint defect.

Recently, it has been reported that expression of a truncated form of human topoisomerase IIIα can partially reverse certain phenotypes associated with ataxia telangiectasia cells, which are defective in the cellular response to DNA damage. Since the transfection of truncated topoisomerase IIIα was shown to influence both radiation responses (including radioresistant DNA synthesis - which is analogous to the intra-S checkpoint in yeast) and hyperrecombination (Meyn et al., 1993; Fritz et al., 1997), it is possible that the model described here might also be applicable to higher eukaryotes.

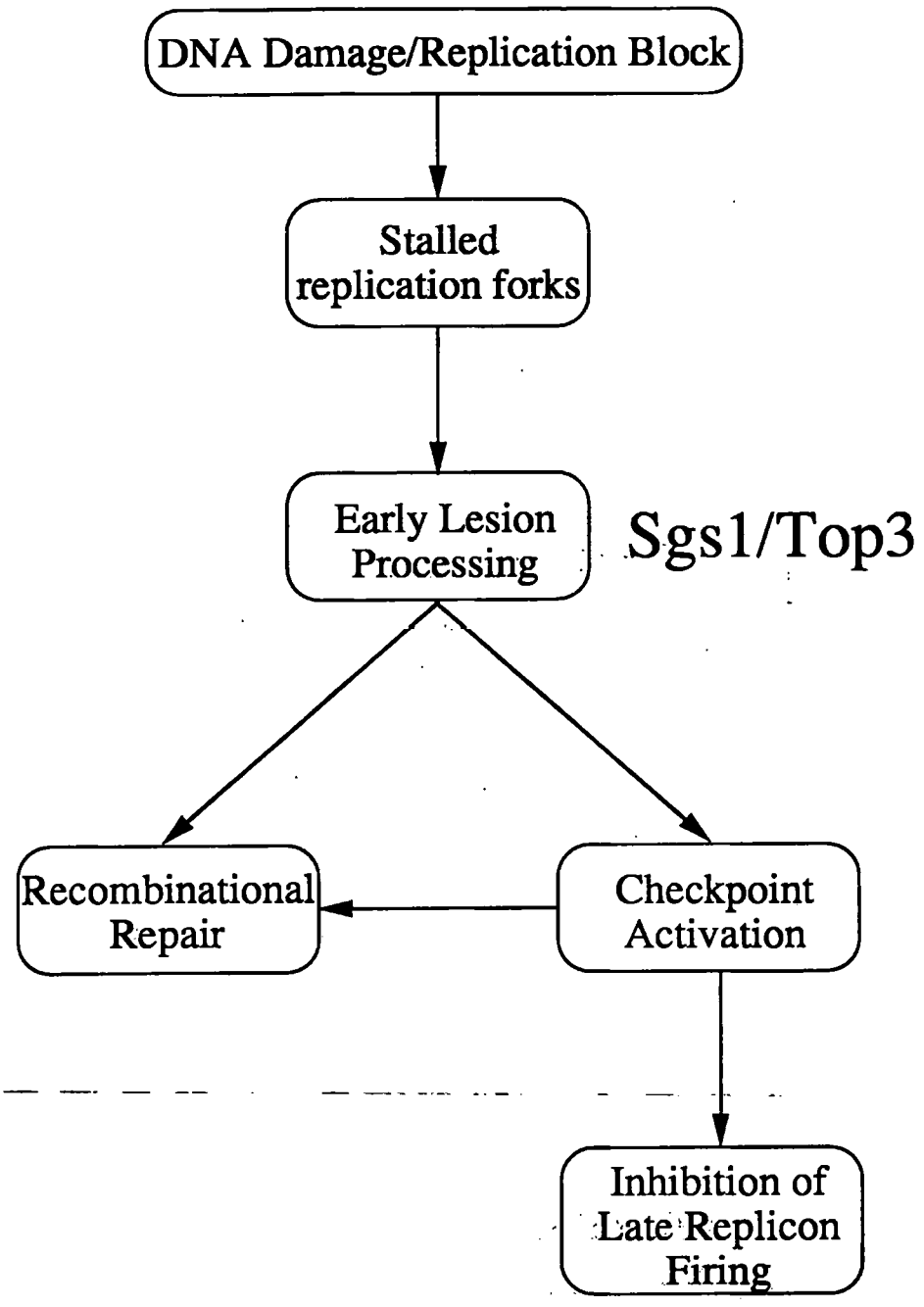


Figure 6.1: A model for the role of Sgs1 and topoisomerase III in mediating responses to replication fork arrest. We propose that Sgs1/Top3 is involved in the early processing of aberrant structures arsing at stalled replication forks. This complex may have at least two functions: first, the preparation of lesions for repair by the recombination machinery and second, the activation of a checkpoint that leads to the inhibition of cell cycle progression. Thus, in the absence of a RecQ helicase, there is a failure to integrate the cellular response to a frequent event, that is, the stalling of replication forks and this leads ultimately to chromosomal instability.

6.4 The role of topoisomerase III in the late stages of replication

As two replication forks converge, a build up of positive supercoils and/or steric hindrance between advancing replisomes could inhibit the final stages of DNA replication (reviewed in Wang, 1996). It has been proposed that Sgs1p could act to unwind the non-replicated region between the advancing replisomes, thus generating intertwinings between the daughter molecules. These catenanes could then be resolved by topoisomerase III prior to the 'filling-in' of the small single-stranded gaps left at the sites of converging forks. A prediction of this model therefore is that *top3* mutants fail to resolve these structures leading to a failure to complete DNA replication or chromosome non-disjunction.

At present it is not possible to refute this model entirely. Thus, it is possible that incompletely replicated DNA is sufficient to cause the arrest of *top3Δ* mutants at G2/M, yet cannot be detected by the pulse field gel electrophoresis study employed in this study. Furthermore, assays to detect chromosome non-disjunction have not been performed. However, the finding that *top3* mutants are sensitive to HU which blocks cells in early S-phase and the demonstration that the *TOP3* transcript is upregulated in G1, both suggest that topoisomerase III is required at a much earlier phase in the cell cycle. Furthermore, if the main activity of topoisomerase III is as a decatenase, it is surprising that the other topoisomerases cannot substitute fully for this function. The failure to observe the accumulation of abnormal replication intermediates in 2D gels derived from *top3Δ* mutants also appears inconsistent with this model.

6.5 Topoisomerase III - a potential therapeutic target?

DNA topoisomerases are important targets for antimicrobial and anticancer therapeutics (Liu, 1994). Anti-cancer drugs acting on type IB topoisomerases (e.g. campothecin) and type II topoisomerases (e.g. etoposide) are now used commonly. The majority of these drugs act by trapping the covalent enzyme-DNA intermediate, thus converting a normal cellular enzyme to a DNA damaging agent (Liu, 1989). In general, the cytotoxicity of this class of agent increases with increasing cellular concentration of the enzyme. Other drugs act by inhibition of the topoisomerase catalytic activity (e.g. merbarone) and perhaps are less dependent upon the concentration of enzyme (Drake et al., 1989). No agents active against type IA topoisomerases have so far been identified,

although it is reported that topoisomerase III expression is much lower than either topoisomerase I or II in mammalian cells. The most likely class of agent to be effective therefore is one that inhibits catalytic activity, rather than one which inhibits the enzyme-DNA covalent intermediate. If mammalian topoisomerase III has a similar role to that of its yeast counterpart in mediating S-phase checkpoint responses, then it is possible to conceive that a topoisomerase III inhibitor and an S-phase specific agent may have synergistic effects in combination.

6.6 Future work

Although the genetic interaction between *SGS1* and *TOP3* is clear cut, the extent to which the proteins encoded by them physically interact has not been determined in great detail. Co-immunoprecipitation analyses could confirm this interaction and assess whether it was cell cycle phase-specific or related to extrinsic effects such as DNA damage. A stepwise dissection of functional domains for both proteins could be performed, linked to 2-hybrid and overexpression analyses. Thus, identification of a minimal Sgs1p-interaction domain in topoisomerase III, could allow its mutation and determination of how it affects the function of topoisomerase III. Dominant-negative effects of overexpression of interaction domains could be determined.

Recently it has been reported that transient inhibition of DNA synthesis leads to a subsequent failure to complete chromosomal replication in *rad53* or *mec1* point mutants (Desany et al., 1998). This aberrant replication may result from a failure to regulate correctly the cellular dNTP pool (Desany et al., 1998; Weinert, 1998; Zhao et al., 1998). The model presented in this chapter would predict that *top3Δ* mutants exposed transiently to HU should also subsequently fail to complete chromosomal replication, as assessed by pulse field gel electrophoresis. In this context, the effect of overexpression of either *RNR1* or *RNR3* on the viability and cell cycle kinetics of *top3Δ* mutants also merits study.

Since this study was initiated, an *S.pombe* gene predicted to encode a protein with substantial similarity to topoisomerase III has been identified. Study of the effect of deleting *S.pombe TOP3* is attractive for a number of reasons. First, it would allow an assessment of the extent to which the phenotypes observed in this thesis are conserved in this evolutionary distant organism. It would be of particular interest to determine whether deletion of *rqh1* had any effect upon the *top3* phenotype. Another advantage to

using *S.pombe*, is that it is easier to identify the position of an individual cell in the cell cycle (Murray and Hunt, 1993). Thus, not only is there a clear relationship between cell length and the point attained in the cell cycle, but also spindle formation occurs only at mitosis. In contrast, a short intranuclear spindle forms earlier in the cell cycle in *S.cerevisiae* cells and the relationship between cell size and cell cycle position is less clear cut, since budding yeast cells divide asymmetrically.

A priority for future studies will be to confirm whether or not the interactions between Sgs1p and topoisomerase III identified in this study, are conserved in humans between BLM and human topoisomerase III. At the time of writing, information derived from two-hybrid, co-immunoprecipitation and far western studies, suggests that this interaction is indeed conserved. These studies could be extended to a comprehensive analysis of cell cycle regulation, immunolocalisation and response to DNA damage, of the respective proteins.

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