

**The role of *RAD51*-like genes in the
repair of DNA damage in
mammalian cells**

**A thesis presented for the degree of Doctor
of Philosophy, University of Oxford**

Catherine A. French

MRC Radiation and Genome Stability Unit, Harwell
and
Wolfson College, Oxford

Trinity Term, 2003

In memory of my Grandad, John Hall

ACKNOWLEDGEMENTS

I would like to thank all the members of the DNA Repair Group, past and present, for their help and support over the course of this D.Phil. Cathryn, Andy, Bryan, Carol, Julie, Caroline, Karen, Enrico, Naoya and Reg have not only been willing to share their technical expertise with me, but have also made the lab a pleasant and fun place to work. I would especially like to thank Cathryn Tambini for introducing me to the lab and taking the time to show me many molecular biology techniques. The assistance of Stuart Townsend (confocal microscopy), David Kestell (radiation services) and David Papworth (statistics) has also been greatly appreciated.

The opportunity to undertake what has been a challenging but rewarding project was given to me by the Medical Research Council who funded this studentship. My supervisor during my time at MRC Harwell has been Prof. John Thacker. John has had to put up with me sharing his office for the past three and a half years. He has also spent many hours showing me cell culture techniques as well as making himself available to explain and discuss many aspects of the project. I feel privileged at having been able to take advantage of his many years of experience in the field, experience that he has readily shared with me. John, I wish you well for the future and hope that you are able to finally enjoy your retirement.

This project would not have been possible without a number of other people. My co-supervisor at Oxford University has been Prof. Ian Hickson who has advised me on progress at several stages. To obtain biochemical data we collaborated with Dr. Jean-Yves Masson and Dr. Steve West, Cancer Research UK, Clare Hall Laboratories, London. I would particularly like to thank Jean-Yves for providing me with detailed protocols for immunoprecipitation experiments and Western blots. Dr. Maria Jasin, Memorial Sloan-Kettering Cancer Center, New York supplied constructs for the homologous recombination repair assay.

I would like to thank my family and friends who have supported me throughout my studies. My parents will be glad to hear that I am now planning to get a 'proper job'. Special thanks go to Som for coping with unexpected experimental over-runs and for putting up with me when writing this thesis. Finally, I would like to thank my Grandad, John Hall, who died while I was completing this study. During his working life and in retirement, he was a committed and enthusiastic scientist who always encouraged me in my scientific career.

The role of *RAD51*-like genes in the repair of DNA damage in mammalian cells

Abstract

Homologous recombination (HR) is essential for the repair of severe forms of DNA damage such as double strand breaks and interstrand cross-links. The highly conserved RAD51 protein is central to this process and forms nucleoprotein filaments on ssDNA that catalyse strand exchange. In mammalian cells, five genes that share sequence homology with RAD51 have been identified; *XRCC2*, *XRCC3*, *RAD51L1*, *RAD51L2* and *RAD51L3*. Interactions amongst the RAD51-like proteins have been demonstrated but at present little is known about their functions in HR. Progress has been hampered in the cases of *RAD51L1*, *-L2* and *-L3*, which were identified through database homology searches, because of a lack of suitable mutant cell lines in which to study them.

Cultured mammalian cell lines, selected for sensitivity to DNA-damaging agents, have been used to clone and characterise the function of genes from several DNA repair pathways. The *irs3* cell line was isolated from the Chinese hamster line V79-4 in a screen for radiosensitive mutants and has a unique genetic defect. *irs3* cells are sensitive to X-rays (2-fold), ethyl methanesulphonate (2.5-fold) and especially mitomycin-C (MMC) (7-fold), as well as showing chromosomal instability. In this study, the sensitivity of *irs3* to DNA-damaging agents was found to be substantially complemented by expression of the human *RAD51L2* gene, but not other *RAD51*-like genes or *RAD51* itself. An increased level of spontaneous chromosome aberrations was also complemented by *RAD51L2* cDNA expression. *irs3* cells were found to have little or no *RAD51L2* cDNA and RAD51L2 protein. This is likely due to a G→T base substitution at the first nucleotide of exon 6 in the hamster *RAD51L2* gene, which encodes an amino acid change of Val to Phe and disrupts the exon 6 3' acceptor splice site.

In collaboration with Dr. Jean-Yves Masson and Dr. Steve West (Cancer Research UK, Clare Hall Laboratories, London), two RAD51-like protein complexes were shown to exist in hamster cells as they do in human cells. Strikingly, neither complex is formed in *irs3*. Site-directed mutagenesis of the invariant lysine in the RAD51L2 Walker A box ATP-binding domain showed that this residue is required for MMC resistance. Consistent with a role in HR, the RAD51L2 protein was found to localise to the nucleus. This localisation was dependent on the eleven amino acids at the C-terminus of the protein.

irs3 cells showed a reduced number of MMC-induced sister chromatid exchanges compared to wild type cells. *irs3* cells were also found to be defective in damage-dependent RAD51 focus formation, providing the first evidence that RAD51L2 is involved in repair via RAD51-dependent HR. Using the *I-SceI* endonuclease to generate a double strand break, it was found that gene conversion is decreased 7-fold in *irs3* compared to wild type cells. In summary, this study provides data which identify *irs3* as a RAD51L2-deficient cell line and demonstrate that RAD51L2 plays a key role in RAD51-dependent HR processes.

CONTENTS

CHAPTER 1: INTRODUCTION

1.1	Sources of DNA damage	1
1.2	DNA damage and replication	6
1.3	Mechanisms of response to severe DNA damage	7
1.3.1	ATM and ATR sense and signal DNA damage.....	9
1.3.2	Functions of BRCA1 and BRCA2 in the DNA damage response.....	14
1.3.3	The MRE11/RAD50/NBS1(XRS2) (MRN/X) complex.....	19
1.3.4	Structural maintenance of chromosomes (SMC) proteins.....	24
1.3.5	The Fanconi anaemia pathway.....	24
1.3.6	The Blooms syndrome helicase.....	27
1.4	Double-strand break repair pathways	29
1.4.1	DNA double-strand breaks.....	29
1.4.2	Non-homologous end joining.....	30
1.5	Homologous recombination repair (HRR)	34
1.5.1	Choice of DSB repair pathway.....	34
1.5.2	Models of gene conversion.....	36
1.5.3	Single-strand annealing (SSA).....	37
1.5.4	Break-induced replication (BIR).....	37
1.5.5	Role of homologous recombination in repairing stalled replication forks.....	41
1.5.6	Homologous recombination proteins.....	43
1.5.7	Mammalian RAD52 mutants exhibit a mild phenotype.....	44
1.5.8	Two RAD54 homologues in mammalian cells.....	46
1.5.9	<i>RAD51</i> is an essential gene in mammalian cells.....	47
1.6	The <i>RAD51</i>-like gene family	48
1.6.1	Cloning the <i>RAD51</i> -like genes.....	48
1.6.2	<i>irs1</i> and <i>irs1SF</i> are defective in HRR.....	49
1.6.3	<i>RAD51</i> -like gene disruption in cell lines and mice.....	51
1.6.4	<i>RAD51</i> -like protein interactions.....	52
1.6.5	Biochemical characterisation of <i>RAD51</i> -like protein function.....	53
1.6.6	Involvement of <i>RAD51</i> -like genes in tumorigenesis.....	56
1.7	Aims of the present study	57

CHAPTER 2: MATERIALS AND METHODS .

2.1	Primary reagents	58
2.1.1	Chemicals and media.....	58
2.1.2	Bacterial strains and media.....	58
2.1.3	Vectors.....	59
2.1.4	Oligonucleotides.....	60
2.1.5	Antibodies.....	64
2.2	Cloning techniques	64
2.2.1	Polymerase Chain Reaction (PCR).....	64
2.2.2	TA and TOPO TA cloning.....	65
2.2.3	DNA agarose gel electrophoresis.....	65
2.2.4	Purification of DNA from agarose gels.....	65
2.2.5	Restriction enzyme digests.....	66

2.2.6	Alkaline phosphatase treatment of vector DNA	66
2.2.7	Ligation of DNA	67
2.2.8	Preparation of electrocompetent cells	67
2.2.9	Transformation of electrocompetent or chemically competent cells ..	67
2.2.10	Direct colony PCR	68
2.2.11	Isolation of plasmid DNA	68
2.2.12	DNA sequencing	69
2.2.13	Analysis and manipulation of DNA sequences	70
2.3	Cell culture	70
2.3.1	Cell lines and culture conditions	70
2.3.2	Transfection of cells.....	71
2.3.3	“Quick” isolation of genomic DNA for PCR	71
2.4	Survival assays	72
2.4.1	Preliminary MMC survival assay.....	72
2.4.2	MMC survival assay	72
2.4.3	X-ray survival assay.....	72
2.5	Identification and isolation of PAC DNA.....	73
2.5.1	PAC filter probing.....	73
2.5.2	Isolation of PAC DNA from bacterial cells.....	74
2.6	Cytogenetic analysis.....	74
2.6.1	Chromosomal mapping of PAC DNA.....	74
2.6.2	Spontaneous chromosome aberration analysis	75
2.6.3	Sister chromatid exchange (SCE) analysis.....	75
2.7	Reverse transcriptase (RT)-PCR.....	75
2.7.1	Isolation of RNA from mammalian cells	75
2.7.2	RT-PCR	76
2.8	Site-directed mutagenesis	77
2.9	Immunofluorescence.....	78
2.10	Cell cycle analysis.....	78
2.11	Western blotting.....	79
2.11.1	Preparation of whole cell protein extracts	79
2.11.2	Polyacrylamide gel electrophoresis.....	79
2.11.3	Western blotting.....	79
2.11.4	RAD51L2 immunoprecipitation.....	80
2.12	Homologous recombination repair assay	80
2.12.1	GFP expression assay.....	80
2.12.2	Southern blotting.....	81

CHAPTER 3: THE *RAD51L2* GENE COMPLEMENTS THE DNA DAMAGE SENSITIVE PHENOTYPE OF THE *IRS3* CELL LINE

3.1	Introduction.....	84
3.2	Results.....	87
3.2.1	<i>RAD51L2</i> cDNA complements <i>irs3</i> MMC sensitivity.....	87
3.2.2	Recloning of <i>RAD51L2</i> into a bicistronic expression vector	89
3.2.3	<i>RAD51L2</i> PAC DNA complements <i>irs3</i> MMC sensitivity.....	93
3.2.4	<i>RAD51</i> cDNA does not complement <i>irs3</i> MMC sensitivity	94
3.2.5	<i>RAD51L2</i> cDNA complements <i>irs3</i> X-ray sensitivity	94
3.2.6	<i>RAD51L2</i> cDNA complements an increased level of spontaneous chromosome aberrations in <i>irs3</i>	95

3.3	Discussion	97
------------	-------------------------	-----------

CHAPTER 4: IDENTIFICATION OF IRS3 AS A *RAD51L2*-DEFICIENT CELL LINE

4.1	Introduction.....	100
4.2	Results.....	101
4.2.1.	Cloning the hamster <i>RAD51L2</i> gene	101
4.2.2	Sequencing the <i>RAD51L2</i> gene in V79 and <i>irs3</i>	105
4.2.3	Little or no <i>RAD51L2</i> protein in <i>irs3</i> cells.....	107
4.2.4	Hamster <i>RAD51L2</i> cDNA partially complements <i>irs3</i> MMC sensitivity	109
4.3	Discussion	111

CHAPTER 5: ROLE OF *RAD51L2* IN *RAD51*-DEPENDENT HRR

5.1	Introduction.....	116
5.2	Results.....	122
5.2.1	Reduced levels of sister chromatid exchange in <i>irs3</i>	122
5.2.2	Damage-dependent <i>RAD51</i> focus formation is reduced in <i>irs3</i>	123
5.2.3	Reduced homologous recombination in <i>irs3</i>	127
5.3	Discussion	134

CHAPTER 6: USE OF IRS3 TO IDENTIFY FUNCTIONAL DOMAINS IN *hs RAD51L2*

6.1	Introduction.....	137
6.2	Results.....	140
6.2.1	Role of the two <i>RAD51L2</i> start codons	140
6.2.2	Site-directed mutagenesis of the <i>RAD51L2</i> Walker A box	145
6.2.3	Localisation of the <i>RAD51L2</i> protein.....	147
6.3	Discussion	150

CHAPTER 7: DISCUSSION

7.1	Summary of main findings	153
7.2	Analysis of <i>irs3</i> transfection experiments	154
7.3	Comparison of <i>irs3</i> with other <i>RAD51</i>-like gene deficient cell lines	158
7.4	Role of the <i>RAD51</i>-like proteins in HRR	162
7.5	Identification and characterisation of damage-response genes; the contribution of mutant cell lines	169
7.6	The future.....	171

BIBLIOGRAPHY

French, C.A., Masson, J.Y., Griffin, C.S., O'Regan, P., West, S.C. and Thacker, J. (2002) Role of mammalian *RAD51L2* (*RAD51C*) in recombination and genetic stability. *J Biol Chem*, 277, 19322-30.

Figures

Figure 1.1	Sources of DNA damage and the major biochemical DNA repair pathways	5
Figure 1.2	Signalling in response to DNA damage	8
Figure 1.3	ATM and ATR signalling in response to DNA damage.....	13
Figure 1.4	Interactions of the BRCA1 and BRCA2 proteins.....	18
Figure 1.5	Structure of the MRN complex	23
Figure 1.6	The Fanconi anaemia pathway	26
Figure 1.7	Model of DSB repair by non-homologous end joining	33
Figure 1.8	Two models of gene conversion.....	38
Figure 1.9	Single-strand annealing.....	39
Figure 1.10	Break-induced replication	40
Figure 1.11	Proposed pathways for the recombinational repair of stalled replication forks	42
Figure 1.12	Proteins involved in the early stages of mammalian homologous recombination	44
Figure 1.13	RAD51-like protein complexes in human cells.....	54
Figure 3.1	<i>RAD51L2</i> cDNA complements the MMC sensitivity of <i>irs3</i>	88
Figure 3.2	Genomic DNA PCR and RT-PCR analysis of drug resistant cDNA transfected clones.....	91
Figure 3.3	MMC survival of V79, <i>irs3</i> and transfected clones	92
Figure 3.4	Localisation of PAC 259-05 DNA to human chromosome 17q.....	93
Figure 3.5	X-ray survival of V79, <i>irs3</i> and <i>RAD51L2</i> cDNA transfected <i>irs3</i> clones	95
Figure 3.6	<i>irs3</i> cells show an increased level of spontaneous chromosome aberrations.....	96
Figure 4.1	Strategy used to clone the hamster <i>RAD51L2</i> gene.....	103
Figure 4.2	Alignment of RAD51L2 protein sequences	104
Figure 4.3	Sequencing the <i>RAD51L2</i> gene in <i>irs3</i>	106
Figure 4.4	The <i>irs3</i> <i>RAD51L2</i> gene mutation	107
Figure 4.5	RAD51L2 immunoprecipitation.....	108
Figure 4.6	Hamster <i>RAD51L2</i> cDNA partially complements the MMC sensitivity of <i>irs3</i>	110
Figure 4.7	<i>irs3</i> and V79 co-immunoprecipitation studies.....	114
Figure 5.1	Sister chromatid exchange	118
Figure 5.2	Mechanisms for repairing a DSB	121
Figure 5.3	Damage-dependent RAD51 focus formation is reduced in <i>irs3</i> cells	125
Figure 5.4	The cell cycle profiles and RAD51 levels in V79 and <i>irs3</i> are similar	126
Figure 5.5	The DR-GFP recombination reporter substrate.....	128
Figure 5.6	Analysis of DR-GFP carrying V79 and <i>irs3</i> clones.....	132
Figure 5.7	HRR of an induced DSB is defective in <i>irs3</i>	133

Figure 6.1	Summary of <i>hsRAD51L2</i> deletion and site-directed mutagenesis constructs	142
Figure 6.2	PCR analysis of <i>RAD51L2</i> transfected clones.....	143
Figure 6.3	Human <i>RAD51L2</i> cDNA lacking the first start codon complements <i>irs3</i> MMC sensitivity	144
Figure 6.4	MMC survival of Walker A box mutants.....	146
Figure 6.5	MMC survival of L2EGFP clones.....	148
Figure 6.6	Localisation of the L2EGFP and L2EGFPNLS fusion proteins	149
Figure 7.1	Role of the RAD51-like proteins in HRR	163
Figure 7.2	Model describing a role for <i>RAD51L2</i> in repairing DNA damage during DNA replication.....	166

Tables

Table 2.1	Constructs	60
Table 2.2	Primers used to make constructs	61
Table 2.3	Primers used to clone hamster <i>RAD51L2</i> gene	62
Table 2.4	Sequencing primers.....	63
Table 2.5	Antibodies	64
Table 3.1	Analysis of <i>RAD51L2</i> transfected clones	90
Table 5.1	Reduced frequencies of spontaneous and MMC-induced SCEs in <i>irs3</i>	123
Table 7.1	Analysis of clones isolated after transfection of <i>irs3</i> with various constructs.....	157

ABBREVIATIONS

6-4PP	6-4 photoproduct
AC	5-azacytidine
ADP	Adenosine diphosphate
AT	Ataxia telangiectasia
AT-LD	Ataxia telangiectasia like disease
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATR	Ataxia telangiectasia and Rad3 related kinase
BASC	BRCA1-associated genome surveillance complex
BER	Base excision repair
BIR	Break-induced replication
BLM	Bloom's syndrome protein
bp	Base pairs
BrdU	5-bromodeoxyuridine
BS	Bloom's syndrome
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CIP	Calf intestinal alkaline phosphatase
CO	Cross over
CPD	Cyclobutane pyrimidine dimer
CRUK	Cancer Research UK
DEB	Diepoxybutane
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNA-PK _{cs}	DNA-dependent protein kinase catalytic subunit
dNTPs	Deoxyribonucleotides
DSB	Double-strand break
dsDNA	Double-stranded DNA
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EMS	Ethyl methane sulphonate
ENU	1-ethyl 1-nitrosourea
ES cells	Embryonic stem cells
EST	Expressed sequence tag
FA	Fanconi's anaemia
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FHA	Forkhead associated domain
G418	Geneticin sulphate
GC	Gene conversion
GFP	Green fluorescent protein
hDNA	Heteroduplex DNA

HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HJ	Holliday junction
HR	Homologous recombination
HRDC	Helicase and RNase D C-terminal domain
HRP	Horseradish peroxidase
HRR	Homologous recombination repair
ICL	DNA interstrand crosslink
IR	Ionising radiation
IRES	Internal ribosome entry site
irs	Ionising radiation sensitive
kb	Kilobase pair
LB	Luria-Bertani medium
LOH	Loss of heterozygosity
mAb	Monoclonal antibody
MEFs	Mouse embryonic fibroblasts
MEM	Minimum essential medium
MMC	Mitomycin c
MMR	Mismatch repair
MMS	Methyl methane sulphonate
MRC	Medical Research Council
MRN	MRE11/RAD50/NBS1 complex
NBS	Nijmegen breakage syndrome
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localisation signal
OD	Optical density
pAb	Polyclonal antibody
PAC	P1 artificial chromosome
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI3K	Phosphoinositol 3-kinase
PIKK	Phosphoinositol 3-kinase like
P-loop	Phosphate-binding loop
PVP	Polyvinylpyrrolidone
RDS	Radioresistant DNA synthesis
RNA	Ribonucleic acid
RNAi	Double-stranded RNA interference
RNase	Ribonuclease
RO	Reverse osmosis
ROS	Reactive oxygen species
RS-SCID	Radiosensitive severe combined immunodeficiency syndrome
RT-PCR	Reverse transcriptase polymerase chain reaction
SCE	Sister chromatid exchange
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i> (budding yeast)
SCID	Severe combined immunodeficiency syndrome
SDS	Sodium dodecyl sulphate
SDSA	Synthesis-dependent strand annealing
SMC	Structural maintenance of chromosomes

<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i> (fission yeast)
SSA	Single-strand annealing
SSB	Single-strand break
SSC	Saline-sodium citrate buffer
ssDNA	Single-stranded DNA
SSPE	Saline-sodium phosphate-EDTA buffer
TBE	Tris/borate/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLS	Translesion synthesis
Tris	Tris[hydroxymethyl]aminomethane
UV	Ultraviolet radiation
V(D)J	Variable Diversity Joining
WRN	Werner's syndrome protein
XP	Xeroderma pigmentosum
XP-V	Xeroderma pigmentosum variant
XRCC	X-ray cross complementing
xrs	X-ray sensitive

CHAPTER 1: Introduction

The mammalian genome is continually exposed to sources of damage that induce a variety of DNA lesions. These lesions alter the genetic code and in some cases block vital processes such as transcription and replication. It is important, particularly in dividing cells, that chromosomes do not undergo replication or segregation whilst carrying damaged DNA, as this can lead to mutations being fixed in the genome. This may result in genetic instability that is known to be associated with cancer development.

To combat this situation, mechanisms have developed to detect and repair DNA damage. Their importance is demonstrated by the fact that many of the pathway components are conserved through evolution. Defects in DNA repair genes are linked to a number of diseases that often include a predisposition to cancer. They also affect the sensitivity of patients or tumour cells to cancer treatments such as radiotherapy. An increased knowledge of DNA repair would lead to a better understanding of cancer and may allow more effective cancer treatments to be developed (Lindahl and Wood, 1999).

1.1 Sources of DNA damage

There is a large range of agents that can damage DNA which originate from both internal and environmental sources. Highly reactive free radicals are generated from oxygen and water during normal cellular processes and external agents such as sunlight, ionising radiation and tobacco smoke are encountered regularly during daily life.

Exogenous damaging agents:

Exogenous agents can be broadly divided into two groups; physical and chemical. The most common physical source of damage that cells are exposed to is radiation. The short-wave ultraviolet (UV) component of sunlight can induce dimerisation of

adjacent pyrimidines in DNA. The main lesion is the cyclobutane pyrimidine dimer (CPD) and a further lesion is the 6-4 photoproduct (6-4PP). Both of these lesions can significantly distort the DNA helix and block transcription and replication. The nucleotide excision repair (NER) pathway is responsible for removing CPDs and 6-4PPs as well as other bulky adducts from DNA. Defects in the NER pathway are responsible for the inherited disease xeroderma pigmentosum (XP) and demonstrate the importance of its function. Patients display an extreme sensitivity to sunlight and a greatly elevated risk of skin cancer (de Laat *et al.*, 1999).

Ionising radiation (IR) exposure can come from both natural and man-made sources. In the UK most people receive the majority of their exposure (85%) from natural sources, mainly radon gas from rocks (50%). Smaller amounts of γ -rays also come from rocks (14%) and some radioactive material is ingested with our food and drink (11.5%). Cosmic radiation from outer space accounts for approximately 10% of our overall dose. About 15% of our exposure to IR is attributed to man-made sources. The use of IR in medicine for radiotherapy and disease diagnosis takes up nearly this entire category (14%). The remaining amount (<1%) is made up from industrial activity and weapons fallout (http://www.nrpb.org/radiation_topics/risks/exposure.htm).

IR can induce a huge range of lesions in DNA including single-strand breaks (SSBs), double strand breaks (DSBs) and various types of base damage. They are caused either directly by the ionisation of DNA or indirectly by the generation of hydroxyl radicals from cellular water that attack DNA. As well as single sites of damage, IR can cause clustered DNA damage. This means that two or more lesions are produced on opposite DNA strands within two helical turns (Goodhead, 1994). The base excision repair (BER) pathway is responsible for the removal of most simple damages eg. modified bases, SSBs and abasic sites that result from insult by IR. However, the simultaneous processing of damages on opposite DNA strands (ie. clustered damage) by BER can generate a DSB, a very genotoxic lesion (see section 1.4.1). It is thought that clustered damages may therefore be repaired sequentially in order to avoid DSB formation (Dianov *et al.*, 2001).

There are a huge variety of chemical compounds that can damage DNA. The genotoxic properties of some of these chemicals are utilised in chemotherapy. The interstrand cross-linking (ICL) agents psoralen, mitomycin-c (MMC), cisplatin, nitrogen mustard and nitrosoureas are all used for this purpose. MMC is frequently used in this study and must undergo a cycle of oxidation and reduction in the cell to become active, a process that is stimulated in the hypoxic environment of many solid tumours. MMC interacts with guanines and forms adducts, intrastrand and interstrand cross-links. In addition, reactive oxygen species released during the activation of the chemical cause further damage to the cell. Although only 5-13% of the adducts induced by MMC are ICLs they are thought to be the main reason for its toxicity (Dronkert and Kanaar, 2001).

ICLs are an extremely toxic lesion because they stop DNA strands from separating and hence block transcription, replication and segregation. The mechanism of ICL repair has been studied extensively in bacteria and yeast but is poorly understood in mammals. Hamster cells mutated in homologous recombination repair (HRR) genes and certain NER genes, namely *ERCC1* and *ERCC4/XPF*, are hypersensitive to ICL agents and are therefore thought to participate in ICL repair. In yeast most NER proteins take part in ICL repair, but in mammals it appears that only *ERCC1* and *ERCC4/XPF* play a role. This suggests there are species differences in ICL repair mechanisms, it is anticipated that repair will be more complex in mammalian cells and more proteins will be involved. It is thought that initial incisions next to the cross-link are made by *ERCC1-XPF* followed by either HRR, break-induced replication (BIR) or single-strand annealing (SSA) (see sections 1.5.3 and 1.5.4), and possible further incisions by *ERCC1-XPF*. It should be noted that as yet there is no direct evidence for BIR in mammals (Dronkert and Kanaar, 2001).

Endogenous damaging agents:

DNA is prone to damage from reactive oxygen species (ROS), such as hydrogen peroxide, superoxide and hydroxyl radicals, which are by-products of normal cellular metabolism. The action of ROS can produce abasic sites, modified bases and SSBs in

DNA. They can also attack other components of the cell such as phospholipid membranes. Aldehydes produced as a result of lipid peroxidation can react with DNA and form adducts (Marnett and Plastaras, 2001). Like all macromolecules, DNA undergoes decay over time, even in the absence of ROS. This occurs spontaneously via hydrolysis, oxidation and nonenzymatic methylation (Lindahl, 1993). Most endogenous DNA damage is repaired by BER.

Internal damage accounts for a significant number of DNA lesions. Approximately 400 uracils and 9000 abasic sites, which are formed by cytosine deamination and hydrolytic depurination respectively, are estimated to be generated in the human genome each day (Kunkel, 1999).

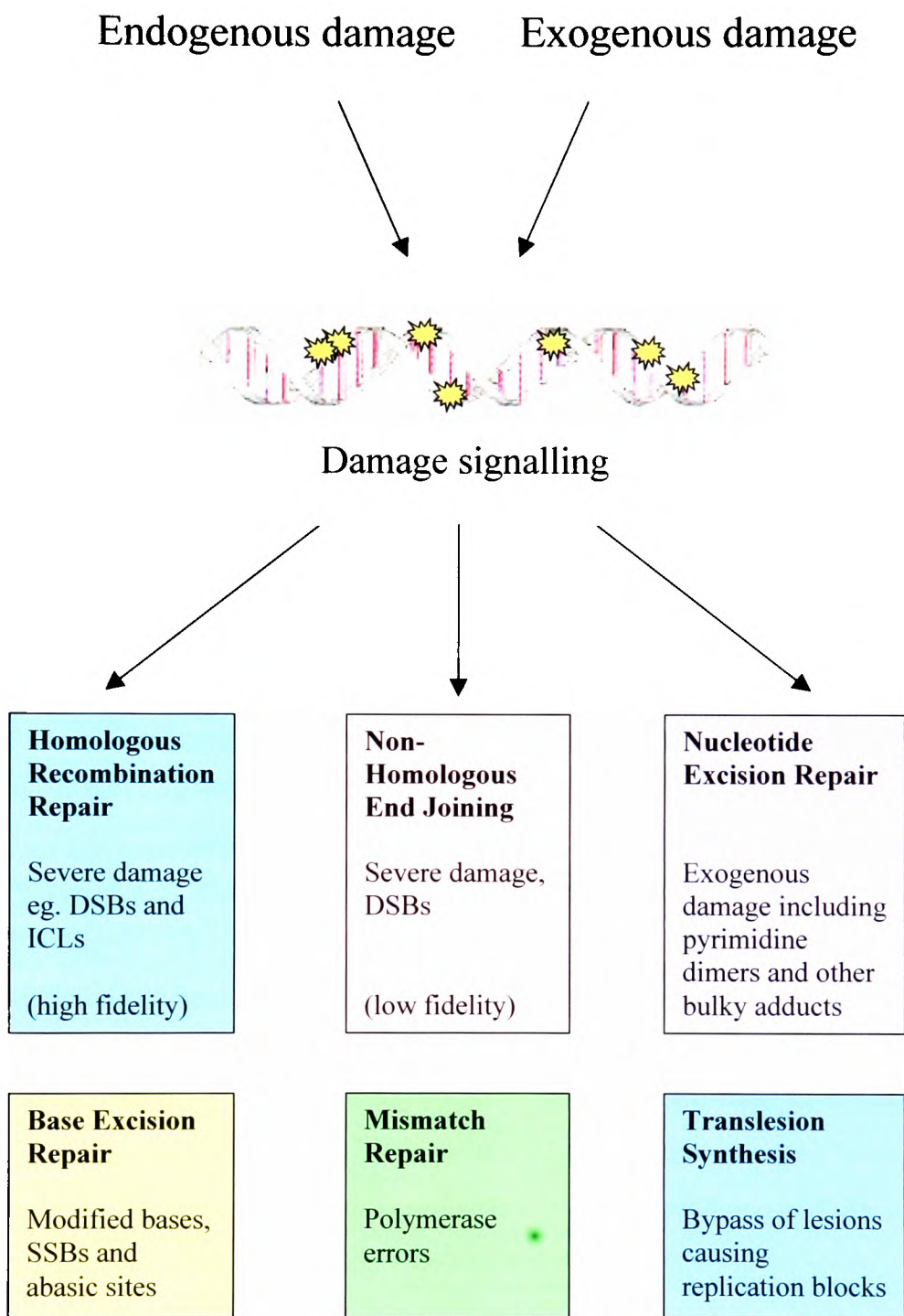


Figure 1.1 Sources of DNA damage and the major biochemical DNA repair pathways. A variety of endogenous and exogenous agents can induce DNA damage. Damage signalling activates six main repair pathways; HRR, NHEJ, NER, BER, MMR and TLS. Failure to remove lesions or inaccurate repair can lead to genome instability that contributes to carcinogenesis.

1.2 DNA damage and replication

Although cells possess a variety of DNA repair mechanisms, some unrepaired lesions are still present when the DNA comes to be replicated. DSBs can arise by replication across a SSB or by rupture of a DNA strand at a stalled replication fork. Human cells are estimated to suffer around 10 DSBs every time a cell divides (Haber, 1999). HR is thought to play a major role in the repair of DNA damage during replication, this is discussed in more detail in section 1.5.5.

It has been known for some time that cells are able to bypass replication blocks in DNA; this is inherently an error-prone process. Recently, enormous progress has been made in the field of translesion synthesis (TLS) and the ‘DNA polymerase switch model’ has emerged to explain its occurrence. When the replicative DNA polymerase encounters a block it is transiently replaced, usually by members of the Y-super-family of polymerases. These enzymes are typically slow when compared to classical polymerases and replicate undamaged templates with low fidelity, but have the ability to copy lesion containing DNA (Pages and Fuchs, 2002). One of these enzymes, Pol η , can replicate accurately through a TT CPD, the major lesion caused by sunlight. The crystal structure of Pol η shows that its active site is quite open and can accommodate two template nucleotides rather than the one that other polymerases hold, making it ideal for this function. About 20% of patients displaying the symptoms of XP do not have a defect in NER and have been designated XP-variants (XPV). A number of these patients have mutations in the *Pol η* gene, showing that error-free replication of UV-damaged DNA prevents cancer formation (Lehmann, 2002; Prakash and Prakash, 2002).

1.3 Mechanisms of response to severe DNA damage

The pathways that respond to DNA damage must be finely tuned and selective. They need to be sensitive enough to be triggered by very small amounts of damage, for example one DSB. Damaged DNA is recognised either directly or indirectly by sensor proteins that signal the presence of abnormalities. Transducer proteins amplify the signal via protein kinase cascades and target a series of effector molecules that initiate the damage response (Fig. 1.2).

One response to DNA damage is to slow progression through the cell cycle. This is thought to provide time for repair pathways to act on lesions and prevents damaged DNA from being replicated. These temporary halts in the cell cycle are referred to as 'checkpoints'. The G1/S and intra-S checkpoints prevent entry into S-phase and slow progress through S-phase respectively, stopping the replication of damaged DNA. The G2/M checkpoint blocks entry into mitosis and stops the segregation of damaged chromosomes.

Another DNA-damage response is an increase in synthesis of deoxyribonucleotides (dNTPs) which are required for the DNA synthesis step of repair. This response is particularly important in non-dividing cells and is achieved partly by transcriptional induction of the ribonucleotide reductase subunit p53R2 by p53. Finally, if the damage inflicted is deemed too great to repair, cells are targeted to the apoptosis pathway. As yet it is not clear how this decision is reached (Jackson, 2002).

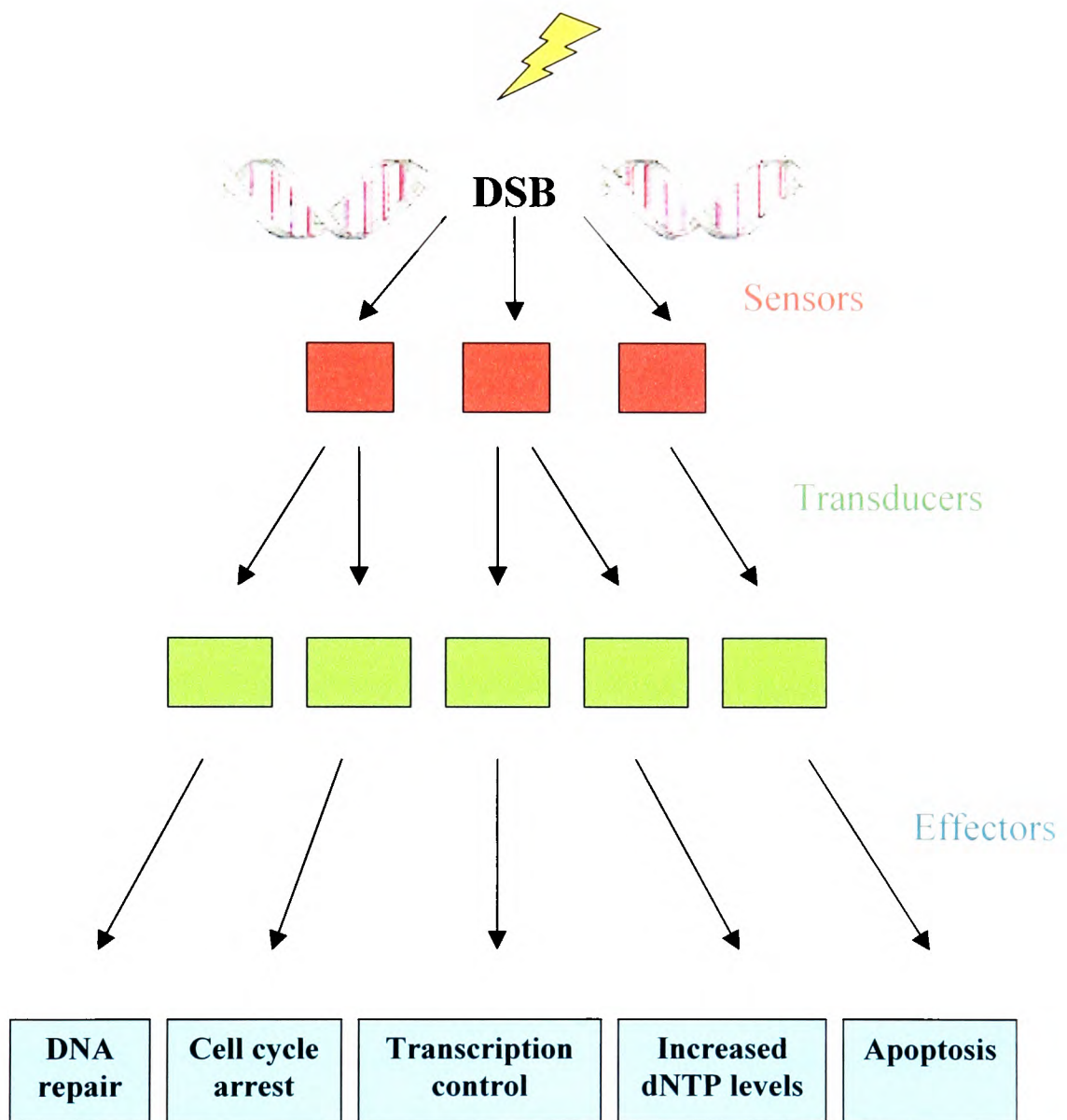


Figure 1.2 Signalling in response to DNA damage. DNA damage, such as a DSB, is recognised by sensor proteins that transmit the signal via a transduction cascade to effector proteins. These molecules activate processes such as DNA repair and cell cycle arrest, or apoptosis if the damage is too severe to repair.

1.3.1 ATM and ATR sense and signal DNA damage

ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3 related) are considered to be at or near the 'start' of the damage-signalling pathway (Abraham, 2001)

ATM has been more extensively studied than ATR because it was discovered first and is associated with the human syndrome AT (ataxia-telangiectasia). AT is a rare, autosomal-recessive inherited disorder with a complex phenotype. Patients exhibit progressive neuronal degeneration, immunodeficiency, sterility, small stature and telangiectasia (dilation of small blood vessels) in the face and ears. They are also clinically radiosensitive and predisposed to cancer (60-180 fold increase), with the majority of the malignancies being lymphomas and T-cell leukemias. AT cells display chromosome breakage, telomere instability, dysfunctional apoptosis and a reduced p53 response. They also show radiosensitivity but are not sensitive to agents such as UV light or replication inhibitors that do not cause DSBs. In addition, AT cells are defective in the G1/S, intra-S and G2/M checkpoints and show radioresistant DNA synthesis (RDS) (Kastan and Lim, 2000; Thompson and Schild, 2002).

The *ATM* gene encodes a large 370kDa S/T kinase that belongs to the phosphoinositol 3-kinase (PI3K) family of proteins. Other members of this family include ATR and DNA-PK, as well as the yeast checkpoint proteins Mec1 from *S.cerevisiae* and Rad3 and Tel1 from *S.pombe*. The catalytic domain is located towards the C-terminus of the protein and is flanked by two FAT (FRAP/ATM/TRRAP) domains.

Atm knockout mice are viable but develop thymic lymphomas at an early age. *Atm*^{-/-} mouse embryonic fibroblasts (MEFs) undergo premature growth arrest and show elevated levels of p21. This growth arrest is not seen in MEFs from *Atm*^{-/-} *Trp53*^{-/-} and *Atm*^{-/-} *p21*^{-/-} double knockout mice. *Atm*^{-/-} *Trp53*^{-/-} animals show an accelerated rate of tumour formation whereas *Atm*^{-/-} *p21*^{-/-} animals show a delay in the rate of tumour formation but increased radiosensitivity. The phenotype of *Atm* heterozygous mice is less severe than that of the *Atm* homozygotes. Treatment of *Atm*

heterozygotes with a low dose of radiation causes premature greying and reduces life span (Barlow *et al.*, 1999).

In contrast to *Atm* knockout mice, *Atr* knockout mice die during the early stages of embryonic development. *Atr*^{-/-} cells show chromosome fragmentation, apoptosis and loss of proliferation potential. Both mouse and chicken DT40 *ATR*^{-/-} cells are not viable. Expression of a dominant negative form of ATR, mutated in the kinase domain, results in radiosensitivity and an ineffective G2/M checkpoint. Unlike ATM-deficient cells, they are also sensitive to UV light and DNA replication inhibitors (Durocher and Jackson, 2001).

ATM and ATR have been shown to phosphorylate a large number of proteins including MDM2, p53, CHK1, CHK2, BRCA1, NBS1, c-ABL, FANCD2, H2AX, and RPA, by either *in vitro* or *in vivo* experiments. These proteins are involved in signalling via many pathways, which can bring about processes such as apoptosis and the stress-mediated transcription of genes. The roles of ATM and ATR in checkpoint signalling and the activation of DNA repair genes is discussed below and outlined in Fig. 1.3. ATM and ATR appear to operate in partially overlapping pathways and are able to act on many of the same substrates. In AT cells the substrates of ATM are still phosphorylated after damage but with slower kinetics, presumably by ATR. Overexpression of ATR can also complement some of the phenotypes of ATM-deficient cells such as RDS. However, although ATM and ATR have overlapping functions they are activated by separate signals. ATM mediates the phosphorylation and stabilisation of p53 in response to IR, whereas ATR is required for the signalling of both UV damage via the rapid phosphorylation of p53 and stalled replication forks.

The way in which ATM and ATR are induced after damage has been a long-standing question. However, the mechanism of ATM activation has recently been elucidated. In unirradiated cells ATM is held as an inactive dimer or multimer, with the kinase domain bound to a region around S1981, which is located in the FAT domain. After treatment with IR rapid autophosphorylation of ser 1981 occurs which causes dimer dissociation and initiates kinase activity. This is a very sensitive mechanism and most ATM molecules in a cell are activated after doses as low as 0.5Gy (Bakkenist and Kastan, 2003).

An important function of ATM and ATR is the activation of checkpoints, which slow down the cell cycle and allow time for lesions to be repaired. There are two mechanisms in which the G1/S checkpoint is activated and it is unclear whether they are used simultaneously or independently. The first mechanism involves the activation and stabilisation of p53 by ATM/ATR. This occurs in three different ways and illustrates how ATM and ATR can act on several members of the same pathway in order to ensure a particular outcome. Firstly, ATM/ATR phosphorylates p53 on ser15, which causes p53 transcriptional activation. Secondly, ATM/ATR act on CHK2, which goes on to phosphorylate p53 on ser20. This modification makes it more difficult for the ubiquitin ligase MDM2 to bind to p53, hence stopping p53 degradation. Thirdly, ATM/ATR phosphorylates MDM2 directly, which prevents it binding to p53. The resulting increase in p53 levels causes transcriptional induction of p21 that inhibits the CDK2-cyclin E complex, which is normally responsible for the G1 to S transition. The second mechanism is rapid and p53 independent. ATM activates CHK2, which then phosphorylates CDC25A. This targets CDC25A for destruction and stops it from activating the CDK2-cyclin E and CDK4-cyclin D complexes (Fig. 1.3) (Nyberg *et al.*, 2002).

Details of the intra-S checkpoint can be found in section 1.3.3. ATM/ATR phosphorylate NBS1 (part of a complex with MRE11 and RAD50), BRCA1 and FANCD2. All of these proteins are thought to play some part in delaying S-phase. The G2/M checkpoint mainly functions to block CDC25C activity, which usually assists entry into mitosis by removing inhibitory phosphatases on CDC2. ATR and ATM phosphorylate CHK1 and CHK2 respectively, which then phosphorylate CDC25C on ser216 creating a binding site for 14-3-3 protein. 14-3-3 moves CDC25C into the cytoplasm and renders it catalytically less active. The protein wee1 can also be phosphorylated by CHK1, this provides a binding site for 14-3-3 and the resulting complex acts to inhibit CDC25C. Finally, p53 induces 14-3-3 σ after DNA damage and triggers it to bind directly to the CDC2-cyclin B complex and sequester it to the cytoplasm (Fig. 1.3) (Nyberg *et al.*, 2002).

ATM is known to regulate several proteins linked to DSB repair. As noted above, ATM phosphorylates NBS1, which as part of the NBS1/MRE11/RAD50

complex has been linked to repair by both HR and NHEJ. It also phosphorylates BRCA1 and can activate BRCA1 indirectly by acting on CHK2 and CtIP to cause their dissociation from BRCA1. BRCA1 and RAD51, a key protein in HRR, have been shown to form a complex in response to DNA damage. In addition, it has been suggested that signalling through ATM may cause c-ABL to phosphorylate RAD51. However, c-ABL is not essential for ATM's chromosome maintenance functions in chicken DT40 cells (Kastan and Lim, 2000; Shiloh, 2001).

The radiosensitive phenotype of AT cells could result from a deficiency in repair rather than just defective checkpoints. There is genetic evidence from DT40 cells that suggests a role for ATM in the HRR pathway. *ATM*^{-/-}*Ku70*^{-/-} double knockout cells show radiosensitivity and a greatly elevated level of IR-induced chromosome aberrations whereas *ATM*^{-/-}*RAD54*^{-/-} show only slightly higher aberrations levels, placing ATM and RAD54 on the same pathway (Morrison *et al.*, 2000).

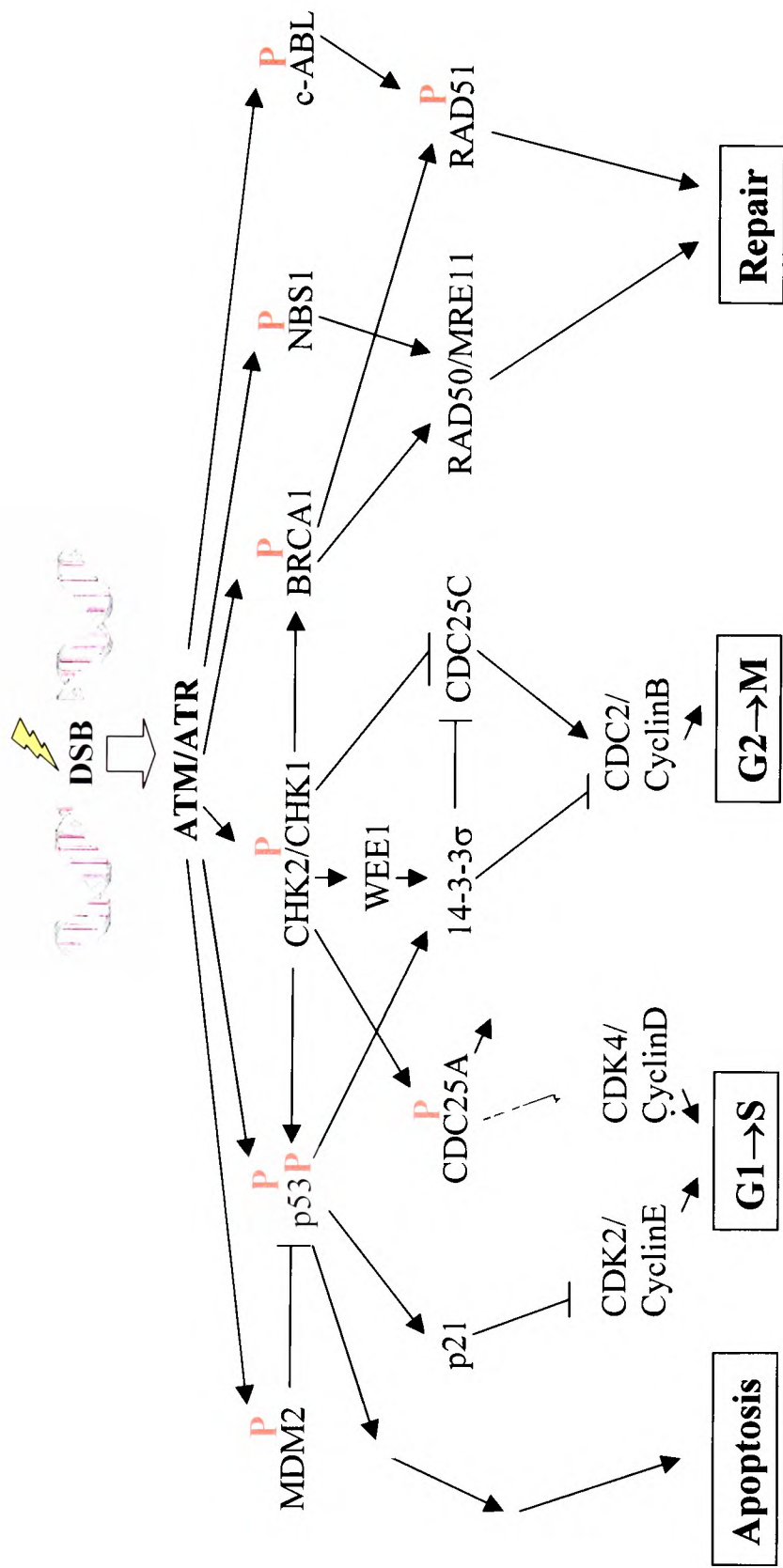


Figure 1.3 ATM and ATR signalling in response to DNA damage. Activation of ATM and ATR leads to the phosphorylation of many cellular target proteins. Some of the main proteins involved in checkpoint signalling and the induction of DNA repair are shown in the diagram above. Details of the S-phase checkpoint are not shown here but can be found in section 1.3.3.

1.3.2 Functions of BRCA1 and BRCA2 in the DNA damage response

Around 10% of all breast cancer cases show a familial pattern of inheritance. Germline mutations in the *BRCA1* or *BRCA2* gene account for a significant number of these cases and have also been associated with other cancers including those of the ovaries and pancreas. *BRCA1* and *BRCA2* mutations are highly penetrant and patients carrying a mutation in one allele of *BRCA2* are associated with a lifetime risk of breast cancer incidence of up to 85% (Nathanson *et al.*, 2001). The roles of BRCA1 and BRCA2 in cancer susceptibility and DNA repair have been recently reviewed (Liu and West, 2002; Venkitaraman, 2002).

Disruption of *Brcal* and *Brca2* in mice leads to embryonic lethality. *Brcal*- and *Brca2*-deficient cell lines show elevated levels of spontaneous and induced chromosome aberrations, increased sensitivity to DNA damaging agents and higher mutation rates. These cell lines are also defective in HR as measured by the I-*SceI* DSB assay (see Chapter 5). In addition *Brcal* cells appear to be impaired in SSA whereas *Brca2* cells are proficient for SSA (Jasin, 2002).

The *BRCA1* and *BRCA2* genes code for two large proteins of 208kDa and 384kDa respectively. The BRCA1 protein has an N-terminal RING domain, which is implicated in several protein-protein interactions. The C-terminus of BRCA1 contains two BRCT domains, which are found in many proteins involved in DNA repair and cell cycle control. The main feature of the BRCA2 protein is eight BRC repeat motifs. They are found in the middle of the polypeptide and provide a binding site for RAD51 (Venkitaraman, 2001).

The BRCA1 and BRCA2 proteins are poorly conserved across mammalian species. For example, the human BRCA1 protein shares just 56% sequence identity with the mouse BRCA1 protein. This is much less than that shown by other comparable proteins such as p53 and RAD51, which show 77% and 99%, sequence identity between human and mouse respectively. However, functional conservation of the BRCA1 protein must exist because human *BRCA1* can rescue the embryonic lethality of *BRCA1*-deficient mice. As yet, no BRCA1 or BRCA2 homologues have been found in budding or fission yeast, although BRCA2 homologues have been

identified in a diverse range of other eukaryotic species and BRCA1 homologues have been identified in chicken, frog and worm (Jasin, 2002).

BRCA1 and BRCA2 localise to the nucleus and are widely expressed during S- and G2-phase. They also co-localise with RAD51 to nuclear foci in S-phase or after treatment of cells with DNA damaging agents. In addition, the BRCA1 protein has been shown to bind to DNA with preference for branched structures and long molecules. At present the significance of this finding is unclear.

The BRCA2 protein interacts directly with RAD51, a protein essential for DSB repair by HR. This interaction occurs primarily via the eight BRC repeats found in BRCA2. The RAD51 protein coats ssDNA substrates to form a nucleoprotein filament that invades homologous templates and initiates strand exchange. Interestingly, RAD51 filament formation is suppressed, and pre-assembled filaments are dissolved, when certain BRC repeat peptides are overexpressed (Davies *et al.*, 2001). Recently, the crystal structure of RAD51 bound to BRC4 showed this to be because the BRC repeat mimics a sequence in RAD51 that is required for oligomerisation. BRCA2 binding therefore prevents filament formation by interfering with a crucial contact between the RAD51 monomers (Pellegrini *et al.*, 2002). The BRCA2 protein is thought to have a direct role in HRR because of its interaction with RAD51. It is possible that BRCA2 binds to RAD51 to carry it to sites of DNA repair or to prevent recombination occurring at the inappropriate times. During the latter stages of HRR, BRCA2 may bind to RAD51 to facilitate the removal of RAD51 filaments from DNA.

Although BRCA1 can interact with BRCA2, and co-localises with BRCA2 and RAD51 in nuclear foci, it should be noted that the majority of BRCA1 does not associate with either protein. BRCA1 is part of BASC (BRCA1-associated genome surveillance complex) (Wang *et al.*, 2000b) and also interacts with numerous other proteins (Deng and Brodie, 2000) (Fig. 1.4). On the whole, proteins that interact with BRCA1 do not interact with BRCA2, suggesting that they exist in mutually exclusive complexes. These complexes could have different functions that overlap at sites of DNA repair. It is thought that BRCA1 does not function directly in HRR but has a

regulatory role, which is facilitated through its many interacting proteins. Some of the possible functions of BRCA1 and its associated proteins are discussed below.

As described in the previous section, BRCA1 is involved in the signalling of DNA damage and checkpoint control. BRCA1 is phosphorylated by ATM, ATR and CHK2 in response to DNA damage. Each of these kinases is activated by different lesions and targets a particular set of BRCA1 residues suggesting that the signalling will be distinct in each case. One of the outcomes of this signalling is the induction of the S-phase and G2/M checkpoints, which are defective in BRCA1-deficient cells. BRCA1 also interacts with the MRE11/RAD50/NBS1 complex, which may be involved in signalling or have a more direct role in the processing of DSB ends (see section 1.3.3) (Venkitaraman, 2002).

BRCA1 also functions as a regulator of transcription and interacts with the RNA polymerase II holoenzyme through RNA helicase A. An example of transcriptional control by BRCA1 is the regulation of expression of the tumour suppressor gene *GADD45*. *GADD45* transcription is normally repressed by a complex of BRCA1 and ZBRK1, a KRAB domain transcription factor. However, after treatment with IR, ATM phosphorylates BRCA1 and this repression is relieved.

BRCA1 interacts with BARD1 through the N-terminal RING finger domain. This complex has been shown to have ubiquitin ligase activity that is far more efficient than that exhibited by either of the component proteins alone. BRCA1 also interacts with BAP1, which is a ubiquitin hydrolase protein. It is possible that BARD1 and BAP1 act in concert to control BRCA1 ubiquitination. If a process such as ubiquitination were used widely by BRCA1 to regulate the functions of substrate proteins it could make interactions involving small proportions of the overall BRCA1 pool biologically significant. It is therefore important to try and identify targets of BRCA1-dependent ubiquitination in order to determine how important this function is (Liu and West, 2002). One potential target is the Fanconi anaemia protein FANCD2. This protein is known to be ubiquitinated in a BRCA1-dependent manner and co-localises with BRCA1 in damage-induced nuclear foci (see section 1.3.4).

Finally, BRCA1 could regulate DNA repair processes at various stages. It has been shown to interact with a SWI/SNF complex and regulators of histone acetylases

and deacetylases, which could function in chromatin remodelling making the area surrounding a break more accessible to repair factors. Recently, BRCA1 has been shown to regulate BACH1 that functions in DNA repair. BACH1 is a putative DNA helicase and was identified because it interacts with the BRCA1 C-terminal BRCT motifs. Over-expression of BACH1 carrying a mutation in the ATPase domain greatly decreases the ability of cells to repair DSBs. This effect can be rescued by adding a second mutation that stops BACH1 interacting with BRCA1. Interestingly, BACH1 and BRCA1 co-localise to nuclear foci during S- and G2-phase. BACH1 focus formation is BRCA1-dependent and does not therefore occur in BRCA1-deficient cells. Together these results show that the repair function of BACH1 is mediated through BRCA1 and suggest that BRCA1 may recruit BACH1 to sites of DNA repair. BACH1 mutations have been identified in 2 out of 65 women with early-onset breast cancer both of whom were normal for BRCA1 and BRCA2 (Cantor *et al.*, 2001).

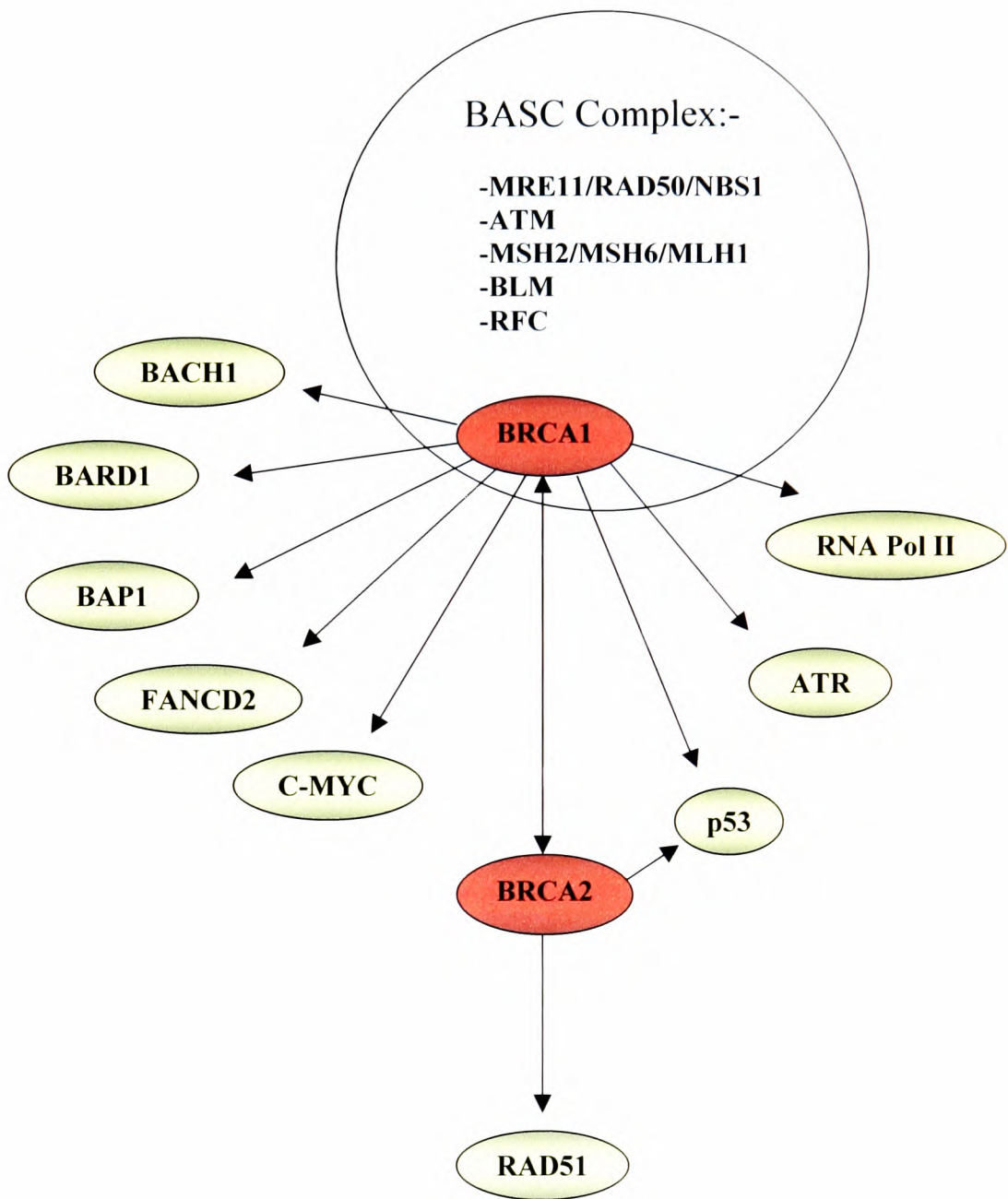


Figure 1.4 Interactions of the BRCA1 and BRCA2 proteins. Proteins that interact with BRCA1 do not generally interact with BRCA2, suggesting different functions for the BRCA1 and BRCA2 complexes. The diagram illustrates a selection of interesting interactions, as determined by yeast two hybrid and immunoprecipitation experiments, but does not provide an exhaustive list of proteins that bind to BRCA1 and BRCA2.

1.3.3 The MRE11/RAD50/NBS1(XRS2) (MRN/X) complex

The three components of the MRN/X complex were first identified in yeast in screens for mutants hypersensitive to DNA damage (*XRS2* and *RAD50*) or defective in meiotic recombination (*MRE11*). Homologues were subsequently identified in humans; the last to be found was *NBS1*, which shares only 29% sequence identity with its yeast homologue *XRS2*. Null mutations in mouse *Mre11*, *Rad50* or *Nbs1* all cause early embryonic lethality (D'Amours and Jackson, 2002).

Hypomorphic mutations in the human *NBS1* and *MRE11* genes have been shown to cause the rare genetic disorders Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disease (ATLD). NBS patients exhibit developmental defects, microcephaly, immunodeficiency and a predisposition to cancer development. NBS cells show chromosomal instability, sensitivity to DNA damaging agents and checkpoint defects. In many ways the phenotype of NBS is very similar to that of AT, but NBS patients do not display ataxia or telangiectasia. ATLD patients were identified as having AT-like symptoms but were found not to have mutations in the *ATM* gene. They were eventually shown to have mutations in the *MRE11* gene. So far no human disorders resulting from mutations in *RAD50* have been identified (Thompson and Schild, 2002).

The MRE11 protein interacts with NBS1 and RAD50 independently and can form dimers or multimers with itself. The protein has an N-terminal portion containing 4 phosphoesterase motifs that form a nuclease domain. MRE11 has 3'-5' dsDNA exonuclease activity and ssDNA and dsDNA endonuclease activity. Nuclease activity is structure specific; the exonuclease acts on substrates with blunt ends or 3' recessed ends but not 3' overhangs, and the endonuclease also acts weakly on hairpin structures. The presence of RAD50 stimulates both exonuclease and endonuclease activity. Endonuclease activity alone is stimulated by NBS1 and ATP. In addition, MRE11 has DNA-binding activity that is stimulated by RAD50 and NBS1. Together these proteins can partly unwind short regions of duplex DNA. This strand-dissociation activity is specific for substrates with 3' overhangs and is stimulated by ATP. MRE11 has also been shown to have strand-annealing activity

and facilitates the formation of duplex DNA from complementary ssDNA ends (D'Amours and Jackson, 2002).

The RAD50 protein has a Walker A and a Walker B motif at its N- and C-terminus respectively, which comprises a bipartite ATP-binding domain. These domains are separated by an extended coil-coiled region that contains a central CXXC motif. RAD50 has homology to members of the SMC (structural maintenance of chromosomes) family which function in chromosome condensation and sister chromatid cohesion. SMC proteins form either homo- or hetero- dimers through intermolecular coiled-coil domain interactions; this brings the Walker motifs together to form a functional domain. Recent structural studies have shown that RAD50 tertiary structure is similar to that of the SMC proteins (Fig. 1.5 A). The RAD50 protein appears to fold back on itself forming a rod-like structure through intramolecular coiled-coil interactions. Dimerisation occurs through the zinc-finger-like hook domain at the centre of the coiled-coil region. MRE11 binds to the RAD50 dimer at the base of the coiled-coil domain. The exact location of NBS1 in the complex is still unclear (de Jager and Kanaar, 2002).

The structure of the RAD50 dimer suggests that its main function is to bind DNA fragments and keep them close together. It could therefore play a role in holding broken chromatids together or could facilitate the search for regions of sequence homology required in HRR. In addition, it has been proposed that RAD50 stimulates the activity of DNA ligases to repair DSBs. It may also restrict the nuclease activity of MRE11 to prevent nucleolytic degradation past a certain point (Fig. 1.5 B) (D'Amours and Jackson, 2002; de Jager and Kanaar, 2002).

The extreme C-terminus of the NBS1 protein is necessary for interaction with MRE11. The NBS1 protein also has an N-terminal forkhead-associated (FHA) domain and BRCT domain. FHA domains bind target proteins in a phosphodependent manner allowing protein-protein interactions to be regulated by phosphorylation. Deletion of the FHA domain stops NBS1 damage-dependent focus formation but does not lead to radiosensitivity. BRCT domains are also known to mediate protein-protein interactions. The NBS1 BRCT domain has not been

identified in budding yeast but is found in fission yeast, making its function unclear (Tauchi *et al.*, 2002b).

The MRN complex has been implicated in many cellular processes. For example, it is required for the maintenance of telomeres in plants, yeast and mammals. Mammalian NBS1 interacts with the telomere binding proteins TRF1 and TRF2 during early S-phase when telomere elongation occurs. MRE11 and RAD50 bind to TRF2 throughout the cell cycle. This suggests that the MRN complex may function in some aspect of telomere replication. Telomeres in NBS1 cells are shorter than those in wild type cells. This can be complemented by co-expression of NBS1 and hTERT (the telomerase catalytic subunit) (D'Amours and Jackson, 2002; Tauchi *et al.*, 2002b).

MRE11 and NBS1 are phosphorylated in response to DNA damage by either ATM or ATR (depending on the type of lesion induced). MRE11 phosphorylation is dependent on NBS1 but the precise residues modified have not been identified yet. NBS1 is phosphorylated in an ATM-dependent manner on S343 and S278. Mutation of S343 results in IR hypersensitivity that is exacerbated when S278 is also mutated. Phosphorylation of these two residues is necessary for type III MRE11 focus formation (see below) and a normal S-phase checkpoint (D'Amours and Jackson, 2002).

NBS cells exhibit RDS, which characterises intra-S phase checkpoint failure. The intra-S phase checkpoint is mediated by 2 separate pathways. The first pathway involves ATM- and NBS1-dependent phosphorylation of SMC1 a component of the cohesin complex that is required for sister chromatid cohesion during S-phase. The second pathway is mediated by CHK2 and CDC25A and appears to be normal in NBS cells. ATLD cells also show RDS suggesting that MRE11 is required in addition to NBS1 for intra-S phase checkpoint function. It appears that MRE11, like NBS1, operates in the SMC1 pathway rather than the CHK2/CDC25A pathway. The involvement of the MRN complex in G1 and G2 checkpoint control remains controversial because only partial deficiencies are seen in NBS1 cells. However, there is reduced p53 and p21 induction (G1 checkpoint proteins) and a defect in

CHK2 activation (G2 checkpoint protein) in NBS1 cells (D'Amours and Jackson, 2002; Tauchi *et al.*, 2002b).

The MRE11 protein has been shown to localise to three types of nuclear foci. Type I foci occur in untreated cells and co-localise with promyelocytic leukaemia (PML) bodies. Type II foci can be observed in damaged cells 10 min to 8 h after treatment whereas type III foci can be seen later, between 4 h and 24 h after treatment. Type III foci are larger and less numerous than type II foci. Neither type II nor type III foci co-localise with the HR protein RAD51 (Mirzoeva and Petrini, 2001). However, MRE11 does co-localise and interact with γ H2AX in damaged cells, and in cells undergoing V(D)J or class switch recombination (D'Amours and Jackson, 2002).

MRE11 foci are also formed during S-phase and co-localise with proliferating cell nuclear antigen (PCNA), an important replication protein. These foci are not formed in Blooms Syndrome (BS) fibroblasts implying a role for BLM in recruiting the MRN complex to sites of DNA damage (see section 1.3.6). Interestingly, MRE11 foci form in XP-V cells (deficient in translesion synthesis) but not in wild type cells after exposure to UV irradiation. These cells are unable to process UV-induced lesions and this unrepaired damage results in DSB production at stalled replication forks. Finally, MRE11-deficient frog replicating extracts show formation of DSBs in the newly replicated DNA. These data illustrate the important role of the MRN complex during DNA replication, in the absence of DNA damage, and provide an explanation for the inviability of vertebrate MRE11 cell lines (D'Amours and Jackson, 2002; Tauchi *et al.*, 2002b).

The role of the MRN complex in DSB repair has been controversial. There is evidence in *S.cerevisiae* that links the complex with the NHEJ pathway but as yet no clear involvement has been shown in mammals. Comparison of *MRE11*^{-/-} *Ku70*^{-/-} DT40 cells with the single mutants has shown that these genes are not epistatic, suggesting that MRE11 does not function in the vertebrate NHEJ pathway (Yamaguchi-Iwai *et al.*, 1999). However, recent evidence does indicate a role for the MRN complex in vertebrate HRR. NBS1 is recruited to sites of DNA damage by direct interaction with γ H2AX (Kobayashi *et al.*, 2002) and may then be involved in repairing this damage by HRR. *NBS1*^{-/-} chicken DT40 cells show a decrease in MMC

induced SCEs and targeted integration frequencies as well as a 100-fold reduction in HR events as measured by the SCneo reporter assay. In contrast, no defects in NHEJ were observed (Tauchi *et al.*, 2002a). This phenotype is very similar to that displayed by other HR deficient mutants.

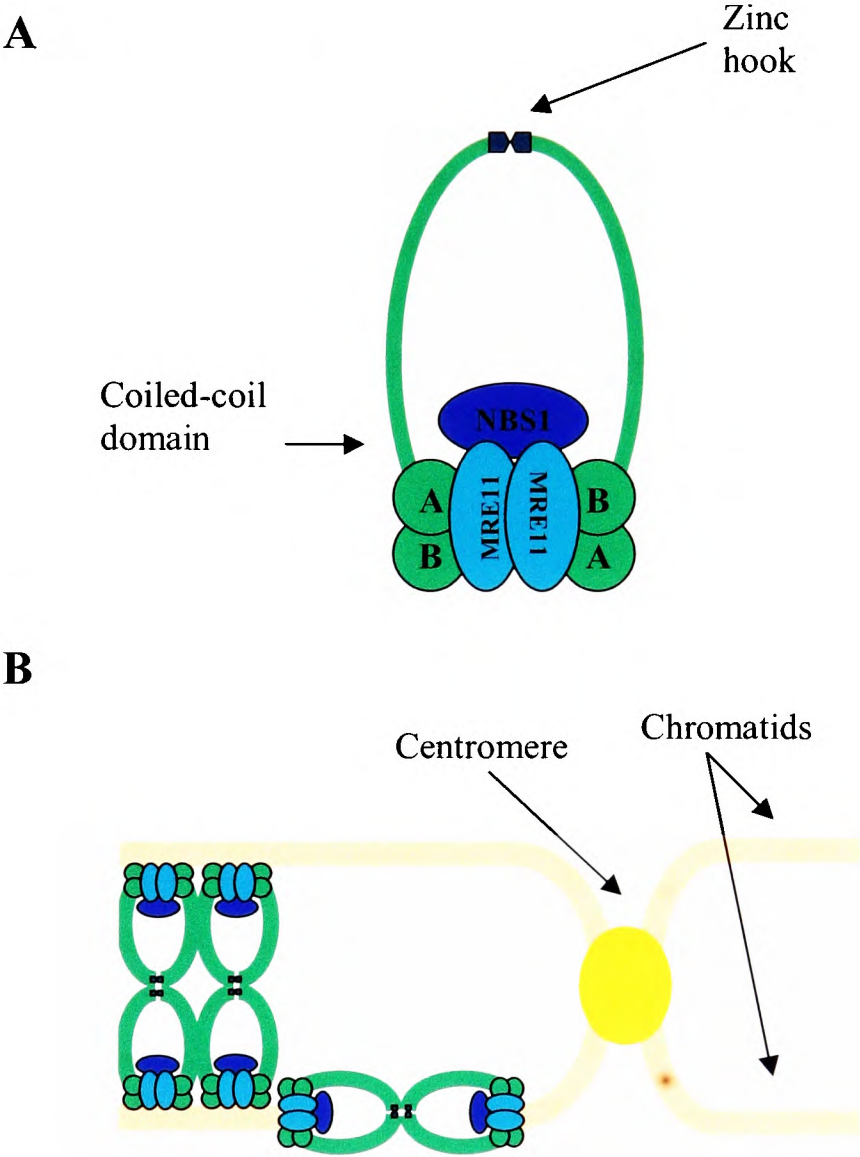


Figure 1.5 Structure of the MRN complex. A, Possible structure of the MRN complex. RAD50 (green) Walker A (A) and B (B) motifs are brought together by an intramolecular coiled-coil. MRE11 binds at the base of the coiled-coil. Speculatively, NBS1 is shown binding to the MRE11 dimer. B, Schematic representation of the way in which the MRN complexes may hold together a broken chromatid. MRN complexes are shown to interact through zinc hooks which co-ordinate a zinc ion between molecules. It has also been suggested that the MRN complex may be flexible and ‘straddle’ the break which may disrupt the MRE11 dimer.

1.3.4 Structural maintenance of chromosomes (SMC) proteins

As noted above, the SMC protein RAD50 has a role in HRR, possibly as a structural component which holds homologous sequences together during the process. However, other SMC proteins have also been implicated in HRR but as yet their precise functions remain unclear. The cohesin complex consists of two SMC proteins (SMC1 and SMC3) as well as SCC1 and SCC3 (Nasmyth, 2002). In yeast, loss of cohesin integrity leads to reduced DSB repair capacity as well as chromosome segregation defects. Similarly, the recently identified SMC proteins Rad18 and Spr18 form a heterodimer that is required for UV damage tolerance. These proteins are not part of the classical NER pathway and are epistatic with Rhp51. They are thought to play a role in HRR by holding broken DNA molecules together (Fousteri and Lehmann, 2000).

1.3.5 The Fanconi anaemia pathway

FA is a rare autosomal recessive disease characterised by progressive aplastic anaemia caused by loss of bone marrow stem cells and cancer susceptibility, mainly acute myeloid leukaemia and squamous cell carcinoma. Around two thirds of patients also suffer from skeletal abnormalities, reduced fertility and hypopigmentation of the skin. Other organs affected include the heart, kidneys, gastrointestinal tract and central nervous system. Many phenotypes have been linked to FA cells the most consistent of which is hypersensitivity to DNA cross-linking agents such as MMC, diepoxybutane (DEB) and cisplatin. In addition, an increased proportion of FA cells contains 4N DNA, which indicates a late S-phase or G2/M delay in the cell cycle. The molecular basis of Fanconi anaemia (FA) has recently been reviewed (D'Andrea and Grompe, 2003; Thompson and Schild, 2002).

The symptoms of FA result from mutations in several different genes. So far eight separate complementation groups have been identified FA-A, B, C, D1, D2, E, F and G. Groups A, C and G account for the majority of FA patients, 65%, 15% and 10% respectively. The genes from all the groups have now been cloned and *FANCA*,

C, E, F, G and *D2* were found to be novel genes. Interestingly, the *B* and *D1* groups have been identified as having biallelic mutations in the *BRCA2* gene and express truncated *BRCA2* proteins. Interallelic complementation probably makes it possible for two complementation groups to be mutated in the same gene (Howlett *et al.*, 2002).

Five FA proteins (*A, C, E, F* and *G*) interact and form a complex in the nucleus. The complex appears to have some chromatin binding activity, which is increased when cells are treated with cross-linking agents. FANCE may also interact with FANCD2, which is separate from the main body of the complex. The FA complex mediates monoubiquitination of FANCD2 at K561 in S-phase or in response to DNA damage, although no catalytic domains have been identified in any of the complex components. FANCD2-Ub is then translocated to chromatin and also to nuclear foci where it co-localises with BRCA1 (Garcia-Higuera *et al.*, 2001) and BRCA2/RAD51 (Fig. 1.6). FANCD2 has been found to coimmunoprecipitate with BRCA1 from irradiated cell extracts. In BRCA1-deficient cells, FANCD2 is not monoubiquitinated and FANCD2 focus formation is reduced.

FANCD2 is phosphorylated on S222 (and other residues) by ATM in response to ionising radiation. S222 phosphorylation also requires the NBS1 protein. The S222 modification is necessary for activation of the S-phase checkpoint that does not occur when cells are transfected with a FANCD2 S222A mutant (Fig. 1.6). The ubiquitination and phosphorylation modifications appear to be part of separate signalling pathways because phosphorylation is not required for MMC resistance or FANCD2 focus formation (Taniguchi *et al.*, 2002).

The phenotype of FA cells and the links between the FA and BRCA pathways suggest that these proteins may operate in HRR. However, at present there is limited direct evidence to support this and the specific role that the FA proteins might play remains unclear. The FA pathway is not essential for HRR because FA knockout mice are viable whereas RAD51- and BRCA2-null mice are embryonically lethal. It should be noted that other functions, such as maintaining redox status, have been suggested for the FA proteins.

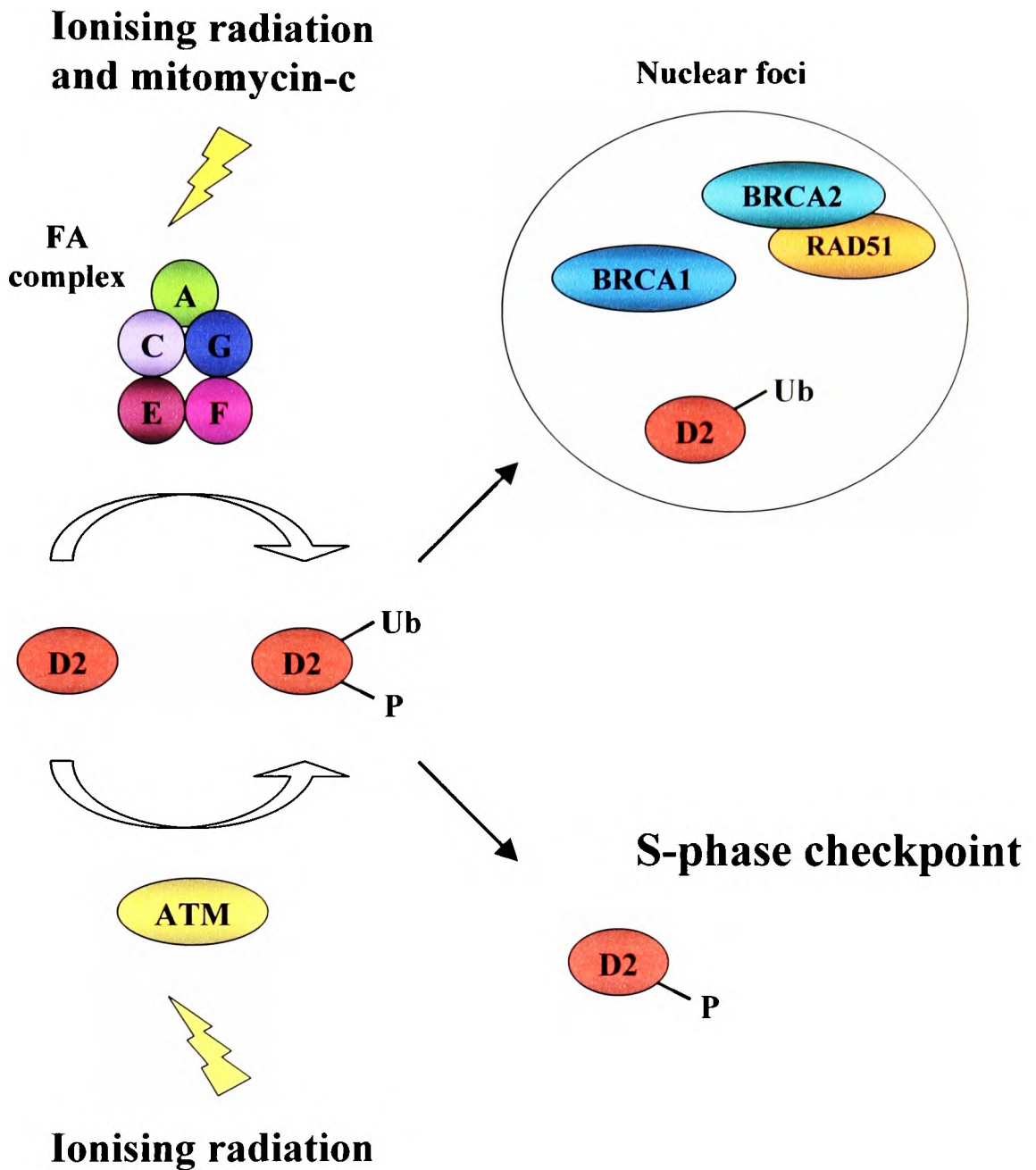


Figure 1.6 The Fanconi anaemia pathway. The FANCD2 protein operates in two separate signalling pathways. In response to MMC or IR, the FA complex monoubiquitinates (Ub) FANCD2 at K561. The activated protein co-localises in nuclear foci with BRCA1 and BRCA2/RAD51. In response to IR, ATM phosphorylates (P) FANCD2 on S222. This modification is required for activation of the S-phase checkpoint.

1.3.6 The Blooms syndrome helicase

Blooms syndrome (BS) is a rare autosomal recessive disorder characterised by growth deficiency, immunodeficiency, infertility in males and reduced fertility in females, susceptibility to diabetes, sun sensitivity, early onset of the menopause and a predisposition to cancer. The types of cancers shown include those displayed by the general population such as leukemias, carcinomas and lymphomas as well as rare cancers such as Wilms tumour and osteosarcoma. The main phenotype of cells from BS patients is an increased number of recombination events, in particular sister chromatid exchanges (SCEs). BS cells also have increased levels of chromosome aberrations and elevated mutation rates. Additionally, they grow slowly and have a protracted S-phase (Mohaghegh and Hickson, 2002).

BS is caused by mutations in the *BLM* gene. In most cases these mutations truncate BLM and the remaining portion of the protein is degraded. It is therefore assumed that most BS patients have no functional BLM protein indicating that it is not required for viability in humans. However, *Blm*^{-/-} mice die at E13.5 (Chester *et al.*, 1998), although in one case viable offspring were obtained. These animals were found to be cancer prone and are thought to be hypomorphs rather than true nulls (Luo *et al.*, 2000).

BLM is a member of the RecQ family of DNA helicases. This family also includes the Werner syndrome protein (WRN) and the protein defective in Rothmund-Thomson syndrome (RecQL4). Like all RecQ family members, BLM has a conserved domain containing 7 motifs commonly found in DNA and RNA helicases. These motifs include a Walker A box (typical of ATP-binding proteins) and a DEAH box (required for ATP hydrolysis). There are also two further regions of sequence similarity towards the C-terminal end of the protein. The RecQ-Ct (C-terminal) domain is immediately downstream of the helicase domain and has an unknown function. The HRDC (helicase and RNase D C-terminal) domain is found in several non-helicase proteins and may be involved in DNA binding or mediating function through protein-protein interactions (Mohaghegh and Hickson, 2002; Nakayama, 2002).

The BLM protein has helicase activity with 3'-5' polarity which can act on several substrates with different structures. BLM can unwind gapped duplex DNA but is not capable of unwinding duplexes from a blunt-ended terminus or from an internal nick. However, it can unwind blunt-ended duplexes with an internal bubble. BLM can also act on joint molecules and synthetic Holliday junction structures. In addition, BLM can unwind G-quadruplex DNA, which forms in G-rich regions of the genome such as telomeres. It can also unwind triple helix DNA if a 3' tail is attached to the third strand. Triple helix DNA is thought to be present under certain circumstances at telomere ends (Mohaghegh and Hickson, 2002; Nakayama, 2002).

It is likely that BLM plays a role in HRR and is involved in resolving stalled replication forks. A partial deletion in the *BLM* gene in chicken DT40 cells results in increased sensitivity to MMS, and elevated levels of SCEs and gene targeting efficiency. These phenotypes return to near normal levels in a *BLM RAD54* mutant, indicating that the increased SCEs seen in the *BLM* mutant are due to events processed by the HRR pathway (Wang *et al.*, 2000a). BLM interacts directly with RAD51 and forms nuclear foci. In untreated cells, a proportion of BLM foci co-localise with RAD51 and RPA foci. The number of BLM foci and the extent to which BLM and RAD51 foci co-localise increases in response to IR. RAD51 focus formation is not dependent on BLM but the number of RAD51 foci is elevated in untreated BS cells (Wu *et al.*, 2001). BLM also interacts with the MMR protein MLH1 that functions in both HRR and MMR. BS cells are not defective in MMR so it would appear that this interaction again links BLM to HRR (Langland *et al.*, 2001; Pedrazzi *et al.*, 2001). BLM can be phosphorylated by both ATM and ATR. After hydroxyurea treatment BLM is phosphorylated by ATR, a modification required for BLM-dependent localisation of the MRN complex to sites of stalled replication forks (Nakayama, 2002; Thompson and Schild, 2002).

There are two mechanisms by which BLM has been proposed to restore stalled replication forks. Firstly, a replication fork stalled at a DNA lesion may regress and form a 'chicken foot structure'. After the damage has been removed by repair proteins or lesion bypass polymerases, BLM may restart replication by reverse branch migration, a recombination-free process. In the absence of BLM, recombination

would have to be used to resume replication leading to higher HR rates and an increased numbers of SCEs (Karow *et al.*, 2000). Secondly, HR may always be used in the restart of replication. In this case BLM could be involved in the removal of Holliday junctions, either by branch migration of the junction towards the end of the chromosome or by recruitment of resolvases (Wu *et al.*, 2001). The hyperecombinagenic phenotype of BS cells is not as easily accounted for with this model.

Finally, the BLM protein has been linked with apoptosis. BLM and p53 interact directly and BS cells show reduced p53-mediated apoptosis after DNA damage. However, the precise way BLM functions in this process remain unclear (Nakayama, 2002; Thompson and Schild, 2002).

1.4 Double strand break repair pathways

1.4.1 DNA double strand breaks

One of the most toxic forms of damage inflicted on DNA by mutagens is the double strand break (DSB). DSBs occur when both strands of the double helix are broken in close proximity and the ends cannot be held together by base-pairing or chromatin structure.

DSBs can be induced by exogenous agents including IR and some chemicals, for example chemotherapeutic drugs. They can also be produced endogenously by reactive oxygen species and mechanical stress on chromosomes. In addition, breaks can occur when a replication fork encounters a DNA lesion, for example a SSB, or collapses. Although DSBs present a serious threat to the cell they are generated as part of specialised biological processes. DSBs are induced during meiosis to enable recombination between homologous chromosomes, and during V(D)J recombination to provide diversity in immunoglobulin and T-cell receptor proteins. They also occur in the processes of immunoglobulin class-switch mutation and somatic hypermutation. DSBs can also be induced artificially in a defined context for experimental purposes using endonucleases such as I-SceI (van Gent *et al.*, 2001).

The nature of DSBs makes them more complicated to repair than other types of DNA damage. Both DNA strands are broken so the complementary strand cannot be used as a template, as for example it is in NER, BER or MMR. The two free ends are also likely to become dissociated from one another making repair more difficult and providing the opportunity for inappropriate recombination with other sites in the genome. Finally, the DNA ends often sustain base damage, which means that they must be processed before the DSB itself can be dealt with.

It is vitally important that DSBs are repaired with high fidelity. Error-prone DSB repair can result in the fusion of broken ends from different chromosomes or nonadjacent regions of the same chromosome. This can lead to chromosome deletions and translocations that can affect many genes simultaneously. This can cause genome instability and carcinogenesis (Haber, 2000a; van Gent *et al.*, 2001).

DSBs are repaired by two main processes, homologous recombination and non-homologous end joining. The features of both pathways are discussed in the next section.

1.4.2 Non-homologous end joining (NHEJ)

NHEJ uses little or no homology to rejoin DNA ends, which can lead to deletions in sequence that are mutagenic. The pathway is required for the repair of DSBs caused by exogenous DNA damaging agents but also processes DSB intermediates generated during V(D)J recombination. A model for NHEJ is outlined in Figure 1.7.

The NHEJ core proteins are well characterised and have been recently reviewed (Jackson, 2002; Khanna and Jackson, 2001). One of the main components of NHEJ is the DNA-dependent protein kinase (DNA-PK). DNA-PK is made up from a Ku heterodimer, consisting of the 69kDa and 83kDa proteins Ku70 and Ku80 respectively, and the 465kDa catalytic subunit (DNA-PK_{CS}). The Ku heterodimer binds DNA ends with high affinity and recruits DNA-PK_{CS} to the site. DNA-PK_{CS} is a S/T protein kinase and its C-terminal region has homology to the catalytic domains of phosphatidylinositol 3-kinase-like (PIKK) family proteins that include the ATM and ATR. It can phosphorylate several target proteins *in vitro* but the significance of

this is not known *in vivo*. Unlike the other core NHEJ proteins DNA-PK_{CS} has no functional homologues in yeast.

DNA-PK_{CS}-deficient mice are hypersensitive to IR and exhibit severe combined immunodeficiency syndrome (SCID) which is due to the defective rejoining of hairpin coding ends during V(D)J recombination. The phenotype of Ku knockout mice is more severe than that of the DNA-PK_{CS} knockout mice and includes growth retardation, features of premature ageing and an inability to rejoin signal ends as well as coding ends. This phenotype could indicate that Ku is required to repair a wider variety of lesions than DNA-PK_{CS} or has other cellular functions not shared with DNA-PK_{CS} (Smith and Jackson, 1999).

The ligase IV/XRCC4 complex is recruited during the latter stages of NHEJ and rejoins the broken DNA ends. *Lig4* and *Xrcc4* deficient mice die during late embryonic development and show extensive neuronal apoptosis as well as other abnormalities. Lethality and neuronal loss can be rescued by *Trp53* deficiency but mice routinely die at approximately 6 weeks of age from progenitor B-cell lymphomas that bear translocations between *c-myc* and the immunoglobulin heavy-chain locus (Barnes *et al.*, 1998; Frank *et al.*, 2000; Gao *et al.*, 2000). The defective neurogenesis observed in *Lig4*^{-/-} mice can also be rescued by *Atm*-deficiency. However, the rescue is less substantial with *Lig4*^{-/-} *Atm*^{-/-} animals surviving for just two days after birth (Lee *et al.*, 2000). These data suggest that in the single mutant it is not just the presence of unrepaired DSBs that cause lethality but also the induction of p53-dependent apoptosis or cell cycle arrest pathways.

Mutations have been found in the *LIG4* gene of humans and these patients are described as suffering from a new disorder called *LIG4* syndrome. The first patient to be identified had leukaemia and was found to be highly sensitive to radiotherapy, but did not appear to be immunodeficient. 180BR cells derived from this patient have a point mutation in the catalytic domain of DNA ligase IV at a highly conserved amino acid (Riballo *et al.*, 1999). *LIG4* mutations have subsequently been found in a further set of patients who display immunodeficiency as well as delayed growth and development (O'Driscoll *et al.*, 2001). The exact symptoms exhibited by each patient will depend on the nature of the *LIG4* mutation carried.

Recently, another group of patients termed RS-SCID (radiosensitive severe combined immunodeficiency syndrome) was identified. Cell lines from these patients are similar to DNA-PK_{CS} defective cell lines in that they can rejoin signal ends but not hairpin coding ends. The defective protein was named Artemis and is a member of the metallo- β -lactamase family, a group of enzymes that use water as a nucleophile to break covalent bonds. Purified Artemis protein possesses single-strand specific 5' to 3' exonuclease activity. It also forms a complex with DNA-PK_{CS}, and is phosphorylated by DNA-PK_{CS}, whereupon it acquires endonuclease activity, which allows it to cleave 5' to 3' overhangs and hairpins (Ma *et al.*, 2002). The Artemis/DNA-PK_{CS} complex has enzymic activities that indicate a role in opening hairpin intermediates in V(D)J recombination (Schlissel, 2002) and processing ends in NHEJ. It is also possible that Artemis is involved in the repair of clustered DNA damage caused by IR by removing base-damaged ends of DSBs (Jeggo and O'Neill, 2002).

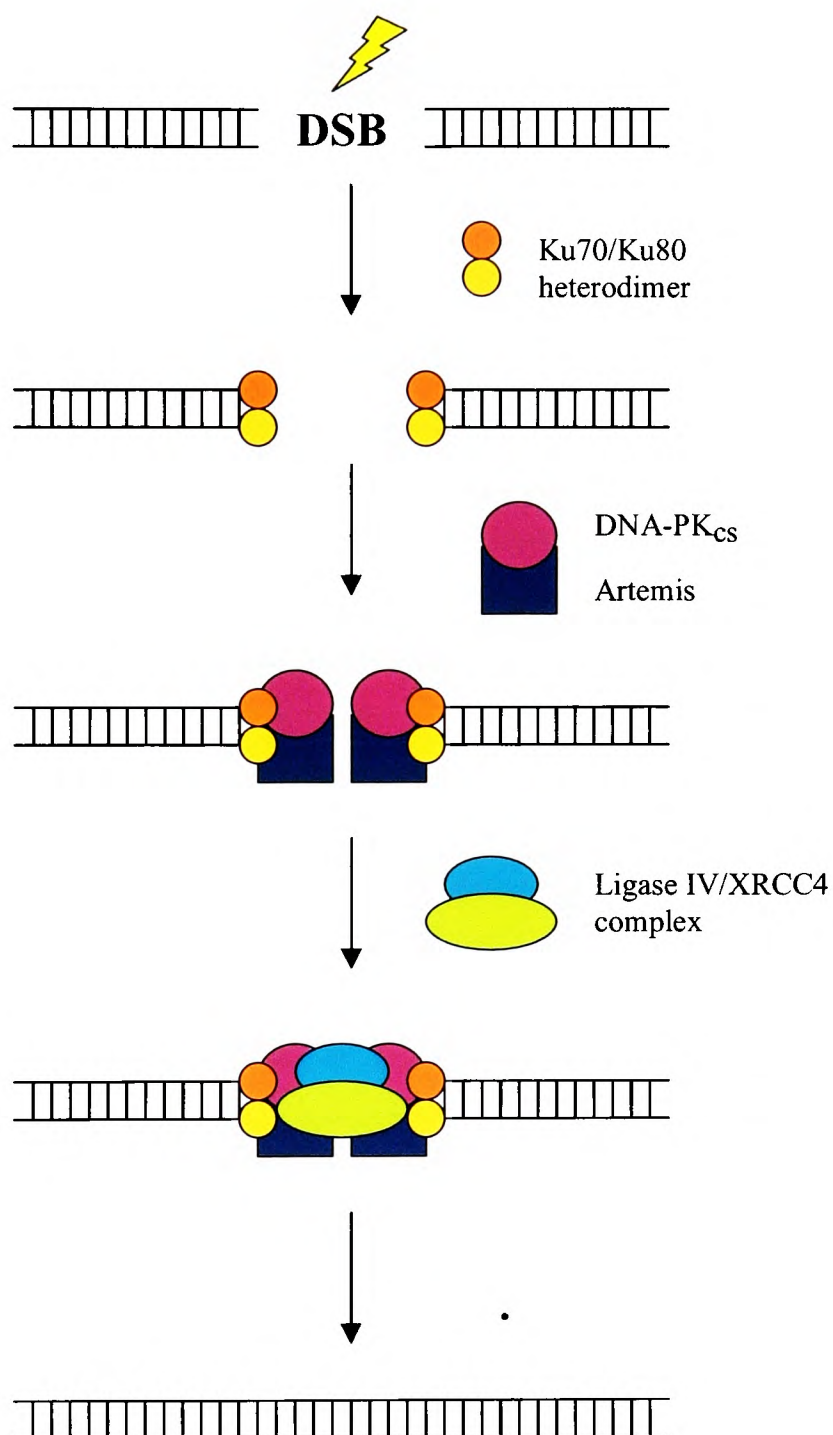


Figure 1.7 Model of DSB repair by non-homologous end joining. After a DSB is formed, the free ends are bound by the Ku heterodimer that then recruits DNA-PK_{CS} and Artemis. Repair is completed by the ligase IV/XRCC4 complex.

1.5 Homologous recombination repair (HRR)

1.5.1 Choice of DSB repair pathway

It has traditionally been thought that HRR predominates in yeast, whereas NHEJ predominates in mammals. However, this appears to be an oversimplification and it is now known that HRR does play an important role in mammalian DSB repair (Johnson and Jasin, 2000). Indeed, in some cases NHEJ cannot compensate for the loss of HRR. Mice with disruptions in several different HRR genes have been found to be embryonically lethal; *Rad51* (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996), *Rad51L1* (Shu *et al.*, 1999), *Rad51L3* (Pittman and Schimenti, 2000) and *Xrcc2* (Deans *et al.*, 2000).

The contribution of each pathway to repair is at least in part determined by cell cycle phase. The initial evidence for this came from the hamster mutant XR-1, which is deficient in the NHEJ protein XRCC4. XR-1 cells are sensitive to γ -radiation in G1- and early S-phase, but in late S-phase survival approaches that of parental cells (Stamato *et al.*, 1983). XR-1 cells are also deficient in DSB repair during the sensitive G1-phase, but repair DSBs normally during the resistant late S-phase (Giaccia *et al.*, 1985). More recently, *Ku70*^{-/-} chicken DT40 cells were shown to be hypersensitive to γ -radiation in G1- and early S-phase, whereas *RAD54*^{-/-} DT40 cells showed hypersensitivity in late S- and G2-phase (Takata *et al.*, 1998). In addition, many HRR proteins have been shown to localise to nuclear foci during S-phase in untreated cells (see section 5.1). HR in G1-phase can be deleterious to the cell because a homologous chromosome must be used as the repair template that can lead to loss of heterozygosity (LOH). In S- and G2-phase the sister chromatid can be used and exact sequence correction can be achieved. It has been shown that the sister chromatid is the preferred template for HRR and is used far more frequently than the homologous chromosome (Johnson and Jasin, 2000).

The relative contribution of the two DSB repair pathways is also affected by developmental stage. Embryonic stem (ES) cells rely heavily on the HRR pathway and are hence used for gene targeting experiments. Interestingly, *Rad54*^{-/-} embryos

are sensitive to IR but adult mice are not (unless NHEJ is also disabled), suggesting that HR is the predominant pathway used in early mouse development. This could be due to the greater availability of the sister chromatid in these rapidly dividing cells. Alternatively, high fidelity repair may be necessary for cells in the early stages of development whereas terminally differentiated somatic cells could tolerate a higher degree of inaccurate repair because much of their genome is non-functional (Essers *et al.*, 2000).

It is likely that HRR and NHEJ have independent as well as overlapping functions. *Rad54*^{-/-} mice are hypersensitive to cross-linking agent MMC regardless of developmental stage, indicating that NHEJ cannot compensate for HRR in the repair of this type of damage (Essers *et al.*, 2000). The exact mechanism by which MMC-induced cross-links are repaired has yet to be determined. However, it is likely that this type of damage does not make an ideal substrate for the NHEJ pathway, or that some of the damage is repaired by a mechanism that avoids the generation of DSBs. Conversely, *Rad54* is not required for V(D)J recombination (Essers *et al.*, 1997) and HR cannot compensate for defects in this process in NHEJ-deficient cells (Frank *et al.*, 2000; Gao *et al.*, 2000).

It has been suggested that in certain situations HRR and NHEJ work cooperatively. An induced DSB on one chromosome can be repaired using limited sequence homology on a heterologous chromosome. It appears that repair is initiated by strand invasion which extends through the homologous region into heterologous sequence. NHEJ terminates the reaction by joining the newly synthesised strands and limits the size of the region that undergoes LOH (Richardson and Jasin, 2000).

The mechanism by which repair is directed down either the HRR or the NHEJ pathway remains largely unclear. It has been suggested that the end-binding proteins RAD52 and Ku might compete for DSB ends and hence channel breaks down one of the two pathways (Van Dyck *et al.*, 1999). In another model, the interaction between Ku and MRE11 was proposed to direct DSBs to the NHEJ pathway (Goeddecke *et al.*, 1999).

1.5.2 Models of gene conversion

There are three major types of HR; gene conversion, single-strand annealing (SSA) and break-induced replication (BIR). All three pathways have recently been reviewed (Haber, 2000b; Paques and Haber, 1999). Gene conversion is defined as “a nonreciprocal transfer of genetic information from one molecule to another” and is considered to be a high fidelity mechanism of DNA repair. The molecular mechanism by which gene conversion occurs is not precisely understood but various models have been proposed. In meiosis, gene conversions are closely associated with crossing-over, a fact that forms the basis of several models including that of Szostak (Szostak *et al.*, 1983). A version of this model is shown in figure 1.8, pathway A.

In this model, the ends of a DSB are resected by 5'-3' exonuclease. The remaining 3' ssDNA overhangs are recombinagenic and invade a homologous template and initiate new DNA synthesis. The homologous template is usually the sister chromatid but can also be a homologous chromosome. This process leads to the formation of two Holliday junctions (HJs) which can be resolved in different ways to form different products. If both HJs are cleaved in the same way gene conversion will not be associated with crossing-over. However, if the noncrossed strands of one HJ and the crossed strands of the other HJ are cleaved then a crossover will occur. In this model, the newly synthesised DNA is semiconservative ie. one new strand is present on the donor molecule and one in the recipient molecule.

Although the Szostak model describes meiosis very well it is not consistent with some observations regarding mitotic gene conversion. One of the main inconsistencies involves the proportion of mitotic gene conversion events associated with crossing-over. Only a small fraction of events actually result in crossovers but the Szostak model would predict an equal outcome unless HJs were resolved preferentially.

These discrepancies led to the development of a further model called synthesis-dependent strand annealing (SDSA) (Fig. 1.8, pathway B). As with the Szostak model, strand invasion and new DNA synthesis is initiated by a 3' overhang at one end of the DSB. Replication occurs in a D-loop and the newly synthesised

strand is displaced from its template. The 3' overhang at the other end of the DSB can then either anneal to the newly synthesised strand or to the D-loop. The latter is less likely and results in HJ formation, which allows SDSA to be associated with crossing-over. Unlike the Szostak model, the newly synthesised DNA is mostly conservative ie. both new strands are found in the recipient molecule.

1.5.3 Single-strand annealing (SSA)

The SSA pathway is used when a DSB occurs between two regions of homologous sequence. 5'-3' resection of the break ends by an exonuclease produces long ssDNA tails and exposes the regions of homology. The complementary sequences then anneal and the two nonhomologous 3' ends are removed. New DNA synthesis and ligation results in two continuous strands (Fig. 1.9). It should be noted that SSA results in a deletion and is therefore an error-prone process. SSA can occur with as little as 30bp homology but is optimal at 200-400bp homology (Paques and Haber, 1999).

1.5.4 Break-induced replication (BIR)

BIR explains the long gene conversion tracts observed in *S.cerevisiae*. Again, 5'-3' resection of the break ends occurs. A 3' end of the DSB then invades a template sequence and establishes a replication fork. If the second end of the DSB does not engage in repair (as it does in gene conversion) the fork can migrate all the way to the end of the chromosome or until a converging replication fork is encountered (Fig. 1.10) (Paques and Haber, 1999).

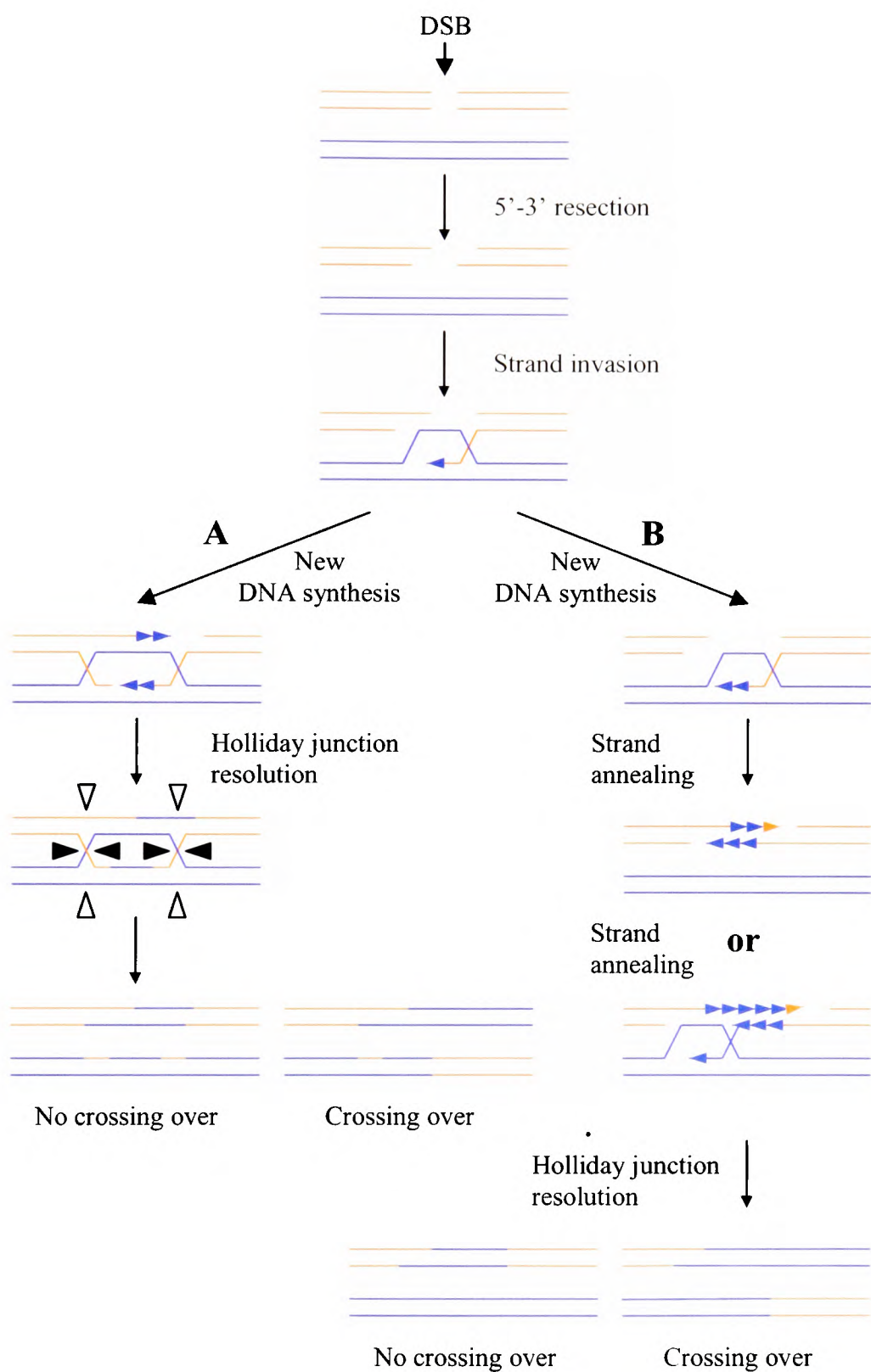


Figure 1.8 Two models of gene conversion. DSB ends are resected by 5'-3' exonuclease allowing 3' overhangs to invade a homologous template and initiate DNA synthesis. Two models predict the resolution of this structure A, Szostak model or B, SDSA (described in text). Red = donor, blue = recipient.

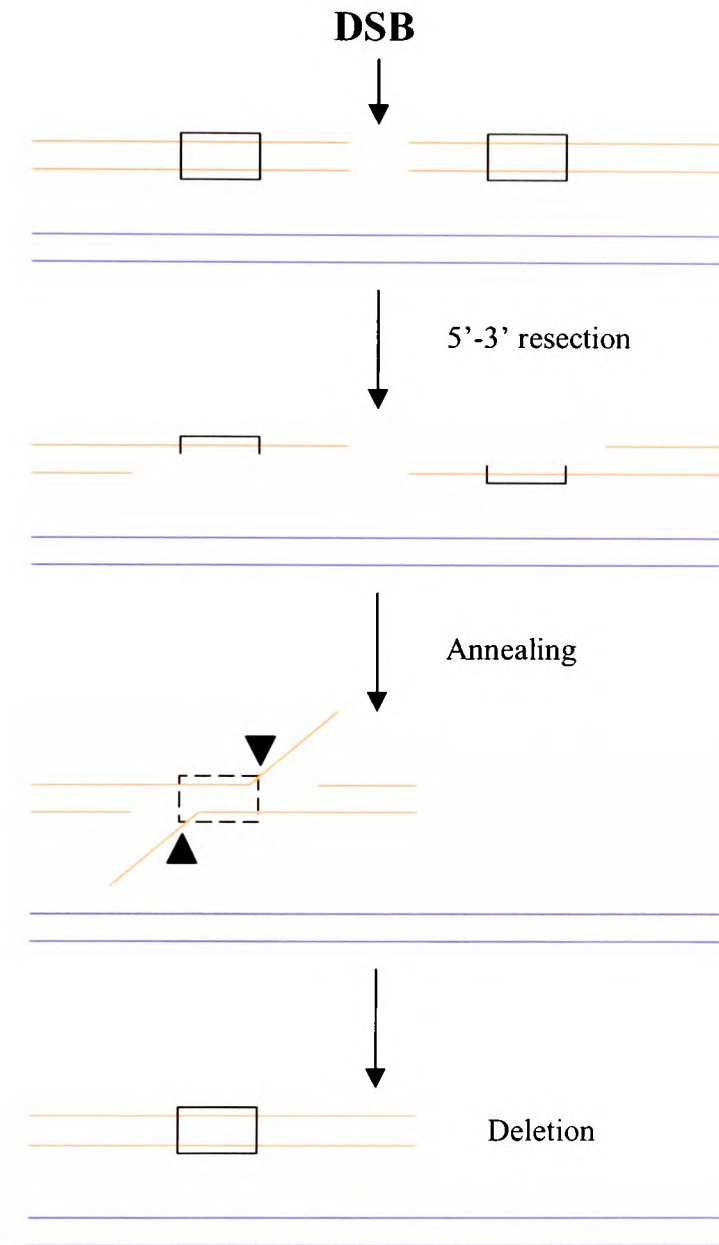


Figure 1.9 Single-strand annealing. Resection of DSB ends exposes regions of sequence homology. The complementary sequences anneal leading to a deletion. Red = damaged DNA, blue = undamaged DNA, black rectangles/ half-rectangles = regions of sequence homology and black triangles = cutting.

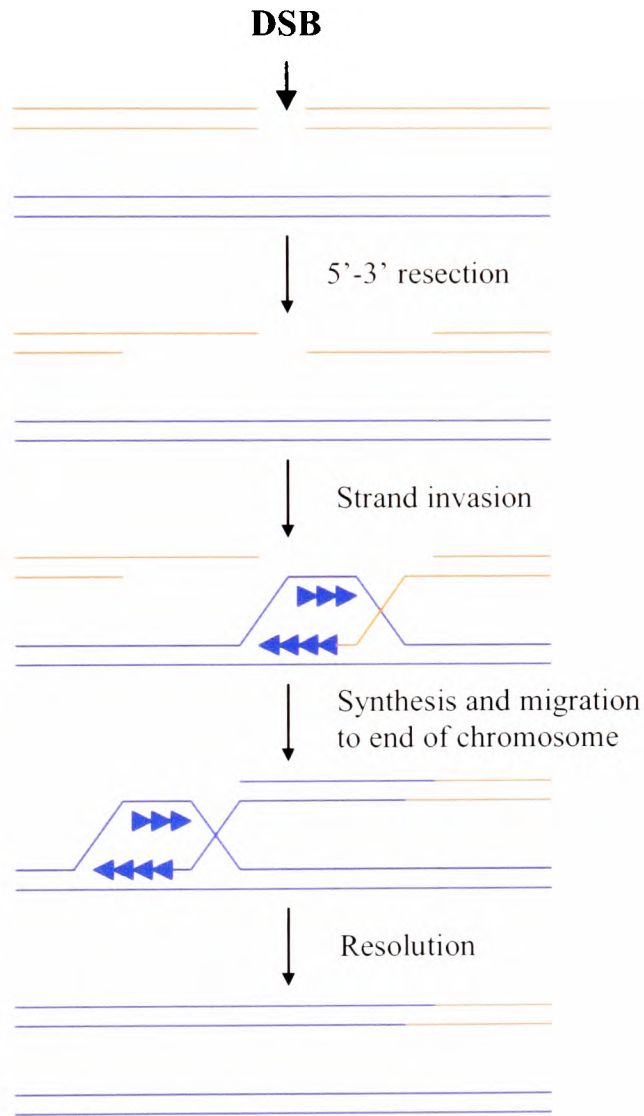


Figure 1.10 Break-induced replication. A 3' DSB end, created by resection of the break, invades a homologous template and establishes a replication fork. The other end of the DSB is not involved in repair and the fork can progress to the end of the chromosome if unhindered. Red = recipient and blue = donor.

1.5.5 Role of homologous recombination in repairing stalled replication forks

Under normal conditions the main function of the HR pathway is thought to be the rescue of stalled replication forks. This process is nonmutagenic because the donor and recipient sequences are identical.

Two repair pathways are utilised depending on the type of DNA damage encountered. If a nick is traversed it is converted into a DSB and the replication fork collapses (Fig. 1.11A). The ends of the break then undergo 5'-3' resection and the resulting 3' overhang invades the homologous template. This reconnects the branch with the rest of the chromosome and creates a HJ that is resolved to restore the fork. If a lesion is encountered which blocks replication then a different pathway is taken (Fig. 1.11B). Collision with such a lesion can leave single-stranded gaps in the fork structure. The fork may then regress forming a 'chicken foot structure' which encompasses a HJ with one short end. Cleavage of this structure results in a DSB which can be repaired via pathway A (Kuzminov, 2001; Lusetti and Cox, 2002).

It should be noted that the chicken foot structure need not be resolved by HR. As mentioned previously, the BLM protein has been implicated in reverse-branch migration of the chicken foot structure to restore replication forks. A replication fork can also be restored by nuclease digestion of the short arm of the HJ.

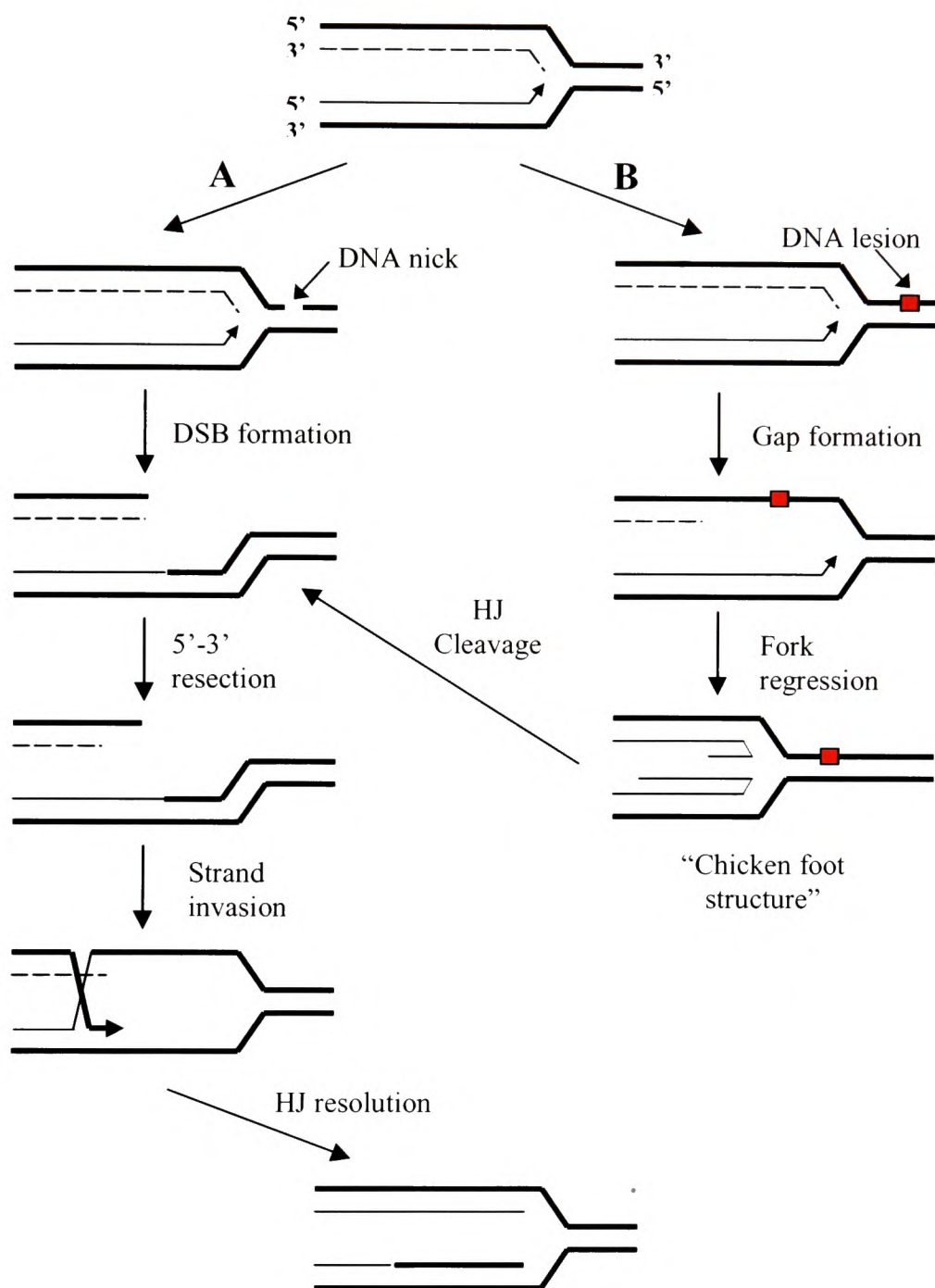


Figure 1.11 Proposed pathways for the recombinational repair of stalled replication forks. The pathway used depends on the type of damage encountered such as A, a single-stranded nick (which is converted to a DSB) or B, a blocking lesion. Both pathways are discussed in more detail in the text.

1.5.6 Homologous recombination proteins

In *S.cerevisiae*, HR is carried out by the protein products of the *RAD52* epistasis group; *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *MRE11* and *XRS2*. Mammalian homologues of nearly all of these genes have now been identified and their roles in HRR are discussed in more detail below. The RAD51 protein is central to HRR and catalyses the strand-exchange reaction which is essential to the process. RAD51 displaces RPA from ssDNA and forms a nucleoprotein filament that enables it to carry out this function. The DNA end-binding protein RAD52 and the DNA-dependent ATPase RAD54 interact directly with RAD51 and stimulate its activity. In addition, there are five RAD51-like proteins in mammalian cells that also play a role in HRR and are discussed in detail below (Fig. 1.12). There is some evidence for the involvement of the MRN complex in HRR but its precise function remains unclear (see section 1.3.3).

Although the proteins involved in the early stages of HRR are relatively well characterised, much less is known about those involved in the later stages. However, a new endonuclease, MUS81, which can act on HJs has recently been identified (Chen *et al.*, 2001). Experiments using human cell-free extracts have shown MUS81 activity to be separate from that of another as yet unidentified resolvase activity, named resolvase A (Constantinou *et al.*, 2001). Resolvase A associates with ATP-dependent branch migration and is highly specific for HJs. It shows sequence selectivity at the incision step and cleaved products can be religated which indicates that cutting occurs by the introduction of symmetrical nicks in strands of opposite polarity. These biochemical features are very similar to those exhibited by the bacterial RuvABC resolvosome. In contrast, MUS81 acts on replication fork and 3' flap structures in preference to HJs. Only a small fraction of MUS81-cleaved products can be religated suggesting that cutting does not occur by a symmetrical nicking mechanism. It has been suggested that MUS81 is a more promiscuous enzyme than resolvase A and may be involved in cleaving stalled replication forks rather than the resolution of HJs in HR (Constantinou *et al.*, 2002).

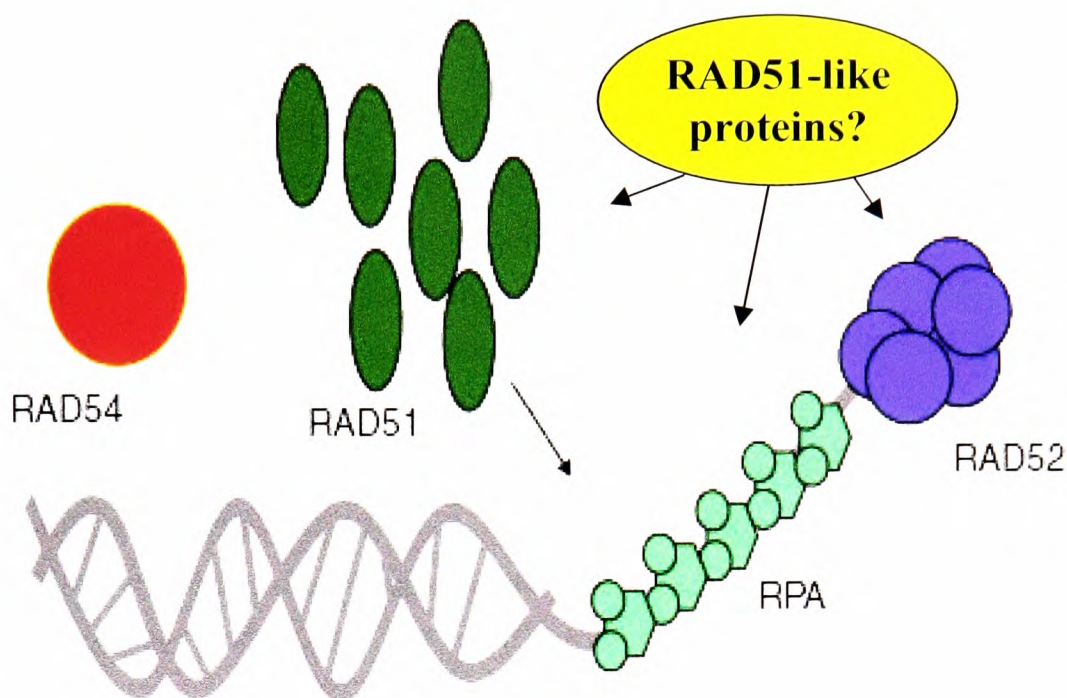


Figure 1.12 Proteins involved in the early stages of mammalian homologous recombination. The RAD51 protein displaces RPA and forms a nucleoprotein filament which catalyses strand exchange. RAD52 and RAD54 bind to RAD51 and stimulate its activity. The role of the five RAD51 like proteins in HRR remains largely unclear.

1.5.7 Mammalian RAD52 mutants exhibit a mild phenotype

Unlike most other RAD52 epistasis group proteins, mammalian RAD52 does not share a high degree of sequence homology with its yeast homologue. In *S.cerevisiae*, Rad52p is a key component of the HRR pathway and mutants show hypersensitivity to DNA-damaging agents and defects in recombination (Game, 1993; Rattray and Symington, 1994). In contrast, inactivation of *RAD52* in mice or chicken DT40 cells results in a very mild phenotype. Mutant cells show normal viability and are not hypersensitive to DNA damaging agents, but do show a reduced frequency of HR (Rijkers *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1998). *Rad52*^{-/-} mice are also fertile and show a normal immune response (Rijkers *et al.*, 1998).

Interestingly, *RAD52*^{-/-} *XRCC3*^{-/-} chicken DT40 cells are inviable and show extensive chromosome breaks suggesting that the RAD51-like proteins might

compensate for the lack of RAD52 in vertebrate cells (Fujimori *et al.*, 2001). Another explanation for the mild RAD52 phenotype could be the existence of other unidentified homologues that compensate for its function. The *S.cerevisiae* Rad59 protein shares significant homology with the N-terminal half of Rad52 (Bai and Symington, 1996). Rad59 interacts with Rad52 and promotes annealing of complementary strands (Davis and Symington, 2001). As yet, no Rad59 homologues have been found in higher organisms. In *S.pombe*, two RAD52 homologues have been identified, *RAD22A* and *RAD22B*. *rad22B* mutant cells are not sensitive to DNA-damaging agents and display normal levels of recombination, a phenotype which resembles that found in mammalian cells. *rad22A* mutants are hypersensitive to DNA-damaging agents and show defects in recombination. This severe phenotype is similar to that of the *S.cerevisiae rad52* mutant. The *rad22A rad22B* double mutant shows a further increase in sensitivity to DNA-damaging agents when compared to the *Rad22A* single mutant (van den Bosch *et al.*, 2001; van den Bosch *et al.*, 2002).

Although there is a high degree of sequence divergence between the human and the yeast RAD52 proteins they appear to have similar biochemical properties. The human RAD52 protein binds ssDNA and promotes annealing of complementary ssDNA molecules. RAD52 also interacts with RAD51 and stimulates RAD51-dependent pairing and strand-exchange activities (Baumann and West, 1999; Benson *et al.*, 1998; Reddy *et al.*, 1997). Under an electron microscope, the RAD52 protein forms ring-like structures (Van Dyck *et al.*, 1998), with each ring containing seven subunits and a large central channel (Stasiak *et al.*, 2000). RAD52 binds specifically to ss-tails produced by resection of DSB ends and protects them from nuclease attack (Van Dyck *et al.*, 1999). Binding occurs via the terminal nucleotide, and it has been proposed that the nature of this interaction leads to the formation of a ssDNA-RAD52 complex, where the ssDNA is bound in a groove on the exposed surface of the RAD52 ring (Parsons *et al.*, 2000). Recently, RAD52 alone has been shown to form filamentous complexes on ssDNA which catalyse homologous pairing (Kagawa *et al.*, 2001). The crystal structure of the RAD52 homologous-pairing domain has been determined and eleven monomers have been shown to make up a closed ring structure (Kagawa *et al.*, 2002).

1.5.8 Two RAD54 homologues in mammalian cells

RAD54^{-/-} mouse ES cells and chicken DT40 cells are hypersensitive to IR and have defects in recombination as measured by targeted integration (Bezzubova *et al.*, 1997; Essers *et al.*, 1997). A study of the repair of an induced DSB in wild type and *Rad54*^{-/-} ES cells showed that *Rad54* promotes gene conversion with predominant use of the sister chromatid as the repair template (Dronkert *et al.*, 2000).

RAD54 is a member of the SWI2/SNF2-like family of proteins that are involved in chromatin remodelling. It is a dsDNA-dependent ATPase which does not have helicase activity in conventional assays (Swagemakers *et al.*, 1998). RAD51 and RAD54 interact (Golub *et al.*, 1997) and RAD54 foci co-localise with RAD51 foci after DNA damage (Tan *et al.*, 1999). Like the *S.cerevisiae* Rad54 protein, binding of the human RAD54 protein to dsDNA induces a change in the topology of the DNA (Tan *et al.*, 1999). RAD54 has been shown to generate unconstrained positive and negative supercoils in dsDNA, which may be the result of RAD54 translocating along the DNA (Ristic *et al.*, 2001). Recent experiments using the *S.cerevisiae* Rad54 protein have shown it to dissociate Rad51 from nucleoprotein filaments formed on dsDNA indicating a role in the turnover of RAD51-dsDNA filaments (Solinger *et al.*, 2002). The yeast Rad54 protein has also been shown to have chromatin remodelling activity stimulated by the RAD51-dsDNA filament which may be required to clear protein from the DNA during the homology search step of HR (Alexeev *et al.*, 2003).

A second RAD54 homologue also exists in mammalian cells and is named RAD54B. RAD54B associates with RAD51, although the interaction is thought to be indirect as it cannot be detected by the yeast two-hybrid assay. Unlike RAD54, whose interaction with RAD51 is induced by IR, RAD54B associates constitutively with RAD51 in immunoprecipitation experiments. RAD54B forms nuclear foci in response to DNA damage which co-localise with RAD51, RAD54 and BRCA1 foci (Tanaka *et al.*, 2000). RAD54B is dsDNA-dependent ATPase, although the rate of ATP hydrolysis is lower than that observed with RAD54 (Tanaka *et al.*, 2002). In *S.cerevisiae*, there is a second RAD54 homologue called Tid1/Rdh54 which interacts

with yeast Rad51 and with the meiosis specific Rad51 homologue, Dmc1 (Dresser *et al.*, 1997; Petukhova *et al.*, 2000). Although the N-terminus of RAD54B shares sequence homology with Tid1/Rdh54 it does not bind directly to DMC1 and may have a different function (Tanaka *et al.*, 2002). Recently, the *RAD54B* gene has been inactivated in a colon cancer cell line. RAD54B-deficient cells are not hypersensitive to DNA damaging agents and have normal levels of SCEs. However, they do show reduced HR as measured by targeted integration frequency (Miyagawa *et al.*, 2002).

RAD54 and *RAD54B* may be involved in the prevention of cancer and homozygous mutations in both genes have been observed in lymphoma, colon cancer and breast cancer (Hiramoto *et al.*, 1999; Matsuda *et al.*, 1999).

1.5.9 RAD51 is an essential gene in mammalian cells

The importance of the RAD51 protein in mammalian HRR is illustrated by the phenotype of *Rad51*^{-/-} mice which show early embryonic lethality (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Embryonic development progresses further in a *Trp53*^{-/-} background but fibroblasts derived from these embryos fail to proliferate in culture (Lim and Hasty, 1996). *RAD51*^{-/-} chicken DT40 cells carrying a *RAD51* transgene under the control of a repressible promoter accumulate in G2/M-phase before dying when expression of the transgene is inhibited. The arrested cells show high levels of isochromatid breaks (Sonoda *et al.*, 1998).

The RAD51 protein shows DNA-dependent ATPase activity and promotes homologous pairing and strand exchange (Baumann *et al.*, 1996). These reactions are stimulated by the addition of RPA (Baumann and West, 1997) and RAD52 (Benson *et al.*, 1998). Unlike the bacterial RecA protein which binds to ssDNA, the preferred substrate of RAD51 is dsDNA with either 5' or 3' ssDNA tails (Mazin *et al.*, 2000; McIlwraith *et al.*, 2000). This property allows it to bind to the resected ends of a DSB and catalyse strand invasion from either the 5' or 3' end. In agreement with the biochemistry data, RAD51 forms nuclear foci after DNA damage which co-localise with RPA and RAD52 foci (Haaf *et al.*, 1999; Liu and Maizels, 2000). RAD51 focus formation is dependent on the presence of several other proteins (see section 5.1).

1.6 The *RAD51*-like gene family

1.6.1 Cloning the *RAD51*-like genes

In humans there are five genes which share sequence homology with *RAD51* and have hence been designated the *RAD51*-like gene family. The family consists of *XRCC2*, *XRCC3*, *RAD51L1* (*RAD51B*, *hREC2*, *R51H2*), *RAD51L2* (*RAD51C*) and *RAD51L3* (*RAD51D*, *R51H3*).

The *XRCC2* and *XRCC3* genes were isolated by their ability to complement the DNA damage sensitive phenotype of the hamster cell lines *irs1* and *irs1SF* respectively. Positional gene candidates were identified by analysis of somatic cell hybrids and the cDNAs were subsequently cloned. When transfected into the appropriate hamster cell line, these cDNAs complemented an extreme sensitivity to DNA cross-linking reagents (Cartwright *et al.*, 1998b; Liu *et al.*, 1998; Tambini *et al.*, 1997; Tebbs *et al.*, 1995).

The *RAD51L1* gene was isolated on the basis of its sequence homology to the recombination repair gene, *REC2*, from the corn smut *Ustilago maydis* (Rice *et al.*, 1997). *RAD51L1* was also independently identified by searching EST databases with human and yeast *RAD51* and *RAD51*-like gene sequences (Albala *et al.*, 1997; Cartwright *et al.*, 1998a). Similar methods were used to identify the *RAD51L2* (Dosanjh *et al.*, 1998) and *RAD51L3* (Cartwright *et al.*, 1998a; Pittman *et al.*, 1998) genes.

Studies looking at the expression patterns of mouse and human *RAD51*-like genes, using Northern blotting and RT-PCR, have yielded varying results. There are no data available on *XRCC3* expression in tissues. Generally, the other four genes are expressed in tissues active in recombination with the highest levels of expression being found in the testis, which could indicate a role in meiotic recombination (Albala *et al.*, 1997; Cartwright *et al.*, 1998a; Cartwright *et al.*, 1998b; Dosanjh *et al.*, 1998). However, high levels of expression in the testis were not reported by Rice *et al.* (1997) and Pittman *et al.* (1998).

The RAD51-like proteins show 20-30% sequence identity with RAD51 and a similar level of sequence identity with each other. It is difficult to say whether any of the RAD51-like proteins are direct counterparts of the yeast Rad55/Rhp55 and Rad57/Rhp57 proteins, but phylogenetic analysis places XRCC2 with Rad55/Rhp55 and the RAD51L proteins and XRCC3 with Rad57/Rhp57, although the confidence in the latter of these relationships is low (Thacker, 1999; Tsutsui *et al.*, 2000).

1.6.2 *irs1* and *irs1SF* are defective in HRR

After the *RAD51*-like family of genes were cloned work began to identify their functions, and in particular their role in HRR. Initially, the only link between the *RAD51*-like genes and HRR was their sequence similarity with the known recombination gene *RAD51*. The transcript expression patterns of the *RAD51*-like genes were also consistent with this role. Work was hampered on the *RAD51L1*, *-L2* and *-L3* genes because no mutant or knockout cell lines existed and most work focused on *XRCC2* and *XRCC3* because of the availability of *irs1* and *irs1SF*.

However, despite these difficulties some work was undertaken with *RAD51L1*. *RAD51L1* is induced by γ -irradiation (Rice *et al.*, 1997) and overexpression of the protein in CHO cells causes G1 delay and hypersensitivity to UV irradiation (Havre *et al.*, 1998) suggesting a possible role in damage detection. A UV-responsive element has also been discovered in the *RAD51L1* promoter region (Peng *et al.*, 1998).

The phenotypes of *irs1* and *irs1SF* are similar. Although both cell lines were isolated on the basis of X-ray sensitivity they are only mildly radiosensitive (Fuller and Painter, 1988; Jones *et al.*, 1987). They were subsequently found to have cross-sensitivity to UV radiation and the monofunctional alkylating agent ethyl methane sulphonate (EMS) (Fuller and Painter, 1988; Jones *et al.*, 1987) as well as extreme hypersensitivity to DNA cross-linking agents such as MMC and cisplatin (Caldecott and Jeggo, 1991; Jones *et al.*, 1987; Jones *et al.*, 1993). This severe sensitivity first made *XRCC2* and *XRCC3* candidates for involvement in HRR.

irs1 and *irs1SF* show elevated levels of chromatid aberrations both spontaneously and after induction with DNA damaging agents (Fuller and Painter,

1988; Jones *et al.*, 1993; Tucker *et al.*, 1991). Microcell-mediated chromosome transfer of complementing or non-complementing chromosomes into each cell line show the chromosome remains intact in complementing clones but frequently undergoes rearrangements in non-complementing clones (Cui *et al.*, 1999). This indicates that *XRCC2* and *XRCC3* are required for chromosome stability in mammalian cells. *irs1* and *irs1SF* show a significant increase in chromosome missegregation, which is associated with fragmentation of the centrosome (Griffin *et al.*, 2000). Similar results have been obtained using *Xrcc2*^{-/-} MEFs and there is also evidence for haploinsufficiency in *Xrcc2*^{+/-} MEFs (Carol Griffin, personal communication). These data demonstrate that *XRCC2* and *XRCC3* are required for correct chromosome segregation and potentially link the two genes with the centrosome defects and aneuploidy often seen in cancer cells. Overall, it can be concluded that *XRCC2* and *XRCC3* contribute to genetic stability and are therefore potential tumour suppressor genes.

Wild type Chinese hamster cells show localisation of RAD51 to discrete nuclear foci after treatment with DNA damaging agents. This process is defective in *irs1* and *irs1SF* but can be restored by transfection of the appropriate complementing cDNA (Bishop *et al.*, 1998; O'Regan *et al.*, 2001). This finding links *XRCC2* and *XRCC3* with the *RAD51*-dependent HRR pathway and suggests that the proteins may be required for the assembly or stabilisation of the multimeric RAD51 complexes.

Both *irs1* and *irs1SF* have now been shown to be defective in HRR directly. *irs1* clones carrying a neomycin recombination reporter substrate show a 100-fold decrease in HRR in response to a single endonuclease-induced DSB compared to wild type cells. NHEJ in these clones is normal and transfection with *XRCC2* cDNA restores HRR to near wild type levels (Johnson *et al.*, 1999). In a similar assay using a GFP recombination reporter construct, a 30-fold reduction in gene conversion was observed in *Xrcc2*^{-/-} mouse ES cells. Again, wild type levels of recombination could be restored by transfection with mouse *Xrcc2* cDNA (Bryan Deans, personal communication). A previous study using the same reporter construct, showed a 25-fold reduction in gene conversion in *irs1SF* cells, which is complemented by transfection with *XRCC3* cDNA (Pierce *et al.*, 1999). An independent study using a

neomycin recombination construct found that transfection of *irs1SF* with *XRCC3* cDNA increased the repair frequency by 34- to 260-fold (Brenneman *et al.*, 2000). Furthermore, a recent study showed that the recombination product spectrum in *irs1SF* is very different to that found in wild type cells. *irs1SF* cells show increased gene conversion tract lengths, increased numbers of discontinuous tracts and frequent local chromosome arrangements associated with HRR (Brenneman *et al.*, 2002). These results suggest that *XRCC3* acts not just at the initiation stages of HRR, but also at the later stages, which involve the formation and resolution of intermediates.

1.6.3 *RAD51*-like gene disruption in cell lines and mice

Mouse models have provided further insight into the function of the *RAD51*-like genes. Disruption of *Rad51l1*, *Rad51l3* and *Xrcc2* in mice results in embryonic lethality (Deans *et al.*, 2000; Pittman and Schimenti, 2000; Shu *et al.*, 1999). The affected homozygous embryos are smaller than those of littermates and often show developmental abnormalities. Lethality usually occurs at early to mid-gestation (Deans *et al.*, 2000; Pittman and Schimenti, 2000; Shu *et al.*, 1999) although some *Xrcc2*^{-/-} embryos survive until the later stages of embryogenesis and die at birth due to respiratory failure (Deans *et al.*, 2000). *Xrcc2*^{-/-} embryos show high levels of apoptosis in the developing nervous system (Deans *et al.*, 2000). *Rad51l1*^{-/-} embryonic development progresses further in a *Trp53*^{-/-} background (Shu *et al.*, 1999). *Trp53*^{-/-} *Xrcc2*^{-/-} mice also show prolonged embryonic development and do not show neural apoptosis but are not viable (Bryan Deans, personal communication). In addition, a recent study has shown that *Atm*^{-/-} *Xrcc2*^{-/-} mice are inviable (Julie Adam, personal communication). The lethality of the knockout mice indicates that the *RAD51*-like genes play an essential role in development and suggests that these genes cannot functionally compensate for one another.

All five *RAD51*-like genes have been disrupted in chicken DT40 cells (Takata *et al.*, 2000; Takata *et al.*, 2001). All the mutant cell lines are viable and have similar phenotypes. They show mild γ -ray sensitivity, severe sensitivity to DNA cross-linking agents and an elevated level of spontaneous chromosome aberrations. They

also have reduced HR, as measured by targeted integration and sister chromatid exchanges, and are defective in RAD51 focus formation after DNA damage. Partial suppression of the mutant phenotype can be achieved in each case by overexpression of the human *RAD51* gene (Takata *et al.*, 2001). When *XRCC2* or *XRCC3* is disrupted in chicken DT40 cells, the pattern of diversification of the immunoglobulin V gene shifts from gene conversion to somatic hypermutation (Sale *et al.*, 2001).

1.6.4 RAD51-like protein interactions

After the *XRCC3* and *RAD51L2* genes were cloned, yeast two hybrid experiments were carried out to look at interactions between these two proteins and the other RAD51-like proteins and RAD51 itself. It was found that *XRCC3* interacts with RAD51 (Liu *et al.*, 1998), and *RAD51L2* interacts with *XRCC3*, *RAD51L1* and very weakly with RAD51 (Dosanjh *et al.*, 1998). A further study by Schild *et al.* (2000) confirmed the previous results and showed that *RAD51L3* interacts with *RAD51L2* and *XRCC2*. The *RAD51L3* interaction with *XRCC2* was also reported by Braybrooke *et al.* (2000), and occurs primarily between the N-termini of the two proteins (Karen Spink, personal communication). Yeast three hybrid experiments showed that expression of *XRCC3* strengthens the weak interaction between *RAD51L2* and RAD51. Expression of *RAD51L1* also enhances the binding of *RAD51L2* to *RAD51L3* and *XRCC3*. Bridging experiments showed that expression of *RAD51L2* allows an indirect interaction between *RAD51L1* and *RAD51L3*. In a similar way, expression of *RAD51L3* allows an indirect interaction between *RAD51L2* and *XRCC2*. It was also found that expression of *RAD51L1* allows *RAD51L2* to interact with itself. This indicates that *RAD51L1* can bind two molecules of *RAD51L2*; there is no other evidence of one RAD51-like protein being able to bind two molecules of a second (Schild *et al.*, 2000). Taken together these results suggest that the RAD51-like proteins may exist as one large complex or as a number of smaller complexes.

Using biochemical methods it has been shown that the RAD51-like proteins exist in complexes in human cells. Co-immunoprecipitation experiments were first

used to demonstrate that XRCC2 and RAD51L3 form a heterodimer (Braybrooke *et al.*, 2000). A further complex consisting of RAD51L2 and XRCC3 also exists (Kurumizaka *et al.*, 2001; Masson *et al.*, 2001a) and is distinct from another complex containing RAD51L1, RAD51L2, RAD51L3 and XRCC2 (Liu *et al.*, 2002; Masson *et al.*, 2001b; Wiese *et al.*, 2002). X-ray treatment does not alter the level of the RAD51-like proteins or alter the interactions between them (Wiese *et al.*, 2002). It should be noted that RAD51L2 is the only protein found in two complexes. There have been several studies in human cells indicating that RAD51 is not present in any of the RAD51-like protein complexes (Masson *et al.*, 2001b; Miller *et al.*, 2002; Wiese *et al.*, 2002). However, one study has shown RAD51 to co-immunoprecipitate with XRCC3 (Liu *et al.*, 2002), and a weak *in vitro* interaction between RAD51 and the RAD51L1-RAD51L2 heterodimer has also been reported (Lio *et al.*, 2003).

Yeast two hybrid screens have been used to look for proteins that interact with XRCC2 and the XRCC2/RAD51L3 heterodimer. Both yeast two hybrid and co-immunoprecipitation data show XRCC2 to interact with SRB7, a component of the RNA polymerase II transcriptional mediator complex (Karen Spink, personal communication). This complex transmits signals from upstream promoter elements and signalling molecules to the basal transcription apparatus via the C-terminus of RNA polymerase II (Malik and Roeder, 2000). Interestingly, the heterodimer appears to behave differently from the XRCC2 and RAD51L3 proteins alone, and expression of RAD51L3 inhibits the interaction between XRCC2 and SRB7 (Karen Spink, personal communication).

1.6.5 Biochemical characterisation of RAD51-like protein function

The purification of the RAD51-like proteins has enabled several groups to investigate their biochemical properties. The RAD51L2-XRCC3 complex has been purified independently by two groups and shows a 1:1 stoichiometry (Kurumizaka *et al.*, 2001; Masson *et al.*, 2001a). Masson *et al.* (2001) find that the complex binds ssDNA but not dsDNA. It also aggregates DNA that may facilitate the homology search between sister chromatids or homologous chromosomes. No evidence for nucleoprotein

filament formation or homologous pairing activity as measured by the D-loop assay was found. However, Kurumisaka *et al.* (2001) find that the RAD51L2-XRCC3 complex does catalyse homologous pairing. It was suggested that the RAD51L2 protein is required for the homologous pairing activity whilst the XRCC3 protein is required for DNA binding. EM images also show the complex to form filamentous structures on circular ssDNA.

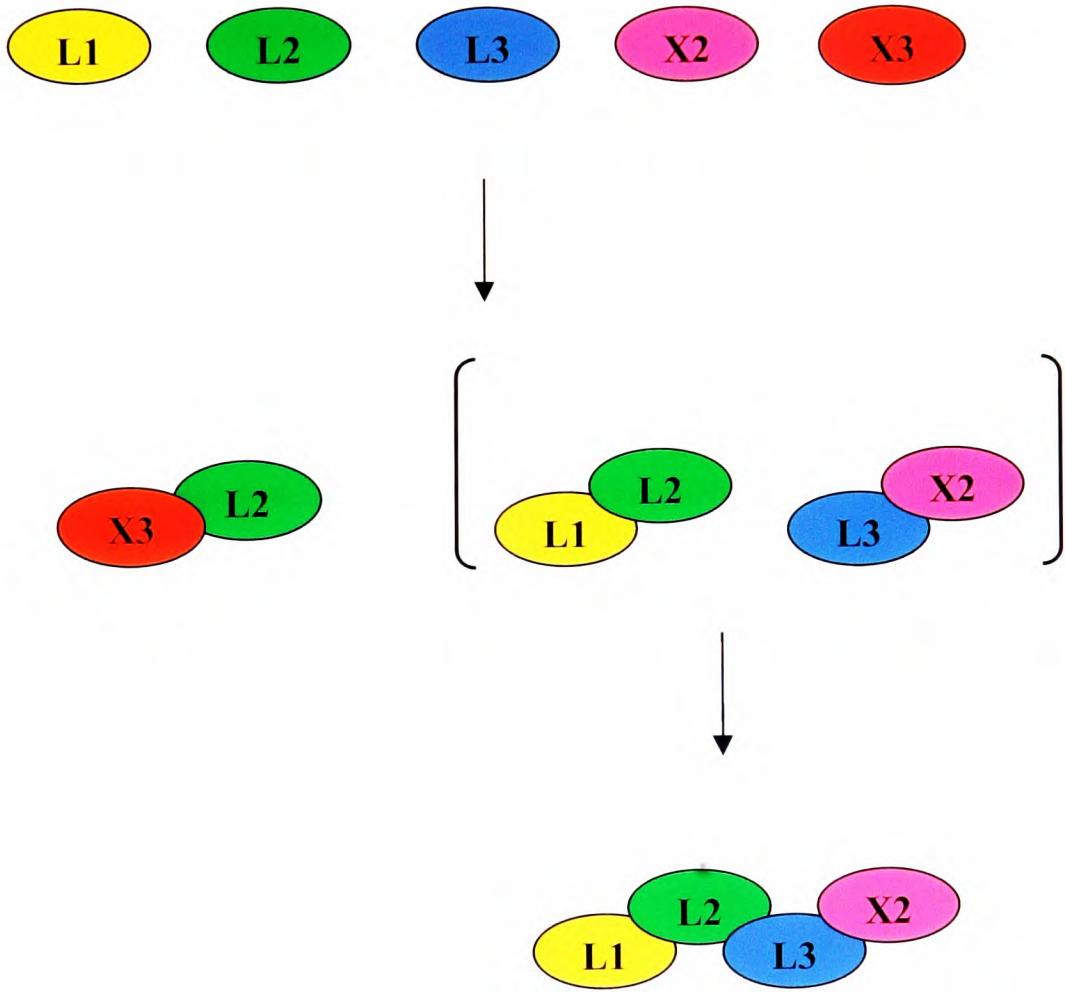


Figure 1.13 RAD51-like protein complexes in human cells. Several studies have shown the RAD51-like proteins to form two distinct complexes, a heterodimer (XRCC3-RAD51L2) and a heterotetramer (RAD51L1-RAD51L2-RAD51L3-XRCC2). There is additional evidence suggesting that the proteins making up the heterotetramer also form two heterodimers (RAD51L1-RAD51L2 and RAD51L3-XRCC2).

The RAD51L1-RAD51L2-RAD51L3-XRCC2 complex (also referred to as the BCDX2 complex) has also been purified by Masson *et al.* (2001) and has a 1:1:1:1 stoichiometry. The complex binds ssDNA and ss gaps in duplex DNA, and exhibits DNA-stimulated ATPase activity at a very low level. Interestingly, the complex also binds nicks in duplex DNA, which could indicate a role in the recognition of incised cross-links.

The RAD51L3 protein was first purified by Braybrooke *et al.* (2000) and was found to bind ssDNA and to have DNA-stimulated ATPase activity. The RAD51L3-XRCC2 heterodimer was purified by Kurumisaka *et al.* (2002) and was reported to have very similar biochemical properties to those described by the same group for the RAD51L2-XRCC3 complex, namely homologous pairing activity and the formation of filaments on ssDNA.

The RAD51L1 and RAD51L2 proteins have also been purified, as has the RAD51L1-RAD51L2 heterodimer. The RAD51L1 protein is reported to have kinase activity (Havre *et al.*, 2000), whilst both proteins bind 3'-end tailed dsDNA in preference to ssDNA or dsDNA and exhibit DNA-stimulated ATPase activity (Lio *et al.*, 2003; Yokoyama *et al.*, 2003). RAD51L1 also binds synthetic Holliday junctions but not replication fork-like structures or B-form DNA (Yokoyama *et al.*, 2003). RAD51L2 but not RAD51L1, shows an apparent strand exchange ability which is actually due to a DNA melting and strand separation activity of the protein (Lio *et al.*, 2003). It is possible that RAD51L2 has a more direct role in HRR, which includes recruiting RAD51 to ssDNA and mediating the separation of duplex DNA to promote strand exchange. The RAD51L1-RAD51L2 heterodimer has an elevated level of DNA binding compared to each protein individually (Lio *et al.*, 2003) and as expected has ATPase activity (Lio *et al.*, 2003; Sigurdsson *et al.*, 2001). The complex allows more RAD51 to bind to DNA, by reducing the amount of binding by the competing RPA protein, and hence is thought to increase the efficiency of the strand exchange reaction (Sigurdsson *et al.*, 2001).

I.6.6 Involvement of *RAD51*-like genes in tumorigenesis

The *RAD51*-like genes are required for genetic stability and may act as tumour suppressor genes in mammalian cells. It could therefore be expected that individuals with mutations of these genes, leading to reduced DNA repair capacity, might have an increased cancer risk (Pierce *et al.*, 2001).

A search for non-conservative amino acid substitutions in DNA repair genes identified the *XRCC3* variant allele Thr241Met in the population (Shen *et al.*, 1998). Several studies have found no association of this allele with lung cancer (Butkiewicz *et al.*, 2001; David-Beabes *et al.*, 2001) or with melanoma (Duan *et al.*, 2002). Cells expressing the variant *XRCC3* protein are also functionally active for DSB-induced HRR and are not sensitive to MMC (Araujo *et al.*, 2002). However, a statistically significant increase in variant allele frequency was reported for melanoma (Winsey *et al.*, 2000), bladder cancer (Matullo *et al.*, 2001) and breast cancer (Kuschel *et al.*, 2002).

Two studies looking at the relationship between the *XRCC2* variant R188H and breast cancer susceptibility have found a positive association of marginal significance (Kuschel *et al.*, 2002; Rafii *et al.*, 2002). Site-directed mutagenesis of *XRCC2* shows non-conservative substitution or deletion of allele 188 to effect cellular sensitivity to DNA damage considerably, whereas a conservative substitution has a weak effect on damage sensitivity (Rafii *et al.*, 2002).

Chromosome translocations resulting in fusions between *RAD51L1* and *HMGIC*, have been reported in uterine leiomyomas (benign solid tumours) and in uterine leiomyomas with associated ascites and pleural fluid (pseudo-Meigs' syndrome) (Amant *et al.*, 2001; Schoenmakers *et al.*, 1999; Takahashi *et al.*, 2001), as well as in a pulmonary chondroid hamartoma (Blank *et al.*, 2001). In addition the 17q23 region, which includes the *RAD51L2* gene, has been shown to undergo amplification in breast cancer although the significance of this is unclear (Barlund *et al.*, 2000; Wu *et al.*, 2000). To date there are no reports of a link between *RAD51L3* and cancer susceptibility.

Taken together these results suggest the possibility of a low penetrance link between the *RAD51*-like genes and susceptibility to certain types of cancer. Further studies using larger sample sizes should help to clarify the picture.

1.7 Aims of the present study

When this study was initiated in late 1999, the five *RAD51*-like genes had been cloned but little was known about their functions. Some yeast two hybrid work had been undertaken to look at interactions between the *RAD51*-like proteins but much of the biochemical data available now had not been published. *XRCC2* and *XRCC3* were cloned from the mutant hamster cell lines *irs1* and *irs1SF* respectively, and studies using these cell lines have been important in determining the function of these genes. However, the *RAD51L1*, *RAD51L2* and *RAD51L3* genes were isolated using database searches so such mutant cell lines have not been available.

At the outset, this study aimed to identify the gene mutated in another hamster cell line, *irs3*. *irs3* was isolated in the same screen as *irs1*, and has a similar but less severe phenotype. Previous work on *irs3* had failed to identify the gene in question, using methods similar to those used to clone *XRCC2* and *XRCC3* (Chapter 3, Introduction). We therefore changed strategy and decided to employ a candidate gene approach. It was hoped that identification of the mutated gene in *irs3* would facilitate functional studies of the gene product using the *irs3* cell line.

This thesis describes the identification of *irs3* as a *RAD51L2*-deficient cell line. It then goes on to explain how experiments with *irs3* have led to the identification of functional domains within the *RAD51L2* protein, and placed *RAD51L2* in the HRR pathway.

CHAPTER 2: Materials and Methods

2.1 Primary reagents

2.1.1 Chemicals and media

Chemicals were purchased from Sigma or BDH unless otherwise stated. Media for cell culture was supplied by Gibco BRL and cell culture plastics were obtained from BD Falcon or Greiner. Radioisotopes were bought from ICN.

2.1.2 Bacterial strains and media

E.coli DH5 α (lab stock), genotype: *supE44 Δ lacU169 (ϕ 80lacZ Δ M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1**

Used to make electrocompetent cells and as a stock strain for maintaining plasmids.

E.coli TOP10F' (Invitrogen), genotype: *F' {lacI^q, Tn10(Tet^R)} mcrA Δ (*mrr-hsdRMS-mcrBC*) ϕ 80lacZ Δ M15 Δ lacX74 *deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL(Str^R) endA1 nupG**

Chemically competent cells supplied with TA and TOPO TA Cloning Kits (Invitrogen).

E.coli INVaF' (Invitrogen), genotype: *F' endA1 recA1 hsdR17 (r_k, m_k⁺) supE44 thi-1 gyrA96 relA1 ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169*

Chemically competent cells supplied with TA and TOPO TA Cloning Kits.

The media used for *E.coli* growth are described below. They were supplemented with antibiotics when required; ampicillin (100 μ g/ml), kanamycin (30 μ g/ml) or zeocin (25 μ g/ml).

LB medium: 10g tryptone, 5g yeast extract (both from Fisher BioReagents) and 10g NaCl made up to 1 litre with Elix RO water (from a Millipore water system) and autoclaved.

Low salt LB medium: As above except 5g NaCl used instead of 10g and pH adjusted to 7.5 with 5M NaOH before autoclaving. This medium was required when zeocin was used as a supplement because zeocin is inactivated by high salt concentrations and acidity or basicity. Zeocin is also light sensitive so plates and media containing this supplement were stored in the dark.

LB agar/ low salt LB agar: 15g Bacto Agar (DIFCO) added to either of the above solutions before the autoclave step.

SOB medium: 20g bacto tryptone, 5g bacto yeast extract, 0.5g NaCl and 10ml of 250mM KCl made up to 1 litre with Elix water and pH adjusted to 7.0 with 5M NaOH before autoclaving.

SOC medium: 5ml of sterile 2M $MgCl_2$ and 20ml of sterile 1M glucose added to 1 litre of SOB.

Freezing medium: Cells were stored at $-20^{\circ}C$ or $-70^{\circ}C$ in LB containing 15% glycerol.

2.1.3 Vectors

A description of the various vectors used is given below:

pZeoSV2(-) (Invitrogen): Mammalian expression vector with zeocin selection.

pIRESneo2 (Clontech): Mammalian expression vector with an internal ribosome entry site (IRES) that permits translation of the neomycin marker and the gene of interest from the same mRNA.

pBluescript II SK (-) (Stratagene): *E.coli* cloning vector used for *RAD51L2* site-directed mutagenesis.

pEGFP-N1 (Clontech): Mammalian expression vector used to express *RAD51L2* as a fusion protein with EGFP

pEGFP-N2 (Clontech): As above except that multiple cloning site is in a different frame with the EGFP gene.

pCR2.1 (Invitrogen): T-vector supplied with the TA Cloning Kit for direct cloning of PCR products.

TOPO2.1 (Invitrogen): Vector supplied with the TOPO TA cloning kit for direct cloning of PCR products.

Several genes were cloned into these vectors to produce the constructs shown in table 2.1.

Table 2.1 Constructs.

Construct	Primers	Restriction sites
hs <i>RAD51L3</i> in pZeoSV2(-)	RAD51L3up RAD51L3down	Nhe1/Kpn1
hs <i>RAD51L2</i> in pZeoSV2(-)	RAD51L2up(b) RAD51L2down(b)	Nhe1/Kpn1
hs <i>RAD51L2</i> in pIRESneo2	RAD51L2up(b) RAD51L2down(b)EcoR1	Nhe1/EcoR1
hs <i>RAD51</i> in pIRESneo2	RAD51upEcoRV RAD51revBamH1	EcoRV/BamH1
Hamster <i>RAD51L2</i> in pIRESneo2	hamL2upfullEcoR1 hamL2revfullBamH1	EcoR1/BamH1
hs <i>RAD51L2</i> (2 nd ATG) in pIRESneo2	hsL22ndATGNhe1 RAD51L2down(b)EcoR1	Nhe1/EcoR1
hs <i>RAD51L2</i> (2 nd ATG) in pBluescript II SK(-)	hsL22ndATGEcoR1 RAD51L2revBamH1	EcoR1/BamH1
hs <i>RAD51L2</i> (2 nd ATG) in pEGFP-N1	hsL22ndATGEcoR1 L2EGFP-N1	EcoR1/BamH1
hs <i>RAD51L2</i> (2 nd ATG) –NLS in pEGFP-N1	hsL22ndATGEcoR1 L2NLSEGFP-N1	EcoR1/BamH1

2.1.4 Oligonucleotides

Primers were designed using OLIGO Analysis Software Version 4.1 (Medprobe, Oslo) and purchased from MWG Biotech. A description of the oligos used during this project can be found in tables 2.2, 2.3 and 2.4.

Table 2.2 Primers used to make constructs. Primers listed 5' to 3' with restriction enzyme sites underlined and start or stop codons in bold.

Name	Sequence	Features	Use
RAD51L3up RAD51L3down	GCAGTGTCTAGCGGGGCTGTTC TTCCGGTACCAACAGCACAGGTCAT	NheI Site. Includes stop codon. KpnI site.	Cloning <i>hsRAD51L3</i> into pZeoSV2(-).
RAD51L2up(b) RAD51L2down(b)	AGGCTAGCATGCGCGGGAAAGACGTT TTGGTACCTTATAATTCTTCCTCTGGGTCTCG	Includes first start codon. <u>NheI</u> site. Includes stop codon. KpnI site.	Amplification of <i>hsRAD51L2</i> from a cDNA library and cloning into pZeoSV2(-).
RAD51L2up(b) RAD51L2down(b)	AGGCTAGCATGCGCGGGAAAGACGTT TTGAATTCCTTATAATTCTTCCTCTGGGTCTCG	Includes first start codon. <u>NheI</u> site. Includes stop codon. <u>EcoRI</u> site.	Cloning <i>hsRAD51L2</i> into pIRESneo2.
RAD51upEcoRV RAD51revBamHI	AGAGGATATCTGCGACCGAGTAATGG AGAGGAGGATCCCAATGATTCAGTCTT	Includes start codon. <u>EcoRV</u> site Includes stop codon. BamHI site.	Amplification of <i>hsRAD51</i> from a cDNA library and cloning into pIRESneo2.
hamL2upfullEcoRI hamL2revfullBamHI	CTCGAATTCACCTGTGTGCTTACG CATCAGGATCCATTCTCCTTCAG	<u>EcoRI</u> site. BamHI site.	Cloning hamster <i>RAD51L2</i> into pIRESneo2.
hsL22ndATGNheI RAD51L2down(b)	GGGAAGGCTAGCCGCTTTGAAATG TTGAATTCCTTATAATTCTTCCTCTGGGTCTCG	Includes 2nd start codon. <u>NheI</u> site. Includes stop codon. <u>EcoRI</u> site.	Cloning <i>hsRAD51L2</i> from 2 nd start codon into pIRESneo2.
hsL22ndATGEcoRI RAD51L2revBamHI	GGGAAGGAATTCGCGCTTTGAAATG TTGGATCCCTTATAATTCTTCCTCTGGGTCT	Includes 2nd start codon. <u>EcoRI</u> site. Includes stop codon. BamHI site.	Cloning <i>hsRAD51L2</i> from 2nd start codon into pBluescript II SK(-).
hsL22ndATGEcoRI L2EGFP-N1	GGGAAGGAATTCGCGCTTTGAAATG TTTCTGGGATCCCAATTCTTCCTCTGGGTCTCG	Includes 2nd start codon. <u>EcoRI</u> site. BamHI site.	Cloning <i>hsRAD51L2</i> from 2nd start codon into pEGFP-N1.
hsL22ndATGEcoRI L2NLSEGF-N1	GGGAAGGAATTCGCGCTTTGAAATG CGTGGATCCGTGCTCAAGGAACCTTCTGT	Includes 2nd start codon. <u>EcoRI</u> site. BamHI site.	Cloning <i>hsRAD51L2</i> from 2nd start codon, without putative NLS, into pEGFP-N1.

Table 2.3 Primers used to clone hamster *RAD51L2* gene. Primers listed 5' to 3'.

Name	Sequence	Use
hamL2F hamL2R	TTGGTGGGTTATCCGCTGTCTC CTTCCTCTGGTTCTCGTGACCG	Used to amplify central section of hamster gene
3'inF 3'inR	CTGCTTCTTCATCACAGACAGA AGGTTGGAATCTAAAATACACAAA	Nested primers used with 3'outF /3'outR to amplify the 3' end of the gene
3'outF 3'outR	ACACCACAGGGATTTAGAGATG GGTCTTCTACCTTCTCTTCTTTT	Nested primers used with 3'inF /3'inR to amplify the 3' end of the gene
5'outF 5'outR	CAACGGGACTTTCCAAAATGTCTG ACACTGCTTCACCTGCCACTCCT	Nested primers used with 5'outerF/5'outerR to amplify the 5' end of the gene
5'outerF 5'outerR	CTCACGGGGATTTCCAAGTCTC CCTCCAAGGCTTCTGCGTTC	Nested primers used with 5'outF /5'outR to amplify the 5' end of the gene
hamL2upfull hamL2revfull	CTCTACGTCACTGTGTGCTTACG CATCATCTTCCATTCTCCTTCAG	Amplification of the full length gene
gapL2F gapL2rev	TGGCAGGTGAAGCAGTGTTTATTG CTTTGCTTTTGCTCCCAGTGAAA	Amplification of exon 6
exon6(b)F exon6(b)R	CTAAATGGCCTTGCCCAACAAA CCCCTAATGCAGGAACAAGCAA	Amplification of the 5' end of exon 6
exon6F exon6R	TCAGATGACAACAAAGATTGATAAAA TGCTCCCAGTGAAAAATAAGTCT	Amplification of the 3' end of exon 6

Table 2.4 Sequencing primers. Primers listed 5' to 3'. Other standard primers such as T7 were also used to sequence vector inserts.

Name	Sequence	Used to sequence:
RAD51L3seq	AGCGGCAAACTCAGGTATG	hs <i>RAD51L3</i>
RAD51L3seqR	GCCTCCCGCTGTCCCTGTCT	hs <i>RAD51L3</i>
RAD51L2seq	TATTCTTGGGGGTGGAGTGC	hs <i>RAD51L2</i>
RAD51L2seqR	TCCCCAACTTTCCCCTAATG	hs <i>RAD51L2</i> and hamster <i>RAD51L2</i>
RAD51L2bF	TAGGGGAAAGTTGGGGACAT	hs <i>RAD51L2</i> and hamster <i>RAD51L2</i>
RAD51L2bR	AACAGAAGGTGATTATGAAG	hs <i>RAD51L2</i> and hamster <i>RAD51L2</i>
hamL2seq	CATTCTTGGGGGTGGAATAC	Hamster <i>RAD51L2</i>
R51INTF (Karen Spink)	GATCTGTCATACGCTAGCTGT	hs <i>RAD51</i>
R51INTR (Karen Spink)	ACAGCTAGCGTATGACAGATC	hs <i>RAD51</i>
pZeoSV2(-)R	GGGAGGGGCAAACAACAGAT	Inserts in multiple cloning site of pZeoSV2(-)
pIRESneo2F	TAGAGAACCCACTGCTTACT	Inserts in multiple cloning site of pIRESneo2
pIRESneo2R	TGGAAACTGACAATCTTAGC	Inserts in multiple cloning site of pIRESneo2

.

2.1.5 Antibodies

The antibodies used in this study are listed in table 2.5.

Table 2.5. Antibodies.

Antibody	Source:
Anti-hRAD51 rabbit polyclonal (Ab-1)	Oncogene Research
Anti-hRAD51 rabbit polyclonal	Novus
Anti- α tubulin mouse monoclonal	Sigma
Biotinylated anti-avidin D	Vector
Anti-rabbit IgG HRP conjugate	Promega
Anti-mouse IgG HRP conjugate	AbCam
Cy5-conjugated anti-rabbit IgG	Jackson ImmunoResearch
Anti-hRAD51L2 monoclonal 2H11	Dr. Steve West, CRUK Clare Hall
Anti-hRAD51L2 polyclonal SWE 31	Dr. Steve West, CRUK Clare Hall

2.2 Cloning techniques

2.2.1 Polymerase Chain Reaction (PCR)

Typically, 5-500ng of template DNA was added to 0.4 μ M forward and reverse primers, 0.2mM dNTPs (Bioline), 1 x Reaction Buffer IV and 0.6U of Thermoprime Plus *Taq* polymerase (both from ABgene) before being made up to a final volume of 25 μ l with UV treated Elix water. If improved proofreading was required the *Taq* polymerase was mixed 10:1 with *Pfu* polymerase (Stratagene). PCR reactions were carried out in either a Perkin Elmer GeneAmp 2400 or 9700 machine using the following standard conditions:

95°C for 4 min 30 secs

(94°C for 40 secs, annealing temperature for 30 secs, 72°C for 90 secs) x 35 cycles

72°C for 5 min

4°C Hold

The annealing temperature used was dependent on the sequence of the primers and varied between 55°C and 65°C.

2.2.2 TA and TOPO TA cloning

PCR products were generated by the method described in section 2.2.1 using *Taq* polymerase and a final extension step of 7 to 10 min. 1-5µl of PCR product was used in a ligation reaction with either pCR2.1 or TOPO2.1 vector according to the manufacturer's protocols supplied with either the TA Cloning Kit or the TOPO TA Cloning Kit respectively (both purchased from Invitrogen). 2µl of each ligation reaction were then heat-shocked into chemically competent INVaF' or TOP10F' cells as described in section 2.2.9.

2.2.3 DNA agarose gel electrophoresis

DNA was run on an appropriate percentage agarose gel (0.4%-2.0%). Gels contained 0.2µg/ml ethidium bromide and were run in 0.5 x TBE buffer (10 x concentrate purchased from BDH). DNA samples were mixed 5 to 1 with loading buffer (25% ficoll, 0.25% Orange G) and electrophoresed with a suitable sized marker at 3-10 V/cm. DNA was visualised by exposing the gel to UV light from a UV transilluminator (Foto/PrepI, Fotodyne).

2.2.4 Purification of DNA from agarose gels

Bands were cut from gels using the preparative setting on the UV transilluminator. DNA was purified from the gel slices using a Qiagen gel extraction kit according to

the manufacturer's protocol. Briefly, gel slices were dissolved in a buffer with high salt concentration and pH <7.5. These conditions enable up to 10µg of DNA to be bound to the silica-gel membrane of a QIAquick spin column while impurities are allowed to pass through. After washing the column with an ethanol containing buffer, DNA was eluted in a buffer with low salt concentration and pH 7.0-8.5.

DNA fragments >10kb in size were purified from agarose gels using a GeneClean II kit (BIO 101) according to the manufacturer's protocol. This kit utilises the same principle as the Qiagen gel extraction kit. DNA is bound to silica beads, rather than the silica membrane of a spin column, at high salt concentration prior to washing and finally eluting at low salt concentration.

2.2.5 Restriction enzyme digests

Restriction enzymes were purchased from New England Biolabs. Typically, 0.5-2µg of DNA were digested with 5-10U of enzyme in a total reaction volume of 10µl for 2-6h. Reaction constituents and conditions were taken from the New England Biolabs catalogue. If the digested DNA was required for further cloning steps it was cleaned up using a Qiagen PCR purification kit according to the manufacturer's protocol. The Qiagen PCR purification kit works on the same principle as the Qiagen gel extraction kit described in section 2.2.4.

2.2.6 Alkaline phosphatase treatment of vector DNA

Digested vector DNA was treated with calf intestinal alkaline phosphatase (CIP) (New England Biolabs) to prevent self ligation. Vector DNA was treated with 0.5U CIP per 1µg DNA, in 1 x NEBuffer 3, for 1 h at 37°C. Reactions were cleaned up using the Qiagen PCR purification kit.

2.2.7 Ligation of DNA

25-200ng of vector and insert DNA were mixed together at an appropriate molar ratio (1:1 – 1:6) along with 1 x T4 DNA ligase buffer and 1U of T4 DNA ligase (both from GibcoBRL). The reaction was made up to a final volume of 10µl with Elix water and incubated for 16-72h at 16°C.

2.2.8 Preparation of electrocompetent cells

A single colony of *E.coli* DH5α was used to inoculate 5ml of LB media and the culture grown for approximately 16h. 1ml volumes of this culture were used to inoculate 4 x 100ml lots of LB media and these cultures were grown until they reached log phase (OD₅₉₅ 0.5-0.7). The bacterial cells were then pelleted by centrifuging for 10 min at 5000 x g. Cell pellets were kept on ice while they were washed 3 or 4 times with sterile, cold Elix water. After each wash the pellets were resuspended in the water by gently tapping the tube before centrifugation for 10 min at 5000 x g. After the final wash step the bacterial pellets were resuspended in 400µl of 10% glycerol and 50µl aliquots were snap frozen in liquid nitrogen and stored at -70°C.

2.2.9 Transformation of electrocompetent or chemically competent cells

Transformation of electrocompetent cells:

Salt was removed from ligation reactions by pipetting them onto nitrocellulose membrane disks (Millipore) which were floated on Elix water for 30 min. 1-3µl of ligation reaction, or cut/uncut vector control, was mixed with 50µl of electrocompetent cells before being transferred to a 0.1cm gap electroporation cuvette (BioRad). Cells were electroporated using a BioRad *E.coli* pulser set at 1.8kV.

Transformation of chemically competent cells:

1-2µl of ligation reaction, or cut/uncut vector control, was mixed with 50µl of chemically competent cells (INVaF' or TOP10F' cells, see section 2.1.2) and incubated on ice for 20-30 min. The cells were then heat-shocked for exactly 30 sec in a 42°C water bath.

Immediately after transformation, by either of the above methods, 250-500µl of SOC media was added to each sample. Samples were incubated for 1 h at 37°C, with shaking at 225rpm before being plated out onto LB agar containing the appropriate antibiotic. If blue/white screening was being used, 4µl of 200mg/ml IPTG and 16µl of 50mg/ml X-gal were mixed together with 100µl LB media and spread onto each plate before the samples were added.

2.2.10 Direct colony PCR

Single bacterial colonies were picked, streaked onto a master plate and added to 20µl Elix water. Samples were heated for 10 min at 100°C, cooled on ice and spun for 5 mins at 14000rpm in an Eppendorf 5415C microcentrifuge. 3µl of the supernatant was immediately added to a PCR reaction that was made up as described in section 2.2.1.

2.2.11 Isolation of plasmid DNA

Isolation of small quantities of plasmid DNA:

10-20µg of plasmid DNA was purified from 1-5ml of bacterial culture using the Qiagen QIAprep miniprep kit according to the manufacturer's protocol. Briefly, bacteria were lysed under alkaline conditions after which the lysate was subsequently neutralised and adjusted to a high salt concentration. The DNA was then bound to and eluted from the silica-gel membrane of a spin column in the same way described for the Qiagen gel extraction kit in section 2.2.4.

Isolation of large quantities of plasmid DNA:

100µg-500µg of plasmid DNA was purified from 25ml-100ml of bacterial culture, using the Qiagen midi or maxi QIAfilter kits respectively, according to the manufacturer's protocol. Briefly, bacteria were subject to alkaline lysis after which the lysate was cleared by filtration. DNA was bound to the anion-exchange resin of a gravity flow column under low salt and pH conditions. The columns then underwent a medium salt wash and DNA was eluted in a high salt buffer before being concentrated and desalted by isopropanol precipitation.

Determination of DNA concentration and purity:

The yield and purity of the DNA isolated was measured using a Pharmacia GeneQuant spectrophotometer as well as by running a sample of the DNA on an agarose gel against a known standard. DNA concentration was determined by measuring the absorbance at 260nm assuming that 50µg/ml dsDNA gives an OD reading of 1. DNA purity was calculated using the OD₂₆₀/OD₂₈₀ ratio and was ideally between 1.7 and 1.9.

2.2.12 DNA Sequencing

0.5-2.0µg of DNA were mixed with 0.2µM primer, 3µl BigDye (ABI Prism terminator cycle sequencing ready reaction kit, Perkin Elmer Applied Biosystems) and made up to a final volume of 10µl with UV treated Elix water. PCR was then carried out using the following conditions:

(96°C for 10 secs, 50°C for 5 secs, 60°C for 4 mins) x 25 cycles
4°C Hold

Extension products were purified by isopropanol precipitation according to the method described in the handbook supplied with the BigDye kit (see above). Briefly,

DNA was precipitated in 60% isopropanol, pelleted by centrifugation, washed in 75% isopropanol and air dried. Samples were then sent to the MRC Mammalian Genetics Unit genotyping, microarraying and sequencing (GAS) core facility where they were run on an ABI Prism 377 sequencer.

Sequencing directly from PCR products:

15µl PCR product was mixed with 4U shrimp alkaline phosphatase (Amersham) and 40U exonuclease 1 (New England Biolabs). Samples were incubated for 30 mins at 37°C after which the reaction was stopped by heating for 15 mins at 80°C. 5µl of this reaction was added to the 10µl BigDye reaction described above.

2.2.13 Analysis and manipulation of DNA sequences

DNA sequences and chromatograms produced by the sequencing facility were viewed and stored on the MRC Mammalian Genetics Unit WISP programme. DNA and protein sequences were obtained, analysed, and manipulated using the GCG general sequence analysis package available at the UK Human Genome Mapping Project Resource Centre (<http://www.hgmp.mrc.ac.uk/>).

2.3 Cell culture

2.3.1 Cell lines and culture conditions

The *irs1*, *irs2* and *irs3* mutant hamster cell lines were previously isolated from the parent line V79-4 in this laboratory (Jones *et al.*, 1987). All cell lines were cultured at 37°C as monolayers in MEM supplemented with 10% (v/v) FBS, 2mM L-glutamine and antibiotics. Cell stocks were stored at -70°C in complete medium containing 7.5% DMSO.

2.3.2 Transfection of cells

Sub-confluent cells were harvested as a single cell suspension, pelleted by centrifugation and resuspended at a concentration of 1×10^6 cells in 0.8ml cytomix buffer (van den Hoff *et al.*, 1992) (10mM K_2HPO_4/KH_2PO_4 , 2mM EGTA, 25mM HEPES, 0.15mM $CaCl_2$, 120mM KCl, 5mM $MgCl_2$, 2mM ATP, 5mM glutathione, pH 7.6). 10 μ g of plasmid DNA was mixed with 0.8ml cell suspension and transferred to a 0.4cm gap electroporation cuvette (BioRad). Cells were electroporated using a BioRad Gene Pulser set at 500 μ FD, 400V. After electroporation cells were put into non-selective medium and incubated for 48 h at 37°C before being seeded into 9cm dishes at a concentration of no more than 5×10^5 cells/dish in the appropriate selective media (800 μ g/ml zeocin, 500 μ g/ml G418, or 5 μ g/ml puromycin). Human *RAD51L2* PAC DNA (see section 2.5) carried no selective marker. 15 μ g of PAC DNA was cotransfected with 2 μ g of the vector pEGFP-N2 and selected in 500 μ g/ml G418. Surviving colonies were pooled and respread in 300 μ g/ml G418 and 4×10^{-8} M MMC.

2.3.3 “Quick” isolation of genomic DNA for PCR

The presence of the appropriate gene (cDNA or PAC) in drug-resistant clones was tested by PCR using cellular genomic DNA as a template according to a previously described method (Aghamohammadi *et al.*, 1992). Approximately 5×10^4 cells were isolated in 100 μ l of medium, pelleted by centrifugation, washed in salts solution A and resuspended in 50 μ l salts solution A (10 x salts solution A: 74g/l NaCl, 2.85g/l KCl, 12g/l $NaHCO_3$, 1.93g/l $Na_2HPO_4 \cdot 2H_2O$, 0.83g/l KH_2PO_4 , 12mg/l phenol red and 1.1g/l glucose). 5 μ l 10% nonidet P-40, 5 μ l 2mg/ml proteinase K and 40 μ l Elix water were added to each sample which was then mixed by thorough vortexing. Samples were incubated at 37°C for 30 mins after which the proteinase K was inactivated by heating to 95°C for 5 mins. Cell debris was removed by centrifuging for 5 mins at 14000 rpm in an Eppendorf 5415C microcentrifuge and 5 μ l of the supernatant was used immediately in a PCR reaction as described in section 2.2.1.

2.4 Survival assays

2.4.1 Preliminary MMC survival assay

This assay was used to get an initial estimate of the MMC sensitivity of several newly isolated clones at one time. Sub-confluent cells were harvested as a single cell suspension and seeded into 24-well tissue culture plates, at a concentration of 300 cells/well for *irs3* and *irs3* cDNA-transfected clones, or 100 cells/well for V79. A chronic treatment of MMC was added to the wells at the following concentrations – no MMC, 1×10^{-8} M, 2×10^{-8} M, 5×10^{-8} M, 1×10^{-7} M and 2×10^{-7} M. Plates were then incubated for 10-12 days after which the MMC was removed and the cells visualised by staining with 0.2% methylene blue in 50% methanol.

2.4.2 MMC survival assay

This assay was utilised when accurate survival data were required. The results of the preliminary assay were used to estimate appropriate concentrations of MMC to use with a particular clone. Sub-confluent cells were harvested as a single cell suspension and a suitable number were seeded into 9cm dishes so as to give approximately 150 surviving cells per dish. MMC was then added and the dishes were incubated for 7 to 11 days to allow colony formation. After this time the MMC was removed and the colonies were stained (as described above) before being counted. Three dishes were used for each dose point and each entire experiment was repeated at least three times.

2.4.3 X-ray survival assay

Sub-confluent cells were harvested as a single cell suspension and diluted to a concentration of 1×10^5 cells/ml. 2ml volumes of this solution were mock treated or irradiated with 2, 4, 6 or 8 Gy 250kVp X-rays. An appropriate number of cells were then seeded into 9cm dishes in order to give approximately 150 surviving cells per dish. The dishes were incubated for 7 to 11 days to allow colony formation after

which colonies were stained (as previously described) and counted. Three dishes were used for each dose point and each entire experiment was repeated at least twice.

2.5 Identification and isolation of PAC DNA

2.5.1 PAC filter probing

Probing:

The full length *RAD51L2* cDNA was used as a probe and was obtained, by digestion with NheI and KpnI, from the pZeoSV2(-) + *RAD51L2* construct (see table 2.1). The probe was labelled by random primer labelling using a Megaprime DNA labelling kit (Amersham) in a similar way to the method described in the manufacturer's protocol. 60ng of *RAD51L2* DNA was mixed with 5µl random nonamer primers and heated to 95°C for 7 min to denature the dsDNA. The reaction was then cooled slowly to allow the primers to anneal to the DNA, after which 10µl labelling buffer (-dCTP), 5µl ($\alpha^{32}\text{P}$) dCTP (50µCi) and 2µl Klenow enzyme were added. The reaction was incubated for 12 mins at 37°C, to allow DNA synthesis and incorporation of the radiolabelled nucleotides, before being stopped by the addition of 2µl 0.5M EDTA. Unincorporated nucleotides were separated from the probe using MicroSpin G-25 columns (Amersham) according to the manufacturer's protocol.

Hybridisation:

High-density gridded filters with human genomic PAC clones (library RPC11) constructed by P. de Jong (Ioannou and de Jong, 1996) and provided by the UK HGMP Resource Centre were pre-hybridised in 5 x SSC for 30 min at 65°C (20 x SSC: 3M NaCl, 0.3M sodium citrate). Filters were incubated with hybridisation solution; 5 x SSC, 5 x Denhart's, 0.5% SDS and purified radiolabelled probe (denatured for 7 min at 100°C and cooled on ice before addition) for 16 h at 65°C (100 x Denhardt's solution: 2% BSA, 2% Ficoll, 2% PVP). After this time a room

temperature wash in 2 x SSC, 0.1% SDS was used to remove excess label. Subsequent washes were carried out at 65°C and used graded reductions in SSC concentration (2 x SSC to 0.1 x SSC) depending on the amount of radioactivity remaining on the filter. The filter was then exposed to X-ray film at -70°C for an appropriate length of time and films were developed using an automatic X-ograph Compact X2 developer. PAC filters were stripped for future use by washing in 0.2M NaOH for 30 min at room temperature followed by a second wash in 0.1 x SSC, 0.1% SDS, 0.2M Tris pH 7.6 for 15 min at room temperature.

2.5.2 Isolation of PAC DNA from bacterial cells

Positive clones 259-05, 259-P8 and 232-L14 were identified on the PAC filters and ordered from the UK HGMP Resource Centre. PAC DNA was isolated from bacterial cells using a Qiagen large construct kit. Briefly, the bacteria were subject to alkaline lysis followed by an ATP-dependent exonuclease digestion which removes contaminating genomic DNA as well as damaged large-construct DNA. DNA was then bound to and eluted from a column containing anion-exchange resin as described in section 2.2.11 for the Qiagen midi and maxi kits. PAC clone 259-05 was selected for further analysis.

2.6 Cytogenetic analysis (Work carried out in collaboration with Carol Griffin)

2.6.1 Chromosomal mapping of PAC DNA

Clone 259-05 DNA was labelled with biotin-dUTP by nick translation (Bionick kit, BRL). The biotinylated probe was hybridised to denatured human metaphase chromosomes (isolation of metaphase cells described in section 2.6.2) using standard methods (Cartwright *et al.*, 1998a). The probe was detected using texas red avidin DCS (Vector) and the signal amplified by adding biotinylated anti-avidin D and a further round of texas red avidin DCS. Cells were visualised using a BX60

fluorescent microscope and metaphases were captured and karyotyped using the Genus Applied Imaging system.

2.6.2 Spontaneous chromosome aberration analysis

Cells were treated with 0.05µg/ml colcemid for 2 h prior to harvesting to increase the number of metaphases obtained. After harvesting, cells were incubated in hypotonic solution (0.038M KCl, 0.017M trisodium citrate) for 20 min at 37°C, and then fixed for approximately 16 h in 75% ethanol, 25% acetic acid. Fixed cells were dropped onto slides and stained with Giemsa for analysis. Chromosome aberrations were scored and classified according to a previously described method (Savage, 1976) from coded slides.

2.6.3 Sister chromatid exchange (SCE) analysis

Cells were seeded in T75 flasks and grown for 48 h to sub-confluent levels. Sister chromatids were differentially labelled by treating the cells with 10µg/ml BrdU in the dark for two cell cycles (24 h). During the first 2 h of this labelling the cells were also exposed to MMC in serum free media. Metaphase preparations were made using the techniques described above and differential staining of the chromatids was achieved using the fluorescence plus Giemsa (FPG, 'harlequin') staining method (Perry and Wolff, 1974).

2.7 Reverse transcriptase (RT)-PCR

2.7.1 Isolation of RNA from mammalian cells

Total RNA was isolated from mammalian cells using a Qiagen RNeasy mini kit. Briefly, up to 1×10^7 cells were lysed in a guanidine isothiocyanate (GITC)-containing buffer that inactivates RNases. Samples were homogenised by passing the lysate through a 20G needle, after which ethanol was added to provide conditions

which allow RNA to bind to the silica-gel membrane of a spin column. Contaminants were removed from the sample by three wash steps before RNA was eluted from the column in RNase free water. The RNA was then treated with 1U per 10µl sample RQ1 RNase-free DNase (Promega) for 15 mins at 37°C and aliquots were stored at -70°C.

Determination of RNA concentration, purity and integrity:

RNA concentration and purity were determined using a spectrophotometer in a similar manner to the method used to determine DNA concentration and purity (see section 2.2.11). Absorbance readings at 260nm were used to measure RNA concentration where an OD reading of 1 corresponds to 40µg/ml RNA. RNA purity was calculated using the OD₂₆₀/OD₂₈₀ ratio and should be between 1.5 and 1.9 when the sample is diluted in water. The integrity of RNA was checked by running 5-10µg of samples on a 0.8% agarose gel. This was carried out in a similar way to that described in section 2.2.3 except that all apparatus were soaked for 10 mins in 3% H₂O₂ before use and solutions were made up in 0.1% DEPC water to limit RNA degradation.

2.7.2 RT-PCR

RT-PCR was carried out using a SuperscriptII RNase H⁻ reverse transcriptase kit (GibcoBRL). 5µg RNA was mixed with 500ng Oligo (dT)₁₅ (Promega) and first strand cDNA synthesis was carried out according to the manufacturers protocol. The resulting cDNA was incubated with 2U RNase H (Invitrogen) per 20µl sample, to remove complementary RNA, for 20 mins at 37°C. 2µl of this reaction was used in a 25µl PCR reaction as described in section 2.2.1.

2.8 Site-directed mutagenesis

Mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). 5-50ng dsDNA template was added to 0.2μM forward and reverse mutagenic primers, 0.2mM dNTPs, 1 x *Pfu* reaction buffer (Stratagene) and made up to a final volume of 50μl with UV treated Elix water. 2.5U of *Pfu* turbo DNA polymerase (Stratagene) was added to each reaction and thermal cycling was carried out as follows:

95°C for 30 secs

(95°C for 30 secs, 55°C for 1 min, 68°C for 13 min) x 18 cycles

68°C for 10 min

4°C Hold

After cycling each reaction was incubated with 5U of *Dpn* I (Stratagene), for 1 h at 37°C to digest non-mutated DNA. 3μl of each reaction was then electroporated into DH5α cells using the methods described in section 2.2.9.

Site-directed mutagenesis primers:

Walker A box K→A:

5'-GCACCAGGTGTTGGAGCAACACAATTATGTATGCAGTTG-3'

Walker A box K→R:

5'-GCACCAGGTGTTGGACGAACACAATTATGTATGCAGTTG-3'

Only the forward mutagenic primers are shown, reverse primers are the exact complement. Bases altered for mutagenesis are underlined.

2.9 Immunofluorescence

Cells were seeded onto coverslips and grown for 48 h to sub-confluent levels before being mock-treated or exposed to 10 Gy X-rays. After a further 5 h incubation at 37°C, cells were washed three times with 4°C PBS and fixed with 1% paraformaldehyde in PBS for 2 h at 4°C. In between each of the following stages cells were washed three times with 4°C PBS. Fixed cells were permeabilised for 10 min at room temperature in 0.1% Triton X-100, 0.1 x SSC and then blocked for 1 h at room temperature in 5% horse serum in PBS. Cells were incubated overnight at 4°C with 1:100 dilution primary antibody, rabbit polyclonal anti-RAD51 (Ab-1) antibody. This was followed by a 1 h incubation at room temperature, in the dark with 1:100 dilution secondary antibody, Cy5-conjugated goat anti-rabbit IgG. Coverslips were then mounted in Vectashield anti-fade medium and cells were analysed using a BioRad MRC600 confocal microscope. Images were selected at random, and were scored from coded slides by two independent scorers, recording the number of discrete strongly fluorescing nuclear foci in each cell. At least 50 cells were scored for each data point, and data were compared statistically to assess homogeneity using the Mann-Whitney U test.

2.10 Cell cycle analysis

Cells were grown for 48 h to sub-confluent levels before being mock-treated or exposed to 10 Gy X-rays. After a further 5 h incubation at 37°C cells were harvested as a single celled suspension. The cells were then pelleted by centrifugation and washed three times in PBS before they were fixed in 1.5ml 50% ethanol in PBS for at least 30 minutes at 4°C. Cells were treated with 0.5% RNase A, and propidium iodide was added to a final concentration of 50µg/ml, prior to flow cytometry analysis using a FACSort (Beckton-Dickinson).

2.11 Western blotting

2.11.1 Preparation of whole cell protein extracts

Cells were grown for 48 h to sub-confluent levels before being mock-treated or exposed to 10 Gy X-rays. After a further 5 h incubation at 37°C the cells were harvested as a single cell suspension, pelleted by centrifugation, washed in PBS and resuspended in Elix water. Cells were lysed by heating for 5 min at 95°C (with frequent vortexing) before being spun for 5 mins at 14000 rpm in an Eppendorf 5415C microcentrifuge. The protein concentration of the supernatant was determined using the BioRad protein assay with a set of standards of known protein concentration. Samples were then mixed with an equal volume of Laemmli SDS sample buffer (10% glycerol, 5% β -mercaptoethanol, 3% SDS, 0.01% bromophenol blue, 100mM Tris-HCl pH 6.8) before being either loaded directly onto a polyacrylamide gel or stored in aliquots at -70°C.

2.11.2 Polyacrylamide gel electrophoresis

Protein samples were separated on SDS-polyacrylamide gels. The running gel contained; 7.5% acrylamide/bisacrylamide solution 37.5:1 (Gibco BRL), 250mM Tris-HCl pH 8.8, 0.1% high grade SDS, 0.2% TEMED and the stacking gel contained; 3.7% acrylamide, 125mM Tris-HCl pH 6.8, 0.1% high grade SDS, 0.2% TEMED. Gels were set by the addition of 0.06% freshly prepared ammonium persulphate. Gels were run in a Hoefer miniVE vertical electrophoresis system, in SDS-PAGE buffer (380mM glycine, 50mM Tris-base, 0.1% SDS) at 100-180V.

2.11.3 Western blotting

Proteins were transferred onto a PVDF Immobilon-P membrane (Millipore) using Flowgen blotting apparatus containing transfer buffer (190mM glycine, 25mM Tris-base, 20% methanol). Protein transfer was carried out for either 2 h at 400 mA or

overnight at 40 mA after which the membrane was incubated with blocking solution (5% milk (Marvel), 5% BSA, 0.1% Tween 20 in PBS) for 2-16 h at 4°C. The membrane was then incubated for 1 h with the appropriate primary antibody diluted to a suitable concentration in blocking solution. After 2 rinses and 3 x 10 min washes in wash buffer (0.1% Tween-20 in PBS), the membrane was incubated for 1 h with secondary horse radish peroxidase (HRP) conjugated antibody diluted to a suitable concentration in blocking solution. After a further set of washes, protein was visualised using an enhanced chemiluminescence (ECL) kit (NEN Renaissance) according to the manufacturer's protocol. Densitometry analysis was carried out on a Kodak image station 440CF. If a membrane was to be reprobed it was incubated for 30 min at 50°C with stripping buffer (2% SDS, 100mM β -mercaptoethanol, 62.5mM Tris-HCl pH 6.8) before being rechecked by ECL.

2.11.4 RAD51L2 immunoprecipitation

Immunoprecipitation analysis was carried out according to the method described by Masson *et al.* (2001b). HSC230 (human Fanconi anaemia cell line), V79 and irs3 cell pellets (2.5×10^8 cells) were resuspended in 3ml lysis buffer (50mM Tris-HCl at pH 7.5, 0.5M NaCl, 0.5% NP-40) containing protease inhibitors, incubated on ice for 30 min, dounced 10 times and sonicated twice for 20 s. Insoluble material was removed by spinning at 47000 rpm for 45 min in a TL-100 ultracentrifuge set at 4°C. Protein complexes in the supernatant were pulled down for 1.5 h at 4°C using an anti-hRAD51L2 pAb (see table 2.5) cross-linked to Aminolink beads (Pierce). Complexes were washed four times in lysis buffer and visualised by Western blotting (see above) using an anti-hRAD51L2 mAb (see table 2.5) at a 1:100 dilution.

2.12 Homologous recombination repair assay

2.12.1 GFP expression assay

The recombination reporter construct used in this assay was kindly provided by Dr. Maria Jasin, Memorial Sloan-Kettering Cancer Centre, New York. V79 and *irs3* cells were stably transfected with *Xho*I linearised p59xDR-GFP6 DNA and independently isolated clones were used in the assay. Sub-confluent cells were harvested as a single cell suspension and diluted to a concentration of 2×10^6 cells per 0.8ml in cytomix (see section 2.3.2). 0.8ml volumes of cell suspension were either transiently transfected with 50µg pCBASce (*I*-SceI expression vector), or transiently cotransfected with 50µg pCBASce and 20µg *RAD51L2* expression vector. Transfected cells were put into non-selective medium for 45 h after which they were harvested and washed twice in PBS. Flow cytometry analysis was carried out on 2×10^5 cells and data were recorded on a green (FL1) versus red (FL3) fluorescence plot.

2.12.2 Southern blotting

Southern blotting was used to determine the integrity and copy number of the DR-GFP construct in stably transfected clones.

Isolation of genomic DNA from mammalian cells:

Genomic DNA was isolated from mammalian cells using the Qiagen genomic DNA midi or maxi kit according to the manufacturer's protocol. Briefly, cells were harvested and lysed after which DNA was bound to the anion-exchange resin of a gravity-flow column under low salt and pH conditions. RNA, proteins and low-molecular-weight impurities were then removed with a medium salt wash and DNA was eluted in a high salt buffer. It was then concentrated and desalted by isopropanol precipitation.

Blotting:

Approximately 10µg of genomic DNA was digested with around 60U of the appropriate restriction enzyme for 8-16h at 37°C. One fifth of this sample was run on

a 0.5% minigel to check for digestion before the rest of the sample was run on a 0.8% gel at 3-5 V/cm with suitable markers. Following electrophoresis the gel was put into 0.25M HCl for 25 min to nick the DNA. The gel was then rinsed with Elix water before being placed in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30 min. After a further rinse with Elix water the gel was placed in neutralisation solution (1.5M NaCl, 0.5M Tris-HCl pH7.2, 0.001M EDTA) for 20 min. The DNA was then transferred overnight to Hybond N+ nylon membrane (Amersham) by capillary action using 20 x SSC as the transfer buffer. The next day the membrane was washed in 2 x SSC and air-dried. DNA was then fixed to the membrane using the optimal crosslink setting on an XL-1500 UV crosslinker (Spectronics Corporation).

Probe preparation:

Probes were labelled using a Megaprime DNA labelling kit and unincorporated nucleotides were separated from the probe using MicroSpin G-25 columns as described in section 2.5.1.

Hybridisation:

This method is similar to that described in section 2.5.1 where some of the solutions and equipment have already been outlined. The membrane was incubated with 25ml pre-hybridisation solution; 5 x SSPE, 5 x Denhardt's solution, 0.5% SDS, 0.02mg/ml herring sperm DNA (pre-heated to 100°C for 5 min and cooled on ice before use) for approximately 5 h at 65°C (20 x SSPE: 3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA pH7.7). After this time the pre-hybridisation solution was removed and replaced with 10ml pre-heated hybridisation solution (the same as the pre-hybridisation solution) containing purified radiolabelled probe (denatured by heating to 100°C for 7 min and cooling on ice). The membrane was incubated with the probe for at least 16 h at 65°C. Following hybridisation the membrane was washed for 15 min at room temperature in 2 x SSPE, 0.1% SDS to remove excess label. Further washes were carried out at 65°C and used reduced concentrations of SSPE depending

on the amount of radioactivity remaining on the membrane. When a suitable number of counts representing specific signal were left on the membrane it was exposed to an imaging screen (Kodak) for an appropriate amount of time. This was then visualised on a phosphorimager (BioRad Molecular Imager FX). Membranes were also exposed to X-ray film at -70°C that was developed using an automatic developer.

CHAPTER 3: The *RAD51L2* gene complements the DNA damage sensitive phenotype of the *irs3* cell line

3.1 Introduction

The *irs3* cell line was isolated with *irs1*, from the Chinese hamster cell line V79-4 in a screen for X-ray sensitive mutants, and shows a 2-fold sensitivity to X-rays. *irs3* cells are also slightly but reproducibly sensitive to UV irradiation as well as 2.5 fold sensitive to EMS. However, the most striking aspect of the *irs3* cell phenotype is a 7-fold sensitivity to MMC (Jones *et al.*, 1987). More detailed investigation into *irs3* cross-sensitivities has shown hypersensitivity to a variety of other DNA cross-linking agents and a milder sensitivity to monofunctional alkylating agents, oxidative mutagens and certain topoisomerase inhibitors including etoposide and camptothecin (Caldecott and Jeggo, 1991; Jones *et al.*, 1993; Thacker and Ganesh, 1990).

Several processes found to be defective in other radiosensitive cell lines have been investigated in *irs3*. *irs3* cells are proficient in the repair of IR-induced DNA ssb and dsb and do not exhibit radioresistant DNA synthesis (Cheong *et al.*, 1992; Thacker and Ganesh, 1990). They show wild type frequencies of mutation in the HPRT gene and undergo V(D)J recombination normally (Thacker *et al.*, 1994). *irs3* cells also show a recovery from the effects of low-dose-rate irradiation which is similar to that observed in wild type cells (Thacker and Wilkinson, 1995).

A notable aspect of the *irs3* phenotype is increased chromosomal instability compared to wild type cells. The cells show elevated levels of spontaneous micronuclei and are particularly sensitive to the induction of micronuclei by cross-linking agents, showing a 2.8-fold increase in percentage cells micronucleated after treatment with 2.5 μ M 1,3 butadiene diepoxide (DEB) (Jones *et al.*, 1993). *irs3* cells are also hypersensitive to the induction of kinetochore-negative micronuclei and to a lesser extent kinetochore-positive micronuclei after X-ray treatment, but are not hypersensitive to the induction of kinetochore-positive or -negative micronuclei after treatment with the aneugen podophyllotoxin or etoposide (Hermine *et al.*, 1997). Analysis of metaphase chromosome aberrations has shown a spontaneous increase in

chromatid deletions compared to wild type cells. A dramatic increase in chromatid aberrations, in particular chromatid exchanges (>50-fold increase), is observed after treatment with 2.5 μ M DEB (Jones *et al.*, 1993).

There have been several genetic studies carried out to divide radiosensitive mutants into different complementation groups. *irs3* cells have been fused to a variety of hamster and mouse mutants and the resulting hybrids tested for IR sensitivity. The *irs3* mutation was found to be recessive (Jones *et al.*, 1988) and the *irs3* cell line complemented the IR sensitivity of all other cell lines tested placing it into a unique complementation group (Jeggo *et al.*, 1991; Jones *et al.*, 1988; Sato *et al.*, 1995; Thacker and Wilkinson, 1991). *irs3* has also undergone genetic testing with a number of MMC sensitive hamster mutants and was again shown to represent a distinct complementation group (Jones, 1994).

A great deal of work has already gone into trying to clone the gene mutated in *irs3*. Transfection of *irs3* with cDNA or genomic DNA, followed by selection for MMC resistance, proved unsuccessful. A positional cloning approach similar to that used to clone *XRCC2* from *irs1* (Tambini *et al.*, 1997) also failed (John Thacker, personal communication). Therefore we decided to employ a candidate gene approach to identify the mutated gene. This method involves transfecting cDNAs, selected by educated guesses, into a cell line and then assaying for complementation of the mutant phenotype, in this case MMC sensitivity.

Initially, there were several promising candidate genes to choose from. *irs3* cells have previously been described as having phenotypic similarities to Fanconi anaemia (FA) cells (Jones *et al.*, 1993). Both show hypersensitivity to DNA cross-linking reagents, little sensitivity to UV and chromosomal instability (Grompe and D'Andrea, 2001). However, FA cells are generally not IR sensitive (Buchwald and Moustacchi, 1998), whereas *irs3* cells are moderately IR sensitive. The Chinese hamster cell line V-H4 has been shown by genetic complementation analysis to belong to the same complementation group as FA-A cells (Arwert *et al.*, 1991). *irs3* complements V-H4 MMC sensitivity and has therefore been placed in a separate complementation group (Jones, 1994). This would suggest that *irs3* cells are not mutated in FA-A but could be mutated in one of the other Fanconi's genes.

Two further candidates were the breast cancer susceptibility genes BRCA1 and BRCA2. Cells deficient in either of these genes show elevated levels of chromosomal aberrations and X-ray sensitivity (Venkitaraman, 2001). Sensitivity to cross-linking agents has also been found in certain cell lines including Brca1-deficient mouse ES cells (Bhattacharyya *et al.*, 2000; Moynahan *et al.*, 2001a) and Brca2-deficient mouse lymphocytes (Yu *et al.*, 2000) and hamster cells (Kraakman-van der Zwet *et al.*, 2002). It should be noted that not all this information was available at the beginning of this study that was initiated in 1999.

It is also possible that mutation of an essential gene that leads to a reduction rather than an abolition of gene function gives rise to the *irs3* phenotype. “Leaky” expression of an essential gene may allow cells to survive when complete disruption of the gene would be lethal. One potential candidate for this type of gene is *RAD51*. Disruption of the *Rad51* gene in mice leads to early embryonic lethality (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). Also, *RAD51*^{-/-} chicken DT40 cells, carrying a human RAD51 transgene under the control of a repressible promoter, accumulate in G2/M before dying when the transgene is inhibited (Sonoda *et al.*, 1998).

At the beginning of this study the five *RAD51*-like genes appeared to be the best choice for a candidate gene experiment. This was based on the observation that the phenotypes of *irs1* and *irs1SF* are very similar to that of *irs3* although they are more severe. *irs1* and *irs1SF* are mutated in *XRCC2* and *XRCC3* respectively (Cartwright *et al.*, 1998b; Liu *et al.*, 1998). However, genetic complementation experiments had previously shown *irs3* to be in a different complementation group to *irs1* and *irs1SF* (Jeggo *et al.*, 1991; Jones *et al.*, 1988; Thacker and Wilkinson, 1991). Therefore, *irs3* cannot be mutated in either *XRCC2* or *XRCC3*. The *RAD51L1* cDNA had also been transfected into *irs3* cells and no change in MMC sensitivity was observed (John Thacker, personal communication). The remaining two *RAD51*-like genes *RAD51L2* and *RAD51L3* consequently became the candidate genes with most potential. The aim of this work hence became to transfect the *RAD51L2* and *RAD51L3* cDNAs into *irs3* and assay stably transfected clones for increases in MMC resistance.

3.2 Results

3.2.1 *RAD51L2* cDNA complements *irs3* MMC sensitivity

The human *RAD51L3* gene had previously been cloned in this lab (Cartwright *et al.*, 1998a). It was recloned into the mammalian expression vector pZeoSV2(-) for this experiment. The human *RAD51L2* gene was amplified from a human testis cDNA library (Clontech), using primers with restriction sites incorporated into their sequence, which enabled subsequent cloning into pZeoSV2(-). The primers, vectors and restriction sites used in all cloning procedures can be found in Materials and Methods, section 2.1.

irs3 cells were stably transfected with the *RAD51L3* and *RAD51L2* constructs and five independent zeocin resistant clones of each were isolated. Genomic DNA was extracted from the clones (see “quick” isolation of genomic DNA for PCR, Materials and Methods section 2.3.3) and used as a template for PCR, with primers to amplify either the full length *RAD51L3* or *RAD51L2* gene. Three of the five *RAD51L3* clones (clones #1, #2 and #3) were found to carry the *RAD51L3* gene but surprisingly only one *RAD51L2* clone (clone #2) was found to carry the *RAD51L2* gene (Fig. 3.2 B and C). All clones were then subjected to a preliminary MMC survival assay (Materials and Methods section 2.4.1) together with untransfected V79 and *irs3* cells. As expected, *irs3* cells were found to be more MMC sensitive than V79 cells, with *irs3* and V79 surviving MMC concentrations of 10-20 nM and 100-200 nM respectively (Fig. 3.1). All *RAD51L3* clones showed a level of MMC sensitivity similar to that of untransfected *irs3* cells, irrespective of whether they carried the *RAD51L3* gene. However, the *RAD51L2* clone shown by PCR to carry the *RAD51L2* gene was more resistant to MMC (surviving to 50-100 nM) than clones that did not carry the gene or untransfected *irs3* cells (Fig. 3.1 and data not shown). *irs3* clones carrying empty pZeoSV2(-) vector showed a similar level of MMC sensitivity to untransfected *irs3* cells (data not shown). These results indicate that *RAD51L2* cDNA, but not *RAD51L3* cDNA, can complement the MMC sensitive phenotype of the *irs3* cell line.

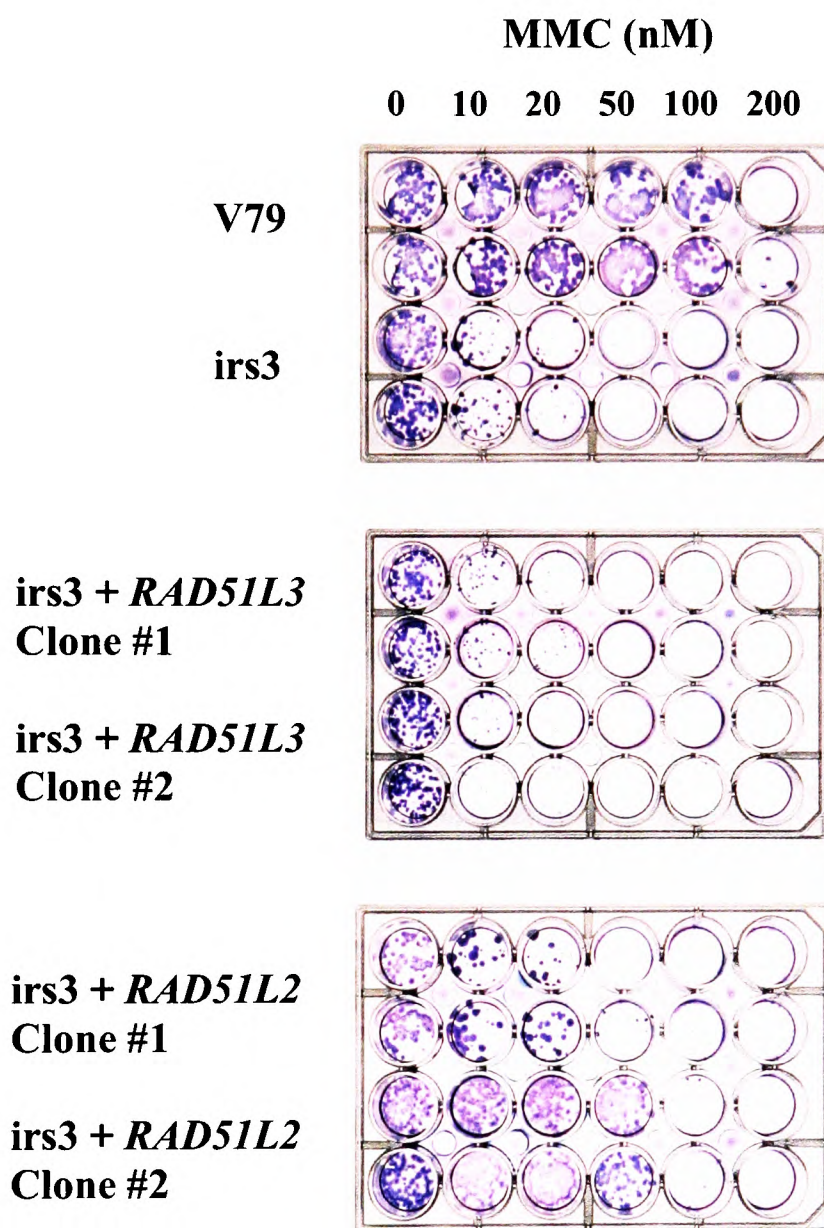


Figure 3.1 *RAD51L2* cDNA complements the MMC sensitivity of *irs3*. Survival of V79, *irs3*, *irs3* transfected with *RAD51L3* cDNA (both clones carry the gene) and *irs3* transfected with *RAD51L2* cDNA (clone #1 does not carry the gene, clone #2 carries the gene). Each cell line is in duplicate.

3.2.2 Recloning of *RAD51L2* into a bicistronic expression vector

The previous section describes data that shows that *RAD51L2* cDNA complements *irs3* MMC sensitivity. However, this result is dependent on data obtained from just one *RAD51L2* transfected clone. To verify the result a further 20 zeocin-resistant clones were isolated. As before, each clone was tested for the presence of the full-length *RAD51L2* gene by genomic DNA PCR and then subject to the preliminary MMC assay. It was found that of the 25 clones isolated in total, 16 contained the full length *RAD51L2* gene by PCR but only 3 showed increased resistance to MMC. All MMC resistant clones carried the *RAD51L2* gene (Table 3.1). This suggests that 13 of the 16 clones shown to carry the *RAD51L2* gene by genomic DNA PCR are not expressing the gene product, and are therefore not resistant to MMC. To overcome this problem it was decided to reclone the *RAD51L2* gene into the pIRESneo2 vector. This vector contains an internal ribosome entry site (IRES) that allows translation of the selective marker (G418) and the gene of interest on the same RNA. *irs3* cells were stably transfected with this construct and 24 G418 resistant clones were isolated. 4 of these clones were found to carry the *RAD51L2* gene by genomic DNA PCR and each of the 4 clones was also shown to express *RAD51L2* by RT-PCR (Fig. 3.2 D). All 4 clones were also found to be MMC resistant (Table 3.1). Clone #2 and clone #13 were used in further experiments and from here on are called clone #1 and clone #2 respectively, which is the notation used in French *et al.*, 2002.

In previous experiments, *irs3* cells transfected with *RAD51L3* in pZeoSV2(-) (Fig. 3.1) or *RAD51L1* in pEBS7 (John Thacker, personal communication) showed no increase in MMC sensitivity. In light of the above results it was decided to check the expression of these clones by RT-PCR. All clones found to carry the full-length *RAD51L1* or *RAD51L3* gene by genomic DNA PCR were also shown to express it by RT-PCR (Fig. 3.2 A and B).

Table 3.1 Analysis of *RAD51L2* transfected clones.

	hs <i>RAD51L2</i> in pZeoSV2(-)	hs <i>RAD51L2</i> in pIRESneo2
Total number of clones isolated	25	24
Clones shown to carry the <i>RAD51L2</i> gene by PCR	16	4
MMC resistant clones	3	4

In order to measure complementation levels more accurately V79, *irs3* and two MMC resistant clones, transfected with the *hsRAD51L2* in pIRESneo2 construct, were subject to a full MMC survival assay (see Materials and Methods section 2.4.2). *RAD51L2* cDNA-transfected clones showed a significant increase in MMC resistance when compared to untransfected *irs3* cells (Fig. 3.3). *irs3* clones carrying empty pIRESneo2 vector showed a similar level of MMC sensitivity to untransfected *irs3* cells (data not shown). It can be concluded from these data that there is a direct correlation between the presence of the *RAD51L2* gene and the restoration of MMC resistance in *irs3* cells.

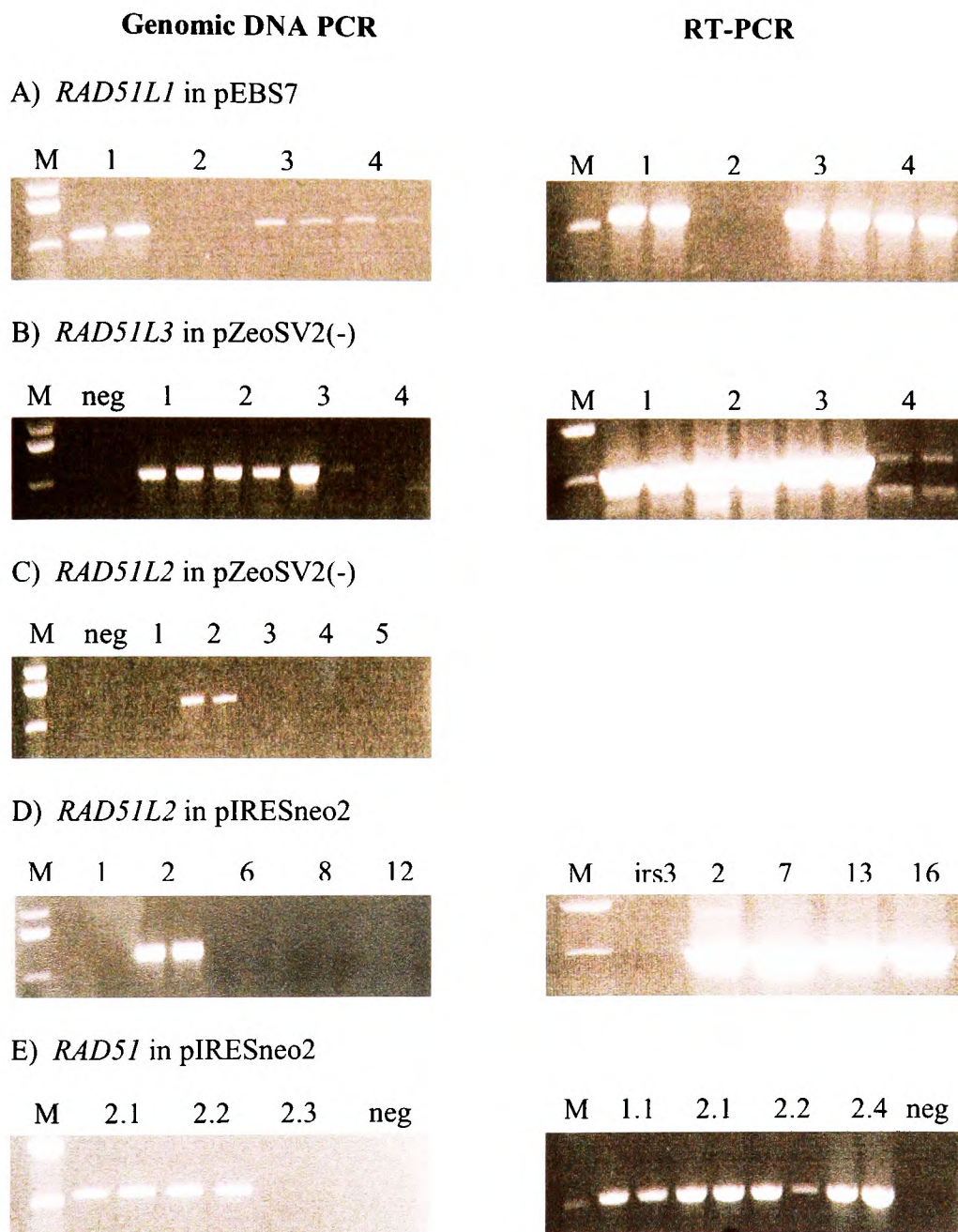


Figure 3.2 Genomic DNA PCR and RT-PCR analysis of drug resistant cDNA transfected clones. Primers used in both types of PCR designed to give full-length gene products except for D, RT-PCR where internal primers were used. All samples run in duplicate appart from D, RT-PCR where samples were run singularly. M, molecular size marker (Invitrogen) with bands equivalent to 1018bp and where shown to 1636bp and 2036bp. Numbers at the top of each gel represent the clone being analysed. Neg, negative.

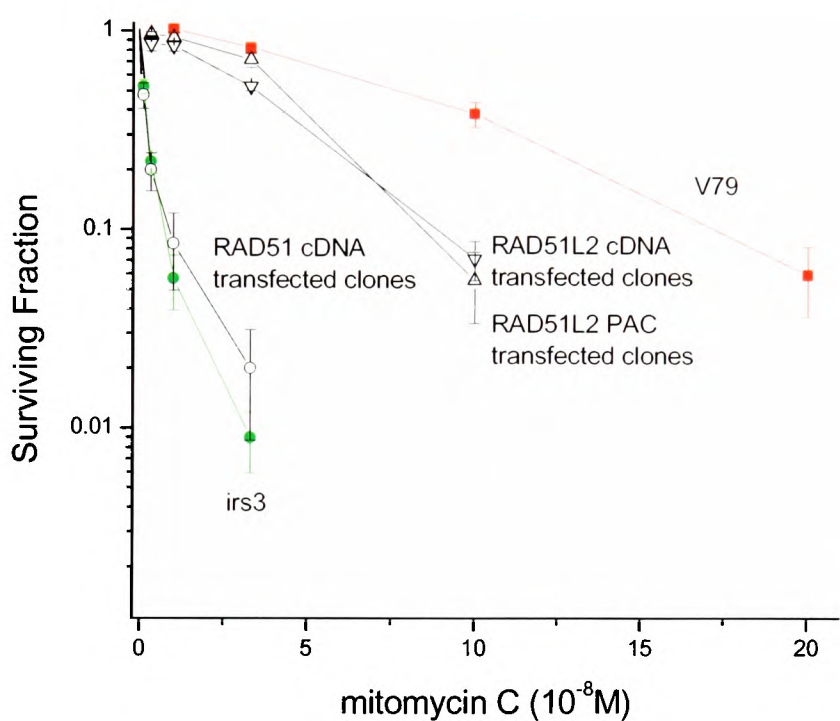


Figure 3.3 MMC survival of V79, irs3 and transfected irs3 clones. Red, V79; green, irs3; black, transfected clones (upright triangle, *RAD51L2* cDNA; inverted triangle, *RAD51L2* PAC DNA, circle *RAD51* cDNA). Results from 2-3 experiments per cell line or clone; transfected clone data shown as mean of 2 independent clones per transfected gene (error bars = SE of the mean).

3.2.3 *RAD51L2* PAC DNA complements *irs3* MMC sensitivity

Although transfection of *irs3* cells with *RAD51L2* cDNA leads to a substantial increase in MMC survival, resistance is not restored to wild type levels. This could be due to species differences between the human and hamster cDNAs or because the human *RAD51L2* cDNA is being overexpressed under the CMV promoter. To address the second point it was decided to try and express *RAD51L2* DNA at levels closer to those normally found in the cell. Filters from the human genomic PAC library RPCI1 were probed with full length *RAD51L2* cDNA and three *RAD51L2* clones were isolated (Materials and Methods section 2.5). One of these clones (259-05) was mapped to chromosome 17q (Fig. 3.4) which is where the human *RAD51L2* gene is reported to be located (Dosanjh *et al.*, 1998). *irs3* cells were then stably transfected with clone 259-05 DNA and MMC survival data were obtained for two independently isolated clones. However, no further increase in MMC resistance was observed in PAC transfected *irs3* cells, with *RAD51L2* cDNA and PAC transfected clones giving very similar results (Fig. 3.3).

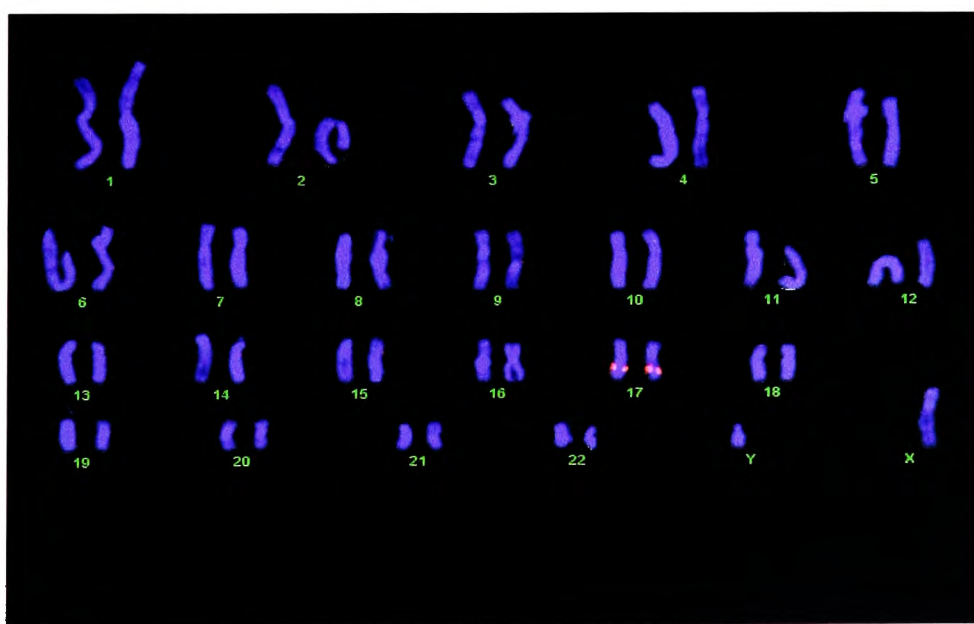


Figure 3.4 Localisation of PAC 259-05 DNA to human chromosome 17q. Pink signals are the labelled PAC probe.

3.2.4 *RAD51* cDNA does not complement *irs3* MMC sensitivity

The *RAD51* gene has previously been shown to complement DNA-damage sensitivity when overexpressed in cell lines that are defective in *RAD51*-like genes. In yeast, overexpression of Rad51 partially suppresses the IR sensitivity of Rad55 and Rad57 mutants (Hays *et al.*, 1995; Johnson and Symington, 1995). In chicken DT40 cells expression of h*RAD51* restores γ -ray and MMC sensitivity to wild type levels in *RAD51L1* mutants (Takata *et al.*, 2000). Furthermore, cisplatin sensitivity is partially complemented and γ -ray sensitivity almost fully corrected by h*RAD51* in *RAD51L2*, *RAD51L3*, *XRCC2* and *XRCC3* mutants (Takata *et al.*, 2001). It was therefore decided to see if the h*RAD51* gene complemented the DNA-damage sensitive phenotype of the *irs3* cell line. The *RAD51* gene was cloned from a cDNA library into the pIRESneo2 vector and transfected into *irs3*. Two independently isolated clones were shown to carry the *RAD51* gene by genomic DNA PCR, and also to express it by RT-PCR (Fig. 3.2 E). The MMC sensitivity of both clones was analysed but neither was found to survive MMC treatment better than untransfected *irs3* cells (See Fig. 3.3).

3.2.5 *RAD51L2* cDNA complements *irs3* X-ray sensitivity

The *irs3* cell line has previously been shown to have a 2-fold increase in X-ray sensitivity when compared to wild type cells (Jones *et al.*, 1987). V79, *irs3* and two *RAD51L2* cDNA transfected clones were assayed for X-ray survival (Materials and Methods section 2.4.3). The survival curves obtained for V79 and *irs3* agreed with previous results (Jones *et al.*, 1987). The *RAD51L2* cDNA transfected clone survival curves were midway between V79 and *irs3* showing partial complementation of the *irs3* X-ray sensitive phenotype (Fig. 3.5).

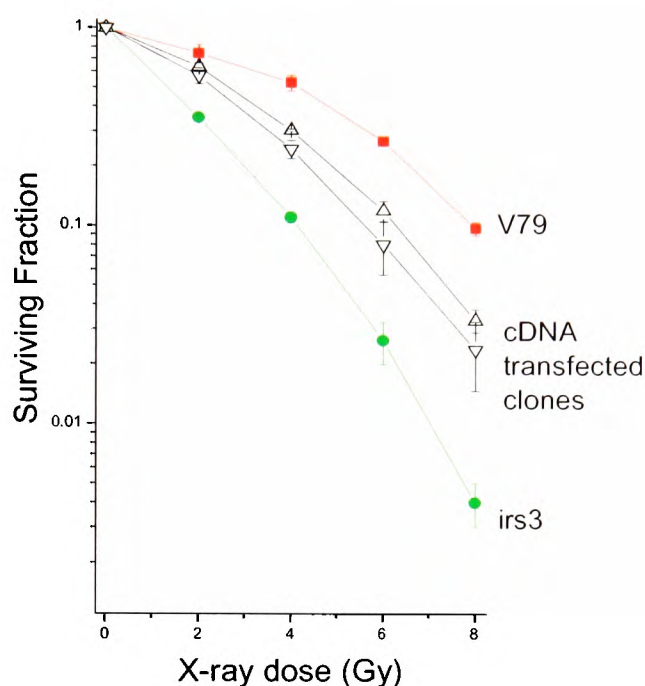


Figure 3.5 X-ray survival of V79, irs3 and *RAD51L2* cDNA transfected irs3 clones. Red, V79; green, irs3; black, *RAD51L2* cDNA transfected clones. Results from 2 experiments per cell line or clone (error bars = SE of the mean).

3.2.6 *RAD51L2* cDNA complements an increased level of spontaneous chromosome aberrations in irs3

The irs3 cell line has previously been described as chromosomally unstable (Jones, 1993). We looked at the levels of spontaneous chromosome aberrations in V79, irs3 and the two *RAD51L2* cDNA transfected clones (See methods section 2.6.1). The level of chromosome aberrations was found to be significantly higher in irs3 than in wild type cells. 1.0% of V79 cells were found to have chromosome aberrations whereas 13.2% of irs3 cells had aberrations (Fig. 3.6 B). The majority of the irs3 aberrations were found to be chromatid type aberrations with 51.5% of all aberrations being scored as isochromatid breaks (Fig. 3.6 A). As with irs3 MMC and X-ray sensitivity, the elevated level of spontaneous chromosome aberrations was largely corrected by transfection with *RAD51L2* cDNA (Fig. 3.6 A and B).

A

Cell line	Cells scored	Chromatid Breaks	Isochromatid breaks	Exchanges	Total aberrations (per 100 cells)
V79	204	0	2	0	2 (1.0)
irs3	249	5	17	11	33 (13.2)
irs3 + <i>RAD51L2</i> clone #1	205	1	3	2	6 (2.9)
irs3 + <i>RAD51L2</i> clone #2	206	4	4	0	8 (3.9)

B

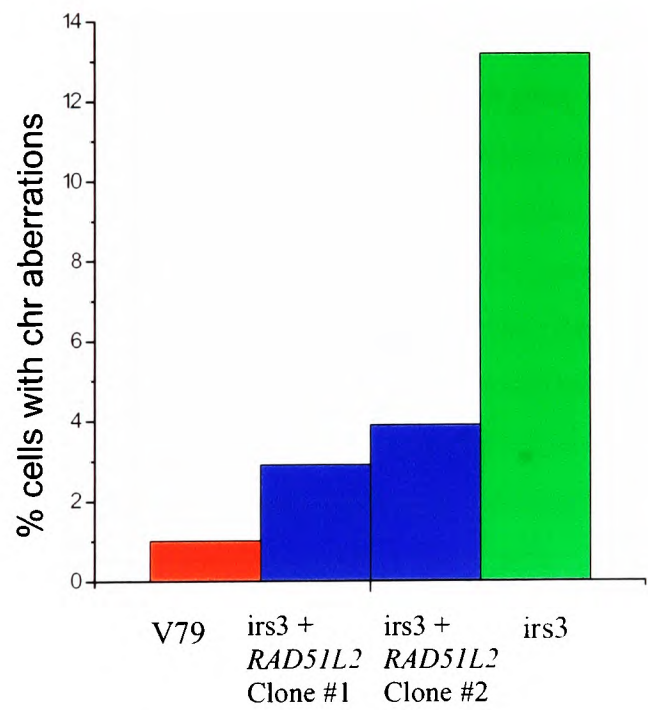


Figure 3.6 *irs3* cells show an increased level of spontaneous chromosome aberrations. A, table showing break down of spontaneous chromosome aberrations in V79, *irs3* and two *irs3* + *RAD51L2* cDNA clones. B, graph showing total number of chromosome aberrations in the same cell lines.

3.3 Discussion

The *irs3* cell line has a phenotype that suggests a defect in some aspect of DNA repair. Here we aimed to identify the gene responsible for this phenotype using a candidate gene approach. *RAD51L2* and *RAD51L3* were chosen as the candidate genes and transfected into *irs3* cells. Clones carrying stably integrated *RAD51L3* cDNA (or *RAD51L1* cDNA, John Thacker, personal communication) had a level of MMC sensitivity similar to that found in untransfected *irs3* cells. However, clones carrying *RAD51L2* cDNA were found to have significantly increased resistance to MMC.

The *RAD51L2* gene was originally cloned into the pZeoSV2(-) vector. However, after transfection of this construct into *irs3* the majority of zeocin-resistant clones isolated were not resistant to MMC even though they carried the full-length *RAD51L2* gene as determined by genomic DNA PCR. In these clones it appears that *RAD51L2* is either not expressed or that expression is down regulated. To overcome this problem *RAD51L2* was recloned into the bicistronic vector, pIRESneo2. In this context, all G418 resistant clones that carried the *RAD51L2* gene also expressed it as determined by RT-PCR and MMC survival. However, the overall number of *RAD51L2*-expressing clones isolated (4/24) was much lower than that seen when *irs3* was transfected with other genes eg. *RAD51L3* in pZeoSV2(-) where 3 out of the 5 zeocin-resistant clones isolated expressed full-length *RAD51L3*. It is possible that overexpression of the *RAD51L2* gene is deleterious to cells. *RAD51L2* expression may only be tolerated in clones that have the construct integrated in a position that results in low expression. In order for other clones to survive *RAD51L2* expression could be suppressed. This point is discussed further in Chapter 7, section 7.2.

In support of the *RAD51L2* cDNA data, *RAD51L2* PAC DNA was also found to complement *irs3* MMC sensitivity. Neither *RAD51L2* cDNA nor genomic DNA fully complemented the sensitivity suggesting that the human gene is not fully functional in hamster cells. Problems associated with overexpression could account for the lack of complete complementation in the case of the cDNA but it would be expected that the genomic DNA would be expressed at a more physiological level.

For this reason human-hamster species differences rather than overexpression appear to be the most likely reason for lack of full complementation.

As well as MMC sensitivity, the *RAD51L2* cDNA complements X-ray sensitivity and an increased level of spontaneous chromosome aberrations in *irs3*, this indicates that the complex *irs3* phenotype is caused by a mutation in one gene rather than mutations in several genes. The *irs3* chromosome aberration data agree broadly with a previous similar study (Jones *et al.*, 1993). Both studies show *irs3* to have an increased level of metaphase aberrations, namely chromatid type aberrations. We also found a very high level of isochromatid breaks in *irs3* (51.5% of all aberrations). However, the Jones study did not score isochromatid breaks as a separate category so it is difficult to tell whether this interesting finding is consistent with previous data. The significance of the elevated level of isochromatid breaks in *irs3* is discussed in Chapter 7, section 7.4.

The results described above link the presence of the *RAD51L2* gene with complementation of the *irs3* DNA damage sensitive phenotype. They also suggest that the *RAD51L2* gene is mutated in *irs3* cells and that this mutation compromises *RAD51L2* gene function. However, there are other interpretations of these results which need to be considered and two possibilities are outlined below.

Firstly, the increase in MMC resistance observed in *RAD51L2* transfected *irs3* cells may be due to reversion. High reversion frequencies have been reported in X-ray sensitive (*xrs*) CHO-K1 mutants after treatment with the DNA methylation inhibitor 5-azacytidine (AC). It is thought that under normal conditions these cells contain two gene copies one mutated copy and one copy that is silenced by hypermethylation. After treatment with AC the silent copy is reactivated leading to a reversion of phenotype (Jeggo and Holliday, 1986). However, *irs3* has previously been tested for reversion and none was detected either spontaneously or after AC treatment (Jones *et al.*, 1987). There was also no evidence for reversion during the current experiments, eg. no background clonal growth in MMC selection that would make this explanation of the results unlikely.

Secondly, it is possible that the *RAD51L2* gene is not mutated in *irs3* cells, but instead that the *RAD51L2* gene product is suppressing the effects of a mutation in

another gene. It would be necessary to identify a mutation in the *irs3 RAD51L2* gene to discount this. An example of a suppressor gene is *RAD51* which has been shown to complement the effects of defective *RAD51*-like genes in yeast and chicken DT40 cells (see section 3.2.4). However, we observed no increase in MMC resistance in *irs3* clones clones expressing *RAD51* cDNA. This differs from the expected result based on the yeast and chick cell data but concurs with the limited evidence available for mammalian cells. *RAD51* overexpression does not suppress the recombination defect observed in *irs1* and *irs1SF* cells (Johnson *et al.*, 1999; Pierce *et al.*, 1999). This suggests that there are subtle differences between the homologous recombination processes of different species.

In summary, this chapter describes the use of a candidate gene approach to identify *RAD51L2* as the most likely gene to be mutated in the *irs3* cell line. To confirm that this is indeed the correct result it will be necessary to find a mutation in *irs3* by comparing the sequence of the *RAD51L2* gene in the wild type and mutant cell lines.

CHAPTER 4: Identification of *irs3* as a *RAD51L2*-deficient cell line

4.1 Introduction

In Chapter 3 a direct correlation between the presence of the *RAD51L2* gene and increased DNA damage resistance in the *irs3* cell line was found. Cells transfected with the *RAD51L2* cDNA showed increased resistance to MMC and X-ray treatment as well as a reduction in the level of spontaneous chromosome aberrations. These data strongly suggest that there is a mutation in the *irs3 RAD51L2* gene. However, as discussed in the previous chapter there are other explanations for these results such as reversion and suppressor gene effects. To eliminate these possibilities the *irs3 RAD51L2* gene was sequenced and compared to the V79 sequence in order to identify any mutations.

A mutation or deletion in any part of the *RAD51L2* gene could potentially affect its function. Such a mutation could be found in either intron or exon sequence, or in the gene promoter. It was decided to sequence the *RAD51L2* cDNA first and to extend the sequencing to other regions if a mutation was not found. The human *RAD51L2* cDNA was previously cloned (Dosanjh *et al.*, 1998), and some genomic and cDNA sequence data for the mouse gene was available in databases. The full mouse *Rad51l2* gene sequence became available towards the end of this study (Leasure *et al.*, 2001). The hamster is not as well studied an organism as the mouse and no *RAD51L2* sequence was available. It was therefore necessary to clone the Chinese hamster *RAD51L2* gene from a cDNA library before any work could begin on sequencing it in *irs3* cells. This Chapter describes the cloning of the Chinese hamster *RAD51L2* cDNA, employing a strategy that makes use of sequence homologies between the human and mouse *RAD51L2* sequences. It also describes the subsequent sequencing of the hamster cDNA in *irs3* and V79 cells and the identification of a mutation in the *irs3 RAD51L2* gene.

4.2 Results

4.2.1 Cloning the hamster *RAD51L2* gene

The Chinese hamster *RAD51L2* gene was cloned from a CHO-K1 cDNA library (Invitrogen). All the primers used in the cloning of the hamster *RAD51L2* gene can be found in Methods, table 2.3. Partial mouse cDNA and genomic DNA *Rad51l2* sequences were obtained from database searches and aligned with the human gene sequence (Dosanjh *et al.*, 1998) to identify regions of homology. Primers were designed to conserved regions, with the 3' end of each primer lying on the first base of a codon where a change would be least likely to occur in the hamster sequence. In this way a central portion of the hamster gene was amplified from the cDNA library (Fig. 4.1, blue primers). The PCR was repeated several times and the products generated were TA cloned (See methods, section 2.2.2) and sequenced. The remainder of the hamster gene was cloned using nested primers designed to the central portion and to the library vector, pcDNA1 (Fig 4.1, 5' end primers = red, 3' end primers = green). Again, the products of several PCR reactions were TA cloned and sequenced. Finally, primers were designed to amplify the full-length gene from the cDNA library. Restriction sites in the primers allowed the gene to be cloned into the pIRESneo2 vector before the construct was verified by sequencing. The hamster *RAD51L2* gene sequence was submitted to the EMBL database, accession number AJ413202.

The *RAD51L2* hamster cDNA is predicted to consist of 9 exons based on the splice sites in the human gene. Translation of the cDNA sequence yields a protein that is 366 amino acids in length. Figure 4.2 shows an alignment of the predicted hamster *RAD51L2* protein sequence with other known *RAD51L2* proteins. It should be noted that both the hamster and the mouse *RAD51L2* sequences lack the start codon that has previously been published for the human sequence (Dosanjh *et al.*, 1998). Instead, the hamster and mouse sequences start 9 amino acids downstream where a second start codon exists in the human sequence. The mouse and hamster proteins show 84% identity to each other, and each shows 77% identity to the human

protein with only one amino acid difference in length (the analysis does not include the 9 amino acids at the start of the human protein). Also included in the alignment is the recently isolated cress (*Arabidopsis thaliana*) protein sequence (Osakabe *et al.*, 2002) and the sequence for rice (*Oryza sativa*) which was obtained from a database search. Both the cress and the rice proteins show a lower 41% identity to the hamster protein, but the Walker box motifs and certain other regions remain highly conserved. Like the human sequence, the cress sequence has two potential start codons. These codons are 22 amino acids apart, with the second codon lying 2 amino acids downstream of the mouse and hamster start codons. The sequence lying between the first and second start codons is not conserved between the human and cress proteins.

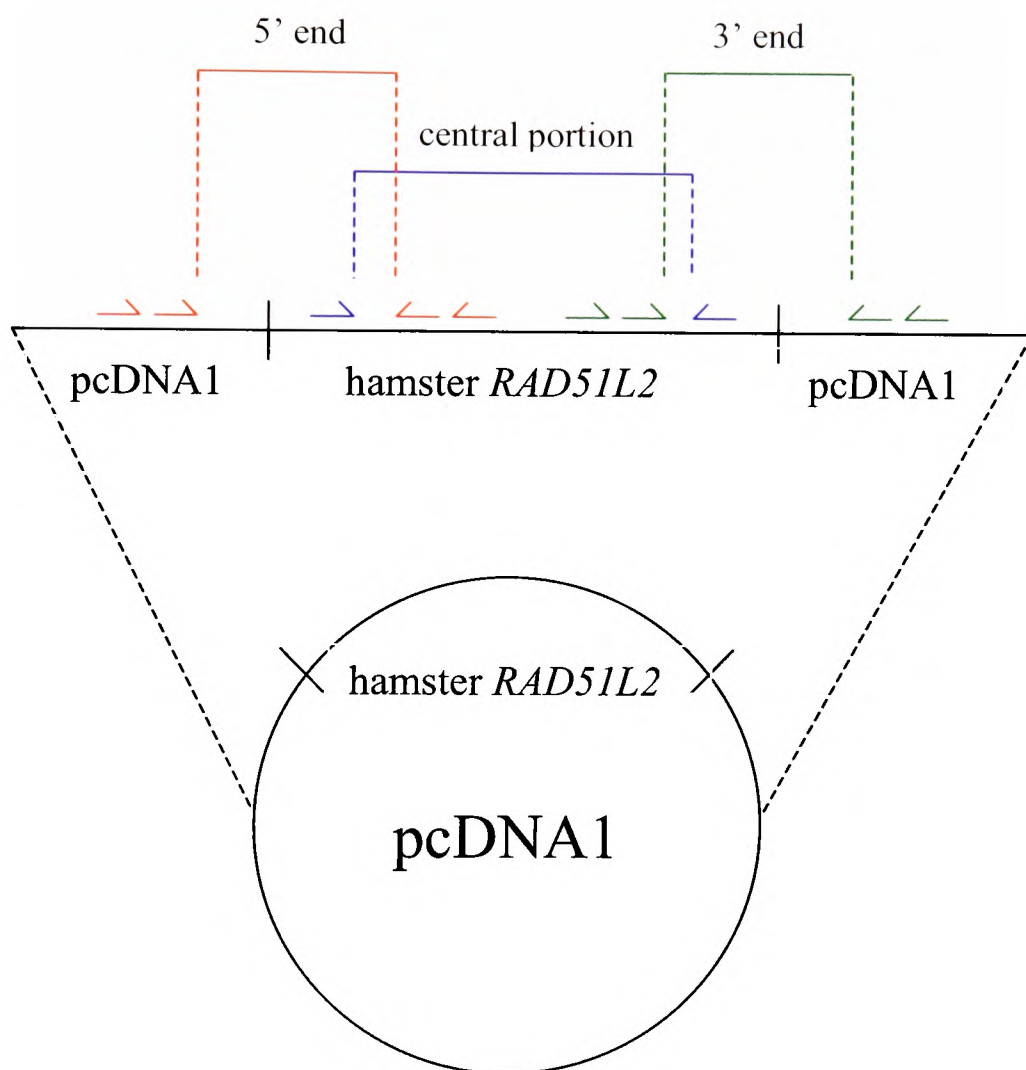


Figure 4.1 Strategy used to clone the hamster *RAD51L2* gene. A central portion of the hamster gene was cloned using primers designed to conserved regions of the human and mouse genes (blue primers). The 5' and 3' ends of the gene were cloned using nested primers designed to the central portion and to the library vector (5' end = red primers, 3' end = green primers).

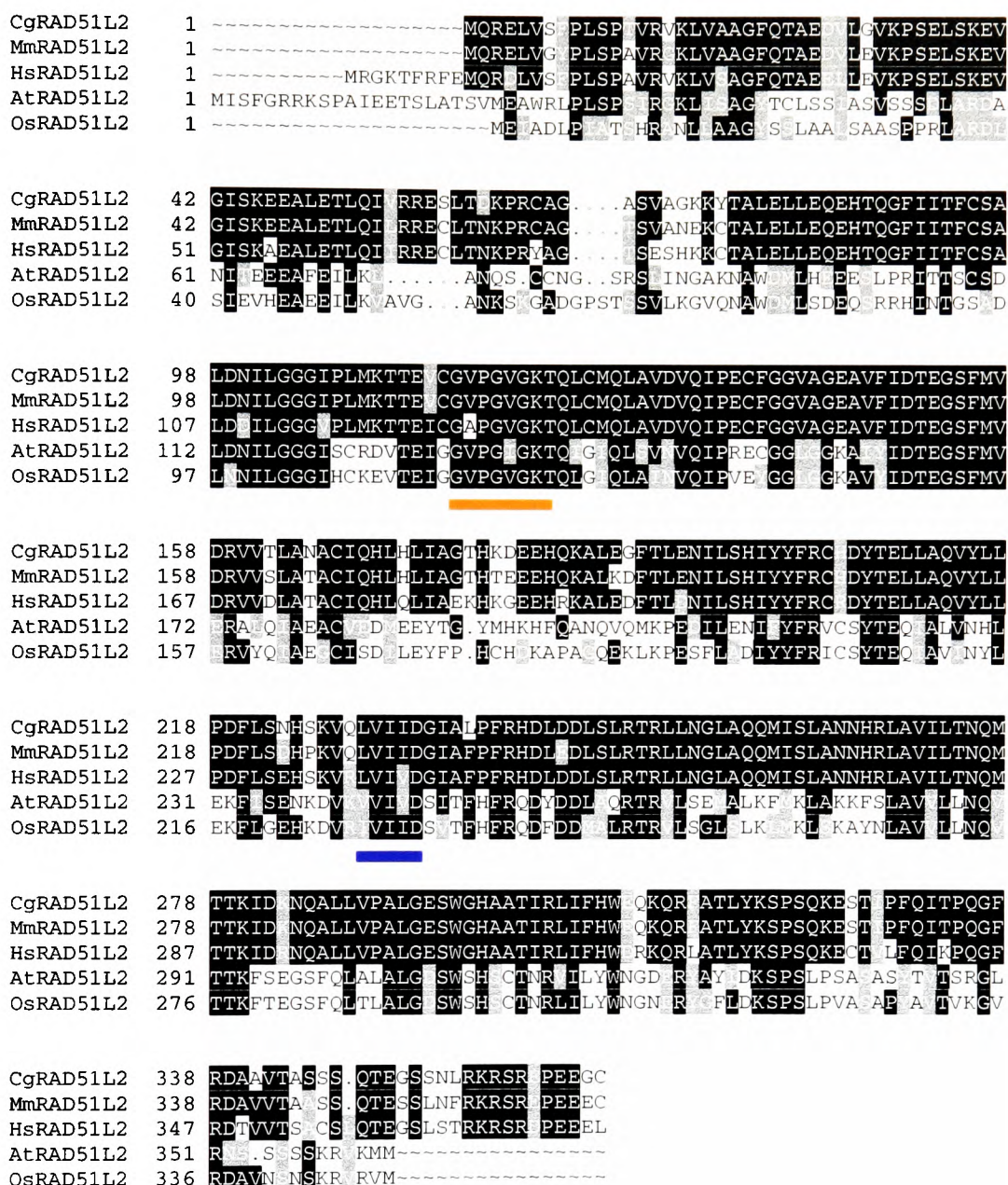


Figure 4.2 Alignment of RAD51L2 protein sequences. Alignment of hamster (AJ413202), mouse (AAK58420), human (O43502), cress (AB062456) and rice (Q9AWX8) RAD51L2 protein sequences. Sequences aligned using PileUp (W2H WEB Interface to GCG, version 4.0) and shaded using Boxshade 3.21 (www.ch.embnet.org/). Black shading indicates identical amino acids and grey shading indicates functionally conserved amino acids. The Walker A and B motifs are underlined in red and blue respectively.

4.2.2 Sequencing the *RAD51L2* gene in V79 and irs3

RNA was extracted from wild type and mutant cells and reverse transcribed to give cDNA. The cDNA was used as a template for PCR using primers designed to amplify the full-length hamster gene. The *RAD51L2* cDNA profiles for V79 and irs3 are shown in Fig. 4.3 A. Both cell lines showed a number of different sized cDNA products and several of the products were isolated and sequenced. The main V79 band was found to be the full-length *RAD51L2* gene and the other products to represent splice variants with different exons missing. The sequence of the V79 *RAD51L2* gene is identical to that of the Chinese hamster *RAD51L2* gene. In repeated amplifications of irs3 cDNA, the full-length gene product was either not present or of very low abundance and could not be cloned. The largest product that could be seen readily lacked exon 6. The sequence of this splice variant was found to be identical to that of V79. irs1 and irs2 are radiation-sensitive cell lines derived from V79 in the same screen as irs3 (Jones *et al.*, 1987). They are included as controls and their profiles are essentially the same as V79.

In an attempt to clone and sequence exon 6 in irs3, primers were designed to either side of the exon. Amplification of V79 cDNA gave predominately a full-length product containing exon 6, whereas amplification of irs3 cDNA gave mostly a product lacking exon 6 (Fig. 4.3 B). Again, it was not possible to clone the very small amount of full-length irs3 product.

In order to obtain sufficient product for cloning it was necessary to “force” the amplification of exon 6 from irs3 cDNA using a combination of primers internal and external to the exon (Fig. 4.3 C). Although the yield of PCR product was very low it was possible to TOPO TA clone it. Sequencing of exon 6 from irs3 cDNA consistently showed a G→T base substitution at the first nucleotide at the 5’ end of exon 6 (Fig. 4.4 A). This mutation changes the 3’ acceptor splice site and gives an amino acid change of Val→Phe (the significance of this is discussed further in section 4.3). This Val residue is conserved amongst all the mammalian and plant *RAD51L2* proteins currently identified (Fig. 4.4 B).

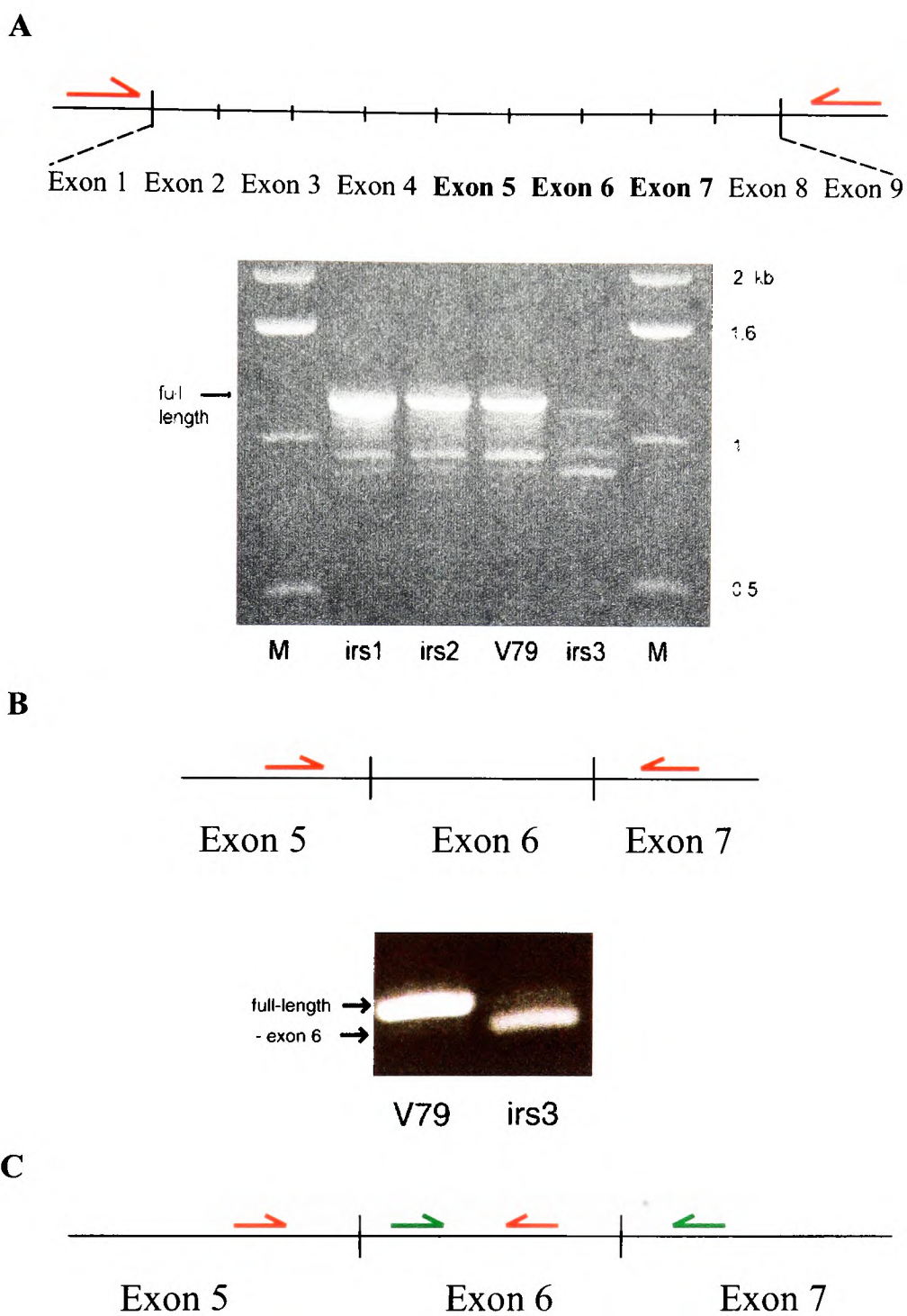


Figure 4.3 Sequencing the *RAD51L2* gene in *irs3* cells. A, PCR from *irs1*, *irs2*, V79 and *irs3* cDNA using primers designed to amplify the full-length *RAD51L2* gene. M, molecular size marker (sizes shown on the right). B, PCR from V79 and *irs3* cDNA using primers designed to either side of exon 6. Full length product is 514bp and product lacking exon 6 is 447bp. C, diagram of the primers used to “force” the amplification of exon 6 from *irs3* cDNA.

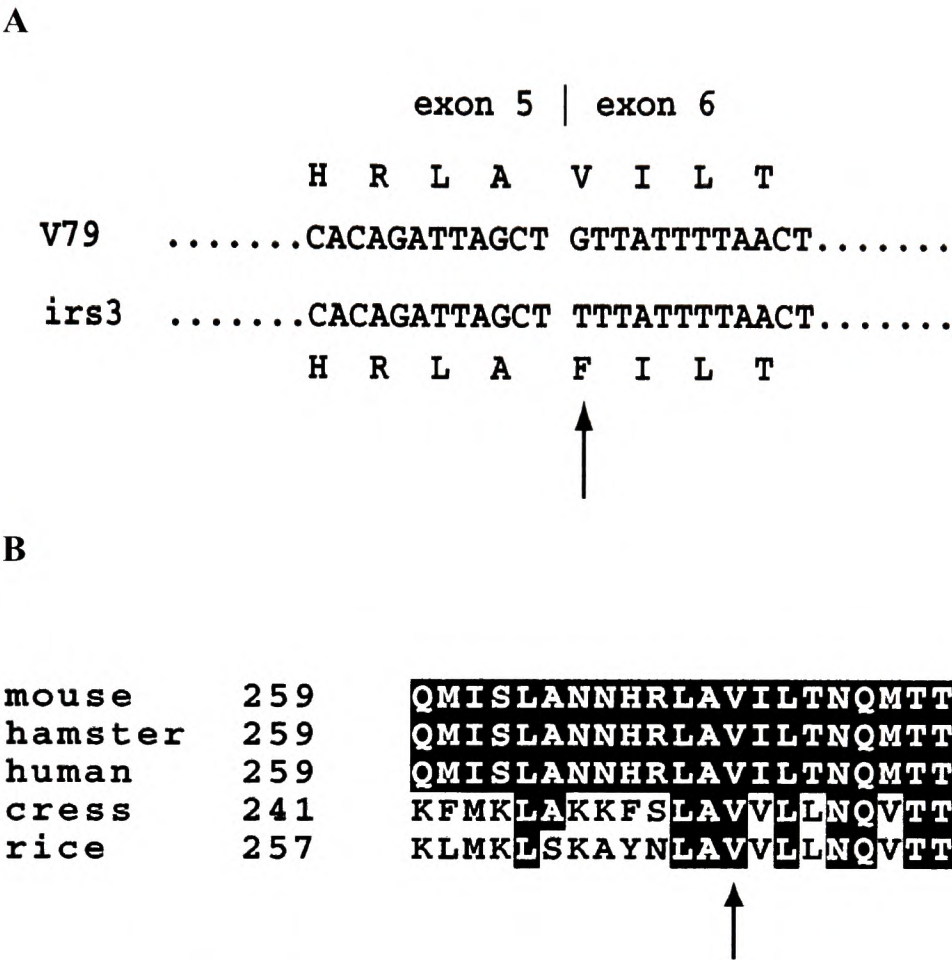


Figure 4.4 The *irs3* *RAD51L2* gene mutation. A, position of the *RAD51L2* gene mutation in *irs3*. B, conservation of the Val residue at the site of the mutation in mammalian and plant *RAD51L2* proteins.

4.2.3 Little or no RAD51L2 protein in *irs3* cells

In order to look at the effects of the *RAD51L2* gene mutation, the level of RAD51L2 protein in *irs3* and V79 was determined. The RAD51-like proteins are thought to be expressed at relatively low levels in mammalian cells so endogenous RAD51L2 protein was first immunoprecipitated from cell-free extracts before being detected by Western blotting. For a detailed description of the RAD51L2 immunoprecipitation protocol see Materials and Methods, section 2.11.4.

RAD51L2 protein was pulled down from *irs3*, V79 and HSC230 (a human Fanconi anaemia cell line included as a positive control) cell-free extracts using an anti-hRAD51L2 pAb (Dr. Steve West, Cancer Research UK, Clare Hall). Protein was visualised by probing a Western blot with anti-hRAD51L2 mAb (Dr. Steve West, Cancer Research UK, Clare Hall). The blot is intentionally overexposed to show the faint RAD51L2 band in the *irs3* lane (Fig. 4.5).

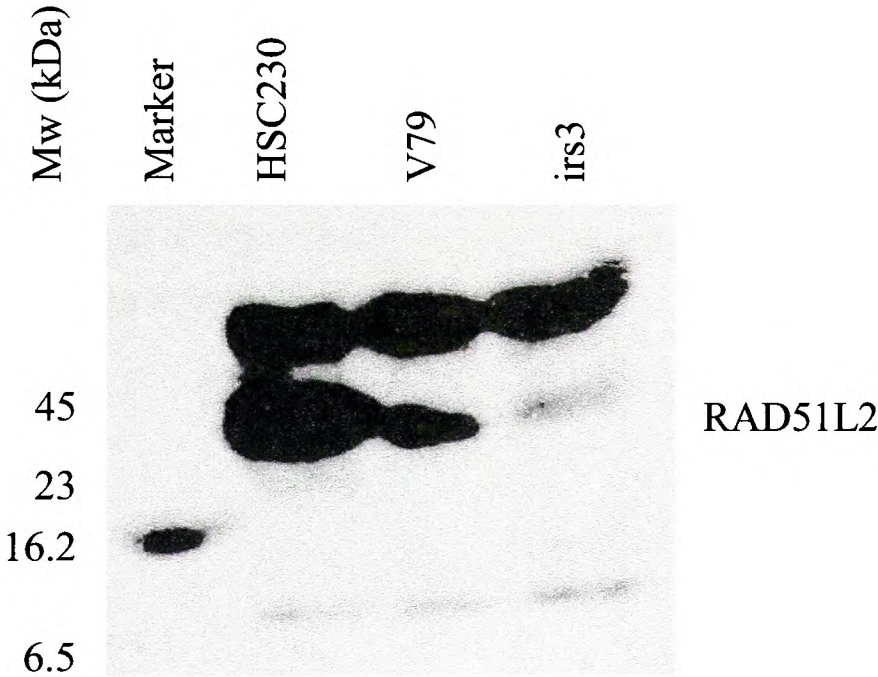


Figure 4.5 RAD51L2 immunoprecipitation. Endogenous RAD51L2 was pulled down from HSC230, V79 and *irs3* cell-free extracts using an anti-RAD51L2 pAb. RAD51L2 protein was visualised by Western blotting using anti-RAD51L2 mAb as the probe. The RAD51L2 protein is approximately 42kDa.

4.2.4 Hamster *RAD51L2* cDNA partially complements *irs3* MMC sensitivity

In Chapter 3 it was found that human *RAD51L2* cDNA or genomic DNA could substantially, but not completely complement *irs3* MMC sensitivity. It was suggested that complete complementation is not observed because of species differences between the human and hamster proteins which mean that the human protein is not fully functional in hamster cells. In order to see if the hamster *RAD51L2* gene could completely complement *irs3* MMC sensitivity, *irs3* cells were transfected with the hamster *RAD51L2* cDNA (construct generated in section 4.2.1). Two independently isolated clones, shown to carry the hamster *RAD51L2* gene by genomic DNA PCR and also to express it by RT-PCR (data not shown), were subjected to the preliminary MMC assay. Unexpectedly, the clones were found to be less resistant to MMC than clones expressing human *RAD51L2* cDNA. However, they were more resistant to MMC than untransfected *irs3* cells (Fig.4.6).

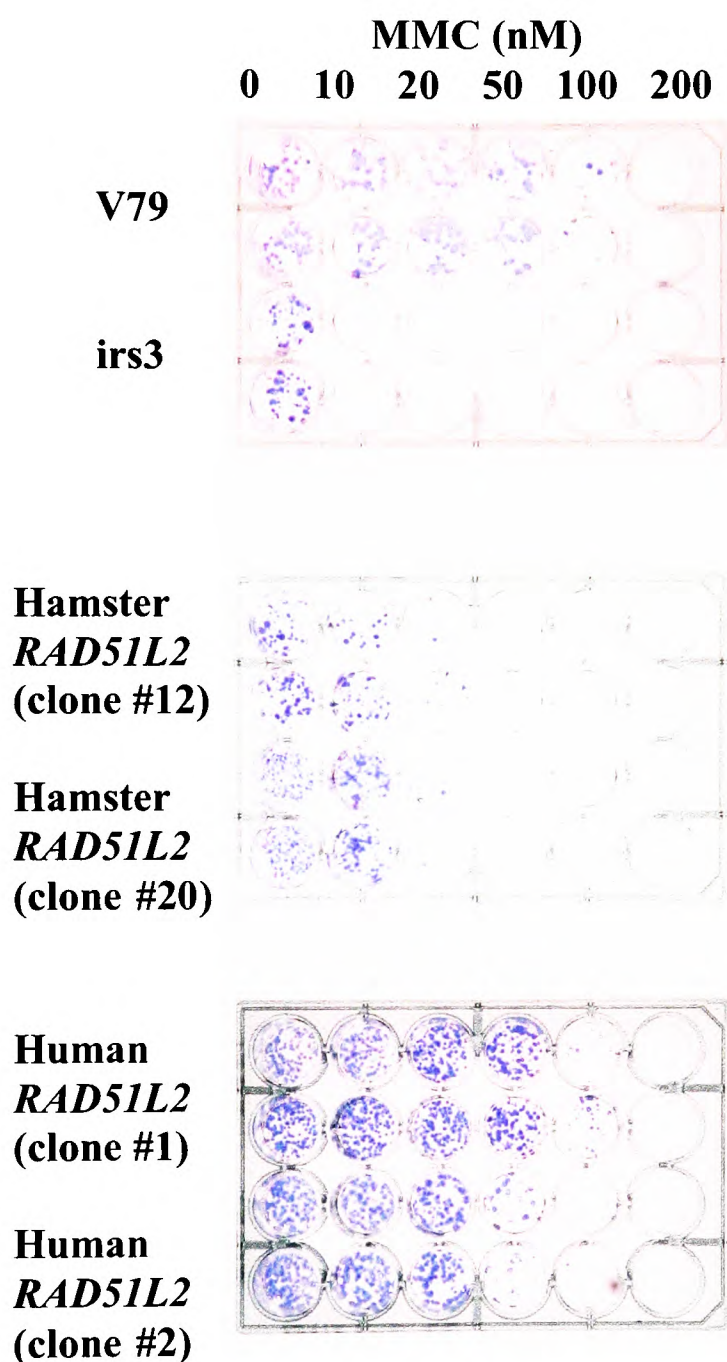


Figure 4.6 Hamster *RAD51L2* cDNA partially complements the MMC sensitivity of *irs3*. Survival of V79, *irs3*, *irs3* transfected with hamster *RAD51L2* cDNA (2 clones) and *irs3* transfected with human *RAD51L2* cDNA (2 clones). Each cell line is in duplicate.

4.3 Discussion

In order to sequence the *irs3 RAD51L2* gene, it was first necessary to clone the hamster *RAD51L2* cDNA. This was completed successfully and the predicted protein sequence was aligned with other previously identified *RAD51L2* proteins to give more information about regions of the protein conserved through evolution.

Sequencing of the *RAD51L2* cDNA in mutant and wild type cells revealed a G→T base substitution at the first nucleotide of exon 6 in *irs3*. It is possible that this substitution may be the result of a deletion in the previous intron. However, 1-ethyl-1-nitrosourea (ENU), the chemical used to derive the *irs3* cell line, is a point mutagen. A G→T base change is consistent with the mutation spectrum of ENU, although it is one of the less common types of substitution found (Justice *et al.*, 1999; Shibuya and Morimoto, 1993).

The *irs3* mutation has two effects, either of which could explain the phenotype of the cell line. Firstly, it causes an amino acid change at position 271 from Val to Phe. This Val residue is conserved in the 3 mammalian and 2 plant *RAD51L2* proteins currently identified and may have an important function. However, an amino acid change can only alter protein function if the protein is expressed, and analysis of *irs3* by immunoprecipitation and Western blotting shows little or no *RAD51L2* protein to be present (Fig. 4.5, also see below). Secondly, the mutation alters the 3' acceptor splice site for exon 6. Point mutations in splice junctions that affect splicing usually occur in the invariant intron GT or AG dinucleotides of the donor or acceptor sites respectively. Mutations in these regions have been demonstrated to be responsible for splicing errors in the *HPRT* gene (O'Neill *et al.*, 1998), and also for human genetic diseases involving defective splicing (Krawczak *et al.*, 1992). The *irs3* mutation, found at position +1 in exon sequence, is not typical of observed splice site mutations but does substitute a G, found in the majority of primate acceptor sites, to a T which is found in only 10% of these sites (Shapiro and Senapathy, 1987). There are some examples of mutations in the exon sequence of splice sites which alter splicing in the *HPRT* gene (O'Neill *et al.*, 1998). It is possible that the *RAD51L2* mutation reduces the "definition" of exon 6 and results in the exon being skipped by the

splicing machinery. Shorter exons have been shown to require stronger splice site consensus sequences for inclusion; exon 6 is 67 bp in length whereas the average vertebrate exon is 137 bp in length (Berget, 1995). This suggestion is supported by the difficulties encountered when amplifying exon 6 from *irs3* RNA by RT-PCR compared to the other exons that are amplified relatively easily.

The RT-PCR analysis revealed the existence of several *RAD51L2* splice variants in both V79 and *irs3*. None of the splice variants sequenced contained exon 6. It is possible that these splice variants are generated from a partially functional copy of the *RAD51L2* gene present in both V79 and *irs3* cells – the other copy would be functional in V79 cells but mutated in *irs3*. However, there is evidence to suggest that many genes are hemizygous in established hamster cell lines (Siminovitch, 1976). The predominant splice variants observed in *irs3* are in some cases of different sizes to those seen in V79. In *irs3*, it is possible that splice variants expressed at a low level and are amplified because of the lack of full-length *RAD51L2* cDNA. Their low abundance would mean that they are not observed in the V79 RT-PCR. The function of the splice variants remains unclear; it appears that they are untranslated as only one full-length protein product is visible by immunoprecipitation and Western blotting (Fig. 4.5, also see below) but they could be involved in regulating the expression of the full-length protein at the RNA level. Two other *RAD51*-like genes have been shown to exhibit alternative splicing. The *RAD51L1* gene occurs in two forms in human cDNA which differ extensively at the 3' end (Cartwright *et al.*, 1998a). Multiple alternative transcripts have also been reported for the *RAD51L3* gene, and northern blot analysis of various tissues has shown that their distribution is not ubiquitous (Kawabata and Saeki, 1999). As yet it has not been determined whether any of these transcripts are translated and their function is not known.

The RT-PCR and immunoprecipitation data show that there is very little or no full-length *RAD51L2* cDNA and *RAD51L2* protein in *irs3* cells. A similar immunoprecipitation experiment was carried out by Dr. Jean-Yves Masson (CRUK, Clare Hall) and no *RAD51L2* protein was detected in *irs3* (Fig. 4.7 B). *irs3* and V79 cell extracts were also probed directly from a Western blot and again no *RAD51L2* protein was detected (Fig. 4.7 A). In the present study a very small amount of

RAD51L2 protein was found in *irs3*. It is possible that this band is due to non-specific binding of the RAD51L2 mAb, or because of cross-reactivity of the antibody with other RAD51-like proteins under the conditions used in the experiment.

In collaboration with Dr. Jean-Yves Masson and Dr. Steve West (CRUK, Clare Hall) further co-immunoprecipitation work was carried out to look at the formation of RAD51-like protein complexes in *irs3* and V79. Two distinct complexes of RAD51-like proteins have been shown to form in human cells; a heterodimer of RAD51L2 and XRCC3, and a heterotetramer of RAD51L1, RAD51L2, RAD51L3 and XRCC2 (see Introduction, section 1.6.4). In V79 cells, anti-RAD51L2 pAb pulled down XRCC3 showing the existence of the heterodimer. Anti-XRCC2 pAb pulled down RAD51L1, RAD51L2 and RAD51L3 but not XRCC3, demonstrating the presence of the heterotetramer (Fig. 4.7 C). This generalises the findings of previous studies from human to hamster cells. In *irs3* cells, neither of these complexes was formed, although anti-XRCC2 pAb pulled down RAD51L3 indicating that the RAD51L3/XRCC2 heterodimer is still present (Fig. 4.7 C). This shows that the endogenous RAD51-like proteins require RAD51L2 for complex formation.

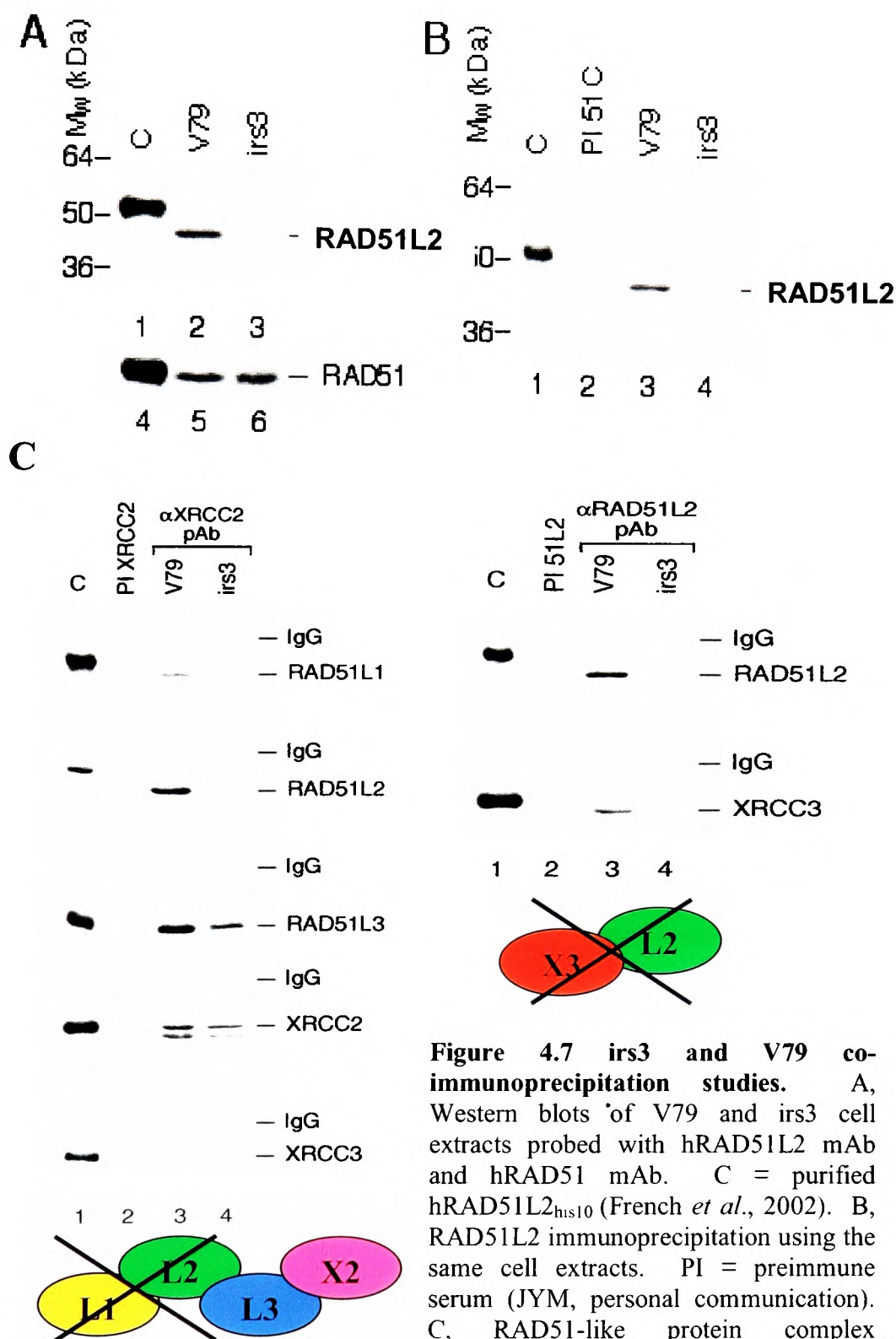


Figure 4.7 *irs3* and V79 co-immunoprecipitation studies. A, Western blots of V79 and *irs3* cell extracts probed with hRAD51L2 mAb and hRAD51 mAb. C = purified hRAD51L2_{his10} (French *et al.*, 2002). B, RAD51L2 immunoprecipitation using the same cell extracts. PI = preimmune serum (JYM, personal communication). C, RAD51-like protein complex formation in V79 and *irs3*. Left, complexes precipitated with hXRCC2 pAb. Right, complexes precipitated with hRAD51L2 pAb (French *et al.*, 2002).

irs3 clones transfected with hamster *RAD51L2* cDNA were found to be less resistant to MMC than clones transfected with the human *RAD51L2* cDNA. This was surprising as it was thought that the hamster gene would be fully functional in hamster cells and would therefore be able to complement the irs3 phenotype better than the human gene. It is unlikely that the moderate level of complementation observed is due to a sequence error in the hamster *RAD51L2* cDNA as the gene has been sequenced multiple times in the CHO-K1 cDNA library and in V79 and irs3. The sequence is also identical to the partial hamster *RAD51L2* gene sequence reported by Godthelp *et al.* (2002). The reduced level of complementation may be due to problems associated with overexpression. Overexpression of DNA repair genes in the corresponding deficient cell lines can have variable effects. The hamster and human *XRCC2* cDNAs driven by the CMV promoter have been shown to complement irs1 MMC sensitivity to similar levels (Cathryn Tambini, personal communication; O'Regan *et al.*, 2001). In contrast, overexpression of mouse *Xrcc2* cDNA is able to completely complement the MMC sensitivity of *Xrcc2*^{-/-} MEFs. Interestingly, this overexpression also results in a higher level of X-ray resistance than that exhibited by wild type cells (Bryan Deans, personal communication). The cDNAs of other DNA repair genes cannot be transfected into the corresponding deficient cells lines, presumably because of the toxic effects of overexpression. For example, no BRCA2 expression was observed when the full-length cDNA was transfected into CAPAN-1 cells, which express a truncated form of BRCA2 (Moynahan *et al.*, 2001b).

CHAPTER 5: Role of RAD51L2 in RAD51-dependent HRR

5.1 Introduction

The RAD51L2 protein is presumed to function in RAD51-dependent HRR. However, this prediction is based solely on the sequence similarity between RAD51L2 and RAD51, and the known interactions amongst the RAD51-like proteins and RAD51 (see Introduction, section 1.6). As yet there is no direct evidence to link RAD51L2 to HRR. In Chapter 4, *irs3* was found to have a mutated *RAD51L2* gene and to lack RAD51L2 protein, confirming the cell line as RAD51L2 deficient. In this Chapter the *irs3* cell line is used to look at the role of RAD51L2 in RAD51-dependent HRR. Three separate approaches have been taken. The levels of sister chromatid exchange and the formation of RAD51 foci in both mutant and wild type cells have been determined after DNA damage. Additionally, an assay that directly measures HRR at the site of an induced DSB has been conducted on both cell lines. Each of these techniques is discussed in more detail below.

The symmetrical exchange of segments of sister chromatids is known as sister chromatid exchange (SCE). In organisms with genetic markers, SCEs can be measured by unequal exchange events at specific loci, but in mammalian cells they are usually measured by cytogenetic methods. SCEs can be seen by allowing chromosomes to replicate twice in the presence of 5-bromodeoxyuridine (BrdU) (Fig. 5.1). After the first round of replication, all duplexes have one labelled and one unlabelled strand. After the second round, one duplex has both strands labelled and the second has only one strand labelled. The BrdU labelled DNA is then visualised using a fluorescence plus Giemsa staining technique. In this way SCEs occurring during both the first and second rounds of replication are detectable in second-division cells. It should be noted that treating cells with BrdU is known to induce SCEs (Perry and Thomson, 1984).

SCEs were first described nearly 50 years ago (Taylor, 1958), but elucidating the mechanism(s) by which they occur has proved difficult. The majority of SCEs are found in S-phase and are associated with replication forks. Homologous

recombination (HR) was one of the first mechanisms suggested for the formation of SCEs but other models such as strand switching at stalled replication forks, possibly mediated by topoisomerases, were subsequently proposed (Tucker *et al.*, 1993). However, recent evidence from HR mutants has reaffirmed the role of HRR in the formation of SCEs, particularly those SCEs whose formation is induced by DNA damaging agents.

Levels of spontaneous SCEs are slightly reduced in mouse *Rad54*^{-/-} ES cells compared to *Rad54*-proficient control cells. After treatment with MMC a greater difference is seen, with m*Rad54*^{+/+} cells showing a 2.6-fold increase in SCEs and m*Rad54*^{-/-} cells showing just a 1.8-fold increase (Dronkert *et al.*, 2000). In addition, hamster cells expressing a dominant negative *S.cerevisiae*/mouse chimeric RAD51 protein, which inhibits gene conversion, show a small decrease in spontaneous SCEs and a less efficient MNU-induced response than m*RAD51*-expressing control cells (Lambert and Lopez, 2001). The level of spontaneous SCEs has also been measured in *irs1*SF and *irs1* hamster cells. The SCE frequency in *irs1*SF cells is essentially normal (Fuller and Painter, 1988), but *irs1* cells show a slightly higher level of SCEs than wild type cells, although the figures are not statistically significant (Tucker *et al.*, 1991). Both spontaneous and MMC-induced SCEs are significantly reduced in *RAD54*^{-/-} chicken DT40 cells. A similar but more pronounced result is found when the expression of a human *RAD51* transgene is down regulated in *RAD51*^{-/-} DT40 cells (Sonoda *et al.*, 1999). Reductions in spontaneous and MMC-induced SCEs have also been reported in chicken DT40 cells lacking each of the *RAD51*-like genes (Takata *et al.*, 2000; Takata *et al.*, 2001).

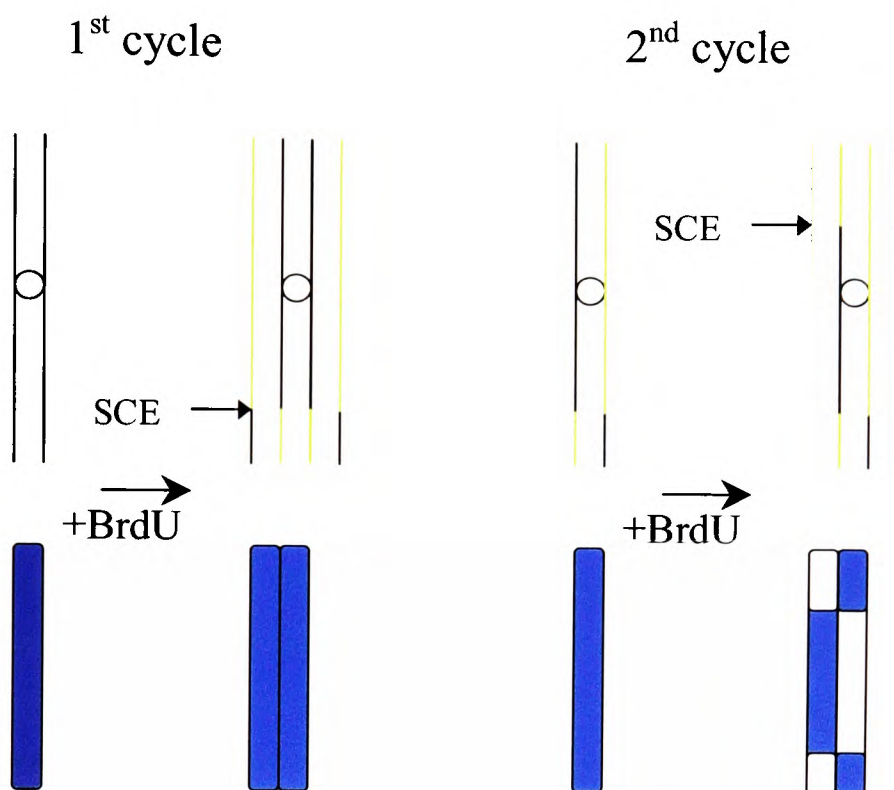


Figure 5.1 Sister chromatid exchange. A, Diagrammatic representation of the differential labelling of sister chromatids with BrdU. — newly synthesised labelled DNA, ■ unlabelled duplex DNA, ■ duplex DNA with one labelled and one unlabelled strand and □ duplex DNA with both strands labelled.

Under certain conditions, cells stained with an anti-RAD51 antibody show localisation of the RAD51 protein to discrete nuclear speckles known as foci. In untreated somatic cells some RAD51 foci are visible in S-phase (Tashiro *et al.*, 1996), but when cells are treated with DNA damaging agents, e.g. X-rays, the numbers of foci increase substantially in a time- and dose- dependent manner (Bishop *et al.*, 1998; Haaf *et al.*, 1995). One study that used shielding to prevent some areas of the cell being irradiated showed RAD51 not to localise with sites of DNA damage (Nelms *et al.*, 1998). However, a more recent study involving micro irradiation of small nuclear areas showed RAD51 foci to be spatially related to sites of damage (Tashiro *et al.*, 2000). RAD51 also shows co-localisation to sites of DNA damage as

represented by ssDNA (Raderschall *et al.*, 1999) and by H2AX phosphorylation (Paull *et al.*, 2000).

Some mutant cell lines have been shown to be defective in the formation of damage dependent RAD51 foci. This indicates that the protein coded by the mutated gene plays a role in assembling the RAD51 nucleoprotein filament on DNA. Cell lines defective in DNA-PK, a component of the NHEJ pathway, do not show a defect in RAD51-focus formation (Bishop *et al.*, 1998). Cells derived from AT patients and ATM^{-/-} chicken DT40 cells show an interesting defect in RAD51 focus formation. In a time course study, the formation of RAD51-foci continues to increase in mutant cells even after such formation has plateaued in wild type cells (Maser *et al.*, 1997; Morrison *et al.*, 2000). Fibroblasts from the eight currently identified Fanconi anaemia complementation groups have also been tested. One study reports that RAD51 focus formation is reduced only in FA-D1 cells (Godthelp *et al.*, 2002a) whereas another study reports that cells from all eight complementation groups show a reduction in RAD51 focus formation (Digweed *et al.*, 2002) after X-ray treatment. Reduced numbers of damage induced RAD51-foci have been observed in several other cell lines mutated in genes associated with HRR including *BRCA1* (Bhattacharyya *et al.*, 2000), *BRCA2* (Yuan *et al.*, 1999), *RAD54* (Tan *et al.*, 1999), *XRCC3* (Bishop *et al.*, 1998) and *XRCC2* (O'Regan *et al.*, 2001). Chicken DT40 cells disrupted in each of the five *RAD51*-like genes also show reduced numbers of damage-induced RAD51 foci (Takata *et al.*, 2000; Takata *et al.*, 2001).

Mammalian cells can repair DSBs using either NHEJ or HR. HRR occurs by two main pathways; SSA and gene conversion, which can be associated with crossing-over (see Introduction, section 1.5). The frequencies of these events can be measured after a single I-SceI-induced DSB, and different recombination reporter substrates can be used to distinguish the events (Johnson and Jasin, 2001). A model of the different repair mechanisms is shown in figure 5.2. The recombination reporter construct contains a selective marker and two recombination reporter genes. The left-hand reporter gene is truncated at the 3' end and the right hand gene has an I-SceI endonuclease site inserted at a position of homology present in the left-hand gene. The alterations made to both genes render them non-functional. A site-specific DSB

is induced by transfection of an I-SceI expressing plasmid into cells. The DSB can be repaired by NHEJ with or without an accompanying deletion or insertion. In some rare case this may result in a functional reporter gene (Fig. 5.2). SSA can also repair the DSB, where resection of the DSB ends exposes regions of homology in the left and right reporter genes. The complementary sequences anneal giving a functional reporter gene and delete the intervening selective marker (Fig. 5.2). Finally the DSB can be repaired by either intrachromatid or sister chromatid gene conversion. If the intermediate is resolved without a crossover, the recombination reporter construct will contain a 3' truncated left hand copy of the reporter gene, a functional selective marker, and a right hand copy of the reporter gene. Alternatively, the intermediate may be resolved with an associated crossover. This results in a functional reporter gene, with loss of the selective marker, which is identical to the outcome of SSA (Fig. 5.2).

Various recombination reporter constructs have been used to look at the frequency of each of the events described above in HRR-deficient cell lines. Both *Rad54*^{-/-} and *Brca2*-deficient cells show an increase in error-prone SSA and a reduction in conservative gene conversion compared to wild type cells (Dronkert *et al.*, 2000; Tutt *et al.*, 2001). These results suggest that gene conversion is the process that suffers most when a HRR gene is lost. This holds for *irs1* and *irs1SF* cells, which show a 100-fold and a 25-fold reduction in gene conversion respectively (Johnson *et al.*, 1999; Pierce *et al.*, 1999). A recombination reporter construct that measures only gene conversion was therefore chosen when analysing recombination in *irs3* cells.

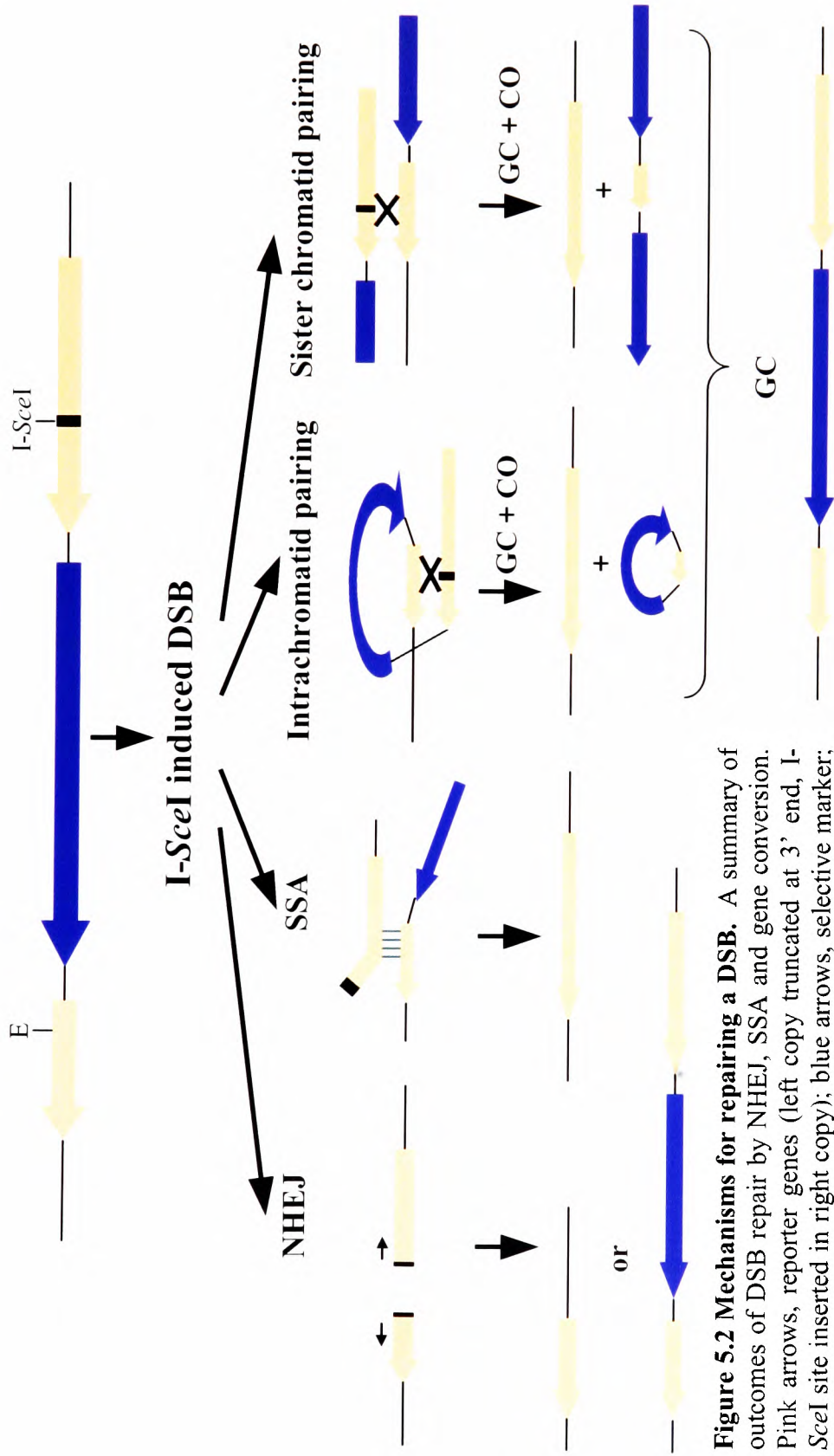


Figure 5.2 Mechanisms for repairing a DSB. A summary of outcomes of DSB repair by NHEJ, SSA and gene conversion. Pink arrows, reporter genes (left copy truncated at 3' end, I-SceI site inserted in right copy); blue arrows, selective marker; green vertical lines, annealing of complementary strands during SSA; E, restriction enzyme site.

5.2 Results

5.2.1 Reduced levels of sister chromatid exchange in *irs3*

Spontaneous and MMC-induced SCEs were measured in V79, *irs3* and two *RAD51L2* cDNA transfected clones. Cells were treated for two cycles (24 h) with BrdU before being fixed and stained. If required, MMC was added during the first 2 h of labelling. Previous experiments have used two methods to compare MMC-induced SCEs in cell lines with differing survivals. The same concentration of MMC can be added to both cell lines i.e. not allowing for their survival difference or equitoxic concentrations of MMC can be added to each cell line. The first approach is usually favoured because each cell line is subjected to the same amount of damage. However, when very MMC-sensitive mutant lines are being tested this method is not possible because the cells would not survive the treatment in sufficient numbers to be tested. For the present experiment it was possible to employ both methods.

irs3 cells showed a small, but non-significant, decrease in spontaneous SCE frequency (Table 5.1). When the same concentration of MMC (4×10^{-7} M) was used for each cell line, *irs3* cells showed a highly significant 30% reduction in SCE frequency compared to wild type cells. The presence of *RAD51L2* cDNA substantially complemented the defect in clone 2 and restored SCE frequencies to wild type levels in clone 1. When equitoxic concentrations of MMC were used the differences observed were even greater, with *irs3* showing a 60-80% reduction in SCE frequency compared to wild type cells.

Table 5.1 Reduced frequencies of spontaneous and MMC-induced SCEs in *irs3*.

Cell line	Cells scored	SCE/cell ($\pm$ se)	P value ¹
V79	101	5.5 $\pm$ 0.2	0.0002
<i>irs3</i>	103	4.4 $\pm$ 0.2	
V79 + MMC (4×10^{-7} M) ²	153	48.0 $\pm$ 1.1	<10 ⁻⁶
<i>irs3</i> + MMC (4×10^{-7} M) ²	127	33.2 $\pm$ 1.1	
<i>irs3</i> + <i>RAD51L2</i> clone 1 (4×10^{-7} M)	98	51.0 $\pm$ 1.2	<10 ⁻⁶
<i>irs3</i> + <i>RAD51L2</i> clone 2 (4×10^{-7} M)	100	42.4 $\pm$ 1.2	<10 ⁻⁶
V79 + MMC (4×10^{-6} M) ³	76	88.2 $\pm$ 2.4	
<i>irs3</i> + MMC (4×10^{-7} M)	127	33.2 $\pm$ 1.1	
V79 + MMC (4×10^{-7} M)	153	48.0 $\pm$ 1.1	
<i>irs3</i> + MMC (4×10^{-8} M) ⁴	105	8.1 $\pm$ 0.3	

¹ based on Mann-Whitney U-tests, comparing either V79 with *irs3* or the *RAD51L2*-transfected clones with *irs3*.

² data summed from 2 independent experiments, where the frequencies for V79 were 47.5 and 48.9 SCE/cell, and for *irs3* were 33.2 and 33.2 SCE/cell, respectively.

³ equivalent survival level to 4×10^{-7} M for *irs3*

⁴ equivalent survival level to 4×10^{-7} M for V79

5.2.2 Damage-dependent RAD51 focus formation is reduced in *irs3*

The RAD51 protein has been shown to localise to discrete nuclear foci following DNA damage. In order to establish a role for *RAD51L2* in this process, RAD51 focus formation was examined in *irs3*. V79, *irs3* and the two *RAD51L2* cDNA transfected clones were either irradiated with 10Gy X-rays or mock treated. After 5 h incubation at 37°C the cells were fixed and stained with an anti-RAD51 antibody. Many RAD51 foci are seen in V79 after irradiation, whereas fewer foci are found in *irs3* (Fig. 5.3 A). This reduction is substantially complemented by the *RAD51L2* cDNA.

In order to quantitate these data accurately random images were selected and the number of strongly fluorescing discrete nuclear foci present in each cell was

recorded. Two independent scorers scored a minimum of 50 cells for each data point, from coded slides. *irs3* cells have on average 4-5 foci/cell after irradiation, which is a substantial reduction from the 14 foci/cell seen in the parent cell line. The two *RAD51L2* cDNA transfected clones show an intermediate level of 8-10 foci/cell (Fig. 5.3 B). The standard error for both *irs3* and V79 irradiated cells is 0.50, and represents an 11.1% and 3.7% error respectively. The 3-fold increase in *irs3* error reflects heterogeneity in the number of foci found in these cells after irradiation. As seen in Fig. 5.3 B, there is little variation between the data recorded by each of the scorers. However, using the Student's t-test it was found that the two scorers differed significantly for both the *RAD51L2* cDNA transfected clones after irradiation.

RAD51 foci are known to form at low frequency in the S-phase of untreated cells and do not form in G1 phase (Tashiro *et al.*, 1996). The present experiment looked at asynchronous populations of V79 and *irs3* cells. It is therefore important to determine whether the cell cycles of the wild type and mutant cell lines differ both in untreated cells and after irradiation. V79 and *irs3* cells were either irradiated with 10Gy X-rays or mock treated, before being fixed after 5 h incubation and analysed by flow cytometry. The cell cycle profiles of both cell lines were found to be very similar, with most cells blocked in G2 phase 5 h after irradiation (Fig. 5.4 A).

The formation of RAD51 foci may also be affected by the relative levels of RAD51 protein in the wild type and mutant cell lines as well as in the cDNA transfected clones. The amount of RAD51 protein in each was investigated using Western blotting. 5 h after either irradiation or mock treatment protein extracts were prepared from each cell line. Aliquots of these extracts were used for a Western blot, which was probed with an anti-RAD51 antibody, and then stripped and reprobed with an anti- α -tubulin antibody as a loading control. Densitometry analysis was used to calculate the ratio of RAD51/ α -tubulin and the resulting figures were normalised against the lowest value. RAD51 protein levels were similar in *irs3*, *RAD51L2* cDNA transfected clones and V79, both before and after irradiation (Fig. 5.4 B).

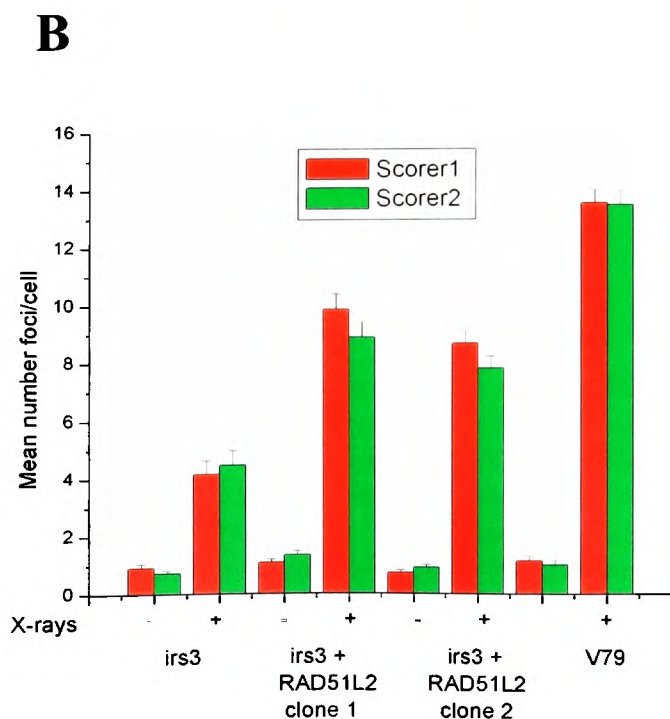
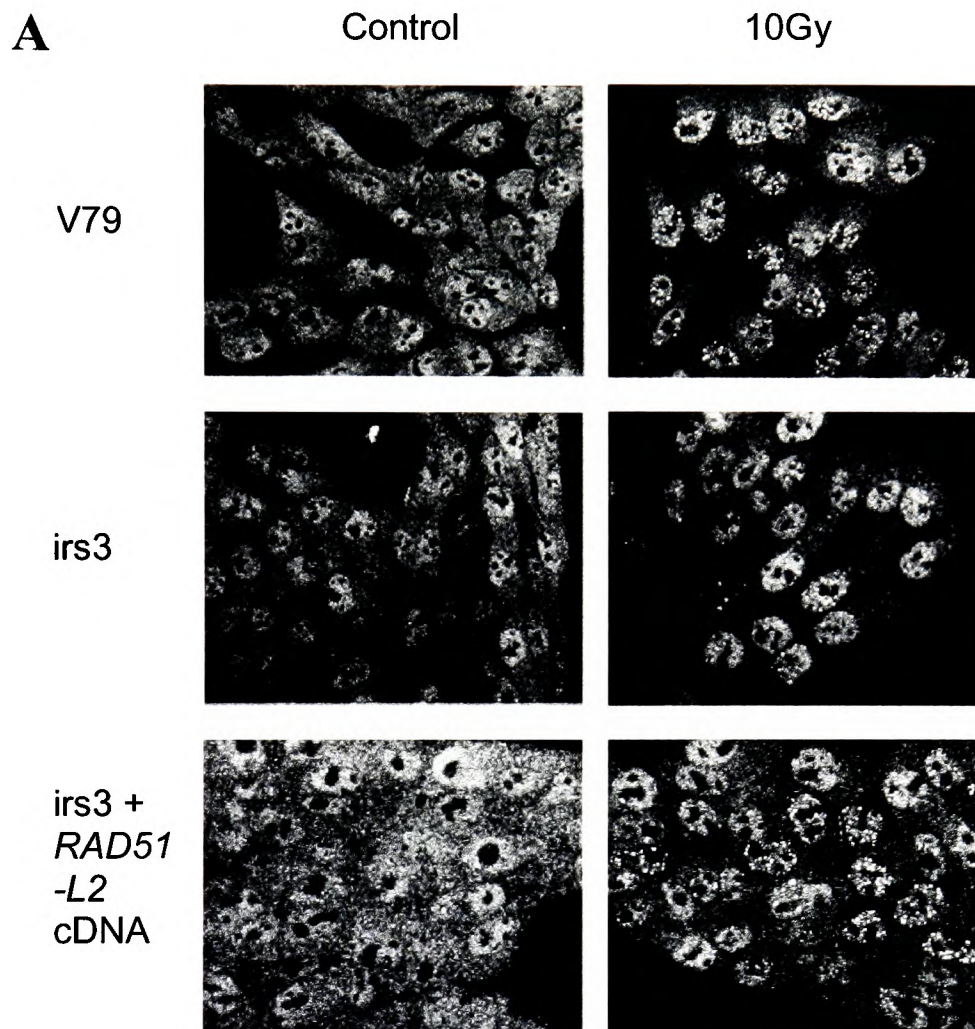
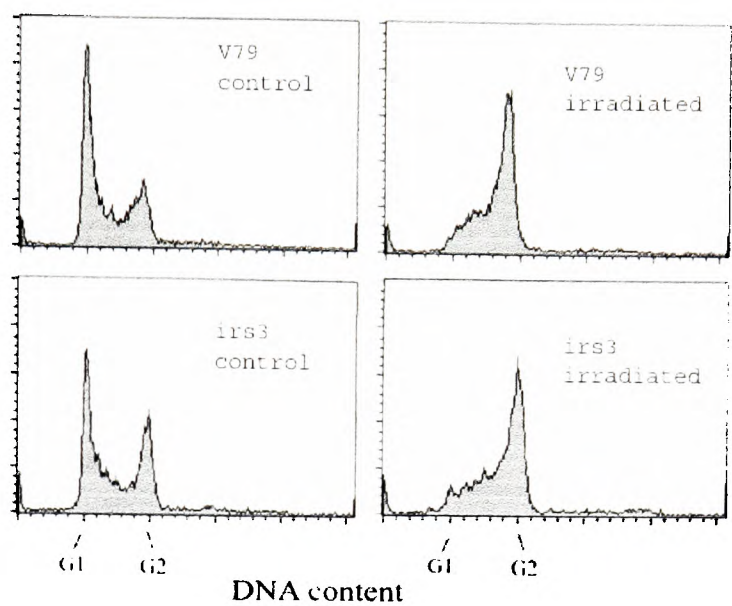


Fig. 5.3 Damage-dependent RAD51 focus formation is reduced in *irs3* cells. A, RAD51 foci in V79, *irs3* and a cDNA-transfected clone, with and without X-ray exposure (measured at 5 h after 10 Gy). Representative fields are shown. B, quantification of focus formation. Data shown for two independent scorers, with standard errors calculated from individual cell counts. All differences between irradiated cell lines were significant ($p < 10^{-6}$) except for the clones transfected with *RAD51L2* ($p = 0.63$).

A



B

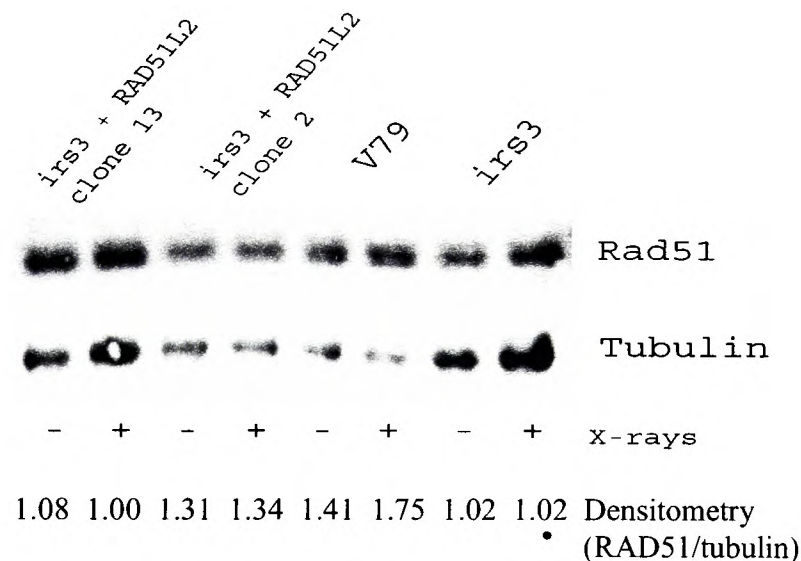


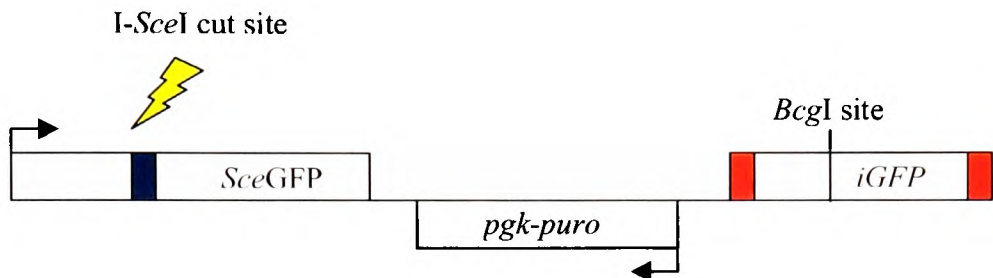
Figure 5.4 The cell cycle profiles and RAD51 levels in V79 and *irs3* are similar. A, flow cytometry analysis of V79 and *irs3*. Analysis carried out 5 h after cells were either mock treated or irradiated with 10Gy X-rays. B, quantification of RAD51 in V79, *irs3*, and two *RAD51L2* cDNA transfected clones by Western blotting. Protein extracts prepared 5 h after cells were either mock treated or irradiated with 10Gy X-rays. Ratio of RAD51:α-tubulin loading control is measured by densitometry and normalised against the lowest value.

5.2.3 Reduced homologous recombination in *irs3*

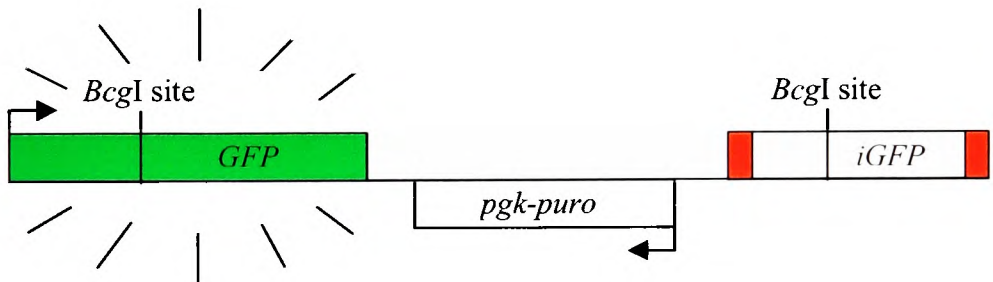
In order to determine if RAD51L2-deficient cells are defective for HRR the I-*SceI* DSB repair system was used. The recombination reporter substrate chosen was DR-GFP (kindly donated by Dr. Maria Jasin, Memorial Sloan-Kettering Cancer Center, New York).

DR-GFP has a tandem repeat of differentially mutated non-functional *GFP* genes. A gene conversion event in DR-GFP leads to expression of an intact GFP protein, which can be simply assayed by flow cytometry. The first *GFP* gene, *SceGFP*, has a *BcgI* site mutated to the recognition site for I-*SceI* endonuclease, whilst the second *GFP* gene is truncated at both ends (Fig. 5.5 A). There are two possible repair products with DR-GFP, a short tract gene conversion (STGC) product (Fig. 5.5 B) and a deletion product (Fig. 5.5 C). The first of these is the result of a non-cross over gene conversion event within the limited length of sequence homology provided by *iGFP*. The second can arise through several mechanisms such as a recombination event with an associated crossover, a long tract gene conversion, or from repair via the SSA pathway. Only the STGC product results in a functional *GFP* gene whereas all other mechanisms result in the deletion product, which has been shown to be non-functional (Pierce *et al.*, 1999).

A DR-GFP recombination reporter substrate



B Short tract gene conversion (STGC) product



C Deletion product

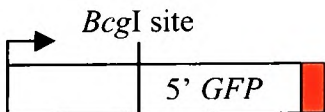


Figure 5.5 The DR-GFP recombination reporter substrate (adapted from Pierce, 1999). A, DR-GFP recombination reporter substrate. B, STGC product. The DSB is repaired using the *iGFP* template and results in a functional *GFP* gene. C, deletion product. The DSB is repaired by other mechanisms which leave a 3' truncated, non-functional *GFP* gene.

The DR-GFP recombination reporter substrate, had previously been sub cloned into a gene-targeting vector for the mouse *Pim1* locus, creating p59xDR-GFP6 (Moynahan *et al.*, 2001b) (Fig. 5.6 A). The selectable hygromycin gene (*hyg*) is fused in frame with *Pim1* coding sequence so that only homologous targeting to the *Pim1* locus or a nonhomologous integration next to random promoter sequence leads to hygromycin resistant cells. In mouse cell lines it is therefore possible to measure gene conversion at a defined genetic locus. Unfortunately, this feature of the construct cannot be utilised when measuring gene conversion in hamster cells.

The p59xDR-GFP6 construct was linearised, using two *XhoI* sites, which flank the targeting arms, and electroporated into V79 and *irs3* cells. 25-30 stably transfected clones were isolated from each cell line by selection in puromycin. Several of these clones were electroporated with 50µg pCBASce plasmid, which transiently expresses I-*SceI*, and analysed by flow cytometry 45 h later (see Methods 2.12.1). Clones with GFP positive cells (approximately half of the clones tested) were assumed to have at least one functional copy of DR-GFP (data not shown). In this way 5 V79 clones and 4 *irs3* clones were chosen for further analysis.

Genomic DNA was isolated from each of the 9 clones and Southern blotting was used to determine how many copies of DR-GFP had been integrated, and whether these copies were intact. Two different digestion and probing strategies were used. Firstly, DNA was digested with *HindIII* and probed with an *iGFP* fragment (Fig. 5.6 B, top). The *HindIII* digest yields one detectable fragment of known size (806bp) that corresponds to *iGFP*. The second detectable fragment corresponds to *SceGFP* and is of unknown size because the second *HindIII* site is found in the hamster genome. If clones carry more than one copy of DR-GFP it is likely that the genomic *HindIII* site will lie at different distances from *SceGFP* in each integrant and more than one band will be seen. A clone showing 2 bands will have a minimum of two DR-GFP copies but may have more if the positioning of the genomic *HindIII* site in certain integrants yields fragments of a similar size, which cannot be resolved on an agarose gel. It should be noted that all the *Pim1* and *hyg* sequence could be lost without affecting the functionality of the construct. If however any of the *SceGFP* fragments are shorter

than 3025bp (indicated by the *SalI/HindIII* cut control) they are likely to be non-functional because they lack the *GFP* promoter.

The *HindIII* digest gives an indication of copy number but does not show that *SceGFP* and *iGFP* are linked. To investigate this question, DNA was digested with *XcmI* and then probed with *pgk-puro* (Fig. 5.6 B, bottom). *XcmI* cuts once in both *SceGFP* and *iGFP* and yields a fragment of known length (3687bp) that is detected by the probe. If *SceGFP* and *iGFP* are integrated as a linked repeat a fragment of 3687bp should be detected (indicated by the *XcmI* cut control).

From the *HindIII* digested blot it appears that V797 and V7915 are single copy clones, V799 and V7910 are double copy clones and V7911 has three copies of DR-GFP. *irs324* appears to be a single copy clone whereas all the other *irs3* clones are double copy. However, one of the *SceGFP* fragments in *irs317* is less than 3025bp and is likely to be non-functional, so *irs317* is in effect a single copy clone.

All the bands on the *XcmI* digested blot appear to be slightly shorter than the expected 3687bp. This might indicate that some of the *pgk-puro* sequence can be lost without affecting functionality, but the size differences may also relate to gel running conditions. It is also possible that some vector sequence has been incorporated into this region during the previous sub cloning procedures. It would appear that all the clones tested have at least one intact DR-GFP substrate (V799 was not determined because the genomic DNA did not digest with *XcmI*). However, a slightly unusual result is observed with V7915, an apparent single copy clone from the *HindIII* digested blot. V7915 yields three bands of different sizes after digestion with *XcmI*. One explanation for this could be that V7915 actually has three integrated copies of DR-GFP but that in two of these copies the 5' end has been degraded into *pgk-puro* so *SceGFP* is not detected. This suggestion is supported by the fact that the *iGFP* band is much stronger on the *HindIII* blot than the *SceGFP* band, indicating that three copies of *iGFP* may exist compared to only one copy of *SceGFP*. The construct is more likely to lose *SceGFP* than *iGFP* because there is less protecting *PimI* targeting sequence at the 5' end than the 3' end.

Clones V797, V7910, V7915, *irs35*, *irs317* and *irs324* were chosen for flow cytometric analysis. Cells were either mock electroporated or electroporated with

50µg pCBASce (I-SceI expression vector). Very few or no GFP positive cells were seen after mock transfection. I-SceI expression in clone V797 led to 3% of cells being GFP positive whereas I-SceI expression in clone irs35 led to just 0.42% of the cells being GFP positive, that is 7- to 8-fold lower than in the wild type clone (Fig. 5.7 A). To test whether the apparent recombination deficiency in irs3 was due to the lack of *RAD51L2* protein 50µg pCBASce was cotransfected into cells with 20µg *RAD51L2* expression vector. 1.42% of the cells from clone irs35 were found to be GFP positive, a 3.3-fold increase over I-SceI expression alone, whereas no significant difference was seen in the wild type clone, V797 (Fig. 5.7 A).

A summary of the results obtained for all 6 clones can be found in Fig. 5.7 B. I-SceI expression experiments were repeated at least twice, and a further cotransfection experiment with *RAD51L2* expression vector was also carried out for each clone. If the I-SceI expression data for all the irs3 and V79 clones is pooled a mean percentage of GFP positive cells can be calculated for both the wild type and the mutant cell lines. This is 0.24% for irs3 and 1.72% for V79, with irs3 cells showing ~7-fold decrease in the ability to repair an induced DSB by HRR. *RAD51L2* expression in irs3 cells partially complements this defect, increasing recombination by ~2.4-fold.

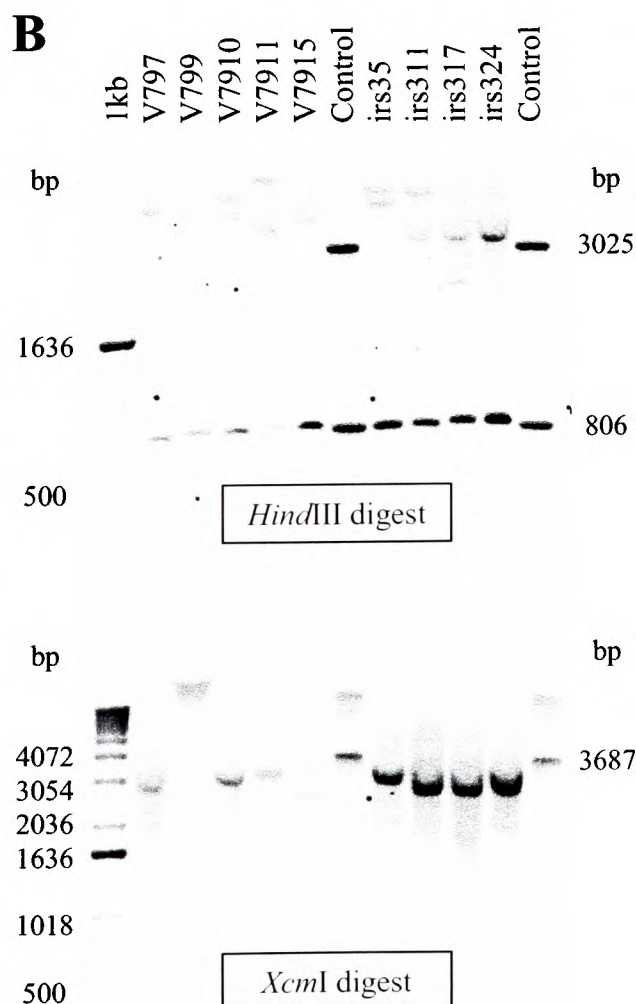
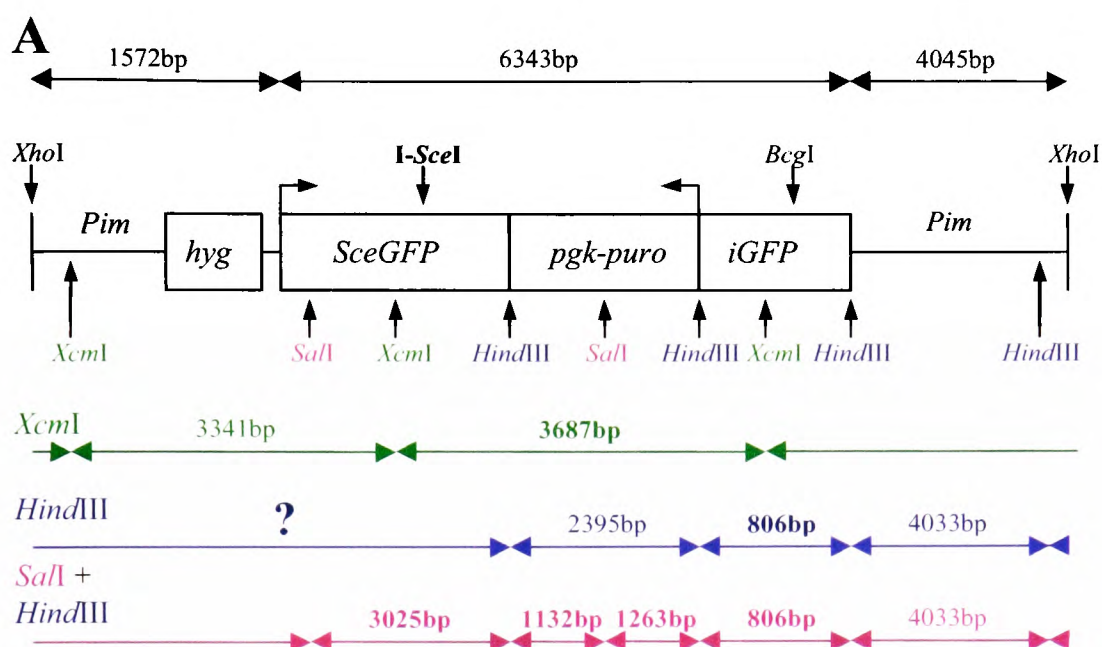


Figure 5.6 Analysis of DR-GFP carrying V79 and irs3 clones. A, map of *XhoI* linearised p59xDR-GFP6 construct.. Sizes of digested fragments are indicated. B, Southern blot analysis of DR-GFP carrying V79 and irs3 clones. Top, DNA digested with *HindIII* and probed with 806bp *iGFP* fragment. Control is 100pg p59xDR-GFP6 cut with *SalI* and *HindIII* to yield two detectable fragments of 3025bp and 806bp. Bottom, DNA digested with *XcmI* and probed with 1132bp and 1263bp *pgk-puro* fragments. Control is 100pg p59xDR-GFP6 cut with *XcmI* to yield one detectable fragment of 3687bp.

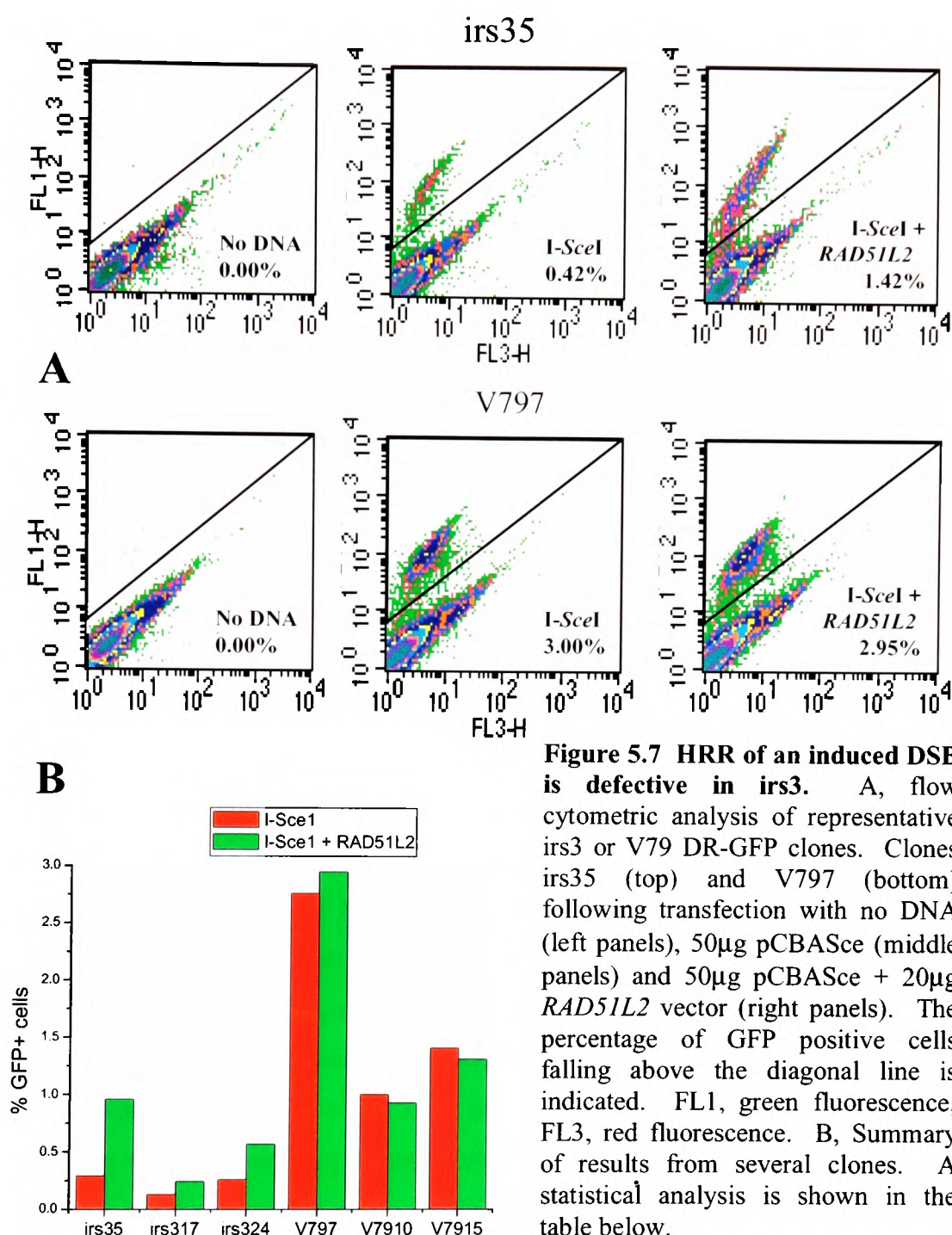


Figure 5.7 HRR of an induced DSB is defective in *irs3*. A, flow cytometric analysis of representative *irs3* or V79 DR-GFP clones. Clones *irs35* (top) and V797 (bottom) following transfection with no DNA (left panels), 50 μ g pCBASce (middle panels) and 50 μ g pCBASce + 20 μ g *RAD51L2* vector (right panels). The percentage of GFP positive cells falling above the diagonal line is indicated. FL1, green fluorescence; FL3, red fluorescence. B, Summary of results from several clones. A statistical analysis is shown in the table below.

Clone	Mean %GFP +ve cells	SE	$\frac{(I-SceI + L2)}{(I-SceI)}$	Mean %GFP +ve cells (<i>irs3/V79</i>)
<i>irs35</i>	0.29%	0.05	3.3	0.24%
<i>irs317</i>	0.13%	0.06	1.8	
<i>irs324</i>	0.26%	0.02	2.2	
V797	2.76%	0.25	1.1	1.72%
V7910	1.00%	0.29	0.93	
V7915	1.41%	0.33	0.93	

5.3 Discussion

In this Chapter three different techniques were used to investigate the role of RAD51L2 in HRR. The results from each technique are discussed below with reference to other relevant studies in the literature.

In order to link RAD51L2 with the RAD51-dependent HRR, the formation of damage-dependent RAD51 foci in *irs3* cells was examined. This response was found to be significantly reduced (3-4 fold) in *irs3* compared to wild type cells, and could not be attributed to reduced levels of RAD51 protein or an altered cell cycle in the mutant cell line.

RAD51 focus formation has also been measured in the XRCC2 deficient cell line *irs1*, which was derived from the same parent cell line, V79, as *irs3*. In the *irs1* study low levels of focus formation (<2 foci/cell) were found in unirradiated cells. Following irradiation, the numbers of foci in V79 cells increased significantly (12-16 foci/cell) whilst little focus formation was observed in *irs1* cells (typically 2-4 foci/cell) (O'Regan *et al.*, 2001). The numbers of foci recorded in unirradiated cells and irradiated V79 cells agree closely with the data obtained here. However, irradiated *irs3* cells show on average 4-5 foci/cell that is slightly more than that reported for *irs1*. *irs3* has a less severe phenotype than *irs1*, showing for example a 7-fold sensitivity to MMC compared to the 60-fold sensitivity displayed by *irs1* (Jones *et al.*, 1987), so this result is not unexpected. There is also some evidence for a slow accumulation of foci during the first 5 h in *irs1* (O'Regan *et al.*, 2001). A time course experiment would be necessary to determine if this was the case in *irs3* cells. Two *irs3* clones transfected with *RAD51L2* cDNA showed partial restoration of RAD51 focus formation (8-10 foci/cell). This is consistent with the levels of complementation found for other end points, and shows that the *RAD51L2* gene specifically is required for correct RAD51-focus formation.

Analysis of 3 *irs3* clones and 3 V79 clones carrying a GFP recombination reporter substrate showed that repair of an induced DSB by gene conversion is reduced on average by ~7-fold in the mutant cell line. This reduction is quite modest compared to those reported for other cell lines, for example hamster cells mutated in

XRCC2 and *XRCC3* show reductions in recombination of 100- and 25-fold respectively (Johnson *et al.*, 1999; Pierce *et al.*, 1999). This could be due to the nature of the mutation in the *RAD51L2* gene and is consistent with the less severe phenotype displayed by the *irs3* cell line. Recombination in *BRCA2* mutant cells is reduced by 6- to >100-fold depending on the nature of the mutation (Moynahan *et al.*, 2001b). It is also possible that the GFP recombination reporter substrate is not as sensitive as neomycin based substrates and further optimisation could be required. Similar methods to those described by Pierce *et al.* (1999) were used in this study but changes in the amount of DNA added to electroporations and the incubation time before flow cytometric analysis may improve results.

A significant amount variation in %GFP positive cells was found between different clones, particularly in V79 DR-GFP clones. However, at no time were the %GFP positive cells in a V79 DR-GFP clone lower than that in an *irs3* DR-GFP clone. It seems that the integration site of the recombination reporter substrate may be more important than the number of copies integrated. Clone V797, which appears to be a single copy clone, shows nearly twice as many GFP positive cells after *I-SceI* expression than the double copy clone V7910.

Expression of *RAD51L2* in *irs3* but not V79 DR-GFP clones increases recombination by ~2.4-fold. This is only a partial complementation of the recombination deficient phenotype and is slightly lower than that observed for other end points. Expression of *XRCC3* in *irsSF* cells completely complements their recombination deficient phenotype (Pierce *et al.*, 1999). In the *irs1SF* study 100µg of *I-SceI* expression vector and 20µg of *XRCC3* expression vector were used in cotransfection experiments, whereas in this study 50µg of *I-SceI* expression vector and 20µg of *RAD51L2* expression vector were used. The full complementation found in the *irs1SF* study is unexpected because it is unlikely that every cell transfected with *I-SceI* vector will also have been transfected with *XRCC3* expression vector. Increasing the amount of *RAD51L2* DNA used in *irs3* electroporations may increase the level of complementation observed.

The frequency of spontaneous and MMC-induced SCEs was measured in V79 and *irs3* cells to gain independent evidence for a *RAD51L2* role in sister chromatid

recombination. The SCE data add to the data obtained from the HRR assay because they show that *RAD51L2* is required for the repair of damage in naturally occurring genomic DNA sequences as well as induced breaks in artificial constructs. The results of the HRR assay and the SCE experiment cannot be compared directly because they measure distinct events. The HRR assay only detects short tract gene conversion events that are not associated with crossing-over, whereas the SCE experiment measures exchanges. Additionally, SCEs can only be detected if they occur on sufficiently long DNA tracts to be visualised cytologically. They may also occur by mechanisms other than gene conversion such as strand switching mediated by topoisomerases, and may be a result of the BrdU treatment (Dillehay *et al.*, 1989).

irs3 cells were found to have a slight but non-significant reduction in spontaneous SCEs and a significant reduction in MMC-induced SCEs compared to wild type cells. This is consistent with results from other mammalian cells lines that lack or have compromised HRR proteins (Dronkert *et al.*, 2000; Fuller and Painter, 1988; Lambert and Lopez, 2001). Chicken DT40 cells lacking *RAD54*, *RAD51* and each of the *RAD51*-like genes show a significant reduction in spontaneous SCEs as well as MMC-induced SCEs (Sonoda *et al.*, 1999; Takata *et al.*, 2000; Takata *et al.*, 2001). This may be because DT40 cells are more reliant on HRR than mammalian cells, a point that is discussed further in the Discussion, section 7.3.

Taken together the data from each of the three experiments described here confirm the suspected role of *RAD51L2* in the repair of DNA damage by *RAD51*-dependent homologous recombination repair.

CHAPTER 6: Use of *irs3* to identify functional domains in *hsRAD51L2*

6.1 Introduction

In Chapter 5 the *RAD51L2* protein was shown to be part of the *RAD51*-dependent HRR pathway. The experiments described in this Chapter were aimed to identify specific functional domains in the *RAD51L2* protein. This type of data will help to elucidate the mechanism of *RAD51L2* action and define its precise role in HRR. *irs3* cells were transfected with constructs containing *RAD51L2* cDNA, with mutations or deletions incorporated, and the ability of each construct to complement *irs3* was determined.

The human *RAD51L2* cDNA has two start codons although neither is in an ideal context for translation initiation when compared to the Kozak consensus sequence $^{-5}\text{CCA/GCCATGG}^{+4}$ (Kozak, 1987). Both retain a purine at the highly conserved position -3 , but the first motif has 3 deviations from the consensus sequence and the second motif 5 deviations; this includes the well conserved G at position $+4$ in both cases. A recent computational analysis of manually verified human mRNA sequences has shown that the majority of transcripts have 3 or 4 mismatches with the Kozak consensus sequence. Also, the frequency of upstream unused AUGs was greater than previously realised; 41% of transcripts have 1 and 24% of transcripts have 2 or more. Furthermore, 30-35% of these unused AUGs have a more favourable context for translation initiation than the actual AUG used (Peri and Pandey, 2001). It has been presumed the 5' most start codon of the *RAD51L2* gene is the one that is used for translation initiation because it is the first AUG encountered and has less mismatches with the Kozak consensus sequence. However, this has not been experimentally determined and it is possible that either codon could be used.

The *RAD51L2* gene was first isolated in a database search because of sequence similarities with the human XRCC3 and yeast Rad51 proteins (Dosanjh *et al.*, 1998). It shares 26.9% sequence identity with the human *RAD51* protein, with the most well

conserved residues being those that make up nucleotide binding or “Walker box” motif. The Walker box was first described in 1982 and consists of two separate sequences, A and B, which come together to form a nucleotide binding site (Walker *et al.*, 1982). Walker boxes are found in many nucleotide binding proteins and are highly conserved in RecA homologues and RAD51-like proteins. ATP allosterically regulates the binding of DNA to the RecA protein; RecA/ATP has a high affinity for ssDNA whereas ATP hydrolysis produces a form (RecA/ADP) with a low affinity for ssDNA (Menetski and Kowalczykowski, 1985). The Walker A motif (also known as the phosphate-binding loop, P-loop) is defined by the consensus sequence G/AXXXGKT/S (X = any amino acid). In RecA this sequence is found at residues 66 to 73 (GPESGKT); mutation of the four residues that identify the P-loop result in a non-functional protein but restricted mutation at other positions is tolerated (Konola *et al.*, 1994; Logan and Knight, 1993). One particularly well characterised mutation is the conservative K72R substitution. Mutant K72R protein is able to bind but not hydrolyse ATP and can promote limited strand exchange *in vitro* (Rehrauer and Kowalczykowski, 1993). However, *in vivo* this mutant has been shown to lack RecA activity (Konola *et al.*, 1994). Data obtained from the RecA crystal structure suggests that Lys72 interacts directly with the β and γ -phosphates of the bound ATP molecule whilst Thr73 interacts with the β phosphate and an associated Mg^{2+} ion. Gly66 and 73 are thought to play critical structural roles but may also form hydrogen bonds with the bound ATP (Story and Steitz, 1992). The Walker B motif consists of an invariant Asp residue at the carboxy terminus of a β -strand. In the RecA protein this sequence is found at residues 140-145 and the D144 residue is proposed to interact with the Mg^{2+} ion (Story and Steitz, 1992).

Mutagenesis has similarly been used to investigate the function of the Walker motifs in the *S.cerevisiae* Rad51p. Rad51p with a K191A Walker A motif mutation is unable to complement the MMS sensitivity and recombination defects of a *RAD51* null strain (Shinohara *et al.*, 1992). Rad51p K191R partially complements the *RAD51* null strain when expressed from a low-copy plasmid (Shinohara *et al.*, 1992) and fully complements it when overexpressed (Sung and Stratton, 1996). The DNA repair defect found when Rad51p K191R is expressed at native levels can be overcome by

mat-ing-type heterozygosity or by overexpression of *RAD54* (Morgan *et al.*, 2002). Additionally, mutation of the Asp in the Walker B box (D280G) of Rad51p leads to a non-functional protein (Chanet *et al.*, 1996). The *S.cerevisiae* data are supported by data from the fission yeast *S.pombe* where mutations in the invariant residues of the Walker A motif (K155A) and Walker B motif (D224Q) give similar non-functional phenotypes (Kim *et al.*, 2002).

The effects of mutating the Walker motifs in the human RAD51 protein have also been investigated using a conditional *RAD51*^{-/-} chicken DT40 cell line. A D222A Walker B motif mutation and a K133A Walker A motif mutation did not rescue the RAD51-deficient lethal phenotype, whereas a K133R mutation gave a partially defective phenotype (Morrison *et al.*, 1999). Mouse ES cells, expressing human RAD51 K133R at comparable levels to the endogenous protein, show increased sensitivity to MMC and IR and decreased spontaneous SCEs. The cells also show a 5-fold reduction in DSB-induced HR (Stark *et al.*, 2002).

Mutations have also been made in the Walker motifs of RAD51-like proteins. In *S.cerevisiae*, mutating the P-loop Lys to Arg/Ala results in increased γ -ray sensitivity and defective sporulation for Rad55p but not for Rad57p (Johnson and Symington, 1995). In addition, K54R, K54A and Δ G53 Δ K54 P-loop mutations have little effect on the function of the human XRCC2 protein (O'Regan *et al.*, 2001), and mutation of the invariant Asp in the Walker B motif (D149N/K) results in only a small loss of MMC resistance (O'Regan, 2002). It has been suggested that because the RAD51-like proteins act as part of dimers or larger complexes that only some of the participating proteins retain the ATP-binding requirement (O'Regan *et al.*, 2001).

It is thought that the RAD51-like proteins play an important role in the early stages of HRR. Consistent with this role, the XRCC2-EGFP fusion protein localises to the nucleus (O'Regan *et al.*, 2001). The RAD51L2 protein is part of the same RAD51-like protein complex as XRCC2 and would therefore also be predicted to localise to the nucleus. Many proteins exhibiting nuclear localisation have a nuclear localisation signal (NLS) which usually consists of one or two short sequences of positively charged lysines and arginines. The NLS is bound by a family of importins or transportins and the resulting complex is actively transported into the nucleus

through the nuclear pore (Cokol *et al.*, 2000). The programs PredictNLS (cubic.bioc.columbia.edu/predictNLS/) and PSORT (psort.ims.u-tokyo.ac.jp/) do not find a nuclear localisation sequence in the RAD51L2 protein sequence. However, a short motif (RKRSR) does exist at the extreme C-terminus of the protein that could constitute a nuclear localisation sequence.

6.2 Results

6.2.1 Role of the two *RAD51L2* start codons

The human *RAD51L2* sequence contains two start codons that are 9 amino acids apart. It has previously been unclear which codon is actually used for translation initiation. However, data obtained from 4 *irs3* clones transfected with the full-length *RAD51L2* cDNA suggest that the second start codon, which is the only codon present in the hamster and mouse sequences, may actually be used at least in hamster cells.

The clones were analysed by genomic DNA PCR and RT-PCR using primers designed to amplify either the full-length gene (forward primer overlapping the first start codon and reverse primer overlapping the stop codon; primers RAD51L2up(b) and RAD51L2down(b)EcoR1, Methods table 2.2) or an internal section (forward primer inside the second start codon and reverse primer inside the stop codon; primers hsL2upintB – 5' TTCCAGACTGCTGAGGAAGCTCCTAGA 3' and hsL2revintB – 5' ACCGTTTCCGGGTGCTCAAG 3'). All 4 clones were shown to carry the *RAD51L2* gene by genomic DNA PCR using both the full-length and internal primers (Fig. 6.2 A). However, when RNA was isolated from the clones and analysed by RT-PCR the internal primers yielded a strong product but the full-length primers yielded little or no product (Fig. 6.2 B). This amplification was repeated several times from different RNA isolates and the same result was obtained. It would appear from these data that the first start codon is either absent or lowly represented at the RNA level. Therefore, it would be anticipated that translation of the RAD51L2 protein would begin at the second start codon because no other translation initiation sites exist between the two sets of primers.

From the above results it would be predicted that a construct containing the *RAD51L2* cDNA with the first start codon removed would be fully functional in *irs3* cells. In order to test this prediction, the *RAD51L2* cDNA was amplified from plasmid DNA using primers with restriction sites placed so as to remove the first start codon and enable cloning into pIRESneo2 (Fig. 6.1 B). *irs3* cells were transfected with this construct and 20 drug resistant clones were isolated, 13 of which were found to carry the 5' truncated gene by genomic DNA PCR (data not shown). 4 of these clones were also analysed by RT-PCR and all were found to express the 5' truncated gene (data not shown). These figures should be compared with those in Table 3.1 where only 4 of 24 clones transfected with the full-length *RAD51L2* construct carried the gene by genomic DNA PCR. The 4 clones shown to express the 5' truncated *RAD51L2* gene were subjected to the preliminary MMC assay together with two previously isolated *irs3* clones expressing the full-length *RAD51L2* gene. It was found that clones carrying both constructs complemented *irs3* MMC sensitivity to a very similar level (Fig. 6.3 and data not shown). It can be concluded that the first start codon is not necessary for *hsRAD51L2* function in hamster cells.

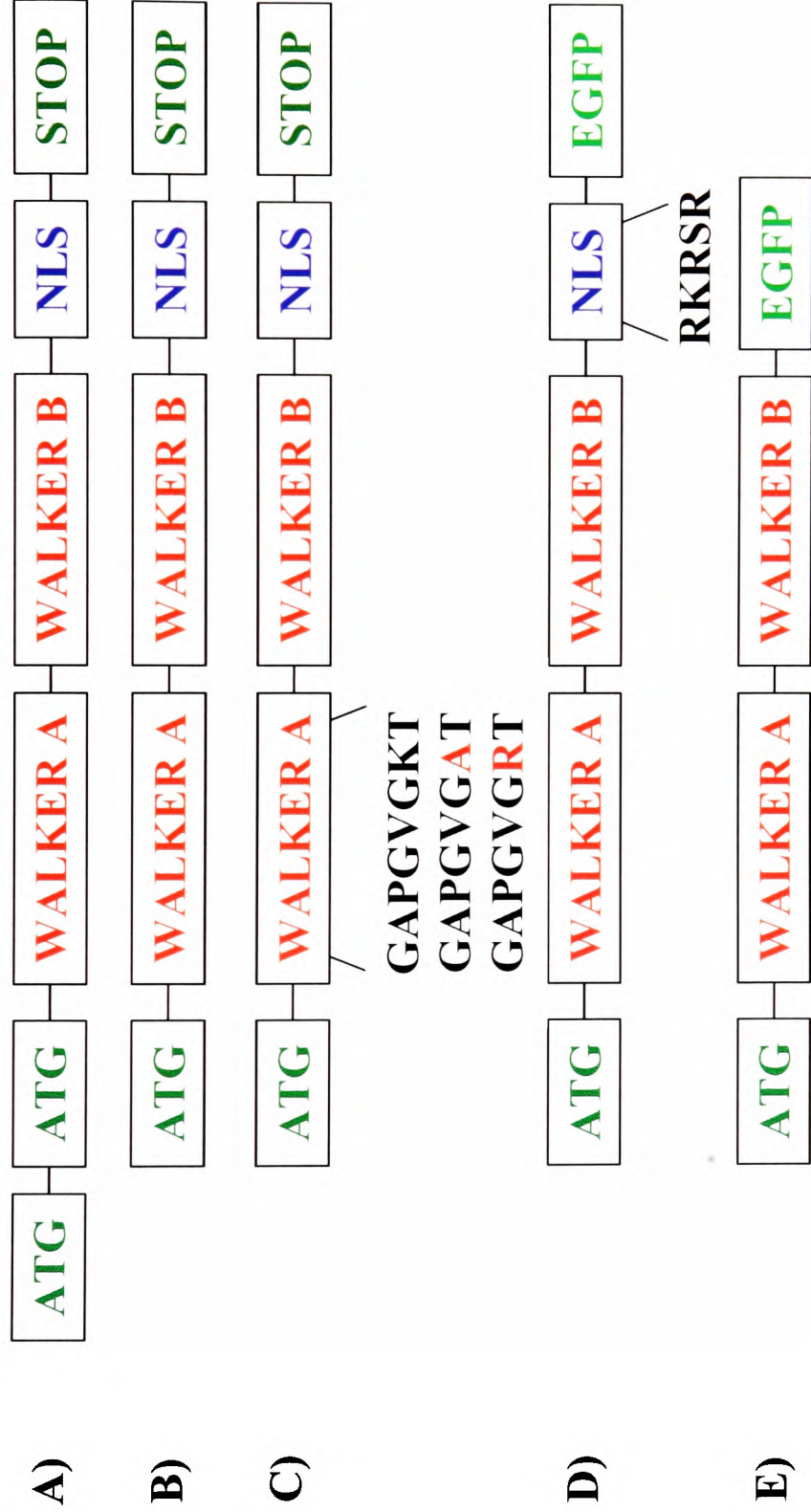


Figure 6.1 Summary of hRAD51L2 deletion and site-directed mutagenesis constructs. A, full-length *RAD51L2* gene; B, 5' deletion removing first start codon; C, non-conservative (K→A) and conservative (K→R) site directed mutagenesis of the invariant lysine residue in the Walker A box (5' deletion removing first start codon); D, 3' EGFP tag (5' deletion removing first start codon); E, deletion of the NLS and 3' EGFP tag (5' deletion removing first start codon).

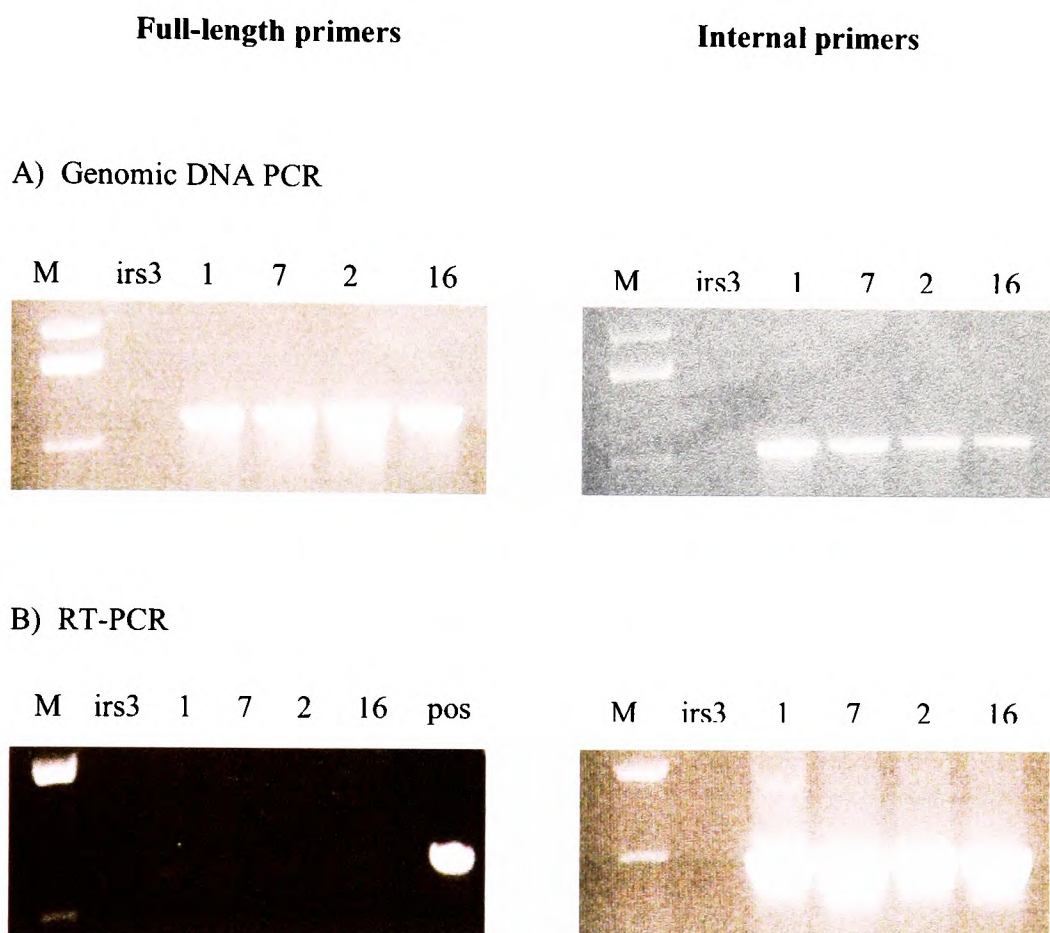


Figure 6.2 PCR analysis of *RAD51L2* transfected clones. A) Genomic DNA PCR and B) RT-PCR analysis of four *irs3* clones carrying the full-length *RAD51L2* gene. Left hand pictures show amplifications using primers to the full-length gene and right hand pictures show amplifications using primers to an internal section (primers described in text above). M, molecular size marker (Invitrogen) with bands equivalent to 1018bp, and where shown to 1636bp and 2036bp. Numbers at the top of each gel represent the clone being analysed. Pos, positive control.

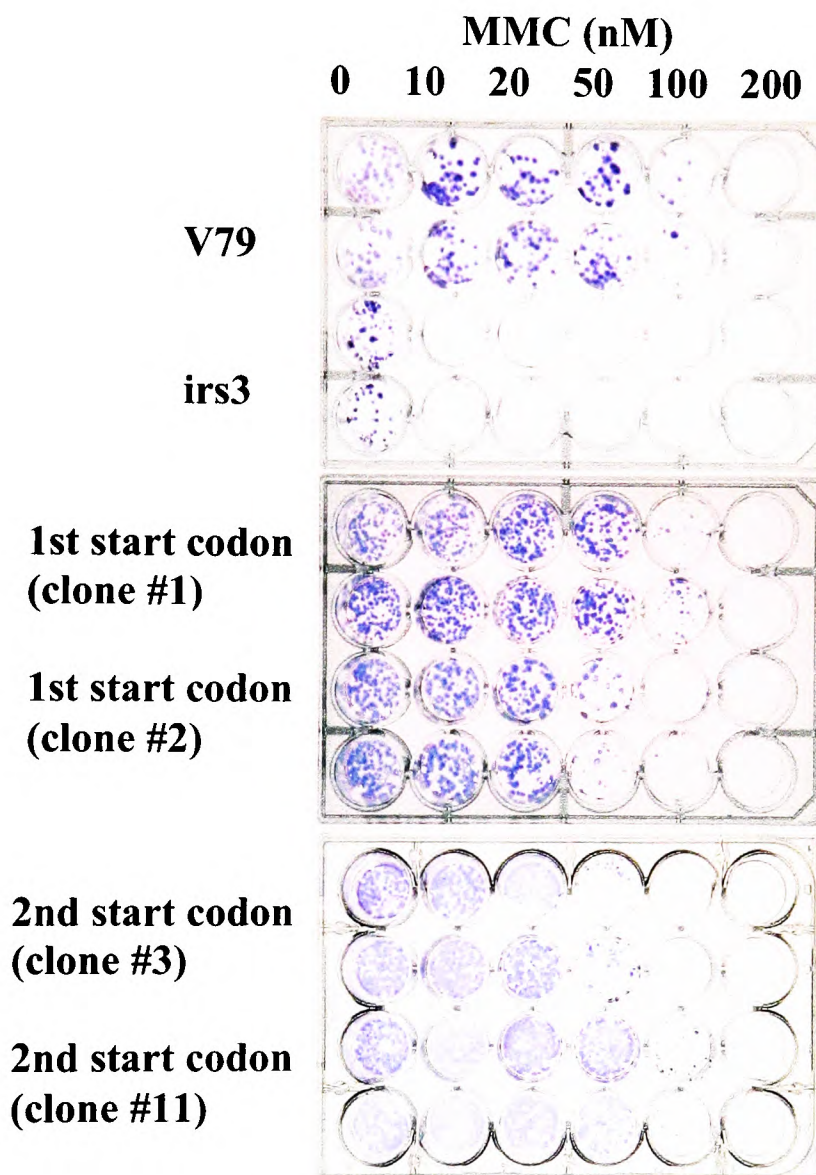


Figure 6.3 Human *RAD51L2* cDNA lacking the first start codon complements *irs3* MMC sensitivity. Survival of V79, *irs3*, *irs3* transfected with full-length *RAD51L2* cDNA (2 clones) and *irs3* transfected with *RAD51L2* cDNA lacking the first start codon (2 clones). Each cell line is in duplicate.

6.2.2 Site-directed mutagenesis of the *RAD51L2* Walker A box

The site-directed mutagenesis protocol had previously been shown to be more effective when the gene of interest was cloned into the pBluescript II SK (-) vector rather than pIRESneo2 (O'Regan, 2002). This may be due to the smaller size of the vector, 3.0kb compared to the 5.2kb pIRESneo2 vector. The *RAD51L2* gene was recloned into pBluescript II SK (-) using primers with restriction sites positioned so as to remove the first start codon. As described above, the first start codon is not required for h*RAD51L2* function in hamster cells and more drug-resistant clones appeared to express the *RAD51L2* gene when the first start codon was deleted.

The invariant lysine residue in the *RAD51L2* Walker A box was targeted for site-directed mutagenesis, which was carried out using the primers and protocol described in Materials and Methods, section 2.8. Mutagenic primers were designed to introduce a non-conservative K→A mutation and a conservative K→R mutation (Fig. 6.1 C). The sequence of each mutagenised *RAD51L2* gene was verified by sequencing before it was cloned back into the pIRESneo2 vector.

irs3 cells were transfected with the *RAD51L2* K→A and K→R constructs and 12 G418-resistant clones of each were isolated. 8 of the 12 K→A clones and 5 of the 12 K→R clones were shown to carry the *RAD51L2* gene by genomic DNA PCR (data not shown). 6 of the K→A carrying clones and 5 of the K→R carrying clones were also tested by RT-PCR and all were shown to express the *RAD51L2* gene (data not shown). The ability of the mutated *RAD51L2* cDNAs to complement irs3 was determined using the preliminary MMC assay. Clones expressing the *RAD51L2* K→A construct were found to survive MMC treatment no better than untransfected irs3 cells (Fig. 6.4, top). Clones expressing the *RAD51L2* K→R construct showed an intermediate level of resistance, being more resistant to MMC than untransfected irs3 cells but less resistant to MMC than irs3 cells transfected with wild type *RAD51L2* cDNA (Fig. 6.4, bottom). It should be noted that the level of MMC resistance exhibited by the *RAD51L2* K→R clones was somewhat variable as illustrated by the differences between clone#5 and clone#9 (Fig. 6.4, bottom). Taken together these

data indicate that the Walker A box lysine residue is important for RAD51L2 protein function.

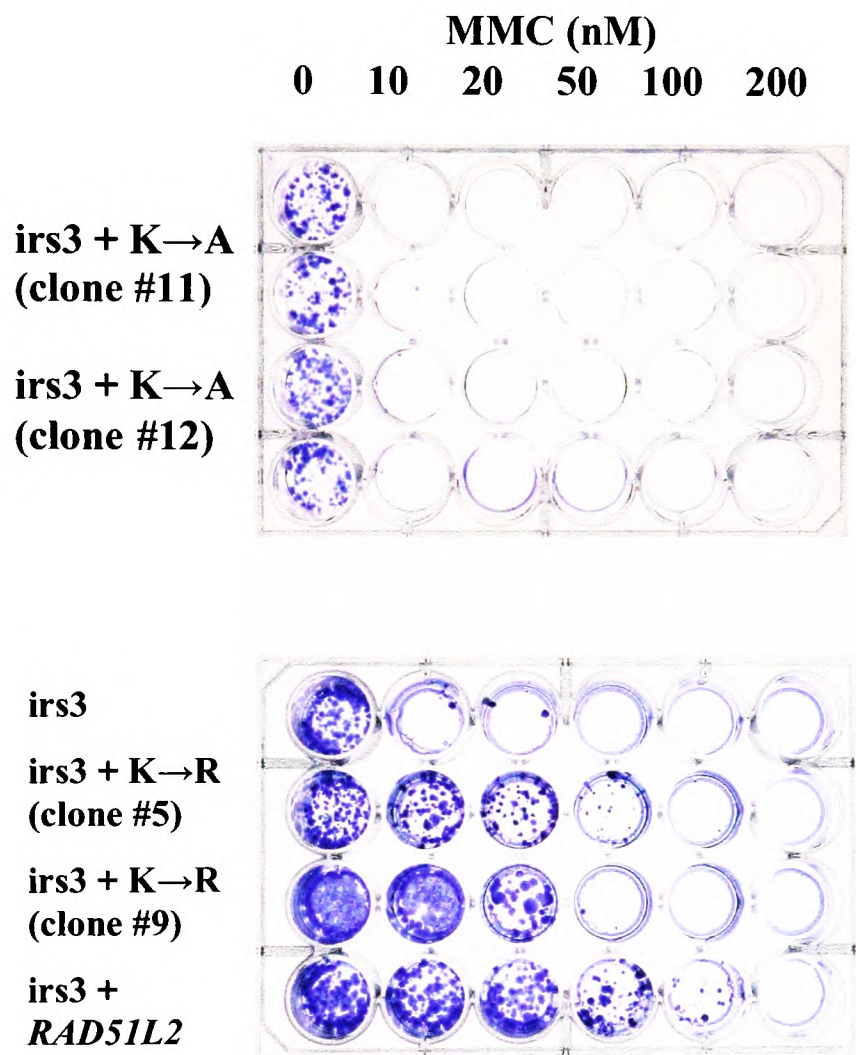


Figure 6.4 MMC survival of Walker A box mutants. Top, survival of *irs3* transfected with *RAD51L2* K→A (2 clones). Each cell line is in duplicate. Bottom, survival of *irs3*, *irs3* transfected with *RAD51L2* K→R (2 clones) and *irs3* transfected with wild type *RAD51L2* cDNA.

6.2.3 Localisation of the RAD51L2 protein

In order to look at the subcellular localisation of the RAD51L2 protein, a fusion construct was generated with EGFP. The *RAD51L2* cDNA was cloned into the pEGFP-N1 vector with primers designed to remove the first start codon and the stop codon, and clone the gene in frame with the downstream *EGFP* gene (Fig. 6.1 D). The resulting fusion protein has the N-terminus of EGFP attached to the C-terminus of RAD51L2. The RAD51L2 protein has a putative C-terminal NLS, RKRSR, positioned at amino acids 366-370. A second EGFP fusion construct was generated in a similar manner to the first, with an 11 amino acid C-terminal deletion that removes the putative NLS sequence (Fig. 6.1 E). From here onwards these two constructs are termed L2EGFP and L2EGFPNLS.

irs3 cells were transfected with L2EGFP and empty pEGFP-N1 vector, and 12 G418 resistant clones of each were isolated. 5 of the 12 L2EGFP clones were found to carry the *RAD51L2* gene by genomic DNA PCR (data not shown) and were subjected to the preliminary MMC assay. 3 were found to survive similarly to untransfected irs3 cells, 1 was found to be partially resistant to MMC (clone #1) and 1 showed a similar level of MMC resistance to irs3 cells transfected with wild type *RAD51L2* cDNA (clone #2) (Fig.6.5 and data not shown). The localisation of the L2EGFP fusion protein in clone #1 and #2 was viewed by confocal microscopy. The best images were obtained with clone #2, where the majority of cells gave a good fluorescent signal. The fluorescence was predominantly nuclear, but excluded from the nucleoli, although some cytoplasmic specks were also visible (Fig. 6.6 A). Clones transfected with pEGFP-N1 vector showed fluorescence throughout the cell (data not shown).

The fluorescence intensity amongst cells in a single clone was often found to be very heterogeneous. Fluorescence intensity also appeared to diminish with passage in EGFP-expressing clones, probably due to the toxicity of the EGFP protein. It was often necessary to alter the gain on the confocal microscope to look at the optimum level of cellular fluorescence. Approximately 20-30% of the cells in clone #1 showed a fluorescent signal. Figure 6.6 C shows three strongly fluorescing cells from clone

#1. Reducing the gain on the confocal microscope reveals nuclear localisation (Fig. 6.6 B).

irs3 cells were also transfected with L2EGFPNLS and 12 G418 resistant clones were isolated. Although 4 of these clones were shown to carry the *RAD51L2* gene by genomic DNA PCR, confocal microscopy showed fluorescence in very few or none of the cells (data not shown). Another 48 clones were therefore isolated and grown in 24 well dishes. The dishes were scanned with the confocal microscope and 10 EGFP-expressing clones were identified (fluorescence in approximately 20-70% of cells). 4 of the 10 clones were chosen for further confocal analysis, and fluorescence was found to be distributed throughout the cell rather than localised to the nucleus (Fig. 6.6 D,E and data not shown; compare with Fig. 6.6 B,C). All 4 clones were shown to express the 3' truncated *RAD51L2* gene by RT-PCR (data not shown). Work is continuing to look at the MMC sensitivity of the L2EGFPNLS clones.

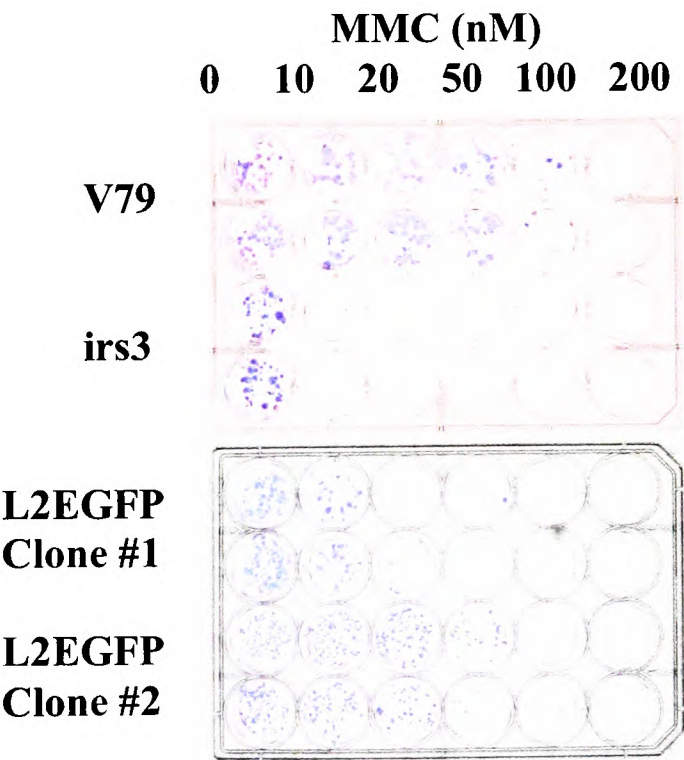


Figure 6.5 MMC survival of L2EGFP clones. MMC survival of V79, irs3 and irs3 transfected with L2EGFP (clone #1 and #2). Each cell line is in duplicate.

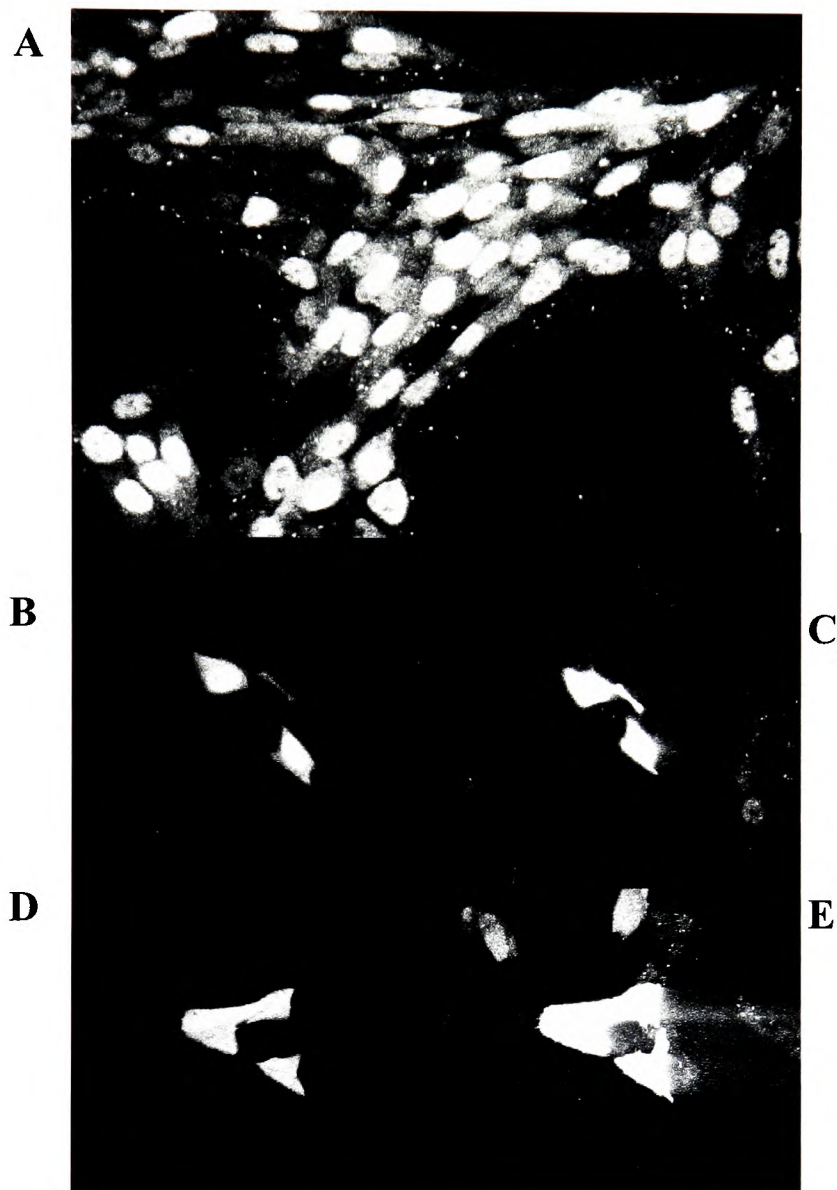


Figure 6.6 Localisation of the L2EGFP and L2EGFPNLS fusion proteins. A, L2EGFP clone #2; B, L2EGFP clone #1 (low gain); C, L2EGFP clone #1 (high gain); D, L2EGFPNLS clone #42 (low gain); E, L2EGFPNLS clone #42 (high gain). Confocal images at excitation 488nm and emission 510nm.

6.3 Discussion

The data obtained from clones transfected with 5' truncated *RAD51L2* cDNA indicate that the first start codon is not required for RAD51L2 protein function, at least in hamster cells. PCR analysis of *irs3* cells transfected with the full-length *RAD51L2* cDNA showed that the first start codon is present in the genomic DNA but appears to be absent or expressed at a low level in RNA. In addition, a higher proportion of G418-resistant clones express *RAD51L2* after transfection with the 5' truncated gene than after transfection with the full-length gene. Taken together these data suggest that the sequence between the first and second start codons may actually be deleterious to cells. It is possible that the region has a specialised function in human cells, which is damaging in a hamster context. The implications of these findings are discussed further in the main Discussion, section 7.2.

Site-directed mutagenesis of the invariant lysine in the Walker A box of RAD51L2 indicates that this residue is important for protein function. No increase in MMC resistance was observed when *irs3* cells were transfected with *RAD51L2* cDNA containing a non-conservative (K→A) mutation, and only partial *irs3* complementation was seen with a conservative (K→R) mutation. This is analogous to the situation found with hRAD51, where a K→A mutation completely ablates protein function and a K→R mutation results in a partially functional protein (Morrison *et al.*, 1999).

The deleterious effect associated with these RAD51L2 mutations is likely due to loss of ATPase activity but could be due to other factors such as disruption of protein structure. Recently, purified RAD51L2 protein was demonstrated to have DNA-stimulated ATPase activity (Lio *et al.*, 2003). In addition, the RAD51L1-RAD51L2 heterodimer and the RAD51L1-RAD51L2-RAD51L3-XRCC2 heterotetramer both exhibit ATPase activity (Lio *et al.*, 2003; Masson *et al.*, 2001b; Sigurdsson *et al.*, 2001). As yet the physiological significance of this ATPase activity is unclear. It is possible that the energy provided by ATP-hydrolysis may be required for the binding or dissociation of the complexes from DNA. It may also be needed to clear RPA from the DNA in preparation for RAD51 binding.

Interestingly, corresponding mutations in the Walker A box of XRCC2 have little or no effect on protein function (O'Regan *et al.*, 2001). In *S.cerevisiae*, equivalent mutations in Rad55p compromise function but have little effect on Rad57p function (Johnson and Symington, 1995). This could suggest that RAD51L2 is functionally more similar to Rad55p and XRCC2 to Rad57p, although phylogenetic analysis places the proteins the other way around (Thacker, 1999). It would be useful to look at the effects of mutating the Walker A boxes of the other 3 RAD51-like proteins.

As predicted, subcellular localisation of the RAD51L2-EGFP fusion protein was found to be predominantly nuclear. This localisation is consistent with a role for the protein in HRR. It appears that the EGFP tag does not affect RAD51L2 protein function because the clone showing nuclear EGFP fluorescence was also found to be resistant to MMC. These results are very similar to those found with the XRCC2-EGFP fusion protein where localisation was predominantly nuclear with some speckles of fluorescence in the cytoplasm (O'Regan *et al.*, 2001). These speckles were also observed with the RAD51L2-EGFP fusion protein and could indicate a cytoplasmic function for the RAD51L2 protein. However, there is no other evidence for a cytoplasmic function and a more likely explanation is that overexpression of the RAD51L2-EGFP fusion protein leads to aggregates of excess protein accumulating in the cytoplasm.

The RAD51L2(-NLS)-EGFP fusion protein was found to be distributed throughout the cell, showing a similar pattern of fluorescence to the vector control. This suggests that the short RKRSR sequence at the RAD51L2 C-terminus may be required for transportation of the protein into the nucleus. The 11 amino acid C-terminal deletion used to eliminate the putative NLS also removes 6 other amino acids. Site-directed mutagenesis of the NLS motif would be necessary to show that the effects observed are due to this sequence. The function of the NLS motif could also be demonstrated by attaching it to a known non-nuclear sequence and looking for evidence of relocalisation. Interestingly, the XRCC2 protein is able to tolerate quite large C-terminal deletions, 30 and 80 amino acid deletions have relatively little effect on protein function (O'Regan, 2002). In contrast, an 11 amino acid deletion has a

marked effect on the function of the RAD51L2 protein, abolishing its nuclear localisation. These results indicate that the extreme C-terminus of the RAD51L2 protein is important for its cellular function.

CHAPTER 7: Discussion

7.1 Summary of major findings

1. *irs3* MMC sensitivity is complemented by *RAD51L2* cDNA or genomic DNA. Complementation is not observed with *RAD51*, *RAD51L1* or *RAD51L3* cDNA.
2. *RAD51L2* cDNA also complements *irs3* X-ray sensitivity and its elevated level of spontaneous chromosome aberrations.
3. Sequencing of the hamster *RAD51L2* gene revealed a G to T base substitution at the first nucleotide of exon 6. This causes an amino acid change of Val to Phe and potentially disrupts the 3' acceptor splice site of exon 6.
4. *irs3* cells have a dramatically reduced level of *RAD51L2* cDNA and little or no *RAD51L2* protein.
5. The hamster *RAD51L2* gene partially complements *irs3* MMC sensitivity.
6. The first *hsRAD51L2* start codon is not required for protein function in hamster cells.
7. The integrity of the highly conserved lysine residue in the Walker A box is required for *RAD51L2* protein function. A non-conservative K to A mutation abolishes protein function whereas a conservative K to R mutation substantially reduces protein function.
8. The *RAD51L2* protein localises to the nucleus and is required for damage dependent *RAD51*-focus formation.

9. *irs3* cells show decreased SCE induction after MMC treatment compared to wild type cells. In addition, *irs3* cells carrying a GFP recombination reporter substrate show a 7-fold reduction in homology-directed repair of a DSB.

7.2 Analysis of *irs3* transfection experiments

During the course of this study the *irs3* cell line was transfected with several constructs. After transfection, independent clones were isolated by drug selection and grown on. Genomic DNA PCR was performed on each of the clones to see if they carried the gene of interest. RT-PCR was then carried out on a number of positive clones to determine if the gene of interest was being expressed. Even though the number of clones tested is relatively small, this analysis yielded some interesting data, a selection of which is shown in table 7.1.

As described in Chapter 3, the full-length *RAD51L2* gene was initially cloned into the pZeoSV2(-) vector before being transfected into *irs3*. 16 of the 25 isolated clones were shown to carry the *RAD51L2* gene by genomic DNA PCR. RT-PCR was not performed on these clones, but 3 of the 25 clones were found to be MMC resistant. This suggests that only 3 of the 16 clones shown to carry the *RAD51L2* gene also expressed it. In contrast, when *irs3* was transfected with *RAD51L3* in pZeoSV2(-), 3 out of the 5 isolated clones both carried and expressed the *RAD51L3* gene as determined by RT-PCR. In summary, when *irs3* is transfected with *RAD51L3* in pZeoSV2(-) 1 in every 1.25 clones isolated expresses the gene of interest whereas transfection with *RAD51L2* in pZeoSV2(-) results in 1 in every 8.3 clones isolated expressing the gene of interest.

At this stage the expression level of the RAD51L2 protein was thought to be important. The pZeoSV2(-) vector has a strong CMV promoter, meaning that under most circumstances RAD51L2 would probably be expressed at a high level. It is known that overexpression of certain genetic stability genes in mutant cell lines is not always successful. For example, no BRCA2 expression was observed when CAPAN-1 cells were transfected with full-length cDNA cloned into an expression vector (Moynahan *et al.*, 2001b). It is possible that expression is suppressed in clones that

can express *RAD51L2* at high levels, whereas clones that have the construct integrated in a position that allow lower expression can tolerate some protein production.

To overcome this problem the *RAD51L2* gene was subcloned into the pIRESneo2 vector where the selective marker (G418) and the gene of interest (*RAD51L2*) are translated on the same RNA. *irs3* was transfected with this construct and 4 of the 24 clones isolated were found to both carry and express the *RAD51L2* gene. Unlike transfection with the *RAD51L2* in pZeoSV2(-) construct, there were no clones that carried the *RAD51L2* gene but did not express it. Data from transfection experiments with the *RAD51* in pIRESneo2 construct are also included for comparison. 1 in 1.5 clones express the gene of interest after transfection with *RAD51* in pIRESneo2, whereas transfection with *RAD51L2* in pIRESneo2 results in just 1 in 6 clones expressing the gene of interest, a substantial reduction (Table 6.1). The *RAD51L3* in pZeoSV2(-) and *RAD51* in pIRESneo2 results are consistent with data reported by O'Regan *et al.* (2001). Various *XRCC2* constructs cloned into the pIRESneo2 vector were transfected into *irs1*; 85% (1 in 1.2) of the G418 clones isolated also expressed the *XRCC2* construct.

Transfection of *irs3* with 5' truncated *RAD51L2* (ie. first start codon deleted) in pIRESneo2 yielded the most interesting findings. 20 clones were isolated and 13 of these clones were found to carry the 5' truncated *RAD51L2* gene by genomic DNA PCR. 4 of the 13 clones were then tested for gene expression by RT-PCR and all were found to be positive. It therefore seems likely that the other 9 clones also express the 5' truncated gene. It appears that a significantly higher proportion of clones isolated after transfected with 5' truncated *RAD51L2* in pIRESneo2 express the gene of interest (1 in 1.5), compared to transfection with *RAD51L2* in pIRESneo2 (1 in 6). Furthermore, other 5' truncated constructs carrying additional Walker A box mutations concur with the above results. 1 in 1.5 and 1 in 2.4 clones isolated after transfection with 5' truncated K→A and K→R constructs express the gene of interest (Table 7.1).

The first start codon is not required for hs*RAD51L2* gene function in hamster cells (Fig. 6.3), but these results suggest that its presence may also be deleterious. 1

in 6 clones isolated after transfection with full-length *RAD51L2* in pIRESneo2 carry the full-length *RAD51L2* gene as determined by genomic DNA PCR. However, analysis of these clones by RT-PCR appears to show that the first start codon is either absent or only expressed at a very low level, whilst internal sequences are highly expressed (Fig. 6.2). It is possible that whilst the full-length primers amplify a genomic DNA template, they may not work effectively in an RT-PCR reaction leading to a false result. If the lack of a PCR product with the full-length primers is a correct result, then it is necessary to explain how this product is lost. It is possible that full-length *RAD51L2* mRNA is recognised as abnormal by the hamster RNA surveillance mechanisms and is targeted for destruction (Butler, 2002). Translational control processes, such as the use of alternative start codons or internal ribosomal entry sites, can be ruled out because the first start codon would still be present in the mRNA and hence detected by RT-PCR (Mignone *et al.*, 2002).

The full-length *RAD51L2* gene is expressed in human cells as seen from cDNA library screening, as well as experimentally (Andy George, personal communication). It is therefore possible that the extra 9 amino acids may play a specific role in regulating the human *RAD51L2* protein. For example, the KARP-1 gene has been shown to use a promoter and additional exons upstream of the Ku86 gene and results in an extra 9kDa of protein being attached to the Ku86 polypeptide. KARP-1 has two repeated leucine motifs that may interact with a similar motif on the catalytic subunit of DNA-PK. Overexpression of the 88 residues specific to KARP in cells results in reduced DNA-PK activity and X-ray sensitivity. KARP-1 is not conserved in hamsters and is thought to be primate specific (Myung *et al.*, 1997).

In summary, these transfection experiments suggest that the overexpression of human *RAD51L2* in hamsters is deleterious. It appears that the presence of transcripts carrying the first start codon may be the reason for the reduction in drug-resistant clones expressing *RAD51L2*.

Construct	hsRAD51L2 in pZeoSV2(-)	hsRAD51L3 in pZeoSV2(-)	hsRAD51L2 in pIRESneo2	hsRAD51 in pIRESneo2	hsRAD51L2 2 nd ATG in pIRESneo2	hsRAD51L2 2 nd ATG K→A in pIRESneo2	hsRAD51L2 2 nd ATG K→R in pIRESneo2
No. clones isolated	25	5	24	6	20	12	12
No. clones carrying genomic DNA of gene of interest	16/25	4/5	4/24	4/6	13/20	8/12	5/12
No. of above clones expressing gene of interest by RT-PCR	N/D, but 3/25 MMC resistant	3/3	4/4	4/4	4/4	6/6	5/5
Proportion of isolated clones expressing gene of interest	1 in 8.3	• 1 in 1.25	1 in 6	1 in 1.5	1 in 1.5	1 in 1.5	1 in 2.4

Table 7.1 Analysis of clones isolated after transfection of irs3 with various constructs. N/D, not determined.

7.3 Comparison of *irs3* with other *RAD51*-like gene deficient cell lines

irs3 vs. *RAD51L2*-deficient hamster cell line, CL-V4B

At around the same time *irs3* was found to carry a *RAD51L2* gene mutation, another hamster cell line was also identified as being *RAD51L2*-deficient. The CL-V4B cell line was isolated from an ENU-treated population of V79B cells on the basis of its sensitivity to MMC (Godthelp *et al.*, 2002b).

When treated chronically with MMC, CL-V4B is reported to be ~32-fold more sensitive than wild type cells whereas *irs3* is ~20-fold more sensitive. These figures are based on a comparison of the doses required to reduce the wild type and mutant cell line survivals to 10% (D_{10} value). The MMC sensitivity of both cell lines is substantially complemented by *RAD51L2* cDNA. At first glance it appears that CL-V4B is significantly more sensitive to MMC than *irs3*. However, when the data from Godthelp *et al.* (2002) and our *irs3* data are plotted on the same scale the MMC sensitivities of CL-V4B and *irs3* are almost identical. The reason that CL-V4B is described as being more MMC sensitive than *irs3* is because V79B is more resistant to MMC than V79-4. It should be noted that our chronic survival data for V79-4 closely agree with those reported by O'Regan *et al.* (2001). There are two possible explanations for this finding. Firstly, the experimental conditions for the chronic MMC treatment may differ between laboratories. For example, different sources of MMC or other reagents such as medium or serum may have been used. Secondly, V79B may recover better during chronic exposure to MMC than V79-4. In conclusion, it is difficult to say whether CL-V4B is more sensitive or equally as sensitive to MMC than *irs3*.

Other aspects of these cell lines phenotypes are very similar. CL-V4B and *irs3* are 1.3-fold and 2-fold more sensitive to X-rays respectively than wild type cells. In this case the survival curves for V79B and V79-4 are very similar. Both cell lines are defective in damage-dependent RAD51 focus formation, although the data cannot be directly compared because the cells were treated with different doses of X-rays and alternative methods of scoring were employed. CL-V4B and *irs3* both show an

increase in spontaneous chromosome aberrations that are elevated to very similar levels. One of the interesting features of *irs3* is the increase in isochromatid breaks. Isochromatid breaks were not scored as a separate category in the CL-V4B study but are likely to be included in the high number of chromatid breaks observed. Like *irs3*, CL-V4B shows a small decrease in spontaneous SCE frequency compared to wild type cells. However, levels of induced SCEs could not be compared because of the very low MMC concentrations used in the CL-V4B experiments. Both studies use a 2 h acute MMC treatment rather than chronic MMC treatment for SCE experiments, which means that higher doses of MMC can be used. Since *irs3* and CL-V4B respond almost identically to chronic MMC treatment it would seem likely that they can tolerate similar acute treatments. In the present study, *irs3* cells were treated with 4×10^{-7} M (400 nM) and 4×10^{-8} M (40 nM) MMC which yielded 33.2 ± 1.1 and 8.1 ± 0.3 SCEs/cell respectively (Table 5.1). CL-V4B cells were treated with 0.5-1.5 ng/ml (1.5-4.5 nM) MMC. Hardly any change in SCEs/cell would be expected when such low MMC concentrations are used. It should be possible to use a higher MMC concentration that would not kill the CL-V4B cells to obtain a more meaningful result.

As described in Chapter 4, the *irs3 RAD51L2* gene has a G to T base substitution at the first nucleotide in exon 6. The CL-V4B *RAD51L2* mutation has not been identified but is not the same as the *irs3* mutation. It gives rise to a 132 bp deletion corresponding to an alternatively spliced transcript that lacks exon 5 and results in loss of the Walker B box. The portion of the hamster gene sequenced by Godthelp *et al.* (2002) is identical to the hamster sequence described here. Western analysis of CL-V4B and *irs3* cell extracts has shown that there is no RAD51L2 protein in either cell line, although the human anti-RAD51L2 antibody used to probe the CL-V4B extracts was reported to poorly recognise the endogenous hamster RAD51L2 protein.

In *BRCA1*-, *BRCA2*-, *XRCC2*- and *XRCC3*-deficient rodent cell lines abnormal centrosomes can lead to incorrect chromosome segregation (Griffin *et al.*, 2000; Kraakman-van der Zwet *et al.*, 2002; Xu *et al.*, 1999). Centrosome experiments have not been carried out using *irs3* cells but CL-V4B cells show normal centrosome

formation. This indicates that in contrast to XRCC2 and XRCC3, RAD51L2 is not required for this function.

Godthelp *et al.* (2002) also report a defect in sister chromatid cohesion in CL-V4B. After treatment with 1µg/ml colcemid, 58% of CL-V4B cells show separated chromatids compared to 26% of V79B wild type cells. When colcemid is not used, 21% of CL-V4B cells and 8% of V79B cells show chromatid separation. In both cases the introduction of *RAD51L2* cDNA partially complements this phenotype. In addition, the study reported 30% and 18% chromatid separation for V79-4 and *irs1* respectively after colcemid treatment. However, these cell lines have been used for many years in our laboratory and no precocious chromatid separation has been observed. For this reason it was decided to repeat the experiment in our laboratory. In this study, V79B and CL-V4B showed 3% and 0% chromatid separation respectively without colcemid treatment. After treatment with 1µg/ml colcemid (a very high concentration compared to the 0.05µg/ml colcemid routinely used in this lab), V79B and CL-V4B showed 4% and 17% chromatid separation respectively. No chromatid separation was observed in V79-4 or *irs3* cells (Carol Griffin, personal communication). Although we find lower levels of chromatid separation in our study, the frequency in CL-V4B is still elevated above that found in wild type cells. This appears to be a unique feature of CL-V4B because increased chromatid separation is not observed in *irs3*.

irs3 vs. RAD51-like gene chicken DT40 mutants

All five *RAD51*-like genes have been disrupted in chicken DT40 cells and all the resulting mutant cell lines were found to have broadly similar phenotypes (Takata *et al.*, 2000; Takata *et al.*, 2001). Similarly to *irs3*, *RAD51L2* mutant DT40 cells are hypersensitive to DNA cross-linking agents such as MMC and mildly sensitive to γ-irradiation. They are also defective in RAD51 focus formation after treatment with γ-irradiation. In addition, chick *RAD51L2* mutant cells are impaired in HR as measured by reduced targeted integration and SCE frequencies (Takata *et al.*, 2001). However, the reduction in both spontaneous and MMC-induced SCEs is greater in the mutant

chick cells than in *irs3*. This could be due to the greater reliance of chick lymphoblastoid cells on HR for repair (Sonoda *et al.*, 2001).

DT40 cells have a much higher gene targeting efficiency than mammalian cells, and a short generation time, making them attractive for phenotypic analysis of single and multiple gene disruptions. They are also relatively easy to handle and have a stable karyotype and phenotype. Seven different selection markers are available for use with DT40 cells and a wide range of assays has been developed for phenotypic analysis. However, there are some disadvantages with DT40 cells. For example, microscopy can be difficult because chicken mini chromosomes obscure the analysis (Winding and Berchtold, 2001). The phenotypes of murine and DT40 mutants are often described as being very similar (Sonoda *et al.*, 2001) but there are some notable differences in HR mutants. Significantly, human *RAD51* cDNA can partially complement the chick *RAD51*-like gene mutants (Takata *et al.*, 2001) but cannot complement the defect in *irs3* (Fig. 3.2) or *irs1* cells (data not shown). In addition, *RAD54* is required for *RAD51* focus formation in mouse cells (Tan *et al.*, 1999) but not in chick cells (Takata *et al.*, 2000). It is therefore important to be cautious when extrapolating HR data from one species to another, and where possible to obtain data from more than one species.

irs3 vs. mutant RAD51-like gene rodent cell lines

The mutant hamster cell lines *irs1* and *irs1SF* are 60- and 100-fold more sensitive to acute MMC treatment than wild type cells (Caldecott and Jeggo, 1991; Jones *et al.*, 1987). In contrast, the *irs3* cell line is only 7-fold sensitive to acute MMC treatment (Jones *et al.*, 1987), and could be described as having a similar but less severe phenotype than *irs1* and *irs1SF*. For example, although *irs3* cells are clearly defective in damage-dependent *RAD51* focus formation, numbers of foci are not reduced to the levels observed in *irs1* (Fig. 5.7; O'Regan *et al.* (2001)). *Xrcc2*^{-/-} MEFs and ES cells are 4-fold more sensitive to acute MMC treatment than wild type cells (Bryan Deans, personal communication), a phenotype that is more similar to *irs3* than *irs1* or *irs1SF*. Given that all five *RAD51*-like gene chicken DT40 mutants are described as

having similar phenotypes (Takata *et al.*, 2001), the different severities in phenotype displayed by the rodent cell lines is unexpected.

There are several possibilities that could explain the observed differences in phenotype. Firstly, a low level of RAD51L2 protein may be present in *irs3*, enough to function in HR but below the threshold detection point of the anti-RAD51L2 antibody. Secondly, *irs3* may have an additional mutation that partially suppresses the effect of the *RAD51L2* mutation. Such a mutation may be necessary for the survival of *RAD51L2* mutant cells. As yet there is no evidence for a secondary mutation in *irs3* but it would be difficult to identify. Alternatively, it is formally possible that both *irs1* and *irs1SF* carry secondary mutations that confer a more severe DNA damage phenotype. The *Xrcc2*^{-/-} knockout MEFs and ES cells are isogenic with the parent lines and would not be expected to carry secondary mutations.

On the other hand, it is possible that none of the above cell lines have residual protein or carry secondary mutations. This would mean that the observed *irs3* phenotype is that of a mammalian RAD51L2 null mutant. The phenotype of CL-V4B is in general very similar to that of *irs3* and no RAD51L2 protein is reported to have been detected in CL-V4B cell extracts (Godthelp *et al.*, 2002b). Although the *RAD51*-like gene chicken DT40 mutants are described as having similar phenotypes, the RAD51L2 mutant is the least sensitive to MMC and X-rays (Takata *et al.*, 2001). It is possible that the differences between the various mutants are less pronounced in DT40 cells than in mammalian cells.

7.4 Role of the RAD51-like proteins in HRR

At the beginning of this study, yeast two hybrid data indicated that the RAD51-like proteins could form one large complex (Schild *et al.*, 2000). If loss of any one of the components stopped the complex from functioning, it would seem likely that all five *RAD51*-like gene mutants would have a similar phenotype. This idea is supported by evidence from the *RAD51*-like gene chick mutants which all show broadly similar phenotypes (Takata *et al.*, 2001). This situation is analogous to that of the FA

complex where loss of FA-A,C,G,E and F give almost identical phenotypes. Double mutants of FA genes also have the same phenotype as single mutants.

However, more recent biochemical evidence has shown that XRCC3 is only immunoprecipitated with RAD51L2, indicating that at least two distinct complexes of RAD51-like proteins must exist (Liu *et al.*, 2002; Masson *et al.*, 2001b; Miller *et al.*, 2002; Wiese *et al.*, 2002) (Fig. 7.1). Furthermore, different biochemical properties have now been described for the various RAD51-like proteins and protein complexes (section 1.6.5).

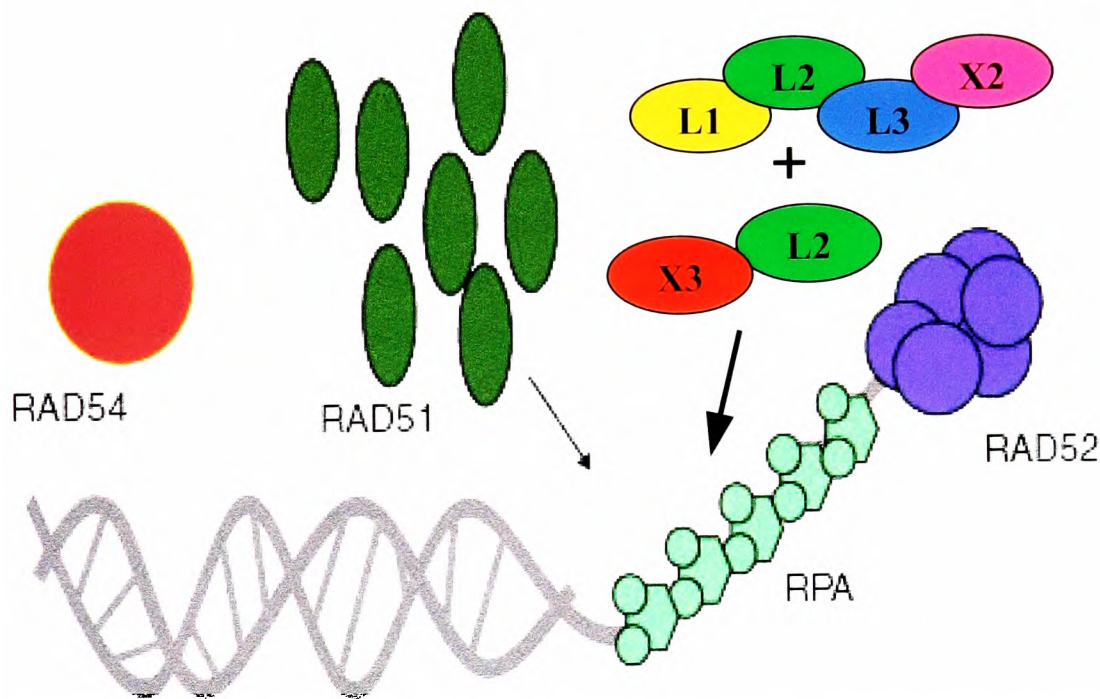


Figure 7.1 Role of the RAD51-like proteins in HRR. The proteins exist in at least two separate complexes with different biochemical properties.

In the present study, *irs3* cells showed reduced SCE frequencies after MMC treatment, defective RAD51 focus formation and impairment in the homology-directed repair of a DSB. These data directly link the mammalian RAD51L2 protein to the HRR pathway for the first time, but the exact role of RAD51L2 and the other RAD51-like proteins in this pathway is still unclear. Some potential functions of the RAD51-like proteins are discussed below.

The human RAD51 protein has very weak strand exchange activity when compared to the bacterial RecA protein (Baumann and West, 1998). However, strand exchange activity can be stimulated by the addition of mediator proteins such as RPA (Baumann and West, 1997) and RAD52 (Benson *et al.*, 1998). The RAD51-like proteins may also act as mediators by assisting the loading of RAD51 onto DNA. This suggestion is based on data from the *S.cerevisiae* Rad55 and Rad57 proteins, which form a stable heterodimer that stimulates Rad51-promoted strand exchange. It is thought they do this by inhibiting RPA, which although an important accessory factor, also competes with Rad51 for binding on the ssDNA template (Sung, 1997). Recently, the RAD51L1-RAD51L2 heterodimer has been shown to promote the assembly of the RAD51-ssDNA nucleoprotein filament in a similar manner (Sigurdsson *et al.*, 2001). In addition, Rad55 and Rad57 mutants display cold sensitivity for DNA repair which suggests they may function in stabilising protein complexes (Game and Mortimer, 1974; Hays *et al.*, 1995; Johnson and Symington, 1995). Rad51 mutations that partially complement Rad55 and Rad57 mutants have been found to increase the stability of Rad51-ssDNA complexes (Fortin and Symington, 2002). It remains to be seen whether the mammalian RAD51-like proteins also stabilise nucleoprotein filaments.

There appear to be functional similarities between the yeast and the human RAD51-like proteins. However, mammalian cells have five RAD51-like proteins whereas yeast only has two. This would suggest that HR in mammals is more complex than in yeast and requires extra factors with additional functions. The yeast Rad55 and Rad57 proteins have only been implicated in the early stages of HR, but there are now several lines of evidence which support a later role for the mammalian RAD51-like proteins in the process. Firstly, EM visualisation of the RAD51L2-XRCC3 heterodimer and the RAD51L1-RAD51L2-RAD51L3-XRCC2 complex shows them to aggregate DNA which may facilitate DNA-DNA contacts during the homology search step of HR (Masson *et al.*, 2001a; Masson *et al.*, 2001b). Secondly, the RAD51L1 protein binds specifically to synthetic Holliday junctions but does not have branch migration activity. This would suggest a role in some aspect of Holliday junction processing (Yokoyama *et al.*, 2003). Thirdly, *irs1*SF cells show an altered

product spectrum with increased gene conversion tract lengths and greater numbers of discontinuous tracts. A defect solely in HR initiation is not consistent with these observations which suggest a role for XRCC3 in the later stages of HR. It is proposed that XRCC3 functions to stabilise heteroduplex DNA (hDNA) either directly or indirectly. In XRCC3-deficient cells, only long regions of hDNA are stable enough to be resolved correctly. Mismatches in unstable hDNA could also be recognised poorly by the MMR machinery leading to the increased number of discontinuous tracts observed (Brenneman *et al.*, 2002).

The present study also provides evidence suggesting that RAD51L2 is involved in the later stages of HR. *irs3* cells show a striking increase in isochromatid breaks as well as a reduction in SCEs. SCEs can arise from crossing-over during HR, whilst isochromatid breaks can be viewed as the result of failed HR during replication. The RAD51L2 protein may be required for the repair of stalled replication forks by HR. In *irs3*, the lack of RAD51L2 protein could lead to the improper resolution of intermediates that would result in unstable structures prone to disintegration. This would yield an increased level of isochromatid breaks and a decreased level of SCEs (Fig. 7.2).

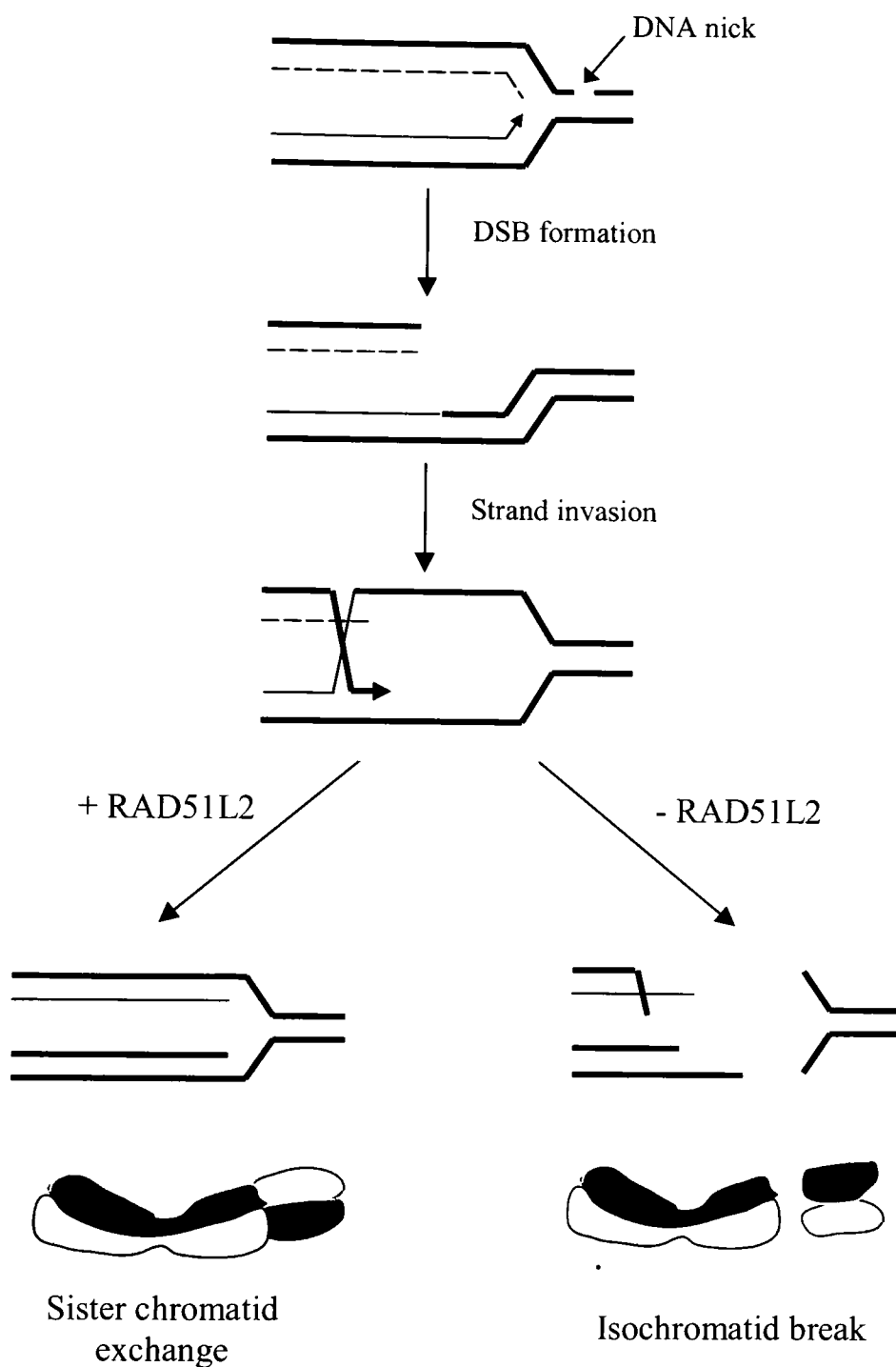


Figure 7.2 Model describing a role for RAD51L2 in repairing DNA damage during DNA replication. Adapted from French *et al.* (2002). The replication fork encounters a single-stranded nick that is converted to a DSB. Strand exchange results in the formation of a recombination intermediate. If RAD51L2 is present, the structure is resolved with a proportion of the products containing crossovers that lead to SCEs. If RAD51L2 is absent, the structure is more likely to disintegrate resulting in an isochromatid break.

There is evidence to suggest that the RAD51-like proteins may play a more direct role in HRR than previously thought. The RAD51L2 protein exhibits strand exchange activity, but this exchange is actually the result of a DNA melting and strand separation activity. As well as facilitating the formation of the RAD51 filament, RAD51L2 may subsequently catalyse the separation of duplex DNA before strand exchange (Lio *et al.*, 2003). In some circumstances, the RAD51-like proteins may be able to compensate for the loss of other HRR proteins. In the brain RAD51 is expressed at very low levels, it has been suggested that the RAD51L2-XRCC3 complex may be involved in the catalysis of homologous pairing in this organ (Kurumizaka *et al.*, 2001). However, the catalysis of homologous pairing is not unique to the RAD51L2-XRCC3 complex and has also been demonstrated for the RAD51L3-XRCC2 complex (Kurumizaka *et al.*, 2002). In addition, chicken DT40 *XRCC3 RAD52* double mutants are inviable whereas the single mutants grow reasonably well. RAD52 can only partially compensate for the loss of XRCC3 whilst XRCC3 almost fully substitutes for lack of RAD52, suggesting that RAD51-like proteins can compensate for the loss of RAD52 (Fujimori *et al.*, 2001). Another interpretation of these results is that in the absence of HRR, a RAD52-mediated SSA pathway is essential.

RAD51-like gene deficient cells are defective in IR- and MMC-induced RAD51 focus formation but form S-phase foci normally (Bishop *et al.*, 1998; Godthelp *et al.*, 2002b; Liu, 2002; O'Regan *et al.*, 2001; Takata *et al.*, 2001) (Fig. 5.3). Similarly, cells expressing truncated BRCA2 (CAPAN-1) have impaired IR-induced but normal S-phase RAD51 focus formation. During S-phase, RAD51 binds specifically to chromatin in an BRCA2-independent manner (Tarsounas *et al.*, 2003). It appears that the RAD51-like proteins and BRCA2 are needed for IR- and MMC-induced RAD51 focus formation but may not play a major role in repairing damage during DNA replication. The binding of RAD51 to chromatin during S-phase may help to target it to sites of fork arrest. The repair proteins used may depend on the type of DNA substrate encountered; for example, stalled replication fork, resected DSB or ICL.

The RAD51L1-RAD51L2-RAD51L3-XRCC2 heterotetramer and the RAD51L2-XRCC3, RAD51L1/-RAD51L2 and RAD51L3-XRCC2 heterodimers all bind ssDNA in preference to dsDNA (Kurumizaka *et al.*, 2001; Kurumizaka *et al.*, 2002; Lio *et al.*, 2003; Masson *et al.*, 2001a; Masson *et al.*, 2001b; Sigurdsson *et al.*, 2001). Therefore it seems likely that these complexes bind to the resected ends of DSBs. Indeed, the RAD51L1- RAD51L2 heterodimer binds to 3'-tails in preference to ssDNA or dsDNA (Lio *et al.*, 2003). Once bound to the ssDNA tails the RAD51-like proteins could stimulate RAD51 filament formation together with RAD52 which binds to ssDNA ends (Parsons *et al.*, 2000). The RAD51-like proteins could carry out this mediator function by competing with RPA for binding to the ssDNA tails, as has been described for the RAD51L1-RAD51L2 heterodimer (Sigurdsson *et al.*, 2001), or by other methods such as stabilisation of the nucleoprotein filament. They would then be in position to carry out further roles later in the HRR process.

One of the main features of cell lines defective in *RAD51*-like genes is their sensitivity to DNA cross-linking agents such as MMC (Caldecott and Jeggo, 1991; Godthelp *et al.*, 2002b; Jones *et al.*, 1987; Jones *et al.*, 1993; Takata *et al.*, 2001). This would suggest that the RAD51-like proteins function in the repair of ICLs. The RAD51L1-RAD51L2-RAD51L3-XRCC2 complex binds to nicks in duplex DNA, a property that has not been identified in any other recombination proteins or complexes. It has been suggested that this activity might allow the complex to bind incised cross-links, generated by NER enzymes, and promote HR at the damaged site (Masson *et al.*, 2001b).

As described above, RAD51L2 is the only RAD51-like protein to be a component of both RAD51-like protein complexes (Fig. 7.1). In *irs3* cells, the lack of RAD51L2 leads to disruption of all complexes except the RAD51L3-XRCC2 heterodimer (Fig. 4.7). Therefore it seems reasonable to assume that RAD51L2-deficient cells would have a more severe phenotype than cells lacking other RAD51-like proteins that would be expected to have at least one complex intact. However, paradoxically *irs3* and CL-V4B appear to have a less severe phenotype than other *RAD51*-like gene deficient cell lines (section 6.3). As stated in section 6.3, one possible explanation for this is that a secondary supressor mutation is necessary for

the survival of *RAD51L2*-deficient cell lines, resulting in an apparently less severe phenotype.

7.5 Identification and characterisation of damage-response genes; the contribution of mutant cell lines

Mutant cell lines have been very important in the identification of novel genes and pathways, as well as in identifying gene function. Components of the all DNA repair pathways have been identified using mutant cell lines. As described previously, much has been learned about the function of the HRR genes *XRCC2* and *XRCC3* using the mutant hamster cell lines *irs1* and *irs1SF* respectively.

Traditionally, genes have been cloned from mutant rodent cell lines by functional complementation or positional cloning. However, in the case of *irs3* these techniques were not successful so a candidate gene approach was employed. The identification of *irs3* as a *RAD51L2*-deficient cell line showed that for this cell line it was a good approach to use. If successful, a candidate gene approach is much less labour intensive than positional cloning methods, but much depends on the amount of information available from which to choose candidate genes (Collins, 1995). More data now exist on the phenotypes of mutant cell lines and a better understanding of DNA repair pathways has enabled more accurate prediction of the phenotypes that are likely to result from defects in DNA repair genes. For these reasons the candidate gene approach is beginning to be used more extensively to clone genes from mutant rodent cell lines. Recently, the hamster cell lines V-C8 and CL-V4B have been shown to be deficient in *BRCA2* and *RAD51L2* respectively using candidate gene approaches (Godthelp *et al.*, 2002b; Kraakman-van der Zwet *et al.*, 2002).

Although mutant cell lines have undoubtedly played an important role in the elucidation of DNA repair pathways there are some inherent problems associated with their use. As described in section 3.3, mutant cell lines can be prone to reversion ie. their phenotype can return to that of wild type cells. Reversion has been described in the *xrs* mutants (Jeggo and Holliday, 1986) and in the *RAD51L2*-deficient cell line CL-V4B (Godthelp *et al.*, 2002b). It should be noted that the *irs3* cell line was tested

for reversion when it was isolated and none was found (Jones *et al.*, 1987). Similarly, no evidence of *irs3* reversion was found during this study.

The isolation of damage-sensitive mutant cell lines involves mutagenising a wild type cell line and selecting for the phenotype of interest. This mutagenesis may cause additional genetic changes that are not related to the damage-sensitive phenotype. A cell line in which this appears to have occurred is the CHO mutant UV40. The *XRCC9/FANCG* gene partially complements UV40 hypersensitivity to DNA-damaging agents and substantially complements an elevated level of chromosome aberrations. However, *XRCC9/FANCG* does not complement a 3-fold increase in SCEs observed in this cell line. This suggests that the SCE increase may be due to the mutation of another gene that is unrelated to *XRCC9/FANCG* (Liu *et al.*, 1997). At present there is no evidence for additional mutations in *irs3*, but this possibility cannot be ruled out (see section 6.3).

Although phenotypic screens involving mutant cell lines have not been exhaustive it is likely that some genes involved in the damage-response will never be identified by this method. Mutations in certain genes may be incompatible with cell growth, or be so deleterious to growth that the mutated cells are lost in culture.

Gene targeting of mouse, human and chicken DT40 cells has been used to identify the function of many genes. Gene knockout experiments provide isogenic cell lines for studies that are potentially free from the secondary mutation problems associated with mutagenised cell lines. There have also been great advances in the field of dsRNA interference (RNAi), although it appears that these type of experiments will yield various degrees of 'knockdown' phenotype rather than a 'knockout' phenotype (Hudson *et al.*, 2002).

However, all the approaches outlined above can only be used with known genes. Knockout experiments can also lead to very severe phenotypes, including lethality at the cellular and animal level, making study difficult. For example, both *Rad51*^{-/-} mice and MEFs are inviable (Lim and Hasty, 1996). Mutant cell lines on the other hand can be used to clone previously unidentified genes. In addition, the induced mutations do not always lead to a null phenotype and may have differing and subtler effects on gene function. The recently identified camptothecin-sensitive cell

lines CM1, CM3 and CM6 have been assigned to the *irs2* complementation group (Johnson *et al.*, 2000). Camptothecin sensitivity appears to be a common feature of the *irs2* complementation group (Johnson *et al.*, 2000; Jones *et al.*, 1993; Thacker and Ganesh, 1990). However, these mutants do not display radiation sensitivity (Johnson *et al.*, 2000), a feature that was used to isolate the other members of the *irs2* group, *irs2*, V-C4, V-E5 and V-G8 (Jones *et al.*, 1987; Zdzienicka and Simons, 1987). In this example, different mutations in the same gene give rise to phenotypic heterogeneity within the *irs2* complementation group.

7.6 The future

This study identifies *irs3* as a RAD51L2-deficient cell line and demonstrates that the RAD51L2 protein plays a key role in RAD51-dependent HRR. The generation of deletions and site-directed mutations has also yielded data on functional domains and sequence motifs in the RAD51L2 protein. However, the deletion and mutation studies described here only begin to address questions about RAD51L2 function.

Further site-directed mutagenesis studies could target other amino acids identified by computer prediction programmes, for example putative phosphorylation sites. The *S.cerevisiae* Rad55p has been shown to be specifically phosphorylated in response to DNA damage and replication blocks. This phosphorylation is dependent on an active DNA damage checkpoint. It has been suggested that Rad55p is the last substrate in the checkpoint response and may provide a link to HRR (Bashkirov *et al.*, 2000). The NetPhos WWW server (www.cbs.dtu.dk/services/NetPhos/) produces predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins (Blom *et al.*, 1999). The programme gives residues a score of between 0.000 and 1.000, and those that score over the threshold value of 0.500 are potential phosphorylation sites. Analysis of the RAD51L2 protein sequence revealed 19 residues that could be phosphorylated; 10 serine, 7 threonine and 2 tyrosine. Of these the highest scoring were all serine residues - S47 (0.974), S329 (0.978), S331 (0.993), S364 (0.982) and S369 (0.995).

Another strategy for identifying functional domains is to align RAD51L2 sequences from several species and look for regions of conserved sequence. It is likely that highly homologous regions will have been conserved because they have an important function within the protein. Fig. 4.2 shows an alignment of 5 mammalian and plant RAD51L2 proteins. There are regions of sequence, other than the Walker box regions, that are highly conserved eg. residues 107-115 and 155-168 (using the human protein numbering). In order to get the best results with this strategy it is important to obtain sequences from as many species as possible. Using PIX (menu.hgmp.mrc.ac.uk/menu-bin/Pix/Pix.pl) several partial putative RAD51L2 sequences have been identified including *Sus scrofa* (pig), *Canis familiaris* (dog), *Xenopus laevis* (clawed frog), *Triticum aestivum* (bread wheat), *Hordeum vulgare* (barley), *Glycine max* (soybean), *Trichinella spiralis* (nematode worm) and *Chlamydomonas reinhardtii* (unicellular green alga). Random mutagenesis (eg. alanine scanning) can also be used to find previously unidentified functional domains.

Disruption of *Rad51l1*, *Rad51l3* and *Xrcc2* in mice results in embryonic lethality (Deans *et al.*, 2000; Pittman and Schimenti, 2000; Shu *et al.*, 1999). Consequently, it seems likely that a *Rad51l2* null mouse would also be inviable. However, even though *Xrcc2* null mice die during embryogenesis, or at birth, much has still been learned about the function of *Xrcc2* from the mouse model. For example, *Xrcc2* was shown to have a role in the developing nervous system (Deans *et al.*, 2000). It could therefore still be beneficial to generate a *Rad51l2* disrupted mouse. Alternatively, a conditional *Rad51l2* mouse could be made which should result in viable offspring.

Biochemical data will undoubtedly play an important role in determining the function of the RAD51-like proteins in HRR. However, biochemical techniques are not as well suited to looking at other aspects of repair. Mutant cell lines, including *irs3* and CL-V4B, could be used to address questions about the usage of DNA repair pathways. Relatively little is known about channelling of damage to the various repair pathways and how this process is controlled.

Ultimately, work on the *RAD51*-like genes will focus on links with human disease. As yet, none of the *RAD51*-like genes have been linked to human cancer

predisposition disorders but mutations, variant alleles, or altered expression in association with specific tumour types have been reported (Pierce *et al.*, 2001). Recent data have shown the central HRR protein, RAD51, to interact with the breast cancer susceptibility gene *BRCA2*. *BRCA2* is thought to be involved directly in HRR because its interaction with RAD51 prevents filament formation on DNA (Pellegrini *et al.*, 2002) (section 1.3.2). There are also connections between HRR factors and proteins associated with disorders such as ataxia-telangiectasia, Nijmegen breakage syndrome, Fanconi anaemia and Blooms syndrome (Introduction, section 1.3.1 to 1.3.6).

Bibliography

- Abraham, R.T.** (2001) Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev*, **15**, 2177-96.
- Aghamohammadi, S.Z., Morris, T., Stevens, D.L. and Thacker, J.** (1992) Rapid screening for deletion mutations in the hprt gene using the polymerase chain reaction: X-ray and alpha-particle mutant spectra. *Mutat Res*, **269**, 1-7.
- Albala, J.S., Thelen, M.P., Prange, C., Fan, W., Christensen, M., Thompson, L.H. and Lennon, G.G.** (1997) Identification of a novel human RAD51 homolog, RAD51B. *Genomics*, **46**, 476-9.
- Alexeev, A., Mazin, A. and Kowalczykowski, S.C.** (2003) Rad54 protein possesses chromatin-remodeling activity stimulated by the Rad51-ssDNA nucleoprotein filament. *Nat Struct Biol*, **10**, 182-6.
- Amant, F., Debiec-Rychter, M., Schoenmakers, E.F., Hagemijer-Hausman, A. and Vergote, I.** (2001) Cumulative dosage effect of a RAD51L1/HMGA2 fusion and RAD51L1 loss in a case of pseudo-Meigs' syndrome. *Genes Chromosomes Cancer*, **32**, 324-9.
- Araujo, F.D., Pierce, A.J., Stark, J.M. and Jasin, M.** (2002) Variant XRCC3 implicated in cancer is functional in homology-directed repair of double-strand breaks. *Oncogene*, **21**, 4176-80.
- Arwert, F., Rooimans, M.A., Westerveld, A., Simons, J.W. and Zdzienicka, M.Z.** (1991) The Chinese hamster cell mutant V-H4 is homologous to Fanconi anemia (complementation group A). *Cytogenet Cell Genet*, **56**, 23-6.
- Bai, Y. and Symington, L.S.** (1996) A Rad52 homolog is required for RAD51-independent mitotic recombination in *Saccharomyces cerevisiae*. *Genes Dev*, **10**, 2025-37.
- Bakkenist, C.J. and Kastan, M.B.** (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*, **421**, 499-506.
- Barlow, C., Eckhaus, M.A., Schaffer, A.A. and Wynshaw-Boris, A.** (1999) Atm haploinsufficiency results in increased sensitivity to sublethal doses of ionizing radiation in mice. *Nat Genet*, **21**, 359-60.
- Barlund, M., Monni, O., Kononen, J., Cornelison, R., Torhorst, J., Sauter, G., Kallioniemi, O.-P. and Kallioniemi, A.** (2000) Multiple genes at 17q23 undergo amplification and overexpression in breast cancer. *Cancer Res*, **60**, 5340-4.
- Barnes, D.E., Stamp, G., Rosewell, I., Denzel, A. and Lindahl, T.** (1998) Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. *Curr Biol*, **8**, 1395-8.
- Bashkirov, V.I., King, J.S., Bashkirova, E.V., Schmuckli-Maurer, J. and Heyer, W.D.** (2000) DNA repair protein Rad55 is a terminal substrate of the DNA damage checkpoints. *Mol Cell Biol*, **20**, 4393-404.
- Baumann, P., Benson, F.E. and West, S.C.** (1996) Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell*, **87**, 757-66.
- Baumann, P. and West, S.C.** (1997) The human Rad51 protein: polarity of strand transfer and stimulation by hRP-A. *Embo J*, **16**, 5198-206.
- Baumann, P. and West, S.C.** (1998) Role of the human RAD51 protein in homologous recombination and double-stranded-break repair. *Trends Biochem Sci*, **23**, 247-51.
- Baumann, P. and West, S.C.** (1999) Heteroduplex formation by human Rad51 protein: effects of DNA end-structure, hRP-A and hRad52. *J Mol Biol*, **291**, 363-74.

- Benson, F.E., Baumann, P. and West, S.C.** (1998) Synergistic actions of Rad51 and Rad52 in recombination and DNA repair [see comments]. *Nature*, **391**, 401-4.
- Berget, S.M.** (1995) Exon recognition in vertebrate splicing. *J Biol Chem*, **270**, 2411-4.
- Bezzubova, O., Silbergleit, A., Yamaguchi-Iwai, Y., Takeda, S. and Buerstedde, J.M.** (1997) Reduced X-ray resistance and homologous recombination frequencies in a RAD54^{-/-} mutant of the chicken DT40 cell line. *Cell*, **89**, 185-93.
- Bhattacharyya, A., Ear, U.S., Koller, B.H., Weichselbaum, R.R. and Bishop, D.K.** (2000) The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. *J Biol Chem*, **275**, 23899-903.
- Bishop, D.K., Ear, U., Bhattacharyya, A., Calderone, C., Beckett, M., Weichselbaum, R.R. and Shinozawa, A.** (1998) Xrcc3 is required for assembly of Rad51 complexes in vivo. *J Biol Chem*, **273**, 21482-8.
- Blank, C., Schoenmakers, E.F., Rogalla, P., Huys, E.H., van Rijk, A.A., Drieschner, N. and Bullerdiek, J.** (2001) Intragenic breakpoint within RAD51L1 in a t(6;14)(p21.3;q24) of a pulmonary chondroid hamartoma. *Cytogenet Cell Genet*, **95**, 17-9.
- Blom, N., Gammeltoft, S. and Brunak, S.** (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol*, **294**, 1351-62.
- Braybrooke, J.P., Spink, K.G., Thacker, J. and Hickson, I.D.** (2000) The RAD51 family member, RAD51L3, is a DNA-stimulated ATPase that forms a complex with XRCC2. *J Biol Chem*, **275**, 29100-6.
- Brenneman, M.A., Wagener, B.M., Miller, C.A., Allen, C. and Nickoloff, J.A.** (2002) XRCC3 controls the fidelity of homologous recombination: roles for XRCC3 in late stages of recombination. *Mol Cell*, **10**, 387-95.
- Brenneman, M.A., Weiss, A.E., Nickoloff, J.A. and Chen, D.J.** (2000) XRCC3 is required for efficient repair of chromosome breaks by homologous recombination. *Mutat Res*, **459**, 89-97.
- Buchwald, M. and Moustacchi, E.** (1998) Is Fanconi anemia caused by a defect in the processing of DNA damage? *Mutat Res*, **408**, 75-90.
- Butkiewicz, D., Rusin, M., Enewold, L., Shields, P.G., Chorazy, M. and Harris, C.C.** (2001) Genetic polymorphisms in DNA repair genes and risk of lung cancer. *Carcinogenesis*, **22**, 593-7.
- Butler, J.S.** (2002) The yin and yang of the exosome. *Trends Cell Biol*, **12**, 90-6.
- Caldecott, K. and Jeggo, P.** (1991) Cross-sensitivity of gamma-ray-sensitive hamster mutants to cross-linking agents. *Mutat Res*, **255**, 111-21.
- Cantor, S.B., Bell, D.W., Ganesan, S., Kass, E.M., Drapkin, R., Grossman, S., Wahrer, D.C., Sgroi, D.C., Lane, W.S., Haber, D.A. and Livingston, D.M.** (2001) BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell*, **105**, 149-60.
- Cartwright, R., Dunn, A.M., Simpson, P.J., Tambini, C.E. and Thacker, J.** (1998a) Isolation of novel human and mouse genes of the recA/RAD51 recombination-repair gene family. *Nucleic Acids Res*, **26**, 1653-9.
- Cartwright, R., Tambini, C.E., Simpson, P.J. and Thacker, J.** (1998b) The XRCC2 DNA repair gene from human and mouse encodes a novel member of the recA/RAD51 family. *Nucleic Acids Res*, **26**, 3084-9.
- Chanet, R., Heude, M., Adjiri, A., Maloisel, L. and Fabre, F.** (1996) Semidominant mutations in the yeast Rad51 protein and their relationships with the Srs2 helicase. *Mol Cell Biol*, **16**, 4782-9.

- Chen, X.B., Melchionna, R., Denis, C.M., Gaillard, P.H., Blasina, A., Van de Weyer, I., Boddy, M.N., Russell, P., Vialard, J. and McGowan, C.H.** (2001) Human Mus81-associated endonuclease cleaves Holliday junctions in vitro. *Mol Cell*, **8**, 1117-27.
- Cheong, N., Wang, Y., Jackson, M. and Iliakis, G.** (1992) Radiation-sensitive *irs* mutants rejoin DNA double-strand breaks with efficiency similar to that of parental V79 cells but show altered response to radiation-induced G2 delay. *Mutat Res*, **274**, 111-22.
- Chester, N., Kuo, F., Kozak, C., O'Hara, C.D. and Leder, P.** (1998) Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom's syndrome gene. *Genes Dev*, **12**, 3382-93.
- Cokol, M., Nair, R. and Rost, B.** (2000) Finding nuclear localization signals. *EMBO Rep*, **1**, 411-5.
- Collins, F.S.** (1995) Positional cloning moves from perdditional to traditional. *Nat Genet*, **9**, 347-50.
- Constantinou, A., Chen, X.B., McGowan, C.H. and West, S.C.** (2002) Holliday junction resolution in human cells: two junction endonucleases with distinct substrate specificities. *Embo J*, **21**, 5577-85.
- Constantinou, A., Davies, A.A. and West, S.C.** (2001) Branch migration and Holliday junction resolution catalyzed by activities from mammalian cells. *Cell*, **104**, 259-68.
- Cui, X., Brenneman, M., Meyne, J., Oshimura, M., Goodwin, E.H. and Chen, D.J.** (1999) The XRCC2 and XRCC3 repair genes are required for chromosome stability in mammalian cells. *Mutat Res*, **434**, 75-88.
- D'Amours, D. and Jackson, S.P.** (2002) The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat Rev Mol Cell Biol*, **3**, 317-27.
- D'Andrea, A.D. and Grompe, M.** (2003) The Fanconi anaemia/BRCA pathway. *Nat Rev Cancer*, **3**, 23-34.
- David-Beabes, G.L., Lunn, R.M. and London, S.J.** (2001) No association between the XPD (Lys751Gln) polymorphism or the XRCC3 (Thr241Met) polymorphism and lung cancer risk. *Cancer Epidemiol Biomarkers Prev*, **10**, 911-2.
- Davies, A.A., Masson, J.Y., McIlwraith, M.J., Stasiak, A.Z., Stasiak, A., Venkitaraman, A.R. and West, S.C.** (2001) Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol Cell*, **7**, 273-82.
- Davis, A.P. and Symington, L.S.** (2001) The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing. *Genetics*, **159**, 515-25.
- de Jager, M. and Kanaar, R.** (2002) Genome instability and Rad50(S): subtle yet severe. *Genes Dev*, **16**, 2173-8.
- de Laat, W.L., Jaspers, N.G. and Hoeijmakers, J.H.** (1999) Molecular mechanism of nucleotide excision repair. *Genes Dev*, **13**, 768-85.
- Deans, B., Griffin, C.S., Maconochie, M. and Thacker, J.** (2000) *Xrcc2* is required for genetic stability, embryonic neurogenesis and viability in mice. *Embo J*, **19**, 6675-85.
- Deng, C.X. and Brodie, S.G.** (2000) Roles of BRCA1 and its interacting proteins. *Bioessays*, **22**, 728-37.
- Dianov, G.L., O'Neill, P. and Goodhead, D.T.** (2001) Securing genome stability by orchestrating DNA repair: removal of radiation-induced clustered lesions in DNA. *Bioessays*, **23**, 745-9.

Digweed, M., Rothe, S., Demuth, I., Scholz, R., Schindler, D., Stumm, M., Grompe, M., Jordan, A. and Sperling, K. (2002) Attenuation of the formation of DNA-repair foci containing RAD51 in Fanconi anaemia. *Carcinogenesis*, **23**, 1121-6.

Dillehay, L.E., Jacobson-Kram, D. and Williams, J.R. (1989) DNA topoisomerases and models of sister-chromatid exchange. *Mutat Res*, **215**, 15-23.

Dosanjh, M.K., Collins, D.W., Fan, W., Lennon, G.G., Albala, J.S., Shen, Z. and Schild, D. (1998) Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes. *Nucleic Acids Res*, **26**, 1179-84.

Dresser, M.E., Ewing, D.J., Conrad, M.N., Dominguez, A.M., Barstead, R., Jiang, H. and Kodadek, T. (1997) DMC1 functions in a *Saccharomyces cerevisiae* meiotic pathway that is largely independent of the RAD51 pathway. *Genetics*, **147**, 533-44.

Dronkert, M.L., Beverloo, H.B., Johnson, R.D., Hoeijmakers, J.H., Jasin, M. and Kanaar, R. (2000) Mouse RAD54 affects DNA double-strand break repair and sister chromatid exchange. *Mol Cell Biol*, **20**, 3147-56.

Dronkert, M.L. and Kanaar, R. (2001) Repair of DNA interstrand cross-links. *Mutat Res*, **486**, 217-47.

Duan, Z., Shen, H., Lee, J.E., Gershenwald, J.E., Ross, M.I., Mansfield, P.F., Duvic, M., Strom, S.S., Spitz, M.R. and Wei, Q. (2002) DNA repair gene XRCC3 241Met variant is not associated with risk of cutaneous malignant melanoma. *Cancer Epidemiol Biomarkers Prev*, **11**, 1142-3.

Durocher, D. and Jackson, S.P. (2001) DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme? *Curr Opin Cell Biol*, **13**, 225-31.

Essers, J., Hendriks, R.W., Swagemakers, S.M., Troelstra, C., de Wit, J., Bootsma, D., Hoeijmakers, J.H. and Kanaar, R. (1997) Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. *Cell*, **89**, 195-204.

Essers, J., van Steeg, H., de Wit, J., Swagemakers, S.M., Vermeij, M., Hoeijmakers, J.H. and Kanaar, R. (2000) Homologous and non-homologous recombination differentially affect DNA damage repair in mice. *Embo J*, **19**, 1703-10.

Fortin, G.S. and Symington, L.S. (2002) Mutations in yeast Rad51 that partially bypass the requirement for Rad55 and Rad57 in DNA repair by increasing the stability of Rad51-DNA complexes. *Embo J*, **21**, 3160-70.

Foster, M.I. and Lehmann, A.R. (2000) A novel SMC protein complex in *Schizosaccharomyces pombe* contains the Rad18 DNA repair protein. *Embo J*, **19**, 1691-702.

Frank, K.M., Sharpless, N.E., Gao, Y., Sekiguchi, J.M., Ferguson, D.O., Zhu, C., Manis, J.P., Horner, J., DePinho, R.A. and Alt, F.W. (2000) DNA ligase IV deficiency in mice leads to defective neurogenesis and embryonic lethality via the p53 pathway. *Mol Cell*, **5**, 993-1002.

French, C.A., Masson, J.Y., Griffin, C.S., O'Regan, P., West, S.C. and Thacker, J. (2002) Role of mammalian RAD51L2 (RAD51C) in recombination and genetic stability. *J Biol Chem*, **277**, 19322-30.

Fujimori, A., Tachiiri, S., Sonoda, E., Thompson, L.H., Dhar, P.K., Hiraoka, M., Takeda, S., Zhang, Y., Reth, M. and Takata, M. (2001) Rad52 partially substitutes for the Rad51 paralog XRCC3 in maintaining chromosomal integrity in vertebrate cells. *Embo J*, **20**, 5513-20.

Fuller, L.F. and Painter, R.B. (1988) A Chinese hamster ovary cell line hypersensitive to ionizing radiation and deficient in repair replication. *Mutat Res*, **193**, 109-21.

- Game, J.C.** (1993) DNA double-strand breaks and the RAD50-RAD57 genes in *Saccharomyces*. *Semin Cancer Biol*, **4**, 73-83.
- Game, J.C. and Mortimer, R.K.** (1974) A genetic study of x-ray sensitive mutants in yeast. *Mutat Res*, **24**, 281-92.
- Gao, Y., Ferguson, D.O., Xie, W., Manis, J.P., Sekiguchi, J., Frank, K.M., Chaudhuri, J., Horner, J., DePinho, R.A. and Alt, F.W.** (2000) Interplay of p53 and DNA-repair protein XRCC4 in tumorigenesis, genomic stability and development. *Nature*, **404**, 897-900.
- Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M. and D'Andrea, A.D.** (2001) Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*, **7**, 249-62.
- Giaccia, A., Weinstein, R., Hu, J. and Stamato, T.D.** (1985) Cell cycle-dependent repair of double-strand DNA breaks in a gamma-ray-sensitive Chinese hamster cell. *Somat Cell Mol Genet*, **11**, 485-91.
- Godthelp, B.C., Artwert, F., Joenje, H. and Zdzienicka, M.Z.** (2002a) Impaired DNA damage-induced nuclear Rad51 foci formation uniquely characterizes Fanconi anemia group D1. *Oncogene*, **21**, 5002-5.
- Godthelp, B.C., Wiegant, W.W., van Duijn-Goedhart, A., Scharer, O.D., van Buul, P.P., Kanaar, R. and Zdzienicka, M.Z.** (2002b) Mammalian Rad51C contributes to DNA cross-link resistance, sister chromatid cohesion and genomic stability. *Nucleic Acids Res*, **30**, 2172-82.
- Goedecke, W., Eijpe, M., Offenberg, H.H., van Aalderen, M. and Heyting, C.** (1999) Mre11 and Ku70 interact in somatic cells, but are differentially expressed in early meiosis. *Nat Genet*, **23**, 194-8.
- Golub, E.I., Kovalenko, O.V., Gupta, R.C., Ward, D.C. and Radding, C.M.** (1997) Interaction of human recombination proteins Rad51 and Rad54. *Nucleic Acids Res*, **25**, 4106-10.
- Goodhead, D.T.** (1994) Initial events in the cellular effects of ionizing radiations: clustered damage in DNA. *Int J Radiat Biol*, **65**, 7-17.
- Griffin, C.S., Simpson, P.J., Wilson, C.R. and Thacker, J.** (2000) Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation. *Nat Cell Biol*, **2**, 757-761.
- Grompe, M. and D'Andrea, A.** (2001) Fanconi anemia and DNA repair. *Hum Mol Genet*, **10**, 2253-9.
- Haaf, T., Golub, E.I., Reddy, G., Radding, C.M. and Ward, D.C.** (1995) Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. *Proc Natl Acad Sci U S A*, **92**, 2298-302.
- Haaf, T., Raderschall, E., Reddy, G., Ward, D.C., Radding, C.M. and Golub, E.I.** (1999) Sequestration of mammalian Rad51-recombination protein into micronuclei. *J Cell Biol*, **144**, 11-20.
- Haber, J.E.** (1999) DNA recombination: the replication connection. *Trends Biochem Sci*, **24**, 271-5.
- Haber, J.E.** (2000a) Partners and pathways repairing a double-strand break. *Trends Genet*, **16**, 259-64.
- Haber, J.E.** (2000b) Recombination: a frank view of exchanges and vice versa. *Curr Opin Cell Biol*, **12**, 286-292.
- Havre, P.A., Rice, M., Ramos, R. and Kmiec, E.B.** (2000) HsRec2/Rad51L1, a protein influencing cell cycle progression, has protein kinase activity. *Exp Cell Res*, **254**, 33-44.

- Havre, P.A., Rice, M.C., Noe, M. and Kmiec, E.B.** (1998) The human REC2/RAD51B gene acts as a DNA damage sensor by inducing G1 delay and hypersensitivity to ultraviolet irradiation. *Cancer Res*, **58**, 4733-9.
- Hays, S.L., Firmenich, A.A. and Berg, P.** (1995) Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. *Proc Natl Acad Sci U S A*, **92**, 6925-9.
- Hermine, T., Jones, N.J. and Parry, J.M.** (1997) Comparative induction of micronuclei in repair-deficient and -proficient Chinese hamster cell lines following clastogen or aneugen exposures. *Mutat Res*, **392**, 151-63.
- Hiramoto, T., Nakanishi, T., Sumiyoshi, T., Fukuda, T., Matsuura, S., Tauchi, H., Komatsu, K., Shibasaki, Y., Inui, H., Watatani, M., Yasutomi, M., Sumii, K., Kajiya, G., Kamada, N., Miyagawa, K. and Kamiya, K.** (1999) Mutations of a novel human RAD54 homologue, RAD54B, in primary cancer. *Oncogene*, **18**, 3422-6.
- Howlett, N.G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G., Ikeda, H., Fox, E.A. and D'Andrea, A.D.** (2002) Biallelic inactivation of BRCA2 in Fanconi anemia. *Science*, **297**, 606-9.
- Hudson, D.F., Morrison, C., Ruchaud, S. and Earnshaw, W.C.** (2002) Reverse genetics of essential genes in tissue-culture cells: 'dead cells talking'. *Trends Cell Biol*, **12**, 281-7.
- Ioannou, P.A. and de Jong, P.J.** (1996) In Dracopoli et al (eds) *Current Protocols in Human Genetics*. John Wiley and Sons, New York, Unit 5.15.
- Jackson, S.P.** (2002) Sensing and repairing DNA double-strand breaks. *Carcinogenesis*, **23**, 687-96.
- Jasin, M.** (2002) Homologous repair of DNA damage and tumorigenesis: the BRCA connection. *Oncogene*, **21**, 8981-93.
- Jeggo, P. and O'Neill, P.** (2002) The Greek Goddess, Artemis, reveals the secrets of her cleavage. *DNA Repair (Amst)*, **1**, 771-7.
- Jeggo, P.A. and Holliday, R.** (1986) Azacytidine-induced reactivation of a DNA repair gene in Chinese hamster ovary cells. *Mol Cell Biol*, **6**, 2944-9.
- Jeggo, P.A., Tesmer, J. and Chen, D.J.** (1991) Genetic analysis of ionising radiation sensitive mutants of cultured mammalian cell lines. *Mutat Res*, **254**, 125-33.
- Johnson, M.A., Bryant, P.E. and Jones, N.J.** (2000) Isolation of camptothecin-sensitive chinese hamster cell mutants: phenotypic heterogeneity within the ataxia telangiectasia-like XRCC8 (irs2) complementation group. *Mutagenesis*, **15**, 367-74.
- Johnson, R.D. and Jasin, M.** (2000) Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. *Embo J*, **19**, 3398-407.
- Johnson, R.D. and Jasin, M.** (2001) Double-strand-break-induced homologous recombination in mammalian cells. *Biochem Soc Trans*, **29**, 196-201.
- Johnson, R.D., Liu, N. and Jasin, M.** (1999) Mammalian XRCC2 promotes the repair of DNA double-strand breaks by homologous recombination. *Nature*, **401**, 397-9.
- Johnson, R.D. and Symington, L.S.** (1995) Functional differences and interactions among the putative RecA homologs Rad51, Rad55, and Rad57. *Mol Cell Biol*, **15**, 4843-50.
- Jones, N.J.** (1994) Genetic analysis of mitomycin C-hypersensitive Chinese hamster cell mutants. *Mutagenesis*, **9**, 477-82.
- Jones, N.J., Cox, R. and Thacker, J.** (1988) Six complementation groups for ionising-radiation sensitivity in Chinese hamster cells. *Mutat Res*, **193**, 139-44.

- Jones, N.J., Cox, R. and Thacker, T.** (1987) Isolation and cross-sensitivity of X-ray-sensitive mutants of V79-4 hamster cells. *Mutat Res*, **183**, 279-286.
- Jones, N.J., Ellard, S., Waters, R. and Parry, E.M.** (1993) Cellular and chromosomal hypersensitivity to DNA crosslinking agents and topoisomerase inhibitors in the radiosensitive Chinese hamster irs mutants: phenotypic similarities to ataxia telangiectasia and Fanconi's anaemia cells. *Carcinogenesis*, **14**, 2487-94.
- Justice, M.J., Noveroske, J.K., Weber, J.S., Zheng, B. and Bradley, A.** (1999) Mouse ENU mutagenesis. *Hum Mol Genet*, **8**, 1955-63.
- Kagawa, W., Kurumizaka, H., Ikawa, S., Yokoyama, S. and Shibata, T.** (2001) Homologous pairing promoted by the human Rad52 protein. *J Biol Chem*, **276**, 35201-8.
- Kagawa, W., Kurumizaka, H., Ishitani, R., Fukai, S., Nureki, O., Shibata, T. and Yokoyama, S.** (2002) Crystal structure of the homologous-pairing domain from the human Rad52 recombinase in the undecameric form. *Mol Cell*, **10**, 359-71.
- Karow, J.K., Constantinou, A., Li, J.L., West, S.C. and Hickson, I.D.** (2000) The Bloom's syndrome gene product promotes branch migration of holliday junctions. *Proc Natl Acad Sci U S A*, **97**, 6504-8.
- Kastan, M.B. and Lim, D.S.** (2000) The many substrates and functions of ATM. *Nat Rev Mol Cell Biol*, **1**, 179-86.
- Kawabata, M. and Saeki, K.** (1999) Multiple alternative transcripts of the human homologue of the mouse TRAD/R51H3/RAD51D gene, a member of the rec A/RAD51 gene family. *Biochem Biophys Res Commun*, **257**, 156-62.
- Khanna, K.K. and Jackson, S.P.** (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet*, **27**, 247-54.
- Kim, W.J., Lee, H., Park, E.J., Hong, S.H. and Park, S.D.** (2002) Role of ATP-binding motifs on DNA-binding activity and biological function of Rhp51, a Rad51 homologue in fission yeast. *Biochem J*, **364**, 869-74.
- Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K. and Komatsu, K.** (2002) NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain. *Curr Biol*, **12**, 1846-51.
- Konola, J.T., Logan, K.M. and Knight, K.L.** (1994) Functional characterization of residues in the P-loop motif of the RecA protein ATP binding site. *J Mol Biol*, **237**, 20-34.
- Kozak, M.** (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res*, **15**, 8125-48.
- Kraakman-van der Zwet, M., Overkamp, W.J., van Lange, R.E., Essers, J., van Duijn-Goedhart, A., Wiggers, I., Swaminathan, S., van Buul, P.P., Errami, A., Tan, R.T., Jaspers, N.G., Sharan, S.K., Kanaar, R. and Zdzienicka, M.Z.** (2002) Brca2 (XRCC11) deficiency results in radioresistant DNA synthesis and a higher frequency of spontaneous deletions. *Mol Cell Biol*, **22**, 669-79.
- Krawczak, M., Reiss, J. and Cooper, D.N.** (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet*, **90**, 41-54.
- Kunkel, T.A.** (1999) The high cost of living. American Association for Cancer Research Special Conference: endogenous sources of mutations, Fort Myers, Florida, USA, 11-15 November 1998. *Trends Genet*, **15**, 93-4.
- Kurumizaka, H., Ikawa, S., Nakada, M., Eda, K., Kagawa, W., Takata, M., Takeda, S., Yokoyama, S. and Shibata, T.** (2001) Homologous-pairing activity of

- the human DNA-repair proteins Xrcc3.Rad51C. *Proc Natl Acad Sci U S A*, **98**, 5538-43.
- Kurumizaka, H., Ikawa, S., Nakada, M., Enomoto, R., Kagawa, W., Kinebuchi, T., Yamazoe, M., Yokoyama, S. and Shibata, T.** (2002) Homologous pairing and ring and filament structure formation activities of the human Xrcc2*Rad51D complex. *J Biol Chem*, **277**, 14315-20.
- Kuschel, B., Auranen, A., McBride, S., Novik, K.L., Antoniou, A., Lipscombe, J.M., Day, N.E., Easton, D.F., Ponder, B.A., Pharoah, P.D. and Dunning, A.** (2002) Variants in DNA double-strand break repair genes and breast cancer susceptibility. *Hum Mol Genet*, **11**, 1399-407.
- Kuzminov, A.** (2001) DNA replication meets genetic exchange: chromosomal damage and its repair by homologous recombination. *Proc Natl Acad Sci U S A*, **98**, 8461-8.
- Lambert, S. and Lopez, B.S.** (2001) Role of RAD51 in sister-chromatid exchanges in mammalian cells. *Oncogene*, **20**, 6627-31.
- Langland, G., Kordich, J., Creaney, J., Goss, K.H., Lillard-Wetherell, K., Bebenek, K., Kunkel, T.A. and Groden, J.** (2001) The Bloom's syndrome protein (BLM) interacts with MLH1 but is not required for DNA mismatch repair. *J Biol Chem*, **276**, 30031-5.
- Leasure, C.S., Chandler, J., Gilbert, D.J., Householder, D.B., Stephens, R., Copeland, N.G., Jenkins, N.A. and Sharan, S.K.** (2001) Sequence, chromosomal location and expression analysis of the murine homologue of human RAD51L2/RAD51C. *Gene*, **271**, 59-67.
- Lee, Y., Barnes, D.E., Lindahl, T. and McKinnon, P.J.** (2000) Defective neurogenesis resulting from DNA ligase IV deficiency requires Atm. *Genes Dev*, **14**, 2576-80.
- Lehmann, A.R.** (2002) Replication of damaged DNA in mammalian cells: new solutions to an old problem. *Mutat Res*, **509**, 23-34.
- Lim, D.S. and Hasty, P.** (1996) A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol Cell Biol*, **16**, 7133-43.
- Lindahl, T.** (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709-15.
- Lindahl, T. and Wood, R.D.** (1999) Quality control by DNA repair. *Science*, **286**, 1897-905.
- Lio, Y.C., Mazin, A.V., Kowalczykowski, S.C. and Chen, D.J.** (2003) Complex formation by the human Rad51B and Rad51C DNA repair proteins and their activities in vitro. *J Biol Chem*, **278**, 2469-78.
- Liu, N.** (2002) XRCC2 is Required for the Formation of Rad51 Foci Induced by Ionizing Radiation and DNA Cross-Linking Agent Mitomycin C. *J Biomed Biotechnol*, **2**, 106-113.
- Liu, N., Lamerdin, J.E., Tebbs, R.S., Schild, D., Tucker, J.D., Shen, M.R., Brookman, K.W., Siciliano, M.J., Walter, C.A., Fan, W., Narayana, L.S., Zhou, Z.Q., Adamson, A.W., Sorensen, K.J., Chen, D.J., Jones, N.J. and Thompson, L.H.** (1998) XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. *Mol Cell*, **1**, 783-93.
- Liu, N., Lamerdin, J.E., Tucker, J.D., Zhou, Z.Q., Walter, C.A., Albala, J.S., Busch, D.B. and Thompson, L.H.** (1997) The human XRCC9 gene corrects chromosomal instability and mutagen sensitivities in CHO UV40 cells. *Proc Natl Acad Sci U S A*, **94**, 9232-7.

- Liu, N., Schild, D., Thelen, M.P. and Thompson, L.H.** (2002) Involvement of Rad51C in two distinct protein complexes of Rad51 paralogs in human cells. *Nucleic Acids Res*, **30**, 1009-15.
- Liu, Y. and Maizels, N.** (2000) Coordinated response of mammalian Rad51 and Rad52 to DNA damage. *EMBO Rep*, **1**, 85-90.
- Liu, Y. and West, S.C.** (2002) Distinct functions of BRCA1 and BRCA2 in double-strand break repair. *Breast Cancer Res*, **4**, 9-13.
- Logan, K.M. and Knight, K.L.** (1993) Mutagenesis of the P-loop motif in the ATP binding site of the RecA protein from Escherichia coli. *J Mol Biol*, **232**, 1048-59.
- Luo, G., Santoro, I.M., McDaniel, L.D., Nishijima, I., Mills, M., Youssoufian, H., Vogel, H., Schultz, R.A. and Bradley, A.** (2000) Cancer predisposition caused by elevated mitotic recombination in Bloom mice. *Nat Genet*, **26**, 424-9.
- Lusetti, S.L. and Cox, M.M.** (2002) The bacterial RecA protein and the recombinational DNA repair of stalled replication forks. *Annu Rev Biochem*, **71**, 71-100.
- Ma, Y., Pannicke, U., Schwarz, K. and Lieber, M.R.** (2002) Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell*, **108**, 781-94.
- Malik, S. and Roeder, R.G.** (2000) Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. *Trends Biochem Sci*, **25**, 277-83.
- Marnett, L.J. and Plataras, J.P.** (2001) Endogenous DNA damage and mutation. *Trends Genet*, **17**, 214-21.
- Maser, R.S., Monsen, K.J., Nelms, B.E. and Petrini, J.H.** (1997) hMre11 and hRad50 nuclear foci are induced during the normal cellular response to DNA double-strand breaks. *Mol Cell Biol*, **17**, 6087-96.
- Masson, J.Y., Stasiak, A.Z., Stasiak, A., Benson, F.E. and West, S.C.** (2001a) Complex formation by the human RAD51C and XRCC3 recombination repair proteins. *Proc Natl Acad Sci U S A*, **98**, 8440-6.
- Masson, J.Y., Tarsounas, M.C., Stasiak, A.Z., Stasiak, A., Shah, R., McIlwraith, M.J., Benson, F.E. and West, S.C.** (2001b) Identification and purification of two distinct complexes containing the five RAD51 paralogs. *Genes Dev*, **15**, 3296-307.
- Matsuda, M., Miyagawa, K., Takahashi, M., Fukuda, T., Kataoka, T., Asahara, T., Inui, H., Watatani, M., Yasutomi, M., Kamada, N., Dohi, K. and Kamiya, K.** (1999) Mutations in the RAD54 recombination gene in primary cancers. *Oncogene*, **18**, 3427-30.
- Matullo, G., Guarrera, S., Carturan, S., Peluso, M., Malaveille, C., Davico, L., Piazza, A. and Vineis, P.** (2001) DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer in a case-control study. *Int J Cancer*, **92**, 562-7.
- Mazin, A.V., Zaitseva, E., Sung, P. and Kowalczykowski, S.C.** (2000) Tailed duplex DNA is the preferred substrate for Rad51 protein-mediated homologous pairing. *Embo J*, **19**, 1148-56.
- McIlwraith, M.J., Van Dyck, E., Masson, J.Y., Stasiak, A.Z., Stasiak, A. and West, S.C.** (2000) Reconstitution of the strand invasion step of double-strand break repair using human Rad51 Rad52 and RPA proteins. *J Mol Biol*, **304**, 151-64.
- Menetski, J.P. and Kowalczykowski, S.C.** (1985) Interaction of recA protein with single-stranded DNA. Quantitative aspects of binding affinity modulation by nucleotide cofactors. *J Mol Biol*, **181**, 281-95.
- Mignone, F., Gissi, C., Liuni, S. and Pesole, G.** (2002) Untranslated regions of mRNAs. *Genome Biol*, **3**, REVIEWS0004.

- Miller, K.A., Yoshikawa, D.M., McConnell, I.R., Clark, R., Schild, D. and Albala, J.S.** (2002) RAD51C interacts with RAD51B and is central to a larger protein complex in vivo exclusive of RAD51. *J Biol Chem*, **277**, 8406-11.
- Mirzoeva, O.K. and Petrini, J.H.** (2001) DNA damage-dependent nuclear dynamics of the Mre11 complex. *Mol Cell Biol*, **21**, 281-8.
- Miyagawa, K., Tsuruga, T., Kinomura, A., Usui, K., Katsura, M., Tashiro, S., Mishima, H. and Tanaka, K.** (2002) A role for RAD54B in homologous recombination in human cells. *Embo J*, **21**, 175-80.
- Mohaghegh, P. and Hickson, I.D.** (2002) Premature aging in RecQ helicase-deficient human syndromes. *Int J Biochem Cell Biol*, **34**, 1496-501.
- Morgan, E.A., Shah, N. and Symington, L.S.** (2002) The requirement for ATP hydrolysis by *Saccharomyces cerevisiae* Rad51 is bypassed by mating-type heterozygosity or RAD54 in high copy. *Mol Cell Biol*, **22**, 6336-43.
- Morrison, C., Shinohara, A., Sonoda, E., Yamaguchi-Iwai, Y., Takata, M., Weichselbaum, R.R. and Takeda, S.** (1999) The essential functions of human Rad51 are independent of ATP hydrolysis. *Mol Cell Biol*, **19**, 6891-7.
- Morrison, C., Sonoda, E., Takao, N., Shinohara, A., Yamamoto, K. and Takeda, S.** (2000) The controlling role of ATM in homologous recombinational repair of DNA damage. *Embo J*, **19**, 463-71.
- Moynahan, M.E., Cui, T.Y. and Jasin, M.** (2001a) Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brc1 mutation. *Cancer Res*, **61**, 4842-50.
- Moynahan, M.E., Pierce, A.J. and Jasin, M.** (2001b) BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell*, **7**, 263-72.
- Myung, K., He, D.M., Lee, S.E. and Hendrickson, E.A.** (1997) KARP-1: a novel leucine zipper protein expressed from the Ku86 autoantigen locus is implicated in the control of DNA-dependent protein kinase activity. *Embo J*, **16**, 3172-84.
- Nakayama, H.** (2002) RecQ family helicases: roles as tumor suppressor proteins. *Oncogene*, **21**, 9008-21.
- Nasmyth, K.** (2002) Segregating sister genomes: the molecular biology of chromosome separation. *Science*, **297**, 559-65.
- Nathanson, K.L., Wooster, R., Weber, B.L. and Nathanson, K.N.** (2001) Breast cancer genetics: what we know and what we need. *Nat Med*, **7**, 552-6.
- Nelms, B.E., Maser, R.S., MacKay, J.F., Lagally, M.G. and Petrini, J.H.** (1998) In situ visualization of DNA double-strand break repair in human fibroblasts. *Science*, **280**, 590-2.
- Nyberg, K.A., Michelson, R.J., Putnam, C.W. and Weinert, T.A.** (2002) TOWARD MAINTAINING THE GENOME: DNA Damage and Replication Checkpoints. *Annu Rev Genet*, **36**, 617-56.
- O'Driscoll, M., Cerosaletti, K.M., Girard, P.M., Dai, Y., Stumm, M., Kysela, B., Hirsch, B., Gennery, A., Palmer, S.E., Seidel, J., Gatti, R.A., Varon, R., Oettinger, M.A., Neitzel, H., Jeggo, P.A. and Concannon, P.** (2001) DNA ligase IV mutations identified in patients exhibiting developmental delay and immunodeficiency. *Mol Cell*, **8**, 1175-85.
- O'Neill, J.P., Rogan, P.K., Cariello, N. and Nicklas, J.A.** (1998) Mutations that alter RNA splicing of the human HPRT gene: a review of the spectrum. *Mutat Res*, **411**, 179-214.
- O'Regan, P.** (2002) Functional analysis of the XRCC2 DNA repair gene. *Clinical medicine*. University of Oxford.

- O'Regan, P., Wilson, C., Townsend, S. and Thacker, J.** (2001) XRCC2 is a nuclear RAD51-like protein, required for damage-dependent RAD51 focus formation without the need for ATP binding. *J Biol Chem*, **11**, 11.
- Osakabe, K., Yoshioka, T., Ichikawa, H. and Toki, S.** (2002) Molecular cloning and characterization of RAD51-like genes from *Arabidopsis thaliana*. *Plant Mol Biol*, **50**, 71-81.
- Pages, V. and Fuchs, R.P.** (2002) How DNA lesions are turned into mutations within cells? *Oncogene*, **21**, 8957-66.
- Paques, F. and Haber, J.E.** (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev*, **63**, 349-404.
- Parsons, C.A., Baumann, P., Van Dyck, E. and West, S.C.** (2000) Precise binding of single-stranded DNA termini by human RAD52 protein. *Embo J*, **19**, 4175-81.
- Paull, T.T., Rogakou, E.P., Yamazaki, V., Kirchgessner, C.U., Gellert, M. and Bonner, W.M.** (2000) A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol*, **10**, 886-95.
- Pedrazzi, G., Perrera, C., Blaser, H., Kuster, P., Marra, G., Davies, S.L., Ryu, G.H., Freire, R., Hickson, I.D., Jiricny, J. and Stagljar, I.** (2001) Direct association of Bloom's syndrome gene product with the human mismatch repair protein MLH1. *Nucleic Acids Res*, **29**, 4378-86.
- Pellegrini, L., Yu, D.S., Lo, T., Anand, S., Lee, M., Blundell, T.L. and Venkitaraman, A.R.** (2002) Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature*, **420**, 287-93.
- Peng, L., Rice, M.C. and Kmiec, E.B.** (1998) Analysis of the human RAD51L1 promoter region and its activation by UV light. *Genomics*, **54**, 529-41.
- Peri, S. and Pandey, A.** (2001) A reassessment of the translation initiation codon in vertebrates. *Trends Genet*, **17**, 685-7.
- Perry, P. and Wolff, S.** (1974) New Giemsa method for the differential staining of sister chromatids. *Nature*, **251**, 156-8.
- Perry, P.E. and Thomson, E.J.** (1984) The methodology of sister chromatid exchanges. In Ramel, C. (ed.) *Handbook of mutagenicity test procedures*. Elsevier Science Publishers.
- Petukhova, G., Sung, P. and Klein, H.** (2000) Promotion of Rad51-dependent D-loop formation by yeast recombination factor Rdh54/Tid1. *Genes Dev*, **14**, 2206-15.
- Pierce, A.J., Johnson, R.D., Thompson, L.H. and Jasin, M.** (1999) XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev*, **13**, 2633-8.
- Pierce, A.J., Stark, J.M., Araujo, F.D., Moynahan, M.E., Berwick, M. and Jasin, M.** (2001) Double-strand breaks and tumorigenesis. *Trends Cell Biol*, **11**, S52-9.
- Pittman, D.L. and Schimenti, J.C.** (2000) Midgestation lethality in mice deficient for the RecA-related gene, Rad51d/Rad51l3. *Genesis*, **26**, 167-73.
- Pittman, D.L., Weinberg, L.R. and Schimenti, J.C.** (1998) Identification, characterization, and genetic mapping of Rad51d, a new mouse and human RAD51/RecA-related gene. *Genomics*, **49**, 103-11.
- Prakash, S. and Prakash, L.** (2002) Translesion DNA synthesis in eukaryotes: a one- or two-polymerase affair. *Genes Dev*, **16**, 1872-83.
- Raderschall, E., Golub, E.I. and Haaf, T.** (1999) Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proc Natl Acad Sci U S A*, **96**, 1921-6.

- Rafii, S., O'Regan, P., Xinarianos, G., Azmy, I., Stephenson, T., Reed, M., Meuth, M., Thacker, J. and Cox, A.** (2002) A potential role for the XRCC2 R188H polymorphic site in DNA-damage repair and breast cancer. *Hum Mol Genet*, **11**, 1433-8.
- Rattray, A.J. and Symington, L.S.** (1994) Use of a chromosomal inverted repeat to demonstrate that the RAD51 and RAD52 genes of *Saccharomyces cerevisiae* have different roles in mitotic recombination. *Genetics*, **138**, 587-95.
- Reddy, G., Golub, E.I. and Radding, C.M.** (1997) Human Rad52 protein promotes single-strand DNA annealing followed by branch migration. *Mutat Res*, **377**, 53-9.
- Rehrauer, W.M. and Kowalczykowski, S.C.** (1993) Alteration of the nucleoside triphosphate (NTP) catalytic domain within *Escherichia coli* recA protein attenuates NTP hydrolysis but not joint molecule formation. *J Biol Chem*, **268**, 1292-7.
- Riballo, E., Critchlow, S.E., Teo, S.H., Doherty, A.J., Priestley, A., Broughton, B., Kysela, B., Beamish, H., Plowman, N., Arlett, C.F., Lehmann, A.R., Jackson, S.P. and Jeggo, P.A.** (1999) Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. *Curr Biol*, **9**, 699-702.
- Rice, M.C., Smith, S.T., Bullrich, F., Havre, P. and Kmiec, E.B.** (1997) Isolation of human and mouse genes based on homology to REC2, a recombinational repair gene from the fungus *Ustilago maydis*. *Proc Natl Acad Sci U S A*, **94**, 7417-22.
- Richardson, C. and Jasin, M.** (2000) Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. *Mol Cell Biol*, **20**, 9068-75.
- Rijkers, T., Van Den Ouweland, J., Morolli, B., Rolink, A.G., Baarends, W.M., Van Sloun, P.P., Lohman, P.H. and Pastink, A.** (1998) Targeted inactivation of mouse RAD52 reduces homologous recombination but not resistance to ionizing radiation. *Mol Cell Biol*, **18**, 6423-9.
- Ristic, D., Wyman, C., Paulusma, C. and Kanaar, R.** (2001) The architecture of the human Rad54-DNA complex provides evidence for protein translocation along DNA. *Proc Natl Acad Sci U S A*, **98**, 8454-60.
- Sale, J.E., Calandrini, D.M., Takata, M., Takeda, S. and Neuberger, M.S.** (2001) Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. *Nature*, **412**, 921-6.
- Sato, K., Chen, D.J., Eguchi-Kasai, K., Itsukaichi, H., Odaka, T. and Strniste, G.F.** (1995) Interspecific complementation between mouse and Chinese hamster cell mutants hypersensitive to ionizing radiation. *J Radiat Res (Tokyo)*, **36**, 38-45.
- Savage, J.R.** (1976) Classification and relationships of induced chromosomal structural changes. *J Med Genet*, **13**, 103-22.
- Schild, D., Lio, Y.C., Collins, D.W., Tsomondo, T. and Chen, D.J.** (2000) Evidence for simultaneous protein interactions between human Rad51 paralogs. *J Biol Chem*, **275**, 16443-9.
- Schlissel, M.S.** (2002) Does artemis end the hunt for the hairpin-opening activity in V(D)J recombination? *Cell*, **109**, 1-4.
- Schoenmakers, E.F., Huysmans, C. and Van de Ven, W.J.** (1999) Allelic knockout of novel splice variants of human recombination repair gene RAD51B in t(12;14) uterine leiomyomas. *Cancer Res*, **59**, 19-23.
- Shapiro, M.B. and Senapathy, P.** (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res*, **15**, 7155-74.

- Shen, M.R., Jones, I.M. and Mohrenweiser, H.** (1998) Nonconservative amino acid substitution variants exist at polymorphic frequency in DNA repair genes in healthy humans. *Cancer Res*, **58**, 604-8.
- Shibuya, T. and Morimoto, K.** (1993) A review of the genotoxicity of 1-ethyl-1-nitrosourea. *Mutat Res*, **297**, 3-38.
- Shiloh, Y.** (2001) ATM and ATR: networking cellular responses to DNA damage. *Curr Opin Genet Dev*, **11**, 71-7.
- Shinohara, A., Ogawa, H. and Ogawa, T.** (1992) Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell*, **69**, 457-70.
- Shu, Z., Smith, S., Wang, L., Rice, M.C. and Kmiec, E.B.** (1999) Disruption of muREC2/RAD51L1 in mice results in early embryonic lethality which can be partially rescued in a p53(-/-) background. *Mol Cell Biol*, **19**, 8686-93.
- Sigurdsson, S., Van Komen, S., Bussen, W., Schild, D., Albala, J.S. and Sung, P.** (2001) Mediator function of the human Rad51B-Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange. *Genes Dev*, **15**, 3308-18.
- Siminovitch, L.** (1976) On the nature of heritable variation in cultured somatic cells. *Cell*, **7**, 1-11.
- Smith, G.C. and Jackson, S.P.** (1999) The DNA-dependent protein kinase. *Genes Dev*, **13**, 916-34.
- Solinger, J.A., Kiianitsa, K. and Heyer, W.D.** (2002) Rad54, a Swi2/Snf2-like recombinational repair protein, disassembles Rad51:dsDNA filaments. *Mol Cell*, **10**, 1175-88.
- Sonoda, E., Morrison, C., Yamashita, Y.M., Takata, M. and Takeda, S.** (2001) Reverse genetic studies of homologous DNA recombination using the chicken B-lymphocyte line, DT40. *Philos Trans R Soc Lond B Biol Sci*, **356**, 111-7.
- Sonoda, E., Sasaki, M.S., Buerstedde, J.M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y. and Takeda, S.** (1998) Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *Embo J*, **17**, 598-608.
- Sonoda, E., Sasaki, M.S., Morrison, C., Yamaguchi-Iwai, Y., Takata, M. and Takeda, S.** (1999) Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. *Mol Cell Biol*, **19**, 5166-9.
- Stamato, T.D., Weinstein, R., Giaccia, A. and Mackenzie, L.** (1983) Isolation of cell cycle-dependent gamma ray-sensitive Chinese hamster ovary cell. *Somatic Cell Genet*, **9**, 165-73.
- Stark, J.M., Hu, P., Pierce, A.J., Moynahan, M.E., Ellis, N. and Jasin, M.** (2002) ATP hydrolysis by mammalian RAD51 has a key role during homology-directed DNA repair. *J Biol Chem*, **277**, 20185-94.
- Stasiak, A.Z., Larquet, E., Stasiak, A., Muller, S., Engel, A., Van Dyck, E., West, S.C. and Egelman, E.H.** (2000) The human Rad52 protein exists as a heptameric ring. *Curr Biol*, **10**, 337-40.
- Story, R.M. and Steitz, T.A.** (1992) Structure of the recA protein-ADP complex. *Nature*, **355**, 374-6.
- Sung, P.** (1997) Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev*, **11**, 1111-21.
- Sung, P. and Stratton, S.A.** (1996) Yeast Rad51 recombinase mediates polar DNA strand exchange in the absence of ATP hydrolysis. *J Biol Chem*, **271**, 27983-6.

Swagemakers, S.M., Essers, J., de Wit, J., Hoeijmakers, J.H. and Kanaar, R. (1998) The human RAD54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase. *J Biol Chem*, **273**, 28292-7.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. and Stahl, F.W. (1983) The double-strand-break repair model for recombination. *Cell*, **33**, 25-35.

Takahashi, T., Nagai, N., Oda, H., Ohama, K., Kamada, N. and Miyagawa, K. (2001) Evidence for RAD51L1/HMGIC fusion in the pathogenesis of uterine leiomyoma. *Genes Chromosomes Cancer*, **30**, 196-201.

Takata, M., Sasaki, M.S., Sonoda, E., Fukushima, T., Morrison, C., Albala, J.S., Swagemakers, S.M., Kanaar, R., Thompson, L.H. and Takeda, S. (2000) The Rad51 paralog Rad51B promotes homologous recombinational repair. *Mol Cell Biol*, **20**, 6476-82.

Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. and Takeda, S. (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *Embo J*, **17**, 5497-508.

Takata, M., Sasaki, M.S., Tachiiri, S., Fukushima, T., Sonoda, E., Schild, D., Thompson, L.H. and Takeda, S. (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Mol Cell Biol*, **21**, 2858-66.

Tambini, C.E., George, A.M., Rommens, J.M., Tsui, L.C., Scherer, S.W. and Thacker, J. (1997) The XRCC2 DNA repair gene: identification of a positional candidate. *Genomics*, **41**, 84-92.

Tan, T.L., Essers, J., Citterio, E., Swagemakers, S.M., de Wit, J., Benson, F.E., Hoeijmakers, J.H. and Kanaar, R. (1999) Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation. *Curr Biol*, **9**, 325-8.

Tanaka, K., Hiramoto, T., Fukuda, T. and Miyagawa, K. (2000) A novel human rad54 homologue, Rad54B, associates with Rad51. *J Biol Chem*, **275**, 26316-21.

Tanaka, K., Kagawa, W., Kinebuchi, T., Kurumizaka, H. and Miyagawa, K. (2002) Human Rad54B is a double-stranded DNA-dependent ATPase and has biochemical properties different from its structural homolog in yeast, Tid1/Rdh54. *Nucleic Acids Res*, **30**, 1346-53.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P.R., Gregory, R.C., Kim, S.T., Lane, W.S., Kastan, M.B. and D'Andrea, A.D. (2002) Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell*, **109**, 459-72.

Tarsounas, M., Davies, D. and West, S.C. (2003) BRCA2-dependent and independent formation of RAD51 nuclear foci. *Oncogene*, **22**, 1115-23.

Tashiro, S., Kotomura, N., Shinohara, A., Tanaka, K., Ueda, K. and Kamada, N. (1996) S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes. *Oncogene*, **12**, 2165-70.

Tashiro, S., Walter, J., Shinohara, A., Kamada, N. and Cremer, T. (2000) Rad51 accumulation at sites of DNA damage and in postreplicative chromatin. *J Cell Biol*, **150**, 283-91.

Tauchi, H., Kobayashi, J., Morishima, K., van Gent, D.C., Shiraishi, T., Verkaik, N.S., vanHeems, D., Ito, E., Nakamura, A., Sonoda, E., Takata, M., Takeda, S., Matsuura, S. and Komatsu, K. (2002a) Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. *Nature*, **420**, 93-8.

- Tauchi, H., Matsuura, S., Kobayashi, J., Sakamoto, S. and Komatsu, K.** (2002b) Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. *Oncogene*, **21**, 8967-80.
- Taylor, J.H.** (1958) Sister chromatid exchanges in tritium-labeled chromosomes. *Genetics*, **43**, 515-529.
- Tebbs, R.S., Zhao, Y., Tucker, J.D., Scheerer, J.B., Siciliano, M.J., Hwang, M., Liu, N., Legerski, R.J. and Thompson, L.H.** (1995) Correction of chromosomal instability and sensitivity to diverse mutagens by a cloned cDNA of the XRCC3 DNA repair gene. *Proc Natl Acad Sci U S A*, **92**, 6354-8.
- Thacker, J.** (1999) A surfeit of RAD51-like genes? *Trends Genet*, **15**, 166-8.
- Thacker, J. and Ganesh, A.N.** (1990) DNA-break repair, radioresistance of DNA synthesis, and camptothecin sensitivity in the radiation-sensitive *irs* mutants: comparisons to ataxia-telangiectasia cells. *Mutat Res*, **235**, 49-58.
- Thacker, J., Ganesh, A.N., Stretch, A., Benjamin, D.M., Zahalsky, A.J. and Hendrickson, E.A.** (1994) Gene mutation and V(D)J recombination in the radiosensitive *irs* lines. *Mutagenesis*, **9**, 163-8.
- Thacker, J. and Wilkinson, R.E.** (1991) The genetic basis of resistance to ionising radiation damage in cultured mammalian cells. *Mutat Res*, **254**, 135-42.
- Thacker, J. and Wilkinson, R.E.** (1995) The genetic basis of cellular recovery from radiation damage: response of the radiosensitive *irs* lines to low-dose-rate irradiation. *Radiat Res*, **144**, 294-300.
- Thompson, L.H. and Schild, D.** (2002) Recombinational DNA repair and human disease. *Mutat Res*, **509**, 49-78.
- Tsutsui, Y., Morishita, T., Iwasaki, H., Toh, H. and Shinagawa, H.** (2000) A recombination repair gene of *Schizosaccharomyces pombe*, *rhpf57*, is a functional homolog of the *Saccharomyces cerevisiae* RAD57 gene and is phylogenetically related to the human XRCC3 gene. *Genetics*, **154**, 1451-61.
- Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y. and Morita T.** (1996) Targeted disruption of the *Rad51* gene leads to lethality in embryonic mice. *Proc Natl Acad Sci U S A*, **93**, 6236-40.
- Tucker, J.D., Auletta, A., Cimino, M.C., Dearfield, K.L., Jacobson-Kram, D., Tice, R.R. and Carrano, A.V.** (1993) Sister-chromatid exchange: second report of the Gene-Tox Program. *Mutat Res*, **297**, 101-80.
- Tucker, J.D., Jones, N.J., Allen, N.A., Minkler, J.L., Thompson, L.H. and Carrano, A.V.** (1991) Cytogenetic characterization of the ionizing radiation-sensitive Chinese hamster mutant *irs1*. *Mutat Res*, **254**, 143-52.
- Tutt, A., Bertwistle, D., Valentine, J., Gabriel, A., Swift, S., Ross, G., Griffin, C., Thacker, J. and Ashworth, A.** (2001) Mutation in *Brca2* stimulates error-prone homology-directed repair of DNA double-strand breaks occurring between repeated sequences. *Embo J*, **20**, 4704-16.
- van den Bosch, M., Vreeken, K., Zonneveld, J.B., Brandsma, J.A., Lombaerts, M., Murray, J.M., Lohman, P.H. and Pastink, A.** (2001) Characterization of RAD52 homologs in the fission yeast *Schizosaccharomyces pombe*. *Mutat Res*, **461**, 311-23.
- van den Bosch, M., Zonneveld, J.B., Vreeken, K., de Vries, F.A., Lohman, P.H. and Pastink, A.** (2002) Differential expression and requirements for *Schizosaccharomyces pombe* RAD52 homologs in DNA repair and recombination. *Nucleic Acids Res*, **30**, 1316-24.

- van den Hoff, M.J., Moorman, A.F. and Lamers, W.H.** (1992) Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res*, **20**, 2902.
- Van Dyck, E., Hajibagheri, N.M., Stasiak, A. and West, S.C.** (1998) Visualisation of human rad52 protein and its complexes with hRad51 and DNA. *J Mol Biol*, **284**, 1027-38.
- Van Dyck, E., Stasiak, A.Z., Stasiak, A. and West, S.C.** (1999) Binding of double-strand breaks in DNA by human Rad52 protein. *Nature*, **398**, 728-31.
- van Gent, D.C., Hoeijmakers, J.H. and Kanaar, R.** (2001) Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet*, **2**, 196-206.
- Venkitaraman, A.R.** (2001) Functions of BRCA1 and BRCA2 in the biological response to DNA damage. *J Cell Sci*, **114**, 3591-8.
- Venkitaraman, A.R.** (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell*, **108**, 171-82.
- Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J.** (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J*, **1**, 945-51.
- Wang, W., Seki, M., Narita, Y., Sonoda, E., Takeda, S., Yamada, K., Masuko, T., Katada, T. and Enomoto, T.** (2000a) Possible association of BLM in decreasing DNA double strand breaks during DNA replication. *Embo J*, **19**, 3428-35.
- Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S.J. and Qin, J.** (2000b) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev*, **14**, 927-39.
- Wiese, C., Collins, D.W., Albala, J.S., Thompson, L.H., Kronenberg, A. and Schild, D.** (2002) Interactions involving the Rad51 paralogs Rad51C and XRCC3 in human cells. *Nucleic Acids Res*, **30**, 1001-8.
- Winding, P. and Berchtold, M.W.** (2001) The chicken B cell line DT40: a novel tool for gene disruption experiments. *J Immunol Methods*, **249**, 1-16.
- Winsey, S.L., Haldar, N.A., Marsh, H.P., Bunce, M., Marshall, S.E., Harris, A.L., Wojnarowska, F. and Welsh, K.I.** (2000) A variant within the DNA repair gene XRCC3 is associated with the development of melanoma skin cancer. *Cancer Res*, **60**, 5612-6.
- Wu, G.J., Sinclair, C.S., Paape, J., Ingle, J.N., Roche, P.C., James, C.D. and Couch, F.J.** (2000) 17q23 amplifications in breast cancer involve the PAT1, RAD51C, PS6K, and SIGMA1B genes. *Cancer Res*, **60**, 5371-5.
- Wu, L., Davies, S.L., Levitt, N.C. and Hickson, I.D.** (2001) Potential Role for the BLM Helicase in Recombinational Repair via a Conserved Interaction with RAD51. *J Biol Chem*, **276**, 19375-81.
- Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T. and Deng, C.X.** (1999) Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol Cell*, **3**, 389-95.
- Yamaguchi-Iwai, Y., Sonoda, E., Buerstedde, J.M., Bezzubova, O., Morrison, C., Takata, M., Shinohara, A. and Takeda, S.** (1998) Homologous recombination, but not DNA repair, is reduced in vertebrate cells deficient in RAD52. *Mol Cell Biol*, **18**, 6430-5.
- Yamaguchi-Iwai, Y., Sonoda, E., Sasaki, M.S., Morrison, C., Haraguchi, T., Hiraoka, Y., Yamashita, Y.M., Yagi, T., Takata, M., Price, C., Kakazu, N. and Takeda, S.** (1999) Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells. *Embo J*, **18**, 6619-29.

- Yokoyama, H., Kurumizaka, H., Ikawa, S., Yokoyama, S. and Shibata, T.** (2003) Holliday junction binding activity of the human Rad51B protein. *J Biol Chem*, **278**, 2767-72.
- Yu, V.P., Koehler, M., Steinlein, C., Schmid, M., Hanakali, L.A., van Gool, A.J., West, S.C. and Venkitaraman, A.R.** (2000) Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev*, **14**, 1400-6.
- Yuan, S.S., Lee, S.Y., Chen, G., Song, M., Tomlinson, G.E. and Lee, E.Y.** (1999) BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. *Cancer Res*, **59**, 3547-51.
- Zdzienicka, M.Z. and Simons, J.W.** (1987) Mutagen-sensitive cell lines are obtained with a high frequency in V79 Chinese hamster cells. *Mutat Res*, **178**, 235-44.

Role of Mammalian RAD51L2 (RAD51C) in Recombination and Genetic Stability*

Received for publication, February 11, 2002, and in revised form, March 18, 2002
Published, JBC Papers in Press, March 23, 2002, DOI 10.1074/jbc.M201402200

Catherine A. French†, Jean-Yves Masson§¶, Carol S. Griffin, Paul O'Regan||, Stephen C. West§, and John Thacker***

From the Medical Research Council, Radiation & Genome Stability Unit, Harwell, Oxfordshire OX11 0RD, United Kingdom and the §Cancer Research UK, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, United Kingdom

The highly conserved RAD51 protein has a central role in homologous recombination. Five novel RAD51-like genes have been identified in mammalian cells, but little is known about their functions. A DNA damage-sensitive hamster cell line, *irs3*, was found to have a mutation in the *RAD51L2* gene and an undetectable level of RAD51L2 protein. Resistance of *irs3* to DNA-damaging agents was significantly increased by expression of the human *RAD51L2* gene, but not by other RAD51-like genes or RAD51 itself. Consistent with a role for RAD51L2 in homologous recombination, *irs3* cells show a reduction in sister chromatid exchange, an increase in isochromatid breaks, and a decrease in damage-dependent RAD51 focus formation compared with wild type cells. As recently demonstrated for human cells, we show that RAD51L2 forms part of two separate complexes of hamster RAD51-like proteins. Strikingly, neither complex of RAD51-like proteins is formed in *irs3* cells. Our results demonstrate that RAD51L2 has a key role in mammalian RAD51-dependent processes, contingent on the formation of protein complexes involved in homologous recombination repair.

The use of cultured mammalian cell lines selected for sensitivity to DNA-damaging agents to identify genes and gene functions has had a major impact on our understanding of DNA repair pathways. In particular, components of the nucleotide excision repair and DNA end joining pathways were identified and their cellular responses characterized using DNA damage-sensitive cell lines (1, 2). Recently, the value of this approach has been reinforced by the identification of genes from the homologous recombination repair pathway, with the cloning of the *XRCC2* and *XRCC3* genes by their ability to complement sensitive mammalian cell lines (3, 4).

The repair of DNA damage by homologous recombination is important for the maintenance of genetic stability in cells. The RAD51 protein is central to the recombination process, and this

protein is highly conserved from yeast to humans (5). Using molecular recombination assays, yeast (*Saccharomyces cerevisiae*) and human RAD51 proteins have been shown to promote DNA strand exchange. In *S. cerevisiae*, two RAD51-like proteins, Rad55p and Rad57p, form a heterodimer and stimulate Rad51-mediated recombination reactions (6). Yeast mutants that lack either Rad51 or these Rad51-like recombination proteins are extremely sensitive to agents causing severe forms of damage to DNA, such as double-strand breaks and interstrand cross-links (7). Rad55p and Rad57p also have counterparts in mammalian cells, including XRCC2, XRCC3, RAD51L1¹ (hREC2, RAD51B, R51H2), RAD51L2 (RAD51C), and RAD51L3 (R51H3, RAD51D) (8). The last three proteins were identified through data base searches using partial homologies to RAD51-like proteins (9–13), and have not as yet been found to be defective in DNA damage-sensitive mammalian cell lines. At present very little is known about the functions of these mammalian RAD51-like proteins, although specific protein-protein interactions have been described which suggest that they form heterodimers and larger complexes that may help recruit RAD51 to sites of DNA damage (14–22).

A series of DNA damage-sensitive hamster cell lines, termed the *irs* mutants, were previously isolated in this laboratory (23). We and others used the *irs1* cell line to clone the *XRCC2* gene by its complementing ability for sensitivity to the potent DNA cross-linking agent, mitomycin-C (24–26). The *irs1* line is also sensitive to other DNA-damaging agents, including ionizing radiation, ultraviolet light, and alkylating agents (23). It shows spontaneous genetic instability, with increased frequencies of mutations (27), chromosomal aberrations (28), and chromosome non-disjunction (29). It has also been shown that the repair of a site-specific double-strand break by homologous recombination is severely reduced in *irs1* compared with the paternal V79 cells (30). We have recently established a functional link between XRCC2 and RAD51 by showing that the *irs1* line is defective in the formation of damage-dependent RAD51 focus formation (31).

The *irs3* cell line is another member of this series of damage-sensitive cell lines. In cell fusions, *irs3* was found to be able to complement damage sensitivity in several other radiation-sensitive mammalian cell lines, including *irs1*, showing that it has a unique genetic defect (32). However, the *irs3* cell line has a similar damage sensitivity profile to *irs1*, being sensitive to x-rays (2-fold), ethyl methanesulfonate (2.5-fold) and especially MMC² (7-fold), and shows chromosomal instability (23, 33). We

* This work was supported in part by the Medical Research Council, Cancer Research UK, and European Commission Contract FIGH-CT1999–00010. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a Medical Research Council studentship.

¶ Supported by a KM Hunter postdoctoral fellowship from the National Cancer Institute of Canada.

|| Supported by a Medical Research Council studentship.

*** To whom correspondence should be addressed: Medical Research Council, Radiation & Genome Stability Unit, Harwell, Oxfordshire OX11 0RD, England. Tel.: 44-1235-834393; Fax: 44-1235-834776; E-mail: j.thacker@har.mrc.ac.uk.

¹ RAD51L is the recommended symbol for the RAD51-like genes/proteins (Human Gene Nomenclature Committee).

² The abbreviations used are: MMC, mitomycin-C; RT, reverse transcriptase; SCE, sister chromatid exchanges; pAb, polyclonal antibody; mAb, monoclonal antibody; IRES, internal ribosome entry site.

have therefore considered that *irs3* may have a similar functional defect to *irs1*, and have characterized the *irs3* cell line further as well as identifying the defective gene responsible for this phenotype.

EXPERIMENTAL PROCEDURES

Gene Cloning and Vector Construction—The cloning of *RAD51L1* and *RAD51L3* was described previously (11). The human cDNAs were cloned into mammalian expression vectors; *RAD51L1* in pEBS7 (34) and *RAD51L3* in pZeoSV2(−) (Invitrogen). Human *RAD51L2* (also called *RAD51C* (12)) was cloned from a human testis cDNA library (CLONTECH) into pZeoSV2(−), and was subsequently recloned into the bicistronic mammalian expression vector pIRESneo2 (CLONTECH). The human *RAD51* gene was cloned from the same cDNA library into pIRESneo2. The Chinese hamster *RAD51L2* gene was cloned from a CHO-K1 cDNA library (Invitrogen); the central portion of the gene was cloned first using primers designed to conserved regions of the human (12) and mouse genes (mouse gene data came from sequencing a mouse IMAGE clone 3598583 found by data base search to have close homology to the human cDNA; data not shown). The remainder of the hamster gene was cloned from the same library using nested primers designed to the central portion and to the library vector, pCDNA1. The accession number of the Chinese hamster *RAD51L2* sequence is AJ413202. All cloned genes were verified by sequencing.

Isolation and Chromosomal Mapping of a Genomic Clone Carrying *RAD51L2*—Gridded filters and clones from the human genomic PAC library RPC11 were supplied by the UK HGMP Resource Centre. Filters were probed with full-length human *RAD51L2* cDNA, and DNA from positive clones was isolated using a Qiagen large-construct kit. One PAC clone (259-O5) was mapped to human metaphase chromosomes using a biotinylated probe, detected with Texas Red avidin. Well spread metaphases were captured and karyotyped using the Genus Applied Imaging system.

Cell Culture and DNA Transfection—Parental V79 and mutant *irs3* cells were cultured at 37 °C as monolayers in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. 10 µg of the *RAD51L1*, *RAD51L2*, *RAD51L3*, or *RAD51* constructs were electroporated into 10⁶ *irs3* cells (BioRad Gene Pulser set at 500 µF, 400 V) and transfected clones were selected in the appropriate drugs. Following clone isolation, resistance to mitomycin-C (MMC) was initially tested in 24-well plates by seeding 100–300 cells/well in graded concentrations of MMC (31). In control experiments we did not see any reversion of the *irs3* phenotype (*i.e.* no background clonal growth under selective conditions; data not shown). PAC 259-O5 was chosen to test complementation with genomic *RAD51L2* DNA. 15 µg of PAC DNA was co-transfected with 2 µg of the vector pEGFP-N2 (CLONTECH) into *irs3* cells as described above, followed by selection in 500 µg/ml G418. Colonies of G418-resistant cells were pooled and reseeded in 300 µg/ml G418 and 4 × 10^{−8} M MMC. The presence of the appropriate *RAD51L* gene (cDNA or PAC) was tested in drug-resistant *irs3* clones by PCR using cellular DNA, and gene expression was checked by reverse transcriptase (RT-) PCR (Superscript II, Invitrogen) of cellular RNA with oligo(dT) as the first-strand primer.

Mitomycin-C and X-ray Survival—Subconfluent cells were harvested as a single-celled suspension. Appropriate numbers of cells were reseeded into 9-cm dishes for MMC treatment, which lasted for the duration of the experiment. Compared with acute treatment (23), chronic MMC treatment increased the survival difference between *irs3* and V79 cells to about 20-fold. X-ray treatment (250 kV) was given to 2 × 10⁵ cells in suspension, followed by reseeding appropriate numbers into 9-cm dishes. Three dishes were used for each dose point.

Immunofluorescence—Cells were seeded onto coverslips and grown for 48 h to subconfluent levels before being mock-treated or exposed to 10 Gray X-rays, a dose previously shown to induce a measurable frequency of foci (31, 35). After a further 5-h incubation at 37 °C, the cells were washed with phosphate-buffered saline and fixed in 1% paraformaldehyde for 2 h at 4 °C. Fixed cells were permeabilized for 10 min at room temperature in 0.1% Triton X-100, 0.1 × SSC and then blocked for 1 h at room temperature in phosphate-buffered saline, 5% normal horse serum. Cells were incubated overnight at 4 °C with 1:100 dilution primary antibody, rabbit polyclonal anti-RAD51 (Ab-1) antibody (Oncogene Research), then incubated with 1:100 dilution secondary antibody, Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) for 1 h at room temperature in the dark. The coverslips were mounted in Vectashield anti-fade medium and the cells analyzed by confocal microscopy at 633 nm (BioRad MRC600). Images were selected at random, and were scored from coded slides by two independent scorers,

recording the number of discrete strongly fluorescing nuclear foci present in each cell. At least 50 cells were scored for each data point, and data were compared statistically to assess homogeneity using the Mann-Whitney *U* test.

Chromosome Analysis—To measure sister chromatid exchanges (SCE), cells were incubated in the dark with bromodeoxyuridine (10 µg/ml) for 2 cell cycles to differentially label sister chromatids. MMC was added for 2 h at the start of the experiment. Colcemid (0.05 µg/ml) was added for 2 h prior to harvesting the cells. Metaphase preparations were made using standard techniques. Differential staining of chromatids was achieved with the fluorescence plus Giemsa (FPG, “harlequin”) staining method (36). Chromatid and chromosome aberrations were scored and classified (37) from coded slides. Chromatid breaks were scored where a physical displacement of the broken fragment was seen.

Hamster *RAD51L2* Gene Analysis—RNA was isolated from parental V79 and from the damage-sensitive lines *irs1*, *irs2*, and *irs3* using an RNeasy Mini kit (Qiagen). This RNA was used in an RT-PCR reaction (as above). The resulting cDNA was used as a template for PCR primers designed to amplify the full-length *RAD51L2* hamster gene. Individual PCR products were sliced out of gels and cloned into pCR2.1-TOPO (Invitrogen) before sequencing. In analyzing PCR products for splice variants, intron/exon boundaries were assumed to be the same as in human genomic sequence (9 exons found using data from accession numbers AC021455 and AC025521).

Antibodies—All antibodies were raised against human recombinant denatured protein as described (17). The monoclonal antibodies 1E11 (*RAD51L1*), 2H11 (*RAD51L2*), 5A8 (*RAD51L3*), 1G4 (*XRCC2*), and 7F12 (*XRCC3*) specifically recognized the corresponding human proteins. The anti-RAD51 mAb (14B4) was purchased from Abcam.

Immunoprecipitation Analysis—V79 or *irs3* cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% Nonidet P-40) containing protease inhibitors, incubated for 30 min on ice, dounced 10 times and sonicated twice for 30 s. Insoluble material was removed by high-speed centrifugation. Protein complexes in the supernatant (equivalent to ~15 mg of extract) were pulled down for 1.5 h at 4 °C using preimmune serum or pAbs raised against *RAD51L2* or *XRCC2* cross-linked to Aminolink beads (Pierce). Complexes were washed four times in lysis buffer and visualized by Western blotting using monoclonal antibodies.

RESULTS

The *RAD51L2* Gene of *irs3* has a G → T Mutation in Exon 6—To establish a link between *RAD51L2* and the *irs3* phenotype, we sought to clone the hamster *RAD51L2* gene and check its integrity in *irs3*. We used sequence homologies between mouse and human *RAD51L2* genes to design primers for the isolation of the hamster gene (see “Experimental Procedures”). Cloning of the full-length hamster cDNA showed that its predicted protein sequence is highly conserved in comparison to the human and mouse *RAD51L2* proteins. The mouse and hamster proteins show 84% identity to each other, and each shows 77% identity to the human protein with only one amino acid difference in length (Fig. 1). Reverse-transcribed RNA from the V79 parent and *irs3* lines was used to make *RAD51L2* cDNA. Using full-length gene primers in PCR, both cell lines showed a number of different sized cDNA products (Fig. 2A). To check that the altered profile of cDNA products in *irs3* was related to the *RAD51L2* gene defect, we assayed the *RAD51L2* cDNA profiles in two other radiation-sensitive mutants isolated in the same screen from mutagenized V79 cells. The *irs1* line (*XRCC2*-deficient) and the *irs2* line (shown by complementation testing to be defective in a different gene from *irs1* or *irs3* (32)) both showed *RAD51L2* profiles that were essentially the same as that for V79 (Fig. 2A).

The largest cDNA product in V79 was cloned and sequenced to show that it was the full-length *RAD51L2* gene; other products were found to represent splice variants with different exons missing. In repeated amplifications of cDNA from *irs3* the full-length gene product was either of very low abundance or not present, but there were also visible levels of some splice variants (Fig. 2A). The largest *RAD51L2* product that could be seen readily from *irs3* preparations was found to lack exon 6.

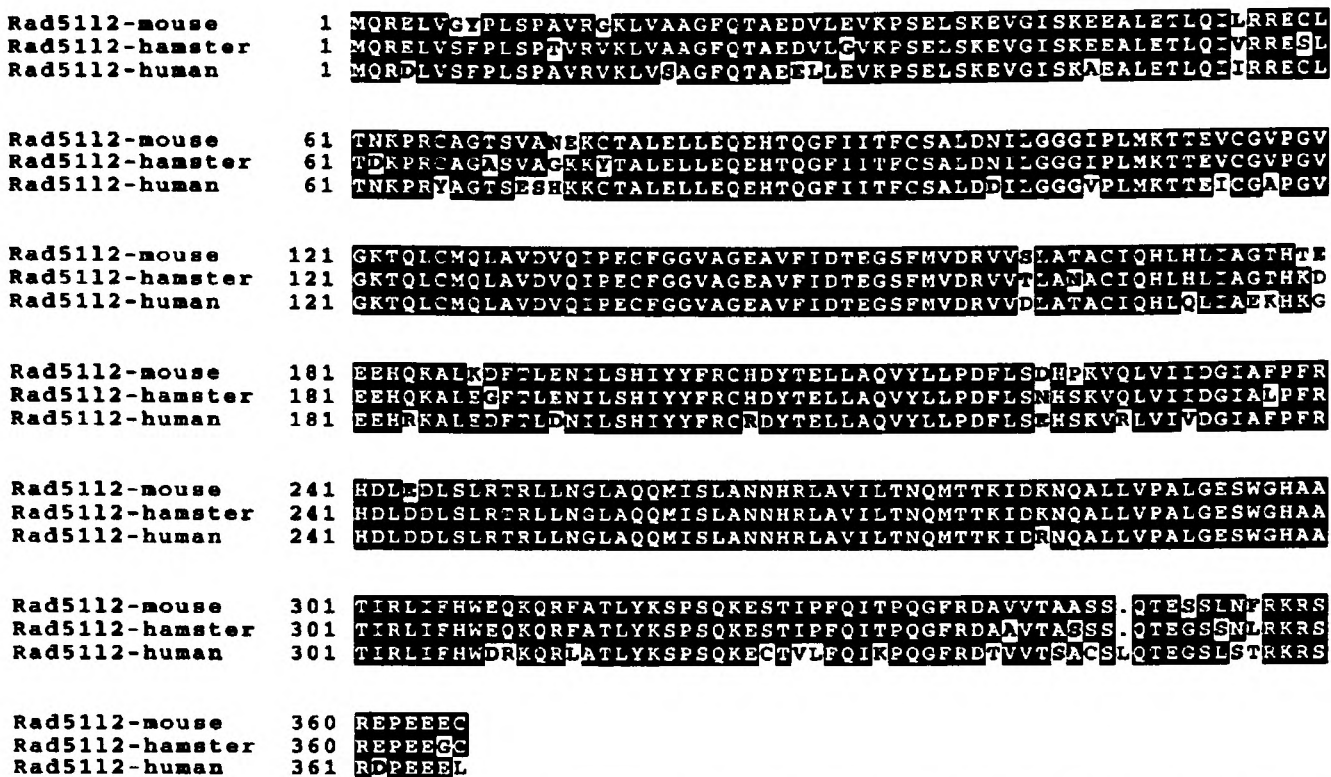
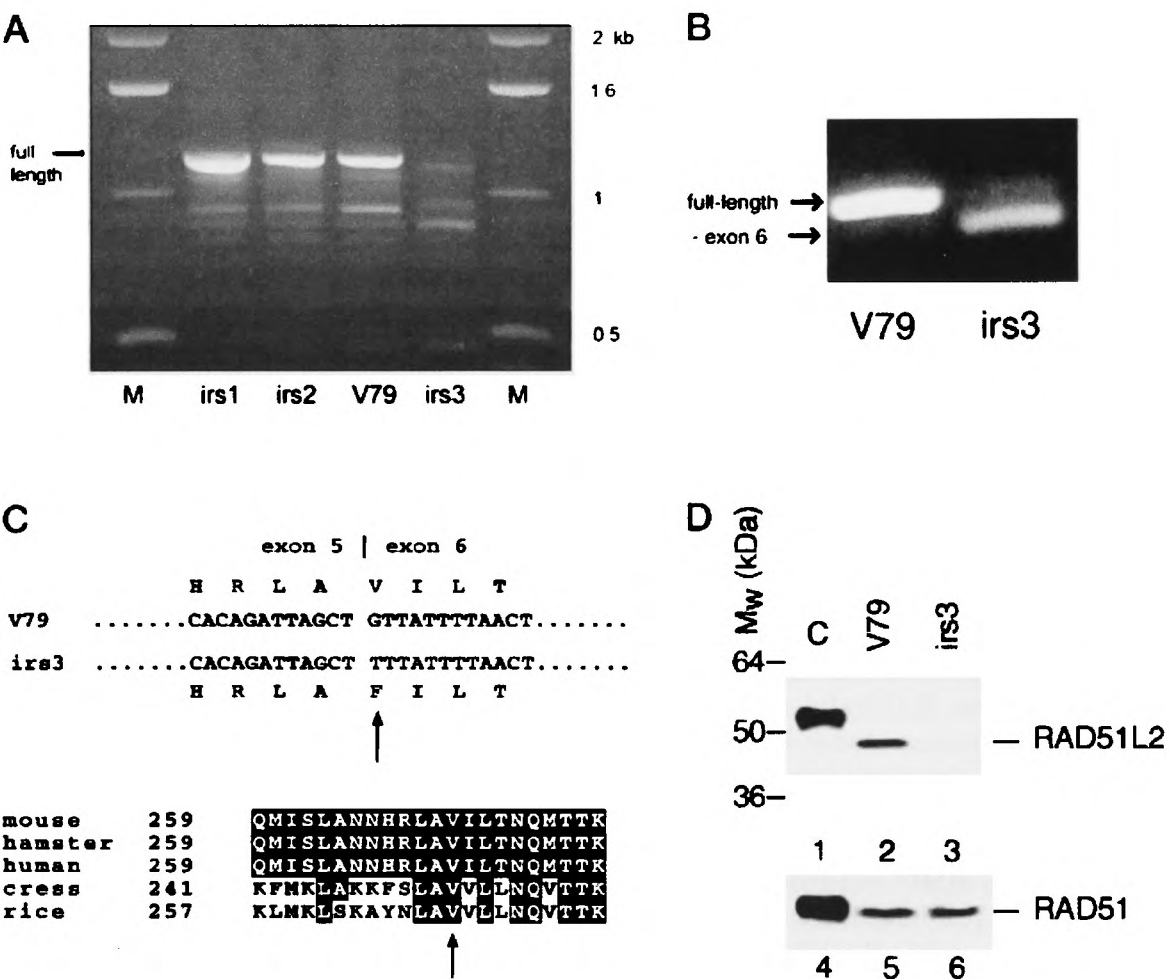


FIG. 1. Conservation of mammalian RAD51L2 protein sequences. RAD51L2 aligned from human, mouse, and hamster (human sequence from accession number O43502 and mouse data³ and accession number AAK58420 (61)). Note that the human sequence is shown starting from a different codon (9 amino acids downstream) than previously published (12), since neither the mouse nor hamster sequence show conservation of that putative start codon. Predicted alignments from PILEUP (GCG version 10), shaded using BOXSHADE (www.ch.embnet.org/).

FIG. 2. RAD51L2 is mutated in *irs3* cells. A, RAD51L2 cDNA profiles amplified from V79 parental and the derived radiation-sensitive lines *irs1*, *irs2*, and *irs3*, using primers designed to give the full-length gene product. M, molecular size marker (Invitrogen), with sizes shown to the right. B, amplification of cDNA from V79 and *irs3* lines with primers placed either side of exon 6 of hamster RAD51L2 cDNA, to give full-length product (514 bp) or product missing exon 6 (447 bp). C, position of the mutation in the RAD51L2 gene of *irs3* (upper diagram, arrow) and conservation of protein sequence in the region of the mutation, in RAD51L2-like proteins from mammalian and plant species (lower diagram, arrow). D, Western blots of V79 and *irs3* whole cell extracts probed with human RAD51L2 mAb (lanes 2 and 3) and human RAD51 mAb (lanes 5 and 6). Lanes 1 and 4 contain purified human RAD51L2^{his10}, migrating more slowly than the endogenous hamster protein, and human RAD51, respectively.

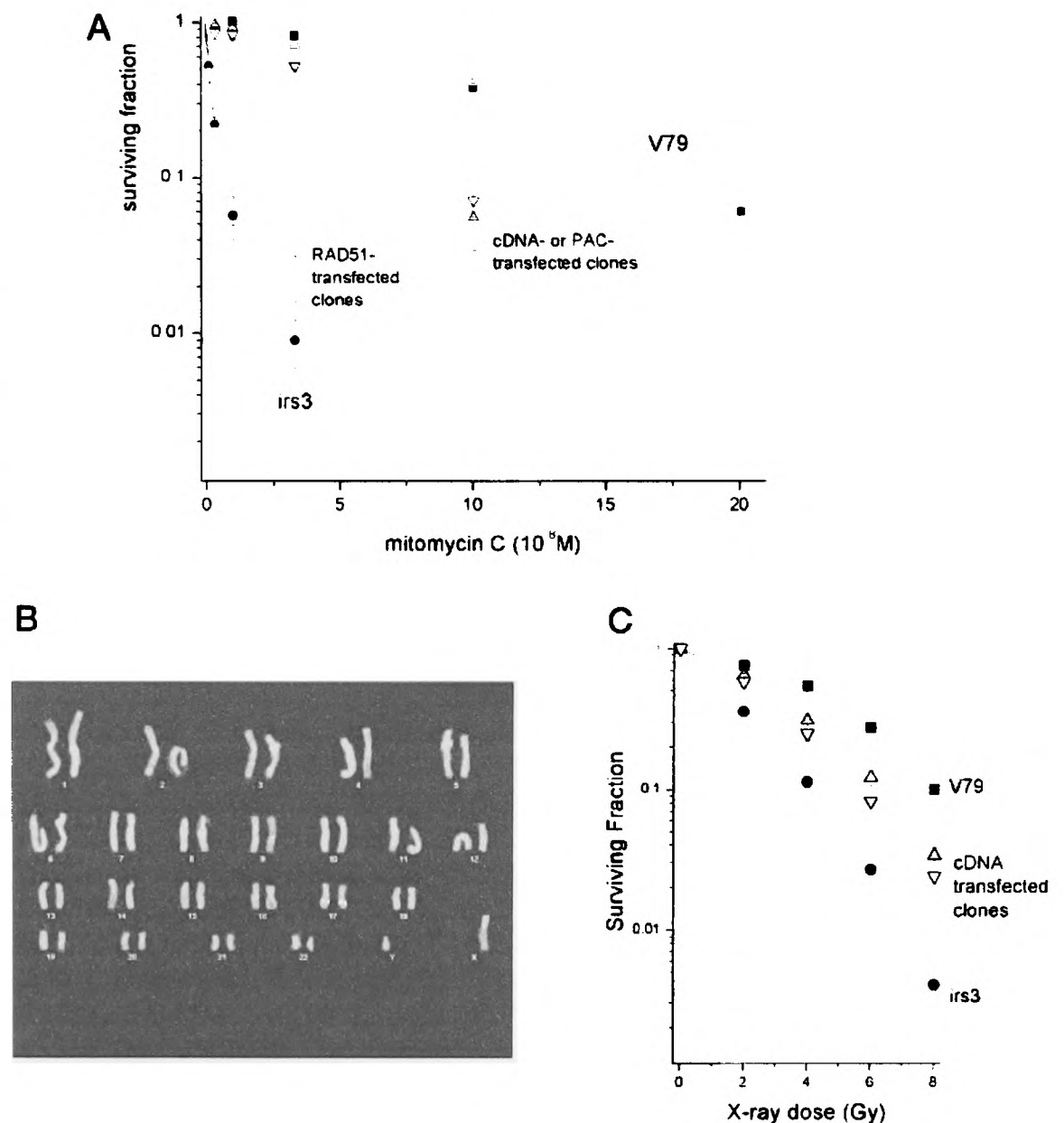


Exon 6 could not be cloned from *irs3* using primers to cDNA sequence either side of the exon (Fig. 2B). However, we were able to obtain sufficient product for cloning by “forcing” the amplification of exon 6 in two pieces, using a combination of overlapping primers internal and external to the exon. The sequence of exon 6 from *irs3* consistently showed a single nucleotide change (G → T) at position +1 (Fig. 2C). This base substitution is consistent with the mutation spectrum of ethyl nitrosourea, which was used to derive the *irs3* cell line, although G → T is one of the less common types of substitution found (38, 39). This change alters the consensus for the 3’

acceptor splice site (see “Discussion”), and gives an amino acid change (Val → Phe). While it is difficult to comment on the importance of this residue for mammalian species, since it is one of many highly conserved amino acids (Fig. 1), comparisons to putative RAD51L2 orthologues in plant species show that this valine is widely conserved (Fig. 2C, which shows comparisons to the cress *Arabidopsis thaliana* and rice *Oryza sativa* where the overall identity to the human protein is reduced to about 40%).

The effect of this mutation on RAD51L2 protein levels is seen in Fig. 2D. We found that polyclonal (not shown) or monoclonal

FIG. 3. Transfection of RAD51L2 into *irs3* increases damage resistance. A, survival of V79, *irs3*, and *irs3*-transfected with RAD51L2 cDNA, RAD51L2 genomic DNA (PAC 259-O5), or RAD51, after chronic treatment with mitomycin C. Closed symbols, *irs3* or V79 cells; open symbols, transfected clones of *irs3* cells (upright triangle, RAD51L2 cDNA; inverted triangle, RAD51L2 PAC; circle, RAD51 cDNA). Results from two to three experiments per cell line or clone; transfected clone data shown as mean of two independent clones per transfected gene (error bars = S.E. of the mean). B, localization of PAC 259-O5 to human chromosome 17q (bright signals). C, X-ray survival of V79, *irs3* (closed symbols), and *irs3* transfected with RAD51L2 cDNA (open symbols). Results from two experiments (error bars = S.E. of the mean).



antibodies raised against human RAD51L2 recognized the hamster protein (V79 extract, lane 2), whereas RAD51L2 was not seen in the *irs3* extract (lane 3). The levels of RAD51 were the same in wild type V79 and the *irs3* cell lines (lanes 5 and 6).

The Human RAD51L2 Gene Restores DNA Damage Resistance to *irs3*—To show that the phenotype of *irs3* is related to the observed RAD51L2 defect, we transfected the human RAD51L2 cDNA into *irs3* cells. To ensure that any responses found were specific to this gene, we also transfected separately the human RAD51L1 and RAD51L3 cDNAs into *irs3*. Cell clones carrying stably integrated cDNAs were selected using dominant drug-resistant markers present in the vectors, and were subsequently tested for resistance to MMC. None of the clones transfected with RAD51L1 or RAD51L3 showed an increase in MMC resistance, despite the presence of transcripts from the full-length gene as seen by RT-PCR (data not shown). However, with RAD51L2, a fraction of the selected clones showed significant MMC resistance. To verify this result under conditions where the RAD51L2 cDNA is expressed coordinately with the dominant selectable marker, we recloned RAD51L2 into an expression vector with an internal ribosome entry (IRES) site. Following transfection of this construct, every clone showing the presence of transcripts from the RAD51L2 gene product was found to be resistant to MMC (Fig. 3A).

In addition to complementation tests with the human RAD51L2 cDNA, we identified human genomic (PAC) DNA carrying the RAD51L2 gene (see "Experimental Procedures"). In Fig. 3B we show that the PAC DNA mapped to the expected chromosomal site of the gene, chromosome 17q (12) (now known to be at 17q23; see genome.ucsc.edu/). Stable PAC-

transfected clones of *irs3* cells gave a similar level of MMC resistance to the cDNA carrying clones (Fig. 3A). We conclude that there is a direct correlation between the presence of the RAD51L2 gene and the restoration of MMC resistance to *irs3* cells.

Cells deficient in homologous recombination genes show sensitivity to a number of DNA-damaging agents, in addition to MMC. We have previously shown an ~2-fold increase in sensitivity to x-rays in *irs3* (23). In the present series of experiments, we confirmed this radiation response and in agreement with the MMC data showed that the RAD51L2 cDNA was able to partially complement the *irs3* X-ray sensitivity (Fig. 3C).

Elevated Isochromatid Breaks and Reduced Sister Chromatid Exchange in *irs3*—Importantly, recombination-deficient cells show spontaneous genetic instability, and *irs3* is no exception to this finding. The frequency of chromosome damage in *irs3* cells was substantially higher than in the V79 parent line, and we found again that the presence of RAD51L2 largely complemented this defect (Table I). It is notable that that the frequency of isochromatid breaks was especially high in *irs3* (77% of total breaks; see "Discussion").

SCE are thought to result from molecular crossing over of DNA strands, especially arising during replication when recombination may rescue forks stalled at sites of damage. A small reduction in spontaneous SCE frequency was found for *irs3* (Table II). However, using the same MMC concentration for both V79 and *irs3* cells (4×10^{-7} M; i.e. not allowing for their survival difference) *irs3* cells gave a highly significant 30% reduction in SCE frequency. If differences in the MMC sensitivity of V79 and *irs3* are taken into account, using approxi-

TABLE I
High levels of spontaneous chromosome damage in *irs3*

Cell line	Cells scored	Chromatid breaks	Isochromatid breaks	Exchanges	Total aberrations (per 100 cells)
V79	204	0	2	0	2 (1.0)
<i>irs3</i>	249	5	17	11	33 (13.2)
<i>irs3</i> + <i>RAD51L2</i> clone 1	205	1	3	2	6 (2.9)
<i>irs3</i> + <i>RAD51L2</i> clone 2	206	4	4	0	8 (3.9)

TABLE II
Reduced frequencies of spontaneous and mitomycin C-induced sister chromatid exchanges in *irs3*

Cell line	Cells scored	SCE/cell (± se)	p Value ^a
V79	101	5.5 ± 0.2	0.0002
<i>irs3</i>	103	4.4 ± 0.2	
V79 + MMC (4 × 10 ⁻⁷ M) ^b	153	48.0 ± 1.1	<10 ⁻⁶
<i>irs3</i> + MMC (4 × 10 ⁻⁷ M) ^b	127	33.2 ± 1.1	
<i>irs3</i> + <i>RAD51L2</i> clone 1 (4 × 10 ⁻⁷ M)	98	51.0 ± 1.2	<10 ⁻⁶
<i>irs3</i> + <i>RAD51L2</i> clone 2 (4 × 10 ⁻⁷ M)	100	42.4 ± 1.2	<10 ⁻⁶
V79 + MMC (4 × 10 ⁻⁶ M) ^c	76	88.2 ± 2.4	
<i>irs3</i> + MMC (4 × 10 ⁻⁸ M) ^d	105	8.1 ± 0.3	

^a Based on Mann-Whitney U-tests, comparing either V79 with *irs3* or the *RAD51L2*-transfected clones with *irs3* at the same dose of MMC.
^b Data summed from two independent experiments, where the frequencies for V79 were 47.5 and 48.9 SCE/cell, and for *irs3* were 33.2 and 33.2 SCE/cell, respectively.
^c Equivalent survival level to 4 × 10⁻⁷ M for *irs3*.
^d Equivalent survival level to 4 × 10⁻⁷ M for V79.

mately equitoxic MMC concentrations, much larger differences in SCE frequency were found. Again, stable clones transfected with the *RAD51L2* cDNA showed a substantial increase in SCE frequencies compared with the *irs3* line (Table II).

Defective Formation of RAD51-like Protein Complexes in *irs3*—It has recently been shown that two distinct complexes of RAD51-like proteins occur in human cells; a heterodimer of RAD51L2 with XRCC3 (16, 17), and a heterotetramer of RAD51L1, RAD51L2, RAD51L3, and XRCC2 (18, 20, 22). To show that hamster V79 cells also contain these complexes, we immunoprecipitated the RAD51-like proteins from extracts using appropriate polyclonal antibodies. As seen in Fig. 4A, anti-RAD51L2 antibodies pulled down XRCC3 from V79, representing the heterodimer (Fig. 4B). Use of anti-XRCC2 antibodies pulled down RAD51L1, RAD51L2, and RAD51L3, but not XRCC3 (Fig. 4C), demonstrating the presence of the heterotetramer in V79 cells (Fig. 4D). In *irs3* extracts, neither of these complexes was formed (Fig. 4, A and C), although RAD51L3 was pulled down by anti-XRCC2 antibodies indicating that the RAD51L3/XRCC2 heterodimer is formed (Fig. 4C).

Damage-dependent RAD51 Focus Formation Is Reduced in *irs3*—The *RAD51L2* gene has been suggested to be involved in RAD51-dependent repair processes in mammalian cells (15), although definitive proof of this is lacking. An important indicator of RAD51 response is the formation of nuclear foci following x-ray damage (35, 40, 41). Measurement of RAD51 foci in *irs3* cells showed that this response was significantly reduced when compared with V79 cells. In *irs3* clones transfected with *RAD51L2* cDNA the RAD51 focus formation was partially restored, in agreement with the other responses measured (Fig. 5, A and B). A low frequency of RAD51 foci forms in unirradiated cells, primarily in S-phase (42), suggesting that differences in cell-cycle distribution may influence the ability to detect foci in *irs3* after irradiation. However, cell-cycle profiles for irradiated *irs3* and V79 cells were very similar, with most cells blocked in G₂ phase at 5 h after a dose of 10 Gray (data not shown). The formation of RAD51 foci may also be affected by the levels of RAD51 protein in *irs3* relative to V79, but we found that RAD51 levels were similar in *irs3*, in *irs3* lines transfected with *RAD51L2*, and in V79, both before and after

irradiation (Fig. 2D and data not shown).

Finally we wished to know whether, as in both yeast (43) and chick (44) cells, the *RAD51* gene itself can partially correct DNA-damage sensitivity when expressed in cell lines defective in RAD51-like genes. To check this possibility, we cloned the human *RAD51* cDNA into the IRES vector and transfected it into *irs3*. However, clones expressing *RAD51* transcript were found to survive MMC treatment no better than *irs3* itself (Fig. 3A).

DISCUSSION

Our data show a direct correlation between the presence of the *RAD51L2* cDNA and resistance to MMC in the *irs3* cells, while the *RAD51L1* and *RAD51L3* cDNAs showed no ability to complement. In support of these results, we also found that human genomic DNA carrying the *RAD51L2* gene significantly complemented the MMC sensitivity of *irs3*. *RAD51L2* cDNA or genomic DNA did not fully complement this phenotype, suggesting that the human *RAD51L2* gene is not fully functional in hamster cells. In addition to the complementation of sensitivity to MMC, we found a similar degree of complementation for x-ray sensitivity, spontaneous chromosomal damage, SCE formation, and damage-dependent RAD51 focus formation. These data reinforce the conclusion that mutation of the *RAD51L2* gene causes the *irs3* phenotype.

The mutation detected in exon 6 of *RAD51L2* in *irs3* (G → T at position +1) is not typical of splicing defects, which commonly alter intronic consensus sites. However, the first and last base of exons do form part of the consensus splice junction sequences, where the base G is generally conserved and the base T is found in less than 10% of splice sites (45). Loss-of-function mutants arising from changes at these sites have been documented in well studied genes, such as human *HPRT* and *TP53* (46, 47). This mutation may reduce “exon definition”; exon 6 is relatively short (67 bp) for mammalian exons (average size 137 bp) and it is known that short exons have an increased chance of differential splicing (48). The loss of exon 6 (Fig. 2) from the full-length transcript provides a possible explanation for the *irs3* phenotype. While other splice variants occur in both *irs3* and wild-type cells, these may not contribute to the *irs3* phenotype (e.g. sequencing of different sized transcripts shows

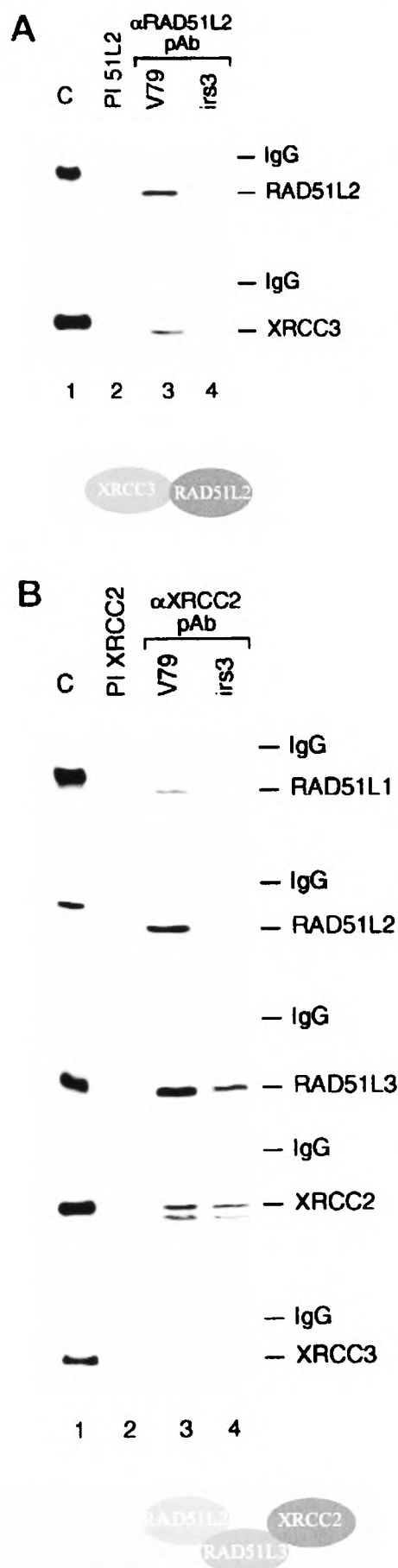


FIG. 4. Lack of RAD51-like protein complex formation in *irs3*. **A**, complex formation between endogenous RAD51L2 and XRCC3 hamster proteins. Protein complexes from V79 or *irs3* were precipitated using preimmune serum (PI; lanes 2) or anti-RAD51L2 pAbs (lanes 3–4), and revealed using anti-RAD51L2 or anti-XRCC3 mAbs as indicated. Lanes 1, marker proteins (human RAD51L2_{his10}, human XRCC3_{his6}). Lower diagram illustrates the complex between RAD51L2 and XRCC3 in V79 cells. **B**, interactions between the RAD51-like proteins in V79 and *irs3* hamster cells. Lanes 1, marker proteins (human RAD51L1_{his6}, RAD51L2_{his10}, RAD51L3_{his6}, XRCC2, XRCC3_{his6}); lanes 2, preimmune serum; lanes 3, co-immunoprecipitation of endogenous RAD51L1, RAD51L2, RAD51L3, and XRCC2 from hamster wild type extract; lanes 4, co-immunoprecipitation of RAD51L3 and XRCC2 from *irs3*. The origin of the doublet detected by XRCC2 antibodies (lanes 3 and 4) remains to be determined. Lower part illustrates the complex between RAD51L1, RAD51L2, RAD51L3, and XRCC2 in V79 cells. In **A** and **B** the His-tagged controls migrate more slowly than the endogenous hamster protein.

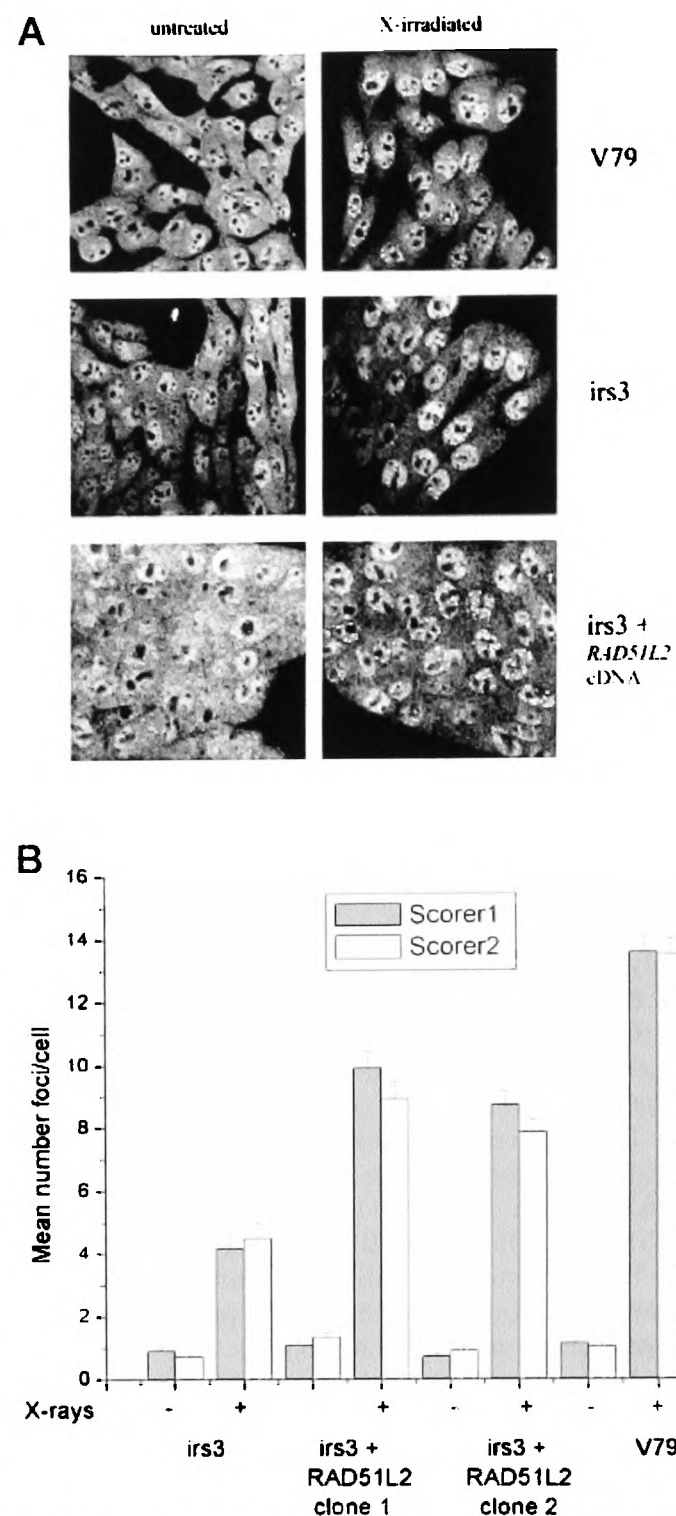


FIG. 5. Damage-dependent RAD51 focus formation is reduced in *irs3*. **A**, foci in wild type V79, *irs3*, and *irs3* cells transfected with *RAD51L2* cDNA, with and without X-ray exposure (measured at 5 h after 10 Gray). Representative fields are shown. **B**, quantitation of focus formation at 5 h following 10 Gray X-rays. Data shown for two independent scorers, with standard errors calculated from individual cell counts. All differences between irradiated cell lines were significant ($p < 10^{-6}$) except for the two clones transfected with *RAD51L2* ($p = 0.63$).

that all have loss of exon 6; data not shown). We detected no other type of *RAD51L2* transcript in *irs3*, consistent with the hemizygous nature of many genes in established hamster lines (49). We presume that these exon-deleted transcripts are not acting as dominant negatives, since the phenotype of *irs3* is recessive in hybrids with wild-type cells (32) and can be complemented by introduction of the wild type *RAD51L2* gene. The exon 6 mutation in *irs3* also gives rise to an amino acid change at position 271 (Val → Phe) which may play a part in the loss of gene function. However, the mutation has clear consequences for the level of RAD51L2 protein; none could be measured by Western blot analysis of *irs3* extracts (Fig. 2D).

Our RAD51-like protein interaction data support inferences initially developed from two-hybrid systems (15) suggesting that the association of RAD51L2 in complexes with

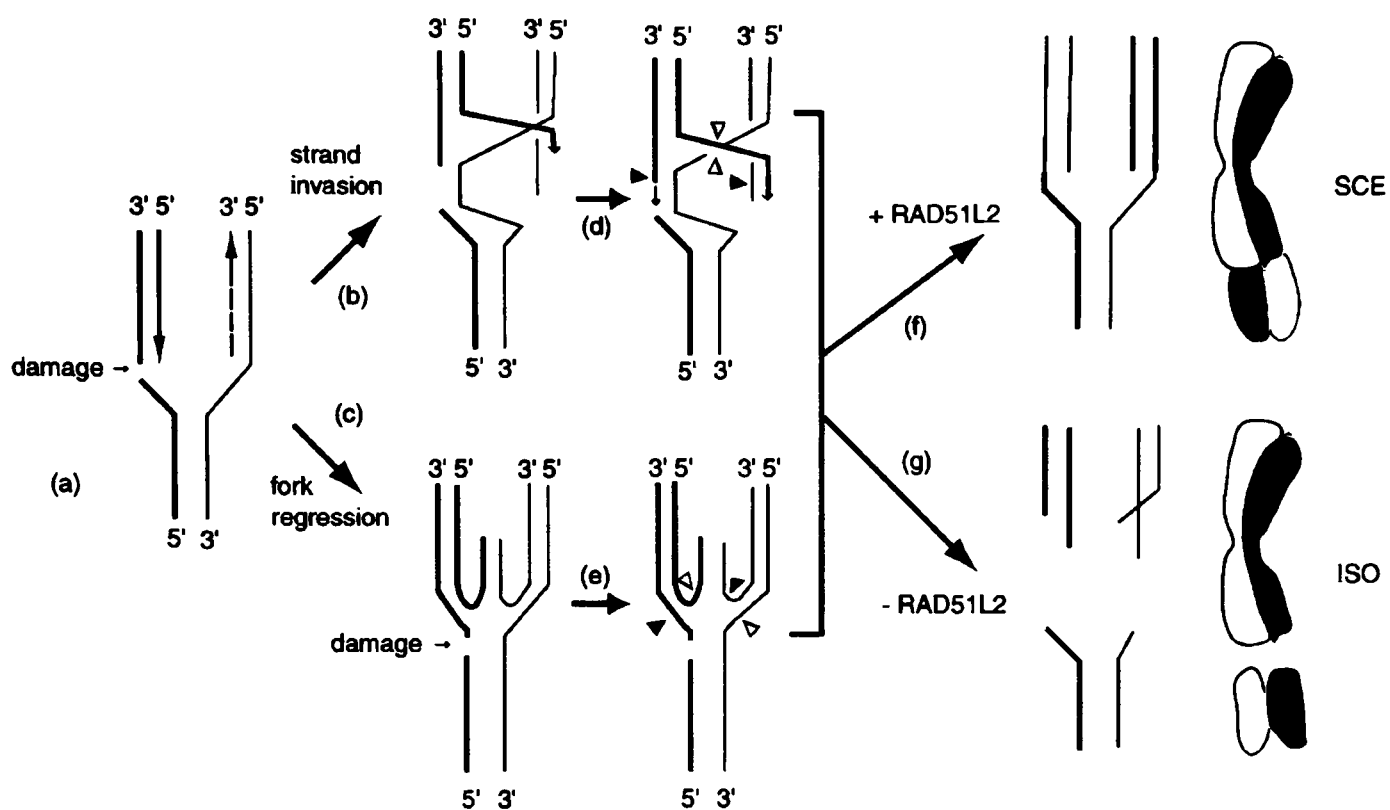


FIG. 6. Scheme for resolution of DNA damage on a replicating chromosome in the presence and absence of RAD51L2, either by strand invasion (a, b, and d) or fork regression (a, c, and e). a, stalled replicating DNA molecule with a strand break (thick and thin lines with large arrows: newly synthesized leading and lagging strands, respectively). b and d, strand exchange to form a recombination intermediate, followed by repair synthesis (small arrows). c and e, regression so that damage now behind fork, while newly synthesized strands can anneal to form a 4-way (Holliday) junction. At d and e the junctions may be resolved to give no crossover (cut at open arrowheads) or crossover (cut at closed arrowheads); however, the intermediate is very vulnerable, having 3 strands with "free" ends, and the fourth strand is broken by cutting (junction resolution) at the open arrowheads. f, RAD51L2 present, SCE can occur in a proportion of chromosomes if correct repair of damage is followed by crossing over; g, RAD51L2 absent, attempted repair in d or lack of repair (e) of the damage can lead to free ends in all strands and separation to give isochromatid breaks (ISO). Note that there would not necessarily be a direct numerical relationship between SCE and ISO formation.

other RAD51-like proteins may be functionally important. The interaction of human RAD51L2 with both XRCC3 (16, 17) and RAD51L1 (19, 21) has recently been confirmed in biochemical studies. Additionally it has been shown that there are two protein complexes containing RAD51L2 in human cells: one containing RAD51L2 and XRCC3, and the other consisting of four proteins, RAD51L1, RAD51L2, RAD51L3, and XRCC2 (18, 20–22). In the present study we have shown that these two RAD51-like protein complexes exist similarly in hamster cells, generalizing previous data. Strikingly, neither of these complexes is formed in *irs3*; the only complex remaining is the RAD51L3-XRCC2 heterodimer (14). Even this heterodimer may be less stable in the absence of RAD51L2, since slightly lower levels of these two proteins were consistently found in *irs3* (Fig. 4C). This is the first demonstration that the native proteins require RAD51L2 for complex formation.

The RAD51-like protein complexes bind preferentially to single-stranded DNA, suggesting that they may assist in the recruitment of RAD51 to sites of damaged DNA. For example, RAD51 filament formation occurs close to the RAD51L1-L2-L3-XRCC2 complex when it is bound to gapped duplex DNA (18). In chronic myeloid leukemia cells, lower levels of the RAD51L2-XRCC3 complex and higher levels of RAD51L1, RAD51L2, and XRCC2 (as well as RAD51) are expressed compared with normal cells (50). Furthermore, in brain tissue, where the key recombination protein RAD51 is in low abundance, it is suggested that the RAD51L2-XRCC3 complex carries out some of the functions of RAD51 (16). These studies suggest that the RAD51L2-XRCC3 complex may have a different role in recombination repair than the RAD51L1-L2-L3-XRCC2 complex. It is clear from our studies that RAD51L2 is a key component of both complexes, and therefore has a major role in homologous recombination processes in mammalian cells.

In eukaryotes there are several different pathways involving homologous recombination that can lead to the repair of DNA damage, and only some of these depend on RAD51 (51). In a number of different cell types it has been shown that RAD51 protein accumulates at nuclear foci following DNA damage, but we found that this process was much reduced in *irs3* cells. This finding links RAD51L2 to RAD51-dependent recombination pathways, joining a growing list of mammalian proteins, including XRCC2 (31), XRCC3 (35), BRCA1 (52), and BRCA2 (53) implicated by lack of RAD51 focus formation.

The *irs3* cells have a phenotype consistent with loss of homologous recombination repair, including their profile of sensitivity to DNA-damaging agents, spontaneous genetic instability, and reduction in SCE frequency. Very recently chick DT40 cells with a disruption of a homologue of the *RAD51L2* gene have also been shown to have these defects, although to different degrees (44). For example, chick *RAD51L2* homologue-deficient cells show similar survival differences to *irs3*, compared with wild type, in response to MMC or X-rays. However, the chick mutant cells have a larger relative reduction in SCE frequency than *irs3*, possibly because of a greater reliance of chick lymphoblastoid cells on homologous recombination for repair (54). The validity of these comparisons will depend on whether the chick gene is an orthologue of the mammalian *RAD51L2* genes (the full coding sequence of the chick gene has not been identified). Significantly, we found that the human *RAD51* cDNA could not complement the defect in *irs3*, while human *RAD51* can partially complement the *RAD51L2*-homologue gene knock-out in chick cells (44). Other differences between the functioning of homologous recombination proteins in chick and mammalian cells have previously been observed: for example, RAD54 is required for RAD51 focus formation in mouse cells (55), but not in chick cells (56). Taken together these data emphasize the need for caution in extrapolating functional

data for homologous recombination from one species to another.

A further feature of the present data that supports the proposed defect in homologous recombination in *irs3* is the reduction in SCE frequency together with an increase in chromosome aberrations (in particular, isochromatid breaks). While SCE can arise from crossing-over during homologous recombination repair, isochromatid breaks (Table I) can be viewed as one outcome of failure to properly carry out homologous recombination during replication. When a replication fork stalls due to a break on one DNA strand, the break may be resolved by RAD51-dependent recombination, including strand transfer and Holliday junction formation (Fig. 6, *a*, *b*, and *d*). Alternatively the fork may regress (57, 58) to allow damage repair against an intact strand, but this allows the annealing of newly synthesized strands and the formation of a Holliday junction at the fork (Fig. 6, *a*, *c*, and *e*). In either case the replicating DNA is in a highly vulnerable state, where incomplete resolution of the junctions could lead to disintegration of the intermediate structure (as shown in Fig. 6g). We suggest that in *irs3*, lack of the RAD51L2 protein increases the probability of disintegration at this stage, yielding isochromatid breaks. Such a failure would also lead to a reduction in the frequency of SCE. Support for these models is seen when a transfected human *RAD51* cDNA is down-regulated in chick *RAD51* knockout cells: a massive increase in chromosomal damage occurs, especially isochromatid breaks (59), associated with an ~50% reduction in SCE frequency (60). The first model (Fig. 6, *a*, *b*, and *d*), however, implies that RAD51-dependent processes are still (at least partially) active in *irs3*. This is supported by our finding that some damage-dependent RAD51 focus formation occurs in *irs3*. RAD51 in these circumstances may not form stable recombination intermediates, leaving these vulnerable to disintegration. In the second model (Fig. 6, *a*, *c*, and *e*) RAD51 is not required for isochromatid formation, consistent with the findings in RAD51-deficient chick cells (see above); in the absence of RAD51L2 the ability of RAD51-dependent recombination to rescue the fork is severely compromised, and isochromatid breaks may result. While the RAD51-like proteins are thought to be involved primarily in early stages of homologous recombination repair, to promote strand exchange by RAD51 (8), the first model suggests that RAD51L2 may normally have an additional role in later stages of recombination repair. This finding is not general for other RAD51-like proteins; for example, the XRCC2-deficient cell line *irs1* does not show a striking increase in isochromatid breaks (31).³

The high degree of conservation of RAD51L2 between mammalian species suggests that the gene has an important function in mammals, although this has yet to be clearly defined. We believe that the availability of a mammalian cell line lacking RAD51L2 activity will be of considerable value in identifying this function.

Acknowledgments—We are grateful to David Papworth, Cathryn Tambini, and Stuart Townsend for assistance and advice.

REFERENCES

- Hoeijmakers, J. H. (1993) *Trends Genet.* **9**, 211–217
- Jeggo, P. A. (1998) *Radiat. Res.* **150**, S80–S91
- Thacker, J. (1999) *Biochimie (Paris)* **81**, 77–85
- Thompson, L. H., and Schild, D. (1999) *Biochimie (Paris)* **81**, 87–105
- Baumann, P., and West, S. C. (1998) *Trends Biochem. Sci.* **23**, 247–251
- Sung, P. (1997) *Genes Dev.* **11**, 1111–1121
- Game, J. C. (1993) *Semin. Cancer Biol.* **4**, 73–83
- Thacker, J. (1999) *Trends Genet.* **15**, 166–168
- Albala, J. S., Thelen, M. P., Prange, C., Fan, W., Christensen, M., Thompson, L. H., and Lennon, G. G. (1997) *Genomics* **46**, 476–479
- Rice, M. C., Smith, S. T., Bullrich, F., Havre, P., and Kmiec, E. B. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7417–7422
- Cartwright, R., Dunn, A. M., Simpson, P. J., Tambini, C. E., and Thacker, J. (1998) *Nucleic Acids Res.* **26**, 1653–1659
- Dosanjh, M. K., Collins, D. W., Fan, W., Lennon, G. G., Albala, J. S., Shen, Z., and Schild, D. (1998) *Nucleic Acids Res.* **26**, 1179–1184
- Pittman, D. L., Weinberg, L. R., and Schimenti, J. C. (1998) *Genomics* **49**, 103–111
- Braybrooke, J. P., Spink, K. G., Thacker, J., and Hickson, I. D. (2000) *J. Biol. Chem.* **275**, 29100–29106
- Schild, D., Lio, Y., Collins, D. W., Tsomondo, T., and Chen, D. J. (2000) *J. Biol. Chem.* **275**, 16443–16449
- Kurumizaka, H., Ikawa, S., Nakada, M., Eda, K., Kagawa, W., Takata, M., Takeda, S., Yokoyama, S., and Shibata, T. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5538–5543
- Masson, J. Y., Stasiak, A. Z., Stasiak, A., Benson, F. E., and West, S. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8440–8446
- Masson, J. Y., Tarsounas, M. C., Stasiak, A. Z., Stasiak, A., Shah, R., McIlwraith, M. J., Benson, F. E., and West, S. C. (2001) *Genes Dev.* **15**, 3296–3307
- Sigurdsson, S., Van Komen, S., Bussen, W., Schild, D., Albala, J. S., and Sung, P. (2001) *Genes Dev.* **15**, 3308–3318
- Liu, N., Schild, D., Thelen, M. P., and Thompson, L. H. (2002) *Nucleic Acids Res.* **30**, 1009–1015
- Miller, K. A., Yoshikawa, D. M., McConnell, I. R., Clark, R., Schild, D., and Albala, J. S. (2002) *J. Biol. Chem.* **277**, 8406–8411
- Wiese, C., Collins, D. W., Albala, J. S., Thompson, L. H., Kronenberg, A., and Schild, D. (2002) *Nucleic Acids Res.* **30**, 1001–1008
- Jones, N. J., Cox, R., and Thacker, J. (1987) *Mutat. Res.* **183**, 279–286
- Tambini, C. E., George, A. M., Rommens, J. M., Tsui, L. C., Scherer, S. W., and Thacker, J. (1997) *Genomics* **41**, 84–92
- Cartwright, R., Tambini, C. E., Simpson, P. J., and Thacker, J. (1998) *Nucleic Acids Res.* **26**, 3084–3089
- Liu, N., Lamerdin, J. E., Tebbs, R. S., Schild, D., Tucker, J. D., Shen, M. R., Brookman, K. W., Siciliano, M. J., Walter, C. A., Fan, W., Narayana, L. S., Zhou, Z. Q., Adamson, A. W., Sorensen, K. J., Chen, D. J., Jones, N. J., and Thompson, L. H. (1998) *Mol. Cell* **1**, 783–793
- Thacker, J., Ganesh, A. N., Stretch, A., Benjamin, D. M., Zahalsky, A. J., and Hendrickson, E. A. (1994) *Mutagenesis* **9**, 163–168
- Tucker, J. D., Jones, N. J., Allen, N. A., Minkler, J. L., Thompson, L. H., and Carrano, A. V. (1991) *Mutat. Res.* **254**, 143–152
- Griffin, C. S., Simpson, P. J., Wilson, C. R., and Thacker, J. (2000) *Nat. Cell Biol.* **2**, 757–761
- Johnson, R. D., Liu, N., and Jasin, M. (1999) *Nature* **401**, 397–399
- O'Regan, P., Wilson, C., Townsend, S., and Thacker, J. (2001) *J. Biol. Chem.* **276**, 22148–22153
- Jones, N. J., Cox, R., and Thacker, J. (1988) *Mutat. Res.* **193**, 139–144
- Jones, N. J., Ellard, S., Waters, R., and Parry, E. M. (1993) *Carcinogenesis* **14**, 2487–2494
- Peterson, C., and Legerski, R. (1991) *Gene (Amst.)* **107**, 279–284
- Bishop, D. K., Ear, U., Bhattacharyya, A., Calderone, C., Beckett, M., Weichselbaum, R. R., and Shinohara, A. (1998) *J. Biol. Chem.* **273**, 21482–21488
- Perry, P., and Wolff, S. (1974) *Nature* **251**, 156–158
- Savage, J. R. (1976) *J. Med. Genet.* **13**, 103–122
- Shibuya, T., and Morimoto, K. (1993) *Mutat. Res.* **297**, 3–38
- Justice, M. J., Noveroske, J. K., Weber, J. S., Zheng, B., and Bradley, A. (1999) *Hum. Mol. Genet.* **8**, 1955–1963
- Haaf, T., Golub, E. I., Reddy, G., Radding, C. M., and Ward, D. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2298–2302
- Raderschall, E., Golub, E. I., and Haaf, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1921–1926
- Tashiro, S., Kotomura, N., Shinohara, A., Tanaka, K., Ueda, K., and Kamada, N. (1996) *Oncogene* **12**, 2165–2170
- Johnson, R. D., and Symington, L. S. (1995) *Mol. Cell. Biol.* **15**, 4843–4850
- Takata, M., Sasaki, M. S., Tachiiri, S., Fukushima, T., Sonoda, E., Schild, D., Thompson, L. H., and Takeda, S. (2001) *Mol. Cell. Biol.* **21**, 2858–2866
- Shapiro, M. B., and Senapathy, P. (1987) *Nucleic Acids Res.* **15**, 7155–7174
- Steingrimsdottir, H., Rowley, G., Dorado, G., Cole, J., and Lehmann, A. R. (1992) *Nucleic Acids Res.* **20**, 1201–1208
- Warneford, S. G., Witton, L. J., Townsend, M. L., Rowe, P. B., Reddel, R. R., Dalla-Poazz, L., and Symonds, G. (1992) *Cell Growth Differ.* **3**, 839–846
- Berget, S. M. (1995) *J. Biol. Chem.* **270**, 2411–2414
- Siminovich, L. (1976) *Cell* **7**, 1–11
- Slupianek, A., Schmutte, C., Tomblin, G., Nieborowska-Skorska, M., Hoser, G., Nowicki, M. O., Pierce, A. J., Fishel, R., and Skorski, T. (2001) *Mol. Cell* **8**, 795–806
- Paques, F., and Haber, J. E. (1999) *Microbiol. Mol. Biol. Rev.* **63**, 349–404
- Bhattacharyya, A., Ear, U. S., Koller, B. H., Weichselbaum, R. R., and Bishop, D. K. (2000) *J. Biol. Chem.* **275**, 23899–23903
- Yuan, S. S. F., Lee, S. Y., Chen, G., Song, M. H., Tomlinson, G. E., and Lee, E. (1999) *Cancer Res.* **59**, 3547–3551
- Sonoda, E., Takata, M., Yamashita, Y. M., Morrison, C., and Takeda, S. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8388–8394
- Tan, T. L., Essers, J., Citterio, E., Swagemakers, S. M., de Wit, J., Benson, F. E., Hoeijmakers, J. H., and Kanaar, R. (1999) *Curr. Biol.* **9**, 325–328
- Takata, M., Sasaki, M. S., Sonoda, E., Fukushima, T., Morrison, C., Albala, J. S., Thelen, M. P., Prange, C., Fan, W., Christensen, M., Thompson, L. H., and Lennon, G. G. (1997) *Genomics* **46**, 476–479

³ C. A. French, J.-Y. Masson, C. S. Griffin, P. O'Regan, S. C. West, and J. Thacker, unpublished data.

J. S., Swagemakers, S. M., Kanaar, R., Thompson, L. H., and Takeda, S. (2000) *Mol. Cell. Biol.* **20**, 6476–6482

57. Seigneur, M., Bidnenko, V., Ehrlich, S. D., and Michel, B. (1998) *Cell* **95**, 419–430

58. McGlynn, P., Lloyd, R. G., and Mariani, K. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 8235–8240

59. Sonoda, E., Sasaki, M. S., Buerstedde, J. M., Bezzubova, O., Shinohara, A.,

Ogawa, H., Takata, M., Yamaguchi-Iwai, Y., and Takeda, S. (1998) *EMBO J.* **17**, 598–608

60. Sonoda, E., Sasaki, M. S., Morrison, C., YamaguchiIwai, Y., Takata, M., and Takeda, S. (1999) *Mol. Cell. Biol.* **19**, 5166–5169

61. Leasure, C. S., Chandler, J., Gilbert, D. J., Householder, D. B., Stephens, R., Copeland, N. G., Jenkins, N. A., and Sharan, S. K. (2001) *Gene (Amst.)* **271**, 59–67