Defects in Homologous Recombination Repair in Mismatch Repair –deficient tumour cell lines

A thesis submitted to the University of Sheffield for the degree of

Doctor of Philosophy

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January 2004

I hereby declare that no part of this thesis has previously been submitted for any degree or qualification of this, or any other, University or Institute of learning.

Acknowledgements

I wish to thank my supervisor Mark Meuth for his perpetual patience, advice and support through out this research project.

I am also extremely grateful to Thomas Helleday, Jason Stone and Jarek Dziegielewski for valuable discussions and for critically analysing my data.

I am indebted to Gerry Meuth, Ian Brock, Gary Rodgers and Sheila Rodgers for their technical advice, without which this project would have been a lot harder.

Furthermore, I wish to thank Yorkshire Cancer Research for funding this research project.

I wish to also thank my family, especially my father, who has advised me and patiently listened to my problems through out my project.

Finally, but not least, I wish to thank Lisa Yates for her support, care and love for which I am eternally grateful.

Publications and Presentations

Publications:

Mohindra, A., Hays, L.E., Phillips, E.N., Preston, B.D., Helleday, T. & Meuth, M. (2002). Defects in homologous recombination repair in mismatch-repair-deficient tumour cell lines. *Hum Mol Genet*, **11,** 2189-200.

Mohindra, A., Bolderson, E., Stone, J., Wells, M., Helleday, T. & Meuth, M. (2004). A tumour-derived mutant allele of XRCC2 preferentially suppresses homologous recombination at DNA replication forks. *Hum Mol Genet*, **13,** 203-12.

POSTER PRESENTATIONS:

Keystone Symposia 2002: Molecular Mechanisms of DNA Replication and Recombination. Salt Lake City, Utah, U.S.A.

Yorkshire Cancer Research Annual Scientific Meeting, 2001-2003

Summary

MMR –deficiency increases the rate of mutations and often sensitizes cells to DSB-inducing agents (e.g. camptothecin and etoposide) as well as MMC (Jacob *et al.,* 2001 and Fiumicino *et al.,* 2000). MMR -deficient tumour cell lines are also sensitive to the cytotoxic effects thymidine (Mohindra *et al.,* 2002). This sensitivity is not a direct consequence of MMR -deficiency or alterations of DNA precursor metabolism. Instead, the results described in the present study suggest that MMR -deficient cells are sensitive to thymidine as a result of defects in HRR.

The ScNeo recombination reporter substrate was used to determine the integrity of the HRR pathway in several MMR -proficient and -deficient tumour cell lines. Four MMR –deficient tumour cell lines were defective in the production of neo^+ recombinants by homology based recombination following the transient expression of a site specific break. Furthermore, all MMR –deficient tumour cell lines tested were sensitive to the cross-linking agent MMC; an effect that is consistent with cells being deficient in HRR (including XRCC2, XRCC3 and BRCA1).

To determine the alterations responsible for such HRR defects, genes known to be required for this pathway were screened for mutations in eight tumour cell lines. This revealed a heterozygous frameshift mutation within the RAD51 paralog, XRCC2, (342delT) in SKUT-1 cells. 342delT was introduced into HRR proficient cells containing the ScNeo substrate. In SW480/SN.3 transfectants, expression of 342delT conferred sensitivity to thymidine and MMC and suppressed HRR induced at the recombination reporter by thymidine but not by DSBs. In the MRC5VA/SN.13 transfectants, expression of 342delT was accompanied by a decreased level of the full-length XRCC2. These cells were defective in the induction of HRR by either thymidine or DSBs. Thus 342delT suppresses recombination induced by thymidine in a dominant negative manner while recombination induced by DSBs appears to depend upon the level of wild-type XRCC2 as well as the expression of the mutant XRCC2 allele. These results suggest that HRR pathways responding to stalled replication forks or DSBs are genetically distinguishable. They further suggest a critical role for XRCC2 in HRR at replication forks, possibly in the loading of RAD51 onto gapped DNA.

Abbreviations

aa	Amino Acid
APRT	adenine phosphoribosyltransferase
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphotase
A-T	Ataxia-Telangiectasia
A-TLD	Ataxia-Telangiectasia Like Disorder
ATM	Ataxia-Telangiectasia Mutated
ATR	ATM and Rad53 Related
BASC	BRCA1-Associated Genome Surveillance Complex
BCDX2	RAD51B-RAD51C-RAD51D-XRCC2 complex
BIR	Break-induced replication
bp	Base Pair
BRCA1	Breast Cancer Associated 1
BRCA2	Breast Cancer Associated 2
BRCT	BRCA1 Carboxy-Terminal Domain
BSA	Bovine Serum Albumin
CHO	Chinese Hamster Ovary
CPT	Camptothecin
Da	Daltons
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dTTP	Deoxythymidine Triphosphate
dNTP	Deoxynucleotide Triphosphate
ddNTP	Dideoxynucleotide Triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid

cDNA	complementary Deoxyribonucleic acid
dsDNA	Double-strand Deoxyribonucleic acid
hDNA	heteroduplex Deoxyribonucleic acid
ssDNA	Single-strand Deoxyribonucleic acid
DSB	double strand break
DSBR	double-strand break repair
E-Coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra-acetic Acid
FAP	Familial Adenomatous Polyposis
FHA	Forkhead Associated Domain
G_0	GAP 0 (non-dividing cells)
G_1	GAP 1
G_2	GAP 2
GC	Gene conversion
GFP	Green Fluorescent Protein
HJ	Holliday Junction
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HPRT	hypoxanthine guanine phosphoribosyltransferase
HRR	Homologous Recombination Repair
IR	Ionising Irradiation
IRD	Infrared Dye
kb	Kilobase
kDa	Kilo-Dalton
LOH	Loss of Heterozygosity
M	Mitosis
MMC	mitomycin C
MMR	Mismatch Repair
MRE11	Meiotic Recombination 11 Homologue A (*S. cerevisiae*)
MSI	Microsatellite Instability
NBS	Nijmegen Breakage Syndrome
Neo	Neomycin Phosphotransferase

NHEJ	Non-Homologous End Joining
nt	Nucleotide
LOH	Loss of heterozygosity
LTGC	Long tract gene conversion
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PIKK	Phosphatidyl-inositol-3-kinase-like Protein Kinase
PMSF	Phenyl Methyl Sulphonyl Fluoride
RDS	Radio-resistant DNA synthesis
RNA	Ribonucleotide Acid
RNAse	Ribonuclease
mRNA	messenger Ribonucleotide Acid
RTase	Reverse Transcriptase
RT-PCR	Reverse transcription PCR
SCE	Sister chromatid exchange
S. cerevisiae	*Saccharomyces cerevisiae*
STGC	Short tract gene conversion
S-phase	Synthesis phase
SDSA	Synthesis-dependent strand –annealing
SSA	Single-strand annealing
Tdr	Thymidine
TEMED	N,N,N',N' Tetramethylethylenediamine
TGFβRII	Transforming Growth Factor β Receptor II
SDS	Sodium Dodecyl Sulphate
v/v	Volume:Volume Ratio
w/v	Weight:Volume Ratio
UV	Ultraviolet

Amino Acid Abbreviations

3 letter code	1 letter code	Amino Acid
Ala	A	Alanine
Arg	R	Arginine
Asp	D	Aspartic Acid
Asn	N	Asparagine
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic Acid
Gly	G	Glycine
His	H	Histidine
Iso	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

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CHAPTER ONE: INTRODUCTION

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1.1 Genomic Instability and Cancer.

The transformation of a somatic cell to a cancerous one is a multi-step process that is characterised by the accumulation of mutations in genes responsible for controlling cell growth and proliferation. The exact number of mutations required for neoplastic transformation is unknown. However, it is proposed that as many a six to eight mutations may be necessary for the development of an invasive tumour (Renan M.J., 1993).

In addition to acquiring numerous mutations, cancerous cells also exhibit increased spontaneous mutation rates. A study used the *hprt* locus to measure mutation rates in untransformed cells and showed that spontaneous mutations occur between 1.4×10^{-10} to 1×10^{-9}/nucleotide/cell generation (Loeb, L., 1991). This frequency is increased by up to 100-fold in neoplastic cells, although the effect seems to be cell type specific (Seshadri *et al.,* 1987; Eshleman *et al.,* 1995 and Bhattacharyya *et al.,* 1994). Cells displaying this 'mutator phenotype' have a high probability of incurring mutations in proto-oncogenes and tumour suppressor genes. Subsequently through its evolution, a cancerous cell may accumulate multiple molecular alterations including gene amplifications, insertions/deletions, rearrangements and point mutations (Cooper *et al.,* 1998).

The gross genetic abnormalities observed in cancerous cells have led to the concept that neoplastic cells are genetically unstable with genomic instability helping to drive tumour development. This observation is illustrated by the increased incidence of neoplasia associated with genetically unstable disorders (reviewed in van Gent *et al.,* 2001 and Levitt and Hickson, 2002). Both abnormal DNA repair (mutational instability) and chromosomal abnormalities (chromosomal instability) can give rise to such

instability. Mutational instability is characterised by point mutations or small deletions and is usually associated with mismatch repair defects (Jacoby *et al.*, 1995; Konishi *et al.*, 1996; Aaltonen *et al.*, 1993 and 1994 and Baba S., 1997). Chromosomal instability, however, involves the loss or gain of whole chromosomes (or fragments of chromosomes), as well as the amplification of chromosomal sequences. This form of instability is often associated with the inactivation of tumour suppressor genes or the activation of proto-oncogenes (reviewed in van Gent *et al.*, 2001). Both of these forms of progressive instability are discussed in detail below.

1.2 Mutational Instability

Eukaryotic cells have a number of repair pathways involved in the repair of abnormal DNA structures. One such pathway is the DNA Mismatch Repair (MMR) pathway. Such a pathway is required to repair errors present in replicated DNA as well as regulating recombination intermediates (reviewed in Buermeyer *et al.*, 1999 and Harfe and Jinks-Robertson, 2000). Effective MMR must not only be able to identify the mismatch present in 'normal' DNA, but it must also be able to identify and correct the 'wrong' base. The existence of several MMR pathways correlates with the repair of various mismatched abnormalities (Karran and Bignami, 1999). For example, the 'long patch' MMR pathway repairs relatively long stretches of DNA, whereas the more specialised, 'short patch' MMR correction pathway is responsible for the specific repair of G: T and A: G mispairs (Karran and Bignami, 1999).

Substrates that initiate MMR processes include: mispaired bases (that can arise during DNA replication) as well as mismatches that are present in heteroduplex recombination intermediates. In addition, MMR proteins also have a direct role in the post-replicative repair of DNA polymerisation errors

that can subsequently cause insertions or deletions of bases within repetitive coding sequences.

Elucidation of the protective role of the MMR system in preventing human cancer has led to a better understanding of the processes involved in the repair of mismatched bases. Cell lines derived from tumours that are deficient in MMR have been shown to exhibit at least three distinct mutator phenotypes. Firstly, the rate of mutations that arise due to the gain or loss of bases in repeated sequences is dramatically increased in a MMR – deficient background (this phenomenon is often termed microsatellite instability) (Gragg *et al.*, 2002; Parsons *et al.*, 1995; Shibata *et al.*, 1994 and Bhattacharyya *et al.*, 1994). Secondly, the rate of spontaneous mutations at specific loci e.g. hprt and aprt, is also dramatically increased in MMR -deficient cells (Battacharyya *et al.*, 1994; Kat *et al.*, 1993 and Eshleman *et al.*, 1995 and Phear *et al.*, 1996). This effect, however, seems to strongly depend on the growth conditions and the cell type. For example, MMR -deficient cells grown at a high density exhibit a more pronounced phenotype than the same cells grown in culture conditions which allows for rapid growth (Richards *et al.*, 1997). Thirdly, biochemical and functional studies have shown that the MMR proteins are also involved in cell cycle checkpoints (in particular the S- and G2/M-checkpoints) and apoptosis events (Yan *et al.*, 2001 and Brown *et al.*, 2003). In addition to the three stated phenotypes, studies have also indicated that MMR –deficient cells are resistant to DNA alkylating agents or base analogues such as 6-thioguanine and cisplatinum (Aebi *et al.*, 1996 and Fink *et al.*, 1996).

1.2.1 The E-coli MutHLS system.

The proteins and mechanisms involved in MMR have been conserved from bacteria through to humans. The *E.coli* MutHLS MMR system has been largely characterized through the analysis of purified proteins. This has

subsequently allowed the MMR reaction to be reconstituted *in vitro* (reviewed in Modrich and Lahue, 1996). Within the *E.coli* 'long-patch' MMR system, the MutS protein (acting as a homodimer) initially recognizes and binds onto base/base mismatches and small (up to 4 nucleotides) insertion/deletion loops that have escaped DNA polymerase proofreading (Parker and Marinus, 1992 and Modrich, P. 1991, 1996 and 1997). Studies with deletion derivatives of MutS have suggested that the N-terminus is responsible for binding onto the mismatched DNA, whilst the C-terminus is responsible for protein dimerisation as well as ATP binding and hydrolysis (Wu and Marinus, 1999). The binding of MutS to DNA subsequently recruits the MutL homodimer, which is responsible for coupling mismatch recognition to downstream processing events. MutL protein dimerisation is also achieved via interactions between the C-terminal regions, whilst the N-terminus contains the ATP binding and hydrolysis domains (Ban and Yang, 1998a and 1998b). Association of MutL with MutS enhances the ATP hydrolysis-dependent translocation properties of MutS and also stimulates the activities of the MutH endonuclease (Au *et al.,* 1992; Ban and Yang, 1998c and Hall and Matson, 1999). MutH is methylation-sensitive and is responsible for discriminating between template DNA strands and newly replicated unmethylated daughter strands (Welsh *et al.,* 1987). In addition, MutH is also responsible for cleaving DNA 5' to such hemimethylated GATC sequences on the nascent strand (Au *et al.,* 1992 and Hall and Matson, 1999). The cleaved DNA is then targeted by the UvrD (MutU) helicase, which unwinds the duplex DNA, beginning at the nick introduced by MutH and proceeding past the mismatched containing DNA (Dao and Modrich, 1998; Hall *et al.,* 1998 and Yamaguchi *et al.,* 1998). Single-strand specific exonucleases (e.g. RecJ, ExoVII, ExoI or ExoX) are thought to subsequently degrade DNA until the mismatch is removed (reviewed in Modrich and Lahue, 1996 and Viswanathan and Lovett, 1998). The MMR process is completed by re-synthesis and re-ligation. Such processes are thought to be predominantly conducted by the Single-strand binding (SSB)

protein, DNA polymerase III holoenzyme and DNA ligase (Modrich, P., 1991 and Modrich and Lahue, 1996).

1.2.2 The Eukaryotic MMR system.

MutS Homologs

All eukaryotic organisms studied to date contain multiple MutS homologs that are often referred to as MSH proteins (termed MSH1-6). However, only three of the six such MutS homologs are thought to participate in the MMR process. Chi and Kolodner (1994) demonstrated that yeast MSH1 encodes a mitochondrial targeting sequence and is specifically involved in the correction of mismatches present in the mitochondria. To date, no MSH1 mammalian homologue has been reported. Furthermore, the MSH4 and MSH5 proteins form a heterodimer that is proposed to be specifically involved in meiotic recombination and not in mismatch recognition (Ross-MacDonald and Roeder, 1994; Hollingsworth *et al.,* 1995 and Hunter and Borts, 1997).

The MSH2 protein is essential for mediating the repair of all types of mismatches that occur in mammalian cells. The MSH3 and MSH6 proteins, however, both confer substrate specificity when complexed with MSH2 (Jiricny, 1998; Kolodner and Marsischky, 1999; Genschel *et al.,* 1998; Macpherson *et al.,* 1998; Palombo *et al.,* 1996 and Acharya *et al.,* 1996). The importance of MSH2 was illustrated in mammalian cell lines deficient in the protein, which exhibited MSI, a six to twelve-fold increase in mutation rate at the *hprt* locus as well as resistance to 6-thioguanine (Reitmair *et al.,* 1997). The mammalian MSH2 protein interacts (via the C-terminus) with either MSH3 or MSH6 to form two distinct heterodimers, termed MutSß and MutSα respectively (Gurrette *et al.,* 1998). The MutSα heterodimer

recognizes single base/base mismatches as well as small and large insertion/deletion loops (Figure 1.1, Page 9). The MutSβ complex, however, recognizes predominantly large (two-eight base pair) insertion/deletion loops (Figure 1). In addition, MutSβ seems to have overlapping substrate specificities with MutSα, with respect to being able to recognize one base pair insertion/deletion loops (Alani, 1996; Habraken *et al.,* 1996 and Marsischky *et al.,* 1996). Interestingly, studies using human MSH2, MSH3 and MSH6 proteins have showed that the MutSα heterodimer is present in a six-fold molar excess over the MutSβ heterodimer at any given time in cultured cells (Drummond *et al.,* 1997 and Marra *et al.,* 1998). Furthermore, the same study also showed that the MSH2 protein was almost entirely (90%) expressed as part of the MutSα complex. These results were consistent with observations that MutSα recognizes all types of mismatches and that inactivation of MSH6 has a greater detrimental effect than the inactivation of MSH3 (Bhattacharyya *et al.,* 1994; Risinger *et al.,* 1996 and Marra *et al.,* 1998).

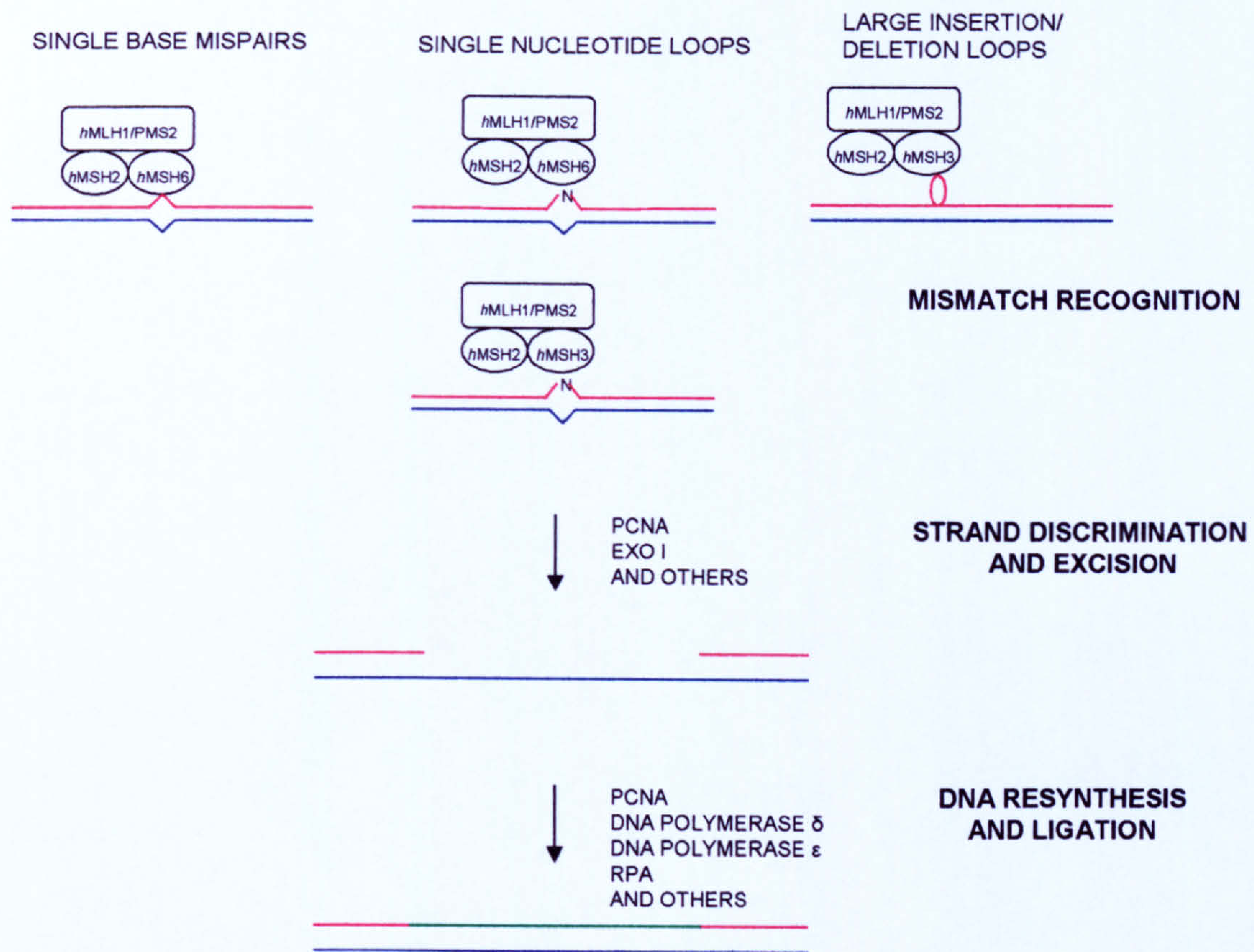

Figure 1.1 MMR processes in eukaryotic cells. *Adapted from Buermeyer et al., 1999*

Recent biochemical studies have indicated that the C-terminus of both MSH2 and MSH6, like the MutS bacterial homolog, contain highly conserved Walker A and B boxes (Hughes and Jiricny, 1992; Iaccarino *et al.,* 1998 and Studamire *et al.,* 1998). These Walker motifs are responsible for ATP binding and hydrolysis, a function that is essential for MMR activity both *in vitro* and *in vivo* (Alani *et al.,* 1997; Iaccarino *et al.,* 1998 and Studamire *et al.,* 1998). Gradia and co-workers (2000) proposed a model in which the MutSα complex exists in a mismatch binding competent ADP-bound state. The recognition of mismatched DNA was subsequently proposed to stimulate an ADP-ATP exchange reaction. Furthermore the addition of ATP in mismatch binding assays has suggested that the MutSα complex undergoes a conformational change that results in the release of

mismatched DNA (Alani *et al.,* 1997; Habraken *et al.,* 1998 and Studamire *et al.,* 1998). This was also confirmed when an ADP-bound form of the MutSα complex was found to be capable of binding to mismatched DNA (Lamers *et al.,* 2000 and Junop *et al.,* 2001). Furthermore, the subsequent ADP-ATP exchange reaction resulted in the release of MutS from a DNA site (Allen *et al.,* 1997). Two models have been proposed to explain the relevance of ATP hydrolysis in MutS and the MSH2/MSH6 complex. Fishel, R. (1998) proposes that the MutS protein is constantly re-cycled, enabling MutS (and MutSα) to translocate away from the mismatch in an ATP hydrolysis-independent manner in order to possibly interact with downstream processing proteins. However Allen and co-workers (1997) have used electron microscopy to suggest that ATP-hydrolysis is required for the translocation of MutS, resulting in a loop-like structure being formed, where the mismatched site is located within the loop, and the MutS homodimer holds the base of the loop together. Thus as yet the precise effect of ATP hydrolysis remains unclear.

MutL Homologs

Eukaryotic cells also possess multiple MutL homologs (termed MLH1, MLH3, PMS2 (PMS1 in yeast) and PMS1 (MLH3 in yeast)) that are all thought to act in a MSH2-dependent pathway (Habraken *et al.,* 1997 and 1998 and Prolla *et al.,* 1994). The MLH1 protein was found to form a pair wise interaction with each of the other three MutL homologs and is therefore central to the MMR 'coupling' process (Wang *et al.,* 1999; Li G. and Modrich, 1995 and Lipkin *et al.,* 2000). All four MutL homologs participate as distinct heterodimers and are involved in the repair of various intermediates. Furthermore, functional and biochemical studies have proposed that the MutLα (MLH1/PMS2) heterodimer has a greater role in MMR than the MutLβ (MLH1/PMS1) heterodimer (Kato *et al.,* 1998 and Raschle *et al.,* 1999). However, unlike the role of MutL in the bacterial

MutHLS system, the roles of the MLH1/PMS2 and MLH1/MLH3 heterodimers have not been fully characterized in the mammalian system, although clear involvement in MMR is proposed (Flores-Rozas and Kolodner, 1998).

The suggested role of MutL in a 'coupling' reaction was confirmed when the C-terminus of MutL was found to directly interact with the MutH endonuclease as well as the UvrD helicase (Hall *et al.,* 1998; Yamaguchi *et al.,* 1998 and Dao and Modrich, 1998). Indeed recent biochemical studies have shown that the human MutLα complex is able to interact with MSH2, MutSα, MutSβ and PCNA (Prolla *et al.,* 1994; Habraken *et al.,* 1998; Habraken *et al.,* 1997 and Gu *et al.,* 1998). Therefore, by analogy to the bacterial MutL protein, this complex is thought to couple mismatch recognition to strand discrimination, excision and re-synthesis steps. The effects of both MLH1 and PMS2 proteins, however, maybe stimulated by ATP binding and/or hydrolysis (which is limited to the NH_3 terminus) (Ban and Yang, 1998).

Strand Discrimination, DNA Excision and Re-synthesis processes

The mechanism of action of additional proteins involved in mammalian MMR processes is currently unknown. Two mechanisms have been proposed by which strand discrimination is achieved in the eukaryotic MMR system. Hare and Taylor (1985) initially suggested, by analogy to the *E.coli* MutHLS system, that methylation of the 5-position of cytosine in CpG islands was a sufficient enough signal to discriminate between the two strands in human MMR. However, biochemical evidence suggests that single-stranded nicks, such as those transiently occurring in Okazaki fragments during semi-conservative DNA replication, provides a better strand discrimination mechanism between daughter and template strands (Karran and Bignami, 1999).

The proliferating cell nuclear antigen (PCNA) protein is also implicated in various MMR steps including strand discrimination, excision and re-synthesis (Johnson *et al.,* 1996 and Umar *et al.,* 1996). This protein can not only directly interact with human MutS and MutL homologs, but is also required in both the excision and re-synthesis steps of the MMR process (Kleczkowska *et al.,* 2001; Gu *et al.,* 1998; Johnson *et al.,* 1996; Kokoska *et al.,* 1999 and Umar *et al.,* 1996). In addition functional studies, in which the yeast PCNA protein was mutated, resulted in increased frameshift mutations (Johnson *et al.,* 1996 and Umar *et al.,* 1996). Aside from PCNA, another proposed MutH homolog is MED1, which is a human methylation-sensitive endonuclease that causes MSI when over expressed (Bellacosa *et al.,* 1999); however the involvement of MED1 in MMR remains unclear.

The excision and re-synthesis steps of the eukaryotic MMR pathway have been proposed to be completed by ExoI, DNA polymerase δ and DNA polymerase ε (Szankasi and Smith, 1995) (Figure 1.1, Page 9). Recent biochemical and functional studies have shown interactions between these proteins and those involved in the MMR process (Tishkoff *et al.,* 1997). Furthermore, an increase in mutation rates was observed in cells expressing inactivated forms of these proteins (Tran et al., 1999 and Qiu *et al.,* 1999).

1.2.3 Mutational instability and predisposition to cancer.

MMR deficiency is correlated with a highly penetrant cancer predisposition syndrome termed hereditary non-polyposis colorectal cancer (HNPCC). HNPCC, otherwise known as Lynch syndrome, is responsible for approximately 5% of all colorectal cases. Such patients usually develop early onset tumours that are predominantly located in the colon. Less common cases have also been noted to have cancer in the endometrium, stomach, small intestine and ovary (Lynch *et al.,* 1998 and Peltomaki and

de la Chapelle, 1997). Mutations in MSH2 and MLH1 are associated with 80-90% of HNPCC cases, although some cases do also have mutations in PMS1, PMS2 and MSH6 (Wagner *et al.,* 2003; Lynch and de la Chapelle, 2003 and Buermeyer *et al.,* 1999). A similar pattern is observed in mice lacking either MSH2 or MLH1. Such mice are predisposed to develop an early onset of tumours ranging from gastro-intestinal tumours to lymphomas, skin tumours and sarcomas (Reitmair *et al.,* 1997 and Prolla *et al.,* 1998). Furthermore, consistent with that seen in humans, mice lacking MSH6, MSH3, PMS1 or PMS2 exhibit milder phenotypes (de Wind *et al.,* 1999; Baker *et al.,* 1995 and Prolla *et al.,* 1998).

A high proportion of HNPCC patients are heterozygous for recessive, germline mutations in MMR genes. A second mutation of the functional allele, predominantly promoted by loss of heterozygosity, inactivates the MMR system and leads to an increase in mutation rates and tumour development (Leach *et al.,* 1993; Shibata *et al.,* 1994; Parsons *et al.,* 1993; Boyer, *et al.,* 1995; Loeb L., 1991; Bhattacharyya *et al.,* 1994; Eshleman *et al.,* 1995; Lazar *et al.,* 1994; Markowitz *et al.,* 1995; Rampino *et al.,* 1997 and Huang, J., *et al.,* 1996). In sporadic cases, however, mutations in MMR genes are rarely observed (approximately 15% of cases, Boland, C., 1997). This indicates that alternative mechanisms are responsible for MMR inactivation. Reports have suggested that the MMR system is inactivated in such cases as a result of gene silencing due to hyper-methylation of the promoter region (Wheeler *et al.,* 1999 and Esteller *et al.,* 1996).

Consistent with the phenotype observed in MMR –deficient cell lines, tumours derived from HNPCC patients frequently exhibit genomic instability which can be detected by changes in the length of repeated sequences (Aaltonen *et al.,* 1993). Furthermore, microsatellite unstable tumours selectively contain frameshift mutations in tumour suppressor genes (e.g.

TGF-βRII, BAX) and DNA repair genes (e.g. MSH3, MSH6 and BLM), thus promoting tumour development (McPherson *et al.,* 1994).

1.3 Chromosomal Instability

Chromosomal instability is characterised by gross rearrangements of chromosomes. Such instability includes the loss or gain of whole chromosomes or fragments of chromosomes as well as the amplification of chromosome fragments. A majority of chromosomal instability disorders arise due to mutations in either tumour suppressor genes or proto-oncogenes. Tumour suppressor genes have been further categorized into two groups, namely 'caretakers' and 'gatekeepers' (reviewed in Levitt and Hickson, 2002). Alterations in expression, or mutations in 'gatekeeper' genes, directly give rise to uncontrolled cell proliferation. Mutations in 'caretaker' genes (e.g. MMR genes), however, give rise to genetic instability by increasing the frequency of mutations occurring within 'gatekeeper' genes.

Within normal living cells, chromosomal DNA is often subjected to mechanical stress as well as chemical modification. Such events have recently been documented to lead to chromosomal instability due to the presence of lethal breaks in one or both strands of the double helix (reviewed in van Gent *et al.,* 2001). Erroneous rejoining of DNA DSBs gives rise to genetic alterations including gene deletion and translocations. Such events can subsequently result in inactivation of tumour suppressor genes or the activation of oncogenes (Weinberg, R., 1988 and Pierotti and Dragani, 1992). Cells are acutely sensitive to DSBs and as few as one or two such lesions are sufficient to trigger the activation of the ATM protein kinase, one of the prime regulators of the DNA damage response (Bakkenist and Kastan, 2003). Generally, DNA damaging agents are classified in two categories, namely endogenous and exogenous agents.

Both forms of agent can subsequently introduce mutations that will affect later generations or even lead to cell death.

Endogenously induced damage can occur at any stage of the cell cycle, e.g. in non-dividing cells, during DNA replication and during mitosis (see Pfeiffer *et al.,* 2000 for review). The major sources of endogenous DNA damaging agents include: water, oxygen, topoisomerases, and errors in base pairing occurring during replication at fragile sites (i.e. micro- or minisatellite sequences) (Sutherland *et al.,* 1998 and Debrauwere *et al.,* 1999). In addition, normal aerobic metabolism gives rise to active oxygen species, such as hydroxyl and super oxide radicals, which particularly target guanine and thymine residues within duplexed DNA. Another source of endogenous DNA damage includes the non-enzymatic methylation of nucleotides which subsequently produce adducts such as O^6-methylguanine and 3-methyladenine (reviewed in Pfeiffer *et al.,* 2000).

Ultraviolet irradiation (UV), ionizing irradiation (IR) and certain chemotherapeutic agents are some of the many examples of exogenous DNA damaging agents (see Pfeiffer *et al.,* 2000 for review). Ionizing irradiation (such as γ-rays and X-rays) is capable of producing reactive oxygen species, as well as producing lethal double- and single-stranded breaks in DNA (Ward, J. 1985). In addition various endonucleases are also capable of inducing DSBs when acting on DNA (Bryant P., 1988 and Thacker J., 1994).

In response to the threat of DNA DSBs, cells have a number of DNA repair mechanisms to ensure the fidelity of the genome and thereby prevent chromosomal instability. In mammalian cells, the Homologous recombination repair (HRR) (which predominates during the late S/early G2 phases of the cell cycle) and Non-Homologous End Joining (NHEJ) (which predominates during the G0, G1 and early S-phases of the cell cycle)

pathways are involved in the repair of such breaks (Takata *et al.,* 1998 and Essers *et al.,* 2000). Biochemical and functional evidence suggests that proteins involved in these two repair pathways not only compete for binding onto the ends of DSBs, but also are capable of acting sequentially (Delacote *et al.,* 2002).

1.3.1. Mechanisms of Non-Homologous End Joining (NHEJ).

The mechanisms involved in NHEJ mediated repair require no homology with a second DNA duplex and little or no homology between the two broken DNA ends. Subsequently NHEJ mediated repair products frequently contain DNA alterations. This fast acting pathway predominates in mammalian cells as indicated by studies using genomically integrated DNA substrates. Such studies showed that NHEJ mediated events occurred between 100-10,000 fold more frequently than HRR events in mammalian cells (Roth *et al.,* 1985). The paradox as to why the 'erroneous' NHEJ pathway predominates over HRR is that, although it frequently results in alterations of DNA sequences around the break point, the chances of these alterations affecting coding regions remains minimal.

The simplest form of the two NHEJ processes is the re-ligation of two blunt or complementary DNA ends. This process does not depend on base pairing interactions and subsequently restores the original sequence (Wilson *et al.,* 1982 and Roth and Wilson, 1986) (Figure 1.2). This process is dependent upon the Ku70/80 heterodimeric complex and, depending on the structure of the ends, results in the production of either 'fill-in' or 'overlap' junctions (Liang and Jasin, 1996; Critchlow and Jackson, 1998 and Feldmann et al., 2000). The production of two non-compatible DNA ends (such as those formed following exposure to irradiation) however, results in DNA re-sectioning to enable ligatable structures to be formed. This process occurs independently of the KU70/80 heterodimer and employs short

sequence homologies in order to direct re-joining (Roth and Wilson, 1986 and Roth *et al.,* 1991). Subsequently this results in an increase in deletion events. The factors involved in this second repair pathway are currently unknown (reviewed in Pfeiffer, 1998).

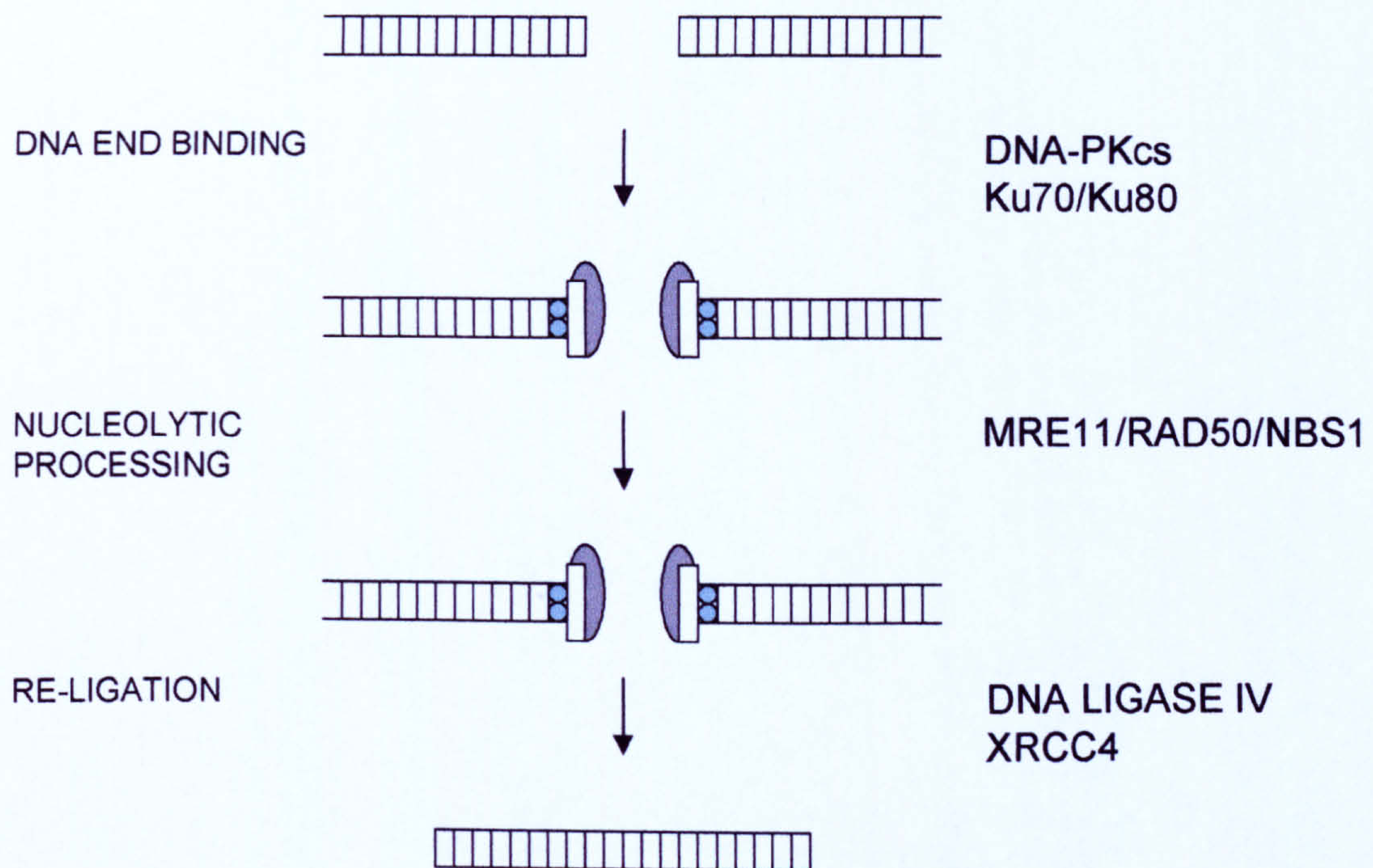

Figure 1.2 NHEJ mediated DNA double-strand break repair. Following formation of a DSB, the DNA-PKcs/Ku70/80 complex initially recognizes and binds onto DNA ends. Such ends are thought to be processed (possibly by the RAD50/MRE11/NBS1 complex), thus resulting in the addition or subtraction of base pairs. This step is followed by end to end ligation by the DNA ligase IV/XRCC4 complex. Therefore, as NHEJ does not make use of a homologous template for repair, this DSB-repair pathway is error prone. *(Adapted from van Gent et al., 2001).*

DNA damaging agents, such as irradiation, frequently cause DNA cross-links or produce DNA ends that are accompanied by additional base changes. These ends are thought to be exonucleolitically processed by the

MRE11/RAD50/NBS1 complex (described on Page 35) which seems not only to be involved in both NHEJ and cell signalling, but also in HRR processes (Goedecke *et al.*, 1999; Petrini, 2000 and Haber, 1998). The RAD50 component of this complex forms two long intra-molecular coiled coils that are thought to facilitate in finding the two ends of a DSB (de Jager *et al.*, 2001a and 2001b and 2002 and Chen *et al.*, 2001).

In mammalian cells, the re-sected DNA ends are subsequently recognized and protected from further degradation by the Ku70/80 heterodimer. The Ku70/80 heterodimer, along with the XRCC4 protein, aligns and stabilizes end-joining intermediates (Liang and Jasin, 1996; Jeggo, P., 1998 and Kabotyanski *et al.*, 1998). Following the alignment and stabilization of DNA ends; the Ku70/80 heterodimer subsequently recruits and binds with a DNA dependent protein-kinase catalytic subunit called DNA-PKcs (Smith and Jackson, 1999). The presence of DNA ends has been demonstrated to activate the kinase domain of DNA-PKcs, thus enabling it to phosphorylate a number of substrates required for repair, including XRCC4 (Critchlow *et al.*, 1997 and Grawunder *et al.*, 1997). However, the significance of such phosphorylation events remains unclear at present. It is possible that the DNA-PKcs protein could assist with trimming and tethering the two DNA ends together while they are re-joined. Alternatively, it could be responsible for regulating other repair molecules such as those involved in signal transduction. Finally the remaining gaps in the DNA sequence are filled, by an as yet unknown polymerase and the ends are ligated by DNA ligase IV. This ligase has been found to strongly interact with the XRCC4 protein which is a substrate for the DNA-PKcs kinase *in vitro* (Critchlow *et al.*, 1997 and Grawunder *et al.*, 1997).

Cells deficient in any gene involved in NHEJ display extremely similar phenotypes. For example, mutation analysis revealed that mutations in the Ku70/Ku80 heterodimer, DNA-PKcs or the XRCC4/DNA ligase IV

heterodimer conferred extreme hypersensitivity to ionizing irradiation (Karanjawala *et al.*, 2002).

1.3.2. Mechanisms of Homologous recombination repair (HRR).

HRR is defined as any exchange of genetic material between homologous DNA sequences. This highly accurate repair mechanism is essential during meiosis (e.g. for the inherent separation of chromosomes) and mitosis (Roeder G., 1997). Several homology dependent pathways exist and can be sub-divided into conservative (e.g. gene conversion and break-induced replication) and non-conservative (e.g. single-strand annealing) processes. Conservative repair processes involve the DSB being accurately repaired through copying sequence information of a homologue or sister chromosome in order to restore the original sequence at the break. Pathways included within this type of repair process include double-strand break repair (DSBR), synthesis-dependent strand-annealing (SDSA) and break-induced replication (BIR) (Figure 1.3, Page 21). Non-conservative repair processes, such as single-strand annealing (SSA), occur when two direct repeats interact with each other such that one repeat copy and the subsequent intervening sequence are lost (Figure 1.4, Page 23). The preference of one pathway over another is dependent on a number of factors including the position of the homologous partner, the initiation event and the length of homology of recombinant molecules.

The double-strand break repair (DSBR) pathway was originally proposed in yeast by Resnick and co-workers (1976) and later expanded by Szostak and colleagues (1983). Similar processes are also proposed to occur in mammalian cells. Within this pathway, recombination events are initiated by a DSB (Sun *et al.*, 1989 and Cao *et al.*, 1990). The DNA ends are proposed to be processed by the MRE11/RAD50/NBS1 complex. However, this complex has 3'-5' exonuclease activity which is the opposite polarity to

what is needed (Usui *et al.,* 1998; Furuse *et al.,* 1998; Paull and Gellert, 1998; Trujillo *et al.,* 1998 and Trujillo and Sung, 2001). The subsequent re-sectioning of DNA produces long 3' single-stranded tails which are used to invade a homologous duplex and initiate synthesis (Sun *et al.,* 1991). Pairing of the invading ssDNA with template dsDNA, results in a strand exchange reaction that subsequently generates heteroduplex DNA (Goyon and Lichten, 1993 and Nag and Petes, 1993). Thus in this DSBR model, both 3' ends are able to prime new DNA synthesis from the two strands of the donor template (McCulloch *et al.,* 2003). The key steps of strand invasion and exchange are mediated by the proteins included within the RAD52 epistasis group. The double-strand exchange reactions lead to the formation of two, four-way junctions (called Holliday junctions) (Collins and Newlon, 1994 and Schwacha and Kleckner, 1994). Such junctions are capable of branch migrating and thus expanding the heteroduplex region. These gene conversion events may not only affect a single gene (short tract), but may cover several contiguous genes (long tract). Subsequent resolution of Holliday junctions, in either the same or opposite direction, results in non-crossover and crossover products being formed respectively.

The SDSA model was initially proposed as an alternative to the DSBR model as a majority of mitotic gene conversion events were found not to be associated with crossover (Schiestl *et al.*, 1988 and McGill *et al.,* 1990). Within this model, following 3' ssDNA invasion, the newly synthesized DNA strands (short tracts) are displaced from the invaded DNA template and allowed to re-anneal to each other. Thus this differs from DSBR, in that all the newly synthesized sequences are on the same molecule.

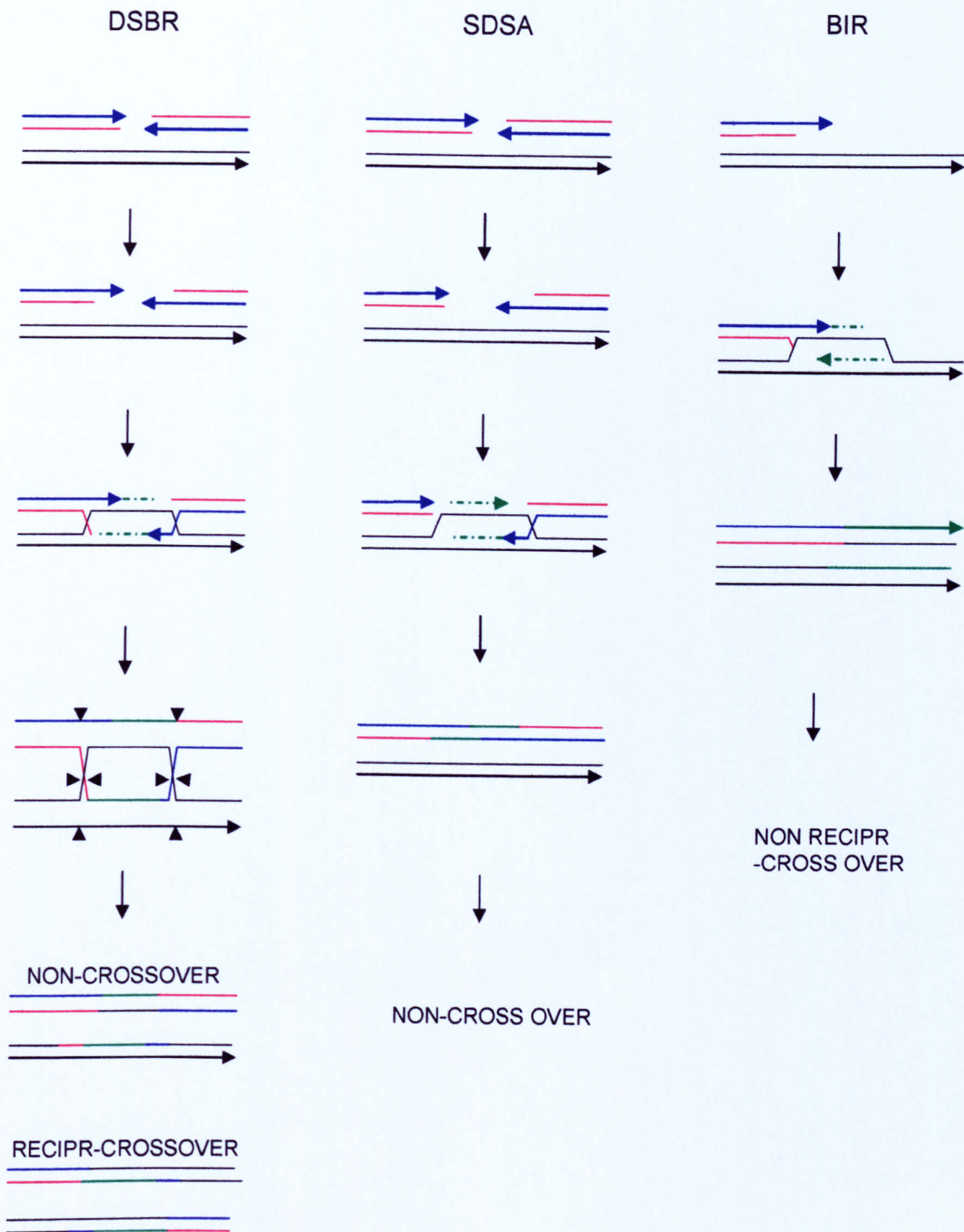

Figure 1.3 Three proposed models for HRR in mammalian cells. *Adapted from* Pfeiffer *et al.*, 2000

The BIR pathway was initially proposed for T4 phage (Mosig, 1987). Within this pathway, only one DSB end invades the homologue or sister chromatid. The invading 3' end subsequently primes DNA synthesis which can cover long DNA fragments. BIR is capable of proceeding to the end of a chromosome or alternatively can be converted into a gap repair (DSBR) if the other DNA end invades the homologous template.

The non-conservative SSA pathway is the simplest of all the homology directed repair pathways. This pathway predominates if the DSB occurs between two flanking homologous DNA repeats and frequently results in a deletion event. The DSB is initially resected by exonucleases, as described above, until large sections of sequence flanking the break are exposed in the form of long 3'single-stranded tails (Figure 1.4, Page 23). The DNA repeats subsequently undergo strand annealing.

The removal of non-homologous 3' ends during both GC and SSA is proposed to require some MMR proteins as well as the ERCC1/XPF heterodimer (Zhang, N. *et al.*, 2002; Chipchase and Melton, 2002 and Adair *et al.*, 2000). *In vitro*, this complex cuts at the junction of duplex DNA and a 3' single stranded extension. SSA events occur independently of RAD51 but requires RAD52 instead (van Dyck *et al.*, 1999 and 2001). This is consistent with the DNA end binding and strand annealing activities of this protein (Mortensen *et al.*, 1996).

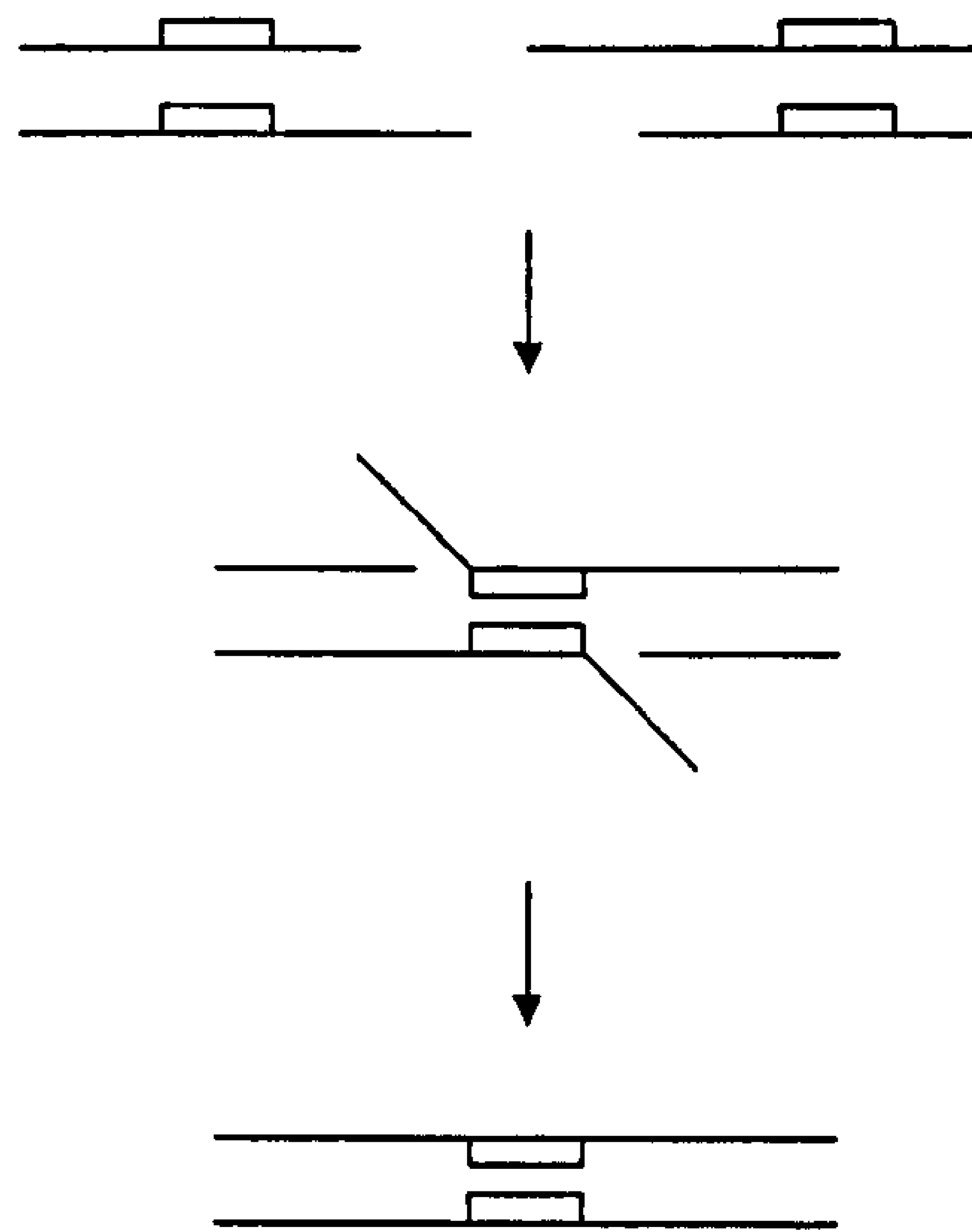

Figure 1.4 Single-strand annealing mediated repair of a DNA DSB. Within this non-conservative form of HRR, two DSB ends interact directly at homologous repeated units. Subsequently, one repeat unit and the intervening sequence are lost due to nucleolytic trimming.

1.3.2 1 Proteins involved in HRR - RAD52 epistasis group

RAD51

The eukaryotic RAD51 protein has important roles during both mitotic and meiotic recombination repair processes (Shinohara *et al.,* 1992). This 37kDa protein belongs to a general family of recombinases, which like the RecA bacterial homolog, forms helical filaments. The formation of such helical filaments enable RAD51 to catalyse homologous DNA pairing in the 'pre-synaptic' phase of recombination and subsequently initiate strand exchange activities (Shinohara *et al.,* 1992 and 1993 and Baumann *et al.,* 1996) (Figure 1.5, Page 25). RAD51 has been shown to interact with many proteins including RPA, RAD52, RAD54, XRCC3, BRCA2, BLM and p53 (reviewed in Thompson and Schild 2001). Furthermore, knockout RAD51

mutations in both chicken and mammalian cells, is lethal (Tsuzuki *et al.,* 1996; Lim and Hasty, 1996 and Sonoda *et al.,* 1998). Taken together, these results therefore underline the importance of this protein to normal cellular function.

Biochemical studies have revealed that members of the recombinase family, from E-coli to eukaryotes, contain highly conserved Walker motifs which are responsible for ATP binding and hydrolysis (Yu et al., 2001; Benson et al., 1994; Ogawa et al., 1993). Mammalian cells expressing an ATP-hydrolysis defective RAD51 protein exhibit increased sensitivity to both MMC and IR, as well as a decreased rate of spontaneous sister chromatid exchange events (Stark *et al,.* 2002). Furthermore, spontaneous inter-chromosomal recombination events were diminished in *Saccharomyces cerevisiae* which expressed an ATP-hydrolysis deficient form of RAD51 (Shinohara *et al.,* 1992). Together these observations imply that ATP hydrolysis is a key step for RAD51 to mediate recombination processes.

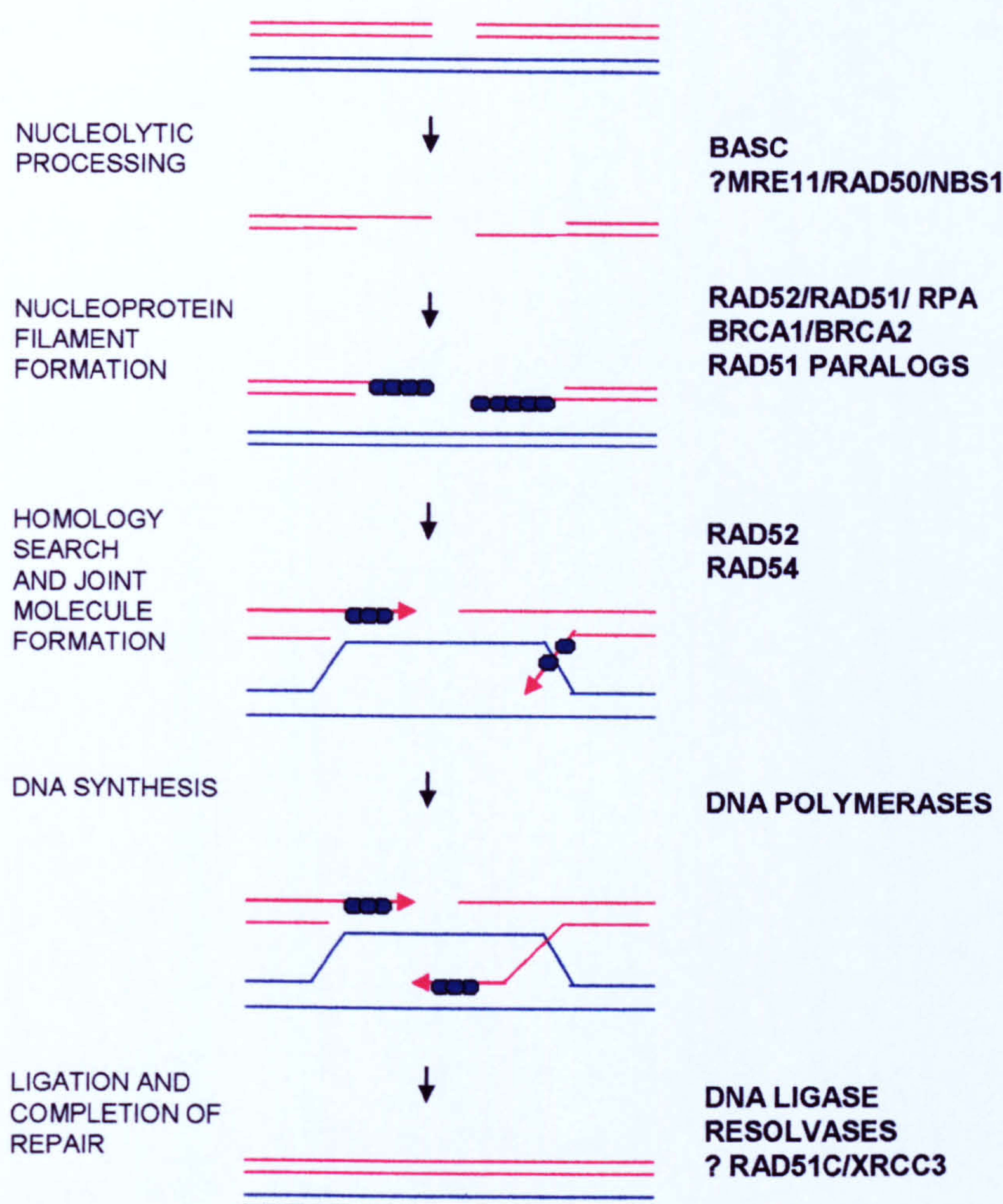

Figure 1.5 Homologous recombination mediated DNA DSB repair. The formation of a DSB activates the ATM kinase (and other proteins included within the BASC complex). Subsequently, the DNA ends are processed (possibly by the RAD50/MRE11/NBS1 complex), resulting in the generation of ss-3' overhangs. Such ends are recognized by RAD52 and/or RPA, which subsequently enable RAD51 to form nucleoprotein filaments. Other proteins involved at this step include the RAD51 paralogs (namely, RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) and BRCA1/BRCA2. The RAD51 nucleoprotein filaments then search for homologous duplex DNA. Following such searching, DNA strand exchange generates a joint molecule between the homologous damaged and undamaged duplexed DNA. This step is thought to be stimulated by the RAD52 and/or RAD54 proteins. DNA polymerases and accessory factors fill in the break. Finally, ligation and resolution of the recombination intermediates complete this accurate repair process. *Adapted from van Gent et al., 2001*

Protein alignment studies between RecA and RAD51 have shown that outside of the homologous core (containing the ATP binding sites), these two proteins display poor homology. RecA has a C-terminal extension that is absent in RAD51 (Shinohara *et al.,* 1992 and 1993). This region of the RecA protein is thought to be responsible for binding onto dsDNA regions (Kurumizaka *et al.,* 1996). Eukaryotic and *Saccharomyces cerevisiae* RAD51 proteins however, have an N-terminal extension that is absent in RecA. This extension has been shown to bind both single- and double-stranded DNA (Aihara *et al.,* 1997 and Yu, X. *et al.,* 2001). Furthermore, as both the N-terminus of RAD51 and the C-terminus lobe of RecA are mobile, this study also demonstrated that ATP mediated 'activation' of RAD51 causes the extension of RAD51 polymers. The eukaryotic RAD51 protein has been shown to assemble into right-handed nucleoprotein helical filaments in the presence of DNA in an ATP binding-dependent manner (Symington, L., 2002; Krejci *et al.,* 2003; Bianco *et al.,* 1998; Yu *et al.,* 2001; Sung, P., 1994 and Sung and Stratton, 1996). Filament formation subsequently catalyzes DNA pairing and strand exchange activities of RAD51, thus yielding heteroduplex DNA joints between homologous regions. The ATP hydrolysis reaction has been shown to cause the dissociation of RAD51 from complexes with DNA (Namsaraev and Berg, 1998). This step seems to be important either for completing the recombination process or for converting the filaments into RAD51 monomeric molecules thus enabling the re-cycling of the protein.

The strand transfer and heteroduplex DNA extension-RAD51 mediated reactions also involve a number of additional proteins, including the heterotrimeric ssDNA binding factor, RPA. RPA sequesters free ssDNA regions, which if left uncovered strongly inhibits the homologous pairing reaction as well as being responsible for the removal of secondary structures that may be present on ssDNA (reviewed in Thompson and

Schild 2001). Following the disruption of DNA secondary structures by RPA, the protein is thought to be displaced by RAD52.

Functional studies showing an involvement of RAD51 in HRR processes have been hampered as disruption of RAD51 in chicken DT40 cells, results in an increase in chromosomal breaks prior to cellular death (Tsuzuki *et al.,* 1996 and Sonoda *et al.,* 1998). A few approaches have, however, been developed to enable a clearer understanding of the expression and function of this protein in HRR. Expression studies have showed that, not only does the RAD51 protein form nuclear foci in the S and G2/M -phases of the cell cycle, but also that these foci rapidly become re-distributed to sites of DNA damage (Tashiro *et al.,* 1996; Yuan *et al.,* 2003 and Haaf *et al.,* 1995). In addition, Kim and co-workers (2001) showed that over-expression of wild type RAD51 and RAD52 in human and hamster cells causes an increase in frequency of spontaneous recombination events but a decrease in frequency of DSB induced HRR events. In addition, a similar approach was used to show that RAD51 prevents illegitimate HRR during DNA replication (Lundin *et al.,* 2003). Therefore it seems that the presence of RAD51 in excess has an inhibitory effect on DSB-induced HRR.

Finally it was shown, using a dominant negative form of RAD51, that this protein does not significantly control global DSBR events, but instead regulates the specific classes of recombination (i.e. strand invasion versus single-strand annealing) (Lambert and Lopez, 2000).

RAD51 paralogs

In addition to RAD51, five RAD51-like genes have also been discovered to be expressed in mammalian cells. These RAD51 paralogs were initially identified either due to their ability to functionally complement the X-ray sensitive hamster *irs1* and *irs1SF* cell lines (in the case of XRCC2 and

XRCC3) (Jones *et al.,* 1987; and Fuller and Painter, 1988), or due to data base searching for sequence similarities with XRCC2 and XRCC3 (in the case of RAD51B, RAD51C and RAD51D) (Albala *et al.,* 1997; Cartwright *et al.,* 1998; Dosanjh *et al.,* 1998 and Pittman *et al.,* 1998). All five paralogs share between 20 to 30% sequence homology with RAD51 and between one another. Such homology, however, is predominantly limited to the two nucleotide binding motifs (termed Walker Box A and B respectively) (Thacker, J., 1999 and Thompson and Schild, 1999). The importance of these motifs was illustrated in a study which showed that mutation of the conserved ATP binding domain in RAD51C severely impaired its function (French *et al.,* 2003). However experiments using *Saccharomyces cerevisiae* Rad55p and Rad57p (RAD51-related proteins) have shown that mutation of Walker box A in Rad55p but not in Rad57p disables the heterodimers function (Johnson and Symington, 1995). Taken together, these results suggest that ATP hydrolysis may be dispensable for function in some of the RAD51 paralogs.

Initially Schild and co-workers (2000) showed (using yeast two-hybrid and three-hybrid screening) a variety of interactions between the RAD51 paralogs. However, more recent biochemical studies have reported that the five paralogs co-precipitated as two distinct complexes both *in vitro* and *in vivo* (Masson *et al.,* 2001; Weise *et al.,* 2002 and Miller *et al.,* 2002). The authors proposed that one complex comprised of RAD51B, RAD51C, RAD51D and XRCC2, whereas the second complex comprised of RAD51C and XRCC3.

All five paralogs seem to play crucial roles in maintaining chromosomal stability as RAD51B, RAD51D or XRCC2 gene disruption in mice often results in early embryonic lethality (Shu *et al.,* 1999; Deans *et al.,* 2000 and 2003 and Pittman and Schimenti, 2000). However, viable knockout mutants of RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 have been generated

in chicken B lymphocyte DT40 cells and mouse embryonic fibroblasts (Deans *et al.,* 2003 and Takata *et al.,* 2001). Such studies have established similar phenotypic effects including elevated spontaneous chromosomal aberrations, extreme sensitivity to DNA cross-linking agents (such as MMC) and mild sensitivity to IR.

Both biochemical and functional studies suggest that the RAD51 paralogs are involved in the homology directed repair of DNA DSBs. For example, purified XRCC2/RAD51D and XRCC3/RAD51C heterodimers have been shown to bind to ssDNA and to catalyse homologous pairing between ssDNA and dsDNA regions (Masson *et al.,* 2001 and Kurumizaka *et al.,* 2003). The same studies also showed that, in a similar fashion to RAD51 and RAD52, both complexes form filamentous structures only in the presence of ssDNA. In addition, Yokoyama and co-workers (2003) purified RAD51B to show that the protein specifically binds to Holliday Junctions in the presence of ATP. Furthermore, the RAD51C/XRCC3 complex is also involved in the processing of Holliday junctions (Liu *et al.,* 2004)

In addition to the biochemical evidence, many functional studies have also been conducted which suggest a possible involvement of the RAD51 paralogs in HRR. Firstly, RAD51B$^{-/-}$ DT40 cells exhibit HRR deficiencies (with respect to intra-chromosomal gene conversion, gene targeting and sister chromatid exchange) in addition to being acutely sensitive to MMC and being unable to form RAD51 foci (Takata *et al.,* 2000). Secondly, French and co-workers (2002) showed that RAD51C$^{-/-}$ hamster cells exhibit a decrease in sister chromatid exchange events, an increase in iso-chromosomal breaks and a decrease in damage dependent RAD51 foci formation. The same group also used site-directed mutagenesis to show that RAD51C mutant cells are deficient in the homology directed repair of DNA DSBs mediated by gene conversion events (French *et al.,* 2003). Finally, both *irs1* (XRCC2 deficient) and *irs1SF* (XRCC3 deficient) cell lines

have been shown to exhibit between a 25 to 100-fold decrease in HRR frequency when recombination events were induced by a site-specific break (Pierce *et al.*, 1999 and Johnson *et al.*, 1999).

In addition, as the BCDX2 complex was shown to specifically bind to ssDNA as well as to single-stranded regions or nicks in duplexed DNA (Masson *et al.*, 2001) and the RAD51C/XRCC3 complex was shown to bind ssDNA (and thus promote DNA-DNA interactions and annealing) (Masson *et al.*, 2001), it is proposed that the human RAD51 paralogs participate in repair as 'pre-RAD51' functional units. These proteins could therefore either promote the assembly of Rad51 nucleofilaments or alternatively stabilize the filaments once formed.

XRCC2 and XRCC3

The X-ray repair cross complementing (XRCC) genes are a diverse set of human genes that complement hamster cell mutants which have the common features of sensitivity to IR, DNA cross linking agents and increased missegregation of chromosomes (Liu *et al.*, 1998; Tebbs *et al.*, 1995 and Griffin *et al.*, 2000). Analysis of XRCC2 and XRCC3 cDNAs and genomic sequences showed that these proteins are RAD51-related (Thacker J., 1999). Furthermore, both gene products are involved in the repair of DSBs (Pierce *et al.*, 1999 and Johnson *et al.*, 1999).

The human XRCC2 gene was identified by positional cloning and mapped to chromosome 7q36.1 (Liu *et al.*, 1998; Tambini *et al.*, 1997 and Thacker *et al.*, 1995). The coding region contains three exons, with Exon III encoding 86% of the coding sequence. The 31.2kb gene encodes a 280 amino acid protein.

Using XRCC2 mouse knockout cells it was possible to show that the XRCC2 protein plays a prominent role in ensuring normal embryonic development and in developing the nervous system (Deans *et al.,* 2000). Furthermore, Johnson and co-workers (1999) used a recombination reporter substrate ScNeo, to show that XRCC2 is essential for the efficient repair of DNA DSBs mediated by HRR between sister chromatids. In addition O'Regan and co-workers (2001) investigated the localisation of XRCC2 in mammalian cells and showed that functional XRCC2 is required for damage-dependent RAD51 focus formation. Further evidence supporting a role for XRCC2 in assisting RAD51 foci formation came from studies which showed that XRCC2 interacted with RAD51D (the DNA-stimulated ATPase that binds to ssDNA) (Braybrooke *et al.,* 2000). This complex may be required for RAD51 foci formation on ssDNA as it has been shown to form a filamentous structure, similar to RAD51, RAD52 and the XRCC3/RAD51C heterodimer, in the presence of ssDNA (Kurumizaka *et al.,* 2002).

The human XRCC3 gene has been mapped to chromosome 14q32.3 (Tebbs *et al.,* 1995). The gene consists of nine exons and spans a 16.8kb region. The encoded XRCC3 protein shares limited homology with Rad51 (Thacker, J., 1999). Furthermore, XRCC3 has been shown to directly interact with Rad51 (Liu *et al.,* 1998). In further support of this observation, RAD51 foci failed to form in the *irs1SF* (XRCC3 deficient) cell line (Bishop *et al.,* 1998). The XRCC3 protein therefore seems to have a pre-RAD51 role as it is required for the assembly or stabilisation of a multimeric form of RAD51 during DNA repair.

A recombination reporter system has been used to demonstrate that error-free homology-directed repair of DNA DSBs is decreased 25-fold in an XRCC3-deficient hamster cell line (Pierce *et al.,* 1999). The authors further demonstrated that this effect was restored to wild type levels through

XRCC3 cDNA expression, thus indicating the direct role of XRCC3 in HRR. Interestingly, Brenneman and co-workers (2002) showed that XRCC3 function was not limited to the initial stages of HRR, but rather also acted in the formation and resolution of HR intermediaries such as heteroduplex DNA. In addition to the role of XRCC3 in DSBR, Henry-Mowatt and co-workers (2003) suggested that the interaction between XRCC3 and RAD51 can function to modulate the speed of replication fork progression in vertebrate cells following DNA damage.

RAD52

The yeast RAD52 gene was initially cloned and sequenced in 1984 (Adzuma *et al.,* 1984) and found to encode a 504 amino acid protein. Electron microscopy studies indicated that both yeast and human RAD52 proteins formed ring shaped structures on DNA in a similar fashion to RAD51 (Shinohara *et al.,* 1998 and van Dyck *et al.,* 1998). Furthermore, functional analysis showed that this protein is involved in HRR, as both spontaneous and DSB induced HRR activities are absent in yeast rad52 mutants (Paques and Haber, 1999).

Studies using purified *Saccharomyces cerevisiae* RAD52 indicated that the protein has at least two important functions during recombination. Firstly, RAD52 has been shown to directly interact with the RAD51 recombinase, where it serves as a mediator in the DNA strand exchange reaction (New and Kowalczykowski, 2002) (Figure 1.5, Page 25). Yeast-two-hybrid screening has suggested that the C-terminus of RAD52 directly interacts with RAD51 (Milne and Weaver, 1993). In addition to this, Krejci and co-workers (2002) showed that residues 409-420, of the *Saccharomyces cerevisiae* RAD52 protein, specifically are indispensable for function and were likely to be sufficient for the interaction with RAD51. The RAD52 protein also facilitates the displacing of RPA in order to stabilize the RAD51

pre-synaptic filament and allows the RAD51 protein to gain access to ssDNA regions (New and Kowalczykowski, 2002). The RAD52 protein mediated stimulation of DNA strand exchange was, however, found to be protein specific. For example, RAD52 had no effect in reactions where RPA and/or RAD51 were replaced with bacterial Single-strand Binding (SSB) protein or RecA respectively (Sugiyama *et al.,* 2002). Furthermore Sugiyama and co-workers (2002) showed that RAD52 forms a co-complex with RPA in the presence of ssDNA in order to recruit RAD51. This event is proposed to lead to the displacement of RPA. Thus in targeting RAD51 to ssDNA regions, RAD52 is also thought to prevent RAD51 from being sequestered by dsDNA segments.

A second activity of RAD52 involves the annealing of ssDNA regions (i.e. single-strand annealing) (Figure 1.4, Page 23). The DNA binding activity of RAD52 is proposed to occur within the N-terminus and such interactions stimulate DNA strand annealing (Mortensen *et al.,* 1996). In addition, biochemical studies have showed that human RAD52 is capable, not only of binding directly to DSBs, but also protecting these regions from further exonuclease resectioning (by the MRE11/RAD50/NBS1 complex) (van Dyck *et al.,* 1999). The authors further suggest that following the occurrence of a DNA DSB, RAD52 competes with the Ku 70/80 complex for the binding of DNA ends, therefore regulating the mechanism of DNA repair.

RAD54

The RAD54 protein is also thought to play a key role in HRR of DNA DSBs. This protein belongs to the Snf2/Swi2 protein family of ATPase's which can utilize the energy produced from ATP hydrolysis to modulate protein to duplex DNA interactions (Eisen *et al.,* 1995). Purified RAD54 protein has been shown to posses both DNA dependent-ATPase activity and DNA

supercoiling activity (Swagemakers *et al.*, 1998; Tan *et al.*, 1999; Ristic *et al.*, 2001). Furthermore a study, in which the ATPase domain of *Saccharomyces cerevisiae* RAD54 was mutated, revealed that ATP hydrolysis promotes conservative DSBR and suppresses spontaneous deletions (Kim P. *et al.*, 2002). In addition, studies using human RAD54 protein also revealed that inactivation of the ATPase activity severely impaired the ability to complete recombination *in vivo* (Tan *et al.*, 1999). Taken together, such studies underline the importance of this domain of RAD54. In addition to RAD54, a distinct homologue, termed RAD54B, has also been cloned although the precise function of this protein remains unclear (Hiramoto *et al.*, 1999).

Mouse knockout RAD54 mutants are viable and display increased sensitivity to DNA damaging agents including ionizing radiation (Essers *et al.*, 2000). Various structure/function studies have indicated that RAD54 is involved in multiple stages of HRR including: pre-synaptic (i.e. nucleoprotein filament binding), 'synaptic' (i.e. homology search and strand invasion) and 'post-synaptic' (i.e. heteroduplex DNA extension) phases.

The pre-synaptic function of this protein was illustrated when both RAD54 and RAD54B foci were shown to co-localize with RAD51 foci following exposure to γ-irradiation (Tanaka *et al.*, 2000). Furthermore, the N-terminus of RAD54 has been shown to bind with RAD51 in a yeast two-hybrid screen (Golub *et al.*, 1997). In addition, by using purified *Saccharomyces cerevisiae* RAD54 protein, the interaction with RAD51 was shown to stabilize the RAD51 nucleoprotein filament complex in a way that is independent of its ATPase activity (Mazin *et al.*, 2003).

The energy produced through ATP hydrolysis is thought to enable the RAD54 protein to produce negative and positive supercoils in duplexed DNA within the 'synaptic' phase of recombination (Petukhova *et al.*, 2000;

Ristic *et al.,* 2001 and van Komen *et al.,* 2000). Such studies have suggested that this activity subsequently leads to the transient opening of DNA strands therefore enhancing the rate at which the incoming duplex molecule can be sampled for homology by the pre-synaptic complex. Thus the RAD54 protein modifies the topology of the dsDNA, making it more accessible for DNA pairing in the 'synaptic' phase. The catalytic ATP hydrolysis activity of RAD54 is essential for its function during this DNA strand exchange reaction *in vitro* (Solinger *et al.,* 2001). Furthermore Sigurdsson and co-workers (2002) showed that the ability of human RAD54 to promote the separation of DNA strands in duplex DNA (via its ATP hydrolysis-driven DNA supercoiling function) is greatly stimulated by its interaction with human RAD51.

The dsDNA-dependent ATPase activity of RAD54 is also thought to facilitate in the 'post-synaptic' phase of heteroduplex DNA extension mediated by RAD51 *in vitro* (Solinger and Heyer, 2001). However, little is currently known about the mechanistic functions of RAD54 in this respect.

The MRE11 Complex

Components of the mammalian MRE11 complex (namely MRE11, NBS1 and RAD50) were initially identified in genetic screens using *Saccharomyces cerevisiae* mutants that were either hypersensitive to UV damage (RAD50) and X-ray induced damage (Xrs2, yeast homolog of NBS1) or deficient in meiotic recombination repair (MRE11) (Cox and Parry, 1968; Suslova *et al.,* 1975 and Ajimura *et al.,* 1993). Cloning studies revealed substantial homology between fungi and vertebrates for the MRE11 and RAD50 genes, indicating the importance of this complex (Tavassoli *et al.,* 1995 and Petrini *et al.,* 1995).

The 80kDa MRE11 protein is central to the function of the MRE11 complex and is capable of interacting independently with either NBS1 or RAD50 (Johzuka and Ogawa, 1995; Carney *et al.,* 1998; Usui *et al.,* 1998; Chamankhah and Xiao, 1999 and Desai-Mehta *et al.,* 2001). Amino acid alignment studies revealed that the N-terminus of MRE11 contains a DNA nuclease domain (Tsubouchi *et al.,* 1998). The authors further suggested that this domain enables the protein to show 3'-5' dsDNA exonuclease activity and ssDNA endonuclease activities. The DNA nuclease domain is not only regulated by RAD50 (which stimulates its endonuclease and exonuclease activities) (Paull and Gellert, 1998 and 1999), but also by NBS1 (which specifically stimulates its endonuclease activity) (Paull and Gellert, 1999) and ATP (in a RAD50 and NBS1 dependent manner) (Paull and Gellert, 1999). Biochemical and functional studies have also reported that the MRE11 protein has intrinsic DNA binding activity that can be stimulated by RAD50 and/or NBS1 (Usui *et al.,* 1998; Furse *et al.,* 1998; Paull and Gellert, 1999; de Jager *et al.,* 2001a and 2001b and 2002). Furthermore, the MRE11 complex has been shown to posses strand dissociation properties (Paull and Gellert, 1998), and can also mediate the annealing of complementary ssDNA (de Jager *et al.,* 2001a and 2001b and 2002). In support of the latter finding, Mirzoeva and co-workers (2003) found that the MRE11 complex is deposited onto chromatin in an S-phase specific manner, where it could possibly play a key role in sister chromatid association and repair.

The RAD50 protein belongs to a family of proteins that are involved in structural maintenance of chromosomes (SMC). Such proteins have predominant functions in sister chromatid cohesion and chromosome condensation (Hirano, T., 2002). In both cultured ES cells and in early developing embryos, a null rad50 mutation is lethal (Luo *et al.,* 1999). This indicates that the MRE11/RAD50/NBS1 complex is essential for viability (Luo *et al.,* 1999). The RAD50 protein, like RAD51 and its paralogs, also

posseses ATPase activity as it contains Walker A and B nucleotide binding motifs. These motifs are located at the N- and C-terminus respectively (Alani *et al.,* 1989). ATP binding and hydrolysis is essential for the function of RAD50, as defects in such domains result in the loss of nuclease activity in the human MRE11/RAD50/NBS1 complex *in vitro* (Alani *et al.,* 1990). Addition of ATP results in a conformational change within the MRE11/RAD50 heterodimer, as it causes the two ATPase motifs of RAD50 to move closer together (Hopfner *et al.,* 2000). This event is subsequently thought to promote the nuclease activity of MRE11 (Hopfner *et al.,* 2001). Both of the nucleotide binding boxes of RAD50 are separated by two heptad-repeat regions, which form an extended coiled-coil structure containing a putative globular domain (Alani *et al.,* 1989 and Dolganov *et al.,* 1996). The MRE11 protein is speculated to bind to this coiled-coil region of RAD50, proximal to the catalytic domain (Anderson *et al.,* 2001). This subsequently enables RAD50 to adopt a V-like structure thus allowing RAD50 to bind onto DNA ends and hold them close together (de Jager *et al.,* 2001a and 2001b and 2002 and Chen L. *et al.,* 2001). However, both the function and significance of holding DNA ends close together remains speculative. It is possible that RAD50 could facilitate searches for homologous regions. Alternatively, it could stimulate the binding of DNA ligase, as proposed by Chen L. and co-workers (2001) using yeast ligase. RAD50 may also mediate cohesion between a broken sister chromatid, stabilize a damaged chromatid section or limit the extent of nucleolytic degradation activity exhibited by MRE11.

The NBS1 protein interacts with MRE11 at the C-terminus and is not thought to be specifically essential for DNA repair (Desai-Mehta *et al.,* 2001). However, disruptions of NBS1 in DT40 cells results in a decrease in gene conversion and sister chromatid exchanges (Tauchi *et al.,* 2002). Such results therefore imply a role for this protein in HRR. The NBS1 protein contains a BRCT domain next to an N-terminal fork head associated

(FHA) domain (Durocher *et al.*, 1999; Tauchi *et al.*, 2001 and Bork *et al.*, 1997). However the significance of such domains is not presently clear. Deletion of the FHA domain does not induce hypersensitivity to ionizing irradiation, but rather inhibits the MRE11 complex re-localization properties *in vivo* (Tauchi *et al.*, 2001).

NBS1 is phosphorylated on the Ser 278 and Ser 343 residues in an ATM-dependent manner as well as mediating the phophorylation of MRE11 in human cells (Dong *et al.*, 1999; Gatei *et al.*, 2000; Lim *et al.*, 2000; Wu *et al.*, 2000 and Zhao *et al.*, 2000). Paull and co-workers (1999) recently described that *in vitro*, NBS1 assists the human MRE11/RAD50 heterodimer in unwinding short stretches of duplex DNA and cleaves fully paired hairpin structures.

The MRE11 complex forms distinct nuclear foci upon DNA damage in an ATM/ATR dependent manner. This indicates that this complex is not only involved in DNA DSB processing, but also in checkpoint signalling during the S-phase (Maser *et al.*, 1997). The authors further showed that damage induced foci formed by both RAD50 and MRE11 are separate, in terms of both localization and time, from those formed by RAD51.

1.3.3 The Sensing and Response to DNA DSB induced damage.

The existence of DNA DSBs in mitotic cells is initially detected by so called 'DNA damage sensors' which are capable of inducing cell cycle arrest at several points. This delay in cell cycle progression ultimately prevents chromosome instability by allowing time for damaged DNA to be repaired. Briefly, the mammalian cell cycle is divided into four well defined stages (Figure 1.6). Within the G1 (gap 1) phase, normal metabolism occurs and the components required for replication are assembled. The G1 phase is followed by the S (Synthesis) phase, where the cell's chromosomal DNA

replicates. This replication process is extremely accurate as the synthesis of a new strand is an exact replica of the template strand. Genes required for initiating and regulating DNA replication are abundant during this phase of the cell cycle. The G2 (gap2) phase of the cell cycle enables the cell to prepare for M (Mitosis) phase following the completion of chromosomal replication.

DNA damage regulatory checkpoints are responsible for not only controlling cell cycle arrest, but also are thought to be involved in (i) controlling the activation of DNA repair pathways, (ii) the movement of DNA repair proteins to the site of DNA damage and (iii) the induction of apoptosis. Arrest in cell cycle progression at the G1/S or G2/M checkpoint provides the indispensable time required to prevent replication (with respect to the G1/S checkpoint) or to prevent entry into mitosis (with respect to the G2/M checkpoint). Failure to arrest or constitutive activation of these processes substantially results in genomic instability and cancer (reviewed in Hartwell and Kastan, 1994).

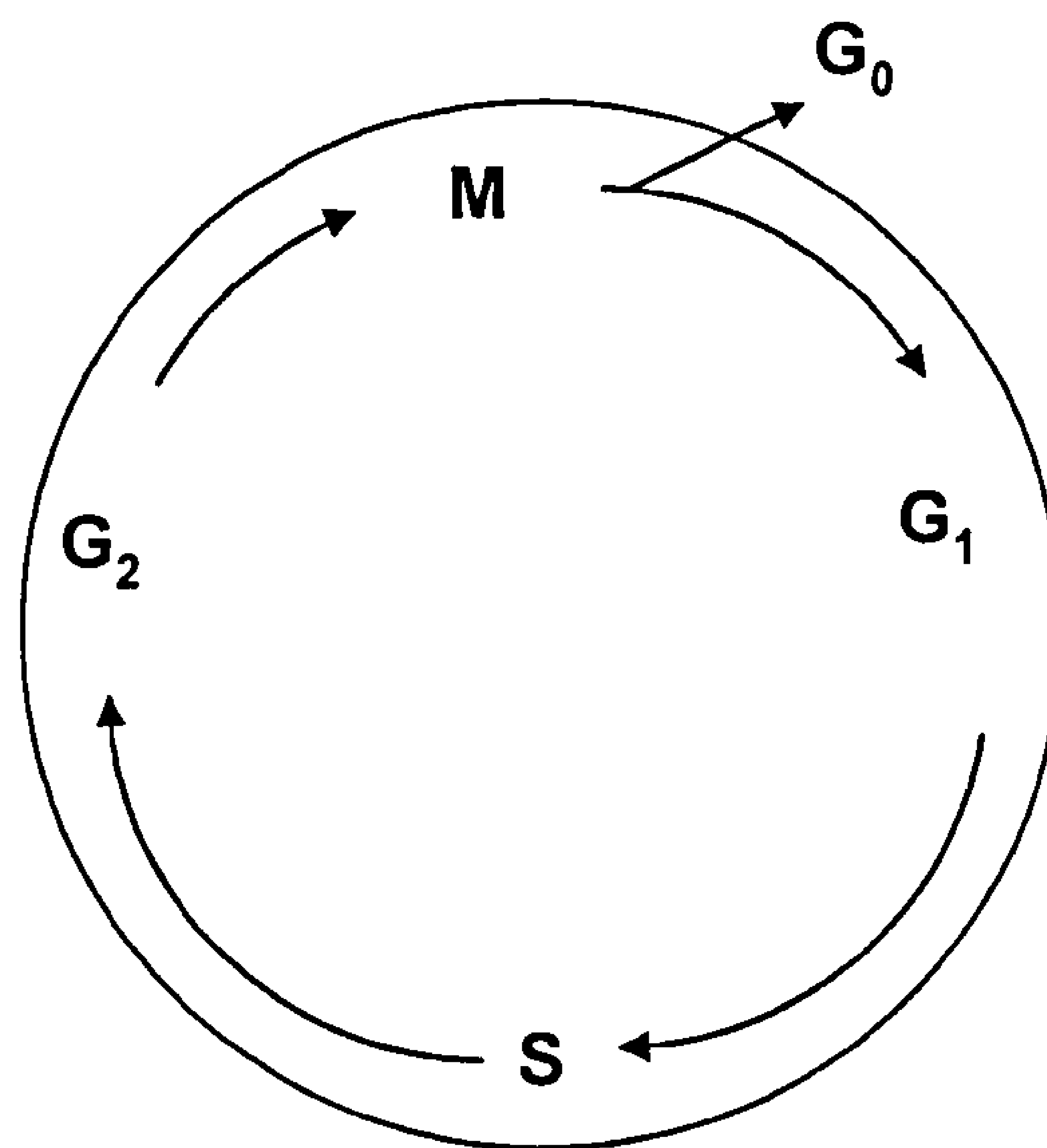

Figure 1.6 The Cell Cycle. The cell cycle is divided into four phases: G1 (Gap1), S (DNA synthesis), G2 (Gap 2) and M (Mitosis). In addition, some cells remain for long periods in a non-dividing state (G0) after completing mitosis.

1.3.3.1 BRCA1 Associated Surveillance Complex (BASC).

The ATM kinase (described below) was recently noted by Wang and co-workers (2000) to exist as part of a 'super BASC complex' that consisted of at least fourteen DNA repair proteins (Figure 1.7). The study used co-immunoprecipitation to reveal that this complex included caretaker proteins, (such as BLM), the MRE11/RAD50/NBS1 complex, some MMR proteins (namely the MSH2/MSH6 heterodimer and the MLH1/PMS2 heterodimer) and replication factor C (RFC). The authors suggested that this complex acted "as a sensor of abnormal DNA structures and/or as a regulator of the post-replicative repair process". Components included within this complex and the possible involvements in HRR are discussed below.

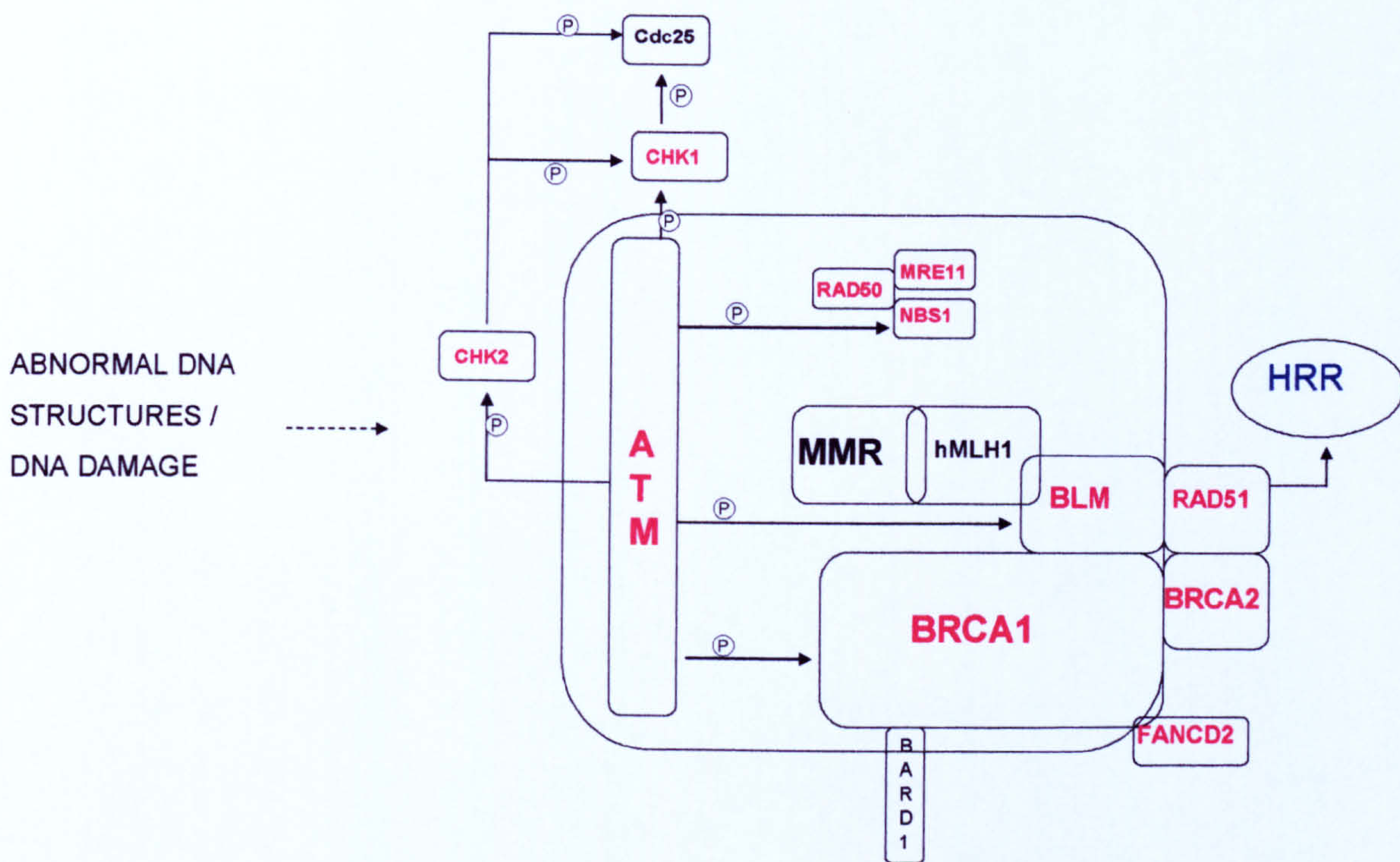

Figure 1.7 The BASC complex: a sensor of abnormal DNA structures and DNA damage. Proteins that have been demonstrated to physically interact are represented as touching boxes. Phosphorylation events are represented as Ⓟ. Not all phosphorylation events are depicted. Furthermore, the significance of some phosphorylation events still remains unclear. The functions of gene products in boxes with red text are described in detail below. *Adapted from Levitt and Hickson (2002)*

The ATM and ATR kinases

DNA damage (induced by agents such as IR, UV irradiation and stalled replication forks) is thought to trigger signal transduction pathways that subsequently effects DNA repair, cell cycle arrest and even apoptosis (Lowndes and Murguia, 2000). Cellular failure to trigger such events frequently results in genomic instability, thus predisposing organisms to cancer. In eukaryotes, two members of the phosphatidylinositol 3-kinase related kinase (PIKK) family, namely ATM and ATR, play crucial roles in

DNA damage recognition and initiation of cellular responses (e.g. cell cycle arrest) (for reviews see: Tibbetts *et al.,* 2000; Zhou and Elledge, 2000; Khanna *et al.,* 2001a and 2001b and Rouse and Jackson, 2002). This super family of protein kinases (which also include Tel1p and Mec1p of *Saccharomyces cerevisiae*, Rad3p of *Schizosaccharomyces pombe* and mammalian DNA-PKcs) all contain a highly conserved phosphatidylinositol 3-kinase domain at the C-terminus. This domain is essential for the signal transduction activities of ATM and ATR (reviewed in Rotman and Shiloh, 1999).

The ATM protein binds directly to DNA following exposure to IR in order to signal downstream effectors (Smith *et al.,* 1999). This gene is approximately 150 kb in length and comprises of 66 exons which encode for a 370kDa protein (Savitsky *et al.,* 1995). Bakkenist and co-workers (2003) showed that the ATM protein is held in an inactive state as a dimer or higher order multimer which becomes dissociated (by intermolecular auto-phosphorylation) following exposure to irradiation. The authors further suggested that this event subsequently initiates the intrinsic kinase activity of ATM through allowing accessibility to substrates of ATM.

The 350 amino acid kinase domain of ATM is able, not only to control cell cycle checkpoints at the G1/S and G2/M boundaries, but also affects the repair of DNA DSBs mediated by HRR. For example, A-T cells were found to be unable to arrest at either the G1/S or G2/M boundaries following exposure to irradiation (Shiloh and Kastan, 2001). Furthermore, A-T cells exhibit radio resistant DNA synthesis (RDS) (i.e. they have a characteristic inability to arrest DNA synthesis after irradiation) (Houldsworth and Lavin, 1980 and Painter and Young, 1980).

ATM mediated phosphorylation of CHK2 has been shown to be crucial for G1, S and G2 arrest following exposure to IR. For example, Falck and co-

workers (2001) showed that the ATM protein is involved in S-phase arrest by noting that irradiation induced degradation of cdc25A required both ATM and CHK2 mediated phosphorylation on the serine 123 residue. In addition, ATM mediated phosphorylation of CHK2 negatively regulates cdc23c activity, which is involved in G2/M arrest (Matsuoka *et al.,* 1998). Aside from the CHK2 substrate, ATM also phosphorylates p53 and Mdm2, both of which have been shown to be specifically involved in G1/S arrest following exposure to IR (Banin *et al.,* 1998; Canman *et al.,* 1998 and Maya *et al.,* 2001). ATM additionally influences S-phase arrest (through its ability to phosphorylate NBS1, BRCA1, FANCD2 and SMC1) and G2/M arrest (through its ability to phosphorylate BRCA1 and hRad17) (Taniguchi *et al.,* 2002; Lim *et al.,* 2000; Kim S. *et al.*, 2002; Xu B., *et al.,* 2001 and 2002 and Bao *et al.,* 2001).

Many lines of evidence have also implicated a function for ATM in HRR. For example, ATM has been shown to interact with and phosphorylate proteins such as RPA, NBS1, BRCA1 and c-Abl (for reviews see: Lavin and Khanna, 1999; Khanna *et al.,* 2001 and Shiloh, 2001). In addition the kinetics of mammalian RAD51 complexes are greatly altered in A-T cells in response to DNA DSBs and in ATM-/- DT40 chicken cells (Chen *et al.,* 1999 and Morrison *et al.,* 2000). Furthermore, ATM -deficient cells not only showed altered kinetics of irradiation induced RAD51 and RAD54 foci formation, but also display defects in RAD54 mediated HRR (Morrison *et al.,* 2000). These findings, along with the observation of general aberrant recombination repair (such as increased intra-chromosomal recombination events) in A-T cells, indicate an important involvement of this kinase in HRR (Meyn S., 1993 and Luo *et al.,* 1996).

The ATR kinase was originally identified in 1996 as a member of the PIKK super family of proteins (Cimprich *et al.,* 1996). At present Seckel syndrome is the only human disease that has been associated with ATR

deficiency (O'Driscoll *et al.,* 2003). However, ATR deficiency in mice is embryonic lethal and cells lacking ATR become non-viable within a few cell divisions (Brown and Baltimore, 2000 and de Klein *et al.,* 2000). Taken together such results demonstrate the importance of this protein for cell survival.

The ATR protein is thought to function by regulating responses to bulky lesions as well as replication inhibitors. Such lesions range from pyrimidine dimmers to stalled replication forks to DNA DSBs (Brown and Baltimore, 2003; Casper *et al.,* 2002 and Pichierri et al., 2003). Techniques predominantly used to study the function of ATR include: over-expression of a dominant negative construct or the use of a *cre-lox*-mediated gene loss system. Recent reports indicate that ATR is required for checkpoint responses to DNA damage induced by agents such as UV light, replication inhibiting agents and hypoxia (Cilby *et al.,* 1998; Cortez *et al.,* 2001; Nghiem *et al.,* 2001 and Hammond *et al.,* 2002). In support of this, substrates for ATR include CHK1, CHK2, p53 an BRCA1 (Tibbetts *et al.,* 1999 and 2000; Xu X. *et al.,* 2002 and Zhao and Piwnica-Worms, 2001). Thus it seems likely that ATR has a key role in G1, S-phase and G2/M arrest. Furthermore, the ATR protein is thought to function in cooperation with an ATR-interacting protein (ATRIP) in order to bind to sites of DNA damage (Cortez *et al.,* 2001).

Casper and co-workers (2002) demonstrated that ATR, and not ATM, is critical for the maintenance of fragile sites which are expressed when DNA replication is inhibited. The authors further suggested that ATR was responsible for not only stabilizing and later restarting stalled replication forks, but also inhibiting late origin firing in the presence of DNA stress, hence blocking M-phase entry before replication is complete. In line with this, deletion of Mec1p function in budding yeast, resulted in genome wide fork stalling followed by chromosome breakage (Cha and Kleckner, 2002).

Interestingly, ATR has recently been shown to also be involved in cell cycle arrest following exposure to irradiation and DNA replication inhibitors (such as aphidicolin) in conjunction with ATM (Tibbetts *et al.,* 1999). Brown and Baltimore (2003) used the *cre-lox*-mediated gene loss system to demonstrate that, following exposure of cells to irradiation, ATR regulated late phase response to M-phase entry, in addition to cooperating with ATM in the early phase. The same study also showed that stalled DNA replication forks were found to instigate cell cycle arrest in an ATR and ATM mediated phosphorylation of the CHK1 and CHK2 (respectively) /cdc2 pathway.

CHK1/CHK2

The ATM and ATR signal transduction control of DNA damage responses is well documented to be mediated in part by the CHK1 and CHK2 kinases (reviewed in Carr, 2002). The human CHK2 kinase is a structural and functional homolog of *Saccharomyces cerevisiae* RAD53 (26% identical and 37% similar) and *Schizosaccharomyces pombe* Cds1 (26% identical and 34% similar). CHK2 is predominantly involved in preventing entry into the synthesis phase and the mitotic phases of the cell cycle (Chebab *et al.,* 2000; Hirao *et al.,* 2000 and Shieh *et al.,* 2000 and Matsuoka *et al.,* 1998). CHK2 becomes phosphorylated and subsequently activated in an ATM and ATR dependent manner (Brown *et al.,* 1999; Chaturvedi *et al.,* 1999; Matsuoka *et al.,* 1998 and 2000 and Xu X. *et al.,* 2002). Phosphorylation specifically occurs within a phospho- amino acid binding motif (known as the FHA domain) and/or a SQ and TQ cluster domain located in the N-terminus (Matsuoka *et al.,* 2000 and Xu X. *et al.,* 2002). Dominant negative and CHK2 knockout mutants fail to arrest in the G1/S phase when responding to IR due to an inability to phosphorylate p53 (Hirao *et al.,* 2000 and Chehab *et al.,* 2000). Such results suggest an involvement of CHK2 in G1 phase cell cycle arrest. The involvement of CHK2 in intra-S phase cell

cycle arrest was initially illustrated when its yeast homolog, RAD53, was shown to be involved in protecting stalled replication forks from pathological rearrangements (Sogo *et al.,* 2002). The authors proposed that active RAD53 prevented the accumulation of abnormal replication intermediates, hence allowing stalled replication forks to restart DNA synthesis. Following on from this study, Falck and co-workers (2001) reported that IR induced ATM dependent CHK2 phosphorylation inhibited the Cdc25A/cdk2 initiation of DNA replication, thus showing the involvement of CHK2 in an intra-S phase checkpoint in human cells. In addition it was noted that this specific checkpoint was also involved in an IR induced ATM dependent phosphorylation of NBS1, which subsequently inhibited DNA synthesis (Falck *et al.,* 2002). Irradiation induced ATM activation of CHK2 was also shown to require the phosphorylation of NBS1 within the MRE11 complex by Buscemi and co-workers (2001). Finally CHK2 mediates G2/M arrest through its ability to phosphorylate Cdc25c on an inhibitory site. This subsequently inhibits Cdc25c's ability to de-phosphorylate and activate Cdc/cyclinB complexes which are involved in the transition of mitotic cells (Matsuoka *et al.,* 1998).

Like CHK2, the CHK1 kinase also becomes phosphorylated and activated in response to IR in mammals, as well as in yeast (Liu Q. *et al.,* 2000; Sanchez *et al.,* 1997 and 1999 and Walworth *et al.,* 1996). This 58kDa protein is implicated in G1/S, intra-S and G2/M cell cycle arresting phases in an ATR predominant manner (Mailand *et al.,* 2000; Zhou and Ellege, 2000; Cliby *et al.,* 2002 and Zhou *et al.,* 2002). Furthermore, Feijoo and co-workers (2001) suggested that mammalian CHK1 was an essential component of the intra-S phase checkpoint. The authors suggested that CHK1 ensures that activation of late replication origins are blocked when early origin synthesis is inhibited. In addition, Xiao and co-workers (2003) demonstrated that CHK1 regulated not only the S-phase arrest, but also

G2/M phase arrest through its ability to target Cdc25A for degradation following camptothecin and doxorubicin treatment.

BRCA1 and BRCA2

Both BRCA1 and BRCA2 gene products are thought to be involved in general genome maintenance as well as regulating HRR. Knockout mutations of either BRCA1 or BRCA2 in mice is lethal, even in early embryogenesis, although truncating mutations remain viable and have been used for functional studies (Ludwig *et al.,* 1997; Gowen *et al.,* 1998 and Shen *et al.,* 1998).

The BRCA1 gene was originally identified by positional cloning (Miki *et al.,* 1994 and Futreal *et al.,* 1994) and found to encode for an 1863 amino acid protein. Analysis of domain structures revealed that the N-terminus of BRCA1 contains a RING-finger domain. This domain regulates protein-protein or protein-DNA interactions via its interaction with zinc ions (Lorick *et al.,* 1999 and Wu *et al.,* 1996). In addition, this domain also has been shown to form a heterodimer with BARD1 (BRCA1-associated RING domain), which together mediates ubiquitin-conjugating enzyme-dependent ubiquitination *in vitro* (Hashizume *et al.,* 2001). It therefore seems that BRCA1 regulates the proteosomal degradation of some proteins. Both FANCD2 and p53 co-localise with BRCA1 and undergo BRCA1-mediated monoubiquitination following the exposure of cells to IR (Garcia-Higuera *et al.,* 2001). The C-terminus of BRCA1 contains 2 BRCT repeats (Koonin *et al.,* 1996; Ekblad *et al.,* 2002 and Williams *et al.,* 2001). Such repeats appear to be multifunctional in that they seem to be involved in, not only transcription control, but also in the repair of DNA DSBs (Gowen *et al.,* 1998 and Moynahan *et al.,* 2001). Furthermore, missense mutations found in BRCA1 almost always occur within either the RING or BRCT domains,

thus underlining the importance of the two domains (Miki *et al.,* 1994 and Futreal *et al.,* 1994).

Many lines of evidence suggest an involvement of BRCA1 in HRR and cell cycle regulation. Firstly, BRCA1 been shown to co-precipitate with RAD51 and BRCA2 (Scully *et al.,* 1997a). In addition, BRCA1, BRCA2 and RAD51 foci co-localize following DNA damage or within the S-phase of the cell cycle (Scully *et al.,* 1997b). Further studies have shown that BRCA1 interacts with RAD51 (Chen *et al.,* 1998). Interestingly, BRCA1 foci co-localize with the MRE11/RAD50/NBS1 complex although BRCA1/RAD51 and BRCA1/RAD50 foci are not observed together (Zhong *et al.,* 1999). Secondly, BRCA1 deficient cells have been shown to be hypersensitive to DNA cross linking agents and have impaired HRR (as measured by gene targeting and homology directed repair of a chromosomal break) (Moynahan *et al.,* 2001). Thirdly, BRCA1 appears to be involved in linking DNA repair and cell cycle arrest. For example, over-expression of BRCA1 can activate the CDK inhibitor p21 which subsequently leads to G1 arrest (Deng and Brodie, 2000). In addition, Yarden and co-workers (2002) showed that BRCA1 also regulates the G2/M checkpoint through its interaction with CHK1 which subsequently acts as a negative feedback for the expression, phosphorylation and cellular localization of Cdc25c and the Cdc2/cyclinB kinase proteins. Thus in the absence of BRCA1, failure to activate the G1 and/or the G2/M checkpoint may result in irreversible chromosomal rearrangement and loss of genomic integrity.

The BRCA2 gene was cloned in a similar fashion to BRCA1 (Wooster *et al.,* 1994 and Wooster *et al.,* 1995) and shown to encode a 3418 amino acid protein. This protein interacts with BRCA1, RAD51 and RPA proteins, the foci of which re-localize in the S-phase or following DNA damage (Sharan *et al.,* 1997; Wong *et al.,* 1997; Marmorstein *et al.,* 1998; Davies *et al.,* 2001; Chen *et al.,* 1998 and 1999). BRCA2 deficient tumour cells show increased

sensitivity to UV light, IR and to DNA cross linking agents (Morimatsu *et al.*, 1998 and Xia *et al.*, 2001). Furthermore, such cells accumulated chromosomal abnormalities including breaks, aberrant chromatid exchanges and translocations (Patel *et al.*, 1998; Tutt *et al.*, 1999 and Yu V., *et al.*, 2000).

Detailed amino acid alignment studies have showed that BRCA2 contains eight highly conserved 'BRC' sequence repeats. These repeats are positioned between residues 990 and 2100 of the C-terminus of human BRCA2 (Wooster *et al.*, 1995; Bignell *et al.*, 1997 and Bork *et al.*, 1996). Each repeat is approximately 30 amino acids in length and appears to be the primary site by which BRCA2 interacts with RAD51 (Wong *et al.*, 1997 and Chen *et al.*, 1999). Furthermore, Pellegrini and co-workers (2002) showed that the BRC4 repeat, within BRCA2, mimics a motif in RAD51 that serves as an interface for oligomerisation between individual RAD51 monomers. Therefore BRCA2 may control the assembly of RAD51 nucleoprotein filaments and therefore the initiation of HRR. Additional experimental data also suggest that BRCA2 is responsible for regulating HRR. For example, BRCA2 is required for the transportation of RAD51 to sites of DNA damage (Davies *et al.*, 2001). Furthermore, RAD51 foci failed to form in BRCA2 deficient cells following IR mediated DNA damage even though they co-localize in BRCA2 proficient cells (Chen *et al.*, 1999 and Yu V., *et al.*, 2000). Additional evidence for the involvement of BRCA2 in HRR was revealed when the C-terminus of BRCA2 was shown to have ssDNA binding activity (Yang H., *et al.*, 2002). Further more, Moynahan *et al.*, (2001) recently showed a severe defect in the repair of induced DNA DSBs in BRCA2 deficient cells.

BLM/WRN/MUS81

Protein alignment studies have suggested that these three gene products share homology with the *E.coli* RecQ family of DNA and RNA helicases (Ellis *et al.,* 1995 and Yu *et al.,* 1996). The BLM gene encodes a 1417 amino acid helicase that, like WRN, unwinds duplex DNA in a 3' to 5' manner in the presence of ATP (Karow *et al.,* 1997 and Neff *et al.,* 1999). The involvement of BLM and WRN helicases in HRR has been demonstrated in many ways, most blatantly perhaps by the observation that both helicases can specifically bind to the crossover regions of Holliday junctions and subsequently assists in its resolution (Yang Q., *et al.,* 2002). Furthermore, BLM has been shown to interact with several other proteins involved in DSB repair or DNA damage signalling, including BRCA1, MRE11 and ATM (Wang *et al.,* 2000). Franchitto and co-workers (2002) demonstrated that the BLM protein is responsible for the re-localization of the MRE11/NBS1/RAD50 complex and BRCA1 to sites of replication arrest. This was supported by the observation that BLM is phosphorylated in an ATM dependent manner following replication arrest (Wang *et al.,* 2000 and Ababou *et al.,* 2000). Thus it seems possible that BLM assists in the loading of these proteins onto abnormal DNA structures, similar to the yeast homolog, SGS1 (Frei and Gasser, 2000). Furthermore, hydroxyurea exposure to human fibroblasts resulted in the co-localization and association of BLM and RAD51 at sites of stalled replication forks (Sengupta *et al.,* 2003).

Amino acid alignment studies have revealed that the WRN helicase shares approximately 41% homology with the BLM helicase (Liu Z., *et al.,* 1999). In addition to its helicase activity, this protein has a unique amino-terminal domain that has exonuclease activity (Kamath-Loeb *et al.,* 1998 and Huang *et al.,* 1998). Furthermore, all WS mutations have been reported to give rise to truncated proteins lacking C-terminal nuclear localization signals,

thus underlining the importance of this region (Matsumoto *et al.*, 1997 and 1998). The WRN helicase, like that reported for BLM, is involved in unwinding four-way structures as well as other abnormal duplex structures including hairpins (Brosh *et al.*, 2001 and Bohr *et al.*, 2000).

The MUS81 protein was originally identified in a yeast-two hybrid screening assay where it was found to interact with RAD54 and Cds1 in budding and fission yeast, respectively (Boddy *et al.*, 2000 and Interthal and Heyer, 2000). MUS81 mutants are sensitive to agents that block replication fork progression (such as methyl methane sulphonate (MMS), hydroxyurea and UV irradiation) but resistant to γ-radiation or DSBs when induced by the HO-endonuclease (Boddy *et al.*, 2000 and Interthal and Heyer, 2000). These results, along with the observation that MUS81 mutants are defective in a late stage of meiotic recombination in *Schizosaccharomyces pombe*, have suggested that MUS81 has an important role in both mitotic and meiotic cells (Boddy *et al.*, 2001). Protein alignment studies have revealed that the C-terminus of MUS81 shares high homology with a 200 amino acid domain in yeast RAD1 and human XPF endonucleases (Interthal and Heyer, 2000 and Mullen *et al.*, 2001). In addition, biochemical studies have revealed that MUS81 acts as a heterodimer with MMS4 (in *Saccharomyces cerevisiae*) and Eme1 (in *Schizosaccharomyces pombe*). Such heterodimers exhibit endonuclease activity and have higher affinity for 3'flap regions or replication fork structures, than for Holliday junctions (Kaliraman *et al.*, 2001 and Doe *et al.*, 2002). The endonuclease activities of the MUS81/MMS4 heterodimer and the RAD1/RAD10 heterodimer are however distinct (Bastin-Shanower *et al.*, 2003). Currently no human MUS81 partner has been reported, although this protein is speculated to share homology with MMS4 and Eme1. In support of the observations in yeast, Constantinou and co-workers (2002) have suggested that human cells possess two distinct endonucleases that cleave Holliday junctions, with the MUS81 containing enonuclease involved in specifically resolving 3'flap

structures and replication fork intermediates. This study therefore supports a predominant role for human MUS81 in replication fork progression.

Fanconi Anemia genes

At least eight complementation groups have been associated with FA, although only six genes have been currently cloned (namely: FANC-A, FANC-C, FANC-D, FANC-E, FANC-F and FANC-G) (de Winter *et al.,* 1998 and 2000a and 2000b; Timmers *et al.,* 2001; Lo Ten Foe *et al.,* 1996 and Strathdee *et al.,* 1992). The FA proteins display little or no homology to other proteins and presently little is known about any functional domains. The highly conserved FANC-D gene encodes for two isoforms, namely FANCD1 and FANCD2 respectively (Timmers *et al.,* 2001). The FANC-A, C, E, F and G gene products have been shown to interact in a nuclear complex (Garcia-Higuera *et al.,* 1999; de Winter *et al.,* 2000 and Medhurst *et al.,* 2001). Furthermore, Siddique and co-workers (2001) showed that FANC-F was specifically involved in stabilizing this complex, as well as being responsible for post-translational modification of FANCD2 in response to IR induced DNA damage. DNA DSBs, caused by IR, results in the ATM-dependent phosphorylation of serine 222 on FANCD2. Inter-strand DNA cross-links, however, caused by MMC, produce the mono-ubiquitination of FANCD2 on lysine 561 also via the FA nuclear complex (Grompe *et al.,* 2002).

The FA gene products maybe involved in HRR as RAD51 foci fail to form in any of the FA deficient cells (Digweed *et al.,* 2002). This effect was reversed when the deficiency was corrected in FA-A, C and G cells. Furthermore, using FA patient derived cells of several complementation groups, Donahue and co-workers (2003) showed that the FA-A, C and G proteins specifically functioned to suppress HRR and elevate end joining activities. Mono-ubiquitination of FANCD2 is therefore thought to generate

a separate signal that overcomes this suppression and activates HRR processes. This is supported by the observation that FANCD2 co-localizes and interacts with BRCA1, NBS1 and RAD51 following IR or MMC exposure in an ATM dependent manner (Taniguchi *et al.,* 2002 and Nakanishi *et al.,* 2002). Fibroblasts derived from FA patients often display genomic instability that is exhibited by increased levels of spontaneous chromosomal breaks and deletions (Zakrzewski and Sperling, 1980; Papadopoulo *et al.,* 1990 and Laquerbe *et al.,* 1999). Furthermore, FA cells are hypersensitive to MMC and are deficient in DNA end joining (Auerbach and Wolman, 1976; Ishida and Buchwald, 1982; Carreau *et al.,* 1999; Escarceller *et al.,* 1997 and 1998; Lundberg *et al.,* 2001 and Donahue and Campbell, 2002).

1.3.4 Chromosomal instability and predisposition to Cancer.

A majority of human cancers develop chromosomal abnormalities. Such abnormalities can range from alterations in chromosome numbers (aneuploidy) to structural aberrations (reviewed in van Gent et al., 2001). Most if not all human genetic disorders that exhibit chromosomal alterations are associated with an increased risk of developing cancer. Furthermore, defects in DNA repair pathways are thought to enhance tumourigenisis as a result of genetic instability. In support of this, mutations in tumour suppressor genes have been well documented to predispose individuals to cancer prone syndromes (reviewed in Levitt and Hickson, 2002).

The human ATM gene is mutated in patients with the genetic pleotropic disorder Ataxia-Telangiectasia. This disorder is characterized by genetic instability, cerebellar degeneration, abnormalities in nervous, immune and reproductive systems, as well as a 30-40% lifetime risk of developing a malignancy (reviewed in Levitt and Hickson, 2002). Cells derived from such patients exhibit high levels of chromosomal aberrations, hypersensitivity to

IR, bleomycin, restriction endonucleases and inhibitors of topoisomerases (Johnson R., *et al.,* 1999). In addition, mutations in the MRE11 or NBS1 genes cause Ataxia-telangiectasia like disorder (ATLD) or Nijmegen breakage syndrome (NBS) respectively (Stewart *et al.,* 1999 and Hall and Angele, 1999). Patients with ATLD exhibit similar symptoms to A-T patients but without telangiectasia. In addition, NBS has a similar phenotype to A-T, however, such patients are frequently mentally retarded as opposed possessing progressive cerebellar ataxia (Hall and Angele, 1999). Patients with A-T, ATLD or NBS frequently develop lymphomas (reviewed in Levitt and Hickson, 2002).

Germline mutations in BRCA1 and BRCA2 account for 66% of breast and ovarian familial cancers and frequently encode a truncated protein (Castilla *et al.,* 1994; Simard *et al.,* 1994 and Friedman *et al.,* 1994). Between 60 to 80% of primary tumours associated with mutations in either BRCA1 or BRCA2 occur in the breast. Prostate and colon cancers make up for the majority of secondary tumours in such patients (reviewed in Levitt and Hickson, 2002).

Mutations in BLM and WRN give rise to Bloom and Werner's syndrome respectively. Both of these syndromes are autosomal recessive disorders that are characterised by immunodeficiency, short stature, male infertility and an increased predisposition to malignancy (reviewed in Levitt and Hickson, 2002). Cells derived from Bloom and Werner's syndrome patient's exhibit chromosomal and DNA instability characterised by an increase in the frequency of sister chromatid exchanges, insertions and deletions and loss of heterozygosity (Thompson and Schild, 2002).

Mutations in the Fanconi genes give rise to Fanconi Anemia (FA) which is a chromosome instability syndrome that is characterized by birth defects, bone marrow failure and cancer susceptibility. FA patients develop several

types of cancer including acute myeloid leukaemia's, squamous cell carcinoma of the head and neck and young onset pancreatic cancer (Alter, B.P., 1996 and van der Heijden *et al.,* 2003). This syndrome affects between 1-5 million people worldwide (Joenje and Patel, 2001), with progressive bone marrow failure being the major cause of death.

Although mutations in genes belonging to the RAD52 epistasis group have not been directly associated with cancer prone syndromes, chicken DT40 cells in which HRR genes have been knocked out exhibit excessive chromosomal breaks and translocations (Thompson and Schild, 2002).

1.4 Regulating roles of MMR proteins in Recombination repair.

In addition to their role in removing replicative base mis-incorporations and polymerase slippage errors, the proteins involved in the eukaryotic MMR system also play a regulatory role in HRR. The importance of the interaction between the MMR system and recombination is illustrated in functional studies using mammalian cell lines deficient in MMR. Such studies have revealed that MMR deficient cells exhibit increased rates of spontaneous recombination as well as being sensitive to the recombination inducing agent, CPT, but resistant to MNNG (Ciotta *et al.,* 1998; Pichierri *et al.,* 2001 and Zhang *et al.,* 2000).

Several biochemical and genetic studies conducted in bacteria and yeast have suggested interactions between the MMR system and various recombination repair processes. Such studies have been based on the ability of MMR proteins to recognize various DNA structures. Firstly, the regulating activity of the MMR system in recombination was illustrated in studies using yeast, which showed that homeologous recombination

frequencies are increased in a MMR –deficient background (Chen W., *et al.,* 1999 and Nicholson *et al.,* 2000). In particular, the disruption of the MSH2 protein (in yeast) resulted in the highest increase in homeologous recombination events; this is consistent with the prominent role of MSH2 in MMR. A similar effect was also confirmed in mammalian cells derived from MSH2 knockout mice (Elliot and Jasin, 2001). Furthermore, Datta and co-workers (1997) also showed that a single mismatch was sufficient to inhibit recombination between otherwise identical sequences. This illustrates the close regulation for controlling recombination events between diverged sequences by the MMR system. Therefore it seems that the MMR system has a role in regulating recombination events between diverged sequences in order to prevent chromosome translocations, deletions and/or insertions.

A second role for the MutS homologs in recombination processes was noted when the MSH2/MSH3 heterodimer was shown to be required to remove non-homologous DNA during both GC and SSA events (Evans *et al.,* 2000). During gene conversion, any non-homologous DNA that may be present at the 3' ends of the invading strand needs to be removed in order to initiate new DNA synthesis. The authors used chromatin immunoprecipitation to show that the MSH2/MSH3 heterodimer associated with these recombination intermediates early in DSBR in order to assist in the rejection of homeologous pairing and to stabilize non-homologous 3' end tails. This was consistent with initial work that showed that the MSH2/MSH3 heterodimer interacted with the RAD1/RAD10 heterodimer to remove any 3' ended ssDNA tails (Sugawara *et al.,* 1997). In addition, it was shown that the MSH2/MSH3 heterodimer, along with RAD1/RAD10, was also involved in the deletion of intervening DNA sequences during SSA, and the resolution of SSA intermediates. However, the requirement for the MSH proteins was found to decrease as the length of the annealed homologous DNA flanking the DSB increases. Furthermore, Studamire and co-workers (1999) proposed that the MSH2/MSH3 heterodimer initially

binds to and stabilizes ssDNA tails before being displaced, in an ATP-dependent manner, to allow access for the RAD1/RAD10 endonuclease. However, the precise function of the MMR proteins in the removal of single-stranded 3' ends remains unclear. It is judged that the MMR proteins either stabilize annealed intermediates by binding to the unpaired ssDNA (thus enabling the RAD1/RAD10 heterodimer to trim off the non-homologous tails), or alternatively, the MMR proteins could recruit the NER proteins to the sites of recombination. Studies conducted in yeast indicated that these four proteins are only required to remove non-homologous ends that are 30 nucleotides or longer, which also required the Srs2 helicase (Paques and Haber, 1997).

Eukaryotic MutS homologs can also interact with mispaired bases occurring in recombination intermediates such as heteroduplex DNA, thereby preventing DNA extension. In *Saccharomyces cerevisiae*, proteins involved in MMR, have been shown to regulate not only heteroduplex tract length, but also the extension of the heteroduplex tract (Alani *et al.,* 1994). Furthermore, in *E.coli*, both of the MutS and MutL proteins have been shown to inhibit RecA-mediated strand exchange activities in conditions where the newly formed heteroduplex DNA contained an excess of mispaired bases (Worth *et al.,* 1994). The MutS homologs have also recently been shown to interact with Holliday junctions, thus triggering their resolution. For example, the *Saccharomyces cerevisiae* MSH2 protein was shown to have a high affinity for Holliday junctions in filter binding and electron microscopic analysis (Alani *et al.,* 1997b). In addition, Marsischky and co-workers (1999) showed that the presence of the MSH6 protein changed the characteristics of the interaction between MSH2 and Holliday junctions in a way that greatly increased the affinity and specificity of MSH2 for Holliday junctions. The importance for the binding of the MMR proteins to such a structure was proposed by the authors to enhance the activity of Holliday junction resolution enzymes. Thus the MMR proteins seem to be

involved in determining the extent of mispaired bases in heteroduplex DNA and in eliminating recombination intermediates.

The final evidence for the involvement of MMR proteins in recombination processes is that the MSH4/MSH5 heterodimer has, to date, not been ascribed a role in MMR, but has rather been shown to be involved in meiotic recombination events (Ross-Macdonald and Roeder, 1994 and Hollingsworth *et al.,* 1995).

1.5 Aim of present study.

Cells that acquire MMR deficiency develop distinct mutator phenotypes that help drive the genetic changes needed for tumourigenisis. Such deficiency often results in cells acquiring an increased number of mutations as well as being hypersensitive to the cytotoxic effects of DSB-inducing agents (e.g. camptothecin and etoposide), MMC and S-phase cell cycle inhibitors (e.g. thymidine) (Mohindra *et al.,* 2002; Jacob *et al.,* 2001 and Fiumicino *et al.,* 2000). Increasing evidence also suggests that components of the MMR system interact with other DNA repair pathways, including NER and HRR, in order to regulate DNA synthesis.

A report published by Lundin and co-workers (2002a) has shown that HRR deficient cell lines also exhibit sensitivity to MMC and thymidine. Thus given the suggested involvement of the MMR proteins in HRR processes, and that such deficiency can confer thymidine sensitivity, the aim of this research project was three fold:

(i) To determine the integrity of the Homologous recombination repair pathway in MMR –deficient tumour cell lines.

(ii) To determine the extent to which a deficiency in the Homologous recombination repair pathway is the result of mutations in genes.

(iii) In the event of gene mutations contributing to Homologous recombination repair deficiency, to determine whether such mutations were responsible for the thymidine sensitivity observed in Mismatch repair tumour cells.

CHAPTER TWO: MATERIALS AND METHODS

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2.1 GENERAL MATERIALS.

MATERIAL	SUPPLIER
6 well tissue culture plates	Greiner Labortechnik
12 well tissue culture plates	Greiner Labortechnik
100 mm tissue culture dishes	Sarstedt Ltd.
2ml, 5ml, 10ml Syringes	Becton Dickinson & Company
Syringe driven filter unit 0.22μm	Millipore
1.2ml Cryovials	Nalgene Ltd.
Genepulser® electroporation cuvette 0.4cm	BioRad
Centrifugal filter tube, 5000 NMWL	Millipore
Rehydration tray	BioRad Laboratories Ltd.
5ml plastic pipettes	Corning Incoporated
10 ml plastic pipettes	Corning Incoporated
10μl,20μl,200μl and 1ml pipette tips	Sarstedt Ltd.
Filtered 10μl,20μl,200μl and 1ml pipette tips	Starlab GMBH
0.5ml,1.5 ml and 2ml eppendorf tubes	Sarstedt Ltd.
96 well PCR plates	Advance Biotechnologies Ltd.
Thin walled PCR tubes	Intermountain Scientific Corporation
PCR flat cap strips	Advance Biotechnologies Ltd.
Sterile disposable Scalpels	Swann - Morton
15ml Falcon® 2059 polypropylene tubes	Becton Dickinson
15 ml centrifuge tubes	Iwakai Ltd.

Table 2.I: Table showing plastics and disposables

EQUIPMENT	SUPPLIER
Ice Machine	Scotsman Ice System Ltd.
P2, P10, P200 and P1000 Pipettes	Finpipette
Co_2 Incubator	Sanyo, Gallenkamp plc.
Hereaus Centrifuge	Sanyo
UV spectrophotometer	Eppendorf
PCR machines	MWG
Water bath	Grant Instruments Ltd.
Vortex	Scientific Industries Inc.
Heating block	Grant Instruments Ltd.
Light Microscope	Olympus
Power Pac	Bio-Rad Laboratories Ltd.
Trans-Blot® Semi-dry Transfer cell	Bio-Rad Laboratories Ltd.
Water bath	Grant Instruments Ltd.
Vortex	Scientific Industries Inc.
Heating block	Grant Instruments Ltd.
Light Microscope	Olympus
Mini horizontal electrophoresis tank	Bio-Rad Laboratories Ltd.
UV Transilluminator	UVP Inc.
800W Microwave	Panasonic
Hotplate Magnetic Stirrer	Chemlab
PH meter	Denever Instruments
Electroporator	Bio-Rad Laboratories Ltd.
Orbital incubator	Gallenkamp
Orbital shaker	Stuart Scientific
ABI 7200 sequence detector	Applied Biosystems
ABI 377 sequencer	Applied Biosystems
Balance	Toledo

Table 2.2: Table showing Laboratory equipment and instruments

MATERIAL	**SUPPLIER**
Para film "M" laboratory film	American National Can Ltd.
Aluminium foil	Terinex Ltd.
Paper tissue	Kimberly-Clark Corporation
Autoclave tape	Rexam Medical Packaging
Latex examination gloves	Ansell Medical

Table 2.3: Table showing miscellaneous disposable laboratory equipment

2.2 METHODS.

2.2.1 Western Blotting

2.2.1.1 Buffers and Reagents.

• RIPA buffer: 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS dissolved in PBS and stored at 4°C.
• Phenyl Methyl Sulfonyl Fluoride (PMSF) (Calbiochem- Novabiochem Corporation) was dissolved in Isopropanol (57mM) at a concentration of 10 mg/ml and stored at -20°C.
• Protein electrolysis (sample) buffer: 1ml glycerol, 0.5ml β-mercaptoethanol, 3ml 10% SDS, 1.25ml 1M Tris-base pH 6.7, 1mg Bromophenol Blue and stored at -20°C.
• Ammonium Persulphate was prepared fresh at a concentration of 10% in ddH_2O and stored at 4°C.
• Acrylamide: 30% acrylogel solution (BDH Merck) was stored at 4°C until use.
• Molecular weight marker: Dual Band Pre-stained protein marker (Bio-Rad) was stored at -20°C until use.
• 10X TGS (Running) buffer: 30g Tris-base, 144g Glycine, 10g SDS adjust up to 1000cc with ddH_2O.
• Sodium Dodecyl Sulphate (SDS) (Sigma). 10g of SDS was dissolved in 100ml of ddH_2O on a heating stirrer and kept at room temperature.
• Towbin (transfer) buffer: 20mM Tris-base, 0.2M Glycine, 200ml Methanol adjust up to 1000cc with H_2O, autoclaved and stored at 4°C.
• PBS-T: Phosphate Buffered Saline mixed with 0.1% Tween- 20.
• Blocking Solution. Dried low fat skimmed milk (Marvel) was dissolved in PBS-T.

2.2.1.2 Experimental Procedure.

Detailed analysis of protein expression was conducted using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). This technique has been well documented to separate proteins in complex mixtures according to their size and molecular weight. The protein samples are

denatured and made monomeric by boiling in the presence of β-mercaptoethanol and negatively charged soap, namely SDS. β-mercaptoethanol is responsible for breaking disulphide bonds which occur between proteins. SDS, however, completely disrupts protein-protein interactions and denatures almost all proteins, thus resulting in the formation of linear molecules. This anionic detergent binds to protein polypeptide chains through its hydrophobic properties, thus imparting all proteins with a relatively equal negative charge.

The acrylamide used in the gels is polymerised with the cross linker, bisacrylamide, which subsequently forms a matrix through which the protein monomers pass when in an electric field. The size of the protein being analysed correlated with the percentage of acrylamide used (Table 2.4). Large molecular weight proteins are separated using low percentage acrlyamide gels as opposed to small molecular weight proteins. The negative charge causes the proteins to migrate towards the cathode and become size fractionated. The smaller protein molecules are able to move faster through the polyacrylamide matrix than the larger molecules. A discontinuous gel system was used which enables the samples to be compressed into a thin starting band, from which finely resolved bands of individual proteins are separated. The discontinuous system has two parts, namely the upper gel (also called the stacking gel) and the lower resolving gel. Protein samples pass easily through the large poured matrix of the stacking gel before becoming concentrated and finely separated in the lower resolving gel.

PROTEIN SIZE RANGE kDa	%GEL USED	30% ACRLAMIDE MIX (ml)	1M TRIS-BASE pH8.8 (ml)	10% SDS (μl)	10% APS (μl)	ddH_20 (ml)	TEMED (μl)
40 - 200	8	2.6	3.75	100	100	3.45	10
15 - 100	12	3.9	3.75	100	100	2.15	10
5 - 50	16	5.2	3.75	100	100	0.85	10

TABLE 2.4 Table showing composition of 8-16% resolving gels. Stacking gels were composed of: 0.83ml (30% Acrylamide mix); 0.63ml (1M Tris pH 6.8); 3.45ml ddH_20; 50μl 10%SDS; 50μl 10% APS and 10μl TEMED.

Protein Extraction and Quantification

All cells were grown in 100mm culture dishes to 80-90% confluence. Proteins of interest were extracted from the respective cell lines by re-suspending the cell pellet in 300μl of ice-cold RIPA buffer, containing the detergents NP-40 and sodium deoxycholate. The protease inhibitor, PMSF (3μl of 10mg/ml) was also added to the suspension. Cells were placed on ice for approximately 60 minutes, after which the protein was extracted by centrifuging at 15,000 x *g* using a bench-top micro-centrifuge at room temperature for 10 minutes to pellet out cell debris. The supernatant containing the extracted protein was aliquoted into 0.5ml tubes and stored at -80°C until required.

Protein samples were quantified based on the Bradford assay (1976), using the commercially available protein reagent Coomassie™ plus-200 (Pierce). Briefly, samples and standards were diluted in PBS to a final volume of 150μl. Bovine serum albumin (BSA) was used as the protein standard within the range of 0-25 μg/μl. Each sample was measured in triplicate. 150μl of Bio-

Rad protein dye was then added to all protein samples and standards. Protein concentrations were determined using an Anthos 2001 automated plate reader (Anthos Labtec Instruments) which measures the absorbance at 595nm. All protein concentrations were calculated relative to the BSA standards.

SDS-Polyacrylamide Gel Electrophoresis

Components of resolving gels were selected in order to give optimal separation of the protein under investigation. Resolving gel mixes were prepared appropriately and poured into pre-assembled gel pouring apparatus. The gels were overlaid with butanol-saturated water and allowed to polymerise for 1 hour at room temperature. The butanol was drained off and the gel surface was briefly rinsed with water. The stacking gel was then poured, and a sample well comb (5.08mm by 0.75mm) inserted. The stacking gel was allowed to polymerise for approximately 20 minutes at room temperature. 50-100μg/ml of protein extracts was mixed with 10μl of reducing buffer and heated for 3 minutes at 100°C prior to loading. 12μl of pre-stained Rainbow molecular weight marker (Bio-Rad) was also loaded on each gel in order to aid the determination of molecular weight. Gels were then subjected to electrophoresis in 1 x TGS running buffer at 125mV until the bromophenol blue tracking dye reached the bottom of the gel.

Immuno-blotting

Proteins resolved on SDS-PAGE gels were then transferred onto nitro-cellulose membrane (Hybond-ECL, Amersham) using a Trans-Blot® Semi-dry Transfer cell (Bio-Rad). Briefly, the gel and nitrocellulose membrane (of similar size) were allowed to soak in cold (4°C) transfer buffer for 10 minutes at room temperature. A blotting sandwich was then set up on the anode

plate of the transfer cell, such that the gel was in contact with a sheet of nitro-cellulose (Figure 2.1). Both were sandwiched between appropriately sized sheets of 3mm filter paper (Bio-Rad) (briefly soaked in cold transfer buffer). Any air bubbles were removed before the cathode plate was placed on top. Transfer was carried out at 10V for 42 minutes.

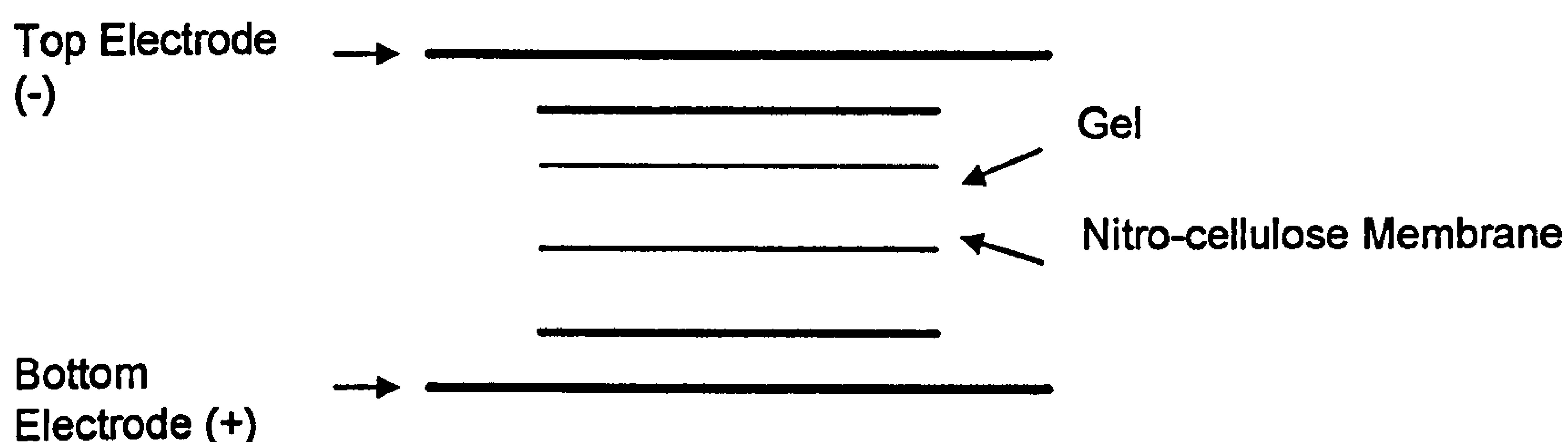

Figure 2.1 Illustration of the immuno-blotting procedure. A blotting sandwich was set up on the anode plate of the transfer cell. Proteins resolved on SDS-PAGE gels were transferred onto nitrocellulose membrane using a Trans-Blot® Semi-dry Transfer cell (Bio-Rad)

Following the transfer of the proteins onto nitro-cellulose, the membrane was incubated on a rocking platform in 5% non-fat milk (w/v) for 1 hour at room temperature to block non-specific sites. The membrane was then incubated with the appropriate primary antibody mixed in 3% non-fat milk (w/v) overnight, shaking at 4°C. On the following day the membrane was washed (5 x 10 minutes, shaking at room temperature) in PBS-T to remove any excess unbound antibody. A species-specific IgG/horseradish peroxidase conjugate secondary antibody (diluted appropriately in 3% milk (w/v)) was then incubated with the membrane for 1 hour, shaking at room temperature. Membranes were finally washed (3 x 5 minutes, shaking at room temperature) in PBS-T.

Protein detection

All proteins were detected using the Amersham Enhanced Chemiluminescence (ECL) system according to manufacturer instructions. The two reagents were mixed in equal volumes (total volume of 3mls) and poured onto the membrane filter, which was agitated for 1 minute. The membrane was subsequently wrapped in cling film and then exposed to Kodak X-Ray film for between 5-15 minutes. Films were developed in the dark by initially immersing them in X-Ray developer (Kodak; diluted according to the enclosed instruction) for 2 minutes, followed by a brief wash in water. Films were then placed in X-Ray fixer (Kodak; diluted according to the enclosed instruction) for a further 2 minutes. Films were finally rinsed in tap water for 5 minutes and allowed to air dry.

2.2.2 Southern Blotting.

2.2.2.1 Buffers and Reagents.

- DNA Lysis Buffer: 100mM Tris-HCl (pH8.5), 5mM EDTA, 0.2%SDS, 200mM NaCl.
- Proteinase K (Sigma) was dissolved in water and used at a final concentration of 100µg/ml.
- Restriction enzymes (Promega). Enzymes were used with supplied buffers and stored at -20°C.
- Low melting agarose (BioWittaker) was dissolved in 1 x TAE at a concentration of either 1 or 2% by heating in a microwave oven (Panasonic).
- 1X TAE: 30mM Tris base, 20mM glacial acetic acid and 2 ml 0.5M EDTA dissolved in 1 litre of water; made to pH:8.0.
- Loading dye: 0.25% Bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water.
- Ethidium Bromide (Sigma). One tablet (100mg) was dissolved in 40ml of ddH_2O.
- 100bp Molecular Weight Marker (Life Technologies Ltd).

- 20 x SSC stock solution: 3M NaCl, 300mM Na_3 citrate salt (pH 7.0) dissolved in distilled water. Distilled water was also used in all subsequent dilutions.
- 20% (w/v) SDS stock solution. 20g of SDS was dissolved in 90mls of distilled water. The SDS was dissolved by heating to 68°C, after which another 100mls of distilled water was added. Distilled water was also used in all subsequent dilutions.
- Hybond-Nylon $^+$ transfer membrane (Amersham) was handled wearing gloves and stored at room temperature.
- Sonicated DNA (Sigma). Was used at a concentration of 1mg/ml and was dissolved in water and incubated overnight at 37°C. DNA was then sheered prior to use.
- Denhardt's Solution: 2% (w/v) bovine serum albumin (Sigma), 2% (w/v) Ficoll™ (Sigma) and 2% (w/v) polyvinylpyrrolidone (Sigma). The components were dissolved by incubating the solution overnight at 37°C.
- Alpha dCTP
- Pre-hybridisation solution: 5 x SSC, 5 x Denhardt's solution and 10% (w/v) SDS. The solution was made up to 25ml with sterile water. 0.5ml of a 1mg/ml solution of sonicated non-homologous DNA was denatured by heating to 100°C for 5 minutes prior to adding to the pre-hybridisation solution.
- Hybridisation solution: 5 x SSC, 5 x Denhardt's solution and 10% (w/v) SDS. The solution was made up to 25ml with sterile water. 0.5ml of a 1mg/ml solution of sonicated non-homologous DNA was denatured by heating to 100°C for 5 minutes and added to the pre-hybridisation solution. 0.1g/ml Dextran sulphate was also added to the hybridisation solution along with a labelled probe.

2.2.2.2 Experimental Procedure.

Southern Blotting is a technique initially developed by Edwin M. Southern in the late 1970s. This technique is often used to definitively ensure a particular fragment of DNA has been successfully integrated into the genome of a host organism. It enables the detection of a specific gene in a genome as well as being able to locate it with respect to restriction sites. Applications for Southern Blotting include the screening of DNA libraries, chromosome walking, in DNA-based disease diagnosis as well as in DNA fingerprinting. Both the integrity and copy number of the recombination reporter substrate, ScNeo was determined using this well established technique.

In brief, Southern Blotting involves the separation of digested DNA fragments on agarose gels followed by denaturation *in situ* (Figure 2.2, Page 75). The fragments are then transferred onto a solid support, e.g. nitrocellulose or nylon membrane, where the DNA fragments becomes fixed and immobilised. The DNA attached to the membrane is hybridised with a labelled nucleic acid probe of interest. The membrane is subsequently washed to remove unbound and weakly bound probe. Bands complementary to the probe are located by an appropriate detection system such as autoradiography. Estimating both the size and number of bands generated after the DNA is digested with different restriction enzymes, solely or in combination, makes it possible to place the target DNA within the context of restriction sites.

Genomic DNA obtained from the MMR -proficient and -deficient cell lines, transfected with ScNeo, was extracted and purified using Proteinase K and phenol. Briefly, 500μl of DNA Lysis buffer was added to the monolayer of cells grown on 100mm culture dishes. Cells were then scrapped off the plates, using a rubber policeman, and placed into 15ml tubes. Proteinase K (final concentration 100μg/ml) was added to all samples and the DNA was incubated overnight at 37°C. The following day, DNA was precipitated using 500μl of 100% Isopropanol and allowed to dry. All samples were then subjected to phenol-chloroform purification. Briefly, samples were re-suspended to a final volume of 500μl in distilled water. An equal volume of buffer saturated Phenol-Chloroform IsoAmyl alcohol mix (at a 25:24:1 ratio; BDH Labs) was added to the re-suspended DNA. Samples were briefly vortexed prior to centrifuging at room temperature at 13.2rpm for 3 minutes. The top aqueous layer was abstracted and placed in a clean Epindorff tube, after which the above step was repeated. DNA was then precipitated by adding 1.5mls of 100% Ethanol and 50μl of 3M Sodium Acetate (pH 5.2), samples briefly mixed and placed at -80°C for 30 minutes. Impurities were discarded by centrifuging all samples for 20 minutes at 13.2rpm at room temperature. The supernatant was then carefully removed and the pellet

obtained washed with ice-cold 70% ethanol. DNA was dried and re-suspended in an appropriate volume of distilled water.

Careful attention was paid to ensure the purity of the DNA as contamination by salt, RNA, protein and DNases could render the DNA resistant to cleavage by restriction endonucleases. Restriction enzymes are protein enzymes that are capable of 'scanning' both single and double stranded DNA molecules and digesting the strands at a particular recognised sequence. Since the sites of cleavage of long DNA molecules are determined by their nucleotide sequence, the long DNAs are broken into discretely sized fragments; which is determined by the distance between restriction enzyme cleavage sites. Most enzymes are affected by high glycerol concentrations (as this can alter the dielectric constant of the mix and therefore affect the specificity of the enzyme). Subsequently the enzymes were used in conjunction with commercially supplied buffers which diluted the enzyme (thus keeping the glycerol concentration to <10%) to avoid this problem. The integrity of the recombination reporter substrate was determined by digesting approximately 10µg of each DNA sample with 50U of Xho I and Hind III in a 60µl reaction supplemented with BSA. This double digest should produce a 4kb sized fragment (Johnson *et al.,* 1999). All digests were performed overnight at 37°C. The DNA samples were mixed with Bromophenol blue tracking dye and size fractionated by gel electrophoresis. 5µl of each sample was run on a 1% agarose gel at 90V in order to ensure that the DNA was completely digested.

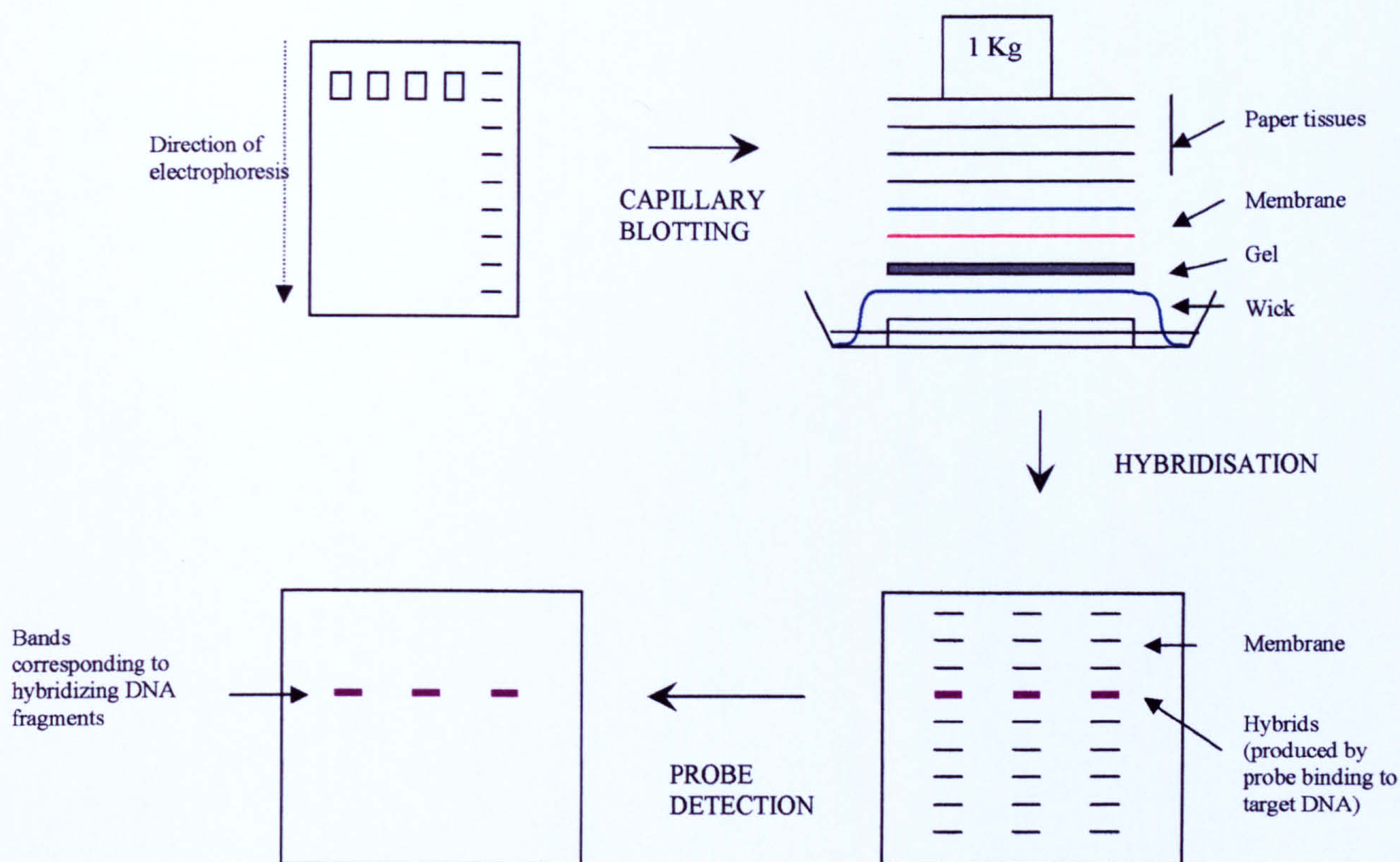

Figure 2.2 Illustration of Southern blotting procedure. Digested genomic DNA, obtained from MMR –proficient and –deficient tumour strains transfected with the ScNeo recombination reporter substrate, was separated by electrophoresis on agarose gels. DNA was subsequently transferred onto a nitrocellulose membrane by capillary transfer. Membranes were then hybridised with a radioactively labelled probe in order to allow for ScNeo detection.

A 1% agarose gel was made using 1 x TAE buffer and poured into horizontally laid casting trays. The gel was allowed to cool to approximately 55°C, after which it was mixed with ethidium bromide (0.5μg/ml). Combs were then inserted, any air bubbles were removed and the gels were allowed to set for 1 hour at room temperature. The combs were then removed and the gel tray loaded into a chamber containing 1 x TAE electrophoresis buffer which just covered the surface of the gel. The samples were then loaded

such that the DNA concentration resembled equal amounts throughout. 20µl of marker was loaded on either side of the samples in order to determine the size of DNA fragments. An electric voltage of 32V was then applied and the DNA fragments were allowed to migrate through the agarose pores overnight. Photographs of the gels were then taken with a ruler placed alongside the gels, both with and without the marker lane. This was performed in order to avoid excess binding of labelled probe to the DNA contained within the marker and to enable the 4Kb fragment to be identified.

Following gel electrophoresis, the DNA was de-purinated in order to render it single stranded. This was done by incubating the gel in 0.25M HCl, shaking at room temperature for 30 minutes, until the bromophenol blue dye front changed colour. The gel was twice washed briefly in distilled water and allowed to re-hydrate for 10 minutes at room temperature. Care was taken not to expose the gel to acid for too long, as this would have resulted in the DNA being cleaved into small fragments which are too short to bind efficiently to the membrane.

An important step in Southern blot hybridisation is the transfer of fragmented DNA to a solid support. DNA is transferred to nylon membranes as the agarose gels used are often too fragile and the DNA within the gel too easily diffusible to withstand the blotting procedure. Supported nylon membranes, however, are far more durable and also have a high DNA binding capacity. During capillary transfer, DNA is carried from the gel in an upward flow of liquid where it attaches to the surface of the membrane. The liquid is attracted through the gel by capillary action which is secured and preserved by a stack of dry absorbent paper towels. The rate at which the DNA is transferred depends mainly on the concentration of the agarose gels used and the size of the DNA fragments. The efficiency of transfer of large DNA fragments is determined by the fraction of molecules that escape from the gel before it becomes de-hydrated. To alleviate this problem, the DNA in the

gels was exposed to HCl followed by denaturation using 0.4M NaOH, which subsequently hydrolysed the phosphodiester backbone at the sites of de-purination. The size fractionated DNA was transferred to a sheet of nylon H+ membrane by capillary action (Figure 2.2). Briefly, a wick was made on a raised platform by saturating three sheets of Whatman 3MM filter paper in 0.4M NaOH. The agarose gel was then placed on top of the wick, with care taken to avoid trapping any air bubbles beneath the gel. A sheet of Hybond-N+ membrane (which was cut to the exact size of the gel) was carefully placed on top of the gel, again avoiding trapped air bubbles. Both the gel and the membrane were then surrounded with cling film in order to prevent the buffer being absorbed directly into the paper towels above. A further three sheets of 3MM filter paper (cut to an appropriate size) were saturated in 0.4M NaOH and placed on top of the membrane. A stack of absorbent paper towels (approximately 5cm high) were then placed on top of the filter paper. A tray and a 1Kg weight were placed on top of the paper towels. The DNA was allowed to transfer for approximately 12-16 hours at room temperature. Following the completion of transfer, the apparatus was dismantled and the membrane was marked with a pencil in order to identify which side the DNA was bound. The membrane was then briefly washed in 2 x SSPE to remove any adhering agarose and was prepared for pre-hybridisation.

Membranes were pre-hybridised in pre-hybridisation solution at 65°C for 24 hours prior to the addition of the Neo probe (Figure 3.1). This pre-hybridisation step prepares the membrane for probe hybridisation by blocking off the non-specific nucleic acid binding sites on the membrane. This subsequently results in a lower signal to noise ratio. The pre-hybridisation solution also contains non-specific DNA, such as sonicated salmon sperm DNA, in order to lower the background. Following pre-hybridisation, the membrane was immersed in hybridisation solution which contained a radioactively labelled, denatured probe. The labelled probe molecules bind onto complementary molecules which are bound onto the membrane. The

subsequent formation of double-stranded DNA strands results in the binding of the probe to the sheet. The probe was labelled using a Prime-It® Random Primer Labelling Kit (Stratagene) following manufacturers instructions. This system makes use of the ability of random hexanucleotides annealing to multiple sites along the length of a DNA template. The primer-template complexes serve as a substrate for the Klenow fragment of DNA polymerase I, which subsequently synthesizes new DNA. The newly synthesised probe is made radioactive by addition of radio labelled ^{32}P-αdCTP to the mixture.

Membranes were incubated in hybridisation solution for approximately 12-16 hours, rotating at 65°C. Hybridisation is often carried out at a low stringency (which refers to the conditions of the hybridisation as compared to the temperature required to separate the DNA strands). This insures that the probe will bind to the target DNA. The membranes were washed repeatedly using low to high stringency conditions such that un-hybridised probe is removed but the hydrogen bonds between the probe and sample DNA are not disrupted. To begin with, all membranes were washed twice in 2 x SSC, 0.1% SDS (w/v) shaking, at room temperature for 10 minutes. The washing solution was then replaced with 1 x SSC, 0.1% (w/v) SDS, and the membranes incubated shaking at 65°C for 15 minutes. In the final step, the membranes were washed with 0.1 x SSC, 0.1% (w/v) SDS at 65°C for 10 minutes. The blots were then wrapped in plastic and of the areas where the labelled probe fragments bound was detected by phosphoimaging (Biorad Machine name, number software). Images were prepared for printing using Adobe Photoshop software.

2.2.3 DNA Amplification and Purification.

2.2.3.1 Uterine Tissue Sample Preparation and microdissection.

Appropriate formalin fixed, paraffin embedded tissue blocks were selected from eight cases of uterine leiomyosarcomas. Normal myometrium and tumour tissue were separately marked by the histopathologist (Dr. Jason Stone) after which 10μm sections were microdissected to give enriched (>80%) normal and tumour tissue. The paraffin wax was removed from the slides in two washes of xylene. Samples were subsequently re-hydrated using consecutive washes in 100% ethanol, 95% ethanol, 70% ethanol and distilled water. Following this, both the tumour tissue and surrounding tissue was micro-dissected out using a scalpel. Both tissue samples from each slide were transferred into respective 1.5ml microcentrifuge tubes. All samples were subsequently purified using a QIAamp® DNA Mini Kit (QIAGEN). In this procedure, samples were lysed in 360μl of tissue lysis buffer and 400μg Proteinase K and incubated at 56°C until the tissue was completely lysed. The rest of the DNA extraction procedure involved binding of DNA onto a silica-gel-membrane and several washing steps to wash away protein and other contaminants. The DNA was eluted with an elution buffer. All DNA samples were quantified and kept at -20°C until use.

2.2.3.2 Polymerase Chain Reaction (PCR).

2.2.3.2.1 Buffers and Reagents.

- 25μl final reaction volume using 2 x PCR Master-mix (ABgene) contains:
- 1.25 units *Taq* DNA polymerase, 75mM Tris-HCl (pH: 8.8), 20mM Ammonium Sulphate, 1.5mM Magnesium Chloride, 0.01% (v/v) Tween 20, 0.2mM each of dATP, dCTP, dGTP and dTTP.

2.2.3.2.2 Experimental procedure.

Genomic DNA and cDNA amplification was obtained using the Polymerase Chain Reaction (PCR) technique. PCR enables the amplification of a specific region of DNA through the use of thermo stable DNA polymerases. Typically, PCR often results in a million fold amplification of the target DNA. This technique is often used in such applications as gene cloning, sequencing and site-directed mutagenesis.

All reactions were prepared according to manufacturers instructions at room temperature using sterile equipment. In all cases 100ng of DNA and 10 pmol of both forward and reverse primers were used unless stated. PCRs contain several components, apart from the template DNA and pairs of synthetic oligonucleotides which prime DNA synthesis, including a thermo stable DNA polymerase, deoxyribonucleoside triphosphates and divalent cations. The DNA *Taq* polymerase used is generally the most frequently used polymerase for routine PCRs. The optimal temperature for this, and most, polymerase enzymes is between 72 and 74°C. The synthetic oligonucleotides used were carefully designed in order to obtain the product of interest in high yield. Careful primer design also suppresses the amplification of unwanted sequences and facilitates subsequent manipulation of the amplified product. The oligonucleotides used, were on average 20 nucleotides in length and, where possible, had a G or a C residue as the last one or two bases on the 3'end. Both of these factors enabled stronger hybridisation of the oligonucleotides to the target DNA and also ensured recognition by the polymerase.

The mechanism by which PCR works involves denaturation of the target DNA by heat; annealing of primers which are complementary to the opposing strands; followed by extension of the annealed primers by DNA polymerases. The length and the G/C content of the dsDNA template determines both the

temperature and the length of time used for the denaturation step of typical PCRs. Denaturation is generally carried out at 94-95°C as this is the highest temperature that most polymerases can endure without being damaged. All PCRs used in the present study were carried out in a DNA thermocycler supplied by MWG. The initial denaturation step was carried out at 94°C for 5 minutes in order to increase the chance of fully separating the DNA strands (Table 2.5). The temperatures used for annealing of synthetic oligonucleotides are described in the Appendix. Generally, annealing was carried out 2°C lower than the calculated melting temperature at which the oligonucleotide primer dissociates from the template. The extension of oligonucleotide primers was carried out at 72°C in all cases.

All PCR products were then purified using either a QIAquick® PCR purification Kit or a QIAquick® Gel Extraction Kit. DNA samples purified using the QIAquick® Gel Extraction Kit were initially separated on 1% agarose gels and the correctly sized DNA fragment was carefully excised using a sharp blade. Both the PCR purification and gel extraction systems make use of a silica gel membrane which binds DNA in the presence of a high salt concentration and a pH of ≤7.5. Once bound, the DNA is washed off impurities (such as unincorporated primers and partially amplified products) by repeatedly washing the columns with an ethanol containing buffer. All DNA was finally eluted in 30µl of sterile water.

STEP	CYCLES	TEMPERATURE	TIME
Denaturation of template DNA	1	94°C	5 mins
Denaturation of template DNA	40	94°C	30 secs
Annealing of oligonucleotides		50-60°C	45 secs
Primer extension		72°C	1min/kb
Final extension step	1	72°C	5 mins

Table 2.5 Table showing typical PCR cycling steps and temperatures used for DNA amplification.

2.2.3.3 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR).

2.2.3.3.1 Buffers and Reagents.

- 25µl final reaction volume using 2 x RT-PCR Master-mix (ABgene) contains:

Thermoprime plus DNA polymerase (Final concentration: 0.625U/25µl, 1.5mM Magnesium Chloride, 0.2mM each of dATP, dCTP, dGTP and dTTP. 0.5µl of *Reverse*-iT™ Blend (50U/µl) was also added to each reaction.

- RQ1 RNase-free DNase Kit (Promega).

2.2.3.3.2 Experimental Procedure.

RT-PCR is a technique frequently used to amplify cDNA copies of mRNA. It allows the detection of gene expression even when the RNA of interest is expressed at low levels and/or when there is only limiting amounts of RNA available. Principally, RT-PCR consists of two reactions, namely the reverse transcriptase reaction which is followed by polymerase chain reaction to

amplify cDNA copies. A reverse gene specific primer is initially hybridised to the mRNA transcript. The primer is extended by an RNA-dependent DNA polymerase called reverse transcriptase, which subsequently converts RNA into a single-stranded cDNA template. Once the first cDNA strand has been synthesised, the mRNA-cDNA hybrid is denatured to allow for second cDNA strand synthesis. The synthesis of the second strand is initiated by the forward primer and is completed with a thermo stable DNA polymerase. The cDNA product is then amplified by a standard PCR which generates multiple copies of desired cDNA. In our studies, this technique was used to screen for mutations in transcribed sequences containing mononucleotide runs.

RT-PCRs contain several components, apart from the template RNA and the reverse transcriptase enzyme, including thermo stable DNA polymerases; pairs of synthetic oligonucleotides (which prime cDNA synthesis); deoxyribonucleoside triphosphates and divalent cations. A variety of primers can be used, apart from gene specific primers, including the use of Oligo (dT) primers (which bind specifically to endogenous poly $(A)^+$ tails of mammalian mRNAs) or random hexanucleotides (which prime cDNA synthesis at random points along RNA templates). Total RNA was extracted from mammalian cell lines using standard procedures (RNeasy® protect mini kit, Qiagen) and subjected to DNase treatment (Promega) in order to eliminate genomic DNA contamination. The deoxyribonuclease I present degrades both single and double stranded DNA molecules in order to produce 3'-hydroxyl oligonucleotides whilst maintaining the integrity of RNA. Briefly, 8µl of extracted RNA was mixed with 1µl of 10X reaction buffer and 1µl of RNase-Free DNase. The mixture was incubated at 37°C for 30 minutes, after which 1µl of DNase stop solution was added and the reaction incubated for a further 10 minutes at 65°C to inactivate the DNase and terminate the reaction.

RT-PCRs were conducted using the Reverse-iT™ ONE-STEP RT-PCR Kit (ABgene) following manufacturer instructions. Between 10pg - 1µg of total

extracted RNA was used along with 10pmol of each forward and reverse primer in a 25µl total reaction volume. Typical reaction conditions are shown in Table 2.6 and were conducted in a DNA thermocycler (MWG). Temperatures used for annealing of synthetic oligonucleotides and respective sequences are described in the Appendix. All cDNA products were purified using either a QIAquick® PCR purification Kit or a QIAquick® Gel Extraction Kit as previously described. 5µl of cDNA product was loaded on a 2% agarose gel and compared to the DNA size marker to ensure that the correct sized product was obtained.

STEP	CYCLES	TEMPERATURE	TIME
First Stand Synthesis	1	47°C	30 mins
RTase Blend inactivation and initial denaturation	1	94°C	2 mins
Denaturation	40	94°C	30 secs
Annealing		50-60°C	30 secs
Extension		72°C	1min/kb
Final Extension	1	72°C	5 mins

Table 2.6 Table showing typical RT-PCR cycling steps and temperatures used for cDNA amplification.

2.2.3.4 DNA Sequencing.

DNA sequencing enables the precise sequence of nucleotides in a sample of DNA to be determined. Both the Maxam and Gilbert (chemical, 1977) and the Sanger (enzyme, 1977) methods of sequencing involve the synthesis of a set of molecules which have a common end and can be separated by size fractionation using polyacrylamide gel electrophoresis. The enzymatic

method of sequencing, initially developed by Sanger (1977), makes use of four specific dideoxynucleotides (ddNTPs) which terminate the synthesis of template DNA. This method is also known as the chain termination method as the ddNTPs used, lack an OH group on the deoxyribose, thus resulting in the termination of chain elongation once incorporated. A primer labelled with a fluorescent tag is annealed to a single-stranded DNA template molecule and a DNA polymerase extends the primer by using ddNTPs. The extension reaction is split into four and each quarter is terminated separately with one of the four specific ddNTPs, thus creating a competition between extension of the chain and base-specific termination. The four samples can be separated by PAGE and the locations of each band can be detected through the emission of fluorescence.

2.2.3.4.1 Experimental Procedure.

cDNA expression studies and sequencing was accomplished using a SequiTerm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies) as described originally by Steffens *et al.,* (1995) which uses the chain termination principle. All sequencing reactions were prepared using sterile equipment, according to manufacturers' instructions. Reagents were thawed on ice and gently vortexed before use. All reactions were completed using a DNA thermocycler (MWG); typical cycling conditions are shown in Table 2.7. Temperatures used for annealing of synthetic oligonucleotides are described in the Appendix. The template DNA was purified extensively using either a QIAquick® PCR purification Kit or a QIAquick® Gel Extraction Kit prior to sequencing, as contamination by salt, RNA, protein and DNases could all obstruct the quality of sequence and read length obtained. The sequencing primer which annealed in a 5' to 3' direction to the template was labelled with a pentamethine carbocyanine dye (IRD700) and had an absorption maximum of 685nm. The complementary sequencing primer was labelled with a heptamethine cyanine dye (IRD800) and had an absorption maximum of

795nm. As both dyes become absorbed and fluoresce near to the infrared region of the spectrum, they enable DNA fragments to be sequenced in both directions.

Upon completion of the sequencing reaction, 3µl of Stop/Loading Buffer (provided) was added to each reaction and samples stored at -20°C until ready for sequencing. All reactions were denatured at 96°C for 3 minutes prior to being subjected to electrophoresis, which was performed using a Li-Cor IR2 420-S DNA Analysis system (Li-Cor Inc.). Between 0.5-1µl of each reaction was then loaded on to a 6% Long rang gel by Dr. Ian Brock (Institute for Cancer Studies) and run at 1500V for between 7-9 hours. Sequences of gene fragments were detected using the AlignIR™ and BaseImagIR™ soft wares (Li-Cor Inc.). Detailed analysis was also conducted using the Sequencher software 4.1 (Gene code corporation) and Adobe Photoshop (7.0).

STEP	CYCLES	TEMPERATURE	TIME
Denaturation of template DNA	1	94°C	5 mins
Denaturation of template DNA	40	94°C	30 secs
Annealing of oligonucleotides		50-60°C	45 secs
Primer extension		72°C	1min/Kb
Final extension step	1	72°C	5 mins

Table 2.7 Table showing typical sequencing reaction steps and temperatures used for DNA amplification.

2.2.4 Agarose Gel Electrophoresis

2.2.4.1 Buffers and Reagents.

- Low melting agarose (BioWittaker) was dissolved in 1 x TAE at a concentration of either 1 or 2% by heating in a microwave oven (Panasonic).
- 1X TAE: 30mM Tris base, 20mM glacial acetic acid and 2 ml 0.5M EDTA mix in 1 litre of water (pH:8.0).
- Loading dye: 0.25% Bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water.
- Ethidium Bromide (Sigma). One tablet (100mg) was dissolved in 40ml of ddH_2O.
- 100bp Molecular Weight Marker (Life Technologies Ltd).

2.2.4.2 Experimental Procedure.

Agarose gel electrophoresis separates DNA fragments according to their size. When agarose is dissolved in boiling water and then cooled it polymerizes thus resulting in a semi-solid matrix containing pores. The pore size can be controlled by the concentration of agarose used, thus different concentrations of gel allows the optimal resolution of fragments in different size ranges. The melted agarose was allowed to cool to approximately 55°C after which 0.5µg/ml Ethidium Bromide was added. Ethidium bromide intercalates into DNA molecules thus staining it and allowing visualization of fragments by UV. The solution was then allowed to solidify in an electrophoresis tray (Bio-Rad) containing an appropriately sized comb. Once the gel had set, the combs were removed and the tray transferred to an electrophoresis tank (Bio-Rad) containing 1 x TAE running buffer. DNA samples were mixed with loading buffer and loaded into the wells. Electrophoresis was performed at 70-100 volts at room temperature until the tracking dye reached the bottom of the gel. DNA bands were visualized on a UV- Transilluminator (UVP Inc.). The sizes of DNA fragments were estimated

by comparison to 5µl of DNA Hyperladder marker (Gibco BRL) which was run on the same gel.

2.2.5 Pulse-Field Gel Electrophoresis.

2.2.5.1 Buffers and Reagents

- Pulse-field low melting agarose (BioWittaker) was dissolved in 0.5 x TBE at a concentration of either 0.8 or 1% by heating in a microwave oven (Panasonic).
- 10 X TBE buffer: 108g Tris-base, 55g Boric acid, 40ml 0.5M EDTA and ddH_20. The pH was adjusted to 8.0 such that the final volume was 1 litre.
- 10 X TE buffer: 10mM Tris-Cl (pH 7.4) and 1mM EDTA (pH8.0) and the final pH was adjusted to 7.4.
- 0.5M EDTA: dissolve 186.1g of dissodium ethylene diamine tetra-acetate into 1 litre of ddH_20. The pH was adjusted to 8.0 with NaOH
- Ethidium Bromide (Sigma). One tablet (100mg) was dissolved in 40ml of ddH_2O.

2.2.5.2 Experimental Procedure

5 x 10^6 cells were plated onto 10cm tissue culture plates 24 hours prior to treatment. Cells were treated with either 10mM thymidine or 100nM CPT. Following treatment, cells were trypsinised from the plates and 1 x 10^6 cells were melted into 0.8% agarose inserts. For IR treatment, 1 x 10^6 cells were melted into 0.8% agarose inserts, after which samples were placed in tubes containing 2mls of D-MEM medium. Such tubes were then placed on ice and exposed to 10Gy IR. All agarose inserts were then incubated at 50°C in 0.5M EDTA, 1% N-laurylsarcosyl and 1mg/ml proteinase K for 48 hours. Inserts were then washed four times for 2 hours in 1 x TE buffer. Samples were then loaded onto 1% agarose gels (made with pulse-field agarose and 0.5 x TBE). Electrophoresis was carried out at 120° angle, 60-240 seconds switch time at 4V/cm for 24 hours (BioRad). Gels were subsequently stained in a solution

of 0.5 x TBE and 1mg/ml ethidium bromide for 2 hours before being visualized on a UV illuminator.

2.2.6 Tissue Culture.

2.2.6.1 Buffers and Reagents.

- Phosphate Buffered Saline (PBS) was prepared by dissolving one tablet (Oxoid Ltd.) in 100ml distilled water and was autoclaved before use.
- Dimethyl Sulphoxide (DMSO) (BDH Merck) was prepared at 10% (v/v) in tissue culture media and used for freezing cells.
- Tissue culture media: Dulbecco's modified eagle's medium (DMEM) supplemented with 4.5g/L Glucose and L-Glutamine was purchased from BioWhittaker Ltd. and stored at 4°C until use. All tissue culture media was supplemented with 10% foetal calf serum and checked for sterility before use.
- Foetal Calf Serum (FCS) (Helena BioSciences) was aliquoted in 50ml tubes and stored at -20°C until use.
- Non-essential Amino Acids was purchased from BioWhittaker Ltd. and stored at 4°C until use.
- Presept was supplied by Johnson and Johnson Medical. One tablet of Presept containing 50% Sodium Dichloroisocyanurate was dissolved in 1 litre of sterilized water and kept in room temperature.
- Trypsin (Gibco BRL) was purchased as a 2.5% solution and diluted at 1:20 with sterile PBS and stored at - 20°C until use.
- Versene/EDTA (ethyelenediamine tetra-acetic acid) was supplied by BDH Merck. 0.2g EDTA was dissolved in 1litre of PBS, aliquoted in 10ml bottles, autoclaved at 10lb/inch^2 for 10 minutes and stored at -20°C until use.
- Geneticin (G418 sulphate) (Invitrogen) was made as a stock concentration of100mg/ml and dissolved in ddH_20.
- Thymidine (Sigma) was made as a stock concentration of 10mM and dissolved in media.
- CPT and MMC (Sigma) were both made at appropriate concentrations and dissolved in DMSO and media respectively.

2.2.6.2 Experimental Procedures.

Maintenance and characteristics of cell lines used

Cell culture was performed in Class II biological safety cabinet (Forma Scientific Inc.) using sterile equipment. All cell lines used (Table 2.8a and 2.8b) were cultured adherently in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum in a humidified 5% carbon-dioxide incubator, at 37°C. All cell lines were routinely passaged on 100mm culture dishes at a ratio of 1:20 approximately every 4 days using a 1:1 dilution of 0.25% trypsin and 0.02% EDTA.

Thawing and Freezing of cell lines.

Cryovials of frozen cells were immediately transferred to a 37°C water bath, to allow rapid thawing and thus limiting the toxicity of the DMSO. Once thawed, cells were centrifuged at 400 x g to remove any traces of DMSO, re-suspended in culture medium and transferred to a 100mm culture dish.

For freezing cells, 1 x 10^7 cells were re-suspended in 10ml of culture media and centrifuged at 400 x g. The culture media was then removed and cells re-suspended in medium supplemented with DMSO (10% DMSO, 20% FCS, and 70% DMEM). The cell suspension was then transferred into 2 ml cryovial tubes and stored at -80°C.

MMR PROFICIENT TUMOUR CELL LINES		
CELL LINE	**TISSUE SOURCE**	**OTHER IDENTIFIED CHANGES**
SW480	Colorectal adenocarcinoma	$p53^{-/-}$; APC truncation
SW620	Colorectal adenocarcinoma	$p53^{-/-}$
MRC-5	Lung foetal fibroblast	SV-40 transformed
LM217E	Immortalised human fibroblast	
HELAS3	Cervical carcinoma	

Table 2.8a Table showing all MMR –proficient tumour cell lines used in the present study. Respective tissue sources and any additional changes are also indicated. All cell lines were obtained from ATCC unless stated otherwise

Cytotoxicity Assays.

The cytotoxic response to DNA damaging agents was measured in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum. The cytotoxic response to thymidine was measured in medium supplemented with 10% dialyzed serum (in which exogenous sources of deoxynucleosides have been removed). Briefly, 500 to 1000 cells were plated into 100mm culture dishes and continuously treated with varying concentrations of thymidine, camptothecin and mitomycin C. Cells were allowed to grow for 10 to 14 days in a humidified 5% carbon-dioxide incubator, at 37°C before staining with 0.4% methylene blue/ 50% methanol (Fisher Scientific). Colonies of >50 cells were scored. The surviving fraction was determined by dividing the average number of colonies for each treatment by the average number of colonies in the control plates. All

experiments were plated in triplicate and were repeated independently two to five times.

The cytotoxic response to irradiation was measured in Dulbecco's Modified Eagle Medium supplemented with 10% foetal calf serum. 5 x 10^5 cells were counted and incubated on ice for 10 minutes, after which they were irradiated at varying doses. The cells were then placed on ice for a further 10 minutes and re-plated at a cell density of 500cells/plate. Cells were incubated for 10 to 14 days in a humidified 5% carbon-dioxide incubator, at 37°C before staining with 0.4% methylene blue/ 50% methanol (Fisher Scientific). Colonies of >50 cells were scored. The surviving fraction was determined by dividing the average number of colonies for each treatment by the average number of colonies in the control plate. All experiments were plated in triplicate and were repeated independently two to five times.

MMR -DEFICIENT TUMOUR CELL LINES			
CELL LINE	**TISSUE SOURCE**	**MMR DEFECT**	**OTHER IDENTIFIED CHANGES**
2774	Ovarian	*h*MSH2$^{+/-}$	p53$^{-/-}$
DLD-1	Colorectal adenocarcinoma	*h*MSH6$^{-/-}$	Polδ$^{+/-}$; p53$^{-/-}$
SKUT-1	Leiyomyosarcoma	*h*MSH2$^{-/-}$	p53$^{-/-}$
HCT116	Colorectal carcinoma	*h*MLH1$^{-/-}$; reduced expression of hPMS2	Activated Ras; TGF-β RII $^{+/-}$
HEC1A	Adenocarcinoma	*h*PMS2$^{-/-}$; reduced expression of *h*MLH1	
RKO	Colon	MutL α	TGF-β RII $^{-/-}$
RKO-E6	Colon	MutL α	p53 null
LS411N	Colorectal carcinoma	MutL α	
SW48	Colorectal adenocarcinoma	*h*MSH6-/-, *h*MLH1-methylation silenced expression	

Table 2.8b Table showing all MMR –deficient tumour cell lines used in the present study. Respective tissue sources, MMR defects and any additional changes are also indicated. All cell lines were obtained from ATCC unless stated otherwise

Transfections.

Transfection is a technique in which a gene of interest is introduced into a mammalian cell line by biochemical or physical methods. Applications for this process include studying gene function, as well as observing the genes regulation and interaction with other host mammalian genes. Various chemical and physical methods have been developed to enable the transfection of DNA into mammalian cells, including calcium phosphate co precipitation, electroporation, and complex formation with DEAE-dextran or cationic lipid reagents.

The ScNeo construct was stably integrated into the host genome by transfecting the various MMR -proficient and -deficient cells using electroporation. This method delivers DNA molecules by exposing cells to a brief electrical pulse of high field strength. The exposure to a cell suspension induces a potential difference across the membrane of the cell which subsequently is thought to instigate temporary hydrophobic and hydrophilic pores in the membrane and enables DNA molecules to enter. Briefly, 15 x 10^6 cells were counted and re-suspended in a total volume of 750μl PBS. The cells were then mixed with 15μg of uncut ScNeo substrate (diluted with ddH_20 to a final volume of 50μl) into pre-chilled 0.4cm electroporation cuvettes (Bio-Rad) and placed on ice for 10 minutes. Cells were then electroplated at 0.4kV/ 50μF using an Easy Jet Plus Electroporator (Gene Flow), placed on ice for a further 10 minutes, after which they were plated on culture dishes containing non-selective medium. Hygromycin (final concentration 0.1mM) was added to the medium after 48 hours and subsequent colonies were isolated and expanded from each cell line.

All other constructs used were transfected into the respective cell lines using Lipofectamine™ 2000 (Invitrogen). This reagent contains positively charged liposomes which are attracted electro statically to the phosphate backbone of DNA as well as to the negatively charged surface of the cell membrane. Briefly, 1 x 10^6 exponentially growing cells were plated into 6 well plates and allowed to attach overnight. The following day, 2μg of purified plasmid DNA was mixed with Lipofectamine (final concentration 10μg/ml) and 400mls of serum-free media. Serum-free medium was used as some serum proteins can interfere with the liposome-DNA complex formation. This mixture was allowed to amalgamate at room temperature for a minimum of 30 minutes. Cells were then washed twice with serum-free medium, after which the DNA containing mixture was added along with 1.6ml of serum-free medium. Cells were incubated at 37°C for either 5 hours or overnight and thereafter re-

plated in selective medium. Six to eight colonies were isolated and expanded from each cell line and plasmid expression analyzed.

Recombination Assays.

Verification for the integrity of the homologous recombination repair pathway, in both MMR -proficient and -deficient cells, was conducted using an assay developed by Jasin and co-workers. The ScNeo reporter substrate (Figure 3.1, Page 104) enables the measurement for homology based recombination of two defective neomycin phosphotransferase genes following the introduction of either a DSB by a transiently expressed restriction endonuclease or a substrate which stimulates homologous recombination events.

I-SceI induced.

Approximately 5 x 10^6 cells were plated onto 100mm culture dishes and allowed to attach overnight at 37°C. The DSB was then induced into the cells by transiently transfecting the pCMV3nls-I-SceI expression vector (10ng) for 5 hours using Liopfectamine, after which the medium was changed to normal medium containing serum. The following day all cells were re-plated onto 100mm culture dishes at varying cell densities, ranging from 2 x 10^4 to 2 x 10^5 cells/plate, and again allowed to attach overnight. The recombination frequencies of Neo^+ recombinants were determined by selection in media containing 1mg/ml G418 (Invitrogen). In addition, two dishes were plated with 500 cells each for cloning efficiency. Cells were allowed to grow for 10-14 days before staining with 0.4% methylene blue/50% methanol (Sigma). Only colonies of ≥ 50 cells were subsequently scored. The frequencies of neo^+ recombinants presented are the number of neo^+ colonies formed/(cells plated x the cloning efficiency of the plated cultures). All experiments were plated in

duplicate and repeated independently four to six times. Recombination frequencies were determined using Microsoft Excell.

Drug induced.

Spontaneous recombination events induced by various agents were diminished by initially plating 1000 cells in 6-well plates. Twelve confluent wells for each dose used were then re-plated onto 100mm culture dishes and allowed to attach. Approximately 1 x 10^6 cells per replica were treated with increasing doses of respective agent for 24 hours after which the cells were allowed to recover for 48 hours post treatment. Cells treated with varying doses of thymidine was performed using medium supplemented with 10% dialyzed serum. Each replica was then re-plated onto 100mm culture dishes (at a cell density of 2 x 10^5 cells/plate) and allowed to attach overnight. The frequency of neomycin resistant colonies was determined by selection in media containing 1mg/ml G418 (Invitrogen). In addition, two dishes were plated with 500 cells each for cloning efficiency for each replica. Cells were allowed to grow for 10-14 days before staining with 0.4% methylene blue/50% methanol (Sigma). Only colonies of ≥ 50 cells were subsequently scored. The frequencies of neo^+ recombinants presented are the number of neo^+ colonies formed/(cells plated x the cloning efficiency of the plated cultures). Each replica was considered as an independent experiment and each dose of drug treatment was repeated at least twice.

Apoptosis Measurements

In order to determine whether MMR –deficient tumour strains possessed an acute sensitivity to cell death following expression of the I-SceI endonuclease, cells were co-transfected with a GFP-plasmid and the pCMV3nls-I-SceI expression vector (10ng). Transfection of DNA was

achieved using Liopfectamine as described above. 24 hours post-transfection, 0.01mg/ml Hoechst 33342 stain was added to the medium in order to assess the number of condensed apoptotic nuclei. After 30 minutes, apoptosing cells were observed by fluorescence microscopy and a minimum of 500 nuclei expressing GFP were counted. Cells were counted as apoptotic if their nuclei were obviously bright blue and condensed.

2.2.7 DNA Cloning

2.2.7.1 Buffers and Reagents.

- The pcDNA3.1/V5-His© TOPO® TA Expression kit (Invitrogen) was used for cloning XRCC2 cDNA.
- The pIRESpuro3 expression vector (Clontech) was used to examine the effects of expressing 342delT on recombination frequencies.
- Luria- Bertani (LB) medium was made by dissolving 25g of LB broth (Merck) in 1 litre of water. pH was adjusted to 7 with 5N NaOH. The medium was autoclaved at 15lb/inch^2 for 20 minutes and kept at room temperature.
- LB agar was made by dissolving 25g of LB broth in 1 litre of water. 15g agar was added and dissolved before autoclaving.

2.2.7.2 Experimental Procedures.

Bacterial Culture.

The *E-coli* strain DH5α was used for maintenance and propagation of all plasmid DNA used. Bacterial cells were grown in L-Broth (supplemented with 100µg/ml Ampicillin), at 37°C in a shaking incubator (225 rpm) in all cases. Transformed bacteria were plated onto agar plates which were made with 1.5% (w/v) agar (in L-Broth) supplemented with 100µg/ml Ampicillin.

Plasmid Vector constructs.

Plasmid vectors are circular, self-replicating DNA molecules that are capable of existing in cells as extra-chromosomal genetic elements. Plasmids are frequently used when investigating genetic functions as they are easy to isolate and manipulate. All plasmids are relatively small in size (2 - 200Kb), contain an origin of replication and have an antibiotic resistance gene. Genes of interest can be cloned into restriction sites contained within the multiple-cloning site of the plasmid.

The pcDNA3.1/V5-His-TOPO® expression vector contains a single 3' thymidine (T) overhang. Gene specific primers were used in an RT-PCR also containing *Taq.* polymerase which has a template independent terminal transferase activity that adds a single deoxyadenosine (A) residue to the 3' ends of the PCR product. Successful cloning reactions can be achieved by ligating the complementary ends together. The pcDNA*XRCC2* vector was constructed by cloning the XRCC2 gene from the SKUT-1 cell line into the PCR site of the pcDNA3.1/V5-His© TOPO® TA expression vector. Briefly, 2μl of the RT-PCR product was mixed with 1μl of salt solution and 1μl of TOPO vector DNA. Sterile water was added to a final volume of 5μl. The reaction was incubated for 5 minutes at room temperature before the plasmid was chemically transformed.

Sub-cloning

Effects of the mutant XRCC2 allele on HRR events were investigated by sub-cloning the gene into the pIRES-puro3 expression vector (Clontech). The pIRES-puro3 vector contains an internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. Therefore after selection with puromycin, nearly all surviving colonies will stably express the gene of

interest. The pIRES-XRCC2 vector was constructed by initially excising the XRCC2 gene from the pcDNA*XRCC2* vector using the BamHI and NotI restriction endonucleases. The insert was separated by agarose gel electrphoresis and purified using a QIAquick® Gel Extraction Kit. of the pIRES-puro vector was also digested using the same restriction endonucleases and purified using a QIAquick® PCR purification Kit. The vector was then treated with shrimp alkaline phosphatase at 65°C for 10 minutes, thus preventing re-ligation of the linear vector. Alkaline phosphatase treatment removes phosphate groups from 5'ends of the DNA molecules. Concentration of both the vector and insert DNA was then estimated using agarose gel electrophoresis along with molecular weight standards of a known concentration. Recombinant DNA was produced by using ligases which catalyse the formation of phosphodiester bonds between the directly adjacent 3'hydroxyl and 5'-phosphoryl termini of nucleic acid molecules. The insert was ligated to the vector in a 3:1 molar ratio of insert:vector. Briefly, 1 *Weiss unit* of T4 DNA ligase (New England BioLabs) was used in a 10μl reaction containing vector DNA, insert DNA, 10 x Ligase buffer and distilled water. The ligation reaction of the protruding ends was conducted overnight at 4°C.

Transformation

Transformation reactions were conducted using sterile equipment and in front of a flame. Briefly, a 50μl vial containing DH5α competent *E-coli* cells were gently thawed on ice. 2μl of the cloning reaction was incubated with the competent cells on ice for 30 minutes. The reaction was then heat shocked at 42°C for 30seconds and immediately transferred to ice for a further 2 minutes. 250μl of room temperature SOC medium was added to the cells and the reaction was incubated in an orbital incubator (225rpm horizontally) at 37°C for approximately 1 hour. Varying cell concentrations were then plated onto LB-agar culture plates (supplemented with 50μg/ml Ampicillin)

and incubated overnight at 37°C. The following day, six to eight well isolated colonies were picked off the plates using sterile pipette tips and were inoculated in 10mls of L-Broth (supplemented with 100μg/ml Ampicillin), at 37°C in an orbital incubator (225 rpm).

Plasmid Extraction and Purification

Bacteria were recovered form overnight cultures by centrifugation at 4000 x g for 10 minutes at 4°C. The supernatant was discarded and plasmid extraction was performed using a QIAprep spin Miniprep kit (QIAGEN). Briefly, this method is based on alkaline lysis of bacterial cells followed by the binding of DNA to silica gel membranes in the presence of a high salt concentration and a pH of ≤7.5. Once bound, the DNA is washed off impurities by repeatedly washing the columns with an ethanol containing buffer. All DNA was finally eluted in 30μl of sterile water. Conformation of both orientation and integrity of the inserted DNA was achieved by direct sequencing and/or restriction digestion and subsequent electrophoresis.

2.2.8 Statistical Analysis

Statistical analysis to measure standard deviation and standard error was conducted using Microsoft Excell. Wilcoxon signed rank test was conducted by Dr. Angie Cox using Stata (version 6) software.

CHAPTER THREE:

HOMOLOGOUS RECOMBINATION REPAIR DEFECTS IN MMR -DEFICIENT TUMOUR CELL LINES

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3.1 INTRODUCTION

Several studies have shown that MMR –deficient tumour cells are sensitive to some DNA damaging agents (e.g. MMC and camptothecin) and S-phase cell cycle inhibitors (e.g. thymidine) (Mohindra *et al.*, 2002; Jacobs *et al.*, 2001 and Fiumicino *et al.*, 2000). In the case of the MMR –deficient tumour cell line, HCT116, the hypersensitive phenotype to thymidine was shown not to be due to the consequence of the MMR deficiency itself. HCT116+3 cells, that were corrected for the *h*MLH1 deficiency (by chromosomal transfer), still exhibited marked sensitivity to this agent (Mohindra *et al.*, 2002). These results therefore suggested that mechanisms other than the loss of MMR could lead to thymidine sensitivity.

Studies conducted by Lundin and co-workers (2002a) showed that the HRR deficient cell line, *irs1SF*, was also sensitive to thymidine when compared to both the wild-type and XRCC3 corrected cell lines. The addition of thymidine induces an imbalance in dCTP and dTTP pools that subsequently results in the slowing of DNA replication forks (Bjursell and Reichard, 1973). It is proposed that this retardation of replication fork progression may result in the accumulation of DNA lesions that specifically requires a proficient HRR pathway for cell survival (Lundin *et al.*, 2002a and Henry-Mowatt *et al.*, 2003).

Increasing evidence suggests that components of the MMR system interact with various recombination repair intermediates. For example, the yeast MMR proteins are thought to assist in the removal of non-homologous 3' ends during both GC and SSA events and the removal of mismatched bases occurring in heteroduplex DNA (Evans *et al.*, 2000; Sugawara *et al.*, 1997; Studamire *et al.*, 1999 and Marsischky *et al.*, 1999). Taken together these studies suggest that proteins involved in both the MMR and HRR processes interact and that both pathways are required in order to maintain

the progression of DNA replication forks. Thus given such interactions, studies conducted in the present chapter investigated the integrity of the HRR pathway in MMR –deficient tumour cell lines.

To determine the integrity of the HRR pathway in MMR –deficient tumour cell lines, a recombination reporter substrate, ScNeo, was used (Figure 3.1). This substrate has effectively been used to demonstrate HRR deficiencies in *XRCC2*, *XRCC3*, *BRCA1* and *BRCA2* -deficient cell lines (Johnson *et al.*, 1999; Pierce *et al.*, 1999, Moynahan *et al.*, 2001a and 2001b). Integration of the ScNeo substrate into cell lines enables HRR events to be assayed by measuring recombination events between two defective neomycin phospho-transferase genes. The first 0.7kb neo repeat, termed 3' neo, contains a 5' truncation of the neo gene and is upstream with respect to the promoter region. The second defective neomycin phospho-transferase gene, termed S2neo, however, is mutated by a small internal deletion that destroys an internal NcoI site and is replaced with an 18 base-pair I-SceI endonuclease site. The two neo repeats are in direct orientation to each other and are separated by a functional 2.1kb hygromycin resistance gene. As this substrate is composed of two defective neo heteroalleles that are placed as direct repeats, it specifically detects DSB-induced recombination events which occur on the same chromatid (intra-chromatid) or on sister chromatids.

Once integrated into cell lines, DSBs can be induced by transiently transfecting a plasmid that expresses the I-SceI endonuclease. I-SceI is a rare cutting endonuclease that can be used to generate a site-specific chromosomal break (reviewed in Dujon, 1989). Homologous recombination between the two neo repeats subsequently results in a functional neomycin phospho-transferase resistance gene. NHEJ recombinants, however, do not contain a functional copy of the neomycin resistance gene and therefore

the frequency of HRR events can be specifically scored by counting colonies grown in G418 selective medium.

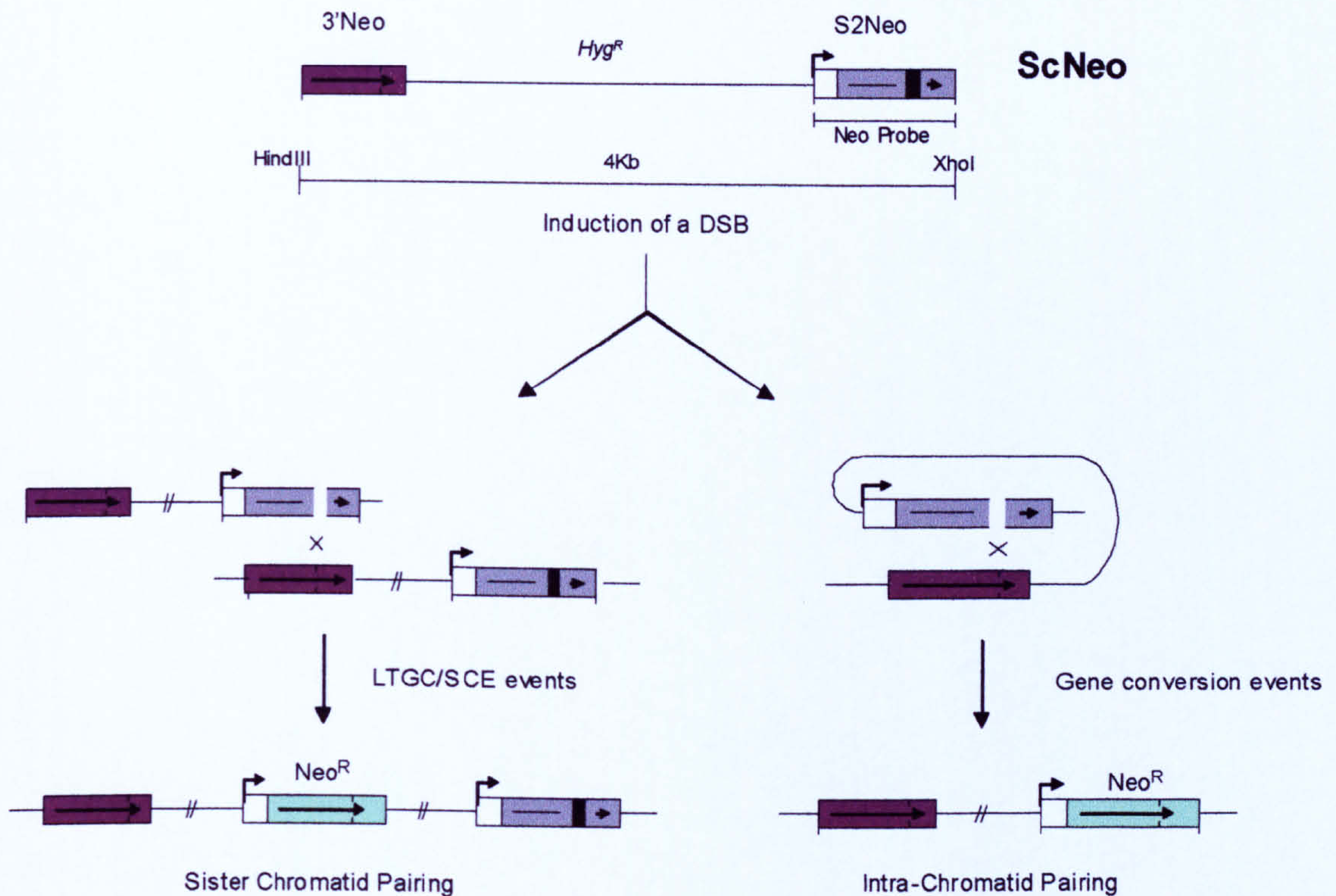


Figure 3.1 Structure of the recombination reporter substrate, ScNeo. Two defective neomycin phospho-transferase genes, namely 3'neo and S2neo, are separated by a functional 2.1kb hygromycin resistance gene. A functional neomycin phospho-transferase gene can be restored following the induction of a DSB. Two types of recombination events are possible. LTGC/SCE events repair the DSB by using the sister chromatid as the repair template and results in expansion of the substrate from 2 and 3 neo containing repeats. STGC events, however, repair DSBs through intra-chromatid pairing and do not alter the overall architecture of the ScNeo substrate.

Two distinct neomycin resistant recombinants are formed depending on the type of repair substrate used. Analysis of both the short tract gene conversion (STGC) and long tract gene conversion (LTGC)/sister chromatid

exchange (SCE) event products revealed that non-crossover gene conversion events occurred as expected when using a direct repeat assay (Moynahan and Jasin, 1997; Richardson *et al.,* 1998 and Johnson and Jasin, 2000). LTGC or SCE events result in the expansion of the substrate from two to three neo containing repeats. The broken end in S2neo invades the homologous 3' neo template for repair and after synthesis is completed, the newly synthesised strand can re-anneal with the non-invading strand. The subsequent repair product contains additional functional neomycin and hygromycin resistance genes. The STGC event occurs when the region immediately surrounding the DSB is repaired using the same chromatid or sister chromatid (following replication), thus not changing the overall length of the 4kb substrate.

In addition to using the I-SceI endonuclease to induce HRR events, the integrity of other HRR mechanisms involved in repairing lesions induced by thymidine, was also investigated in the present chapter. Accumulating evidence from bacteria and yeast suggest that proteins involved in HRR are required to repair collapsed or stalled replication forks (Cox, 2001; Kraus *et al.,* 2001 and Michel *et al.,* 2001). In addition, Lundin and co-workers (2002a) have suggested that HRR is specifically required for mammalian cell survival following thymidine treatment. This suggests that thymidine induces DNA lesions that are repaired by HRR events.

3.2 RESULTS

3.2.1 Integration of the ScNeo recombination substrate.

The proficiency of the HRR pathway was initially investigated in both MMR –proficient and –deficient tumour cell lines. To test the integrity of this pathway, the ScNeo recombination reporter substrate was used to measure homology based recombination of two defective neomycin phosphotransferase genes following the introduction of a DNA DSB (Johnson *et al.*, 1999). The vector containing ScNeo was electroporated into three MMR –proficient (SW480, SW620 and MRC5VA) and four MMR –deficient tumour cell lines (DLD-1, Hec-1-A, HCT116 and SKUT-1). The integrity of the integrated substrate was confirmed by screening hygromycin resistant colonies. Southern blot analysis, using S2neo as the labelled probe, revealed that all strains used in the present study contained intact copies of ScNeo (Figure 3.2b).

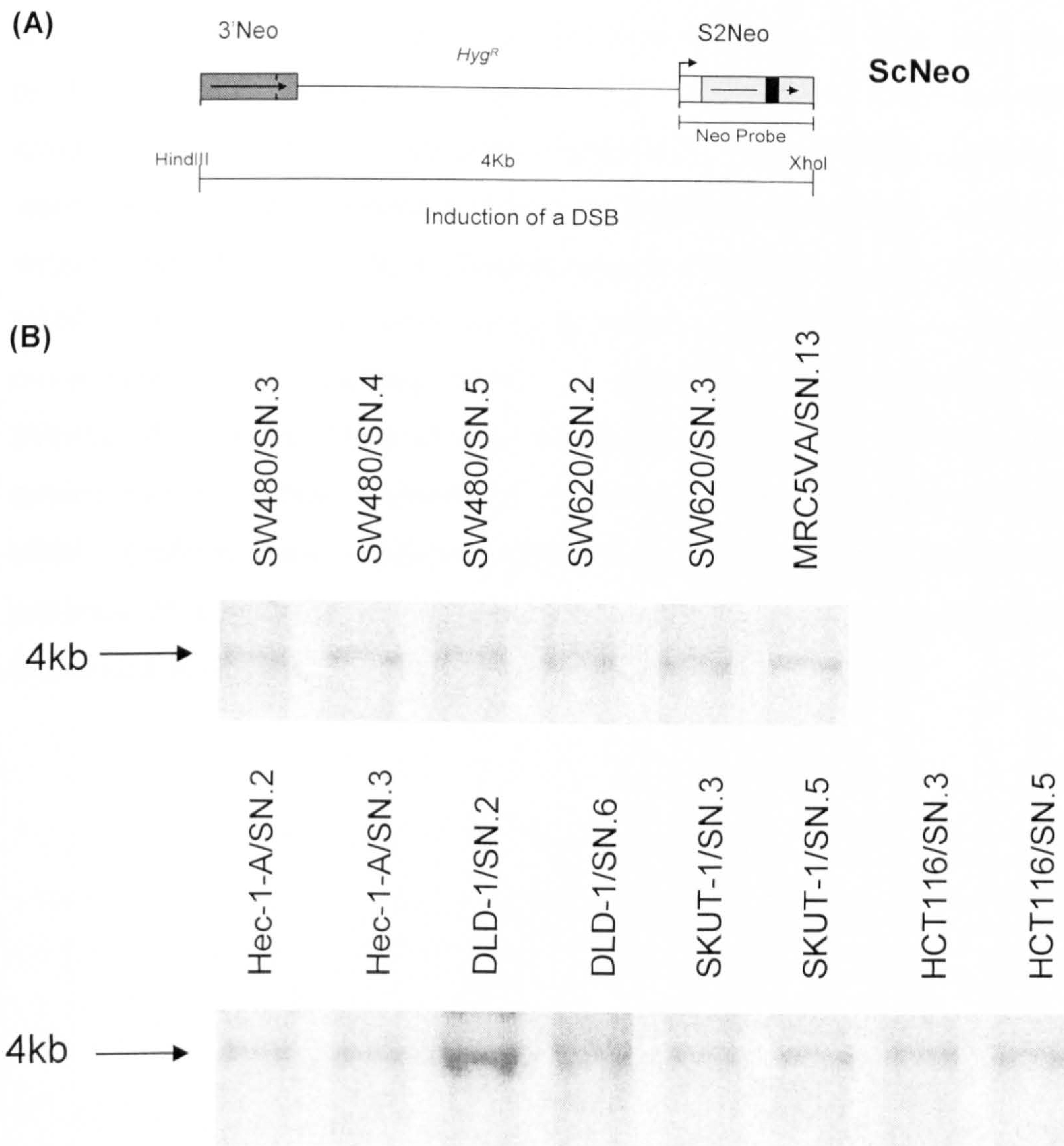


Figure 3.2 Strains derived from both MMR –proficient and –deficient cells contain intact copies of ScNeo. (A) S2neo was used as a labelled probe to detect the recombination substrate in genomic DNA extracted from the various cell types. (B) Southern blot showing that all strains contained intact copies of ScNeo. SW480/SN.3, 4 and 5 and SW620SN.2 and 3 are strains derived from the MMR –proficient cell lines, SW480 and SW620, respectively. The MRC5VA/SN.13 strain was derived from the MMR –proficient fibroblast MRC5VA cell line. The DLD-1/SN.2 and 6 strains were isolated from the *h*MSH6-deficient cell line DLD-1. The Hec-1-A/SN.2 and 3 strains were isolated from the *h*PMS2-deficient cell line Hec-1-A. The HCT116/SN.2 and 3 strains were derived from the *h*MLH1-deficient cell line HCT116 and the SKUT-1/SN.3 and 5 strains were isolated from the *h*MSH2-deficient SKUT-1 cell line.

To investigate whether expression of the ScNeo recombination substrate altered the phenotype of MMR –proficient and –deficient cells, with respect to the cytotoxic effects of thymidine, the sensitivity of ScNeo-containing strains to this agent was examined (Figure 3.3). Exponentially growing cells were treated with increasing doses of thymidine and cell survival was determined after 10-14 days. Studies revealed that at 10% survival, the four MMR -deficient tumour cell lines investigated were between 3.2 to 4.6-fold more sensitive to cytotoxic effects of thymidine when compared to the SW480/SN.3 strain. Furthermore, the extent of sensitivity to thymidine was determined to be dose dependent. Taken together these results show that MMR –proficient and –deficient strains, expressing ScNeo, exhibit similar patterns of sensitivity to thymidine as those reported for parental cells (Mohindra *et al.*, 2002).

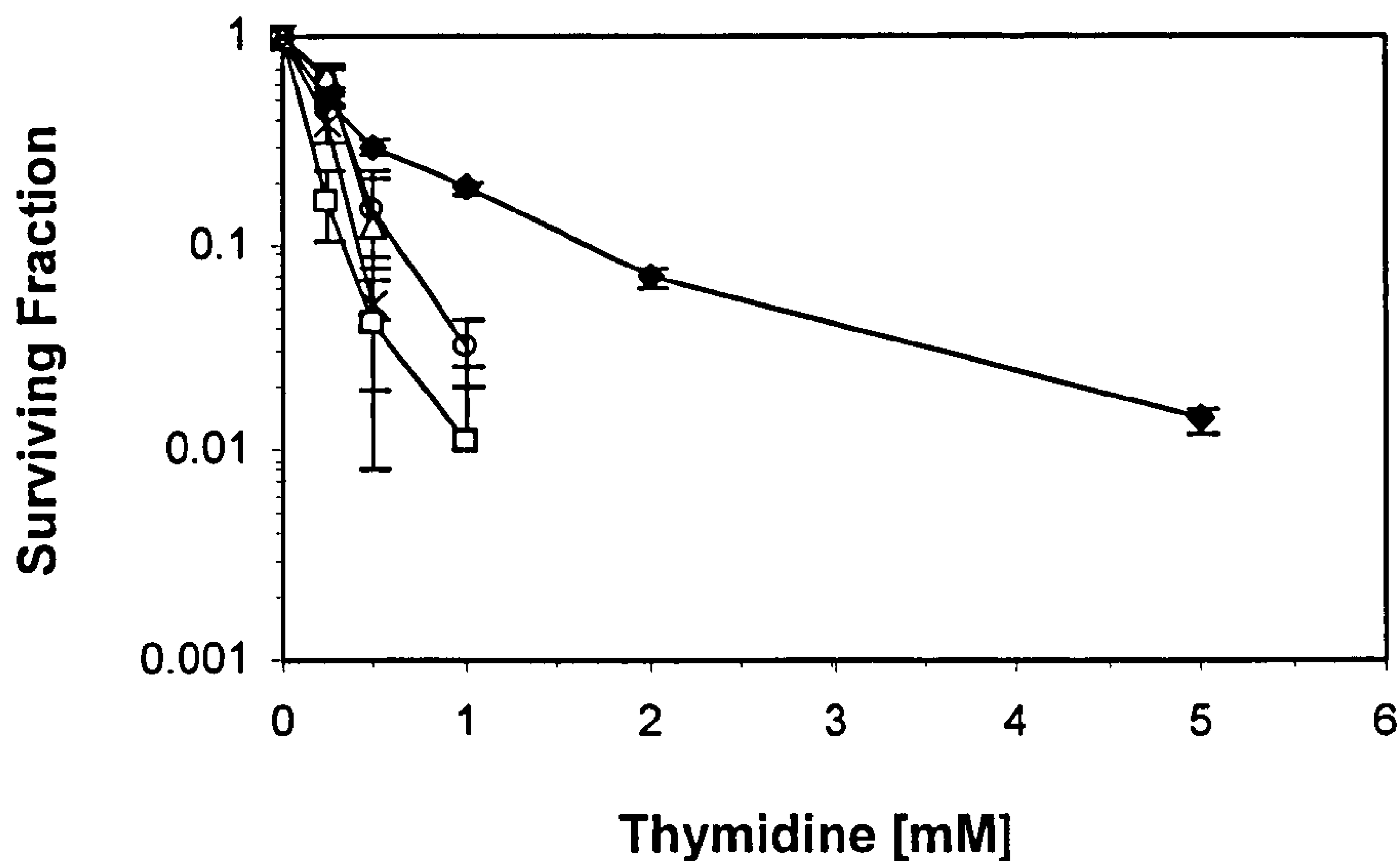


Figure 3.3 MMR –deficient strains, transfected with ScNeo, are hypersensitive to the cytotoxic effects of thymidine. MMR -deficient/ScNeo transfected clones have mutations in *h*MLH1 (HCT116, □), *h*MSH2 (SKUT-1, Δ), *h*PMS2 (Hec-1-A, ○) and *h*MSH6 (DLD-1, X). The MMR –proficient/ScNeo transfected clone, SW480/SN.3 (♦), is derived from the colorectal carcinoma SW480 cell line. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in triplicate are presented.

3.2.2 Analysis of the HRR pathway in MMR –proficient cells.

The integrity of the HRR pathway was examined in MMR –proficient strains transfected with the ScNeo construct. A DSB was induced in all strains by transiently transfecting cells with a plasmid expressing the I-SceI endonuclease. Following the selection of cells in medium supplemented with G418, the MMR -proficient (SW480 and SW620) colon cancer cell lines produced neo^+ recombinants at a frequency of $0.8 - 3.5 \times 10^{-3}$ (Figure 3.4). This was between 370 to 1000-fold higher than frequencies obtained when the same strains that were not transfected with the expression vector ($p=0.0077$, Wilcoxon signed rank test). The frequency of neo^+

recombinants in a MMR -proficient fibroblast, MRC5VA, was also investigated. A strain stably expressing the ScNeo substrate produced neo^+ recombinants at a frequency of 4.38×10^{-3}. This was 110-fold higher when compared to the results obtained from mock transfected cultures. Taken together, these results suggest that MMR –proficient cells also contain a proficient HRR pathway.

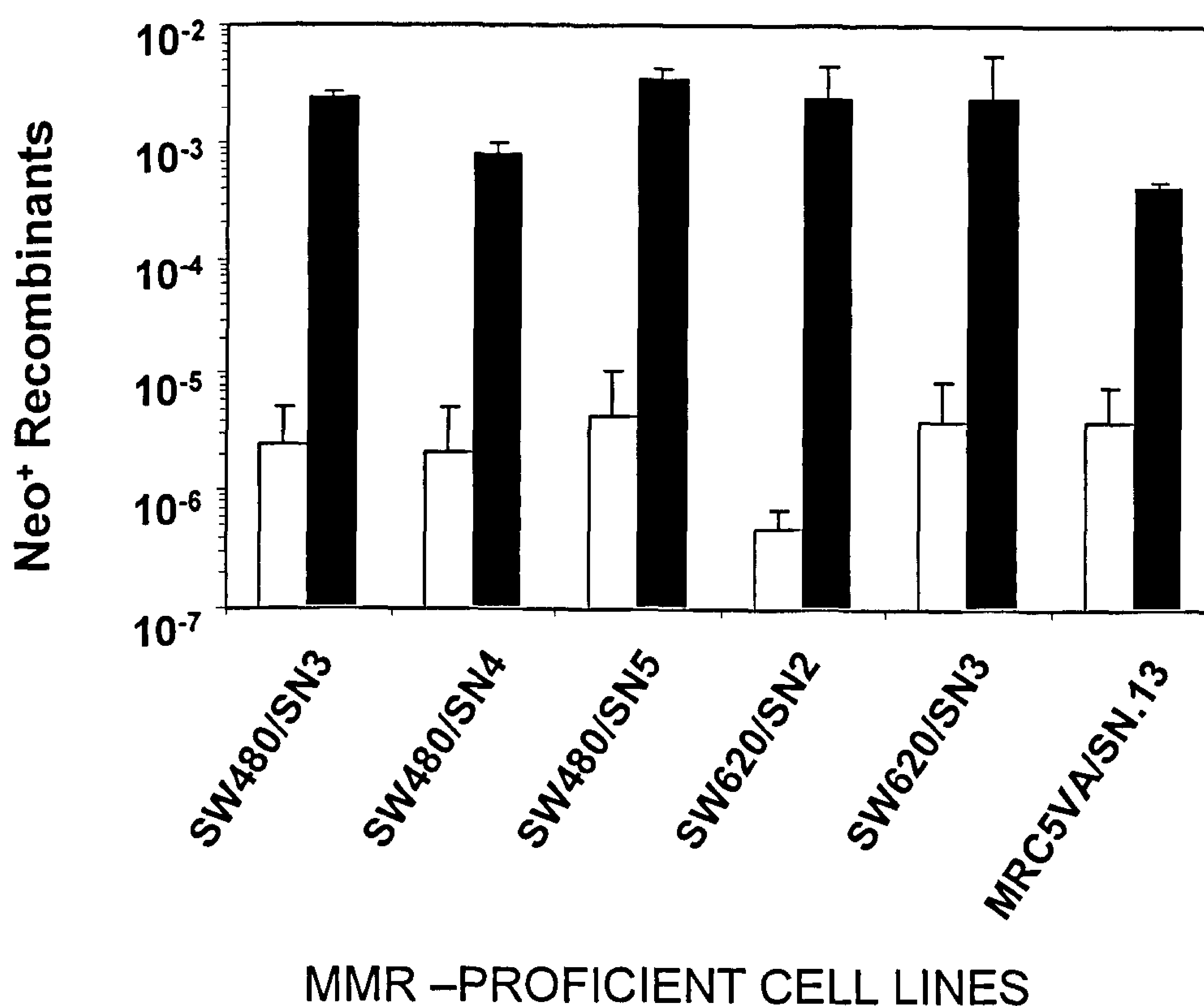


Figure 3.4 Induction of a DSB results in an increase in the frequency of neo+ recombinants in MMR –proficient cells. Induction of a DNA DSB (solid bars) in multiple strains derived from three parental MMR –proficient cell lines (namely SW480, SW620 and MRC5VA) resulted in an increase the frequency of neo+ recombinants by 110 to 1000-fold when compared to mock transfected cultures (empty bars). The results shown are an average of three independent experiments and standard deviation is indicated by error bars.

3.2.3 Analysis of the HRR pathway in MMR –deficient cells.

Having determined the integrity of the HRR pathway in three MMR –proficient cell lines, the proficiency of this pathway was also investigated in MMR –deficient tumour cell lines. The frequency of neo^+ recombinants in four MMR deficient cell lines containing the ScNeo substrate, namely, DLD-1 ($hMSH6^{-/-}$), Hec-1-A ($hPMS2^{-/-}$), SKUT-1 ($hMSH2^{-/-}$) and HCT116 ($hMLH1^{-/-}$) was measured. As shown in Figure 3.5, induction of a DSB did not increase the frequency of neo^+ recombinants in any strain investigated when compared to strains that were not transfected with the endonuclease (p=0.58, Wilcoxon signed rank test). Typically, both DSB -induced and spontaneous values ranged from between 0.21 to 2.72 x 10^{-6} (Figure 3.5). This range is between 200 to 15000-fold lower than the frequencies obtained in the MMR -proficient strains. These results suggest that MMR -deficient cells are defective in the production of neo^+ recombinants in a homology based recombination assay.

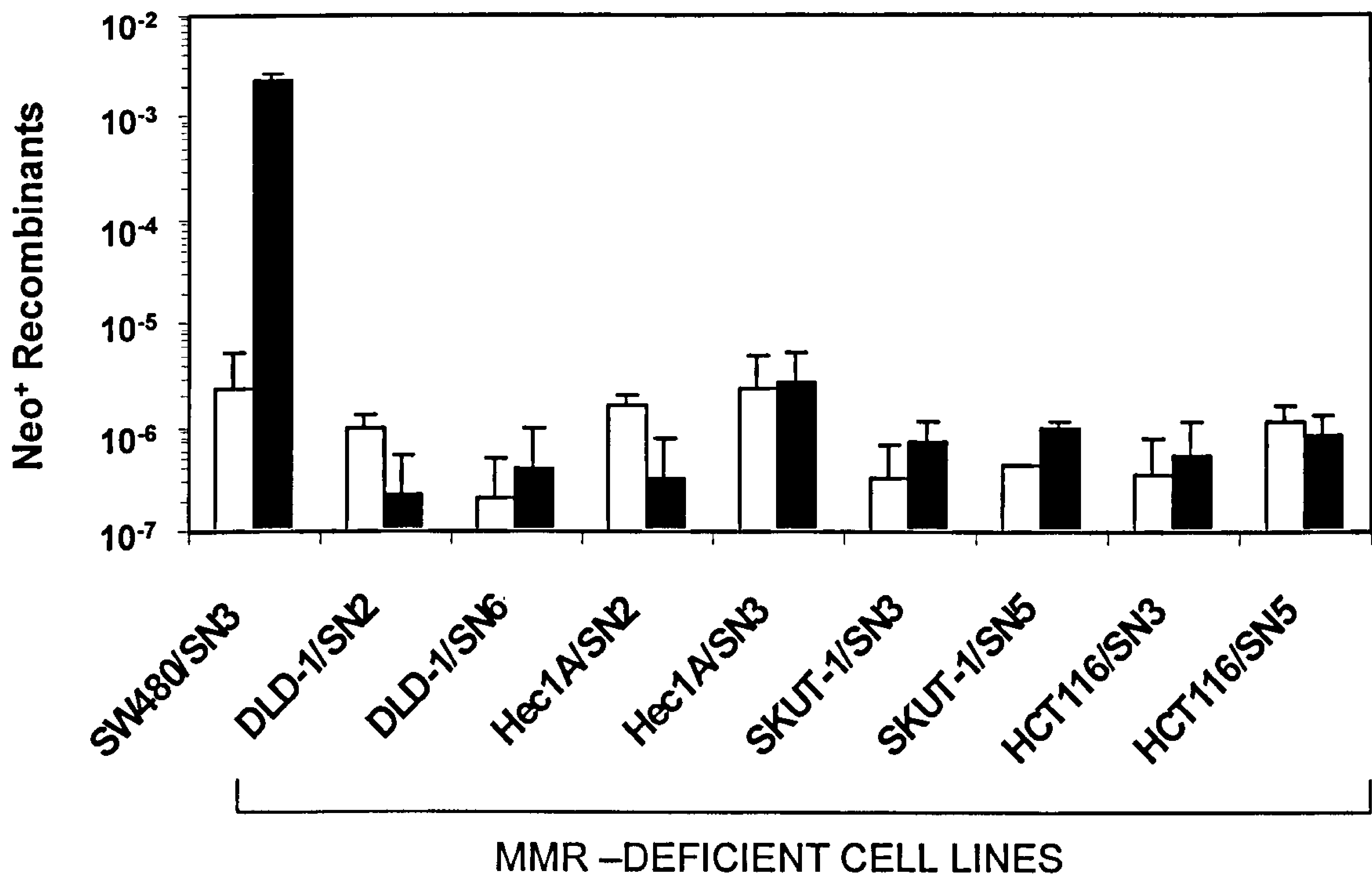


Figure 3.5 MMR –deficient tumour cells are defective in the production of neo+ recombinants in a homology based assay. Induction of a DNA DSB (solid bars) in strains derived from four parental MMR –deficient cell lines showed no increase in the frequency of neo+ recombinants when compared to mock transfected cultures (empty bars). Results obtained from the same experiments using a MMR –proficient, SW480/SN.3 strain, is included for reference. The results shown are an average of three independent experiments and standard deviation is indicated by error bars.

3.2.4 Transfection controls.

The observation that MMR –deficient strains are defective in the production of neo$^+$ recombinants in a homology based recombination assay could however be the result of an inability of these strains to take up and express the plasmid expressing the I-SceI endonuclease. To test this hypothesis, the percentage of cells taking up a construct expressing a green fluorescent tagged protein (GFP) from the same cytomegalovirus promoter in the expression vector pcDNA3 was determined. The number of cells emitting a

green fluorescence was determined by FACS analysis in three ScNeo transfected strains (one MMR -proficient and two MMR –deficient). Such repeated experiments (n=3) showed no differences between the three strains, with respect to the percentage of cells expressing GFP, 24 hours post transfection (Figure 3.6). On average, 12.8% of MMR –proficient SW480/SN.3 cells expressed the green fluorescent protein as opposed to 11.2% of DLD-1/SN.2 and 8.2% of Hec-1-A/SN.3 MMR –deficient cells. These results therefore suggested that there is no significant difference in the uptake and expression of a transgene from a CMV promoter between the various cell lines.

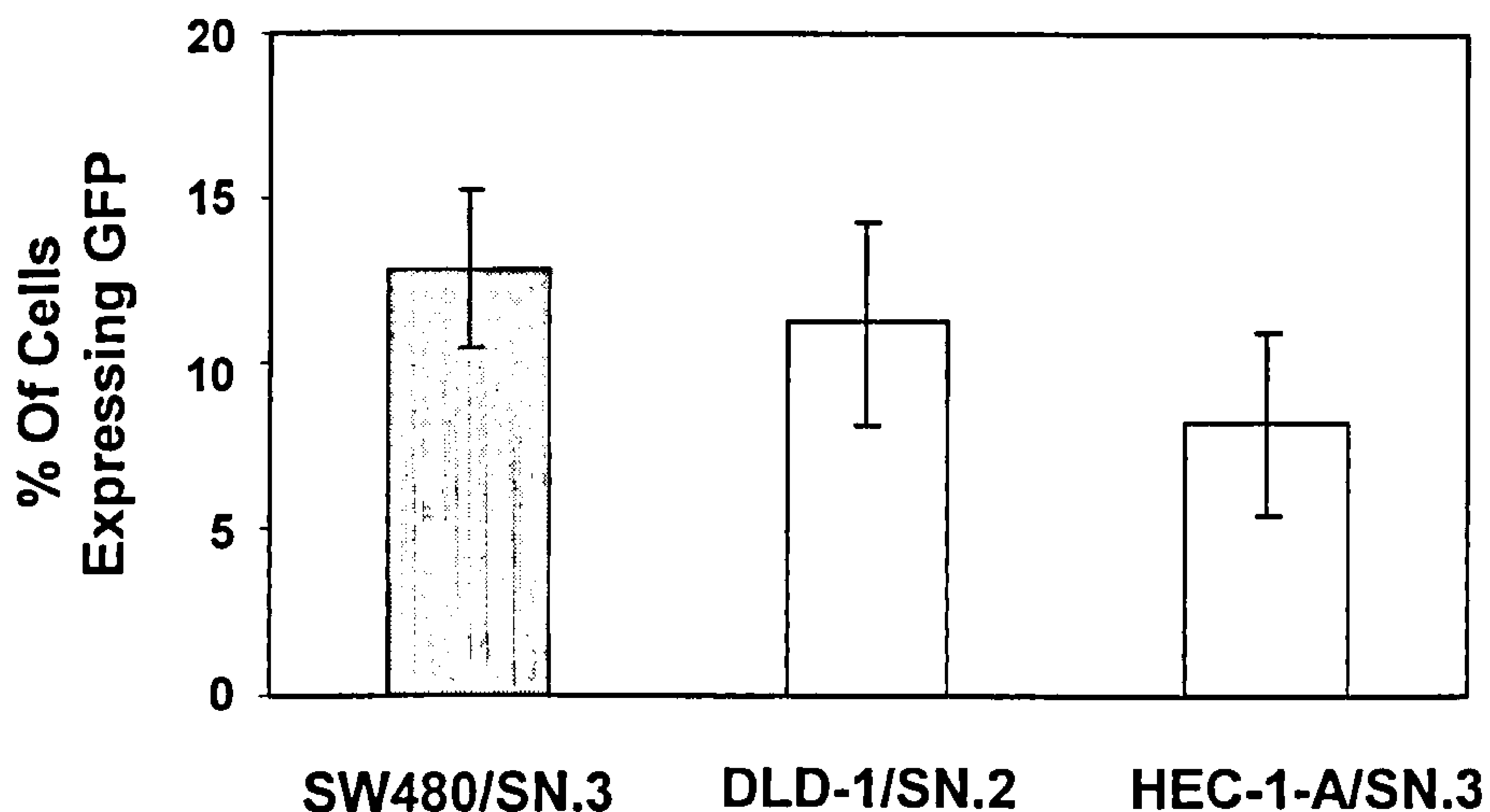


Figure 3.6 Both MMR –proficient and –deficient tumour cells express similar amounts of GFP. No significant difference in the percentage of GFP⁺ cells was observed when the GFP construct was transfected into a MMR –proficient (SW480/SN.3, shaded box) strain and two MMR –deficient (DLD-1/SN.2 and Hec-1-A/SN.3, empty boxes) strains. GFP expression was determined by FACs analysis 24 hours post transfection. The results are an average of three independent experiments and standard deviation is indicated by error bars.

3.2.5 The decrease in frequency of neo+ recombinants is not a consequence of apoptosis.

The possibility that the ScNeo transfected, MMR -deficient strains possessed an acute sensitivity to cell death, following expression of the I-SceI endonuclease, was also determined. As explained in the Materials and Methods, during the transfection process cells are able to take up multiple copies of plasmid DNA. Therefore cells expressing GFP were also likely to express the I-SceI endonuclease. Based on this concept, both the GFP and I-SceI constructs were co-transfected into MMR -proficient and -deficient strains, as GFP^+ cells are also likely to express the I-SceI endonuclease. The fraction of GFP^+ cells undergoing apoptosis was then determined 24 hours post transfection by Hoechst staining. Such staining specifically detects condensed apoptotic nuclei (refer to Page 97). As shown in Figure 3.7, in all strains tested, between 27 and 33% of cells expressing GFP underwent apoptosis; with no significant difference being observed between the MMR –proficient and –deficient strains, post I-SceI expression. Therefore the low levels of neo^+ recombinants produced by the MMR -deficient strains does not seem to be due to the result of an induction of cell death following expression of the I-SceI endonuclease.

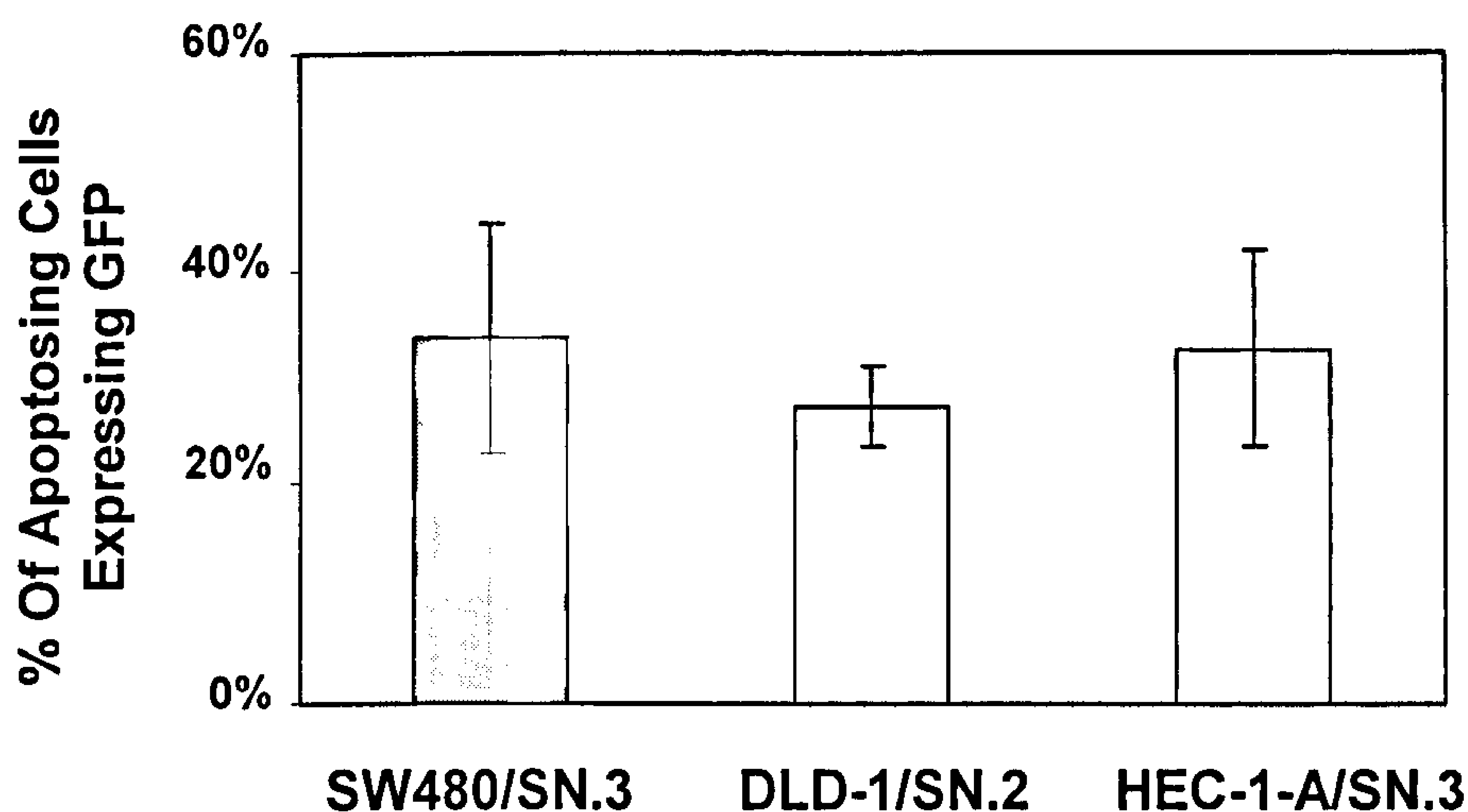


Figure 3.7 A similar percentage of MMR –proficient and –deficient, GFP$^+$ cells undergo apoptosis. No significant difference was observed when the number of apoptosing cells were counted after a GFP construct was co-transfected with the I-SceI expression vector into a MMR –proficient (SW480/SN.3, shaded bar) strain and two MMR –deficient (DLD-1/SN.2 and HEC-1-A/SN.3, empty bars) strains. Apoptosis events were defined as condensed nuclei following Hoechst staining 24 hours post transfection. The results are an average of three independent experiments and standard deviation is indicated by error bars.

3.2.6 Effects on Recombination frequency in cells corrected for MMR defect.

To determine whether a lack of MMR protein expression is responsible for recombination defects observed in Section 3.2.3, Hec-1-A/SN.3 cells were corrected for the *h*PMS2 deficiency. To complement the MMR deficiency in Hec-1-A cells, human *PMS2* cDNA (cloned into the pIRESpuro3 expression vector) was stably introduced into a Hec-1-A strain containing the ScNeo construct, Hec-1-A/SN.3. As this strain was initially cloned from a single cell, we were able to reduce potential genotypic heterogeneity within the MMR –deficient cell population.

3.2.6.1 Confirmation of *h*PMS2 expression

As determined by Western Blot analysis of whole cell lysates, the *h*PMS2 protein was weakly detected in 2 out of 7 transfectants examined (Figure 3.8a). The *h*PMS2 protein was also detected in the SW480, MMR – proficient, cell line. Furthermore, no *h*PMS2 protein was detected in Hec-1-A/SN.3 (*h*PMS2$^{-/-}$) extracts.

The *h*PMS2 protein is well documented to interact with and be required to stabilize another MMR protein, *h*MLH1. Furthermore, cells deficient in *h*PMS2 also have reduced levels of *h*MLH1 protein, presumably as a result of turnover of the un-complexed *h*MLH1 protein (Li *et al.,* 1995). Thus the level of the *h*MLH1 protein was also investigated in the same transfectants in order to confirm that the MMR defect was corrected. As shown in Figure 3.8b, the two transfectants in which *h*PMS2 was detected also expressed relatively higher levels of the *h*MLH1 protein when compared to parental Hec-1-A/SN.3 extracts.

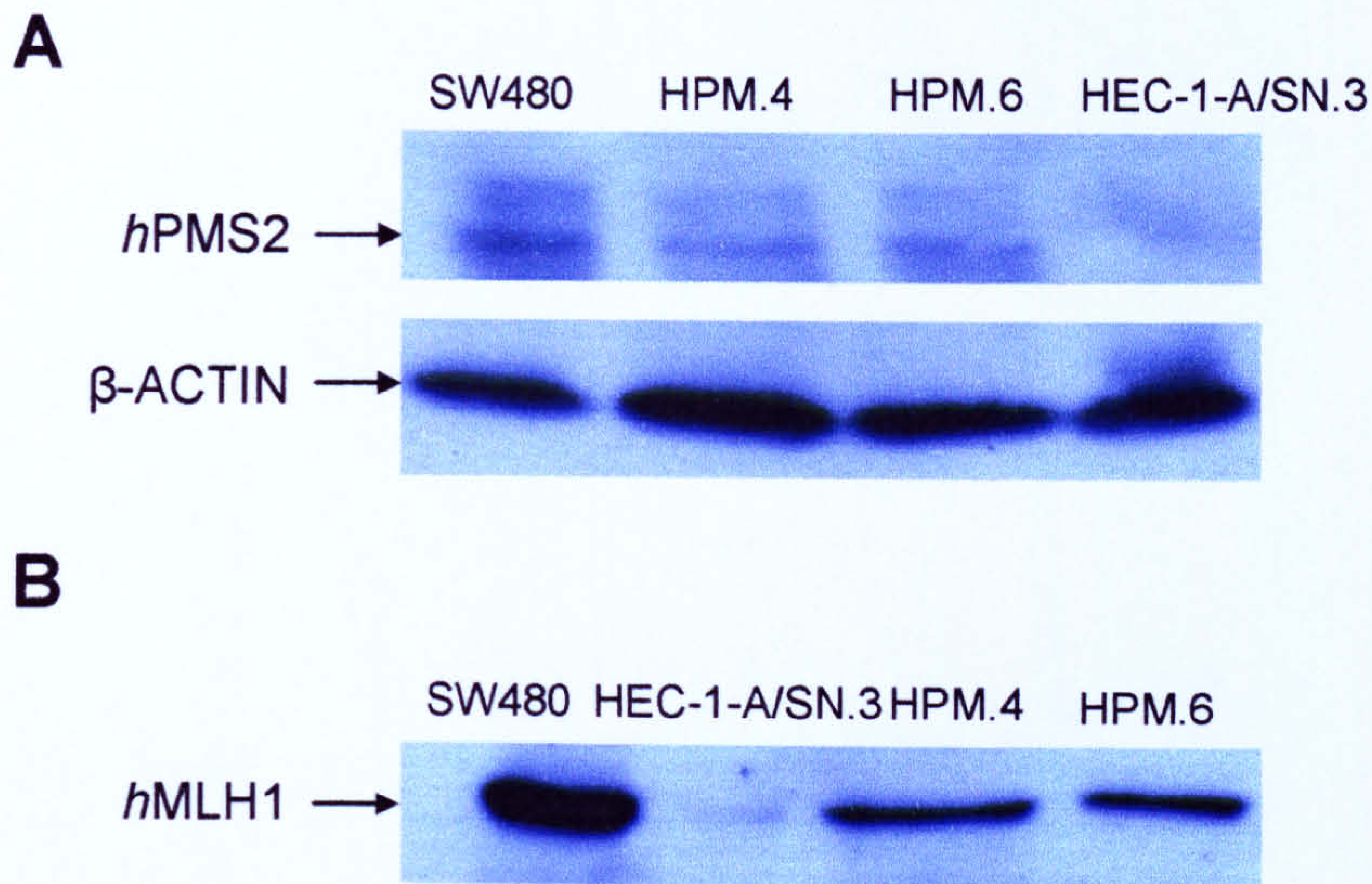


Figure 3.8 Stable transfection of *h*PMS2 cDNA into Hec-1-A/SN.3 cells results in increased levels of both *h*PMS2 and *h*MLH1. Two transfectants, namely HPM.4 and HPM.6, both expressed increased levels of (A) *h*PMS2 and (B) *h*MLH1 protein following stable transfection of *h*PMS2 cDNA. Protein expression was detected by Western Blot analysis of whole cell lysates. β-Actin was used as a loading control for all samples.

In order to further establish the expression of wild type *h*PMS2 cDNA in Hec-1-A/SN.3 cells, the effects of 6-thioguanine (6-TG) was tested. MMR – deficient cells have been reported to be resistant to the cytotoxic effects of 6-TG when compared to cells expressing a functional MMR system (Hawn *et al.,* 1995). 6-TG is a purine analogue that is incorporated into DNA and becomes either phosphorylated (by HPRT) or methylated (by S'-adenosylmehionine) (Waters and Swann, 1997 and Elion 1989). Following DNA replication, 6-meTG residues on the parental strand are matched with thymine or cytosine residues to form DNA mismatches. The MMR system recognises these mismatches and attempts are made for repair. Since both the parental and newly replicated strands contain the incorrect residue, the MMR system undergoes a futile cycle that is thought to eventually result in the formation of single stranded breaks. Such breaks subsequently result in

activation of the G2/M checkpoint and apoptosis (Yan *et al.,* 2003). The loss of a functional MMR system results in cells being resistant to 6-TG due to an impaired ability to generate an appropriate signal that can inhibit cell growth.

Cells were treated with increasing doses of 6-TG and allowed to grow for 10-14 days in order to form viable colonies. The 6-TG cytotoxicity curves for the two transfectants that displayed highest levels of *hPMS2* protein are shown in Figure 3.9. At 10% survival, both the HPM.4 and HPM.6, *h*PMS2-corrected, strains were between 2.6 to 2.8-fold more sensitive to 6-TG when compared to parental cells. These results therefore show that increased expression of wild-type *h*PMS2 confers increased sensitivity to 6-TG.

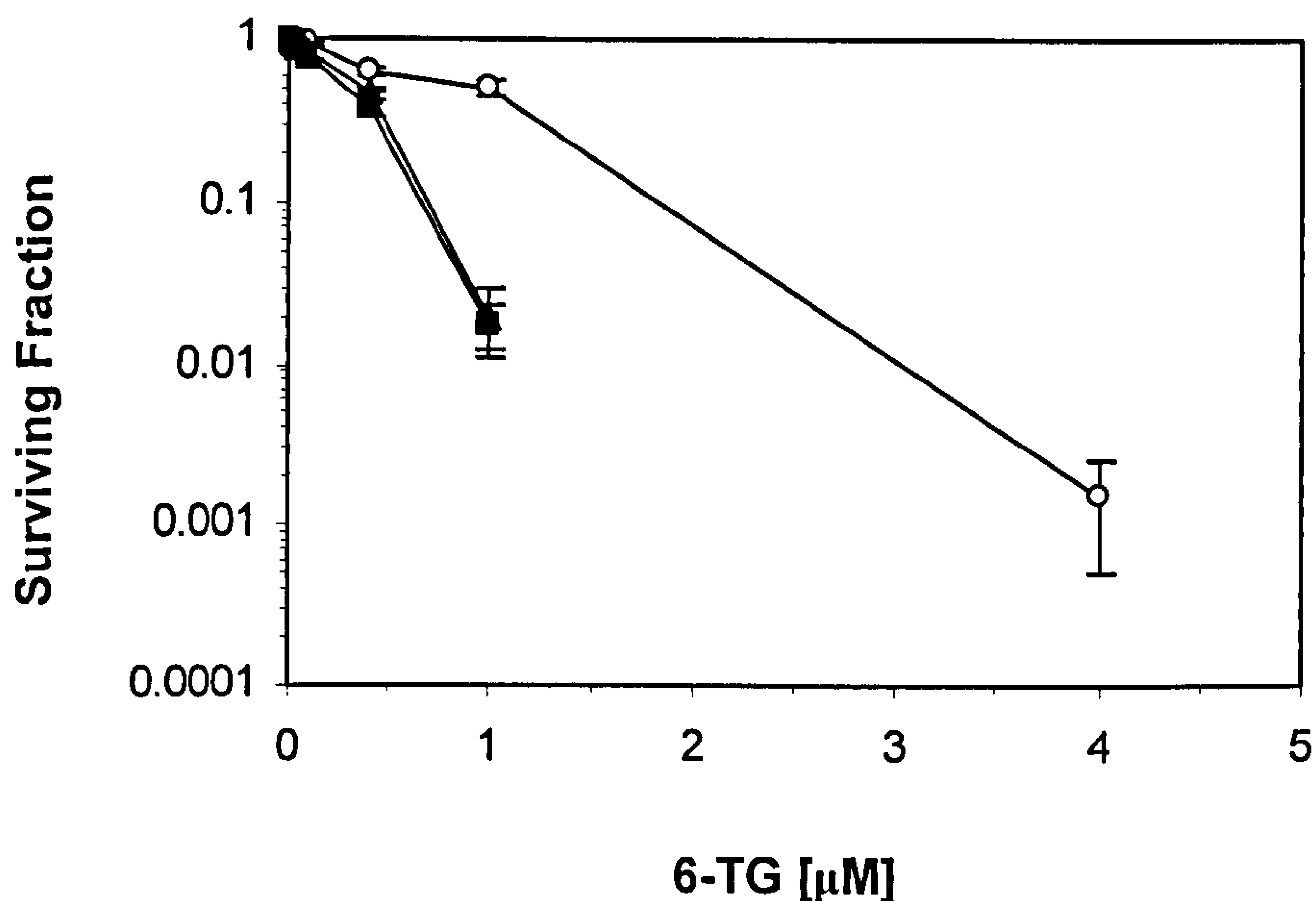


Figure 3.9 Cells expressing increased levels of *h*PMS2 protein are sensitive to 6-TG. Two Hec-1-A/SN.3 transfectants, expressing the *h*PMS2 protein, are sensitive to 6-TG when compared to parental Hec-1-A/SN.3 cells. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented: Hec-1-A/SN.3 (○), HPM.4 (■) and HPM.6 (▲).

Previous studies have shown that HCT116+3 cells, corrected for the MMR -deficiency, remain sensitive to the cytotoxic effects of thymidine (Mohindra *et al.,* 2002). Therefore the effect of thymidine treatment on strains expressing increased levels of *h*PMS2 protein was also investigated in order to confirm that protein expression did not alter the sensitivity. Cells were treated with increasing doses of thymidine and allowed to form colonies for 10-14 days. The parental Hec-1-A/SN.3 strain remained sensitive to thymidine following continuous exposure when compared to the MMR –proficient, SW480 cell line. In addition, both the *hPMS2* corrected transfectants, namely HPM.4 and HPM.6, exhibited the same degree of sensitivity to thymidine as parental Hec-1-A/SN.3 strains (Figure 3.10). At 10% survival, the two transfectants showed only a 1.4-fold difference in sensitivity to thymidine when compared to parental Hec-1-A/SN.3 cells. Thus correction of the *h*PMS2 defect, in Hec-1-A/SN.3 strains, results in increased 6-TG sensitivity but does not alter the thymidine sensitivity.

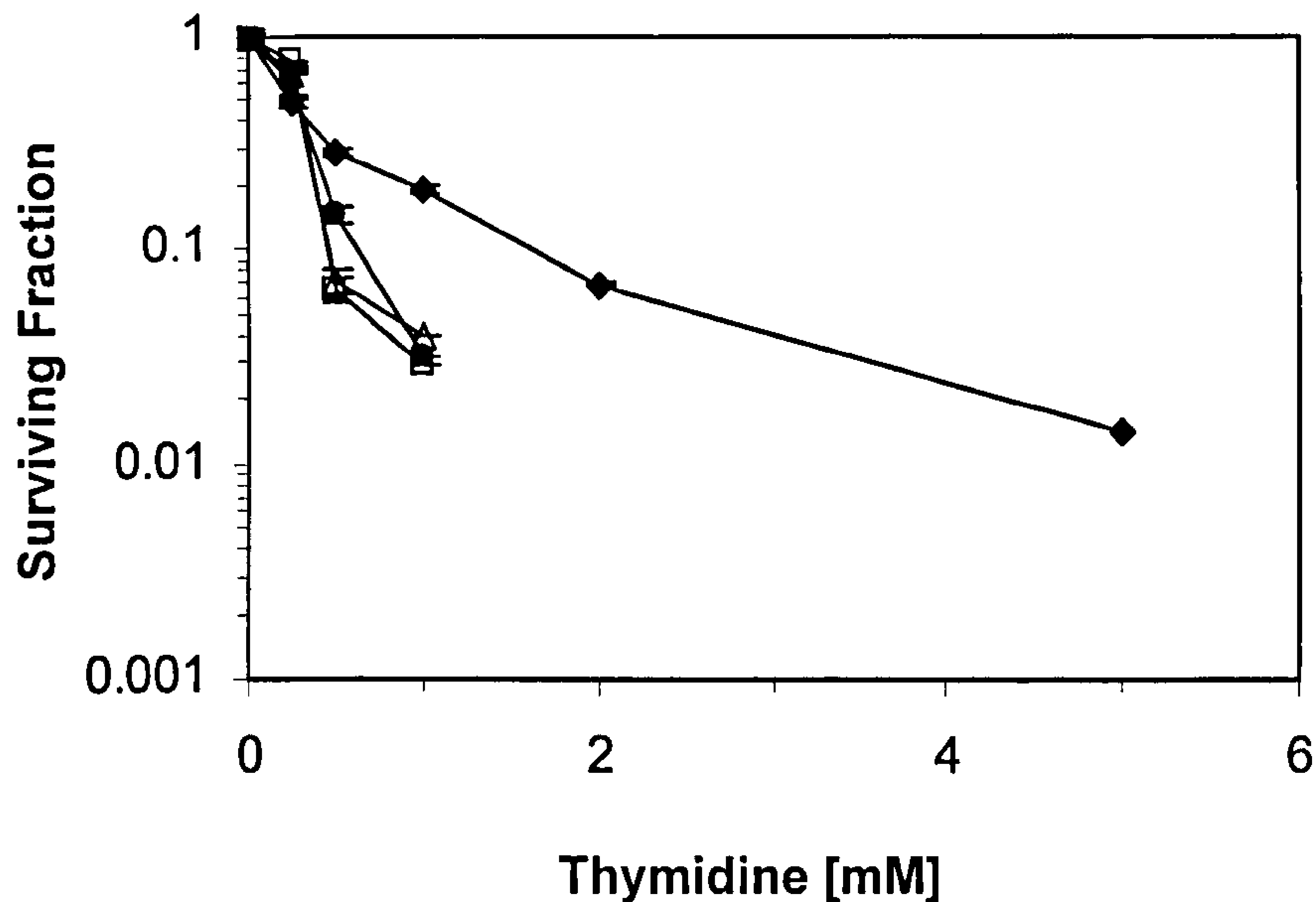


Figure 3.10 Cells expressing increased levels of *h*PMS2 protein remain sensitive to thymidine. Two Hec-1-A/SN.3 transfectants, expressing increased levels of the *h*PMS2 protein, remain sensitive to thymidine when compared to untransfected parental cells. Results showing sensitivity to thymidine of MMR –proficient, SW480 cells (♦) is included for reference. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented: Hec-1-A/SN.3 (●), HPM.4 (□) and HPM.6 (Δ).

3.2.6.2 DSB induced Recombination Assay

The frequency of neo$^+$ recombinants formed following the induction of a DSB was next determined using the two transfectants that displayed increased levels of *h*PMS2 protein. As shown in Figure 3.11, transient transfection of an I-SceI endonuclease resulted in no significant increase in the frequency of neo$^+$ recombinants formed in either of the two transfectants tested. Interestingly, induced recombination frequencies were in fact lower (although not statistically significant) in the HPM.4 strain that displayed the highest level of *h*PMS2 protein. Frequencies of neo$^+$ recombinants obtained following induction of a DSB were however similar in the HPM.6 strain to that observed in the parental Hec-1-A/SN.3 strain. Spontaneous

recombination frequencies were also similar in all strains tested. Taken together these results suggested that the *h*PMS2 deficiency in Hec-1-A cells is not responsible for such HRR defects.

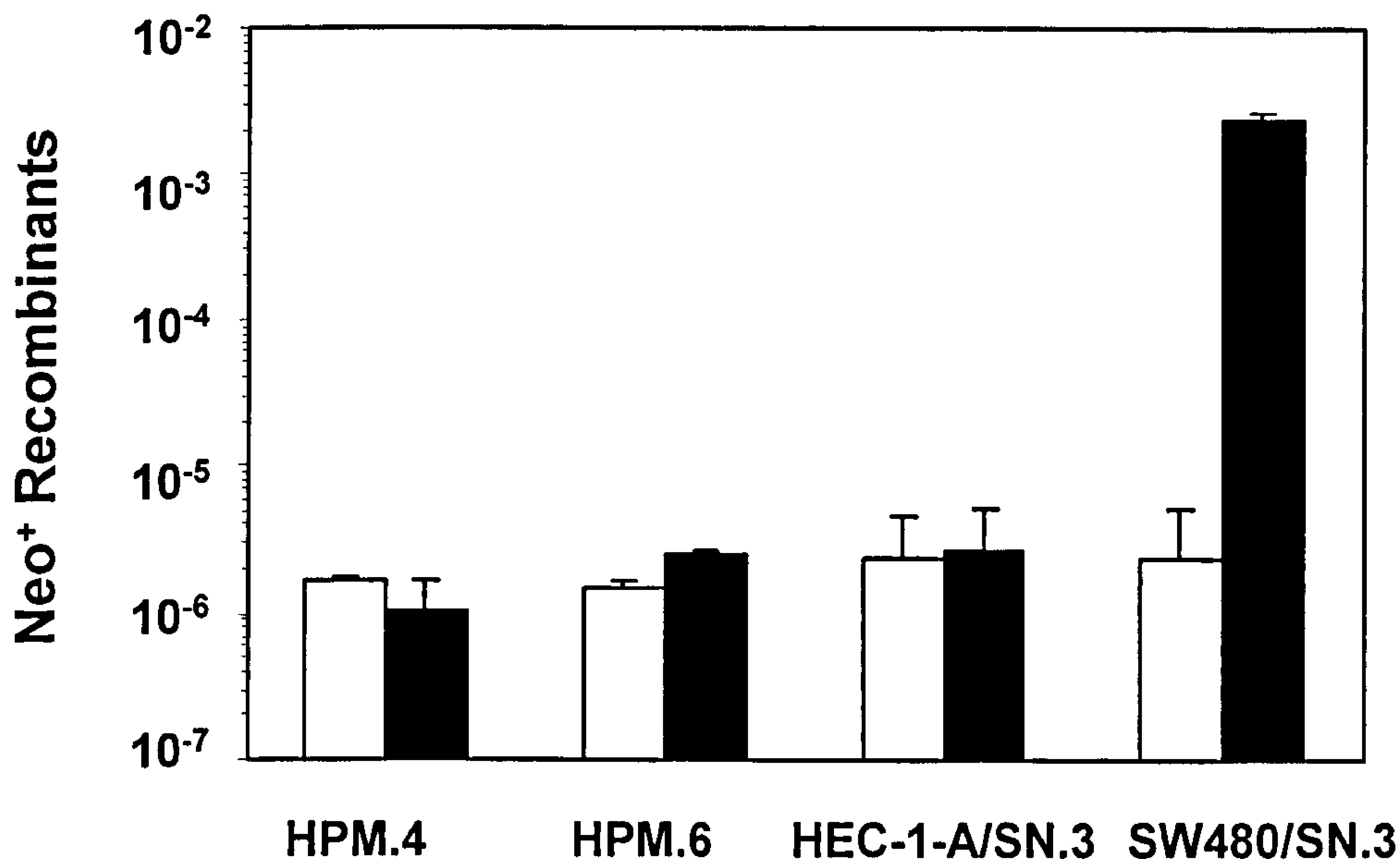


Figure 3.11 Both HPM.4 and HPM.6 strains show no increase in frequency of neo+ recombinants following expression of a DSB. Induction of a DNA DSB (solid bars), in two strains expressing increased levels of *h*PMS2 protein, showed no increase in the frequency of neo+ recombinants when compared to mock transfected cultures (empty bars). Results obtained from the same experiments using a MMR –proficient, SW480/SN.3 strain, are included for reference. The results shown are an average of three independent experiments and standard deviation is indicated by error bars.

3.2.7 MMR –deficient cells are hypersensitive to mitomycin C (MMC).

Sensitivity of various MMR –deficient cells to the DNA cross-linking agent, MMC, was also determined. The HRR deficient hamster cell lines *irs1* and

irs1SF (deficient for XRCC2 and XRCC3 respectively) were initially characterized on the basis of their sensitivity to MMC and IR (Jones *et al.*, 1987; and Fuller and Painter, 1988). MMC is a DNA cross-linking agent which reacts with DNA to form mono-adducts that are mainly thought to occur at the N2 position of guanine residues (for review see Lawley and Phillips, 1996). This subsequently causes the residue to rearrange and develop both inter-strand and intra-strand cross-links. Recent evidence suggests that proteins involved in excision repair pathways, such as the XPF/ERCC1 heterodimer, are initially required for repair by incising the DNA strands 5' with respect to such adducts (de Silva *et al.,* 2000). These incisions are usually single-stranded breaks that maybe subsequently converted into DSBs following a round of DNA replication. Using both *Saccharomyces cerevisiae* and mammalian cells, it has been shown that proteins involved in HRR are required for the repair of DSB substrates induced by cross-linking agents (Mchugh, *et al.*, 2000 and Liu *et al.*, 1998).

The effects of MMC on MMR –proficient and –deficient tumour cell lines was investigated by treating cells with increasing doses of this agent and allowing viable cells to form colonies (Figure 3.12). Results showed that all five MMR –deficient cell lines tested were significantly more sensitive to the cytotoxic effects of MMC when compared to the two MMR –proficient cell lines used. At 10% survival, both the *h*MLH1 deficient HCT116 and SW48 cell lines were greater than 4.7-fold more sensitive than the MMR –proficient, SW480, cell line. The SKUT-1, Hec-1-A and LS411N, MMR -deficient cell lines, however, were up to 2.6-fold more sensitive to MMC when compared to SW480 cells. Thus taken together, this data suggests that MMR –deficient cells, like HRR –deficient cells, are sensitive to the DNA cross-linking agent, MMC.

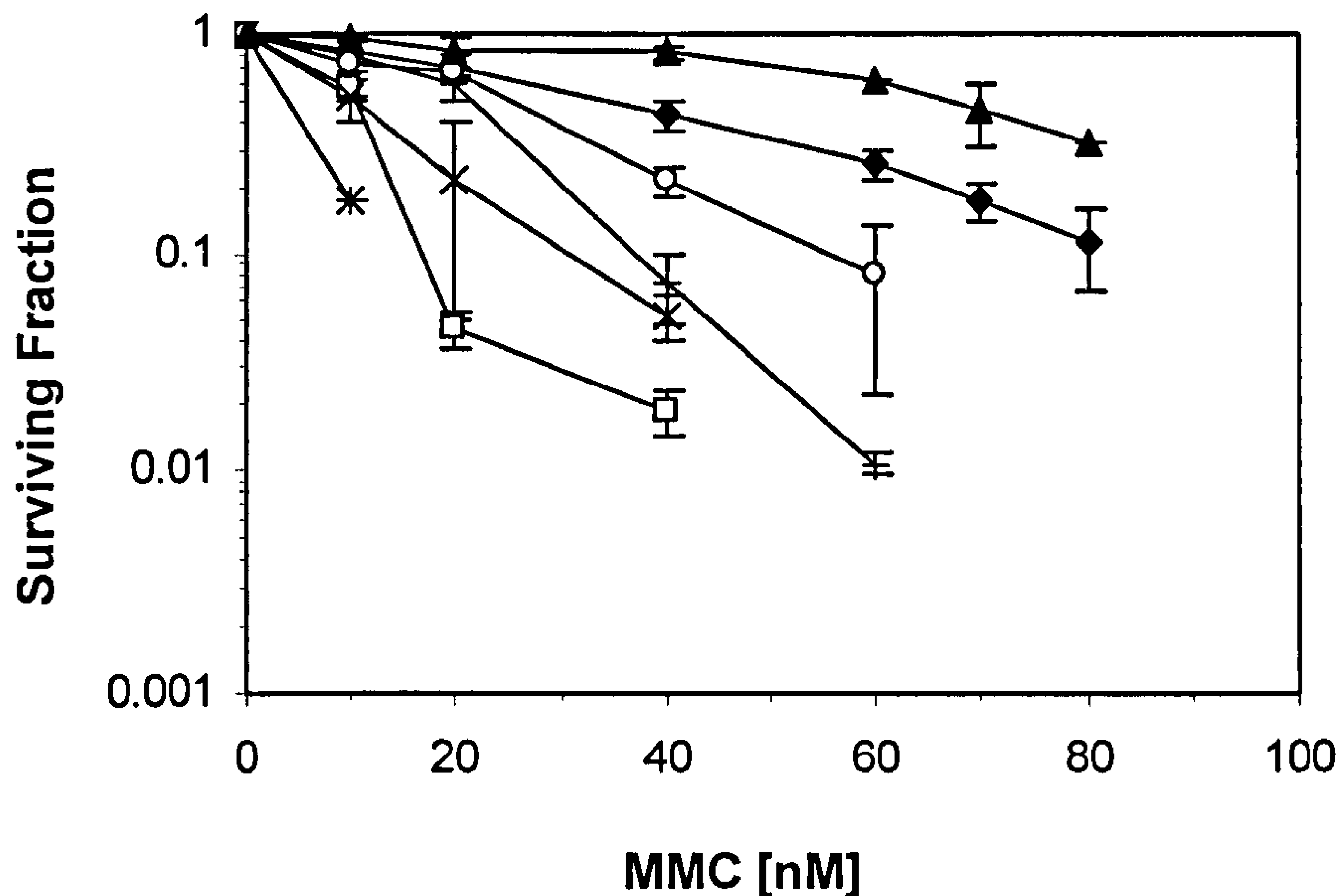


Figure 3.12 MMR –deficient cells are sensitive to the cytotoxic effects of MMC. The MMR –deficient cell lines tested have mutations in *h*MLH1 (HCT116, □; SW48,*; LS411N, o), *h*MSH2 (SKUT-1, X) and *h*PMS2 (Hec-1-A, +) respectively. The MMR –proficient cell lines used were the colorectal carcinoma cell line SW480 (♦) and the fibroblast, MRC5VA (▲). The mean (symbols) and standard deviation (error bars) of three independent experiments performed in triplicate are presented.

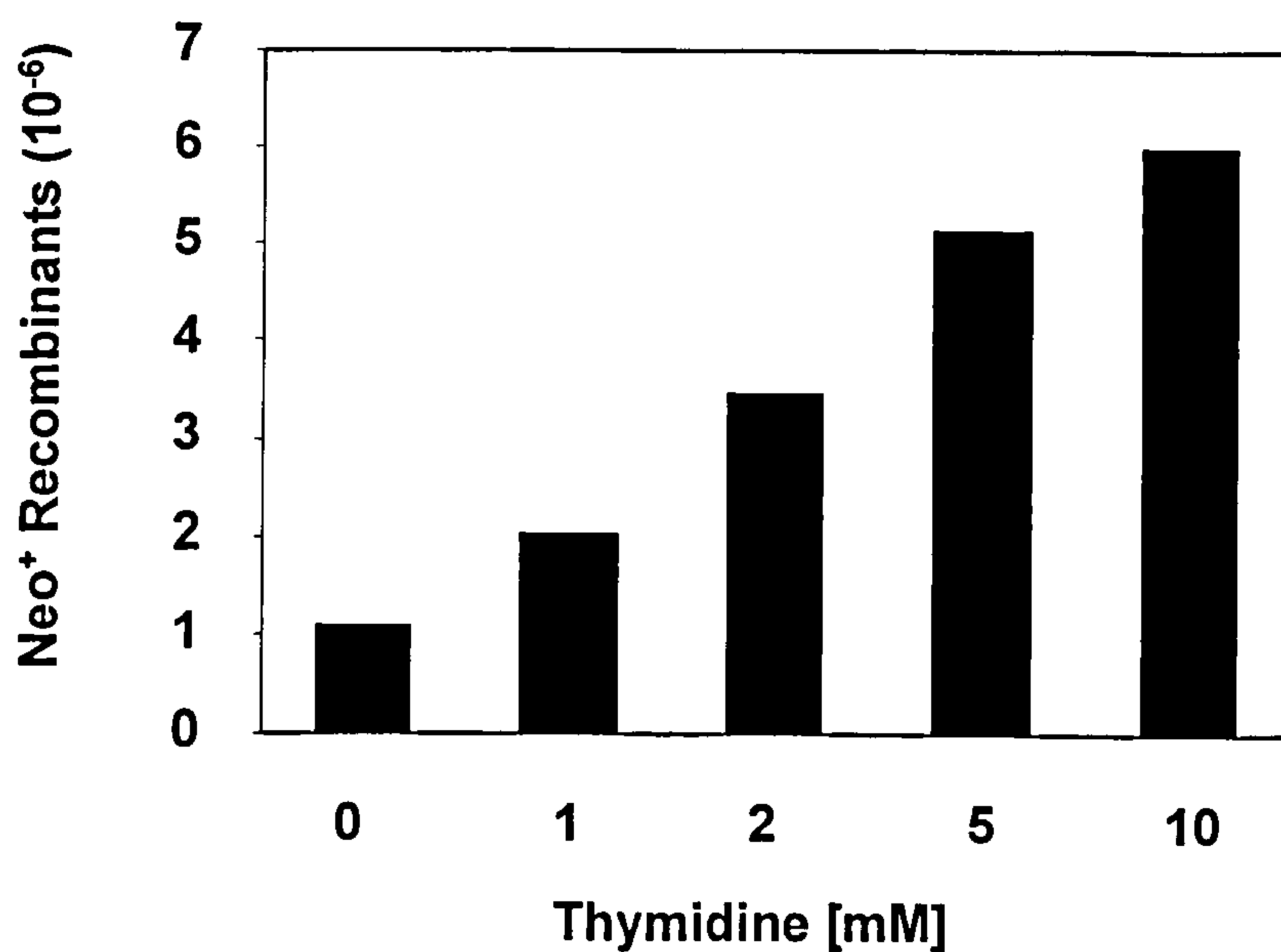
3.2.8 MMR -deficient tumour cells are defective in homology based recombination induced by S-phase cell cycle inhibitors.

The above experiments suggest that DLD-1 (*h*MSH6$^{-/-}$), Hec-1-A (*h*PMS2$^{-/-}$), SKUT-1 (*h*MSH2$^{-/-}$) and HCT116 (*h*MLH1$^{-/-}$) cells are defective in the production of neo^{+} recombinants when a DSB is initiated by an endonuclease. The integrity of the HRR pathways involved in repairing recombinogenic lesions induced by thymidine was next investigated in order to ascertain whether such processes are also defective in a MMR -deficient background.

The DNA lesion induced by thymidine is proposed to be recombinogenic as HRR processes are required for mammalian cell survival (Lundin *et al.*, 2002a). The effect of exposing cells to increasing doses of thymidine on recombination frequency was therefore determined in both MMR –proficient and –deficient cells. MMR –proficient, SW480/SN.3 strains, exposed to 10mM of thymidine, exhibited an induction in the frequency of neo$^+$ recombinants by up to 6-fold (Figure 3.13a). Furthermore, the effects observed were found to be dose-dependent with highest levels of recombinants occurring at 10mM thymidine.

The recombinogenic effect of thymidine in a MMR, *h*MSH6 –deficient, DLD-1-ScNeo transfected strain was also tested (Figure 3.13b). However, following thymidine treatment, no neo$^+$ recombinants were recovered, even when up to 36 million cells were plated in total. As DLD-1/SN.2 cells are sensitive to the cytotoxic effects of thymidine, it was not possible to treat these cells with high thymidine doses. However, even when 12 million cells were treated with 2mM thymidine, no recombinants were recovered. A similar observation was also noted when DLD-1/SN.2 cells were treated with hydroxyurea and camptothecin (data not shown). Taken together these results suggest that *h*MSH6 -deficient, DLD-1SN.2, cells are unable to repair either thymidine-induced lesions or DSBs.

(A)



(B)

CELL LINE	Thymidine [mM]	Recombination frequency
DLD-1/SN.2	0	< 7.22×10^{-7}
	1	< 2.59×10^{-7}
	2	< 2.41×10^{-7}

Figure 3.13 Thymidine induces the frequency of neo+ recombinants in the MMR -proficient, SW480/SN.3 strain, but not in the DLD-1/SN.2, *h*MSH6 -deficient strain. All cells were exposed to various concentrations of thymidine for 24 hours, before being plated in G418 selective media. Thymidine induced the frequency of neo+ recombinants in the SW480/SN.3 strain. However, no recombinants were retrieved from the DLD-1/SN.2 strain (indicated by less than values).

3.3 DISCUSSION

MMR –deficient cells acquire an additional series of mutations that provide a selective growth advantage and contribute to the mutator phenotype seen in such cells. Both MMR –deficient and HRR –deficient cell lines have been reported to be sensitive to the cytotoxic effects of thymidine (Mohindra *et al.,* 2002 and Lundin *et al.,* 2002a). Treatment with thymidine depletes cells of dCTP and subsequently results in the retardation of replication fork progression. Furthermore there is an increasing amount of evidence from bacteria and yeast for the involvement of HRR proteins in rescuing stalled/collapsed replication forks (Cox, 2001; Kraus *et al.,* 2001 and Michel *et al.,* 2001). In the present study, the integrity of the HRR pathway was therefore determined in a MMR –deficient background.

The recombination reporter substrate, ScNeo, was used to measure homology based recombination events between two defective neomycin phospho-transferase genes. Recombination events were induced using a variety of recombination inducing agents. The results showed that all four MMR –deficient cell lines tested, namely DLD-1 (*h*MSH6$^{-/-}$), Hec-1-A (*h*PMS2$^{-/-}$), SKUT-1 (*h*MSH2$^{-/-}$) and HCT116 (*h*MLH1$^{-/-}$), were defective in the production of neo^{+} recombinants by homology based recombination following the expression of a site-specific DSB. This contrasted to the three MMR –proficient cell lines SW480, SW620 and MRC5VA tested which produced neo^{+} recombinants at a frequency of 0.8 – 4.38 x 10^{-3}. These results are consistent with a study published by Slebos and Taylor (2001) which described the use of a host cell reactivation assay to measure HRR events based on the reactivation of a green-fluorescent protein in various MMR –proficient and –deficient cell lines. This study also provided evidence that the MMR –deficient, HCT116 cell line, showed no increase in recombination events.

The HRR defects observed in MMR –deficient cells was not due to a lack of ability of cells to express the I-SceI endonuclease, nor was it due to an increase in an I-SceI induced apoptosis event. Furthermore, correction of the MMR –deficiency in the Hec-1-A/SN.3 strain did not alter the frequency of neo^+ recombinants formed following the induction of a site-specific break. Such results therefore suggest the *h*PMS2 deficiency in Hec-1-A cells is not directly responsible for the HRR defects.

In addition, all five MMR –deficient tumour cell lines tested were variably sensitive to the cross-linking agent MMC. These results are in agreement with a study conducted by Fiumicino and co-workers (2000) where a sensitivity of various MMR -deficient cells to MMC was also noted. Cells deficient in HRR (including XRCC2, XRCC3 and BRCA1) are also known to be acutely sensitive to MMC (Jones *et al.,* 1987; Fuller and Painter, 1988 and Moynahan *et al.,* 2001). However, the sensitivity to MMC observed in such cells is much greater than that observed in the tumour cell lines. Taken together these results suggest that HRR defects occur in MMR -deficient tumour cells.

To address the question of whether DLD-1 cells were specifically defective in the processing of substrates induced by I-SceI or were also defective in the HR mediated repair of structures associated with collapsed or stalled replication forks, thymidine was used to induce recombination events. The results showed that potentially lethal substrates formed following exposure to thymidine could not be repaired in the DLD-1/SN.2, *h*MSH6 –deficient, strain. Such treatment presumably led to cell death and therefore no detectable neo^+ recombinants were observed.

As described previously, the thymidine sensitivity observed in MMR –deficient cells was not directly due to the loss of MMR, as HCT116+3 cells (corrected for the *h*MLH1 deficiency) remained sensitive to thymidine

(Mohindra *et al.,* 2002). In addition, Elliot and co-workers (2001) reported, using mouse MSH2 knockout ES cells, that deficiency in MMR does not directly result in defects in HRR. Consistent with the above studies, correction of the *h*PMS2 –deficiency in the Hec-1-A/SN.3 strain did not alter thymidine sensitivity or the frequency of neo^+ recombinants formed following the induction of a site-specific break. Taken together these studies suggest that both the loss of HRR and thymidine sensitivity appear to be the result of events that occur downstream from the initial loss of MMR.

The addition of thymidine induces an imbalance in dCTP and dTTP pools that subsequently results in the slowing of DNA replication forks (Bjursell and Reichard, 1973). Increasing evidence from both prokaryotic and eukaryotic cells suggest that the loss of HRR could account for the thymidine sensitivity observed in various cell types (Cox, 2001; Kraus *et al.,* 2001 and Michel *et al.,* 2001). In addition, it is proposed that this retardation of DNA replication fork progression may result in the accumulation of DNA lesions that specifically require a proficient HRR pathway for mammalian cell survival (Lundin *et al.,* 2002). Therefore the findings in the present study, which show HRR defects in MMR –deficient cells, are consistent with an involvement of HRR processes in conferring thymidine sensitivity. The absence of both MMR and HRR processes may therefore result in the loss of ability to form colonies instead of S-phase arrest.

There are a number of possible advantages gained by HRR deficiency in terms of tumour progression in this subset of MMR –deficient tumours. Components of the MMR system interact with various HRR processes including: regulating homeologous recombination events, removing non-homologous DNA during both GC and SSA processes and regulating heteroduplex-DNA extension (Evans *et al.,* 2000; Sugawara *et al.,* 1997;

Studamire *et al.,* 1999 and Marsischky *et al.,* 1999). Therefore the loss of MMR and the subsequent loss of HRR pathways may provide a selective growth advantage as cells maybe unable to regulate homeologous recombination events between diverged sequences. The occurrence of chromosome translocations, deletions and/or insertions events would therefore be encouraged. In addition, as HRR processes seem to be involved in maintaining the integrity of replication forks, the loss of such a pathway would impair the S-phase cell cycle checkpoint (Henry-Mowatt *et al.,* 2003). In further support of this argument, Morrison and co-workers (2000) used chicken DT40 cells to show that the DNA damage 'sensing' ATM protein and HRR act on the same pathway; although this study only used irradiation induced chromosomal aberrations to measure defects in HRR in various knock out cell lines.

Phenotypic characterization of MMR –deficient cells revealed that such cells are resistant to DNA alkylating agents such as 6-thioguanine and cisplatin. Resistance to such classes of chemotherapeutic agents has subsequently hindered the treatment of tumours displaying microsatellite instability. The loss of HRR and the hypersensitivity of MMR –deficient cells to thymidine raise the possibility of using agents that induce dNTP pool imbalances in conjunction with current methods to improve the treatment of this subset of tumours.

CHAPTER FOUR:

SCREENING OF HOMOLOGOUS RECOMBINATION REPAIR GENES IN MISMATCH REPAIR –DEFICIENT TUMOUR CELL LINES

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4.3 **DISCUSSION** **155**

4.1 INTRODUCTION

The results obtained in the previous chapter suggest that MMR –deficient tumour cell lines contain defective HRR pathways. In order to determine whether this deficiency was the result of mutations in genes involved in HRR (and/or the signalling of DNA damage), cDNA sequences encoding a selection of these genes were screened for mutations.

Eukaryotic genomic DNA consists of simple repetitive DNA regions which are each composed of a small number of repeated bases and are considered to be hotspots for mutations in a MMR –deficient background (Tautz and Schlotterer, 1994 and Strauss, 1999). These repeated bases range from between 1-13 base pairs (microsatellites) to greater than 15 base pairs (minisatellites) in length. Approximately 100,000 microsatellite repeats are scattered throughout the human genome (Weber and May, 1989). Experimental data indicates that in a MMR –deficient background, the rate of microsatellite instability at these repetitive sequences is dramatically elevated (reviewed by Kolodner, 1996 and Modrich and Lahue, 1996). Such instability is thought to predominantly arise due to DNA polymerase slippage errors which occur during replication, rather than base mis-incorporations or recombination processes (reviewed by Kunkel, 1992 and Sia *et al.,* 1997). Streisinger and co-workers (1985) used T4 bacteriophage to propose a model in which it was suggested that misalignment of re-associated primer and template strands occurred, possibly due to polymerase slippage (Figure 4.1). Such misalignment subsequently forms a loop containing one or more unpaired repeated unit which, if left un-repaired, results in alterations of the number of repeats following replication.

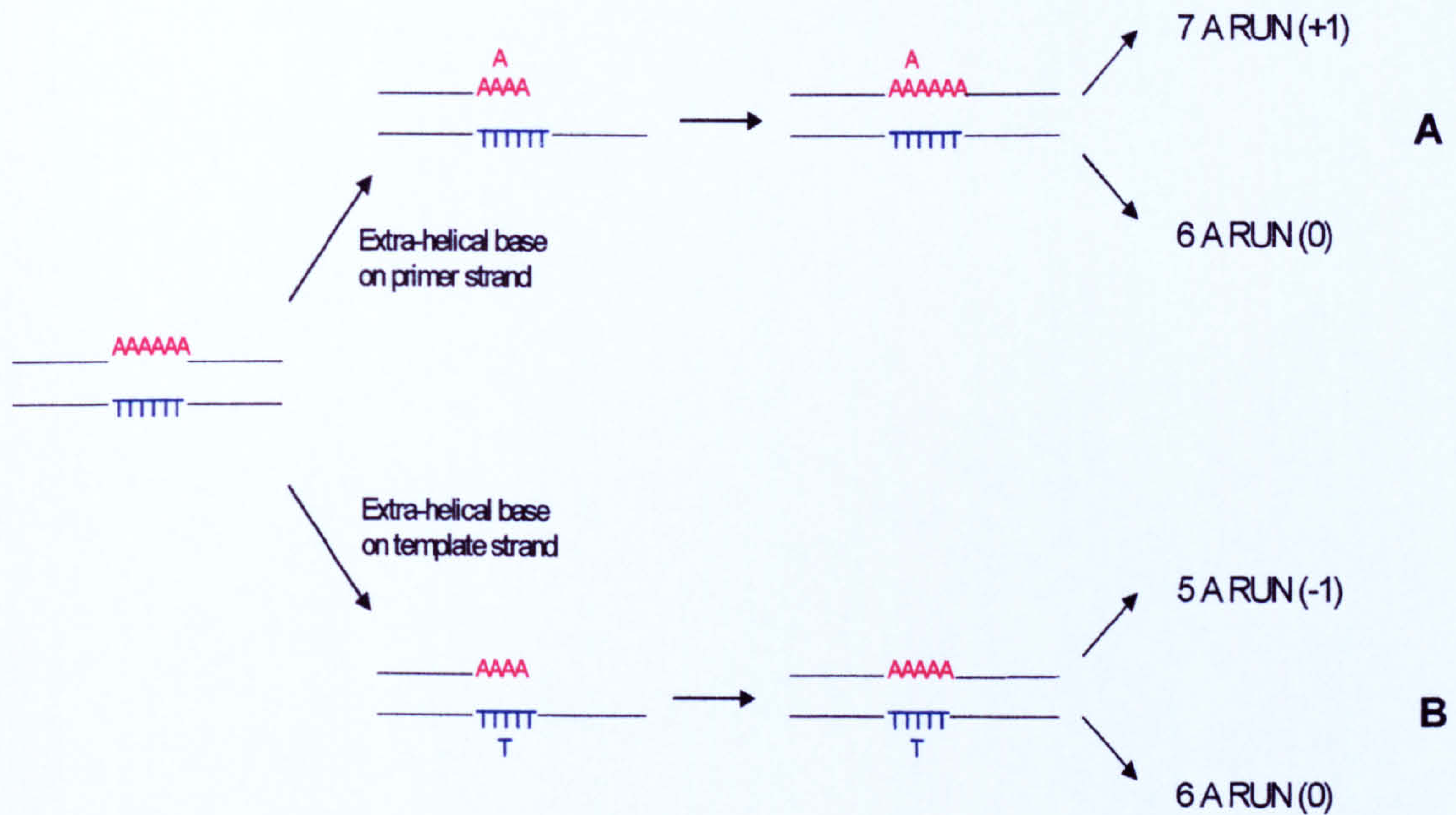

Figure 4.1 Generation of frameshift mutations due to DNA polymerase slippage on mononucleotide repeats. Such events involve a transient dissociation of the template and primer strands followed by misaligned annealing within the repeat tract. This subsequently results in the formation of a frameshift intermediate that is composed of an extra-helical loop. If this mismatch remains uncorrected, the repetitive tract will contain (A) more repeats or (B) fewer repeats, following replication. *Adapted from Gragg et al., 2002*

The addition or subtraction of bases in non-multiples of three within such repeats contained in coding sequences frequently generates frameshift mutations. Frameshift-type mutations predominate in MMR –deficient cells and almost always result in the alteration of the protein product and therefore protein function. Therefore the microsatellite instability (MSI) phenotype, in which alterations in mono- and di-nucleotide repeats of microsatellites occur, most likely reflects the accumulation of frameshift mutations.

MSI is well documented to be suppressed by the 3' to 5' exonucleolytic proofreading of DNA polymerases as well as by the post-replicative MMR system (Kroutil *et al.*, 1996; Sagher *et al.*, 1999; Strand *et al.*, 1993 and Tran *et al.*, 1997). Mutation analysis studies show that MMR defective cells frequently display an increased rate of mutations both spontaneously and at specific loci (Gragg *et al.*, 2002; Parsons *et al.*, 1995; Shibata *et al.*, 1994; Bhattacharyya *et al.*, 1994; Kat *et al.*, 1993; Eshleman *et al.*, 1995 and Phear *et al.*, 1996). Furthermore, the rates of frameshift mutations occurring in MMR –deficient cells have been shown to be increased by between 200 to 1000-fold when compared to MMR –proficient cells (Bhattacharyya *et al.*, 1994 and Malkhosyan *et al.*, 1996). Correction of the MMR deficiency was found to reduce the mutator phenotype by more than 90% (Umar *et al.*, 1997; Koi *et al.*, 1994 and Glaab *et al.*, 1997). Such results therefore confirm a direct involvement of MMR with MSI.

The types of mutations accumulating in MMR –deficient cells, however, appear to be a function of the component of the MMR pathway that is altered (Bhattacharyya *et al.*, 1994 and Malkhosyan *et al.*, 1996). For example HCT116 (*h*MLH1 deficient) and DLD-1 (*h*MSH6 deficient) cells both display high rates of spontaneous mutations at the *hprt* locus. Mutation spectra analysis, however, showed that in HCT116 cells frameshift mutations constituted a large proportion of such mutations at the *hprt* locus. The results of mutation spectra analysis studies in HCT116 cells are the opposite of that observed in DLD-1 cells, where point mutations were prominent. This is consistent with the role of *h*MLH1 in repairing all types of replication errors, whereas *h*MSH6 is specifically involved in the repair of single base-base mispairs.

The inability of MMR –deficient cells to form neo$^+$ recombinants, reported in the present study could be due to mutations in genes that are members of the RAD52 epistasis group. Alternatively mutations in genes that are

involved in the signalling of DNA damage could also be responsible for the HRR defects. As described above, in a MMR –deficient background, cells have a higher probability for incurring frameshift mutations specifically at mononucleotide runs. The sequence of genes involved in HRR and DSB signalling were therefore initially screened for the frequency of mononucleotide runs occurring within the coding sequence. Genes containing a high frequency of such runs were subsequently sequenced in the search for mutations.

4.2 RESULTS

4.2.1 Analysis of genes involved in DNA damage signalling and HRR in MSI$^+$ tumour cell lines.

Mononucleotide runs were initially screened for in several genes that belong to the *RAD52 epistasis* group. As shown in Table 4.1, screening of coding sequences revealed that the RAD51 gene and the RAD51 paralogs, XRCC3, RAD51B, RAD51C and RAD51D, did not contain a significant number of mononucleotide runs that are greater than 6 base pairs in length. Analysis of the coding sequence within the XRCC2 gene, however, revealed three mononucleotide runs that were eight base pairs in length. The other significant genes of interest, namely RAD52 and RAD54B, had coding sequences that coded for a 9 base pair and two seven base pair mononucleotide runs, respectively.

In addition to the above genes, further sequence analysis was conducted on genes involved in the signalling of DSBs. As shown in Table 4.1 the coding sequences for the checkpoint signalling gene, CHK1, and the DNA helicases, WRN and BLM, all contained a high frequency of mononucleotide runs. These genes were therefore also considered to be potential candidates for incurring frameshift mutations.

Genes Sequenced	Number of mono-nucleotide runs				
	5 bp	6 bp	7 bp	8 bp	9 bp
XRCC2	4			3	
XRCC3	4	2			
RAD51		1			
RAD52	4	4			1
RAD54	4				
RAD54B		4	2		
RAD51B	2				
RAD51C	2				
RAD51D	1				
	Number of mono-nucleotide runs				
FANCD2		5	2		
MUS81	2	1	1		
WRN	20	1	1	1	
BLM	12	4	2	1	
CHK1	2	3	1		1
CHK2	15	2			

Table 4.1 Table showing the number of mononucleotide runs in some of the genes involved in HRR and/or signaling of DNA damage. Details of primers sequences and annealing temperatures used for the respective gene are stated in Appendix I-III (pages 236-238).

4.2.2 The MMR –deficient, SKUT-1, cell line has a frameshift mutation in the XRCC2 gene.

Sequence analysis of all genes shown in Table 4.1 was limited to mononucleotide runs that were 6 base pairs in length as these regions have a higher probability for incurring frameshift mutations. Specific regions encoding such mononucleotide runs were subsequently amplified, sequenced and analyzed for mutations in SW480, HelaS3, LM217E (MMR –proficient) and HCT116, SKUT-1, 2774, Hec-1-A, DLD-1 (MMR –deficient) cell lines.

Sequencing of amplified cDNA products revealed a frameshift mutation in the XRCC2 gene of the SKUT-1, MMR –deficient cell line. As shown in Figure 4.2a, this mutation was a single nucleotide deletion in a run of eight thymine residues between nucleotides 342 and 350 of the XRCC2 coding sequence. The mutation appeared to be heterozygous as both wild-type and 342delT mutant allele sequences could be detected in the sequencing image (Figure 4.2b). Further confirmation for the presence of the mutant allele was achieved when the amplified fragment covering this region was sub-cloned into the pcDNA3.1 expression vector and sequenced (Figure 4.2b).

Analysis of cDNA amplified sequences derived from the XRCC3, RAD51, RAD52, RAD54, RAD54B, RAD51B, RAD51C or RAD51D genes, however, revealed no additional mutations in any of the MMR –proficient or –deficient cell lines used. In addition, sequence analysis of the CHK1, CHK2, WRN or BLM genes, did not reveal any further frameshift mutations in any of the MMR –proficient or –deficient tumour cell lines used.

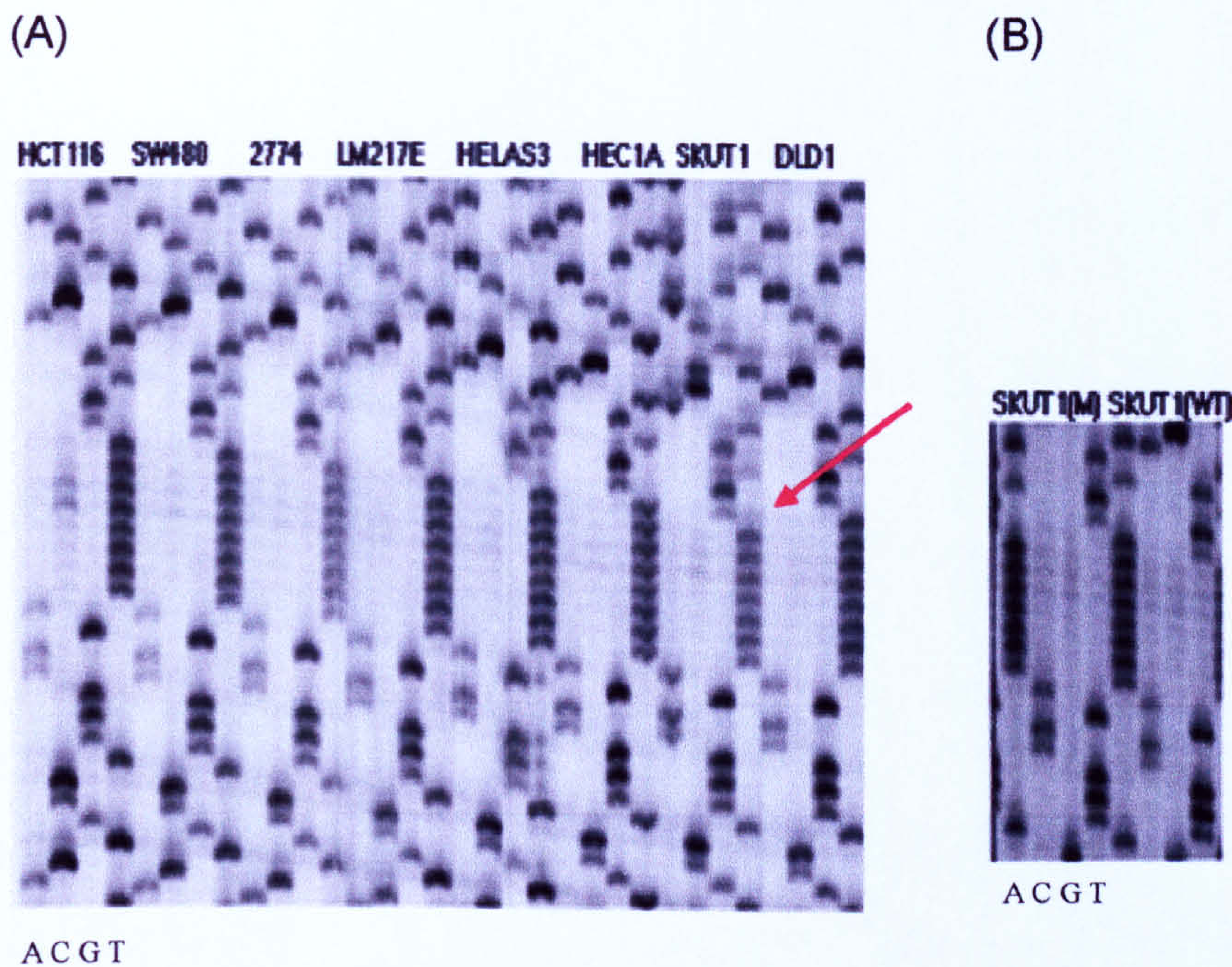

Figure 4.2 A MMR –deficient tumour cell line, SKUT-1, encodes for a frameshift mutation within the XRCC2 gene. (A) XRCC2 sequence analysis of amplified cDNAs from both MMR –proficient and –deficient tumour cell lines revealed a frameshift mutation within the SKUT-1 cell line. (B) Further confirmation for the presence of a heterozygous -1 frameshift in the run of eight thymine residues (indicated by arrow) was achieved through cloning the gene into a pcDNA3.1 expression vector.

As frameshift mutations are well documented to completely alter translated protein sequence, amino acid sequences of both wild-type XRCC2 and 342delT were therefore aligned. This alignment showed that the frameshift mutation of XRCC2 in SKUT-1 was a nonsense mutation as it introduced a premature stop codon 48 nucleotides downstream from the frameshift (Figure 4.3). This subsequently resulted in a loss of the wild type amino acid sequence after amino acid 116 out of the 280 encoded by the gene. In addition, the mutation found in the XRCC2 gene is predicted to encode a peptide that retains the Walker Box A but loses Walker Box B.

```
                                                                    WALKER BOX A
XRCC2      1    MCSAFHRAESGTELLARLEGRSSLKEIEPNLFADEDSPVHGDILEFHGPEGTGKTEMLYH
342delT    1    MCSAFHRAESGTELLARLEGRSSLKEIEPNLFADEDSPVHGDILEFHGPEGTGKTEMLYH

                                                                         ↓
XRCC2     61    LTARCILPKSEGGLEVEVLFIDTDYHFDMLRLVTILEHRLSQSSEEIIKYCLGRFFLVYC
342delT   61    LTARCILPKSEGGLEVEVLFIDTDYHFDMLRLVTILEHRLSQSSEEIIKYCLGRFFWCTA
                                       WALKER BOX B
XRCC2    121    SSSTHLLLTLYSLESMFCSHPSLCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQCL
342delT  121    VVAPTYFLHFTH*

XRCC2    181    EKLVNDYRLVLFATTQTIMQKASSSSEEPSHASRRLCDVDIDYRPYLCKAWQQLVKHR
342delT  181

XRCC2    241    MFFSKQDDSQSSNQFSLVSRCLKSNSLKKHFFIIGESGV
342delT  241
```

Figure 4.3 The frameshift mutation in XRCC2 alters the wild type protein sequence after amino acid 116 and produces a premature stop at codon 133. Wild-type XRCC2 amino acid sequence (red) is aligned with mutant peptide amino acid sequence (blue) to show that Walker box B is deleted in the mutant XRCC2 allele. An arrow indicates the location of the deleted residue.

4.2.3 All cells in the SKUT-1 population carry the heterozygous XRCC2 mutation.

The possibility that the frameshift mutation found in the XRCC2 gene only occurred in a subset of the SKUT-1 cell population was next investigated. To test this, cDNA was amplified and sequenced from well isolated colonies derived from single cells (Figure 4.4). Such sequencing revealed that all of the twelve such colonies isolated (six of which are shown), were heterozygous for this mutation, as both wild type and mutant XRCC2 alleles could be detected. However, a majority of the isolates predominantly displayed the mutant XRCC2 allele.

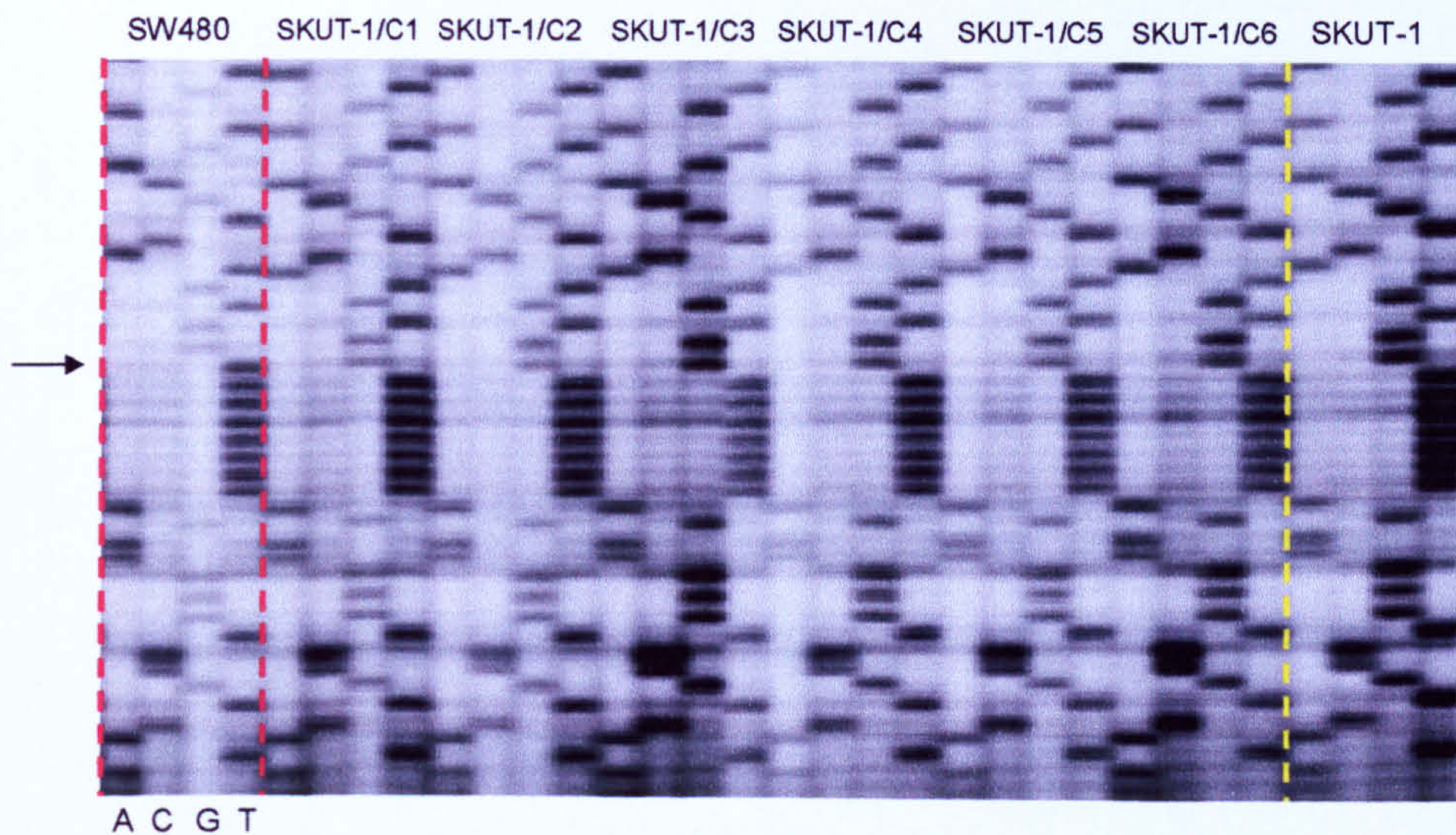

Figure 4.4 All cells in the SKUT-1 population carry the heterozygous XRCC2 mutation. Amplified sequences from colonies derived from single cell isolates of the MMR –deficient, SKUT-1 cell line, show the presence of a frameshift mutation of XRCC2 (indicated by arrow). A heterozygous -1 frameshift in a run of eight thymine residues (indicated by arrow) is evident in all cell isolates of SKUT-1.

4.2.4 XRCC2 mutations in gynaecological tumours

The SKUT-1 cell line was derived from a uterine sarcoma (Risinger *et al.*, 1995). The presence of the mutation in this cell line was therefore investigated in several similar gynaecological tumours. Malignancies of the uterus (excluding the cervix) are common. The vast majority of uterine malignancies are endometrial carcinomas. Endometrial carcinomas are the most frequent malignancy of the female genital tract in the Western world and account for 7% of all invasive cancers in women (Kumar *et al.*, 2003 and Fletcher, 2000). Conversely, uterine sarcomas are very rare and only account for between 1 to 5% of all malignancies of the uterus. The essential difference between carcinomas and sarcomas is their embryological tissue of origin and differentiation. Carcinomas arise from epithelial tissues whereas sarcomas derive from mesenchymal connective tissues. The three

main categories of uterine sarcomas include leiomyosarcomas, endometrial stromal sarcomas and mixed Mullerian tumours.

Leiomyosarcomas constitute 25% of uterine sarcomas. These tumours arise from smooth muscle cells within the myometrium and exhibit histological features that phenotypically resemble this path of differentiation towards smooth muscle. These tumours usually start as a rapidly growing, painless swelling in the wall of the uterus; with the exact cause being unknown. These tumours are usually solitary and appear as a large, poorly circumscribed mass having a soft flesh consistency with necrosis. The risk of malignancy of these tumours is graded by the number of cells undergoing cell division. Tumours having >10 mitotic figures/10 microscopic high-power fields (hpf) are classed as being highly aggressive (an example of which is shown in Figure 4.5), whereas tumours that are of intermediate risk of malignancy have 5-9 mitotic figures/10hpf. Other histological factors used to assess malignancy include nuclear pleomorphism and the presence of necrosis. Treatment for these tumours mainly includes surgery and/or chemotherapy. However, the survival prognosis for 5 years is only between 40 to 50%.

Another 15% of uterine sarcomas arise from the supporting stromal cells that surround the glands within the lining of the uterus (i.e. the endometrium) and are called endometrial stromal sarcomas. The endometrial stroma gives rise to a variety of sarcomas which can vary from low to very high grade. Endometrial stromal sarcomas are also treated with surgery and/or chemotherapy.

The remainder of uterine sarcomas comprises the mixed-Mullerian tumours. These are characterised by both a neoplastic epithelial and stromal component (Kempson and Hendrickson, 2000). As the presence of a

neoplastic epithelial component provides a second variable in the analysis of the tumours, these were excluded from the study.

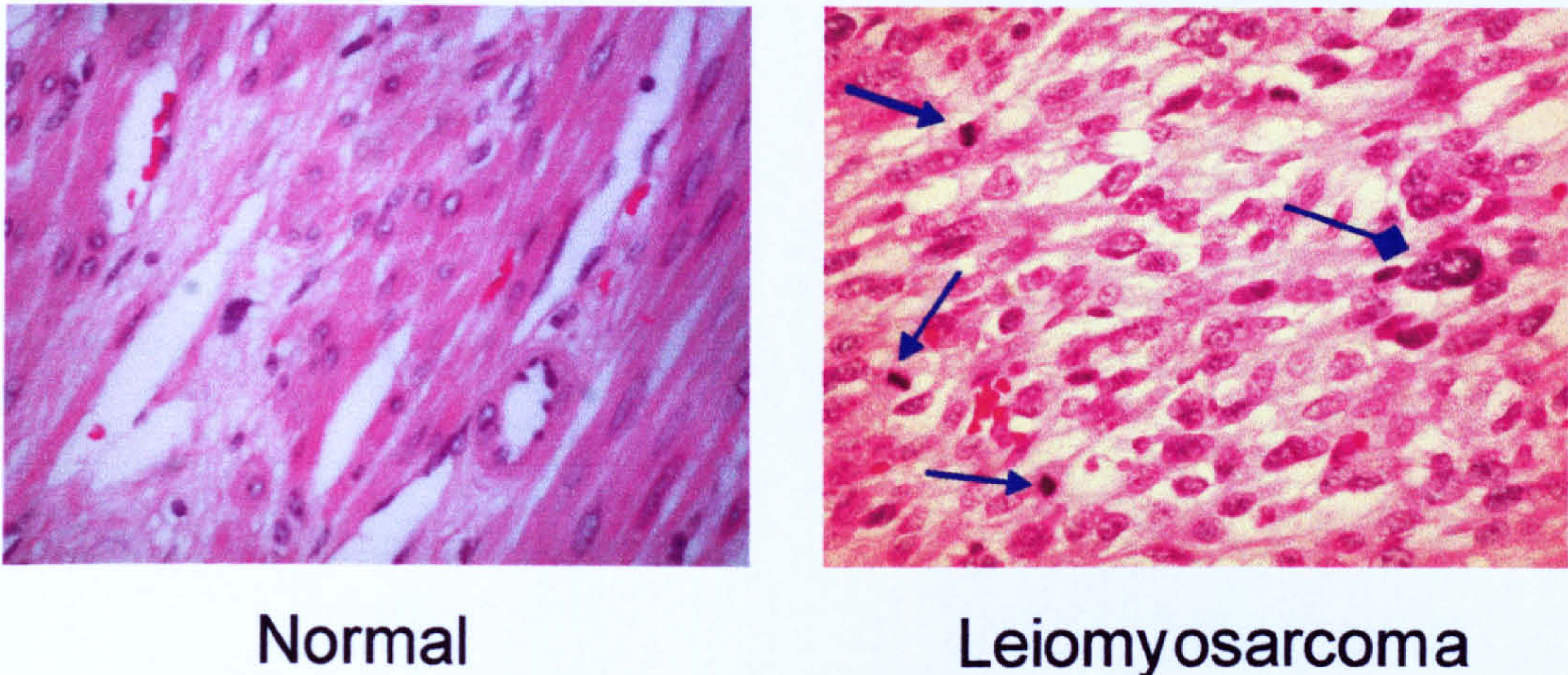

Figure 4.5 Image showing samples of normal and leiomyosarcomas tissue. The presence of marked nuclear pleomorphism (blue diamond arrowhead) and three mitotic figures (arrowed) in the leiomyosarcoma are displayed in the image on the right. Image supplied by Dr. Jason Stone.

Only 4 endometrial stromal sarcomas and 8 leiomyosarcomas of the uterus could be sourced from the Sheffield area from the last six years. Benign mesenchymal neoplasias of the uterus (and therefore, by definition, not sarcomas) e.g. leiomyomas (fibroids) were not included in the study. Although these figures are small, these numbers are in keeping with those observed at other cancer centers (Goff *et al.,* 1993; Amant *et al.,* 2003; Melilli *et al.,* 1999; Gonzalez-Bosquet *et al.,* 1997 and Chauveinc *et al.,* 1999). Genomic DNA was extracted and purified from all microdissected tumour samples along with appropriate control tissue from each patient. All exons of the XRCC2 gene were amplified using the primer sequences and annealing temperatures indicated in the Appendix (Page 241). Sequence analysis of amplified regions containing the mononucleotide run of eight thymine residues in exon III revealed the 342delT frameshift in one of the uterine leiomyosarcomas (Figure 4.6). This specific tumour was classified

as a high-grade uterine leiomyosarcoma and furthermore, sequencing gels showed equal expression of both wild type and mutant alleles. Analysis of the four endometrial stromal sarcomas, however, did not reveal any mutations; an example of which is shown in Figure 4.8 (sample number 9).

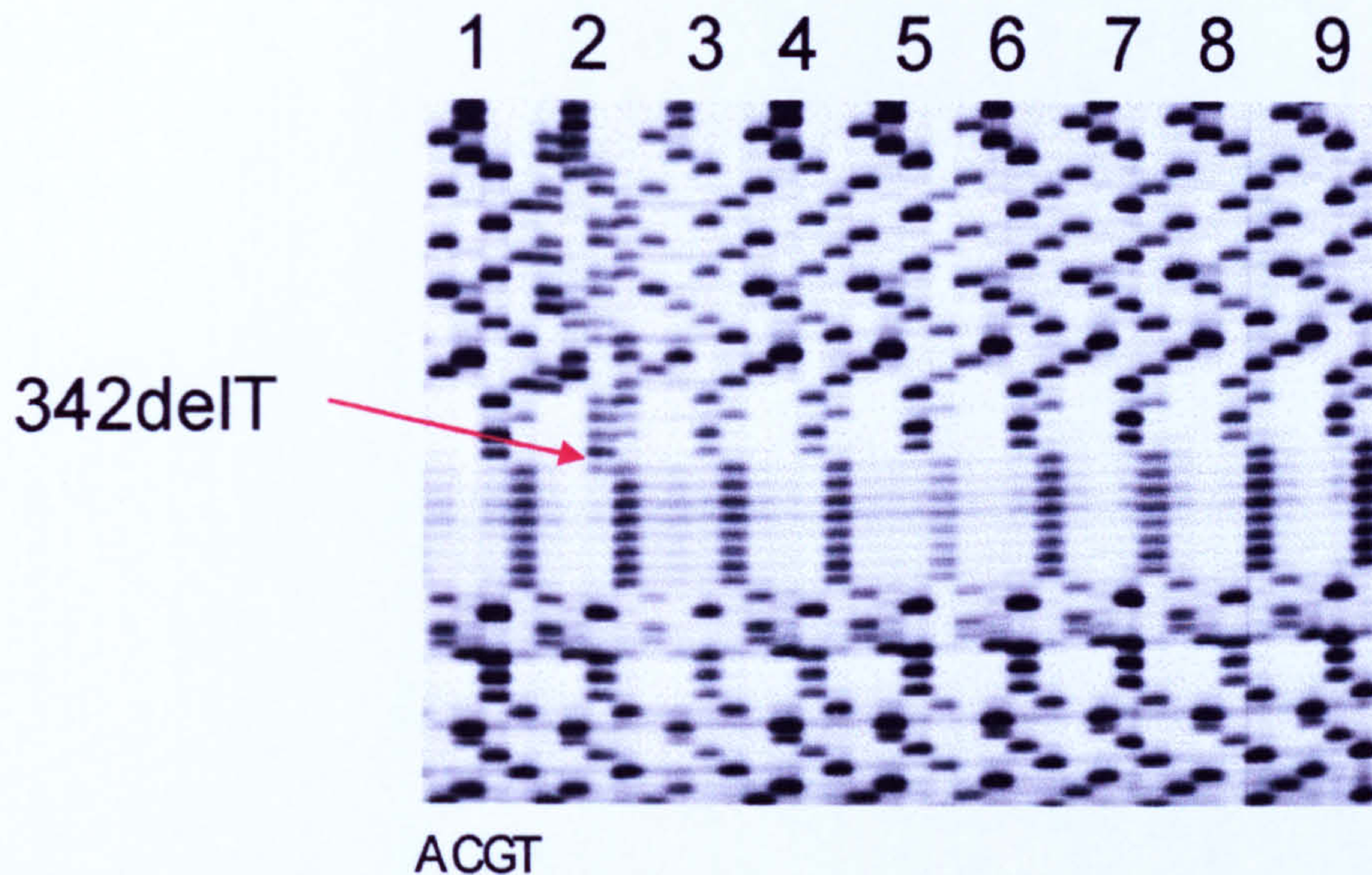

Figure 4.6 Sequence analysis of eight independent leiomyosarcoma samples revealed the same frameshift mutation of XRCC2 found within the SKUT-1 cell line. A heterozygous -1 frameshift in a run of eight thymine residues (indicated by arrow) was evident in a high grade leiomyosarcomas tissue sample (Sample 2). No such mutations were found in any of the endometrial stromal sarcomas examined.

4.2.5 SKUT-1 cells express decreased levels of wild-type XRCC2 protein.

The MRE11 protein has previously been shown to be expressed at low levels in HCT116 and SW48, MMR -deficient cells, which both encode for a frameshift mutation in an intronic run of eleven thymine residues (Giannini *et al.,* 2002). Therefore, by analogy to MRE11, XRCC2 protein expression levels were investigated in the MMR –deficient SKUT-1 cell line.

Western blot analysis of whole cell lysates extracted from both MMR –proficient and –deficient tumour cell lines revealed that the level of XRCC2 protein was depressed in SKUT-1 cells (Figure 4.7). In addition, a rapidly migrating protein was also detected in the SKUT-1 cell line that interacted with the XRCC2 antibody. This peptide had approximately the same molecular weight (17.2kDa) predicted to be encoded by the mutant XRCC2 allele and was not detected in any other cell line examined (data not shown).

Figure 4.7 The SKUT-1, MMR –deficient, cell line expresses reduced levels of wild type XRCC2 protein. Western blot analysis of whole cell lysates was used to measure XRCC2 protein expression levels (indicated by arrow) in the MMR –proficient SW480 cell line and the MMR –deficient SKUT-1, cell line. β-Actin was used for loading control.

4.2.6 Functional analysis of 342delT

As SKUT-1 cells expressed reduced levels of wild-type XRCC2 protein as well as a mutant peptide, the possibility that this could account for the thymidine sensitivity found in such cells was investigated.

The mutant XRCC2 allele was cloned into a pcDNA3.1 expression vector (Appendix 3) and stably transfected into the MMR –proficient cell line, SW480. The presence of the mutant construct was confirmed through sequencing of amplified XRCC2 cDNA. As shown in Figure 4.8, three such transfectants appeared to display approximately equal levels of both mutant and wild-type XRCC2 alleles. However, the degree of mutant expression in the three transfectants was found not to be as high as that found in the SKUT-1, MMR –deficient cell line.

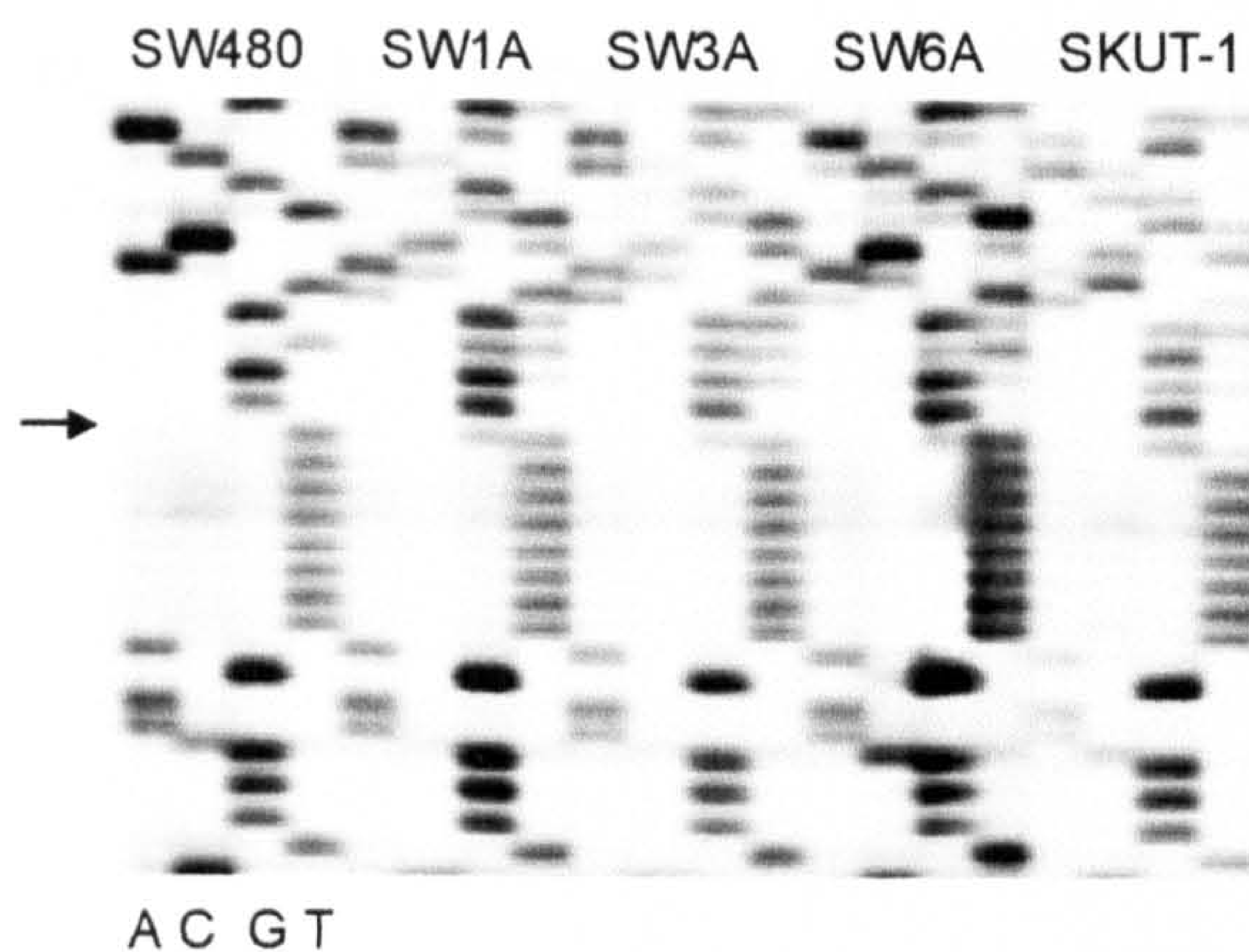

Figure 4.8 Expression of 342delT (indicated by arrow) can be detected in several SW480 clones transfected with the pcDNA*XRCC2* construct. The sequence patterns in three transfectants (SW1A, SW3A and SW6A) is compared with un-transfected SW480 (containing only wild type XRCC2) and SKUT-1 (which carries both the frameshift mutation of XRCC2 as well as wild type) cells.

4.2.6.1 Cells expressing 342delT become sensitive to thymidine.

The results obtained in the previous chapter indicated that defective HRR mechanisms could be responsible for the thymidine sensitivity observed in MMR –deficient cell lines. Therefore, the possibility that expression of the mutant allele could confer sensitivity to thymidine in SW480, MMR –

proficient, cells was investigated. The viability of plated cells was measured in the presence of increasing doses of thymidine after 10-14 days. The MMR –deficient, SKUT-1 cell line was 3-fold more sensitive to thymidine when compared to SW480 cells at 10% cell survival (Figure 4.9a). At 10% survival, the two MMR –proficient strains expressing the mutant XRCC2 allele were up to 2-fold more sensitive than SW480 cells.

In addition, the effect of expressing an empty vector into SW480 cells was also investigated as a control. As shown in Figure 4.9b, no increase in sensitivity was observed when cells expressing the empty vector were treated with increasing doses of thymidine. Taken together, these results suggest that the thymidine sensitivity observed in MMR –deficient, SKUT-1 cells, could be due to the depressed level of XRCC2 protein.

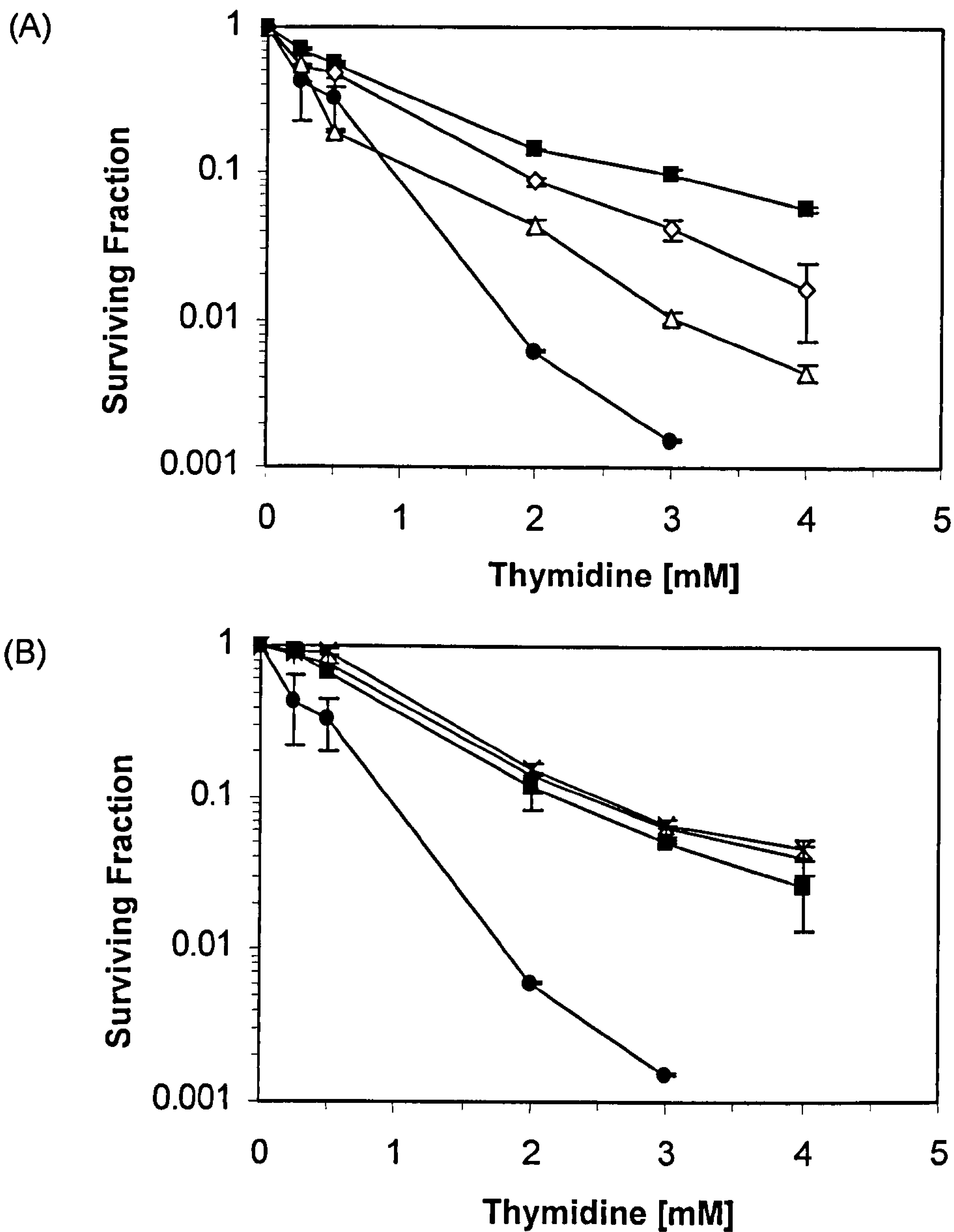

Figure 4.9 Expression of 342delT in SW480, MMR –proficient cells confers sensitivity to thymidine. (A) Transfectants of SW480 expressing the mutant XRCC2 allele (SW1A, ◊ and SW6A, Δ) are sensitive to thymidine when compared to untransfected SW480 cells (■). Sensitivity of SKUT-1 cells to thymidine is included for reference (●). (B) Control experiments using empty-vector expressing transfectants (SWPC.2, † and SWPC.5, X) showed no significant difference to thymidine sensitivity when compared to SW480 cells (■). Sensitivity of SKUT-1 cells to thymidine is included for reference (●). The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

4.2.6.2 Restoration of wild-type XRCC2 protein levels in SKUT-1 cells results in thymidine resistance.

The results obtained above suggest that strains expressing the mutant XRCC2 allele are more sensitive to the cytotoxic effects of thymidine when compared to parental cells. The effect of increasing the level of wild type XRCC2 protein in SKUT-1 cells was therefore investigated next. Increased levels of wild-type XRCC2 protein were obtained through stably transfecting the pEBS7-XRCC2 construct (kindly provided by Prof. John Thacker) containing wild type XRCC2 cDNA into the MMR –deficient cell line. Whole cell extracts obtained from the transfectants, were analysed by Western blotting in order to confirm increased XRCC2 protein expression. Two colonies that were isolated following transfection of this construct, both showed increased levels of the wild type XRCC2 protein (Figure 4.10a). Furthermore it was observed that exposure of such strains to thymidine restored resistance to this agent in the MMR –deficient, SKUT-1, cell line (Figure 4.10b). Thus increased level of wild-type XRCC2 protein restores resistance to thymidine.

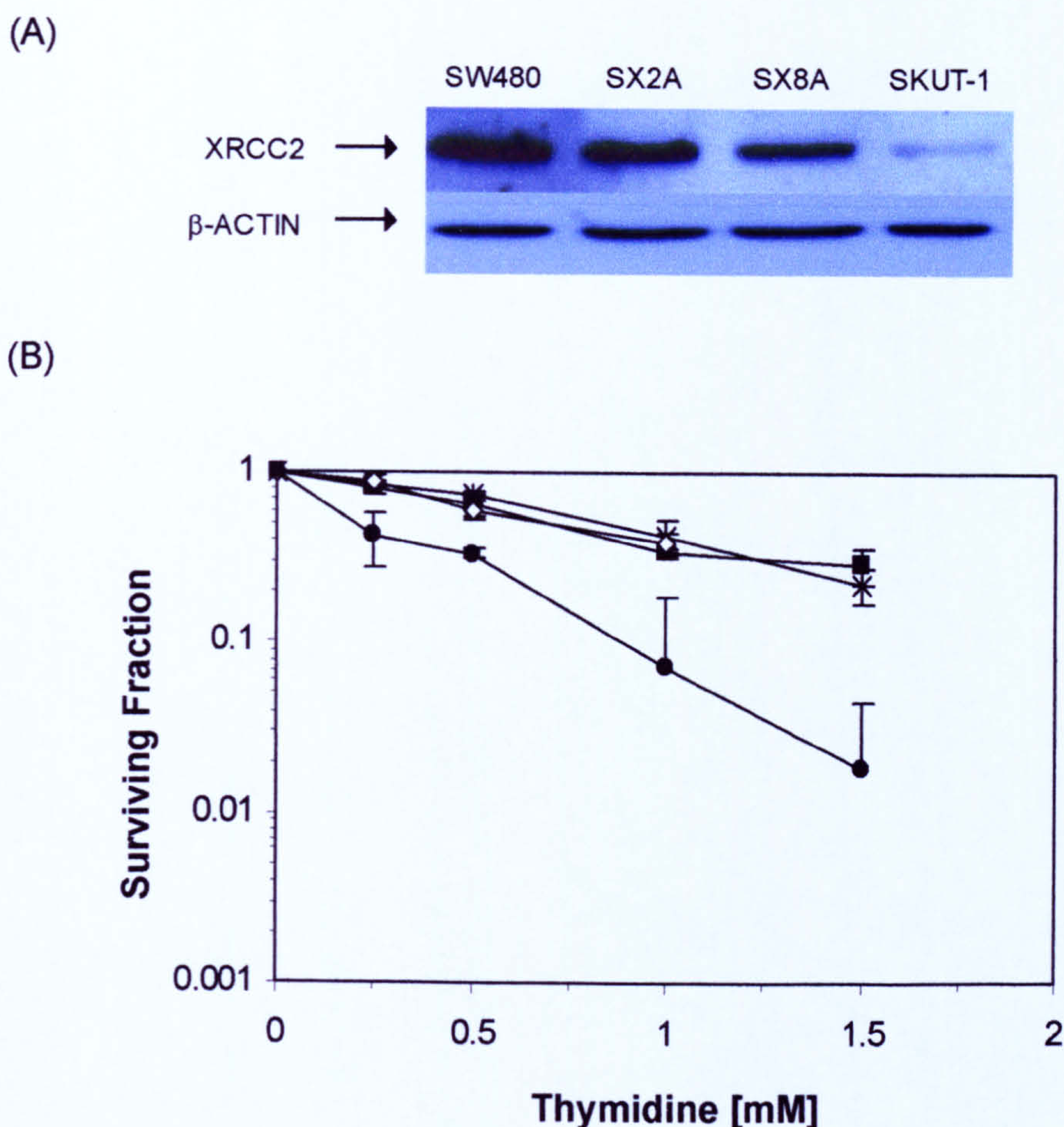

Figure 4.10 Restoration of wild type XRCC2 protein levels in SKUT-1 cells results in thymidine resistance. (A) Western blot analysis of whole cell extracts obtained from SKUT-1 cells transfected with wild type XRCC2 cDNA (arrow). Two transfectants (SX2A and SX8A) both expressed increased levels of wild type XRCC2 protein. The XRCC2 protein levels in cell extracts obtained from SW480 and SKUT-1 cells are included for reference. (B) Restoration of wild type XRCC2 protein levels in SKUT-1 cells resulted in thymidine resistance in two transfectants (SX2A, * and SX8A, ◊). The sensitivity to thymidine of SKUT-1 cells (●) and SW480 cells (■) are included for reference. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

4.2.6.3 342delT expression in SW480 cells confers MMC sensitivity.

The HRR deficient *irs1* (XRCC2-deficient) and *irs1SF* (XRCC3-deficient) cell lines have been well documented to be acutely sensitive to the DNA cross linking agent MMC, but only mildly sensitive to irradiation damage (Jones *et al.,* 1987; Fuller and Painter, 1988; Caldecott and Jeggo, 1991 and Tebbs *et al.,* 1995). The effects of both of these agents on cells expressing the mutant XRCC2 allele were therefore investigated.

The viability of plated cells was measured in the presence of increasing doses of MMC after 10-14 days. At 10% survival, two strains expressing the mutant XRCC2 allele, exhibited sensitivity to MMC when compared to parental SW480 cells (Figure 4.11a). The MMR –deficient, SKUT-1, cell line remained significantly more sensitive to the cytotoxic effects of MMC at all doses used when compared to either transfected cells or SW480, MMR –proficient cells. The empty pcDNA3.1 vector was also transfected into the MMR –proficient, SW480, cell line in order to observe whether the sensitivity of both transfectants to MMC was influenced by vector expression or due to clonal variation. As shown in Figure 4.11b, no increase in sensitivity was observed when cells expressing the empty vector were treated with increasing doses of MMC. These results suggest that a functional XRCC2 protein (and therefore HRR pathway) is involved in the repair DNA damage induced by MMC.

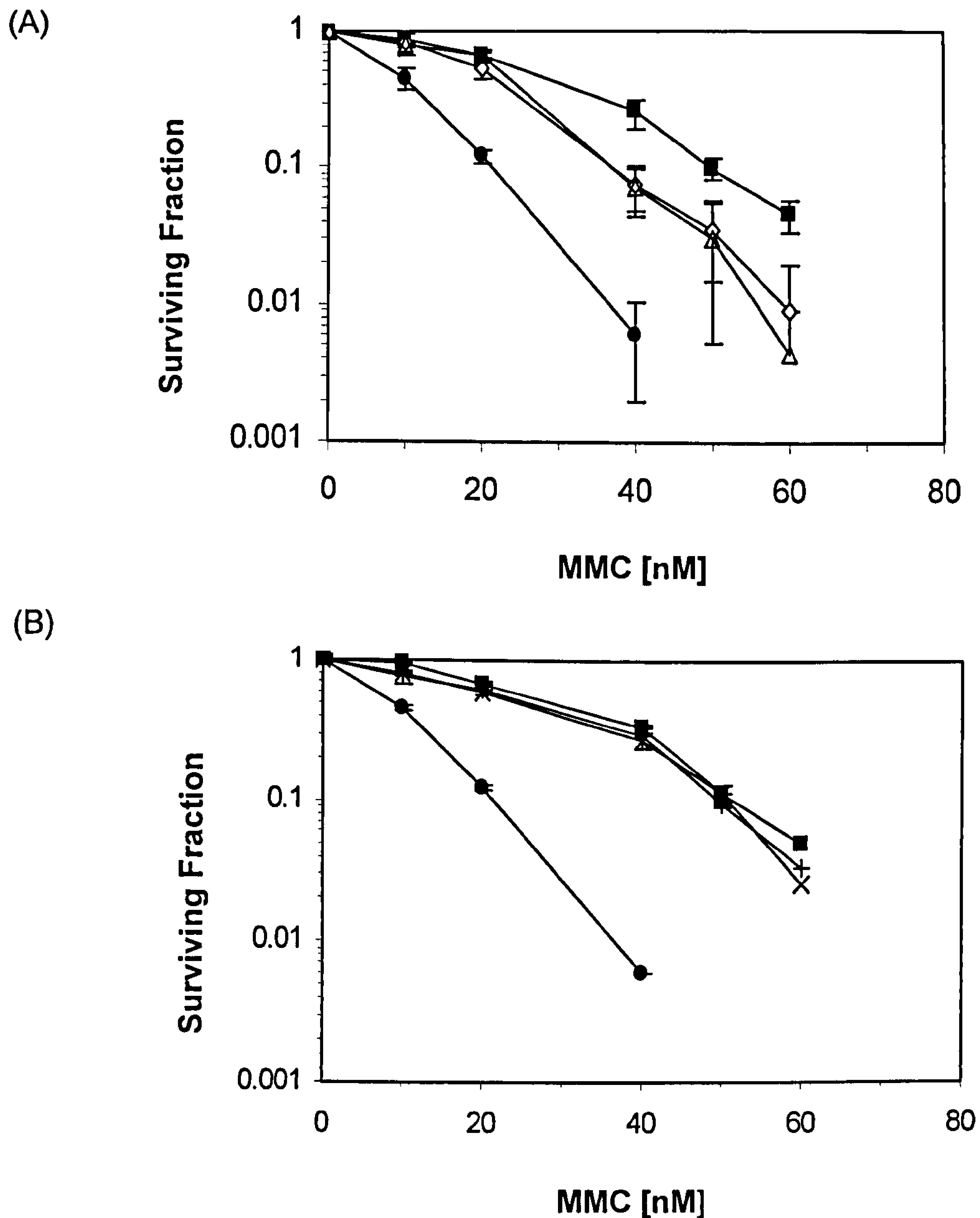

Figure 4.11 Expression of 342delT in SW480, MMR -proficient cells confers sensitivity to MMC. (A) Transfectants of SW480 expressing the mutant XRCC2 allele (SW1A, ◊ and SW6A, Δ) are sensitive to MMC when compared to parental SW480 cells (■). Sensitivity of SKUT-1 cells to MMC is included for reference (●). (B) Control experiments using empty-vector expressing transfectants (SWPC.2, † and SWPC.5, X) showed no significant difference to MMC sensitivity when compared to SW480 cells (■). Sensitivity of SKUT-1 cells to MMC is included for reference (●). The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

4.2.6.4 342delT expression in SW480 cells does not confer sensitivity to irradiation.

The sensitivity of the strains expressing the mutant XRCC2 allele to irradiation was also investigated as this agent has previously been observed to have a weak toxic effect on HRR deficient cells (Jones *et al.*, 1987; Fuller and Painter, 1988; Caldecott and Jeggo, 1991 and Tebbs *et al.*, 1995). SW480, SKUT-1 and two strains expressing the mutant XRCC2 allele were exposed to increasing doses of irradiation and allowed to form colonies for 10-14 days. As shown in Figure 4.12a, SKUT-1 cells were 4-fold more sensitive to irradiation (at 10% survival) when compared to the MMR –proficient, SW480, cell line. The two strains expressing the mutant XRCC2 allele were not, however, significantly more sensitive to irradiation when compared to parental SW480 cells. Furthermore, SW480 cells transfected with an empty vector control exhibited similar resistance to irradiation as un-transfected cells (Figure 4.12b).

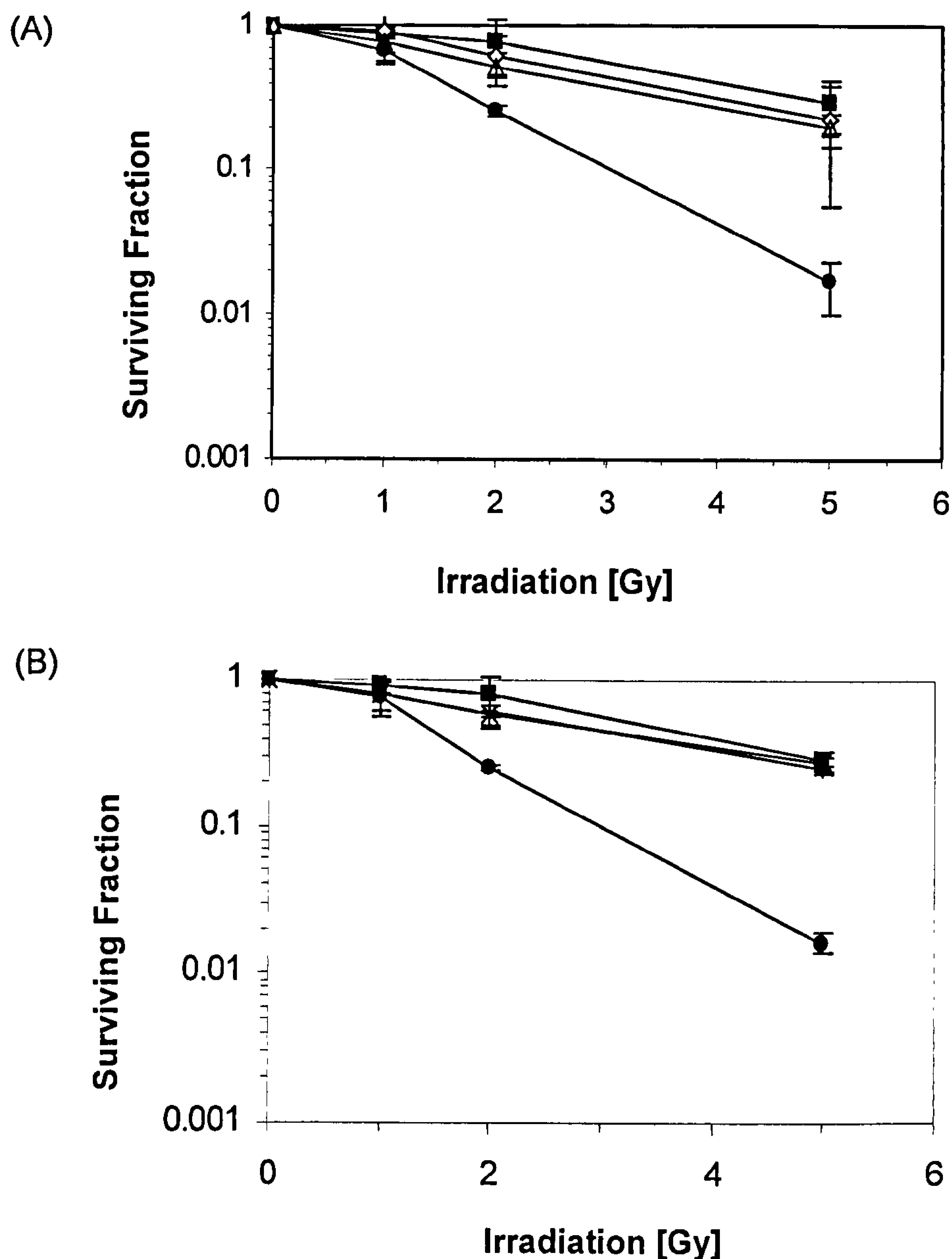

Figure 4.12 Expression of 342delT in SW480, MMR -proficient cells does not confer sensitivity to Irradiation. (A) Transfectants of SW480 expressing the mutant XRCC2 allele (SW1A, ◊ and SW6A, Δ) are not sensitive to irradiation when compared to parental SW480 cells (■). Sensitivity of SKUT-1 cells to irradiation is included for reference (●). (B) Control experiments using empty-vector expressing transfectants (SWPC.2, † and SWPC.5, X) showed no significant difference to irradiation sensitivity when compared to SW480 cells (■). Sensitivity of SKUT-1 cells to irradiation is included for reference (●). The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

4.3 DISCUSSION

The aim of the present study was to determine whether the apparent HRR defects observed in MMR –deficient cells was due to mutations in genes known to be involved in the pathway. Screening of eight MMR –proficient and –deficient tumour cell lines revealed a frameshift mutation within XRCC2 in the SKUT-1, MMR –deficient tumour cell line. Although the mutation identified was heterozygous, transfection of the mutant allele into a MMR –proficient tumour cell line conferred thymidine sensitivity. Furthermore, increasing the level of the wild-type protein in SKUT-1 cells, restored resistance to thymidine. Therefore thymidine sensitivity observed in the SKUT-1, MMR –deficient, cell line can be attributed, at least in part, to the presence of the mutant allele. Transfectants expressing the mutant XRCC2 allele were, however, found to be more resistant to MMC and to irradiation when compared to SKUT-1 cells. This can possibly be attributed to other unknown alterations within the SKUT-1 cell population which can further contribute to the sensitivity.

Genes that contain small repeated sequences within the coding sequence are particularly susceptible to mutations in cells that express a microsatellite mutator phenotype (reviewed by Kolodner, 1996 and Modrich and Lahue, 1996). Furthermore, in a MMR –deficient background, frameshift mutations have been shown to predominantly occur. This is probably due to an inability to repair polymerase slippage errors (reviewed by Kunkel, 1992 and Sia *et al.,* 1997). Such mutations subsequently contribute to the MSI phenotype observed in HNPCC derived tumours. For example in MMR -deficient tumour cells, the transforming growth receptor factor β receptor II (TGF- βRII) is frequently inactivated due to targeted frameshift mutations in the A_{10} repeat (Markowitz *et al.,* 1995 and Parsons *et al.,* 1995). In addition, Rampino and co-workers (1997) showed that approximately 50% of MSI+ colon adenocarcinomas were found to have frameshift mutations in

the G_8 tract within the coding sequence of the apoptosis promoting BAX gene. Therefore the XRCC2 frameshift mutation found in the T_8 tract of SKUT-1 cells may be coupled with selection, as other mononucleotide runs in XRCC2 are not mutated. Furthermore, assuming that the loss of MMR occurs as an early event, the XRCC2 frameshift mutation further supports the argument that loss of HRR in MMR –deficient cells occurs as a downstream event with respect to tumour progression.

Although the XRCC2 mutation was found in only one of the MSI+ tumour cell lines, such a mutation does not appear to be common in MSI+ or microsatellite stable colon cancers. Other studies conducted in our lab on a collection of 16 MSI+ and 92 microsatellite stable colon cancers did not detect the mutant allele (J. Scorah and M. Meuth). However, the same study revealed that a further intronic frameshift mutation in the MRE11 gene occurred in a high proportion (85%) of these MSI+ colon cancers. As the MRE11 protein seems to be implicated in HRR, the tumour data implies that a defective HRR pathway may further contribute to tumour development in MSI^+ colon cancers.

As the SKUT-1 cell line was originally derived from a uterine sarcoma (Risinger *et al.*, 1995) the possible significance of XRCC2 frameshift mutations in gynaecological tumours was also investigated. In this particular study 4 endometrial stromal sarcomas and 8 leiomyosarcomas of the uterus were examined. Sequencing analysis revealed that one of the 8 leiomyosarcomas of the uterus also carried the same XRCC2 frameshift mutation as that found in the SKUT-1 cell line. Interestingly, another study detected a translocation breakpoint which affects the last exon of a uterine specific isoform in RAD51B in the more common uterine leiomyomas (Schoenmakers *et al.*, 1999). Taken together these results imply that there is a selective pressure of gynaecological tumours to acquire mutations in

genes encoding the RAD51 paralogs with respect to enhancing tumour progression.

The advantage gained by acquiring such mutations in these tumours, with respect to tumour development, can be explained in context to the reported role of HRR in delaying of DNA replication fork progression following DNA damage (Henry-Mowatt *et al.*, 2003). Cells losing part of the HRR pathway, for example through mutations in XRCC2 (and possibly RAD51B), may be unable to regulate DNA synthesis on damaged templates. This would subsequently result in higher levels of genetic instability and tumourigenesis. In support of this, Griffin and co-workers (2000) recently showed an increase in chromosome missegregation associated with fragmented centrosomes in XRCC2 deficient cells which, the authors suggested, could subsequently lead to genetic instability and cancer.

The difference in acquired mutations observed between the MSI+ colon cancers and the gynaecological samples could be due to a number of reasons. For example, the two types of tumours might undertake different pathogenic routes during progression with selective advantages being gained either through the loss of MRE11 with respect to colon cancers (and possibly other checkpoint related genes) or the loss of the RAD51 paralogs with respect to uterine leiomyosarcoma. Alternatively, the different rates of exposure to, and types of carcinogens in the colon, as opposed to that exposed in the uterus, may account for such differences that occur.

CHAPTER FIVE:

EFFECTS OF THE MUTANT XRCC2 ALLELE ON DSB AND THYMIDINE –INDUCED RECOMBINATION

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5.3 DISCUSSION **180**

5.1 INTRODUCTION

The observation that MMR –deficient tumour cells are sensitive to thymidine, in addition to the results obtained in Chapter 3, which demonstrated HRR defects in such cells, is consistent with the hypothesis that HRR is involved in repairing lesions that are induced by thymidine. Furthermore, cDNA sequence analysis in Chapter 4 revealed a frameshift mutation within the RAD51 paralog gene, XRCC2, in MMR –deficient SKUT-1 cells. The resulting peptide encoded a 132 amino acid protein that retained the ATPase Walker Box A but lost the highly conserved Walker Box B. Expression of this mutant allele into a MMR –proficient, SW480 tumour cell line conferred sensitivity to both thymidine and, to a lesser extent, MMC. Given these results, the effects of the mutant XRCC2 allele on both DSB and thymidine-induced recombination was examined in the present chapter.

The HRR pathway is required for the accurate repair of DNA breaks induced by agents such as MMC, as well as being involved in the rescue of stalled/collapsed replication forks. This repair process frequently utilizes interactions between the sister chromatids, which act as templates for DNA synthesis (van Gent *et al.,* 2001). Such observations suggest that this pathway is particularly effective in the S-phase of the cell cycle. In eukaryotic cells, it has been noted that the RAD51 protein (a functional homologue of the *E-coli* RecA recombinase) plays an influential role in homologous recombination mediated repair. This protein forms nucleoprotein filaments on ssDNA fragments, thus promoting homologous pairing and DNA strand exchange (Baumann *et al.,* 1996; Gupta *et al.,* 1997 and Sung *et al.,* 1994). In human cells, efficient HRR requires other related genes, in addition to RAD51, that belong to the RAD52 epistasis group.

These genes include RAD52, RAD54 and the RAD51 paralogs amongst others (reviewed in Thompson and Schild, 2001).

Currently, five mammalian RAD51 paralogs (namely XRCC2, XRCC3, RAD51B, RAD51C and RAD51D) have been isolated and shown to share between 20-30% sequence homology to the RAD51 protein (Thacker, J., 1999a and 1999b). These RAD51 paralogs were initially identified either due to their ability to functionally complement X-ray sensitive hamster cells (in the case of XRCC2 and XRCC3), or through data base searching for sequence similarities with XRCC2 and XRCC3 (in the case of RAD51B, RAD51C and RAD51D) (Albala *et al.,* 1997; Cartwright *et al.,* 1998; Dosanjh *et al.,* 1998 and Pittman *et al.,* 1998). The RAD51D protein is most closely related, in terms of structure and homology, to the yeast RAD57 protein; whereas the XRCC2 protein shares highest homology with the yeast RAD55 protein (Thacker J., 1999a and 1999b).

It is proposed that the RAD51 paralogs may act as accessory factors in assisting with the 'loading' of RAD51 onto DNA, similar to the RAD55 and RAD57 gene products in *Saccharomyces cerevisiae* (Sung, P. 1997; Takata *et al.,* 2001; Braybrooke *et al.,* 2000; O'Regan *et al.,* 2001 and Masson *et al.,* 2001). Further evidence for the 'pre-synaptic' role of these paralogs, comes from the observation that the XRCC3 protein interacts with RAD51 and that its presence is required for the assembly of RAD51 complexes *in vivo* (Bishop *et al.,* 1998). Although the five paralogs share less than 30% sequence homology with each other (Thacker, J. 1999), knockouts of any paralogs exhibit extremely similar phenotypes. Studies using chicken DT40 cells, in which each paralog was individually knocked out, showed that such strains all displayed reduced growth rates, exhibited chromosomal instability and frequently accumulated spontaneous breaks (Takata *et al.,* 2001). Cells defective in any of the paralogs also displayed marked sensitivity to DNA damaging agents including MMC and IR (Tambini *et al.,* 1997;

Cartwight *et al.,* 1998; Liu *et al.,* 1998 and Takata *et al.,* 2001). Furthermore, XRCC2, XRCC3, RAD51C and RAD51B deficiency has been shown to directly result in impaired HRR (Johnson *et al.,* 1999; Pierce *et al.,* 1999; French *et al.,* 2002 and Takata *et al.,* 2000).

Recent studies have reported that the five paralogs co-immunoprecipitate as two distinct complexes as well as individual heterodimers (Masson *et al.,* 2001; Kurumizaka *et al.,* 2002; Wiese *et al.,* 2002 and Liu N., *et al.,* 2002). One such complex comprises of RAD51B, RAD51C, RAD51D, and XRCC2 (the BCDX2 complex), whereas the second complex consists of the RAD51C and XRCC3 proteins. The BCDX2 complex was shown to specifically bind to ssDNA as well as to single-stranded regions or nicks in duplexed DNA (Masson *et al.,* 2001). The RAD51C/XRCC3 complex, however, was shown to bind ssDNA and thus promote DNA-DNA interactions and annealing (Kurumizaka *et al.,* 2002 and Masson *et al.,* 2001).

Given the proposed 'pre-synaptic' involvement of XRCC2 in both DSB-induced HRR and the requirement of HRR in maintaining the integrity of collapsed/stalled replication forks, the effects of expressing the mutant XRCC2 allele on both DSB and thymidine-induced recombination are investigated in the present chapter.

5.2 RESULTS

5.2.1 Expression of 342delT in SW480/SN.3 and MRC5VA/SN.13 strains

Having determined the phenotypic effects of the mutant XRCC2 allele on both thymidine and MMC sensitivity in the SW480 cell line, the effects of 342delT expression on DSB-induced HRR were investigated. To achieve this, the mutant XRCC2 allele was sub-cloned into the pIRESpuro3 expression vector (see Appendix 4, Page 240) in order to allow for its introduction into the MMR –proficient, SW480/SN.3 and MRC5VA/SN.13 strains containing the ScNeo recombination reporter substrate. Details of all cell lines and transfectants used in the present chapter are described in Table 5.1. Expression of the mutant construct was confirmed through sequencing of amplified XRCC2 cDNA.

Cell line	Parent	Description
SW480/SN.3	SW480	MMR+ cell line carrying ScNeo recombination reporter
SXIR.1-4	SW480/SN.3	Derivatives of above expressing XRCC2 mutant allele
MRC5VA/SN.13	MRC5VA	MMR^+ fibroblast carrying ScNeo recombination reporter
MX.1 AND .2	MR5VA/SN.13	Derivatives of above expressing XRCC2 mutant allele
SKUT-1		MMR –deficient uterine sarcoma cell line
SKUT-1/SN.3	SKUT-1	Derivative of above carrying ScNeo

Table 5.1 Table showing parental lines and transfectants used in the present chapter.

As shown in the sequencing images presented in Figure 5.1a, SW480/SN.3 transfectants appeared to display both wild type and mutant alleles of XRCC2 in approximately equal quantities. MRC5VA/SN.13 transfectants, however, appeared to display predominantly the mutant allele (Figure 5.1b). A similar effect was observed in the MMR –deficient, SKUT-1/SN.3 strain, which also appeared to predominantly display the mutant XRCC2 allele (Figure 5.1b).

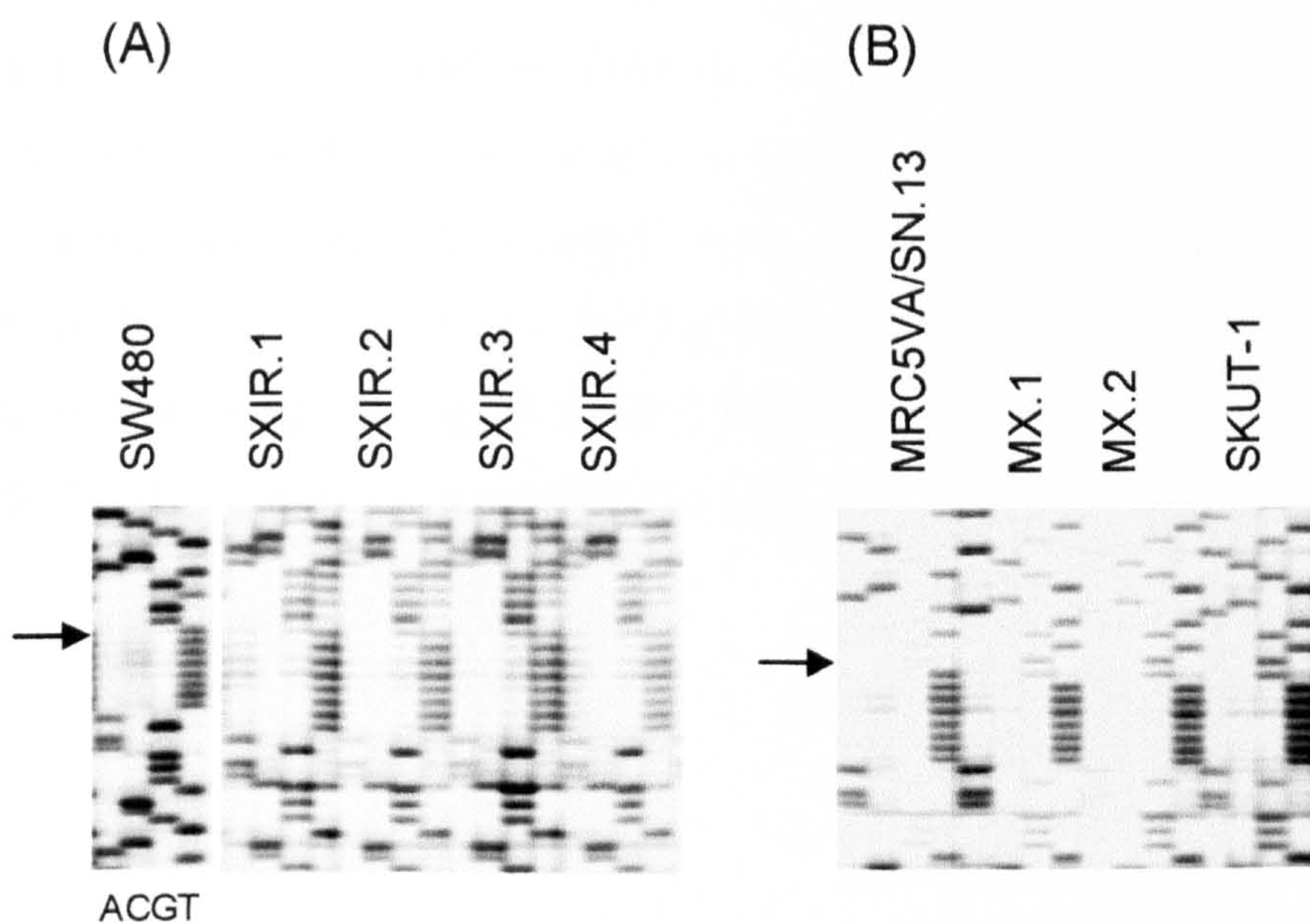

Figure 5.1 Sequencing gel images showing expression of 342delT. (A) Expression of the mutant XRCC2 allele (indicated by arrow) in the SW480/SN.3 strain transfected with mutant XRCC2 cDNA. The sequence pattern for four such transfectants (SXIR.1, SXIR.2, SXIR.3 and SXIR.4) is compared with un-transfected SW480/SN.3 cells (containing only wild type XRCC2). (B) Expression of the mutant XRCC2 allele (indicated by arrow) in the MRC5VA/SN.13 strain transfected with the mutant XRCC2 cDNA sub cloned into pIRES-puor3 expression vector. The sequence patterns in two MRC5VA transfectants (MX.1 and MX.2) is compared with parental MRC5VA cells (containing only wild type XRCC2) and SKUT-1 (which carries both the frame shift mutation of XRCC2 as well as wild type).

Western blot analysis of whole cell extracts, obtained from transfected strains, revealed a peptide having approximately the same molecular weight as that predicted to be encoded for by 342delT in all transfectants (Figure 5.2). This 17.2kDa peptide was not observed in any other MMR –proficient or –deficient cell lines examined, other than in SKUT-1 cells and transfectants, indicating that it had been translated from the mutant XRCC2 allele. Consistent with results from the sequencing gels, the ratio of the levels of peptide and full length XRCC2 protein varied between the two strains transfected. The SW480/SN.3 transfectants retained equal levels of wild type XRCC2 protein to that found in parental cells, and showed clear expression of the peptide (Figure 5.2). MRC5VA/SN.13 transfectants, however, showed reduced levels of wild type XRCC2 protein but high levels of mutant peptide. The effect seen in MRC5VA/SN.13 transfectants is similar to that noted in the SKUT-1/SN.3 strain. Thus, all strains express 342delT although the level of wild type XRCC2 protein in the MRC5VA and SKUT-1/SN.3 strains is greatly reduced.

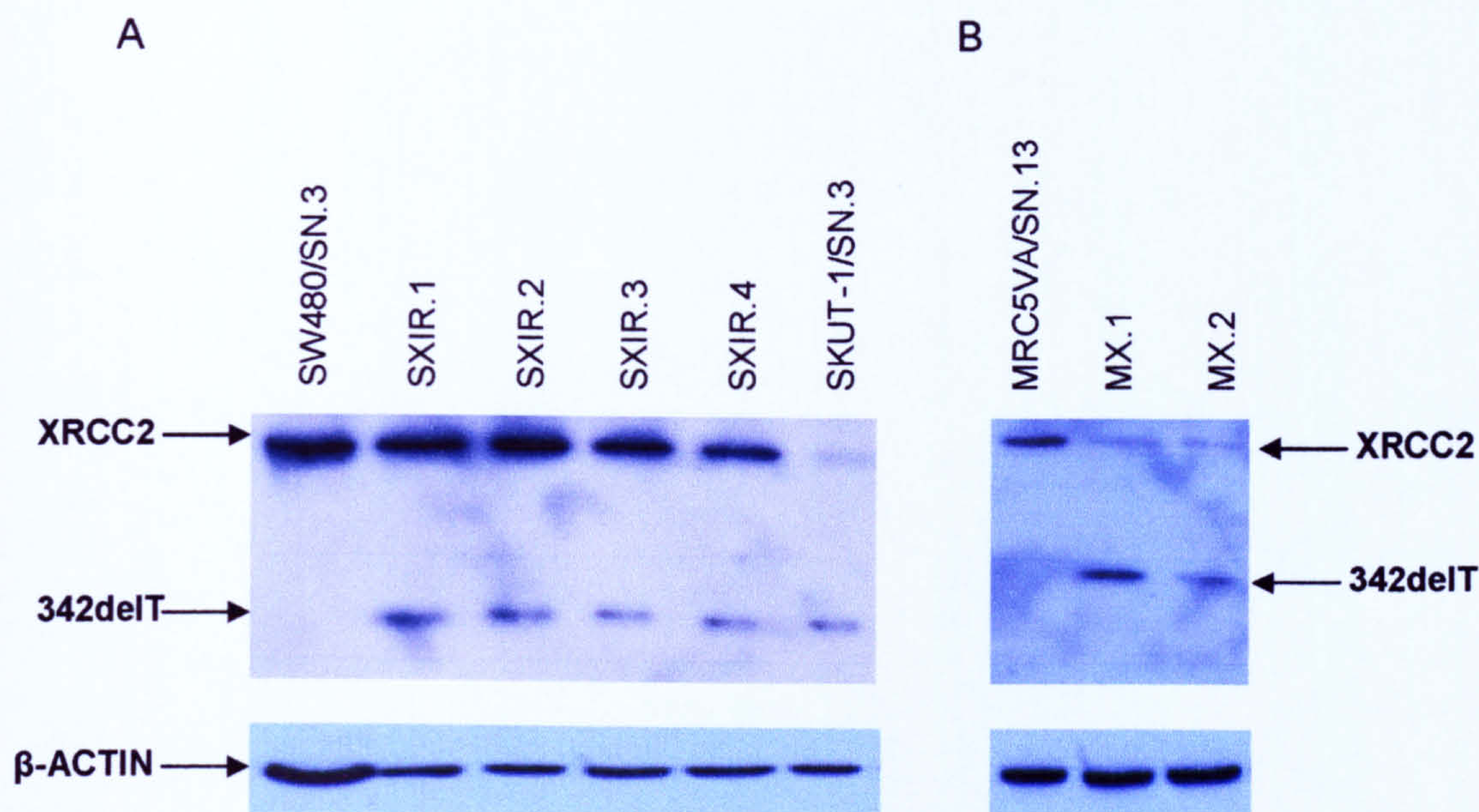

Figure 5.2 Detection of 342delT protein expression in transfected cell lines by Western blotting. Both the wild type XRCC2 protein and the mutant peptide are indicated by arrows in both SW480/SN.3 (SXIR.1, SXIR.2, SXIR.3 and SXIR.4) and MRC5VA/SN.13 (MX.1 and MX.2) transfectants. SW480/SN.3 transfectants appear to express equal levels of both wild-type and mutant XRCC2 protein. MRC5VA/SN.13 transfectants, like the SKUT-1/SN.3 strain, appear to display increased levels of mutant XRCC2 protein when compared to wild type. β-actin was used for loading control.

5.2.2 ScNeo containing strains expressing 342delT become sensitive to thymidine and MMC.

Experiments conducted in Chapter 4 showed that expression of 342delT could confer thymidine sensitivity when expressed in SW480 cells. Therefore to investigate whether expression of the pIRESpuro3-342delT construct altered the phenotype of SW480/SN.3 and MRC5VA/SN.13 cells, with respect to thymidine, the sensitivity of transfectants to this agent was examined (Figure 5.3). Exponentially growing cells were continuously treated with increasing doses of thymidine and cell survival was determined after 10-14 days. Consistent with previous data, strains expressing 342delT

became more sensitive to the cytotoxic effects of thymidine when compared to the respective parental cell line. At 10% survival, SW480/SN.3 transfectants were up to 2-fold more sensitive to the cytotoxic effects of thymidine than parental cells (Figure 5.3a). The MRC5VA/SN.13 transfectants (which predominantly expressed 342delT) were observed to exhibit a greater degree of sensitivity than SW480/SN.3 transfectants. At 10% survival such strains were between 3.8 to 4.8-fold more sensitive to thymidine than parental MRC5VA/SN.13 cells (Figure 5.3b). The MMR –deficient, SKUT-1/SN.3 strain remained significantly more sensitive (2-fold) when compared to both SW480/SN.3 and MRC5VA/SN.13 transfectants at all doses tested. These results therefore suggested that 342delT conferred thymidine sensitivity when expressed in MMR –proficient cells.

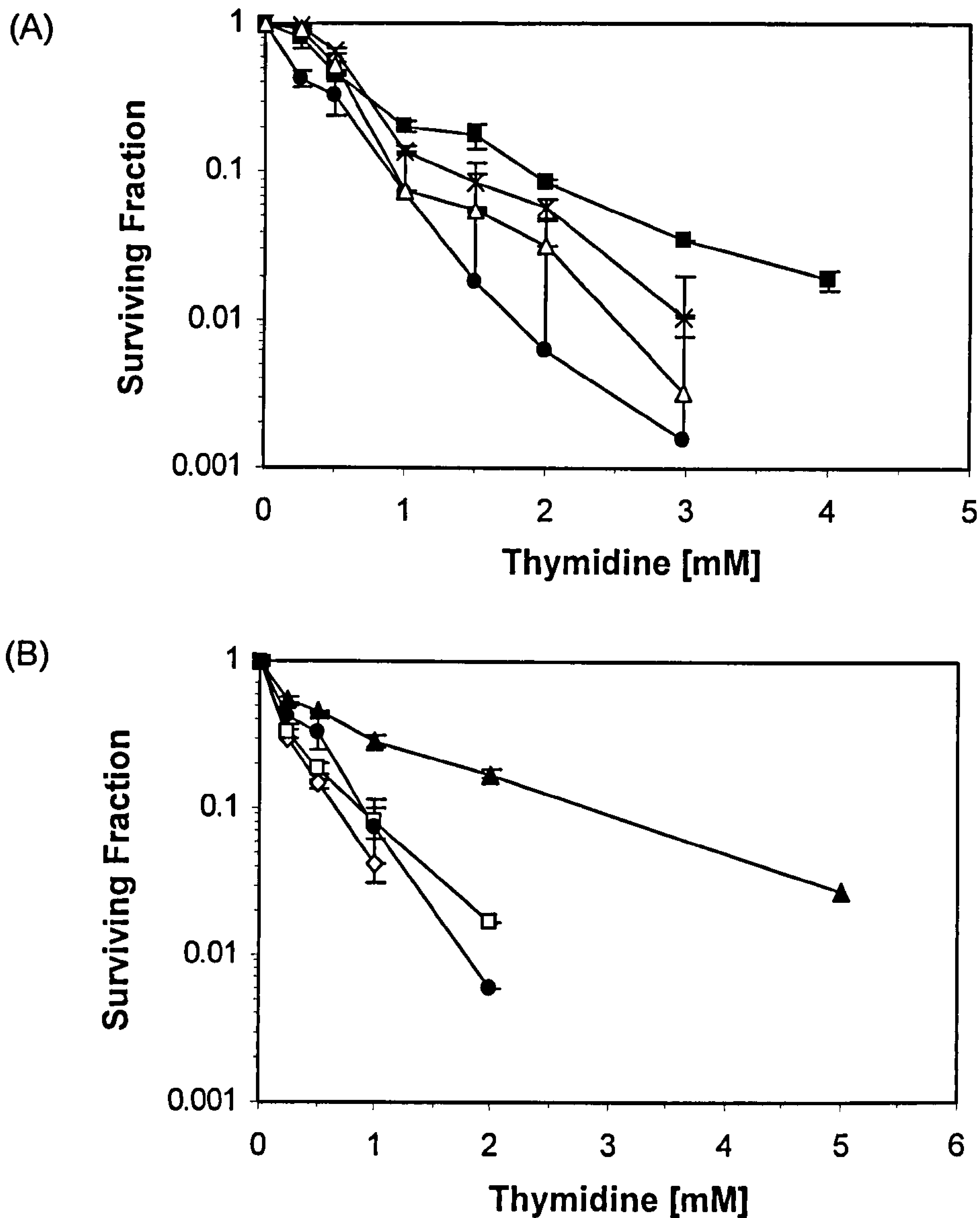

Figure 5.3 Expression of 342delT confers thymidine sensitivity. (A) Transfectants of SW480/SN.3 expressing the mutant XRCC2 allele (SXIR.3, ◊ and SXIR.4, X) are sensitive to thymidine when compared to untransfected SW480/SN.3 cells (■). The sensitivity of the SKUT-1/SN.3 strain (●) to thymidine is included for reference. (B) Transfectants of MRC5VA/SN.13 expressing the mutant XRCC2 allele (MX.1, ◊ and MX.2, □) are sensitive to thymidine when compared to untransfected MRC5VA/SN.13 cells (▲). The sensitivity of the SKUT-1/SN.3 strain (●) to thymidine is included for reference. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

Findings from previous published studies suggest that the proteins involved in HRR proteins are involved in the repair of DNA damage induced by MMC (Jones *et al.,* 1987; Fuller and Painter, 1988; Caldecott and Jeggo, 1991 and Tebbs *et al.,* 1995). The sensitivity to this agent was therefore investigated using exponentially growing cells transfected with the pIRESpuro3-342delT construct (Figure 5.4). Results showed that the SKUT-1/SN.3 cells, SW480/SN.3 and MRC5VA/SN.13 transfectants expressing the mutant XRCC2 allele, all displayed increased sensitivity to MMC relative to SW480/SN.3 and MRC5VA/SN.13 parental strains. At 10% survival, all SW480/SN.3 (Figure 5.4a) and MRC5VA/SN.13 (Figure 5.4b) transfectants were found to be up to 2-fold more sensitive to MMC when compared to parental SW480/SN.3 and MRC5VA/SN.13 cells. At 10% survival, the SKUT-1/SN.3 strain was also 2-fold more sensitive to MMC when compared with either SW480/SN.3 or MRC5VA/SN.13 cells. Therefore, consistent with results presented in the previous chapter, expression of 342delT conferred sensitivity to MMC when expressed in MMR –proficient cell lines.

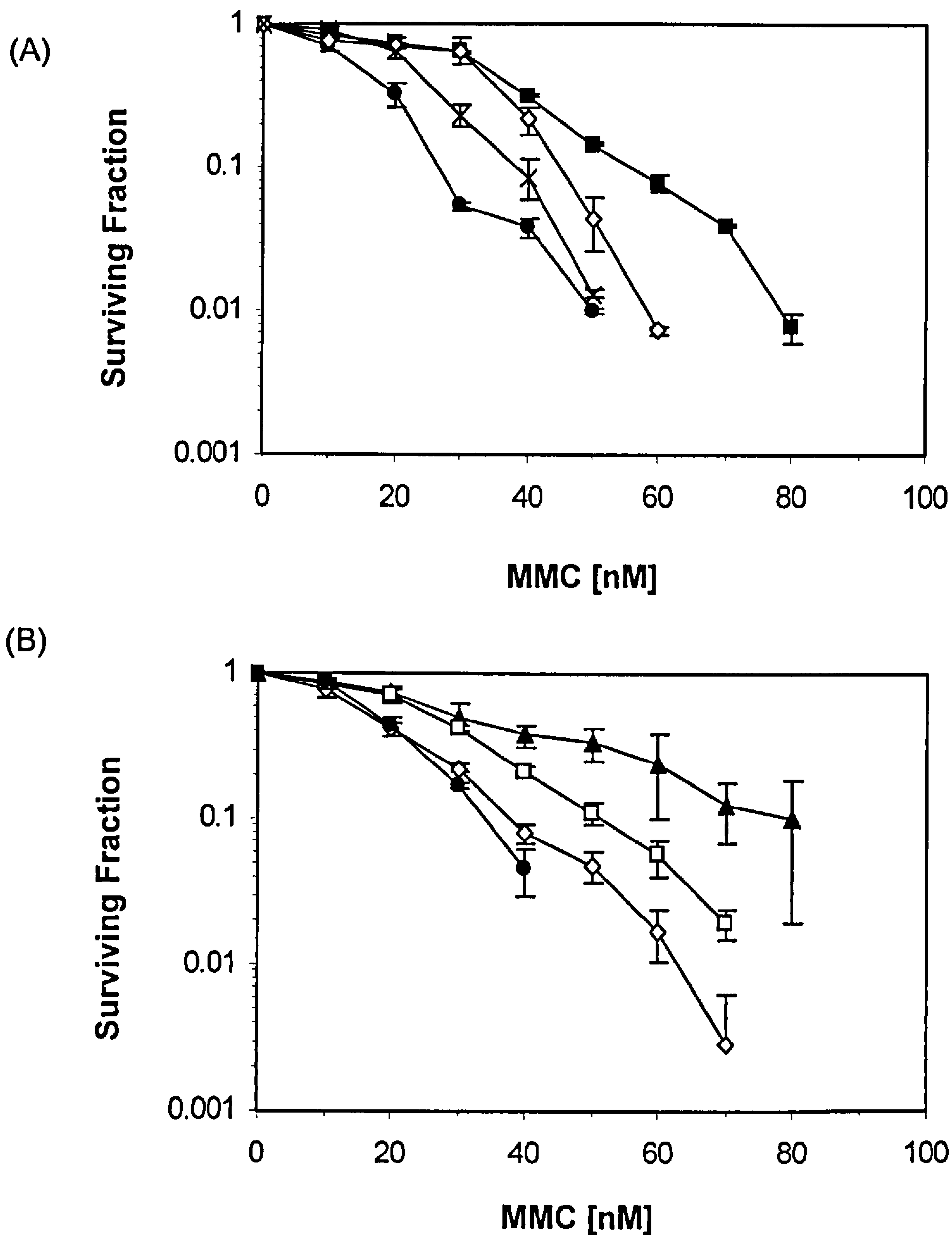

Figure 5.4 Expression of 342delT confers sensitivity to MMC. (A) Transfectants of SW480/SN.3 expressing the mutant XRCC2 allele (SXIR.3, ◊ and SXIR.4, X) are sensitive to MMC when compared to untransfected SW480/SN.3 cells (■). The sensitivity of the SKUT-1/SN.3 strain (●) to MMC is included for reference (B) Transfectants of MRC5VA/SN.13 expressing the mutant XRCC2 allele (MX.1, ◊ and MX.2, □) are sensitive to MMC when compared to parental MRC5VA/SN.13 cells (▲). The sensitivity of the SKUT-1/SN.3 strain (●) to MMC is included for reference. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

5.2.5 342delT exerts a cell line specific effect on I-SceI induced recombination.

In agreement with the involvement of XRCC2 in HRR, Johnson and co-workers (1999) showed that XRCC2 deficient hamster cells exhibit a marked decrease in the production of neo$^+$ recombinants in a homology based recombination assay. As described on Page 103, this assay measures homology based recombination events that occur between two defective neomycin phospho-transferase genes following the induction of a site specific DSB.

In order to determine the effect of the mutant XRCC2 allele on DSB-induced recombination, SW480/SN.3 and MRC5VA/SN.13 cells stably transfected with the ScNeo recombination substrate were used. As shown in Figure 5.5, induction of a DSB in a MMR –proficient, SW480/SN.3, strain resulted in an 850-fold increase in the frequency of neo$^+$ recombinants when compared to mock transfected cultures. Following transfection of the I-SceI endonuclease in SW480/SN.3 transfectants expressing 342delT, a 600-1500-fold increase in neo$^+$ recombinants was also observed when compared to mock transfected cultures. This frequency is similar to that observed in the SW480/SN.3 strain.

In contrast to this observation, induction of a DSB in MRC5VA/SN.13 transfectants (expressing predominantly 342delT) resulted in only a 1.7 to 2.4-fold increase in the frequency of neo$^+$ recombinants (Figure 5.5). This was a much weaker inductive effect when compared to the MRC5VA/SN.13 strain not expressing 342delT, which showed a 110-fold increase in the frequency of neo$^+$ recombinants. The results obtained in the MRC5VA/SN.13 transfectants are similar to that observed in the SKUT-1/SN.3 clone, which also showed no increase in the frequency of neo$^+$ recombinants following the induction of a site-specific DSB.

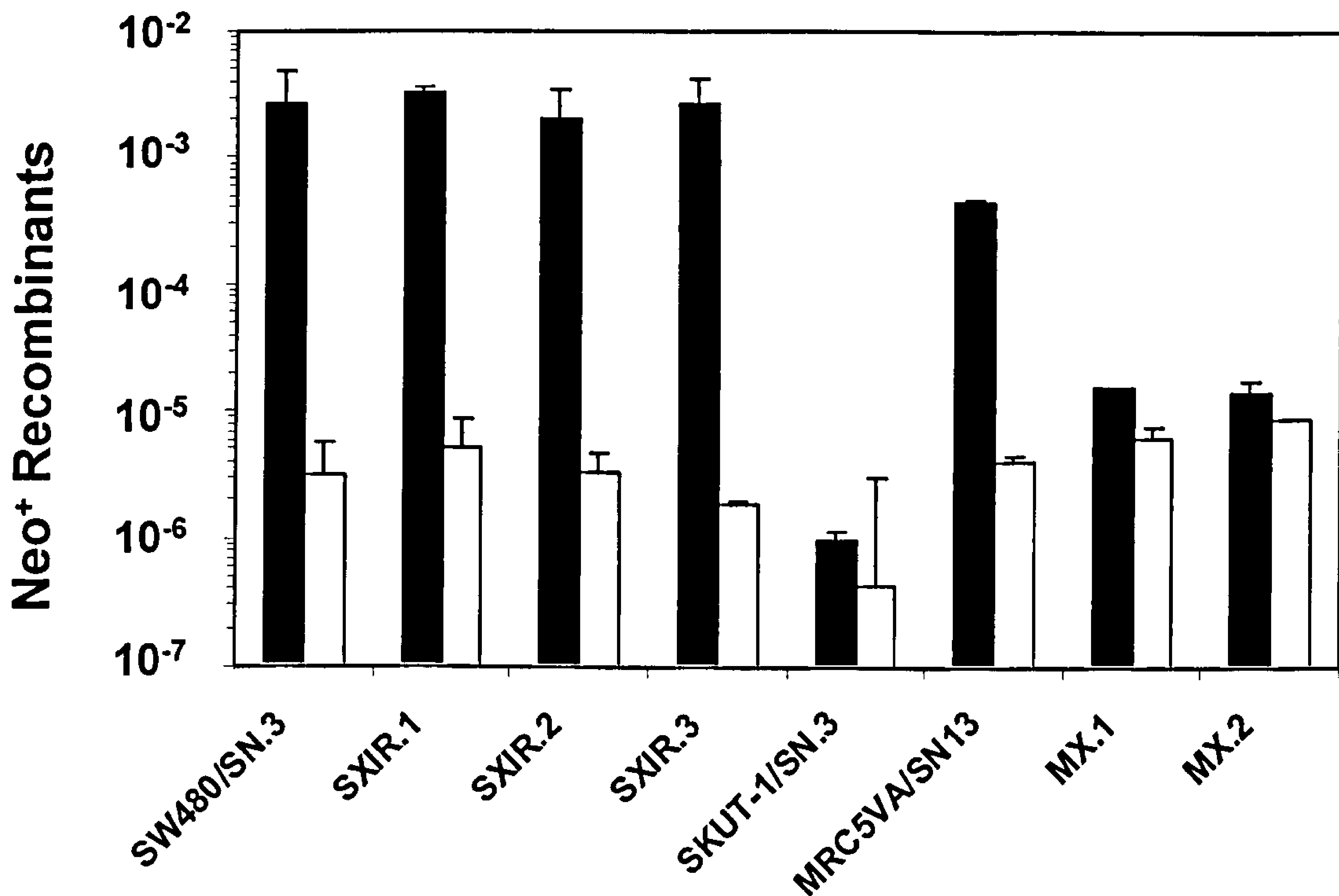

Figure 5.5 Cell –specific effects of 342delT on homology directed repair of a site-specific DSB. SW480/SN3 cells expressing 342delT (namely SXIR.1 and SXIR.2) show a robust induction of neo+ recombinants, following transient transfection of the I-SceI expression construct (solid bars), when compared to mock transfected cultures (empty bars). In contrast the induction of neo+ recombinants is suppressed to levels similar to those found in SKUT-1 cells in MRC5/SN13 transfectants expressing the mutant XRCC2 allele (namely MX.1 and MX.2). The results shown are an average of three independent experiments and standard deviation is indicated by error bars.

Taken together, these results suggest that SW480/SN.3 transfectants expressing 342delT *and* wild-type XRCC2 protein remain proficient in the repair of DSBs. The combination of depressed wild-type XRCC2 levels and the presence of the mutant XRCC2 allele in MRC5VA/SN.13 strains, however, causes a much weaker inductive effect in the frequency of neo^+ recombinants. Thus the effect of recombination events induced in the ScNeo recombination reporter by a site-specific DSB is dependent on the level of wild-type XRCC2 protein expression.

5.2.6 Both SW480/SN.3 and MRC5VA/SN.13 strains expressing 342delT are defective in thymidine induced recombination.

Studies reported by Lundin and co-workers (2002) suggested that exposure of cells to thymidine subsequently results in the production of lesions at replication forks that are specifically repaired by proteins involved in HRR. The effect on recombination events induced by thymidine treatment was therefore investigated in clones expressing the mutant XRCC2 allele (Figure 5.6). Replica cultures were treated for 24 hours with increasing doses of thymidine and the frequency of neo^+ recombinants determined. Such replica cultures were initially inoculated from 1000 cells in order to eliminate pre-existing neo+ cells and to obtain a more precise measurement of the inductive effects of thymidine. This approach is deemed to be more sensitive than the assay used to measure thymidine-induced recombination events in Chapter 3 where mass cultures were inoculated and exposed to thymidine. Treatment of the MMR proficient, SW480/SN.3 and MRC5VA/SN.13, parental strains resulted in a 6-fold increase in the frequency of neo^+ recombinants (Figure 5.6a and 5.6b, respectively). Furthermore, the effects observed were found to be dose-dependent with maximal levels of recombinants occurring at 10mM thymidine treatment.

SW480/SN.3 transfectants, expressing 342delT, were treated with 2mM or 10mM thymidine as they showed an intermediate sensitivity to thymidine. Using such doses, no increase in the frequency of neo^+ recombinants was observed. This is in marked contrast to the results obtained when using the

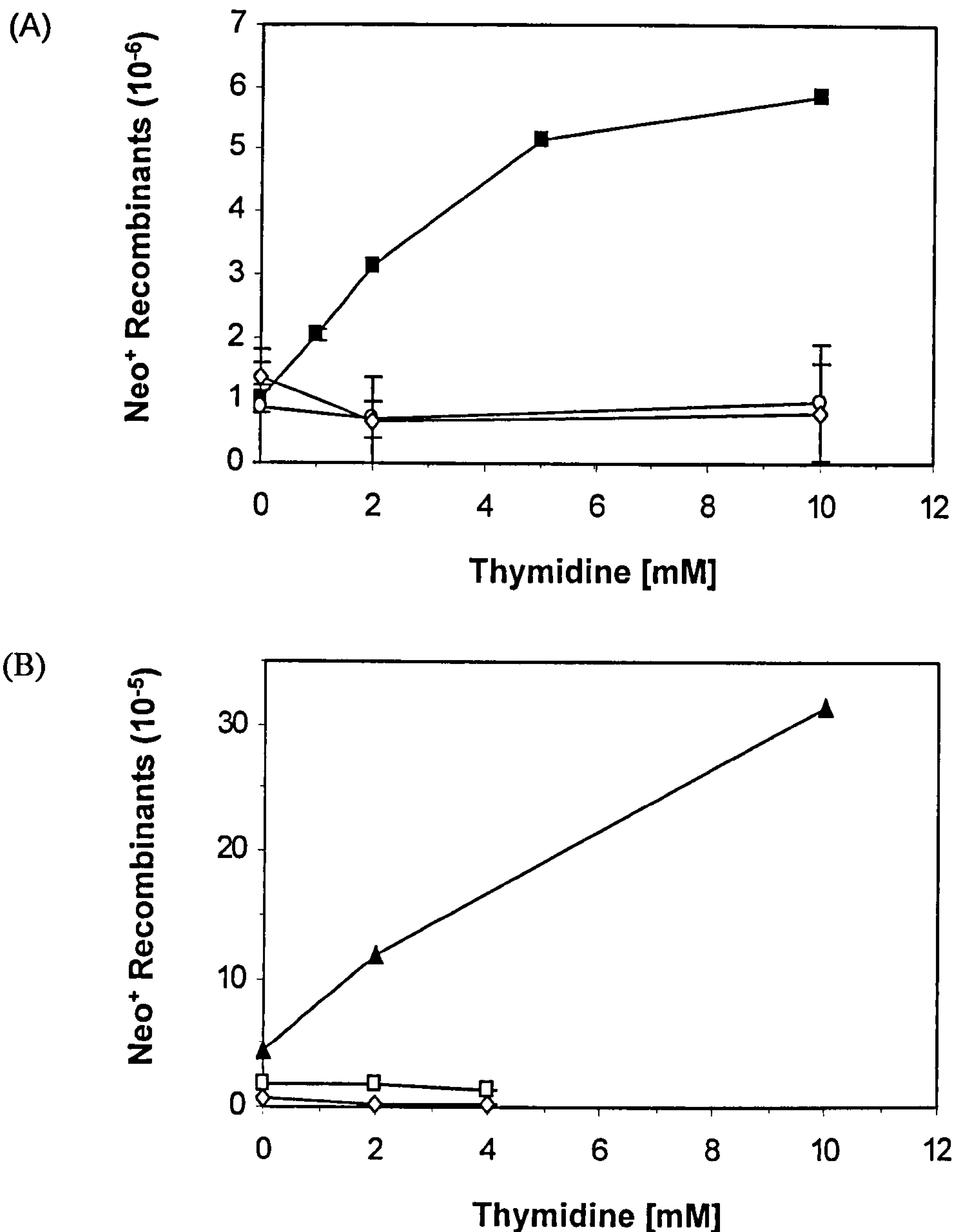

Figure 5.6 Cells expressing 342delT are defective in HRR induced by thymidine. (A) 24 hour thymidine treatment induced recombination events in SW480/SN.3 cells (■) but not in the transfectants (SXIR.1, ○ and SXIR.2, ◊). (B) 24 hour thymidine treatment also induces recombination events in MRC5VA/SN.13 cells (▲ -data supplied by Emma Bolderson) but not in cells expressing the mutant XRCC2 allele (MX.1, ◊ and MX.2, □). The frequencies of neo^+ recombinants induced by thymidine in SKUT-1/SN.3 cells were: 0mM = 1.01×10^{-7}; 2mM = $< 1.81 \times 10^{-7}$ and 4mM = $< 2.27 \times 10^{-7}$. Six independent replica cultures were treated for each experiment and the results are an average of six independent experiments. The standard deviation for each treatment is indicated by error bars

parental SW480/SN.3 strain. Additionally no increase in the frequency of neo^+ recombinants was observed in MRC5VA/SN.13 transfectants when treated with 2mM or 4mM thymidine. The parental strain, however, displayed a 3 to 4-fold increase in the frequency of neo^+ recombinants at the same doses. The SKUT-1/SN.3 strain was also investigated using this approach as it had previously been demonstrated that the DLD-1/SN.2 (MMR –deficient) strain was also defective in thymidine-induced HRR. Since SKUT-1/SN.3 cells are sensitive to this agent, these cells could not be treated with 5 or 10mM thymidine. However, even when 12 million cells were treated with 2mM thymidine, no neo+ recombinants were retrieved (frequency $<1.8 \times 10^{-7}$). Treating SW480/SN.3 cells with this same concentration of thymidine, however, resulted in a 3-fold increase in the frequency of neo+ recombinants (Figure 5.6a).

5.2.7 Thymidine fails to induce any detectable DSBs in treated cells.

Thymidine treatment is suggested to induce DNA lesions at stalled replication forks. To determine whether thymidine induced DSBs at such replication forks, all strains were treated with thymidine as well as camptothecin and irradiation (which are known to induce DSBs). Cells were treated with either 10mM thymidine or 100nM camptothecin for 24 hours, or exposed to 10Gy of irradiation. After the respective treatment, cells were harvested and analyzed for DSBs by pulse field gel electrophoresis.

Consistent with previous reports, no DSBs were detected in any strain when treated with 10mM thymidine (Figure 5.7). This was in contrast to that observed when cells were exposed to either 100nM of CPT or 10Gy of irradiation, which both induced large amounts of DSBs compared to untreated cells.

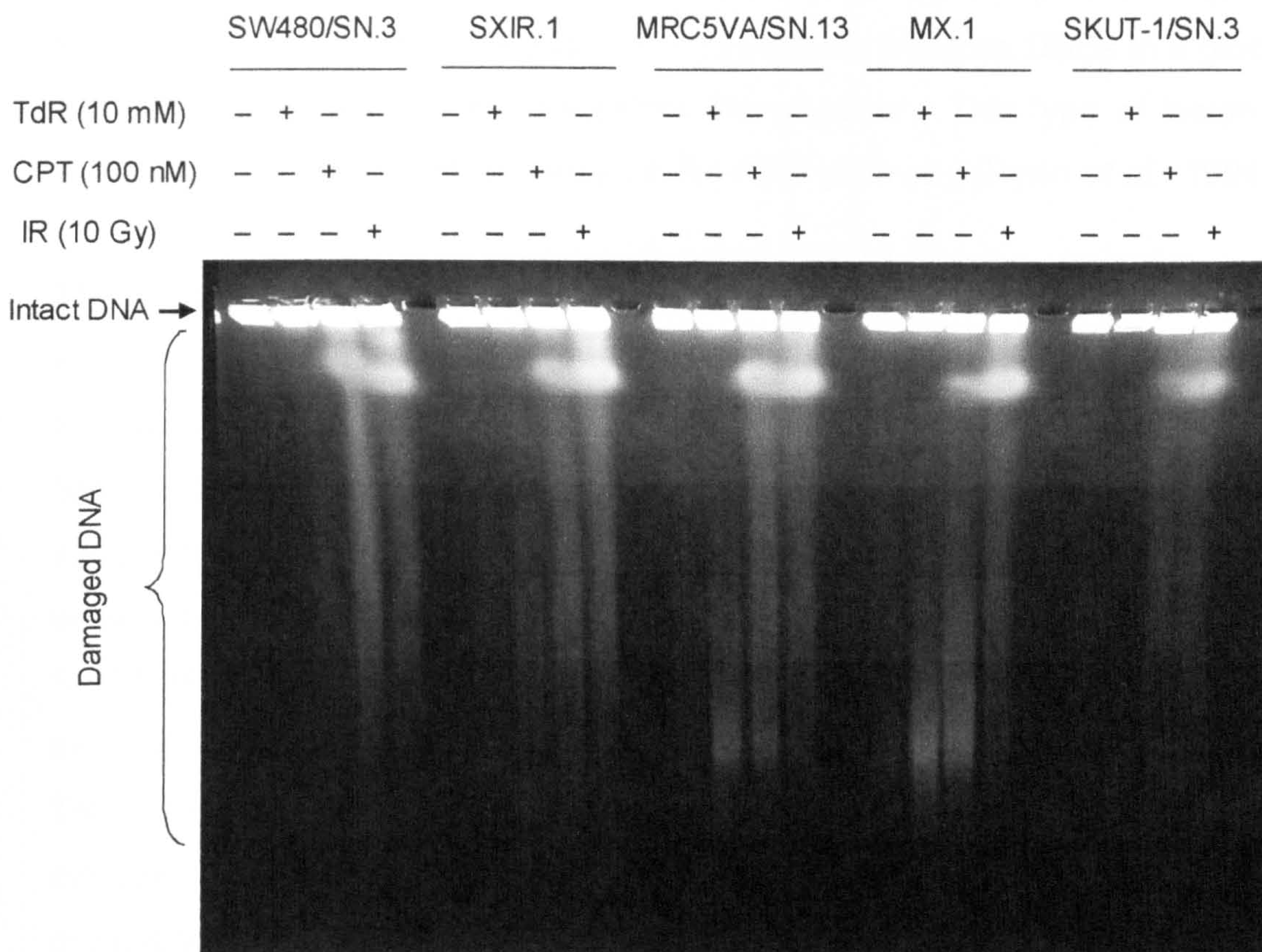

Figure 5.7 Thymidine fails to induce any detectable DSBs in treated cells. The indicated cells were treated with 10 mM thymidine, 20 nM camptothecin for 24 hours or exposed to10 Gy of irradiation before being harvested for analysis of DSBs by PFGE. DSBs are readily detectable in all cells treated by camptothecin or exposed to irradiation but not thymidine.

5.2.8 Cells expressing 342delT are sensitive to camptothecin.

The results presented above suggest that SW480/SN.3 strains expressing 342delT remain competent for repairing I-SceI induced DSBs but are unable to repair lesions induced by thymidine. One reason for such results could be that cells expressing 342delT are unable to repair DNA lesions specifically induced by thymidine. An alternative explanation for such

results is that the HRR pathway is saturated by thymidine treatment as this agent is capable of inducing HRR events at every replication fork. To investigate this, the DNA Topoisomerase I inhibitor, camptothecin (CPT) was used to initiate HRR events. CPT specifically induces DSBs in a global manner at replicating forks throughout the genome. This type of lesion is suggested to be repaired primarily by the HRR pathway (Ryan *et al.*, 1994).

The sensitivity of strains expressing the mutant allele to CPT was initially investigated. Exponentially growing cells were continuously treated with increasing doses of CPT and cell survival was determined after 10-14 days. SW480/SN.3 transfectants expressing 342delT showed intermediate sensitivity and at 10% survival were at least 1.2-fold more sensitive to CPT when compared to the parental strain (Figure 5.8a). At 10% survival, MRC5VA/SN.13 strains expressing 342delT were also found to be 1.4-fold more sensitive to CPT when compared to the parental strain (Figure 5.8b). The MMR –deficient, SKUT-1/SN.3 strain was 1.4-fold more sensitive to the cytotoxic effects of this agent when compared to either of the MMR –proficient, SW480/SN.3 and MRC5VA/SN.13, strains tested.

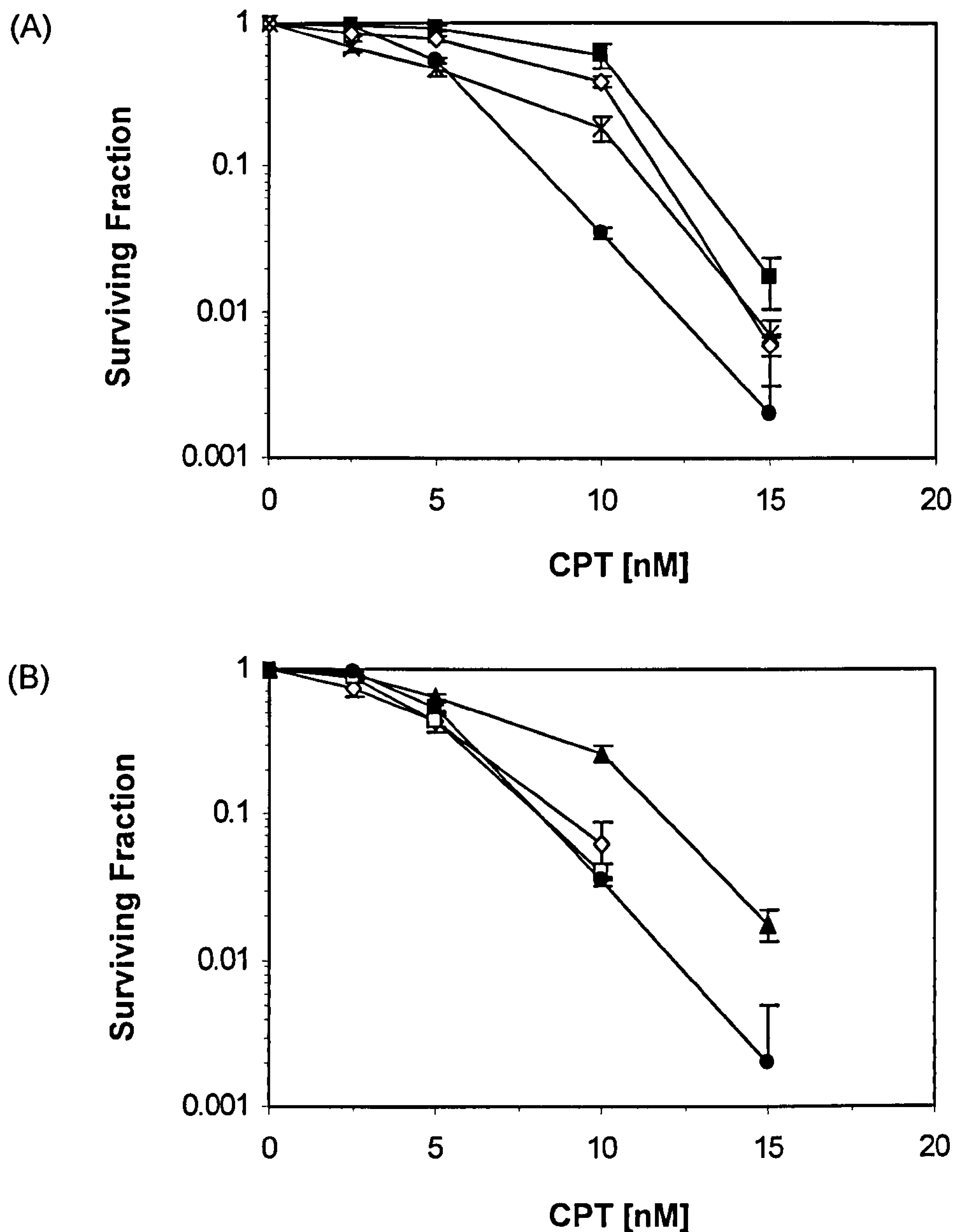

Figure 5.8 Expression of 342delT confers sensitivity to camptothecin (CPT). (A) Transfectants of SW480/SN.3 expressing the mutant XRCC2 allele (SXIR.3, ◊ and SXIR.4, X) are sensitive to CPT when compared to untransfected SW480/SN.3 cells (■). The sensitivity of the SKUT-1/SN.3 strain (•) to CPT is included for reference (B) Transfectants of MRC5VA/SN.13 expressing the mutant XRCC2 allele (MX.1, ◊ and MX.2, □) are sensitive to CPT when compared to parental MRC5VA/SN.13 cells (▲). The sensitivity of the SKUT-1/SN.3 strain (•) to CPT is included for reference. The mean (symbols) and standard deviation (error bars) of three independent experiments performed in duplicate are presented.

5.2.9 SW480/SN.3 cells expressing 342delT are proficient in HRR induced by CPT.

Previous published studies have shown that exposure of cells to CPT induces HRR events in mammalian cells (Ryan *et al.,* 1994). Therefore the ability of CPT to induce HRR events in cells expressing the mutant XRCC2 allele was investigated. Approximately 18 million cells were treated for 24 hours with increasing doses of CPT in replica cultures and the frequency of neo+ recombinants determined. Such replica cultures were initially inoculated from 1000 cells in order to eliminate pre-existing Neo^+ recombinants and to obtain a more precise measurement of the recombinogenic effects of CPT. As shown in Figure 5.9, CPT induced the frequency of neo^+ recombinants up to 10-fold in a dose dependent manner in the SW480/SN.3 parental strain. Furthermore, the two transfectants expressing 342delT also exhibited an increase in the frequency of neo^+ recombinants to levels that were similar to the parental strain. These results therefore show that SW480/SN.3 strains expressing 342delT are able to repair recombination events following CPT treatment but not following thymidine treatment.

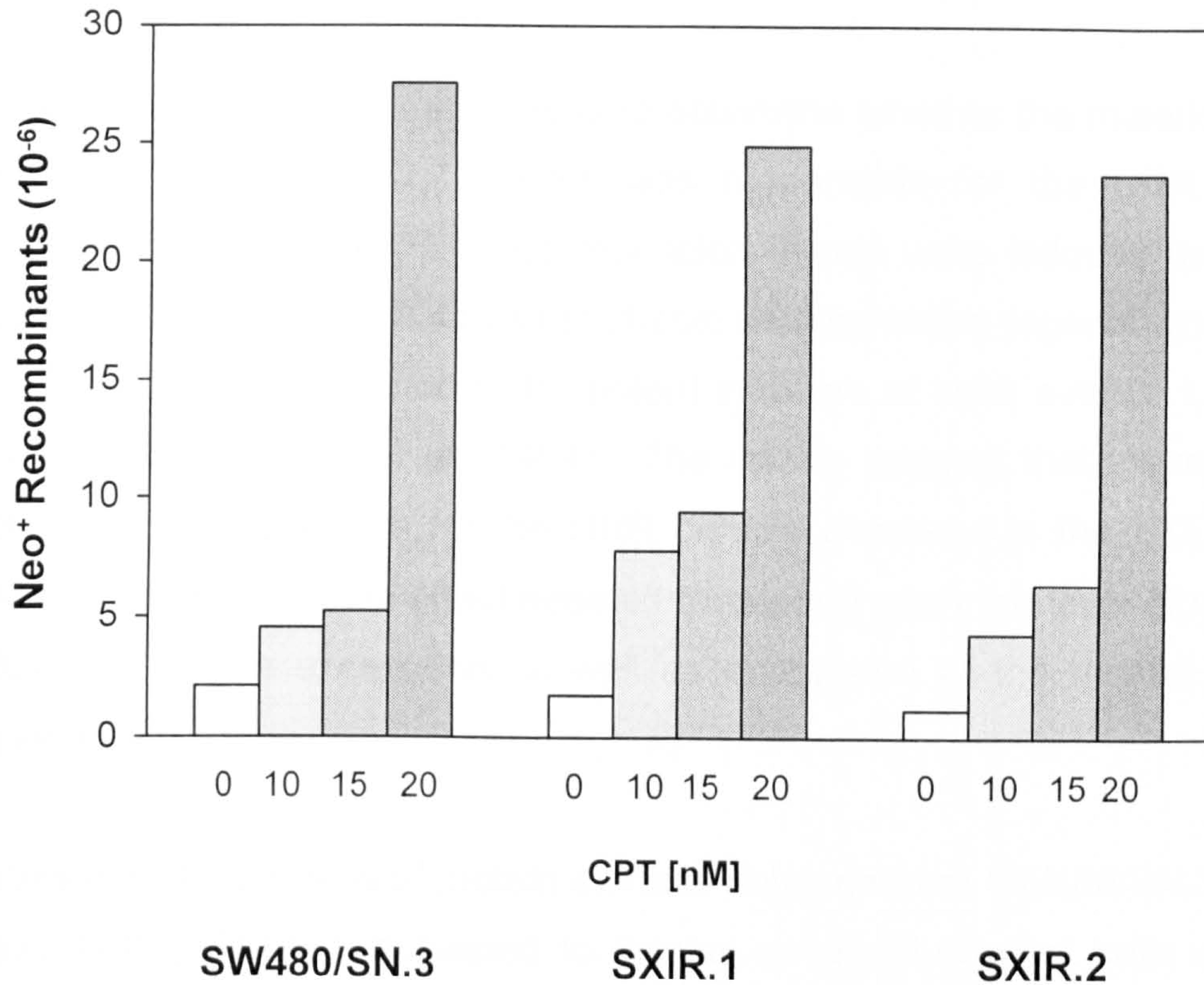

Figure 5.9 Cells expressing 342delT are proficient in HRR induced by CPT. CPT treatment for 24 hours induced recombination events in SW480/SN.3 cells as well as in the SXIR.1 or SXIR.2 transfectants (labeled). Six independent replica cultures were treated for each experiment and the results are an average of six independent experiments. The standard deviation for each treatment is indicated by error bars.

5.3 DISCUSSION

The aim of the present study was to determine whether the mutation found within XRCC2 of SKUT-1 cells was responsible for the HRR defects observed in the cell line. Recombination events were induced using site-specific breaks, as well as camptothecin and thymidine (agents which have all previously been noted to be potent inducers of such events, Lundin *et al.,* 2002 and Ryan *et al.,* 1994). The results suggest that expression of 342delT is responsible for the HRR defects observed in the SKUT-1 cell line. Furthermore this effect seemed to depend upon the level of wild-type XRCC2 protein expression as well as expression of the mutant XRCC2 allele.

Western blot analysis of protein extracts obtained from SW480/SN.3 strains expressing 342delT appeared to display an equal level of both wild-type and mutant allele. MRC5VA/SN.13 strains expressing 342delT, however, appeared to display reduced levels of the wild-type XRCC2 protein but higher levels of the mutant peptide. A similar observation was noted when the SKUT-1/SN.3 strain was examined. The mechanism responsible for the depression in the level of the full length XRCC2 protein in MRC5VA/SN13 cells and SKUT-1 is not clear. MRC5VA/SN.13 transfectants and SKUT-1 cells appear to produce an excess of the mutant transcript as judged by the level of the cDNAs amplified from these cells. However it is unlikely that enhanced expression of the mutant allele would suppress expression of the wild-type. Nonsense mediated decay (which alters the stability of messenger RNAs containing nonsense mutations) should specifically affect the mutant gene in these cells and not the wild-type (reviewed in Wilusz *et al.,* 2001). One possible explanation is that the decreased level of wild-type XRCC2 protein is the result of increased turnover of the protein. It is known that XRCC2 forms a complex with other RAD51 paralogs (Masson *et al.,* 2001 and Liu N., *et al.,* 2002). The mutant peptide may associate more

effectively with the complex than the full length protein and the un-complexed protein may be degraded. This would be similar to the situation with the MMR proteins *h*MLH1 and *h*PMS2 which are normally found in cells in the form of heterodimers (Li *et al.*, 1995). Nonsense mutations that eliminate the full length wild-type *h*MLH1 also result in a decline in the level of the un-complexed partner protein *h*PMS2 (Drummond *et al.*, 1996).

The differences in the ratio of the levels of wild type: mutant protein expression in the two cell lines appears to be reflected in the ability to form recombinants following induction of DNA DSBs. SW480/SN.3 transfectants, expressing equal levels of both wild-type and mutant allele 342delT, repaired both I-SceI and camptothecin induced DSBs as effectively as the parental strain. MRC5VA/SN.13 strains however, expressing reduced levels of wild type XRCC2 protein, displayed a reduced ability to repair I-SceI induced DSBs via a homology based repair assay when compared with the parental strain. This latter effect is similar to that observed in the SKUT-1/SN.3 strain, in which the level of wild type XRCC2 protein is also depressed when compared to the level of mutant peptide.

The results obtained using I-SceI and CPT contrast to those obtained when HRR events were induced using thymidine. Both SW480/SN.3 and MRC5VA/SN.13 strains expressing 342delT showed no increase in the frequency of neo^+ recombinants following thymidine exposure. These results suggest that 342delT specifically acts in a dominant negative manner to suppress HRR events induced by thymidine. This suppression is further highlighted when the cytotoxic effect of thymidine was measured in transfectants expressing 342delT. All strains expressing 342delT were between 1.4 to 4.8-fold more sensitive to this agent when compared to parental strains.

Taken together these results suggest that the wild type XRCC2 protein is involved in the specific repair of DNA DSB lesions induced by agents including an I-SceI endonuclease and CPT. This is consistent with suggestions that a functional wild-type XRCC2 protein is required in the repair of DNA DSBs in hamster cells (Johnson *et al.,* 1999). Either expression of 342delT and/or the reduced level of wild-type XRCC2 protein may interfere with thymidine-induced recombination events in both SW480/SN.3 and MRC5VA/SN.13 cells.

To date, the structure of the lesion induced by thymidine is unknown. Evidence from bacteria and yeast suggest that one such structure could involve a four-way DNA junction that forms due to the reversal of stalled replication forks (Postow *et al.*, 2001). Such lesions are proposed to form substrates for HRR as they involve long tracts of gapped DNA. Given the involvement of the XRCC2 protein in HRR processes, expression of 342delT can potentially impair the resolution of such structures at several steps. Firstly, the BCDX2 complex has recently was shown to specifically bind to gapped DNA structures (Masson *et al.*, 2001). The function of this complex is proposed to be within the 'pre-synaptic' phase of HRR in assisting with the 'loading' of the RAD51 protein in order to initiate recombination events. By analogy, in bacterial cells the RecFOR complex loads the RecA recombinase protein onto gapped sequences (Morimatsu *et al.*, 2003). Furthermore Kurumizaka and co-workers (2003) found that the XRCC3 fragment containing amino acid residues 63-346 is essential for RAD51C binding. This region contains the Walker-type ATPase motifs. Both of XRCC3 and XRCC2 have some sequence homology and display similar phenotypes with respect to DNA damaging agents. Therefore, it is possible that loss of the last 164 amino acids from XRCC2 may inhibit binding with RAD51D, by analogy with XRCC3 and RAD51C. Such an inability to bind may therefore inhibit the function of the BCDX2 complex. Thus cells expressing the mutant peptide would be unable to 'load' RAD51

onto these gapped DNA structures (Figure 5.10). 342delT, however, may not interfere with the repair of DNA DSBs as effectively and the presence of relatively high levels of wild type protein may be sufficient to load RAD51 onto DNA ends of DSBs and promote repair (as observed in SW480/SN.3 transfectants).

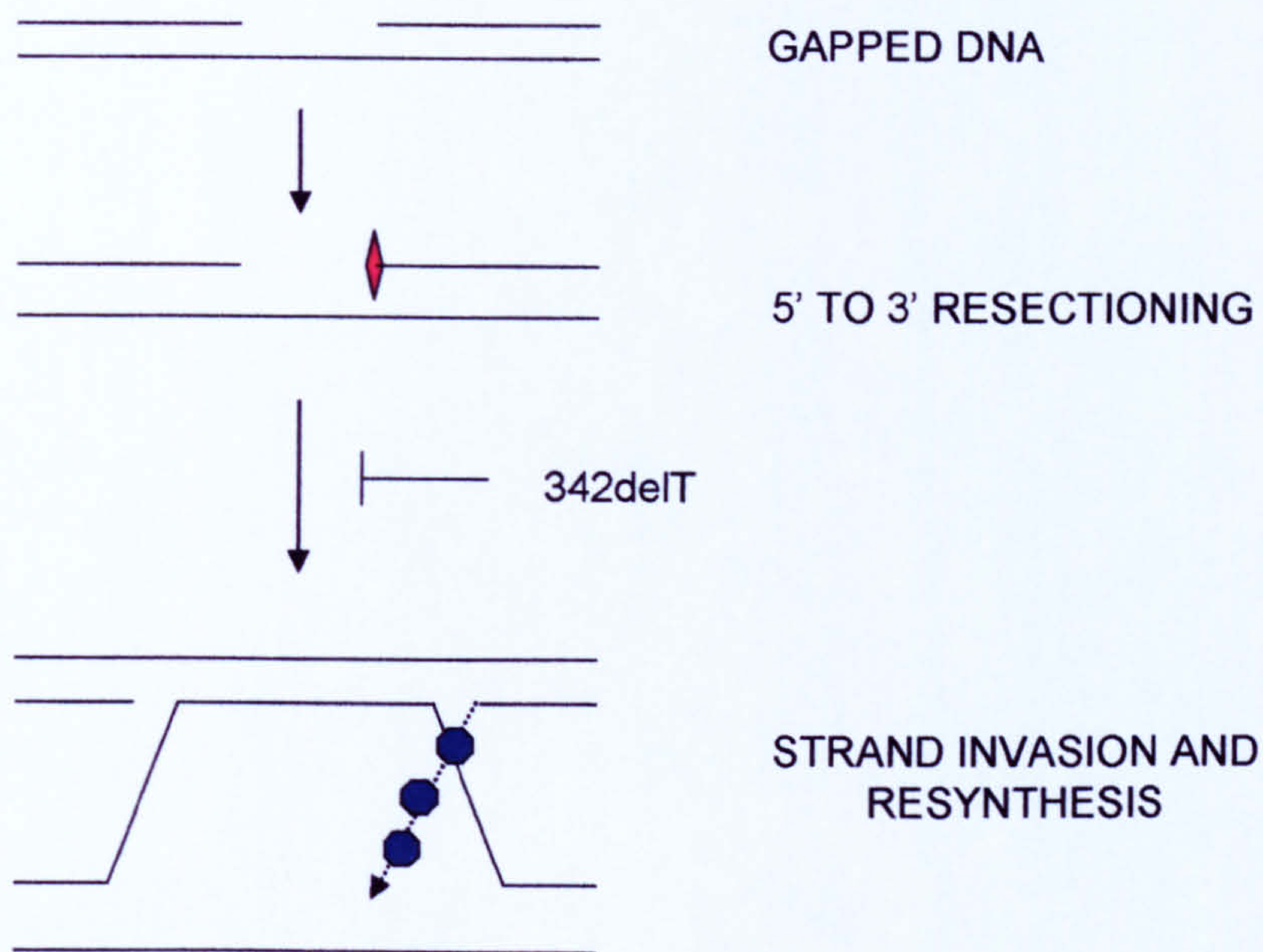

Figure 5.10 Expression of 342delT may inhibit the binding of the BCDX2 complex to gapped DNA structures. Four-way DNA junctions (formed following the reversal of stalled replication forks) may include gapped DNA structures. The BCDX2 complex specifically binds to gapped DNA structures (Masson *et al.,* 2001) in order to assist in the loading of RAD51 (indicated as blue circles). Expression of 342delT may therefore inhibit the processing of lesions induced by thymidine by inhibiting the function of the BCDX2 complex.

An alternative explanation to the effects of 342delT is made clear from a recent report that implicates the BCDX2 complex in being necessary for branch migration within Holliday junctions (Liu *et al.,* 2004). Furthermore it

was suggested that within the BCDX2 complex, the RAD51D/XRCC2 heterodimer acted as an 'ATP-dependent motor' which drives the branch migration process. This heterodimer is proposed to be initially recruited by RAD51B or the RAD51B/RAD51C heterodimer in a similar manner in which the bacterial RuvA protein targets the RuvB protein (Figure 5.11). Hence deletion of Walker Box B from XRCC2 may inhibit 'ATP-dependent motor' activity of the RAD51D/XRCC2 heterodimer. Such a situation may result in the accumulation of unresolved Holliday junctions which are thought to occur during the repair of DSBs or when stalled replication forks reverse.

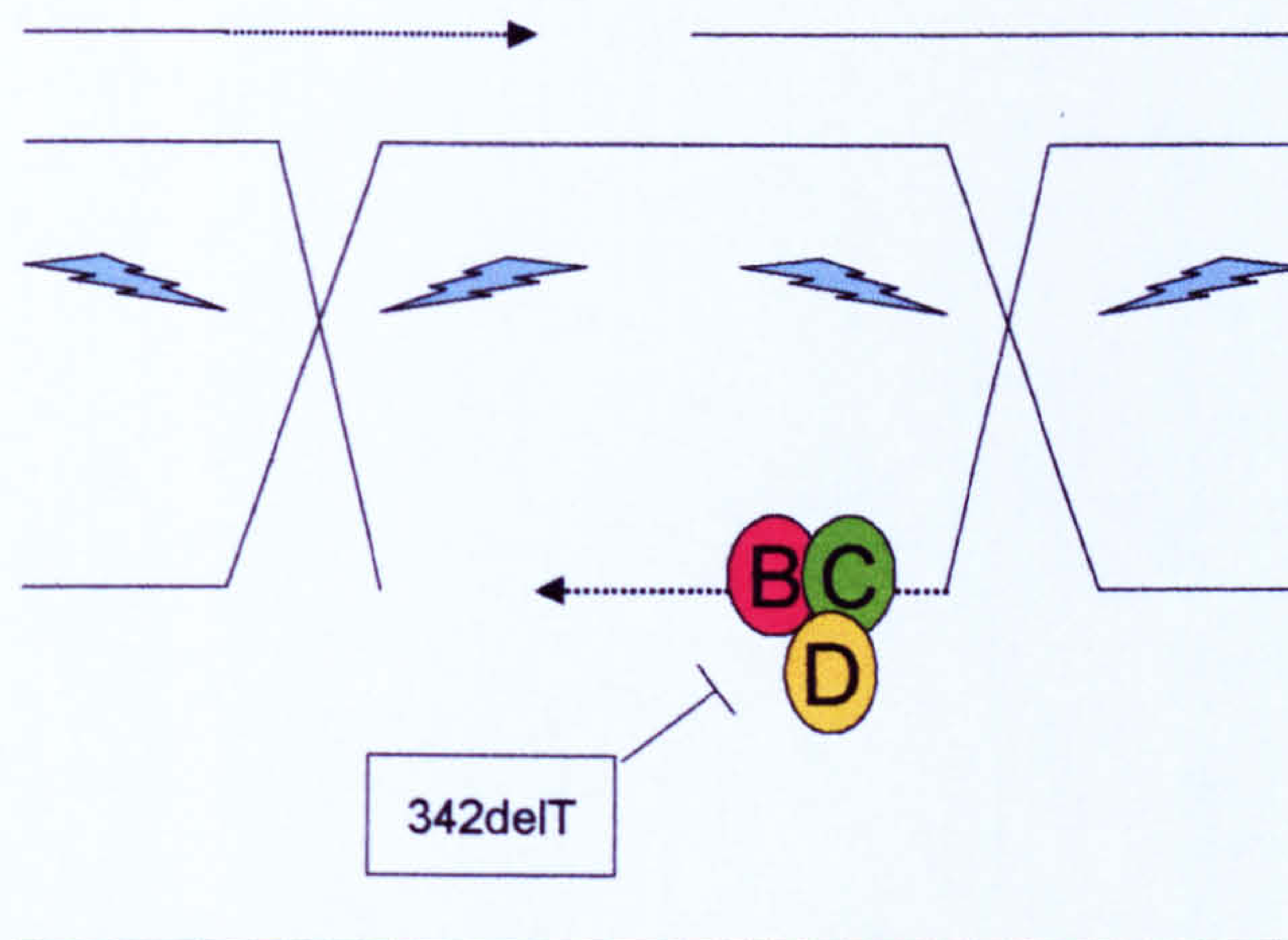

Figure 5.11 Expression of 342delT may alter the kinetics of Holliday Junction resolution. The 'ATP-dependent motor' activity of the RAD51D/XRCC2 heterodimer, within the BCDX2 complex (indicated) may be inhibited by the presence of 342delT. Such events may subsequently lead to unresolved Holliday Junctions.

In additional support to the present study, the RAD51C/XRCC3 complex has also recently been shown to regulate eukaryotic replication fork progression following DNA damage induced by cisplatin (Henry-Mowatt *et al.,* 2003). These observations therefore suggest a strong involvement of the RAD51 paralogs in maintaining the integrity of stalled replication forks

(that are induced by agents such as thymidine and cisplatin). Furthermore, these results suggest that HRR induced by either DSBs or by thymidine are genetically distinguishable.

CHAPTER SIX:

GENERAL DISCUSSION

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6.1 Overview and discussion of results

The aim of this research project was three fold:

(i) To determine the integrity of the Homologous recombination repair pathway in MMR –deficient tumour cell lines.

(ii) To determine the extent to which a deficiency in the Homologous recombination repair pathway was the result of mutations in genes.

(iii) In the event of gene mutations contributing to Homologous recombination repair deficiency, to determine whether such mutations were responsible for the thymidine sensitivity observed in Mismatch repair tumour cells.

The results described in the present study show that the HRR pathway is defective in MMR –deficient tumour cell lines. In support of this, four MMR –deficient tumour cell lines were defective in the production of neo^+ recombinants by homology based recombination following the transient expression of a site specific break. Furthermore, DLD-1/SN.2 cells were also defective in the production of neo^+ recombinants when recombination events were induced using the replication inhibitor, thymidine. These results are consistent with a study published by Slebos and Taylor (2001) which described the use of a host cell reactivation assay to measure HRR events based on the reactivation of a green-fluorescent protein in various cell lines. This study also provided evidence that the MMR –deficient, HCT116 cell line, showed no increase in recombination events. In addition, all five MMR –deficient tumour cell lines tested were sensitive to the cross-linking agent MMC. These results are in agreement with a study conducted by Fiumicino and co-workers (2000) where a sensitivity of various MMR -deficient cells to MMC was also noted. Cells deficient in HRR (including

XRCC2, XRCC3 and BRCA1) are also known to be acutely sensitive to MMC (Jones *et al.*, 1987; and Fuller and Painter, 1988 and Moynahan *et al.*, 2001). Taken together these results suggest that HRR defects occur in MMR -deficient tumour cells.

To further our understanding of suggested defects in HRR acquired in these specific tumour cell lines, genes involved in HRR and DSB signalling were sequenced. Screening of eight tumour cell lines revealed a frameshift mutation within the RAD51 paralog, XRCC2, of the MMR –deficient SKUT-1 cell line. Such frameshift mutations occur predominantly in a MMR defective background. This provides further evidence to support the argument that the loss of HRR is a downstream event, following the initial loss of MMR in these specific cell lines.

The experiments conducted in the present study further showed that the XRCC2 mutation could account for the thymidine sensitivity observed in SKUT-1 cells. Transfection of 342delT into two different MMR –proficient cell lines, conferred both thymidine sensitivity and suppressed HRR events induced by thymidine. Increasing evidence from both prokaryotic and eukaryotic cells suggest that the loss of HRR may account for the thymidine sensitivity observed in various cell types (Cox, 2001; Kraus *et al.*, 2001 and Michel *et al.*, 2001). The addition of thymidine induces an imbalance in dCTP and dTTP pools which subsequently results in the slowing of DNA replication forks (Bjursell and Reichard, 1973). Exposure of cells to thymidine has previously been shown to specifically require a proficient HRR pathway for cell survival (Lundin *et al.*, 2002a). In addition, other studies have used CHO cells deficient in HRR, to show that such cells exhibit delayed cell cycle progression (Fuller and Painter, 1988; Griffin *et al.*, 2000 and Tebbs *et al.*, 1995), hypersensitivity to agents known to stall DNA replication forks (Lundin *et al.*, 2002a), increased mutation rates (Thacker *et al.*, 1994 and Fuller and Painter, 1988) and chromosome

instability (Griffin *et al.,* 2000 and Cui *et al.,* 1999). RAD51 foci have also been shown to form in post-replicative DNA and at stalled replication forks (Tashiro *et al.,* 2000 and Sengupta *et al.,* 2003).

MMR –deficient cell lines have been previously shown to become hypersensitive to the cytotoxic effects of thymidine. This sensitivity was documented not to be due to the loss of MMR (Mohindra *et al.,* 2002). Therefore, it appears that the thymidine sensitivity, like the loss of HRR, is also a result of events downstream from the initial loss of MMR.

To date, the precise structure of the lesion induced by thymidine is unknown, although it seems unlikely to involve the formation of DSBs (as determined by pulse field gel electrophoresis – Figure 5.7, Page 175). However, the present and a previous published study (Lundin *et al.,* 2002) have shown that treatment of cells with thymidine and hydroxyurea (agents proposed to stall DNA replication forks) can potently induce HRR events. Therefore it appears that HR proteins repair additional substrates other than DSBs which may form at stalled replication forks. In support of this argument, expression of the mutant XRCC2 allele specifically suppressed recombination events induced by thymidine in a dominant negative manner. The BCDX2 complex has recently been reported to preferentially bind to gapped DNA structures (Masson *et al.,* 2001). Therefore it seems possible that expression of the mutant XRCC2 allele may inhibit the BCDX2 complex from binding onto the long tracts of gapped DNA regions which may form following thymidine exposure.

A replication fork falters when it encounters an un-repaired DNA lesion or when its progress is blocked by a DNA bound protein. Emerging evidence from both bacteria and yeast implies that stalled replication forks can be bypassed and/or restored via a so-called fork reversion reaction. A similar phenomenon is proposed to occur in mammalian cells, however, at present

no evidence exists to suggest that such an event occurs. Restoration of stalled replication forks is initially proposed to occur either due to direct nuclease action or alternatively due to positive torsional strain within DNA (Postow *et al.,* 2001). Initially, the newly replicated daughter strand is thought to reverse in direction and subsequently produce a so-called 'half chicken foot' substrate (Figure 6.1). Such substrates are thought to be recognised and cleaved by the Mus81/Eme1 endonuclease in human cells and subsequently initiate HRR events using a single end for repair (Ciccia *et al.,* 2003 and Chen *et al.,* 2001). Continual reversal of replication forks, however, produce a four-way junction that includes a Holliday junction (termed a 'chicken foot'). Such structures are well documented to require HRR proteins for processing and are proposed to be either resolved directly in order to form a DSB or (if HRR is activated prior to Holliday junction resolution) restore replication. As Holliday junctions are predominantly resolved in non-crossover events in mitotic mammalian cells, the products formed following the resolution of a chicken foot are thought to be almost always the result of a gene conversion event (Figure 6.1).

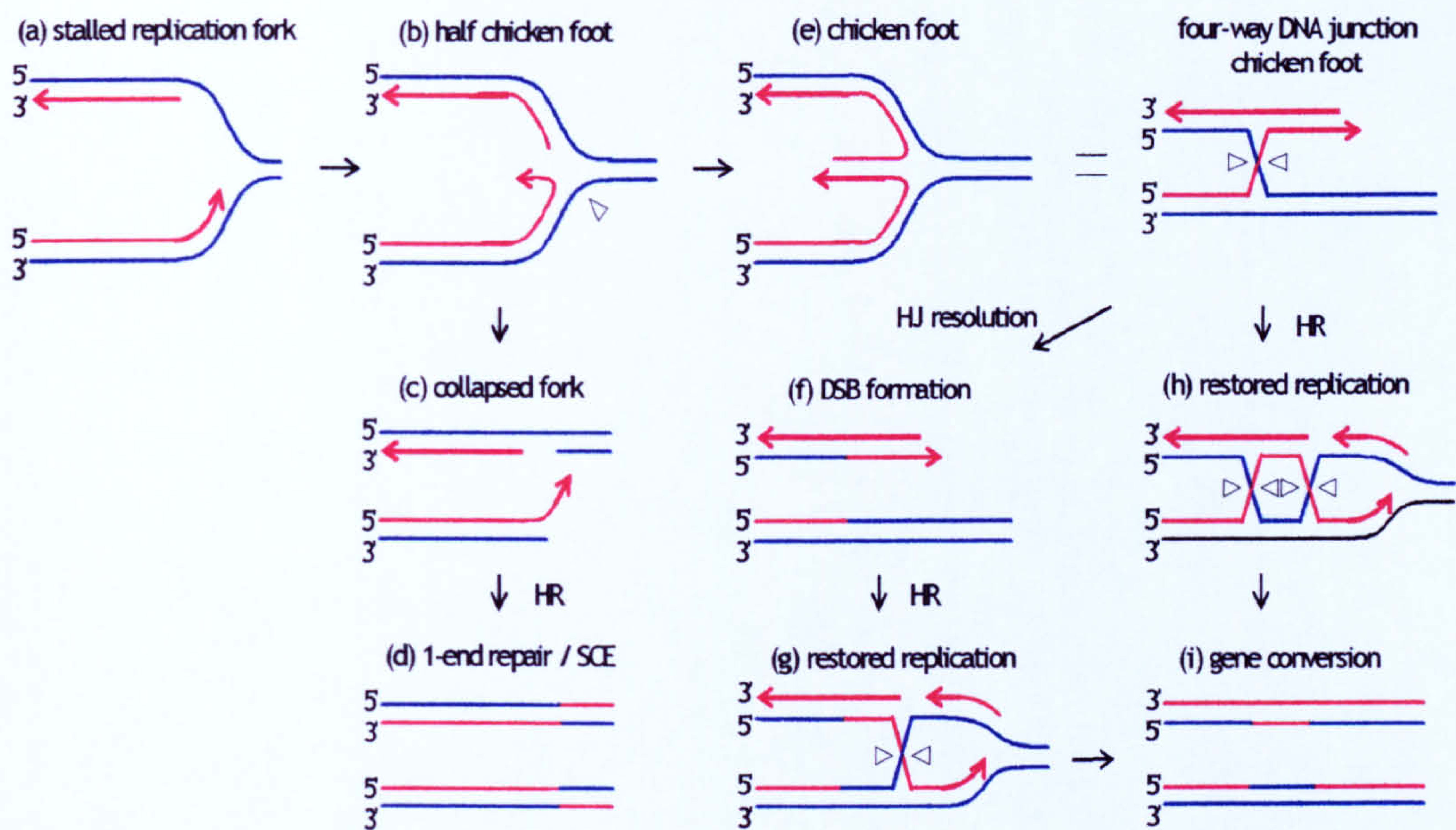

Figure 6.1 HR mediated repair of stalled replication forks. (A) Treating cell with thymidine may stall replication forks. (B) Such stalled replication forks may reverse either due to positive torsional strain in DNA or due to enzymatic action (Postow *et al.,* 2001). (C) The intermediary structure formed when only one strand reverses (half chicken foot), may be cleaved by endonucleases therefore, resulting a collapsed replication fork with one free end. (D) Such structures will result in a sister chromatid exchange (SCE) when repaired by HRR. (E) Alternatively, a stalled replication fork may fully reverse to form a four-way DNA junction (chicken foot). (F) The subsequent Holliday junction may be cleaved resulting in a 1-end DSB that can be repaired by proteins involved in HRR in order to restore replication. (G) However, the 3' end may also directly be used to initiate recombination events and thus also restore replication. Such an event avoids the formation of a DSB. (I)The resolution of the two Holliday junctions with crossing over would result in a gene conversion event, as predicted in the Szostak model (Szostak *et al.,* 1983). Arrows indicate the direction of DNA synthesis, blue lines indicate template DNA and red lines indicate newly synthesised DNA. *Adapted from Helleday, T. (2003).*

6.2 Future Studies

Future experiments are required in order to confirm the precise type of lesion induced by thymidine and the subsequent substrate generated which initiates HR mediated repair. In addition, the signalling steps required for HRR activation following thymidine exposure need to be investigated. Evidence from other studies conducted in the laboratory suggest a possible role for the ATM kinase in the initial steps of damage signalling induced by thymidine, although the mechanisms involved remain unknown. A truncation of the MRE11 gene (which is a substrate for ATM when part of the MRE11/RAD50/NBS1 complex) has also been reported to give rise to thymidine sensitivity and act in a dominant negative manner with respect to thymidine induced recombination (Scorah *et al.,* unpublished data). Therefore the direct involvement of ATM, MRE11 and XRCC2 in the repair of thymidine induced lesions could be achieved using co-transfected combinations of the genes into the SW480, MMR proficient cell line and observing any effects on thymidine induced recombination events.

Biochemical alterations which may occur as a result of expressing 342delT need to be investigated with respect to the processing of lesions induced by thymidine. Recent studies conducted by Masson and co-workers (2001) suggested that the RAD51 paralogs are present as two distinct complexes (termed the BCDX2 and RAD51C/XRCC3 complexes). The RAD51C/XRCC3 complex has been reported to regulate eukaryotic replication fork progression following cisplatin exposure (Henry-Mowatt *et al.,* 2003). However, the BCDX2 complex may be involved in regulating stalled replication forks induced by thymidine in mammalian cells. Furthermore, to improve our understanding for the requirement of the RAD51 paralogs in the maintenance of replication forks, a clearer insight into the factors responsible for determining which of these two complexes act on a particular lesion is needed.

Alterations in the XRCC2 amino acid sequence (as noted in the SKUT-1 cell line) may inhibit the formation of, or alter the alignment of proteins involved in the BCDX2 complex. Co-immunoprecipitation experiments, using mammalian extracts, could be conducted to ascertain any such alterations both pre- and –post thymidine treatment. The phenotypic effects of mutating the other RAD51 paralogs, with respect to thymidine induced recombination events, could also be investigated. A similar mutation to that present in XRCC2 in SKUT-1 cells can be introduced using techniques such as site-directed mutagenesis. These studies would provide a better understanding of the requirement of ATP-hydrolysis within the BCDX2 complex's function, as deletion of Walker Box B seems to have detrimental effects on the function of XRCC2.

6.3 Implications to Cancer

To date, published studies provide evidence that strongly link MMR -deficiency to the MSI phenotype observed in HNPCC cases. However, alterations in other proteins which are required for the maintenance of the genome may also contribute to this phenotype. Genes encoding proteins which appear to be involved in HRR (as well as genes that encode for proteins that regulate the S-phase checkpoint) may be potential targets for such alterations. In agreement with this, other studies have shown that the MRE11 gene is mutated in 85% of MSI+ colon cancers (Scorah, J. *et al.*, unpublished). Although the XRCC2 frameshift mutation reported in the present study does not seem to contribute to the MSI phenotype, data obtained from functional studies do support a role for XRCC2 in maintaining the integrity of the mammalian genome. Future work, however, is required to establish whether the mutations which give rise to the various genetic instability disorders promote the disruption of a specific step within a

common pathway. Such studies should also determine whether these events are unrelated.

It is possible that the XRCC2 mutation acquired in the SKUT-1 (*h*MSH2-deficient) cell line is a random event, with respect to the development of gynaecological uterine tumours. However, the frameshift mutation found in the T_8 tract of SKUT-1 cells is likely be coupled with selection, as other mononucleotide runs in XRCC2 are not mutated. Furthermore, the RAD52 and RAD54B genes (which encode for 9 base pair and two seven base pair mononucleotide runs, respectively) were not mutated, even though the probability for incurring mutations is expected to be higher in longer runs.

The XRCC2 frameshift mutation found in SKUT-1 cells and leiomyosarcomas seems to confer a dominant negative phenotype when expressed in either SW480 or MRC5VA cells. A dominant negative phenotype is often observed when the product of the defective gene inhibits the function of the wild-type gene product within the same cell (Herskowitz, 1987). Therefore, expression of the mutant XRCC2 peptide may confer thymidine and MMC sensitivity by destroying the activity of the BCDX2 complex.

Such a dominant negative effect exerted by the XRCC2 frameshift mutation may also stimulate genetic instability and therefore cancer. Dominant negative mutations are documented to promote various types of cancer. Bertrand and co-workers (2003) injected a dominant negative form of RAD51 in mice in order to measure the tumourigenic effect. The study concluded that such mice exhibited a higher frequency of tumours as well as a faster growth rate when compared with control mice. Furthermore, expression of a dominant negative form of TGFβ –RII in transgenic mice increased the amount of tumour metastasis with respect to both prostate and mammary tumour formation (Tu *et al.*, 2003 and Gorska *et al.*, 2003).

Finally, dominant negative p53 mutations have also been observed in serous adenocarcinomas (Sakuragi *et al.,* 2001).

A greater number of leiomyosarcoma tumour samples would need to be examined in order to ascertain whether the XRCC2 mutation is indeed a random event. In the present study, the XRCC2 mutation occurred in one out of eight tumour samples. However, in addition to these results, Schoenmakers and co-workers (1999) published the finding of additional mutations in RAD51B in uterine leiomyomas. The advantage gained by such events can be explained in context with the reported role of HRR proteins in delaying DNA replication fork progression following cisplatin or UV treatment (Henry-Mowatt *et al.,* 2003). Hence MMR -deficient cells that acquire further mutations in the HRR genes may be unable to regulate correct DNA synthesis on damaged templates which would lead to a higher level of genetic instability and promote tumourigenisis.

Furthermore, phenotypic characterization of MMR –deficient cells revealed that these cells are resistant to DNA alkylating agents such as 6-thioguanine and cisplatin. Resistance to such classes of chemotherapeutic agents has subsequently hindered the treatment of tumours displaying microsatellite instability. The loss of HRR and the hypersensitivity of MMR –deficient cells to thymidine raise the possibility of using agents that induce dNTP pool imbalances in conjunction with current methods to improve the treatment of this subset of tumours.

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Appendix 1 Table showing primer sequences and annealing temperatures used to analyze mononucleotide runs in genes involved in HRR.

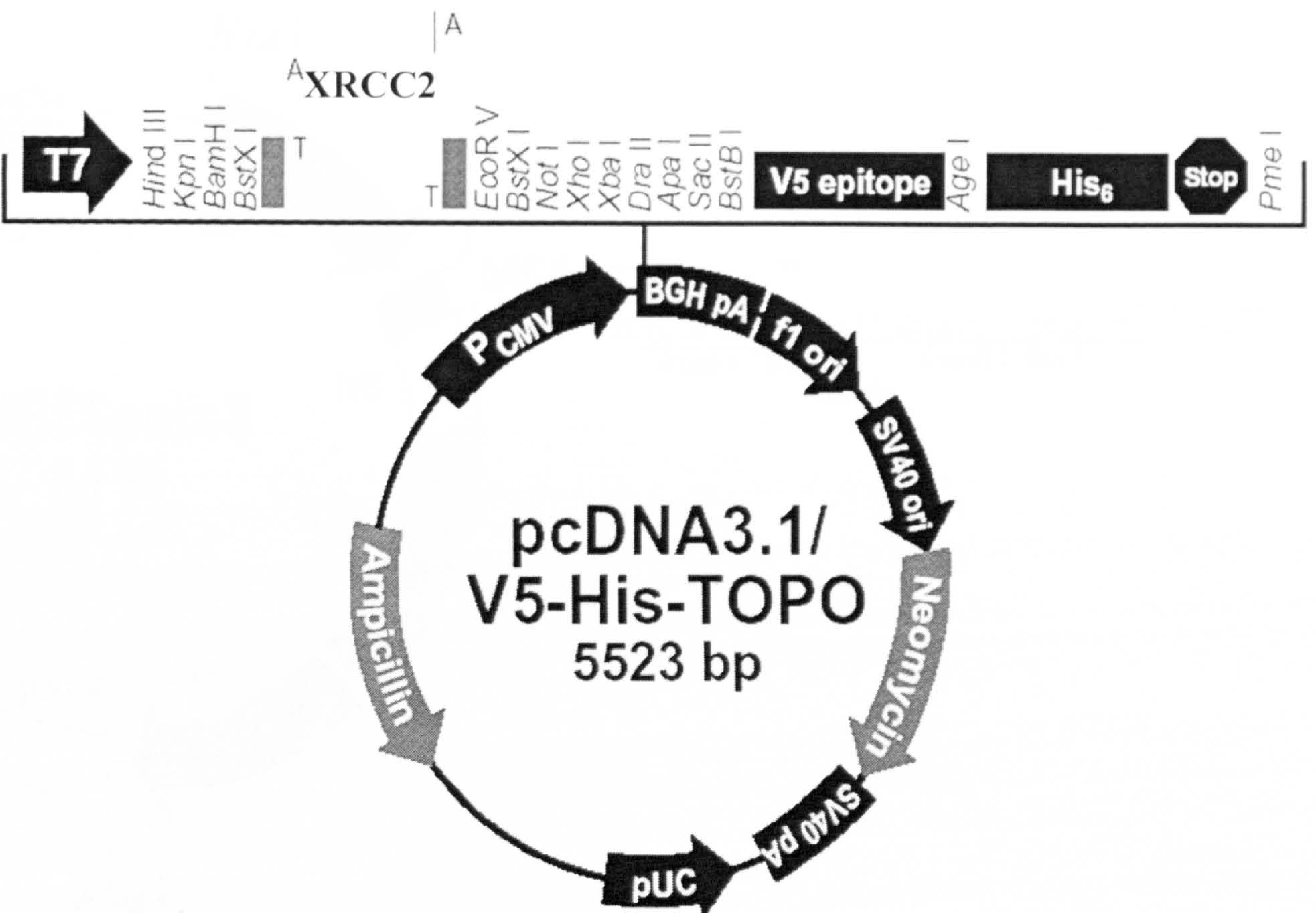
GENE	RT-PCR PRIMERS (5'-3')	TEMP (°C)	PRODUCT SIZE (bp)	IRD-LABELLED SEQUENCING PRIMERS	TEMP (°C)
XRCC2	F: AGTTGGTGAATGGCGTTGGT R: CGTAGTACCCTGCAAAAGAC	53	901	F: AGTTGGTGAATGGCGTTGGT R: CGTAGTACCCTGCAAAAGAC	53
XRCC3					
FRAGMENT 1	F: TGAATTGAAGGCGAGTGCCT R: AGCAGTACGGGGACCTTCTT	55	654	F: TGAATTGAAGGCGAGTGCCT R: AGCAGTACGGGGACCTTCTT	55
FRAGMENT 2	F: CCAGGAGAGCTGCTTCAGAA R: GCAGACGCGTTTTAAAGGCC	55	581	F: CCAGGAGAGCTGCTTCAGAA R: GCAGACGCGTTTTAAAGGCC	55
RAD51	F: GATCATACAGATTACTACTGG R: ACTCCATCTGCATTAATGGC	53	667	F: ATACGCTAGCTGTCACCTGC R: ACTCCATCTGCATTAATGGC	52
RAD52	F: R:				
RAD54					
FRAGMENT 1	F: GTTTAAACTCCTAGGCCCA R: CATCAATGTGATGCACTGCA	52	573	F: GTTTAAACTCCTAGGCCCA R: CATCAATGTGATGCACTGCA	52
FRAGMENT 2	F: ATCATGGCTGATGAGATGGG R: ATTCTTCTGCCGGTTTGGCT	53	732	F: ATCATGGCTGATGAGATGGG R: ATTCTTCTGCCGGTTTGGCT	53
FRAGMENT 3	F: AGGTCGTTTGTTGTAGGCTG R: GGAAGACGAAGGTGATAGCA	52	938	F: AGGTCGTTTGTTGTAGGCTG R:GGAAGACGAAGGTGATAGCA	52
RAD54B	F: TGGTACTCCAATTCAGAATGA R: TCACTATGTGCCAGTAGCTTG	55	1318	F: AATCACCCCTGCCTTTTGTT R: TGTGGACCAAGCTGACAATC	51
RAD51B	F: AGTTGGATGCTGCAGACC R: TGGAACTGGTATTTAACCG	50	1152	F: AGTTGGATGCTGCAGACC R: TCTCTGTGTCAATGTACA	48
RAD51C	F: TTAGCAGGTGAGCCTGCG R: ATTTGTACACTTTGAGA	42	1143		
RAD51D	F: GAACATGGGCGTGCTCA R: TCATGTCTGATCACCCTG	52	866	F: GAACATGGGCGTGCTCA R: TCATGTCTGATCACCCTG	52

Appendix 2 Table showing primer sequences and annealing temperatures used to analyze mononucleotide runs in genes involved in DNA damage signaling and repair.

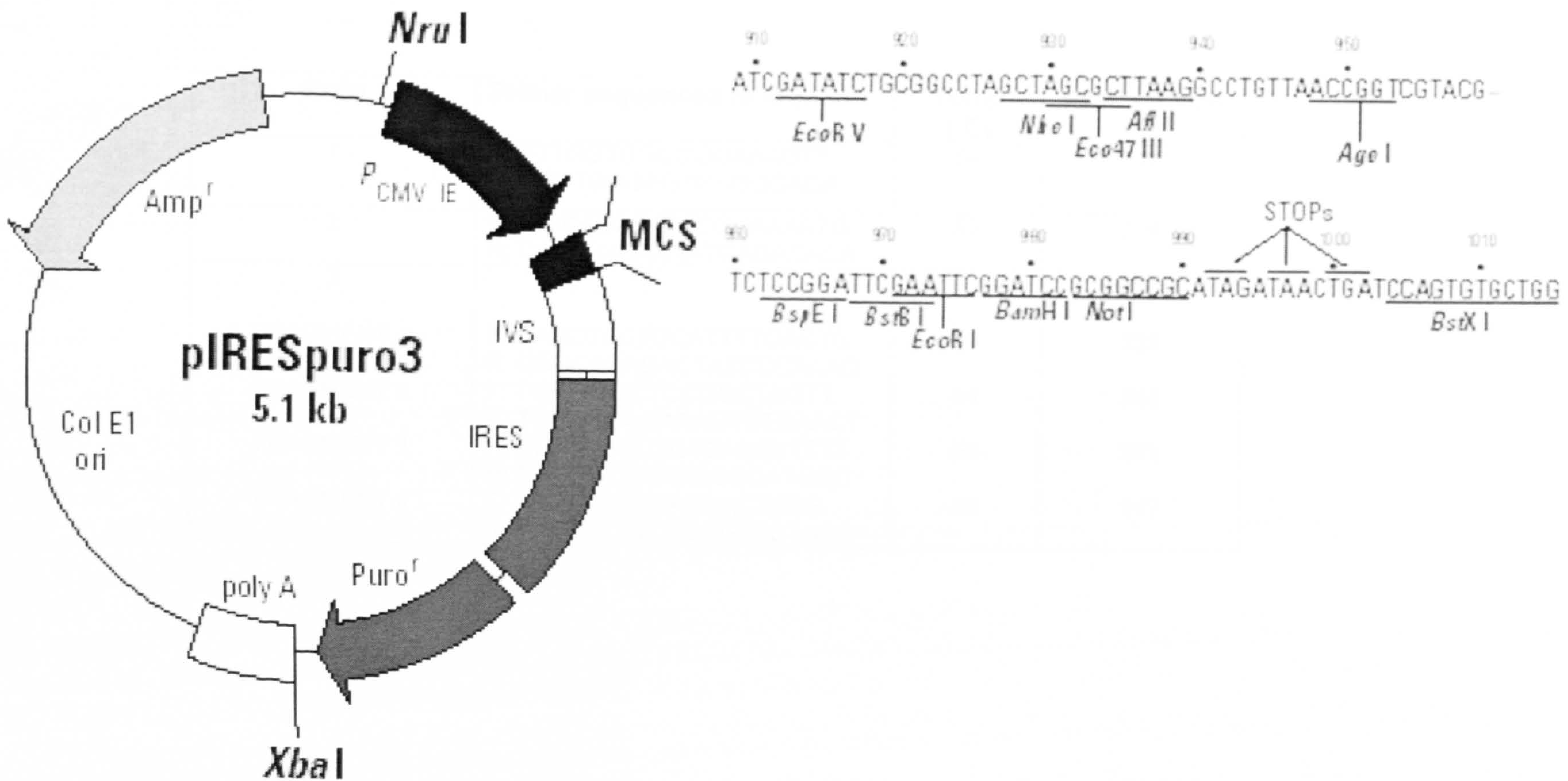
GENE	RT-PCR PRIMERS (5'-3')	TEMP (°C)	PRODUCT SIZE (bp)	IRD-LABELLED SEQUENCING PRIMERS	TEMP (°C)
CHK1	F:TGGAGTCATGGCAGTGCC R: TTCTGGCTGAGAACTGGAGTA	54	928	F:TGGAGTCATGGCAGTGCC R: TTCTGGCTGAGAACTGGAGTA	55
				F: GATCTCAGCTTACTGCAACC R: AGGCGAGGTGGCTCACACCT	53
CHK2 FRAGMENT 1	F: GTCAGGTGCCTGCAGCGCT R: ACATCACGACCGCGTGAGC	58	503	F: GTCAGGTGCCTGCAGCGCT R: ACATCACGACCGCGTGAGC	58
FRAGMENT 2	F: AGCTCCTTAGAGACAGTGTC R: AATTGTACATCAGTGACTGT	48	1660	F: AGCTCCTTAGAGACAGTGTC R: AATTGTACATCAGTGACTGT	48

GENE	RT-PCR PRIMERS (5'-3')	TEMP (°C)	PRODUCT SIZE (bp)	IRD-LABELLED SEQUENCING PRIMERS	TEMP (°C)
FANCD2					
FRAGMENT 1	F: GAGTCTTACATTGAGGATGAA R: GAGCAATACTGATCAGCTGCA	54	268	F: GAGTCTTACATTGAGGATGAA R: GAGCAATACTGATCAGCTGCA	54
FRAGMENT 2	F: TCATCCATTCTGTCGCTGGC R: GCATTCATCATCATAGCAGAT	54	195	F: TCATCCATTCTGTCGCTGGC R: GCATTCATCATCATAGCAGAT	54
FRAGMENT 3	F: AGACTATGTCCCTCCTCTTGGA R: AGGAGGTGTCAGCATACTCTC	58	403	F: AGACTATGTCCCTCCTCTTGGA R: AGGAGGTGTCAGCATACTCTC	58
FRAGMENT 4	F:ACTACTCAGCCAGAGCGTCGT R: CATGCAGAACAGGATGACTAT	55	522	F:ACTACTCAGCCAGAGCGTCGT R: CATGCAGAACAGGATGACTAT	55
FRAGMENT 5	F: CTTACTGGAAACCTTCCAGTTG R: ACAGTGCCAGACTCTGGTG	55	297	F: CTTACTGGAAACCTTCCAGTTG R: ACAGTGCCAGACTCTGGTG	55
MUS81	F: ATCCTACAGCACTTCGGAGAC R: GTTTCACGGCATAAGCTCAGG	55	1439	F: TCCTTCACAGGAACCTGGTC R: AGTGAGCAGAGAGGGTTTG	55
WRN					
FRAGMENT 1	F: TTGGACTCTGCAAATAGGACA R: TCCTGAGACTGCTGTTCCAA	53	1220	F: TTGGACTCTGCAAATAGGACA R: TCCTGAGACTGCTGTTCCAA	53
FRAGMENT 2	F: TTGGAACAGCAGTCTCAGGA R: ACTTGCAGTAGCAGTAAGTGC	54	868	F: TTGGAACAGCAGTCTCAGGA R: ACTTGCAGTAGCAGTAAGTGC	54
FRAGMENT 3	F: AGGCTGATATTGGTATCACGC R: CCATATCCACCAGTATCTTGT	55	1551	F: AGGCTGATATTGGTATCACGC R: CCATATCCACCAGTATCTTGT	55
FRAGMENT 4	F: CAAACAAGATACTGGTGGATA R: GATGAAGTCTCAGTATTGATG	55	619	F: CAAACAAGATACTGGTGGATA R: GATGAAGTCTCAGTATTGATG	55
BLM					
FRAGMENT 1	F: GTCTGCGTGCGAGGATTAT R: GATCTTTCACAGCAGTGCTTGT	54	1573	F: TGATTCACTTGATGGCCCTA R: TGCCAAGAGCTTCCTCTCAT	52
FRAGMENT 2	F: CCTGCCCTACAGGGAATTCTAT R: CAGTCTGGTCACATCATGAT	52	1634	F: GTTCATCACAAGGATGAGAA R: GATTCGGTTGTTATGAGAAT	52
FRAGMENT 3	F: CCTCAGTGATTCTGCCAGAGA R: ACGGTCTATTTATAGGCTTCG	58	1412		

Appendix 3 Figure showing map of the pcDNA3.1/V5-His-TOPO vector .



Appendix 4 Figure showing map of the pIRESpuro3 expression vector .



Appendix 5 Table showing XRCC2 primer sequences and annealing temperatures used to sequence genomic DNA derived from uterine tumours.

Exon	Primer sequences (5'-3')	Temp (°C)	Product size
1	F: GTTGGTGGCGGGAAAGTT R: GGTTGGGAGTGAGGGAGA	54	118
2	F: TTACAGACTTTCGGAAAATG R: CAATTGGAAAATGAGACACA	52	204
3			
FRAGMENT 1	F: GAGCTACTGCATTTTGACTG R: GAGCACAGACTATCCCAAAG	56	228
FRAGMENT 2	F: TGATATGCTCCGGCTAGTT R: TGGAGGAGAAAGTGTGAACT	54	242
FRAGMENT 3	F: GGATAGCCTGTCAGCTTTTT R: TTATCTCTGTAAGGCATGGC	56	251
FRAGMENT 4	F: TGCCTCTCGACGACTGTG R: GTCTTTTGCAGGGTACTACG	58	247

Appendix 6: Addresses of Companies

ABgene,
Blenheim Road, Epsom, Surrey, KT19 9AP

American Type Culture Collection (ATCC),
P.O. Box 1549, Manassas, VA 20108 U.S.A.

Amersham Pharmacia Biotech UK Ltd,
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA

Ananchem Ltd,
Anachem House, Charles Street, Luton, Bedfordshire, LU2 0EB

Becton Dickinson,
Between Twins Road, Cowley, Oxford, OX4 3LY

Bethyl Laboratories Inc,
25043 West FM 1097, Montgomery, TX 77356

Bennet Scientific Ltd,
1 Tenterk Close, Bleadon, Weston-Super-Mare, Somerset, BS24 0BJ

Bibby Sterlin Ltd,
16 The Edge Business Centre, Humber Road, London NW2 6EW

Bio-Rad Laboratories Ltd,
Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TP

Britannia Pharmaceuticals,
41-51 Brighton road, Redhill, Surrey, RH1 6YS

Calbiochem,
10394 Pacific Center Court, San Diego, California 92121 USA

Cambrex Bioscience Inc. (formerly BioWhittaker),
8830 Biggs Ford Road, Walkersville, MD 21793-0127, USA

Cancer Research UK,
P.O. Box 123, Lincoln's Inn Fields, London, WC2A 3PX

Cell Signalling Technology Inc,
166B Cummings Center, Beverly, MA 01915, USA

CIS Biointernational,
RN306-91400 Saday, BP32, F-91132, Gif/Yvette, Cedex, France

Continental Lab. Products (CLP),
5648 Copley Drive, San Diego, CA 92111, USA

Corning Inc.,
HP-AB-03, Corning, NY 14831, USA

Denver Instrument Company,
1855 Blake Street, Suite 201, Denver, CO 80202, USA

Epicentre,
726 Post Road, Madison, WI 53713, USA

Eppendorf AG,
Barkhausenweg 1, 22331 Hamberg, Germany

Eurogentec,

Parc scientifique du Sart Tilman, 4102 Seraing, Belgium

Gene Codes Corporation,

640 Avis Drive, Ann Arbor, MI 48108, USA

Gilson Medical Electronics,

BP45, F95400, Villiers-le-Bel, France

Grant Instruments (Cambridge) Ltd,

Shepreth, Cambridge, SG8 6GB

Greiner Labs,

Maybach Strasse 2, Postfach 1162, D-7M3, Frickenhausen, Germany

GRI Ltd,

Gene House, Queensborough Lane, Rayne, Braintree, Essex, CM77 6TZ

Harvard Apparatus Inc.,

Fircroft Way, Edenbridge, Kent, TN8 6HE

Hirschmann Laboratory,

D-72606, Nuertingen, Germany

ILFORD Imaging UK Ltd,

Town Lane, Mobberley, Knutsford, Cheshire, WA16 7JL

International Equipment Company,

300 Second Avenue, Needham Heights, MA 02494, USA

Invitrogen Life Technologies,
3 Fountain Drive, Inchinnan Business Park, Paisley, UK

Kodak Scientific Imaging Systems,
LabTech International, 1 Acorn House, The Broyle, Rigmer, East Sussex, BN8 5NW

MediCell International Ltd.,
239 Liverpool Road, London, N1 1LX

Mettler-Toledo Ltd.,
64 Boston Road, Beaumont Leys, Leicester, LE4 1AW

MWG-Biotech AG.,
Anzinger Strasse 7, D-85560, Edersberg, Germany

Nalgene,
Unit 1A, Thorn Business Park, Hereford, HR2 6JT

New England Biolabs Inc.,
32 Tozer Road, Beverly, MA 01915-5599

Nikon,
Nikon House, 380 Richmond Road, Kingston-upon-Thames, Surrey, KT2 5PR

Oncogene Research Products,
10394 Pacific Center Court, San Diego, California 92121, USA

Perbio,
Knutpunkten 34, SE-25278 Helsingborg, Sweden

Philip Harris Scientific,

Lynn Lane, Shenstone, Lichfield, Stafford

Promega UK,

Delta House, Chilworth Science Park, Southampton SO16 7NS

Qiagen Ltd,

Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 9AX

Roche,

F.Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland

Sanyo Gallenkamp,

Monarch Way, Belton Park, Loughborough, LE11 5XG

Sanyo Scientific,

1062 Thorndale Avenue, Bensenville, IL 60106, USA

Scaleman,

10 Peabody Street, Bradford, MA 01835, USA

Scientific Industries,

70 Orville Drive, Bohemia, NY 11716, USA

Scotsman Ice System Ltd.,

20010 Bettonlino di Pogliano, Milan, Italy

Sigma-Aldrich Company Ltd.,

Fancy Road, Poole, Dorset, BH12 4QH

SLS Ltd.,

Wilford Industrial Estate, Nottingham, NG11 7EP

Starstedt Ltd.,

68 Boston Road, Beaumont Leys, Leicester, LE4 1AW

STATA Corporation,

702 University Drive East, College Station, TX 77840, USA

Stratagene,

11011 N. Torrey Pines Road, La Jolla, CA 92037, USA

Stuart Scientific,

21 Holmethorpe Avenue, Redhill, Surrey, RH1 2NB

Thermo Forma Scientific,

401 Millcreek Road, P.O. Box 649, Marietta, OH 45750, USA

UVP Inc.,

Upland, CA, USA

Volac International Ltd.,

Orwell, Royston, Hertfordshire, SG8 5QZ

VWR International Ltd.,

Merck House, Poole, Dorset, BH15 1TD

Whatmann Biometra,

Rudolf-Wissel-Strasse 30, 37079, Gottingen