Double-Strand Break Repair by Non-homologous End-Joining

by

Claire Joanna Chappell

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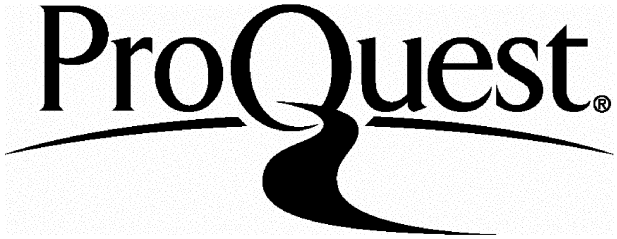

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Abstract

Non-homologous end-joining (NHEJ) is an important mechanism of double-strand break repair (DSBR) which is largely conserved from yeast to humans. Mammalian NHEJ is dependent on the Ku, DNA-PK_{cs}, XRCC4 and DNA ligase IV proteins. NHEJ-defective rodent cells are highly sensitive to ionising radiation, implicating NHEJ as a critical mechanism for the repair of radiation-induced double-strand breaks (DSBs). DNA termini at sites of radiation-induced DSBs exhibit modifications, including the presence of 5'-hydroxyl and 3'-phosphate or phosphoglycolate groups. During DSBR, these termini must undergo enzymatic processing to return the normal 5'-phosphate and 3'-hydroxyl groups required for ligation. Despite the activities of the core NHEJ proteins being well documented, little is known about the auxiliary processing factors required for repair of ionising radiation-induced breaks.

Using an *in vitro* DNA end-joining assay catalysed by human cell-free extracts I have investigated the wider protein requirements of NHEJ and the ability of extracts to join certain modified DNA termini. In contrast to the essential role for the Mre11 and Xrs2 proteins in the joining of complementary termini in *Saccharomyces cerevisiae*, the human homologues, MRE11 and NBS1 were not required for the joining of complementary protruding 5' termini. Joining of modified and non-complementary termini implicated requirements for exonuclease and polymerase activities during extract-catalysed end-joining. Moreover, end-joining reactions with DNA molecules containing 5'-hydroxyl termini demonstrated a role for polynucleotide kinase (PNK) in the repair of radiation-induced breaks. The defective end-joining observed with PNK-depleted extracts was complemented by addition of physiological amounts of purified human PNK, but not T4 PNK. In addition, phosphorylation of 5'-hydroxyl termini was not observed when DNA end-joining was blocked, either by use of XRCC4 antibodies or DNA-PK_{cs}-defective extracts, implicating co-ordination between the phosphorylation and end-joining reactions. Finally, gel filtration and coimmunoprecipitation experiments indicated that PNK might be associated with the NHEJ proteins *in vivo*.

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Abbreviations

aa	amino acid
AFM	atomic force microscopy
AT	ataxia telangiectasia
ATLD	ataxia telangiectasia-like disorder
bp	base pair
BRCT	BRCA1 carboxy-terminus
BSA	bovine serum albumin
ccc DNA	covalently closed circular DNA
CIP	calf intestinal phosphatase
C-terminus	carboxy-terminus
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
$DNA\text{-}PK_{CS}$	DNA-dependent protein kinase - catalytic subunit
DSB	double-strand break
DSBR	double-strand break repair
dsDNA	double-strand DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ES	embryonic stem
FHA	fork-head associated
GST	glutathione S-transferase
H	hydrogen
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethenesulfonic acid)
HLB	hypotonic lysis buffer
HR	homologous recombination
hrs	hours
IP	immunoprecipitation
IR	ionising radiation
kb	kilobase pair
kDa	kilodalton

LB	Luria broth
MEFs	mouse embryonic fibroblasts
MDa	mega dalton
min	minutes
MMS	methyl methane sulphonate
MMTV	mouse mammary tumour virus
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
mRNA	messenger RNA
NBS	Nijmegen breakage syndrome
NHEJ	non-homologous end-joining
NRE	negative regulatory element
nt	nucleotide
N-terminus	amino-terminus
OH	hydroxyl
oligo	oligonucleotide
P	phosphate
^{32}P	phosphorous-32
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PG	phosphoglycolate
PI 3-kinase	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol 3-kinase-related kinase
PNK	polynucleotide kinase
Q	glutamine
RNA	ribonucleic acid
rpm	revolutions per minute
RSS	recombination signal sequence
S	serine
SCID	severe combined immune-deficient
SDS	sodium dodecyl sulphate
SSA	single-strand annealing

SSB	single-strand break
SSBR	single-strand break repair
ssDNA	single-strand DNA
SV40	simian virus 40
T	threonine
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
Tween-20	polyoxyethylenesorbitan monolaurate
UV	ultraviolet
V(D)J	variable, diversity and joining
vol	volume(s)

Nucleotides and cofactors

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMP-PNP	5'-adenylylimidodiphosphate
ATP	adenosine 5'-triphosphate
ATPγS	adenosine 5'-O-(3-thiotriphosphate)
CTP	cytidine 5'-triphosphate
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythimidine 5'-triphosphate
dGMP	deoxyguanosine 5'-monophosphate
dGTP	deoxyguanosine 5'-triphosphate
diADE	P^1,P^4-di(adenosine-5')tetraphosphate

dTTP	deoxythimidine 5'-triphosphate
GTP	guanosine 5'-triphosphate
IP_6	inositol hexakisphosphate
NAD^+	nicotinamide adenine dinucleotide
NaP	sodium phosphate
NaPPi	sodium pyrophosphate
NaTriP	pentasodium tripolyphosphate
UTP	uridine 5'-triphosphate

I. Non-homologous end-joining as a double-strand break repair mechanism

DNA double-strand breaks (DSBs) present a serious threat to the integrity of the genome. These lesions result from exposure of cells to exogenous agents such as ionising radiation (IR) and the anticancer drugs bleomycin, etoposide and adriamycin. They are also produced endogenously by free radicals, a by-product of normal oxidative metabolism, and stalled DNA replication forks. Moreover, DNA DSBs are specifically induced in certain cells to facilitate rearrangement of the genome. For example, DSBs are generated during meiosis to initiate recombination between homologous chromosomes. DSBs are also induced during V(D)J recombination; a specialised process of immunoglobulin gene rearrangements used in lymphocyte development to create different antigen-recognition sites.

Unrepaired DSBs can have fatal consequences for cells. In unicellular organisms such as yeast, a single unrepaired break is sufficient to cause the death of the cell (Resnick and Martin, 1976; Bennett *et al.*, 1993). Furthermore, the ends of broken chromosomes are highly recombinogenic and in multicellular organisms the resulting chromosomal aberrations such as gene deletions and translocations may induce cancer.

Mechanisms of double-strand break repair

In response to the threat of DSBs, eukaryotic cells have developed three different mechanisms for repair of these lesions: homologous recombination (HR), single-strand annealing (SSA) and non-homologous end-joining (NHEJ).

Homologous recombination

Homologous recombination accurately repairs double strand breaks using the undamaged sister chromatid or homologous chromosome as a template for repair (see Fig. 1.1). Mammalian proteins identified as being required for HR include the products of the *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2*, *XRCC3*, *MRE11* and *NBS1* genes (reviewed in Khanna and Jackson, 2001). On the whole, the mechanism of homologous recombination has been conserved from yeast[1] to humans with the same key proteins required in both organisms (Baumann and West, 1998b; Kanaar *et al.*, 1998). The first step in HR is the processing of the DSB and generation of 3' single-strand (ss) tails. In yeast this is catalysed by the Mre11, Rad50 and Xrs2 proteins which associate to form a heterotrimeric complex (Ivanov *et al.*, 1994; Johzuka and Ogawa, 1995; Tsubouchi and Ogawa, 1998; Usui *et al.*, 1998). The mammalian homologues, MRE11, RAD50 and NBS1 are also thought to be required for the

[1] In this thesis any reference to yeast indicates *Saccharomyces cerevisiae* unless stated otherwise.

Figure 1.1. Different mechanisms of double-strand break repair.

DSBs caused by ionising radiation, free radicals or chemical damage can be repaired by homologous recombination, single-strand annealing or non-homologous end-joining. Homologous recombination is catalysed by proteins encoded by the *RAD52* epistasis group of genes, of which the RAD51 protein (indicated by yellow ovals) is essential for strand invasion and homologous pairing. Single-strand annealing occurs within repetitive gene sequences. This process requires RAD52 (green octagons) in mammals and Rad52 and Rad59 in yeast. The single-strand flaps are removed by ERCC1 and XPF (represented by red triangles). The third DSB repair mechanism, NHEJ, is catalysed by the Ku heterodimer (yellow circles), DNA-PK_{cs} (dark blue ovals), XRCC4 (purple diamonds) and DNA ligase IV (light blue hexagon).

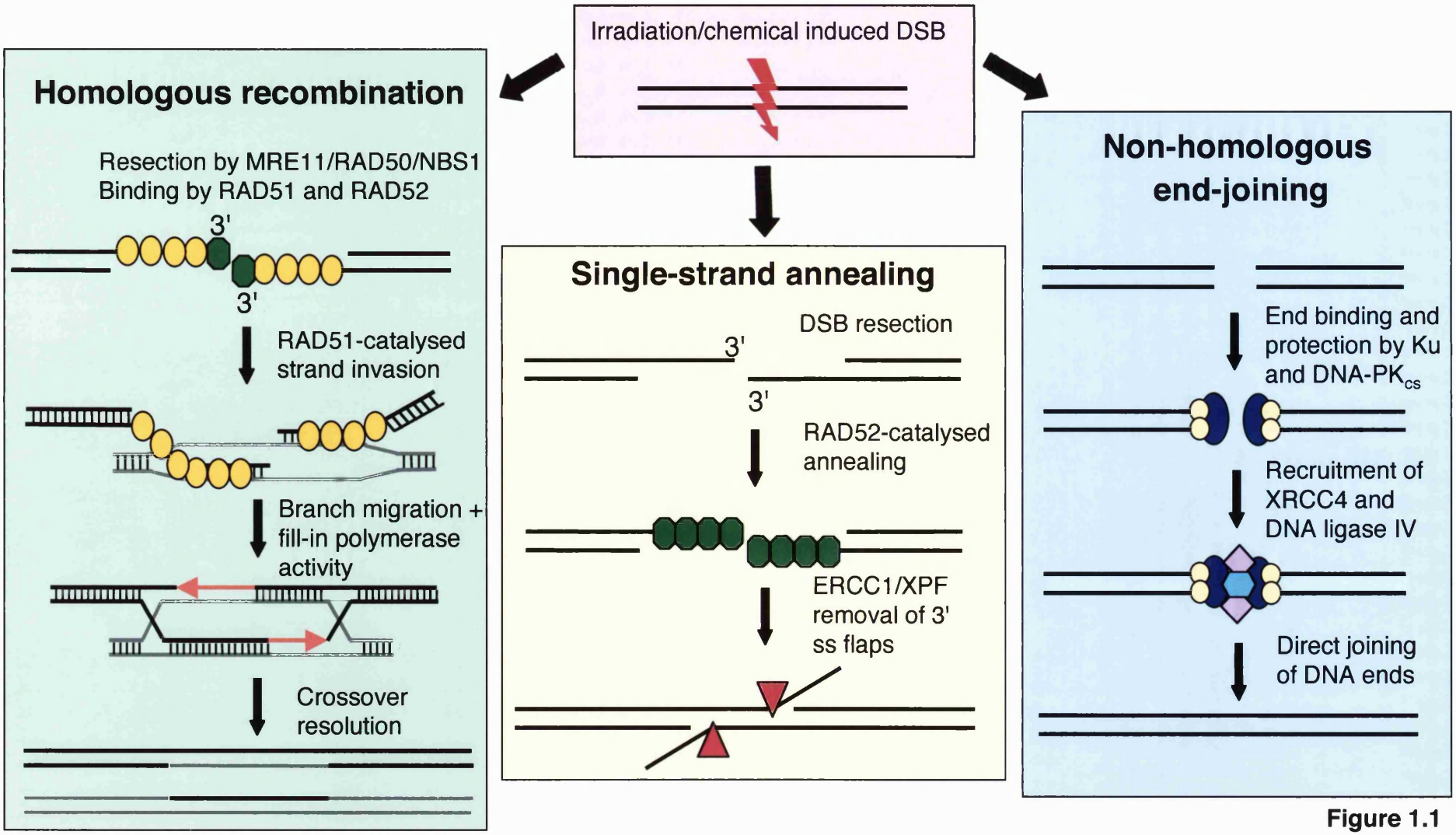

Irradiation/chemical induced DSB
Homologous recombination
Resection by MRE11/RAD50/NBS1
Binding by RAD51 and RAD52
3'
3'
RAD51-catalysed
strand invasion
Branch migration +
fill-in polymerase
activity
Crossover
resolution
Single-strand annealing
DSB resection
3'
3'
RAD52-catalysed
annealing
ERCC1/XPF
removal of 3'
ss flaps
Non-homologous
end-joining
End binding and
protection by Ku
and DNA-PKcs
Recruitment of
XRCC4 and
DNA ligase IV
Direct joining
of DNA ends

Figure 1.1

initial processing of the DSB. Whilst the NBS1 protein shares little similarity with Xrs2, both proteins form complexes with Rad50 and Mre11 and thus appear to be functional homologues (Carney *et al.*, 1998).

The single-stranded regions are then bound by RAD51 and this protein catalyses invasion and pairing of the ss tail with a sister chromatid (Sung, 1994; Baumann *et al.*, 1996). The RAD52, RAD54, RAD51B, RAD51C, RAD51D and XRCC2 and XRCC3 proteins are thought to facilitate the loading of RAD51 (Benson *et al.*, 1998; Bishop *et al.*, 1998; New *et al.*, 1998; Shinohara and Ogawa, 1998; Petukhova *et al.*, 1999; Sonoda *et al.*, 2001). RAD51 and RAD52 appear to have similar roles in both yeast and mammals, however there are differences in the auxiliary factors used during HR. Whilst human cells contain five RAD51 paralogs, (RAD51B, RAD51C, RAD51D and XRCC2 and XRCC3) yeast contain only two, Rad55 and Rad57 (Albala *et al.*, 1997; Sung, 1997; Cartwright *et al.*, 1998a; Cartwright *et al.*, 1998b; Dosanjh *et al.*, 1998; Liu *et al.*, 1998; Pittman *et al.*, 1998). Mammalian cells do not contain obvious homologues of the yeast *RAD55* and *RAD57* genes; however, the RAD51 paralogs show as much similarity with Rad57 as they do with RAD51 itself (reviewed in Thacker, 1999). The paralogs are, therefore, thought to substitute for Rad55 and Rad57 as the "accessory factors" for mammalian HR. Finally, studies in prokaryotes suggest that in the latter stages of HR further processing of the recombination intermediates generated by strand invasion includes branch migration and resolution of the DNA crossovers (Holliday junctions), DNA synthesis and nick ligation (West, 1997).

Single-strand annealing

An alternative pathway called single-strand annealing can be used when the DSB occurs in repetitive DNA sequences (reviewed in Haber, 2000; Sung *et al.*, 2000). If 5' to 3' resection of the DSB exposes direct repeat sequences, then these complementary sequences can anneal leading to loss of one of the two repeats and the intervening DNA (see Fig. 1.1). SSA is, therefore, a non-conservative type of recombinational repair. Repair is completed by nucleolytic removal of any non-homologous 3' tails and nick ligation. This process requires the products of the *RAD1*, *RAD10*, *RAD52* and *RAD59* genes in yeast, and presumably the homologues *XPF*, *ERCC1* and *RAD52* (no *RAD59* homologue has yet been identified) in mammalian cells (Sugawara and Haber, 1992; Ivanov and Haber, 1995; Bai and Symington, 1996).

Non-homologous end-joining

In comparison to HR and SSA, non-homologous end-joining does not require a homologous chromosome for repair, nor extensive sequence homology at the broken DNA termini. During NHEJ there may be limited processing of the DNA termini, but ultimately the two DNA ends are rejoined directly (Fig. 1.1). Although NHEJ can repair DSBs accurately, small deletions are often observed around the break site, and because of this, NHEJ is classed as an inaccurate or illegitimate repair pathway.

In mammalian cells, the proteins of the NHEJ pathway have been identified as Ku; the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs});

XRCC4 and DNA ligase IV (reviewed in Weaver, 1995; Jeggo, 1998; Lieber, 1999; Karran, 2000). The Ku protein and DNA-PK_{cs} make up the DNA-dependent protein kinase (DNA-PK), a serine/threonine kinase that is activated at DNA termini (Carter *et al.*, 1990; Dvir *et al.*, 1992). The Ku protein, consisting of a heterodimer of 70- and 80-kDa subunits, exhibits high affinity for DNA ends, allowing recruitment and activation of DNA-PK_{cs} (Mimori and Hardin, 1986; Blier *et al.*, 1993; Gottlieb and Jackson, 1993). DNA-PK is implicated in the protection of DNA ends against exonuclease attack, the alignment of the broken ends, and the initiation of intracellular DNA-damage-signalling pathways (Jeggo, 1997; Smith and Jackson, 1999). XRCC4 is a DNA-binding phosphoprotein, which forms a tight complex with DNA ligase IV and is a substrate for DNA-PK *in vitro* (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997; Leber *et al.*, 1998; Modesti *et al.*, 1999).

Yeast also possess a NHEJ pathway that utilises many of the same proteins as mammalian NHEJ (reviewed in Critchlow and Jackson, 1998; Tsukamoto and Ikeda, 1998). Yeast homologues of Ku70 and Ku80 have been identified as yKu70/Hdf1 and yKu80/Hdf2 (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a; Feldmann *et al.*, 1996). The homologues of XRCC4 and DNA ligase IV have also been identified as Lif1 (ligase-interacting factor 1) and Lig4 or Dnl4 (Schär *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997; Herrmann *et al.*, 1998). There does not appear, however, to be a *S. cerevisiae* homologue of DNA-PK_{cs}. Other proteins implicated as playing a role in NHEJ in yeast include Rad50, Mre11 and Xrs2. Repair assays designed to monitor NHEJ in

yeast cells, have demonstrated that not only does disruption of *RAD50*, *MRE11* or *XRS2* impair NHEJ, but that this inhibition is epistatic with the *KU70* or *LIG4* genes (Milne *et al.*, 1996; Boulton and Jackson, 1998).

Relative contributions of homologous recombination and non-homologous end-joining to repair of double-strand breaks

In yeast, mutation of the genes required for HR (*RAD51*, *RAD52*, *RAD54*, *RAD55* or *RAD57*) resulted in hypersensitivity to ionising radiation, whilst inactivation of the NHEJ protein yKu70 did not (Thompson and Schild, 1999). Disruption of *YKU70*, however, caused additional ionising radiation sensitivity in *rad52* mutants (Boulton and Jackson, 1996a; Milne *et al.*, 1996; Siede *et al.*, 1996). In yeast, therefore, NHEJ does contribute to DSB repair and genomic stability, but its effects are secondary and only apparent in the absence of HR.

Mammalian cells differ from yeast in the balance of the two major DSB repair pathways. Initially, the relative radiosensitivities of *rad52* mutants indicated that homologous recombination was of less importance in vertebrate cells than in yeast cells. Disruption of *RAD52* in yeast cells led to extreme sensitivity to ionising radiation, with between one and two DSBs inducing cell death in these cells (Resnick and Martin, 1976). Comparatively, *RAD52* knockouts in both mouse and chicken cells did not confer an increased sensitivity to IR or any other DNA damaging agents (Rijkers *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1998). Conversely, mutation of the NHEJ genes in mammals results in severe ionising radiation sensitivity (Lees-Miller *et al.*, 1995; Gu *et al.*, 1997;

Singleton *et al.*, 1997; Errami *et al.*, 1998; Gao *et al.*, 1998; Grawunder *et al.*, 1998a; Taccioli *et al.*, 1998; Kurimasa *et al.*, 1999b; Riballo *et al.*, 1999; O'Driscoll *et al.*, 2001). Indeed many of these genes were identified by their ability to complement the ionising radiation sensitivity of mutant rodent cells (Smider *et al.*, 1994; Taccioli *et al.*, 1994; Blunt *et al.*, 1995; Li *et al.*, 1995; Miller *et al.*, 1995; Peterson *et al.*, 1995). Additionally, transfection studies using mammalian cells showed that exogenous DNA was incorporated predominantly by non-homologous integration, rather than homologous integration (Ivanov and Haber, 1997). Together these data indicated that NHEJ was the primary DSB repair pathway in mammalian cells.

This hypothesis has been modified, however, in light of more recent studies. A rare-cutting endonuclease was used to generate a single DSB in one of two direct chromosomal repeats in hamster cells (Liang *et al.*, 1998). Analysis of the repair products, in brief, found that SSA accounted for ~30% of the repair events, whilst 14% of the products were formed by homologous recombination. The remaining repair products were generated by NHEJ, the majority of which displayed small deletions or insertions at the break site. The assay is limited because accurate repair by the NHEJ proteins was not scored, as it could not be distinguished from the failure of the endonuclease to cut at its recognition sequence. Additionally, the very fact that the DSB was induced in one of a pair of direct repeats may favour recombinational repair by the SSA pathway. Despite this, however, these results demonstrated that homologous recombination is used to repair DSBs in mammalian cells.

The use of knockouts in mice and the chicken B lymphocyte cell line, DT40, have also contributed to our understanding of the overlapping and distinct roles of different DSB repair pathways in vertebrate cells. In contrast to the viability of yeast *rad51* mutants, *RAD51*$^{-/-}$ mice die early in embryonic development, demonstrating a requirement for RAD51 for normal cell proliferation in mammalian cells (Lim and Hasty, 1996; Tsuzuki *et al.*, 1996). However, cells derived from a 3.5 day *RAD51*$^{-/-}$ mouse embryo exhibited IR sensitivity, as did mouse cells in which *RAD51* expression was transiently inhibited using antisense oligos (Lim and Hasty, 1996; Taki *et al.*, 1996). Furthermore, DT40 cells in which *RAD51* was placed under the control of a repressible promoter accumulated IR-induced chromosomal breaks when the *RAD51* transgene was inhibited (Sonoda *et al.*, 1998). Homologous recombination therefore contributes to DSB repair and ionising radiation resistance in vertebrate cells.

Mutation of *RAD54* in both chicken DT40 and mouse embryonic stem (ES) cells also resulted in radiation sensitivity (Bezzubova *et al.*, 1997; Essers *et al.*, 1997). Extending the investigation, DT40 *KU70*$^{-/-}$ and *RAD54*$^{-/-}$ single and double knockouts were established to determine the relative importance of HR and NHEJ in this chicken lymphocyte cell line (Takata *et al.*, 1998). Results from the irradiation of synchronised cells suggested that the relative contribution of the two DSB repair pathways varied across the cell cycle. Wild type cells showed increased radiation resistance in late S – G_2 phase that was not observed with *RAD54*$^{-/-}$ cells, which exhibited a reasonably uniform radiosensitivity across the whole cell cycle. In agreement with previous studies using SCID, XR-1 (XRCC4-

defective or *xrs* (Ku-defective) cells (Jeggo, 1990; Lee *et al.*, 1997) *KU70*$^{-/-}$ DT40 cells showed significant radiation sensitivity during G_1 and early S phase. In contrast to yeast cells, therefore, in which HR appears to be the predominant DSB repair pathway throughout the cell cycle, in mammalian cells, Rad54-mediated homologous recombination is more active in the late S and G_2 phases of the cell cycle. During G_1 and early S phase DSB repair is catalysed primarily by the NHEJ proteins. Furthermore, *RAD54*$^{-/-}$/*KU70*$^{-/-}$ cells were significantly more sensitive to γ-radiation, X-rays and MMS than either single mutant (Takata *et al.*, 1998). This would suggest that despite the apparent cell cycle segregation of recombinational and non-homologous repair, in the absence of one repair pathway the other can contribute to DNA damage repair.

Similar observations have been made with mouse knockouts (Essers *et al.*, 2000). Whilst, *Rad54*$^{-/-}$ ES cells are radiosensitive, *Rad54*$^{-/-}$ mice display variable sensitivity to ionising radiation. The mice are hypersensitive to IR at the embryonic stage, but not as adults, where the radiation sensitivity is only observed in the absence of NHEJ (*RAD54*$^{-/-}$/SCID double knockout). This suggests that the relative contribution of the two major DSB repair pathways may also change during different developmental stages.

HR may be favoured in *S. cerevisiae* because the yeast genome contains relatively little noncoding DNA, and it is therefore important that any breaks be repaired accurately. By comparison, mammalian cells contain a higher percentage of noncoding DNA, and the probability of causing a fatal mutation by the inaccurate rejoining of breaks is lower. Following DNA replication when the

sister chromatids are in close proximity, however, mammalian cells still favour HR for the repair of double-strand breaks.

II. Components of the non-homologous end-joining pathway

Identification of defective NHEJ proteins using radiation sensitive rodent cell lines.

The characterisation of mammalian mutant cell lines that are hypersensitive to ionising radiation has helped to advance the DNA repair field. Rodent cells lines were isolated which displayed significant sensitivity to X-rays and bleomycin, with little or no cross sensitivity to UV-radiation or the alkylating agent MNNG (Jeggo and Kemp, 1983). Subsequently the radiation sensitive rodent cell lines were divided into 11 complementation groups, called IR groups, and the genes defective in these groups designated X-ray cross complementing (XRCC) genes (for review see Zdzienicka, 1995; Jeggo, 1998). Of the eleven IR groups, IR1 was found to be defective in single-strand break (SSB) repair, whilst IR2 and IR3 were defective in homologous recombination (the role of *XRCC2* and *XRCC3* in HR having been discussed above). Cells of IR groups 4, 5, 6 and 7 displayed not only defects in DSB repair, resulting in the observed ionising radiation hypersensitivity, but were also defective V(D)J recombination – the process by which antigen diversity is generated during lymphocyte development (see Table 1.1 and Weaver, 1995; Zdzienicka, 1999).

Table 1.1. Properties of mammalian NHEJ-defective cells.

IR group	Gene	Mutated cell line	Phenotype
IR4	*XRCC4*	Rodent – XR-1	Radiation sensitive Defective DSB rejoining, but normal DNA end-binding and DNA-PK activities Defective V(D)J recombination
		Knockout mouse	Embryonic lethal
IR5	*XRCC5/KU80*	Rodent – *xrs*-2,4,5,6,7	Radiation sensitive Defective DSB rejoining Lacking DNA end-binding and DNA-PK activities Defective V(D)J recombination
		Knockout mouse	Exhibits immunodeficiency, growth retardation and loss of cellular proliferation
IR6	*XRCC6/KU70*	Mouse *KU70*$^{-/-}$ ES cells	Same as for IR5
		Knockout mouse	Same as for *KU80*$^{-/-}$ mice
IR7	*XRCC7/DNA-PK*$_{cs}$	Rodent - SCID, V-3, SX9	Radiation sensitive Defective DSB rejoining Lack DNA-PK activity despite normal DNA end-binding Defective V(D)J recombination (coding joints only)
		Human – M059J	Radiation sensitive Defective DSB repair Defective DNA-PK activity
	DNA ligase IV	Human – 180BR, 411BR, LB2304, FB2303	Radiation sensitive Defective DSB rejoining Reduced fidelity of signal joints during V(D)J recombination
		Knockout mouse	Embryonic lethal

Adapted from (Jeggo, 1998).

Cells of the IR5, IR6 and IR7 complementation groups were found to lack components of DNA-PK. IR group 7 mutants displayed double-strand (ds) DNA end-binding activity but no protein kinase activity, indicating that *XRCC7* encoded the catalytic subunit of the DNA-dependent kinase (Rathmell and Chu, 1994; Blunt *et al.*, 1995). In comparison, the IR group 5 mutants lacked the dsDNA end-binding activity characteristic of the Ku protein (Getts and Stamato, 1994; Rathmell and Chu, 1994). Ultimately, expression of Ku80 and not Ku70 was found to complement the group 5 mutants, therefore the *KU80* gene corresponded to *XRCC5* (Smider *et al.*, 1994; Taccioli *et al.*, 1994; Finnie *et al.*, 1995). In contrast, *KU70* was first identified as the gene encoding the smaller subunit of the Ku heterodimer. Only later, after *KU70*$^{-/-}$ ES cells were established and it was confirmed that they displayed the expected radiation sensitivity and defects in V(D)J recombination, was *KU70* designated as *XRCC6* (Gu *et al.*, 1997).

IR group 4 mutants display wild-type levels of dsDNA end-binding and DNA-PK activity, suggesting that the mutated protein was not a component of the DNA-PK complex (Getts and Stamato, 1994; Rathmell and Chu, 1994; Blunt *et al.*, 1995). Complementation studies identified *XRCC4* as the gene defective in IR group 4 mutants (Li *et al.*, 1995). The XRCC4 protein at that time had no obvious domains, motifs or role in NHEJ.

One component of the NHEJ pathway not identified by characterisation of radiation sensitive cell lines is DNA ligase IV. This ligase was assumed to play a role in NHEJ after it was found to form a tight association with XRCC4 (Critchlow

et al., 1997; Grawunder *et al.*, 1997). More recently cell lines derived from patients exhibiting either immunodeficiency or radiation sensitivity were shown to have mutations in the *LIG4* gene (Riballo *et al.*, 1999; O'Driscoll *et al.*, 2001).

The Ku protein

Ku structure

The Ku protein was originally discovered as an autoantigen recognised by the sera of patients with certain autoimmune diseases (Tuteja and Tuteja, 2000). The protein is a heterodimer composed of a ~70 kDa and a ~80 kDa subunit (termed Ku70 and Ku80, respectively). Compatible with an essential role for Ku in DSB repair, this protein has been conserved from yeast to humans (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a; Feldmann *et al.*, 1996). Moreover, sequence analysis has shown that the two Ku subunits share significant homology, which may indicate that they arose through duplication and divergence of a single ancestral gene (Gell and Jackson, 1999). Analysis of deletion mutants determined the domains of both subunits required for formation of the heterodimer (summarised in Fig. 1.2A – B; Wu and Lieber, 1996; Osipovich *et al.*, 1997; Cary *et al.*, 1998; Wang *et al.*, 1998a; Wang *et al.*, 1998b; Gell and Jackson, 1999). Using baculovirus expressed fragments of Ku, the central region of the Ku80 protein, amino acids (aa) 371 – 510, were found to be critical for binding Ku70 (Wang *et al.*, 1998b). Other studies, in which the dimerisation domains were analysed by the yeast two-hybrid assay or *in vitro* pull-down assays using GST-fusion proteins, defined slightly different regions of

Figure 1.2. Structure of the Ku heterodimer.

(A, B) Schematic representations of the different domains of Ku70 (A) and Ku80 (B), as defined in the crystal structure and during mutational analyses.

(C) Ribbon diagram of Ku-DNA complex. Ku70 is coloured red, Ku80 yellow and the DNA grey. Taken from (Walker *et al.*, 2001).

(D) Space-filling model of Ku-DNA complex, again taken from (Walker *et al.*, 2001).

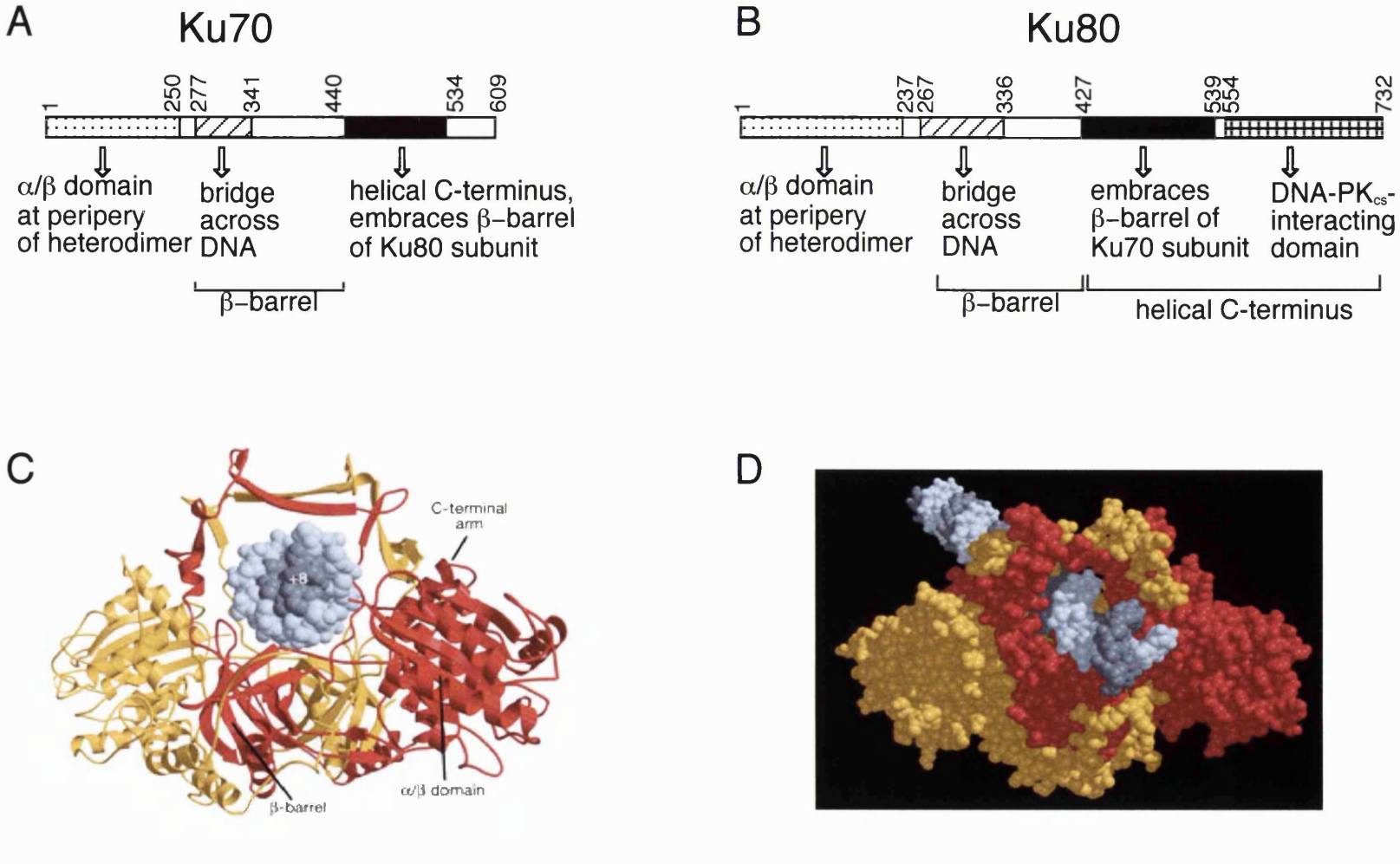

A
Ku70
1
250
277
341
440
534
609
α/β domain at peripery of heterodimer
bridge across DNA
helical C-terminus, embraces β–barrel of Ku80 subunit
β–barrel
B
Ku80
1
237
267
336
427
539
554
732
α/β domain at peripery of heterodimer
bridge across DNA
embraces β–barrel of Ku70 subunit
DNA-PKcs-interacting domain
β–barrel
helical C-terminus
C
C-terminal arm
+8
β-barrel
α/β domain
D

Figure 1.2

Ku80 required for interaction with Ku70 (Wu and Lieber, 1996; Osipovich *et al.*, 1997; Cary *et al.*, 1998; Gell and Jackson, 1999). In general, however, the N-terminal was found not to be required for dimerisation, but the degree to which the central and C-terminal domains were required for Ku70-interaction varied. One study defined the minimal domain of Ku80 required for Ku70-binding as being only 28 amino acids long, from residues 449 to 477 (Osipovich *et al.*, 1997). Accordingly, point mutations at residues 453 and 454 were sufficient to disrupt heterodimer formation.

The dimerisation domain of Ku70 was, if anything, less clearly defined. The smallest fragment of Ku70 that retained Ku80-binding in the yeast two-hybrid assay contained residues 243 to 609 (Osipovich *et al.*, 1997). Whilst in coimmunoprecipitation experiments two separate Ku70 fragments displayed dimerisation activity (aa 1 – 115 and 430 – 482), both domains apparently bound independently to the same Ku80 molecule (Wang *et al.*, 1998a; Wang *et al.*, 1998b). Expression of Ku polypeptides fused to GST, however, identified the Ku80-binding domain of Ku70 to be located in residues 449 – 578 (Gell and Jackson, 1999).

The Ku heterodimer has some distinctive DNA binding properties. Ku binds to linear dsDNA with high affinity and this binding is not competed by circular dsDNA, tRNA, mRNA or viral genomic RNA (Mimori and Hardin, 1986; de Vries *et al.*, 1989). The lack of binding to circular dsDNA demonstrates that the Ku protein requires free ends for dsDNA binding. The structure of the DNA end, however, is not important as similar binding is observed with dsDNA fragments

containing blunt, 3' protruding or 5' protruding termini (de Vries *et al.*, 1989). In contrast, Ku will bind to both circular and linear ssDNA as well as a variety of discontinuities in dsDNA including nicks and ss hairpin loops (de Vries *et al.*, 1989; Paillard and Strauss, 1991; Blier *et al.*, 1993). Moreover, DNA-bound Ku is not static; the Ku protein is able to transfer directly between DNA molecules with complementary termini and Ku can also translocate along DNA to occupy internal sites (de Vries *et al.*, 1989; Bliss and Lane, 1997). Binding of multiple Ku heterodimers to a single DNA molecule has been observed as a ladder of bands by gel electrophoresis analysis, and as a series of 'beads on a string' by electron microscopy (de Vries *et al.*, 1989; Blier *et al.*, 1993). By analysing the number of Ku heterodimers bound to DNA fragments of different lengths, each Ku heterodimer was estimated to bind to between 27 and 32 base pairs of DNA (de Vries *et al.*, 1989; Paillard and Strauss, 1991; Blier *et al.*, 1993). In comparison to the rapid binding of Ku to DNA ends, the inward translocation of Ku is rate-limiting. Neither end-binding nor translocation, however, requires ATP or divalent cations (de Vries *et al.*, 1989; Blier *et al.*, 1993).

The Ku protein has been reported to bind DNA in a sequence-specific manner, especially to sequences in transcriptional regulatory elements (reviewed in Tuteja and Tuteja, 2000). A clear consensus recognition sequence for Ku binding has not emerged from these studies, however, putting into doubt a possible role for Ku in transcriptional regulation. Despite this, the binding of Ku to a negative regulatory element (NRE1) in the long terminal repeat of the mouse mammary tumour virus (MMTV) has been demonstrated to have some functional

basis. In association with DNA-PK_{cs}, Ku binding to NRE1 has been shown to repress MMTV transcription (Giffin *et al.*, 1996).

The recent resolution of the structure of the Ku heterodimer bound to DNA has confirmed which residues of each subunit are involved in dimerisation, and also revealed how the unique DNA binding properties of Ku are achieved (Walker *et al.*, 2001). The two subunits share a common topology and fit together, almost as mirror images of each other, to form a wide base or cradle to support duplex DNA, with a preformed bridge that encircles the DNA (see Fig. 1.2C – D). The bridge encompassing the DNA is composed of short β-hairpins from both subunits, and maintains the same conformation in the absence of DNA. The composition of both the bridge and the base structure from the Ku70 and Ku80 subunits explains why both subunits are required for high affinity DNA binding. The structure also explains why in mutational analyses the DNA binding domains of each subunit were found to coincide with the dimerisation domains (Wang *et al.*, 1998a; Wang *et al.*, 1998b). The reality that Ku binds to DNA by threading it through a central hole, rather like threading cotton through a needle, does raise the question as to how the Ku protein is unloaded after two DNA ends have been rejoined? Without a free DNA end for unloading, alternative methods for the release of bound Ku might include disruption of the heterodimer, or limited proteolysis of the protein. It remains to be determined how Ku unloading occurs *in vivo*, and whether it involves reversible or irreversible changes to the Ku heterodimer. Finally, the Ku heterodimer makes no contact with the bases of an encircled DNA fragment thus excluding the possibility of Ku having any sequence

specific DNA binding activity (Walker *et al.*, 2001). It has been suggested that the apparent specificity of Ku binding to a transcriptional regulatory element in MMTV may be due to the DNA sequence adopting an altered configuration to which Ku binds (Walker *et al.*, 2001).

Ku function

By far the most extensively characterised function of Ku is its role as the DNA targeting subunit of DNA-PK. Initially, three polypeptides were found to copurify with the DNA-dependent kinase activity, a large subunit of around 350 kDa (the catalytic subunit) and two smaller subunits, demonstrated to be Ku70 and Ku80 by peptide sequencing (Lees-Miller *et al.*, 1990). Additionally, biochemical characterisation has revealed that the Ku protein is required for DNA-PK activity. Separation of DNA-PK into the individual constituents was accompanied by a loss of kinase activity, which could subsequently be reconstituted by mixing the Ku- and DNA-PK_{cs}-containing fractions (Dvir *et al.*, 1992). During phosphorylation of Sp1, Ku was found to stimulate kinase activity by recruiting DNA-PK_{cs} to DNA ends (Gottlieb and Jackson, 1993). Furthermore, both Ku- and DNA-PK_{cs}-defective cell lines were shown to lack DNA-PK activity (Blunt *et al.*, 1995; Finnie *et al.*, 1995; Peterson *et al.*, 1995). When short fragments of DNA were used (<26bp) some Ku-independent DNA-PK_{cs} kinase activity was observed, chiefly because the DNA was too short to allow the loading of both Ku and DNA-PK_{cs} (Yaneva *et al.*, 1997; West *et al.*, 1998). In addition, some kinase activity was observed with DNA-PK_{cs} alone when longer DNA fragments were used, but only

at a low salt concentration (Hammarsten and Chu, 1998). Under these low salt conditions, addition of Ku still stimulated DNA-PK_{cs} kinase activity by 5- to 10-fold. From these results, a picture has emerged of the Ku protein stabilising the low affinity DNA binding of DNA-PK_{cs} and thus stimulating kinase activity.

In agreement with a role for Ku in stimulating DNA-PK activity, there is evidence of a direct interaction between DNA-PK_{cs} and the Ku heterodimer. Visualisation of protein-DNA interactions by atomic force microscopy (AFM) has shown that large protein complexes are formed on DNA fragments in Ku, DNA-PK_{cs} and DNA mixtures (Cary *et al.*, 1997; Yaneva *et al.*, 1997). Whilst there is some disagreement as to whether these large complexes are found exclusively at DNA ends or also at internal sites, it is clear that these complexes are much larger than those seen when Ku and DNA-PK_{cs} are incubated separately with DNA. These complexes are, therefore, thought to represent ternary complexes of DNA, DNA-PK_{cs} and Ku. In addition, biochemical analyses have determined that the C-terminus of Ku80 mediates the interaction of the Ku heterodimer with DNA-PK_{cs} (Gell and Jackson, 1999; Singleton *et al.*, 1999). *In vitro* translated Ku80 lacking the C-terminal 178 aa was able to dimerise with Ku70 and displayed similar DNA-binding activity to full length Ku80 (Singleton *et al.*, 1999). Consequently, expression of truncated Ku80 protein rescued the DNA binding defect and defective signal joint formation of the Ku80-defective *xrs*-6 cell line. In contrast to expression of full length Ku80, however, truncated Ku80 was unable to complement DNA-PK activity and coding joint formation defects of *xrs*-6 cells. In this respect expression of C-terminally truncated Ku80 turned the Ku-defective

phenotype of *xrs*-6 cells into a SCID-like phenotype, confirming the role of the Ku80 C-terminus in the activation of DNA-PK activity.

The Ku protein is also thought to have more direct roles in DNA repair, independent of DNA-PK stimulation. A repair assay using linear plasmids transfected into mammalian cells found that extrachromosomal DNA was subject to increased degradation in Ku80-deficient *xrs*-6 cells but not DNA-PK_{cs}-deficient SCID cells (Liang and Jasin, 1996). These results were thought to show that Ku acts to prevent exonucleolytic degradation of broken DNA ends. In contrast, however, V(D)J intermediates generated in Ku80-deficient mice are relatively stable, arguing against a role for Ku in the protection of DNA ends, at least in V(D)J recombination (Zhu *et al.*, 1996). In addition to binding dsDNA ends, the Ku protein has been shown by AFM to form loops in DNA fragments and to join two DNA molecules (Cary *et al.*, 1997; Pang *et al.*, 1997). Furthermore, Ku stimulates the activity of mammalian ligases by promoting the association of linear DNA molecules (Ramsden and Gellert, 1998). Ku, therefore, functions not only as the targeting subunit of DNA-PK, but also as a bridging factor, holding together broken DNA ends whilst allowing access by other repair enzymes.

The Ku protein is reported to have DNA-dependent ATPase and ATP-dependent helicase activities (Cao *et al.*, 1994; Tuteja *et al.*, 1994; Ochem *et al.*, 1997). Ku appears not to contain the classical helicase motifs, however, and mutations in the putative ATP-binding domains have no detectable effect on Ku-mediated repair (Jin and Weaver, 1997; Singleton *et al.*, 1997).

Additional roles of Ku implicated by the phenotypes of knockout mice

Generation of Ku70- and Ku80-knockout mice has in general, confirmed the observations seen with defective cell lines. Both Ku70- and Ku80-knockout mice are immunodeficient due to an absence of mature B and T lymphocytes (Nussenzweig *et al.*, 1996; Zhu *et al.*, 1996; Gu *et al.*, 1997). Aside from impaired V(D)J recombination, however, the Ku-deficient mice have other interesting phenotypes. Both Ku70- and Ku80-knockout mice are much smaller in comparison to heterozygous littermates (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). Furthermore, the deletion of Ku80 has been reported to result in an early onset of senescence and a shortened lifespan in mice (Vogel *et al.*, 1999). Fibroblasts isolated from the knockout mice also display prolonged doubling times and giant polyploid cells indicative of premature senescence (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). These phenotypes are not shared by SCID or DNA-PK_{cs}-knockout animals and cells, indicating that Ku may have a DNA-PK_{cs}-independent role in growth control. Recently, shortened telomeres have been observed in Ku-deficient mouse embryonic fibroblasts and embryonic stem cells (d'Adda di Fagagna *et al.*, 2001). A role for Ku in telomere length maintenance may, therefore, account for the premature senescence of Ku-null cells.

DNA-PK_{cs}

DNA-PK_{cs} as a member of the phosphatidylinositol 3-kinase superfamily

Prior to the cloning of the *DNA-PK_{cs}* gene, biochemical assays had identified DNA-PK as a serine/threonine protein kinase, composed of the catalytic subunit

(originally identified as p350) and the Ku heterodimer (Carter *et al.*, 1990; Lees-Miller *et al.*, 1990; Suwa *et al.*, 1994). It was surprising therefore, when sequence analysis identified DNA-PK_{cs} as containing a catalytic domain conserved within phosphatidylinositol (PI) 3-kinases (Hartley *et al.*, 1995). Despite containing this PI 3-kinase domain, DNA-PK did not exhibit any lipid kinase activity (Hartley *et al.*, 1995). Consequently DNA-PK_{cs} has been categorised along with related protein kinases as a subgroup of the PI 3-kinase family, termed the PI 3-kinase-related kinases (PIKK). The PI 3-kinase-related kinases (reviewed in Smith and Jackson, 1999) include ATM (and its *S. cerevisiae* homologue Tel1); ATR (Mec1 in *S. cerevisiae* and Rad3 in *S. pombe*); FRAP (yeast homologues Tor1 and Tor2) and TRRAP (Trap1p in *S. cerevisiae*). These kinases are distinguished from the classical PI 3-kinases by being serine/ threonine protein kinases and not lipid kinases. The PIKKs are also much larger in size, all over 200 kDa, in comparison to an average size of 95 – 110 kDa for the PI 3-kinases. All the members of the PI 3-kinase superfamily share a conserved kinase domain at their carboxy terminus. In addition, the PIKKs contain a further region of homology at the extreme C-terminus that is not seen in the PI 3-kinases (see Fig. 1.3A).

The *DNA-PK_{cs}* gene encodes for a 4,096 amino acid polypeptide with a predicted molecular mass of 465 kDa (Hartley *et al.*, 1995). As described above, the C-terminal kinase domain is made up of around 400 conserved residues. Coimmunoprecipitation of certain DNA-PK_{cs} fragments by Ku antibodies identified the Ku-binding domain of DNA-PK_{cs} as also being located at

Figure 1.3. Structure of DNA-PK_{cs}.

(A) Schematic representation of DNA-PK_{cs} protein showing the PI 3-kinase and PIKK domains conserved between DNA-PK_{cs}, ATM and ATR.

(B) Three-dimensional image reconstruction of the DNA-PK_{cs} molecule. A and B represent two open channels, C indicates the potentially flexible arm that surrounds channel A, and the arrows D and E indicate two openings into an enclosed cavity. Taken from (Leuther *et al.*, 1999).

(C) Cross-sectional view of DNA-PK_{cs}, showing the configuration of the enclosed cavity. Also taken from (Leuther *et al.*, 1999).

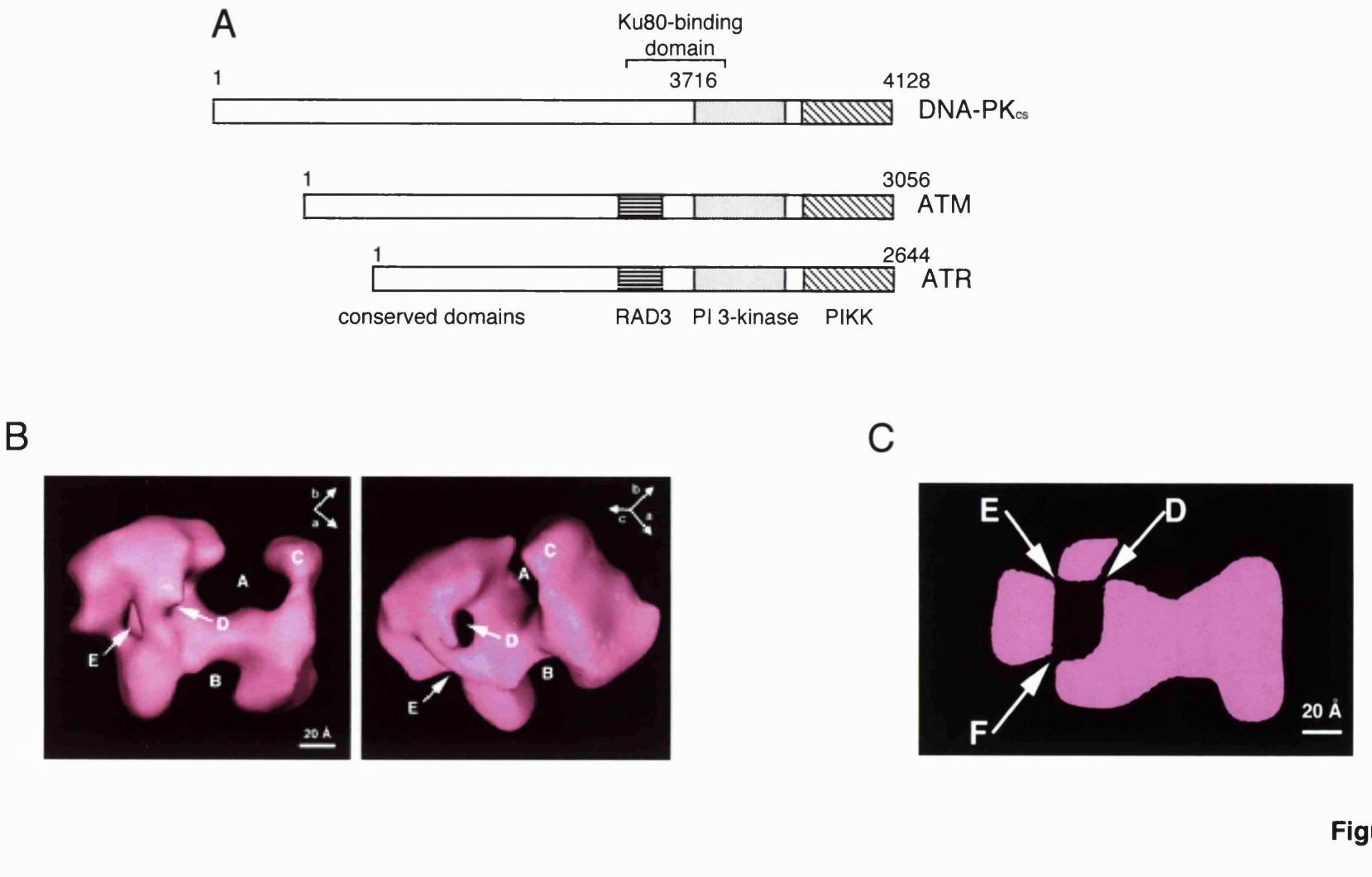

A
Ku80-binding
domain
1
3716
4128
DNA-PKcs
1
3056
ATM
1
2644
ATR
conserved domains
RAD3
PI 3-kinase
PIKK
B
A
C
D
E
B
20 Å
C
E
D
F
20 Å

Figure 1.3

the C-terminus of the protein, partially overlapping with the protein kinase homology domain (Jin *et al.*, 1997). The remaining central and N-terminal regions of DNA-PK_{cs}, comprising ~3500 aa, have no significant homology to any other proteins. It is possible, however, that the remainder of the protein is important in maintaining the distinct architecture of DNA-PK_{cs}, as observed by cryo electron microscopy and electron crystallography (Chiu *et al.*, 1998; Leuther *et al.*, 1999). One striking feature of DNA-PK_{cs} is that the interior of the protein is largely hollow. DNA-PK_{cs} contains an open channel (marked A in Fig.1.3B) similar to those seen in other double-stranded DNA-binding proteins (Leuther *et al.*, 1999). In addition there is an enclosed cavity with three openings large enough to accommodate single-stranded DNA (marked D, E and F in Fig.1.3C). Biochemical analyses demonstrated that DNA-PK_{cs} has separate binding sites for single- and double-stranded DNA, the dsDNA binding site having lower affinity for ssDNA and vice versa (Leuther *et al.*, 1999). In addition, Ku-independent activation of DNA-PK_{cs} kinase activity appeared to require both ds- and ssDNA binding, which may represent a mechanism for the DNA structure to control the DNA-PK kinase activity.

DNA-PK substrates

The true *in vivo* targets of DNA-PK have not yet been identified, however, a number of proteins have been shown to be substrates for DNA-PK *in vitro.* DNA-PK predominantly phosphorylates serine or threonine residues that precede a glutamine residue, giving short S-Q or T-Q consensus sequences (Smith and

Jackson, 1999). Several transcription factors and the C-terminal domain of RNA polymerase II are included in the *in vitro* targets of DNA-PK (reviewed in Anderson, 1993). This, together with the apparent sequence specific binding of Ku to transcription regulatory elements led to speculation that DNA-PK might play a role in transcription. Resolution of the Ku structure has, however, shown that the Ku protein makes no contact with the bases in a DNA helix, excluding the possibility of Ku binding to specific DNA sequences (Walker *et al.*, 2001). Moreover, as yet none of the transcription factors or RNA pol II have been shown to be *in vivo* targets of DNA-PK, putting into doubt the role of Ku and DNA-PK_{cs} in transcriptional regulation.

Another protein identified as an *in vitro* target of DNA-PK was the tumour suppressor protein p53, which was phosphorylated at serine 15 and serine 37 (Lees-Miller *et al.*, 1990; Lees-Miller *et al.*, 1992). Phosphorylation of serine 15 has been shown to block the interaction of p53 with its negative regulator Mdm2 (Shieh *et al.*, 1997). In addition, the Mdm2 protein was also shown to be a substrate for DNA-PK *in vitro*, and phosphorylation of Mdm2 also prevented p53-binding (Mayo *et al.*, 1997). These observations led to the idea that DNA-PK may act in the DNA damage-signalling pathway and induce cell cycle arrest through stabilisation and activation of p53. However, the DNA-PK related proteins ATM and ATR are also capable of phosphorylating serine 15 of p53. Furthermore, fibroblasts lacking DNA-PK_{cs} induce a normal p53 response and cell cycle arrest following irradiation, indicating that DNA-PK phosphorylation of

p53 is not essential for activation of p53 *in vivo* (Araki *et al.*, 1999; Burma *et al.*, 1999; Jimenez *et al.*, 1999).

The 34 kDa subunit of replication protein A (RPA) is also a DNA-PK target *in vitro*, with the phosphorylation sites mapped to threonine 21 and serine 33 (Niu *et al.*, 1997). Again, there are conflicting reports as to whether RPA serves as an *in vivo* target for DNA-PK (Boubnov and Weaver, 1995; Fried *et al.*, 1996). Other more plausible DNA-PK substrates include the Ku heterodimer, DNA-PK_{cs} and another component of the NHEJ pathway, XRCC4 (Chan and Lees-Miller, 1996; Critchlow *et al.*, 1997; Leber *et al.*, 1998). The effect of DNA-PK phosphorylation of Ku is unknown. Disruption of consensus SQ motif at residues 51 and 52 of Ku70 did not alter the DNA end-binding activity of Ku, nor the ability of the mutant protein to complement the IR-sensitivity of *KU70*$^{-/-}$ cells (Jin and Weaver, 1997). In a conflicting study, however, it was suggested that Ku70 is predominantly phosphorylated at serine 6 which does not correspond to a canonical DNA-PK consensus site (Chan *et al.*, 1999). In comparison, phosphorylation by DNA-PK has been shown to disrupt the DNA binding activity of XRCC4 (Modesti *et al.*, 1999). Moreover, autophosphorylation of DNA-PK_{cs} leads to dissociation of the catalytic subunit from DNA-bound Ku, and consequently inactivation of DNA-PK (Chan and Lees-Miller, 1996). As such phosphorylation of the key components of the NHEJ pathway by DNA-PK may act a negative regulatory mechanism, initiating the release of the repair proteins from rejoined breaks.

Role for DNA-PK$_{cs}$ in DSB repair

As with the Ku protein, evidence for a role for DNA-PK$_{cs}$ in DSB repair comes largely from the radiosensitivity of defective cell lines. DNA-PK$_{cs}$-defective rodent cells exhibit radiation sensitivity similar to that observed with Ku-defective cells, which is in agreement with the proteins acting together during DSB repair (Biedermann *et al.*, 1991; Fukumura *et al.*, 1998). A human DNA-PK$_{cs}$-defective cell line, M059J, has also been identified which is highly radiosensitive (Lees-Miller *et al.*, 1995). However, the exact role of DNA-PK$_{cs}$ in NHEJ remains unclear. Based on the structure of DNA-PK$_{cs}$ the protein could simply act as a scaffold opening up DNA ends to allow pairing of strands from opposite sides of the break (Leuther *et al.*, 1999). Despite functional substrates for DNA-PK having not yet been identified *in vivo*, there is evidence, however, to suggest that DNA-PK kinase activity is required for DSB repair and that DNA-PK$_{cs}$ does not simply play a structural role. Wortmannin is a fungal metabolite that inhibits DNA-PK$_{cs}$ activity by binding covalently to the ATP binding site (Izzard *et al.*, 1999). Addition of wortmannin to human cells in culture results in defective double strand repair and an associated radiation sensitivity (Boulton *et al.*, 1996; Rosenzweig *et al.*, 1997; Sarkaria *et al.*, 1998). Although the concentration of wortmannin required to radiosensitise cells correlates closely with inhibition of DNA-PK$_{cs}$ activity, wortmannin has also been demonstrated to inhibit the kinase activity of other PIKKs, such as ATM and ATR (Sarkaria *et al.*, 1998). In addition, wortmannin has also been shown to partly sensitise both DNA-PK$_{cs}$-defective and AT (ATM-defective) cells to ionising radiation (Rosenzweig *et al.*,

1997). Thus, inactivation of both DNA-PK_{cs} and ATM may contribute to the IR sensitivity of wortmannin treated cells.

To investigate the requirement for the kinase activity of DNA-PK in DSBR more rigorously, site directed mutations were created within the kinase domain of DNA-PK_{cs} (Kurimasa *et al.*, 1999a). The ability of the mutated DNA-PK_{cs} protein to complement the radiosensitivity of DNA-PK_{cs}-defective cell lines was then assessed. Whereas expression of wild type DNA-PK_{cs} rescued the radiation sensitivity and DSB repair defect of DNA-PK_{cs}-defective cells, expression of kinase-inactive DNA-PK_{cs} did not. These data indicate that the kinase activity of DNA-PK is required for DSB repair.

XRCC4

The *XRCC4* gene was identified when a cDNA library was screened for sequences, which when expressed would complement the V(D)J recombination defect of the IR group 4 mutant cells, XR-1 (Li *et al.*, 1995). The gene encodes a 334 aa protein with a predicted molecular weight of 38 kDa, although it has been noted that during SDS-PAGE, XRCC4 migrates anomolously with an apparent molecular mass of ~55 kDa (Li *et al.*, 1995; Critchlow *et al.*, 1997). The sequence of the XRCC4 protein showed no significant homology to any previously described proteins, and therefore did not give any clues as to the function of this protein (Li *et al.*, 1995). The only identifiable domains included a nuclear localisation signal, and a number of potential phosphorylation sites,

suggesting that XRCC4 may be a substrate for DNA-PK, casein kinase and other cytosolic protein tyrosine kinases.

Subsequently, biochemical characterisation of XRCC4 *in vitro* has established that this protein binds DNA, associates with DNA ligase IV and is a substrate for DNA-PK (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997; Leber *et al.*, 1998; Modesti *et al.*, 1999). The interaction between XRCC4 and DNA ligase IV was initially identified by coimmunoprecipitation (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). The two proteins were also shown to interact in yeast two-hybrid studies, and they extensively copurified during chromatography of HeLa nuclear extracts. Separation of the nuclear extracts by gel filtration demonstrated that within the cells, XRCC4 and DNA ligase IV are found not as monomers, but within larger protein complexes with a molecular mass of approximately 200 kDa. Furthermore, both the coimmunoprecipitation and co-elution during gel filtration were observed at 1 M NaCl, indicating that XRCC4 and DNA ligase IV are tightly associated.

The interaction of XRCC4 and DNA ligase IV stimulates the *in vitro* activity of DNA ligase IV (Grawunder *et al.*, 1997). In addition, XRCC4 is thought to help stabilise DNA ligase IV. In support of this hypothesis, expression of recombinant DNA ligase IV in the baculovirus system is unsuccessful unless XRCC4 is coexpressed (Chen *et al.*, 2000). Furthermore, the XRCC4-defective XR-1 cells have virtually undetectable levels of DNA ligase IV despite displaying normal levels of DNA ligase IV mRNA (Bryans *et al.*, 1999). This DNA ligase IV deficiency is rescued by introduction of *XRCC4* cDNA into XR-1 cells.

Functional domains of XRCC4

To establish the different functional domains of the XRCC4 protein, the biochemical activities of various deletion mutants were either analysed *in vitro* or assessed for their ability to complement the XRCC4-defective XR-1 cells (Grawunder *et al.*, 1998b; Leber *et al.*, 1998; Modesti *et al.*, 1999). The central region of XRCC4, from residues 100 – 250, was found to be required for *in vivo* interaction with DNA ligase IV (Grawunder *et al.*, 1998b). Indeed, residues 29 – 200 were critical for the stimulation of DNA ligase IV activity *in vitro* (Modesti *et al.*, 1999). Binding and stimulation of DNA ligase IV could, however, occur in the absence of XRCC4 DNA-binding, suggesting that XRCC4 does not act by targeting DNA ligase IV to DNA, but rather by stimulation of adenylation of the ligase (Modesti *et al.*, 1999). A larger fragment of XRCC4 (encompassing residues 1 – 250) was required for restoration of X-ray resistance and functional V(D)J recombination, demonstrating that DNA ligase IV-binding was necessary but not sufficient for the *in vivo* function of XRCC4 in both DSB repair and V(D)J recombination (summarised in Fig. 1.4A; Grawunder *et al.*, 1998b; Modesti *et al.*, 1999).

Analysis of XRCC4 phosphorylation *in vitro* demonstrated that purified DNA-PK was able to phosphorylate XRCC4 (Critchlow *et al.*, 1997; Leber *et al.*, 1998). In addition, XRCC4 was also phosphorylated by a DNA-cellulose bound fraction of cellular extract (Leber *et al.*, 1998). The kinase activity of the DNA-bound fraction was largely inhibited by addition of the DNA-PK_{cs} inhibitor wortmannin, and greatly reduced when SCID extracts were used, indicating that DNA-PK_{cs}

Figure 1.4. Structure of XRCC4.

(A, B) Schematic representation of the different XRCC4 domains as defined by mutational analysis (A), and in the crystal structure of XRCC4 (Junop *et al.*, 2000; B).

(C, D) Ribbon diagrams of the XRCC4 dimer (C) and tetramer (D). Taken from (Junop *et al.*, 2000)

(E) Ribbon diagram of the XRCC4 dimer and associated DNA ligase IV polypeptide. Taken from (Sibanda *et al.*, 2001).

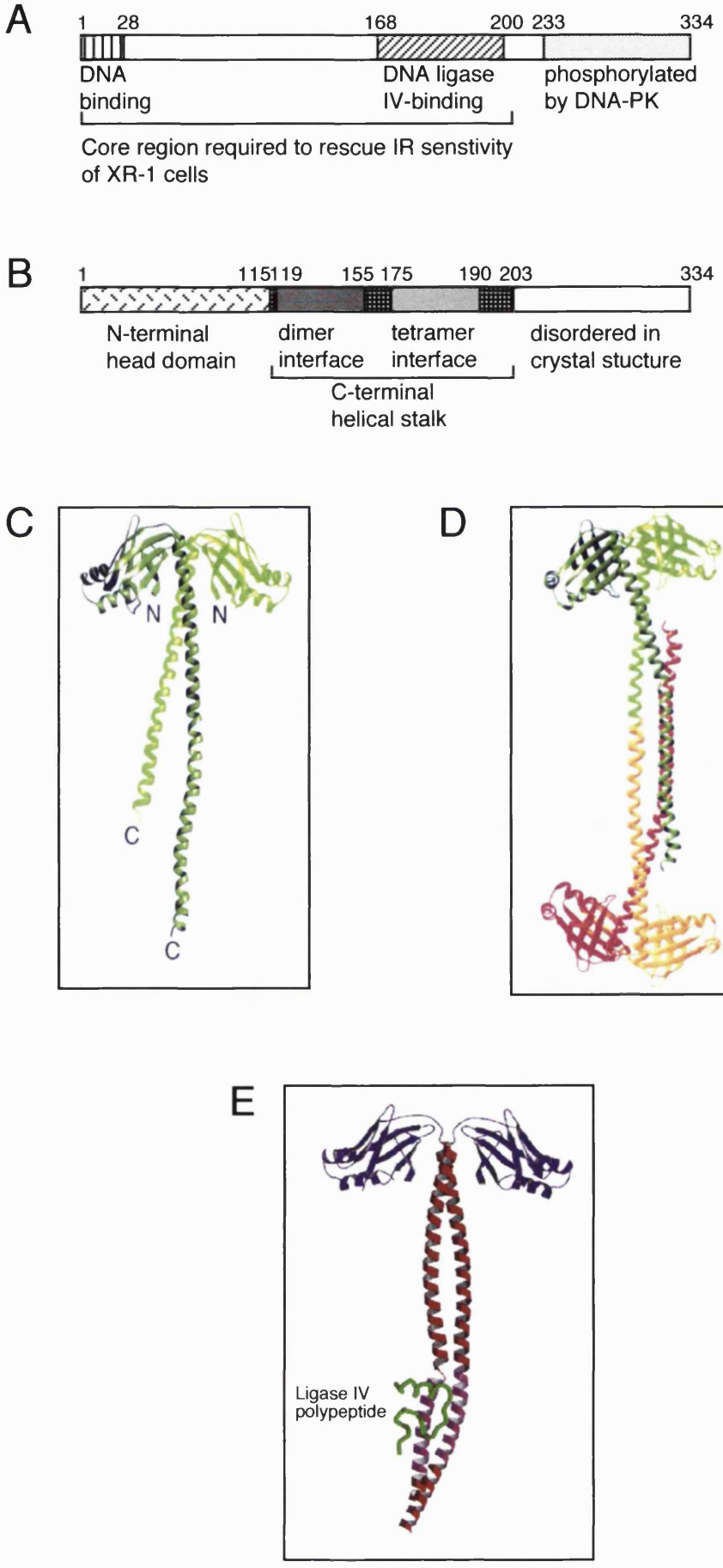

A
1
28
168
200
233
334
DNA binding
DNA ligase IV-binding
phosphorylated by DNA-PK
Core region required to rescue IR senstivity of XR-1 cells
B
1
115
119
155
175
190
203
334
N-terminal head domain
dimer interface
tetramer interface
disordered in crystal stucture
C-terminal helical stalk
C
N
N
C
C
D
E
Ligase IV polypeptide

Figure 1.4

was responsible for phosphorylation of XRCC4 in cellular extracts. The residual phosphorylation observed in the absence of DNA-PK_{cs}, however, suggests XRCC4 is also a target for other kinases. The *in vivo* phosphorylation sites of XRCC4 have been determined to lie within the C-terminus of the protein, between amino acids 264 and 334 (Leber *et al.*, 1998). This region of XRCC4 has been shown to be dispensable for V(D)J recombination, indicating that DNA-PK phosphorylation of XRCC4 is not required for this process (Modesti *et al.*, 1999).

XRCC4 structure

The crystal structures of XRCC4 either alone or bound to a DNA ligase IV polypeptide have recently been resolved (Junop *et al.*, 2000; Sibanda *et al.*, 2001). In the isolated state, the N-terminal domain of XRCC4, from residues 1 – 115, forms a compact head domain, whilst the C-terminal residues 119 – 203 assemble into a long helical stalk (see Fig. 1.4C – D; Junop *et al.*, 2000). The extreme C-terminal residues, aa 204 onwards, were disordered in the crystal structure, however, preventing mapping of this region. Within the crystals XRCC4 existed as a tetramer, or what could be described as a dimer of dimers. The XRCC4 dimer was said to resemble a palm tree, with the two head domains at one end of the molecule and the C-terminal stalks extending to the opposite end. Residues 119 to 155, the section of the helical stalk adjacent to the head domains, were found to mediate dimerisation, and the DNA binding surface was

suggested to lie within the N-terminal head domain, each subunit containing one DNA-binding domain.

In the isolated state, two XRCC4 dimers interact via the C-terminal stalks to create a tetramer, such that the overall structure has two globular heads at either end of the tetramer separated by a long helical bundle. When the crystal structure of XRCC4 bound to the DNA ligase IV polypeptide was resolved, the N-terminal globular head domains and the site of interaction between two XRCC4 monomers were found to be the same (Sibanda *et al.*, 2001). The binding to the DNA ligase IV polypeptide, however, induced a large rearrangement of the helical tails of XRCC4 (Fig. 1.4E), such that the formation of XRCC4 tetramers was precluded.

XRCC4-defective cells and mice

The underlying mutation in the rodent XR-1 cells resulted in deletion of the complete *XRCC4* gene (Li *et al.*, 1995). In cell culture, therefore, an *XRCC4*-null mutation causes X-ray sensitivity and V(D)J recombination defects, but the cells are viable. In contrast, *XRCC4*$^{-/-}$ knockout mice die during late embryonic development (Gao *et al.*, 1998). The embryonic lethality can be rescued by disruption of p53, however, along with the impaired cellular proliferation and neuronal apoptosis displayed by *XRCC4*$^{-/-}$ mice (Gao *et al.*, 2000). This suggests that these phenotypes are due to a p53 response to unrepaired DNA damage, rather than an intrinsic requirement for XRCC4 for viability. In contrast, *XRCC4*$^{-/-}$

/p53$^{-/-}$ mice still exhibited impaired V(D)J recombination and lymphocyte development, showing an absolute requirement for XRCC4 for lymphogenesis.

DNA ligase IV

The gene encoding DNA ligase IV (*LIG4*) was originally identified because it contained a conserved sequence found in all known eukaryotic DNA ligases (Wei *et al.*, 1995). The corresponding 96 kDa protein product exhibited ATP-dependent ligase activity that was distinguished from the other mammalian ligases by the ability to join oligo(dT)·poly(rA) but not oligo(rA)·poly(dT) hybrid substrates (Robins and Lindahl, 1996). All mammalian ligases display significant homology in the catalytic domains, especially within the active site and conserved peptide motifs (Tomkinson and Mackey, 1998). Outside of this conserved catalytic domain, however, DNA ligase IV has a long C-terminal extension that shows little homology to DNA ligase I or III (Wei *et al.*, 1995). Two BRCA1 C-terminus (BRCT) domains are located within this carboxyl terminus of DNA ligase IV, and this region was identified early on as supporting the interaction of DNA ligase IV with XRCC4 (Critchlow *et al.*, 1997). The hypothesis that the BRCT motifs were involved in protein-protein interactions was disproved later, when the XRCC4 interaction was shown to be mediated by a motif situated between the two BRCT domains rather than within them (Grawunder *et al.*, 1998c).

Initially, the only evidence for a role of mammalian DNA ligase IV in NHEJ was the specific interaction between this protein and XRCC4. Targeted

disruption of *LIG4* in mice and human cells has, however, confirmed the requirement for DNA ligase IV during NHEJ. Similar to the *XRCC4* knockout, inactivation of the *LIG4* gene in mice leads to embryonic lethality (Barnes *et al.*, 1998; Frank *et al.*, 2000). Indeed, the comparable phenotypes displayed by *LIG4*$^{-/-}$ and *XRCC4*$^{-/-}$ embryos indicate that the two proteins have similar functions *in vivo*. Furthermore, *LIG4*$^{-/-}$ human pre B cells and mouse embryonic fibroblasts (MEFs) display ionising radiation sensitivity and defective V(D)J recombination, phenotypes that are common to all NHEJ mutants (Frank *et al.*, 1998; Grawunder *et al.*, 1998a).

A number of DNA ligase IV-defective human cell lines have been derived from patients exhibiting either radiosensitivity or immunodeficiency (Riballo *et al.*, 1999; O'Driscoll *et al.*, 2001). Whilst all the underlying mutations have been shown to either disrupt the catalytic domain of DNA ligase IV or prevent association with the XRCC4 protein, none are predicted to confer a null phenotype as this causes embryonic lethality in mice. As expected, the cell lines exhibit pronounced radiosensitivity, defective DSB repair, and chromosomal instability (Badie *et al.*, 1995; Badie *et al.*, 1997; O'Driscoll *et al.*, 2001). The DNA ligase IV-defective cells do not, however, exhibit any cell cycle defects, and arrest at the G_1/S and G_2/M checkpoints following irradiation. With respect to V(D)J recombination, the frequency of joining is unaltered in the *lig4* mutants, but a decrease in the fidelity of signal joints is detected (O'Driscoll *et al.*, 2001; Riballo *et al.*, 2001). Despite the *lig4* mutations resulting in very similar cellular phenotypes, some differences were observed in the clinical characteristics

displayed by the DNA ligase IV-defective patients. One cell line was established from a developmentally normal leukaemia patient who overresponded to radiation therapy, but who exhibited no overt immunodeficiency (Plowman *et al.*, 1990). The other patients identified as having defects in DNA ligase IV displayed immunodeficiency, microcephaly (abnormally small head) and growth and developmental delays (O'Driscoll *et al.*, 2001). The symptoms exhibited by these patients have been used to define a new syndrome, called LIG4 syndrome (see Table 1.2).

Non-homologous end-joining in yeast

Although yeast predominantly repair DSBs by homologous recombination, they are also able to rejoin broken DNA ends by direct ligation. In general, the mechanism of NHEJ is conserved from yeast to man. Homologues of most of the mammalian NHEJ proteins have been identified in yeast; one notable exception is the absence of a DNA-PK_{cs} homologue in *S. cerevisiae*. The properties of NHEJ-defective yeast cells are summarised in Table 1.3. In general, whilst the yeast factors show only limited sequence homology with their mammalian counterparts, the biological function of each protein is highly conserved.

Table 1.2. Clinical and cellular phenotypes of LIG4 syndrome, Ataxia Telangiectasia, Nijmegen Breakage Syndrome and Ataxia Telangiectasia-Like Disorder.

Characteristic	LIG4	NBS	AT	ATLD
Clinical				
Predisposition to malignancy	-	++	+	+
Radiosensitivity		+	+	+
Immunodeficiency	+	+	+	+
Growth retardation	+	+	(+)	(+)
Microcephaly	+	+	-	-
Mental retardation		(+)	-	-
Ataxia	-	-	+	+
Cerebellar degeneration		-	+	+
Ocular telangiectases	+	-	+	-
Cellular				
Chromosome breakage	+/-	+	+	+
Radiosensitvity	+	+	+	+
Radioresistant DNA synthesis	-	+	+	+
Defective cell cycle checkpoints	-	+	+	+
Reduced p53 response		-	+	-
Normal V(D)J recombination	-	+	+	

Adapted from (Digweed *et al.*, 1999)

Table 1.3. Properties of NHEJ-defective yeast cells.

Yeast gene	Mammalian homologue	Phenotype of defective yeast cells
YKU70/HDF1	*XRCC6/KU70*	Radiation sensitive in *rad52* background Defective NHEJ Defective in telomere length maintenance and telomeric silencing
YKU80/HDF2	*XRCC5/KU80*	Same as for *ku70*
LIF1	*XRCC4*	Radiation sensitive in *rad52* background Defective NHEJ Display normal telomere length maintenance and telomeric silencing
LIG4/DNL4	*DNA ligase IV*	Radiation sensitive in *rad52* background Defective NHEJ Display normal telomere length maintenance and telomeric silencing
MRE11	*MRE11*	Radiation sensitive in wild-type background Defective NHEJ Defective meiotic recombination, retarded resection of DSBs Display increased mitotic recombination Defective in telomere length maintenance but display normal telomeric silencing
RAD50	*RAD50*	Same as for *mre11*
XRS2	*NBS1*	Same as for *mre11*
SIR2,3 and 4	-----	Radiation sensitive in *rad52* background Defective NHEJ Defective in telomere length maintenance and telomeric silencing
NEJ1	------	Defective NHEJ Reportedly exhibit retarded DSB resection or loss of preferential nuclear localisation of Lif1

Adapted from (Jeggo, 1998)

Yeast Ku70 and Ku80

The *S. cerevisiae* Ku homologues are variously called yKu70 or Hdf1 and yKu80 or Hdf2. Although the yeast Ku subunits display only ~20% identity with human Ku70 and Ku80, like the mammalian homologues, yeast Ku exhibits DNA end-binding activity (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a; Feldmann *et al.*, 1996; Milne *et al.*, 1996). In addition, the use of *yku70* and *yku80* mutants has confirmed that these proteins act in NHEJ (Boulton and Jackson, 1996a; Milne *et al.*, 1996; Tsukamoto *et al.*, 1996b). Due to the predominance of DSB repair by HR, in yeast inactivation of yKu70 or yKu80 does not result in hypersensitivity to ionising radiation unless in a *rad52* mutant background (Boulton and Jackson, 1996b; Boulton and Jackson, 1996a; Siede *et al.*, 1996). This demonstrates, however, that in the absence of homologous recombination NHEJ can contribute to DNA repair and genomic stability in yeast.

To study yeast NHEJ *in vivo*, a plasmid-based rejoining assay was devised (Boulton and Jackson, 1996b). A plasmid encoding a metabolic marker was linearised by restriction endonucleases in a region lacking sequence homology to the chromosomal DNA. After transformation of the linear DNA into various yeast strains, DSB repair activity was scored as the number of colonies arising after growth on selective medium. In wild type cells cohesive ends were joined with high efficiency. This repair was catalysed predominantly by NHEJ as a 40- to 400-fold reduction in joining was seen when either the *YKU70* or *YKU80* genes were inactivated (Boulton and Jackson, 1996b; Boulton and Jackson, 1996a). Strains carrying disruptions in both *YKU70* and *YKU80* showed the same repair

defect as the *yku70* or *yku80* single mutants, demonstrating that, as expected, the two yeast Ku proteins act in same DSB repair pathway. Despite the break site not carrying sequences homologous to the yeast genome some repair was catalysed by HR, such that *rad52* mutants exhibited a slightly lower DSB repair activity in comparison to wild type cells.

Mutation of *YKU70* or *YKU80* not only resulted in a reduced end-joining efficiency but also more inaccurate repair. In the presence of Ku, rejoining of complementary ends was mostly by simple ligation, which preserved the sequence of the break site. In contrast, the rare repair events detected in *yku70* and *yku80* mutants exhibited large deletions, with as much as 800 bp lost from the break site (Boulton and Jackson, 1996b; Boulton and Jackson, 1996a; Wilson *et al.*, 1997). In conclusion, in yeast, rejoining of restriction endonuclease-induced DSBs in plasmid DNA is predominantly by Ku-dependent NHEJ, resulting in accurate rejoining of the broken DNA ends. In the absence of Ku, however, some breaks are rejoined by an inaccurate, Ku-independent repair mechanism.

Yeast cells carrying *yku70* and *yku80* mutations displayed three unexpected phenotypes; they were unable to grow at 37°C, exhibited substantial telomere shortening and were defective in telomeric silencing (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a; Feldmann *et al.*, 1996; Porter *et al.*, 1996; Boulton and Jackson, 1998). Subsequently, crosslinking studies have shown that Ku associates with telomeric chromatin *in vivo*, and yeast two-hybrid studies have demonstrated an interaction between Ku and Sir4; a telomere-binding

protein that activates transcriptional silencing through establishment of a condensed chromatin state (Tsukamoto *et al.*, 1997b; Gravel *et al.*, 1998). In brief, the yeast Ku protein is thought to act in telomere maintenance by protecting the telomere against recominogenic and nucleolytic activities (reviewed in Bertuch and Lundblad, 1998; Weaver, 1998).

Lig4

A yeast homologue of mammalian DNA ligase IV, Lig4, was identified independently three times in yeast genomic screens using the conserved catalytic domain common to all eukaryotic ligases (Schär *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997). The gene, called either *LIG4* or *DNL4*, encodes a ligase possessing a large C-terminal extension that is homologous throughout its length to the distinctive C-terminus of mammalian ligase IV. Overall, the 944 aa Lig4 protein displays 25% identity to human DNA ligase IV.

In agreement with a role for Lig4 in yeast NHEJ, yeast *lig4* mutants only display radiation sensitivity in a *rad52* mutant background (Teo and Jackson, 1997; Wilson *et al.*, 1997). Furthermore, the *lig4*/*rad52*/*yku70* triple mutant displays the same IR sensitivity as *lig4*/*rad52* or *yku70*/*rad52* double mutants, indicating that yKu70 and Lig4 act in the same DSB repair pathway. As expected, using the *in vivo* plasmid-based assay *lig4* cells demonstrate a repair defect similar to that observed in *yku70* cells (Schär *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997). In contrast to yKu70 and yKu80, however,

there is no evidence that Lig4 functions in telomere length maintenance (Teo and Jackson, 1997).

Biochemical analyses of yeast Lig4 showed that it shared many characteristics with human DNA ligase IV. A GST-Lig4 fusion protein isolated from yeast cells exhibited higher levels of adenylation following preincubation with sodium pyrophosphate (Wilson *et al.*, 1997). This indicated that, like human DNA ligase IV, yeast Lig4 is isolated as a stable ligase-adenylate complex. Moreover, Lig4 showed the same substrate specificity as DNA ligase IV on DNA/RNA hybrid duplexes (Schär *et al.*, 1997).

Lif1

Lif1 is a functional homologue of the mammalian XRCC4 protein. Lif1 has been shown to specifically interact with Lig4 by coimmunoprecipitation and yeast two-hybrid analyses (Herrmann *et al.*, 1998; Teo and Jackson, 2000). The yeast Lif1 protein shares 22% identity with XRCC4, with the highest degree of sequence conservation in the region of Lif1 that mediates Lig4 binding (Herrmann *et al.*, 1998). In keeping with the function of XRCC4 in mammals, Lif1 in yeast: i) is required for NHEJ; ii) stabilises the Lig4 protein; iii) stimulates the ligation activity of Lig4 by assisting adenylation of the ligase; iv) binds DNA co-operatively and v) helps target Lig4 to DSBs (Herrmann *et al.*, 1998; Teo and Jackson, 2000).

Other factors implicated as acting in yeast NHEJ

Sir proteins

The plasmid-based end-joining assay described earlier and an alternative assay, in which a single DSB is generated at the mating-type (MAT) locus by HO endonuclease, have been used to determine if additional factors are required for yeast NHEJ (Moore and Haber, 1996; Tsukamoto *et al.*, 1997b). After yKu70 was found to interact with Sir4, a protein involved in the transcriptional silencing of mating type genes and telomeric sequences, the role of this and other silencing proteins in NHEJ was investigated. Mutations in *SIR2*, *SIR3* and *SIR4* were found to confer increased radiation sensitivity to *rad52* mutants and to show substantial defects in Ku-mediated end-joining, which led to the suggestion that the Sir proteins are directly involved in NHEJ (Tsukamoto *et al.*, 1997b). This hypothesis was revised, however, after it was shown that the level of NHEJ in yeast was influenced by the mating type status of the cells (Astrom *et al.*, 1999).

Haploid yeast cells (which express either MAT**a** or MATα) exhibit significantly higher levels of NHEJ than the MAT**a**/MATα diploid cells (Astrom *et al.*, 1999). Indeed, the end-joining activity of diploid cells can be increased by disruption of one mating type alleles (i.e. generation of mat**a**Δ/MATαdiploid). This demonstrates that NHEJ is downregulated in response to the simultaneous expression of MAT**a** and MATα. Presumably, NHEJ activity is high in haploid cells, because the lack of pairs of homologous chromosomes for much of the cell cycle precludes homology-driven repair. In contrast, homologous recombination is probably favoured in diploid cells because it is a more accurate mechansim of

DSB repair, and consequently NHEJ is downregulated. In *sir* mutants however, silencing of the cryptic **a** and α genes at the HML and HMR loci is alleviated and haploid cells exhibit the properties of a MAT**a**/MATα diploid, including repression of NHEJ (Astrom *et al.*, 1999; Lee *et al.*, 1999). The Sir proteins therefore do not have a direct role in NHEJ, but instead indirectly influence NHEJ activity through altering the mating-type phenotype of *sir* mutants.

Nej1

Following the discovery that NHEJ was repressed by the MAT**a**/MATα phenotype screens were set up to identify proteins that were highly expressed in haploid cells and absent in diploid cells. Of the putative haploid-specific genes one was shown to be required for NHEJ, non-homologous end-joining regulator 1 or *NEJ1* (Kegel *et al.*, 2001; Valencia *et al.*, 2001). Disruption of *NEJ1* impaired plasmid recircularisation and the repair of HO-induced DSBs to a similar extent as mutation of *YKU70* or *LIG4*. Moreover, generation of a *nej1/lig4* double mutant demonstrated that these two genes were in the same epistasis group (Valencia *et al.*, 2001). Constitutive expression of *NEJ1* restores NHEJ activity in *sir* mutant strains and MAT**a**/MATαcells, indicating that mating type regulation of NHEJ in yeast is mediated by transcriptional repression of *NEJ1* (Kegel *et al.*, 2001; Valencia *et al.*, 2001).

Nej1 has been shown to interact with Lif1 in yeast two-hybrid screens and by coimmunoprecipitations (Frank-Vaillant and Marcand, 2001; Kegel *et al.*, 2001; Valencia *et al.*, 2001). The Nej1 protein contains two putative

transmembrane helical domains in the N-terminus, but the significance of these has not yet been established. Whilst immunoflourescent studies showed that Nej1 localised to the nucleus, it did not colocalise with nuclear pores (Kegel *et al.*, 2001). Conflicting reports suggest that mutation of Nej1 may prevent the transport of Lif1 to the nucleus or delay the resection of DSBs during repair (Kegel *et al.*, 2001; Valencia *et al.*, 2001). Hence it is currently ambiguous exactly how Nej1 regulates NHEJ activity.

Mre11, Rad50 and Xrs2

Initially Mre11, Rad50 and Xrs2 were identified as being members of the *RAD52* epistasis group, which catalyse homologous recombination (Paques and Haber, 1999). Indeed, in many ways the phenotypes of *mre11*, *rad50* and *xrs2* cells resemble those of other HR mutants. They display acute sensitivity to ionising radiation in contrast to the NHEJ *ku* and *lig4* mutants, which only exhibit IR sensitivity when HR is disabled (see Table 1.3 on page 58; Siede *et al.*, 1996; Wilson *et al.*, 1997; Paques and Haber, 1999). Furthermore, *mre11*, *rad50* and *xrs2* cells are defective in meiotic recombination, another phenotype characteristic of HR mutants (Alani *et al.*, 1990; Ivanov *et al.*, 1992; Johzuka and Ogawa, 1995). Yet overwhelming evidence from a number of different *in vivo* assays pointed towards these proteins also participating in NHEJ. Disruption of *MRE11*, *RAD50* and *XRS2* cause defects in: recircularization of linear plasmid DNA (Milne *et al.*, 1996; Boulton and Jackson, 1998); repair of HO endonuclease-induced DSBs (Moore and Haber, 1996); illegitimate integration of

transforming DNA (Schiestl *et al.*, 1994; Tsukamoto *et al.*, 1996a) and the repair of DSBs induced by dicentric plasmids (Tsukamoto *et al.*, 1997a). In these processes the *mre11*, *rad50* and *xrs2* mutations were epistatic with mutations in Ku, confirming a requirement for *MRE11*, *RAD50* and *XRS2* in NHEJ as well as HR.

MRE11 encodes a 78 kDa protein with conserved phosphoesterase motifs at the amino-terminus, and two distinct DNA binding domains towards the carboxy-terminus (see Fig. 1.5; Haber, 1998). It was not a surprise therefore, when Mre11 was demonstrated to be a nuclease. Mre11 alone exhibits ATP-independent, Mn^{2+}-dependent 3' to 5' exonuclease activity on dsDNA and ss endonuclease activity (Furuse *et al.*, 1998; Usui *et al.*, 1998; Trujillo and Sung, 2001). Yeast two-hybrid experiments and coimmunoprecipitations have demonstrated that Mre11 interacts with both Rad50 and Xrs2 to form a large protein complex, often referred to as the M/R/X complex (Johzuka and Ogawa, 1995; Usui *et al.*, 1998). Association with Rad50 appears not to significantly alter the exonuclease activity of Mre11, but does stimulate Mre11 ss endonuclease activity in an ATP-dependent manner (Trujillo and Sung, 2001).

One phenotype of cells mutated in the M/R/X complex, which is not shared by other HR mutants, is that following induction of DSB in mitotic cells by the HO endonuclease, the 5' to 3' resection of the break is retarded (Sugawara and Haber, 1992; Ivanov *et al.*, 1994; Tsubouchi and Ogawa, 1998). This observation along with the identification of Mre11 as a nuclease, led to the hypothesis that the M/R/X complex was responsible for processing of DSBs,

Figure 1.5. Putative structure of the Mre11/Rad50 complex.

(A) Functional domains of Mre11. The N-terminal region of Mre11, which is more highly conserved between yeast and humans, contains a consensus phosphoesterase (p'esterase) motif which is essential to the nuclease activity of the protein. By comparison, mutational analyses have determined the C-terminus to be important for the DNA-binding activity of Mre11.

(B) Schematic representation of Rad50. The architecture of the protein, having two globular domains containing the nucleotide binding motifs separated by a central coiled-coil domain, is shared by members of the structural maintenance of chromosome (SMC) proteins.

(C) Model of the *S. cerevisiae* Rad50 dimer determined by electron microscopy. The antiparallel coiled-coil domains bend sharply at the hinge domain allowing the association of the globular N- and C-termini to form the catalytic domain. Taken from (Anderson *et al.*, 2001).

(D) Model of DNA-bound Rad50/Mre11 complex. Mre11 associates with the catalytic domain of Rad50. This complex is proposed to act as and end-bridging factor, binding opposite ends of a double-strand break. Also taken from (Anderson *et al.*, 2001).

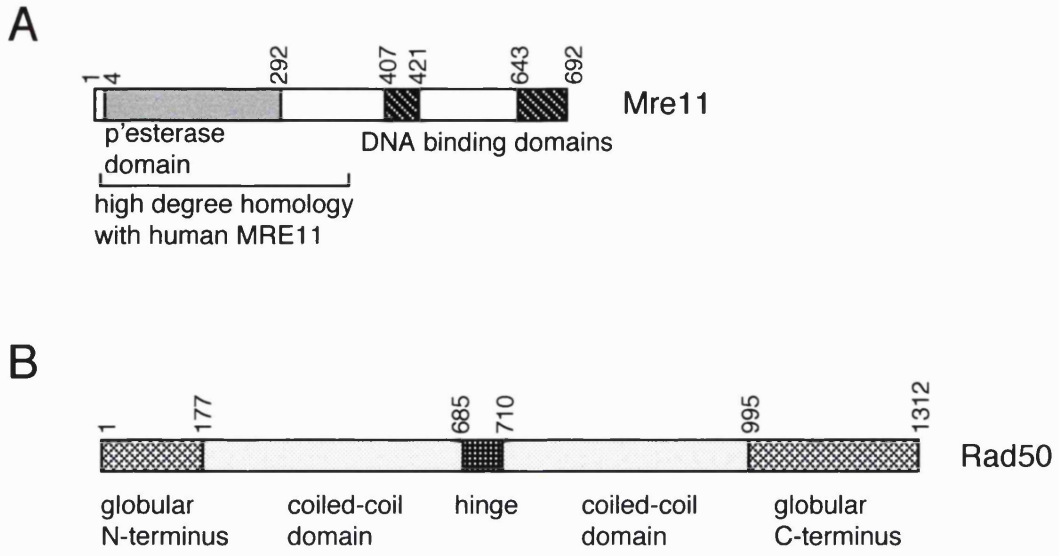

A
1
4
292
407
421
643
692
Mre11
p'esterase
domain
DNA binding domains
high degree homology
with human MRE11
B
1
177
685
710
995
1312
Rad50
globular
N-terminus
coiled-coil
domain
hinge
coiled-coil
domain
globular
C-terminus
C
NH2
COOH
Hinge
D
NH2
COOH
Mre11
Hinge

Figure 1.5

even though the 3' to 5' *in vitro* exonuclease activity of Mre11 seemed inconsistent with the 5' to 3' degradation observed *in vivo*. To clarify this point various groups have made mutations in the phosphoesterase domain of Mre11 (Furuse *et al.*, 1998; Usui *et al.*, 1998; Bressan *et al.*, 1999; Moreau *et al.*, 1999). These mutations eliminated the *in vitro* nuclease activity of Mre11 and resulted in a meiotic phenotype in which the Spo11-induced DSBs were generated, but did not undergo the normal exonucleolytic processing. In contrast, mutations within the C-terminal DNA binding domain prevented DSB formation (Furuse *et al.*, 1998; Usui *et al.*, 1998). With respect to meiotic recombination, therefore, the nuclease activities of Mre11 are required for the processing but not the formation of DSBs.

The role of Mre11 nuclease activity in mitotic repair, or more specifically NHEJ is more ambiguous. Two nuclease-defective *mre11* mutants were found to exhibit MMS sensitivity, however the mutant that also lacked Rad50 binding exhibited a more extreme sensitivity (Usui *et al.*, 1998). This would suggest that mitotic DSB repair is made up of nuclease-dependent and nuclease-independent mechanisms. In contrast, wild type levels of plasmid end-joining were observed in a different nuclease-defective *mre11* mutant (Moreau *et al.*, 1999).

Rad50 is a large (153 kDa) protein containing coiled-coil and nucleotide binding domains (Alani *et al.*, 1989; Anderson *et al.*, 2001b). ATP binding has been shown to be essential to Rad50 function; mutations in the nucleotide-binding site confer a null phenotype, and the DNA binding activity of the protein is ATP-dependent (Alani *et al.*, 1990; Raymond and Kleckner, 1993). The distinct

architecture of the Rad50 protein, having two terminal globular domains containing the ATP-binding motifs separated by a long coiled-coil domain, is shared by the structural maintenance of chromosome (SMC) proteins (Hirano *et al.*, 1995; Hopfner *et al.*, 2000). The SMC proteins form antiparallel dimers such that the catalytic domains are assembled from the N and C termini from opposing monomers. Biochemical and structural analyses of the *Pyrococcus furiosus* Rad50 terminal domains indicated that both the N and C termini were required to form a single catalytic domain with ATP- and DNA-binding activities (Hopfner *et al.*, 2000). Furthermore, investigation of yeast Mre11-Rad50 complexes by electron microscopy has shown yeast Rad50 to have the same anti-parallel coiled-coil structure as the SMC proteins (Anderson *et al.*, 2001b). The dimer appears to bend sharply in the middle of the coiled-coil domain, such that the two catalytic domains are brought in close proximity (see Fig. 1.5). Moreover, Mre11 is bound to the globular domains and thus closely associated with the ATPase and DNA binding domains of Rad50.

The role of MRE11, RAD50 and NBS1 (the human Xrs2 homologue) in mammalian NHEJ will be discussed later.

III. Role of NHEJ proteins in V(D)J recombination

V(D)J recombination is a mechanism of DNA rearrangement used by lymphocytes to assemble the variable portion of immunoglobulin and T-cell receptor genes. In this process the component variable (V), diversity (D) and joining (J) gene sequences are recombined to generate a unique chromosomal

sequence. In brief, V(D)J recombination consists of a DNA cleavage reaction catalysed by the products of the recombination-activating genes (RAG proteins), followed by a rejoining reaction mediated by the NHEJ proteins (illustrated in Fig. 1.6 and reviewed in Schatz, 1997; Smider and Chu, 1997; Weaver and Alt, 1997). The lymphoid-specific RAG1 and RAG2 proteins initiate V(D)J recombination by binding to conserved recombination signal sequences (RSS) which flank the different gene segments and by bringing two RSS into close proximity (McBlane *et al.*, 1995; Hiom and Gellert, 1998). DSBs are then introduced between the coding and signal sequences by the RAG proteins, generating blunt signal ends and covalently closed hairpins at the coding ends (Roth *et al.*, 1993; Schlissel *et al.*, 1993; Ramsden and Gellert, 1995). During the second stage of V(D)J recombination, the two signal ends are joined by accurate ligation (Smider and Chu, 1997). In a separate end joining reaction, the hairpin coding ends are opened and two coding ends joined, frequently accompanied by nucleotide addition or loss. In general, this second phase of V(D)J recombination requires Ku, DNA-PK_{cs}, XRCC4, DNA ligase IV and terminal deoxynucleotidyl transferase (TdT), in addition to RAG1 and RAG2 (Gilfillan *et al.*, 1993; Komori *et al.*, 1993; Taccioli *et al.*, 1993; Grawunder *et al.*, 1998a). TdT is responsible for the addition of non-templated nucleotides into opened hairpins and as such is only involved in coding joint formation (Gilfillan *et al.*, 1993; Komori *et al.*, 1993). Evidence from DNA-PK_{cs}-defective SCID mice, which are defective in coding joint but not signal joint formation, suggest that that DNA-PK_{cs} may also be required only for the joining of coding ends (Lieber *et al.*, 1988). In summary, V(D)J

Figure 1.6. Summary of V(D)J recombination.

V(D)J recombination is initiated by the binding of RAG1 and RAG2 (shown in green) to recombination signal sequences (RSS, indicated as coloured triangles). RAG1 and RAG2 cleave the DNA between the RSS and the coding sequence (coloured rectangles), generating blunt signal ends and hairpin coding ends. In the second phase of the reaction, the coding ends and signal ends are joined in separate end-joining reactions catalysed by the NHEJ proteins. Signal joint formation occurs via precise fusion of the blunt ends and requires Ku (yellow), XRCC4 (purple) and DNA ligase IV (blue). In comparison, coding joint formation is often inaccurate. The opened hairpin ends are often subject to nucleotide loss; alternatively TdT (red) can add nontemplated nucleotides (N) to the 3' ends of the coding region. The processed coding ends are finally joined in a reaction requiring DNA-PK_{cs} in addition to Ku, XRCC4 and DNA ligase IV. This model is adapted from (Schatz, 1997).

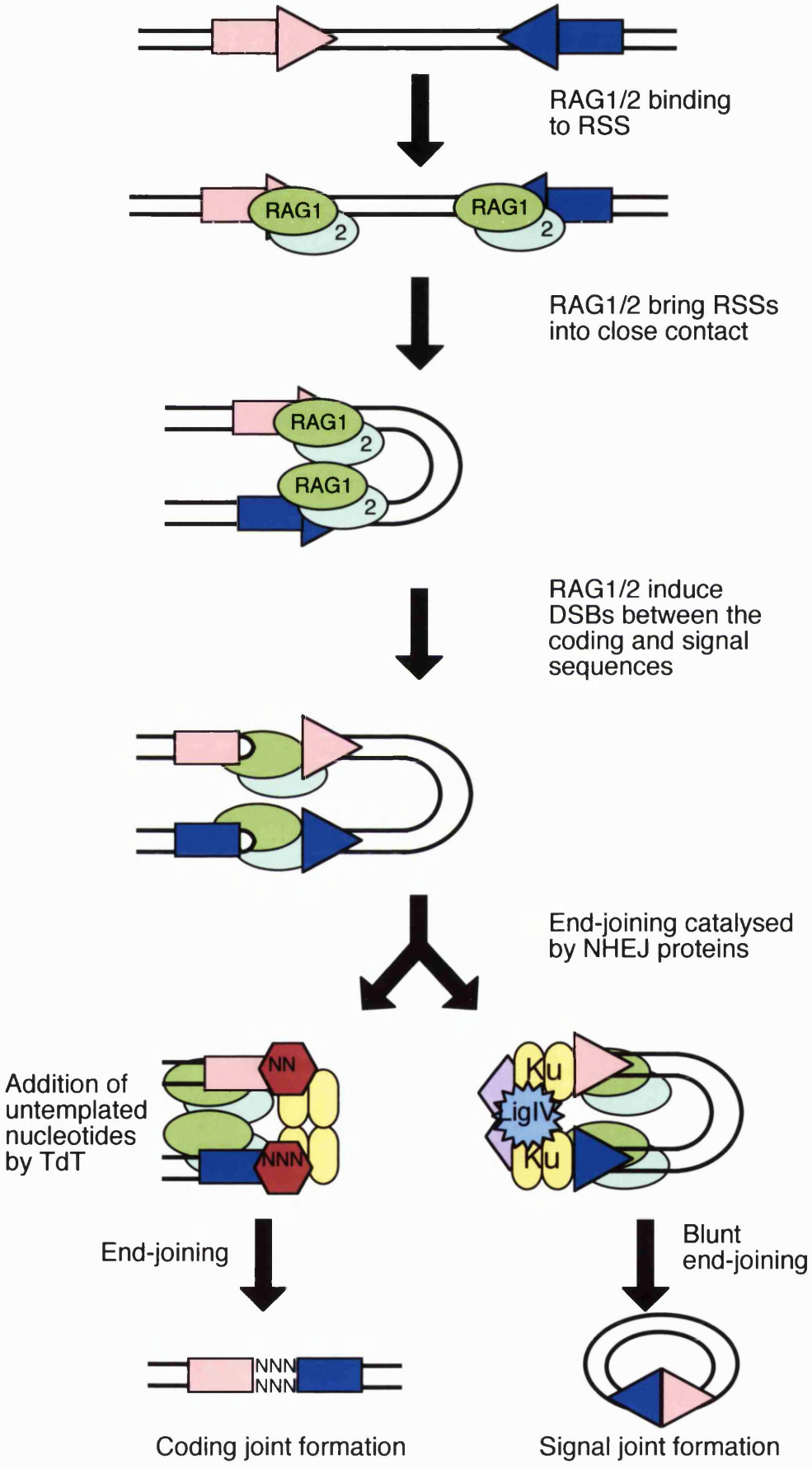

RAG1/2 binding
to RSS
RAG1
2
RAG1
2
RAG1/2 bring RSSs
into close contact
RAG1
2
RAG1
2
RAG1/2 induce
DSBs between the
coding and signal
sequences
End-joining catalysed
by NHEJ proteins
Addition of
untemplated
nucleotides
by TdT
NN
NNN
Ku
LigIV
Ku
End-joining
Blunt
end-joining
NNN
NNN
Coding joint formation
Signal joint formation

Figure 1.6

recombination is a mechanism of DNA rearrangement that combines the activities of general DNA repair proteins with a unique method of DSB initiation to create a lymphoid specific process.

IV. Repair of ionising radiation-induced double-strand breaks

Structure of IR-induced breaks

Ionising radiation induces a large spectrum of DNA lesions, either inducing the damage by direct interaction of the radiation energy with DNA, or indirectly through the formation of reactive oxygen species, such as hydroxyl radicals (reviewed in Friedberg *et al.*, 1995). Due to the predominance of water in biological systems, however, the majority of DNA damage is believed to be due to hydroxyl radical attack. This in itself is an important factor in determining the type of damage produced, because IR does not bring about an even distribution of hydroxyl radicals. Instead, clusters of free radicals are produced and when these occur within close proximity to DNA they result in the formation of multiply damaged sites. The different types of DNA lesion induced by IR, including base alterations, sugar damage and single-strand breaks can therefore be found in different combinations at multiply damaged sites. When two SSBs are located close to each other (within about 10 – 20 bp) and on opposing strands then a double-strand break results.

The chemical structure of DNA termini at the sites of radiation-induced breaks has been investigated by irradiation of aqueous DNA solutions or

mammalian cells from which the DNA was subsequently extracted. Radiation-induced breaks displayed two types of 3' termini, with 3'-phosphate and phosphoglycolate groups occurring at the same frequency (Henner *et al.*, 1982; Henner *et al.*, 1983). In contrast, 5' termini were classified as one of three types, those with the normal phosphate groups (~20%), those with hydroxyl groups (~15%) and those with neither phosphate nor hydroxyl groups (65%; Coquerelle *et al.*, 1973). In short, these data indicate that the majority of the 3' and 5' termini at irradiation-induced DNA breaks exhibit modified termini, and cannot be joined by simple ligation. Accordingly, repair of IR-induced DSBs must include processing of the modified termini and restoration of ligatable 5'-P and 3'-OH groups. The mammalian Ku, DNA-PK_{cs}, XRCC4 and DNA ligase IV proteins have not displayed any enzymatic activities which might be compatible with a role in DNA end processing. Additional proteins are, therefore, predicted to act in the repair of radiation-induced DSBs by NHEJ.

One set of proteins which are possible candidates for catalysing the processing of modified termini are MRE11, RAD50 and NBS1. Plasmid-based repair assays have shown that the homologues of these proteins act in NHEJ in yeast (Milne *et al.*, 1996; Boulton and Jackson, 1998). The yeast homologues are also thought to act in the resection of DSBs during homologous recombination, and purified Mre11 protein from both yeast and humans exhibits nuclease activity (Cao *et al.*, 1990; Ivanov *et al.*, 1994; Furuse *et al.*, 1998; Paull and Gellert, 1998; Tsubouchi and Ogawa, 1998; Moreau *et al.*, 1999). In the

next section I will discuss what is known about MRE11, RAD50 and NBS1 and what evidence there is to support a role for these proteins in mammalian NHEJ.

MRE11, RAD50 and NBS1

In general the MRE11 and RAD50 proteins are highly conserved between yeast and humans. Both proteins are comparable sizes in the two organisms ~150 kDa for RAD50 and ~80 kDa for MRE11 (Petrini *et al.*, 1995; Dolganov *et al.*, 1996). Furthermore, the amino acid sequences of hMRE11 and *Sc*MRE11 share 50% identity in the N-terminal region, which encompasses the phosphodiesterase domains, although the C-termini are more divergent (Petrini *et al.*, 1995). In contrast, the hRAD50 and *Sc*RAD50 proteins share the highest conservation (again >50% identity) at both the N and C termini, with an intermediate portion exhibiting much lower sequence homology (Dolganov *et al.*, 1996). This is not surprising considering that the Walker A nucleotide binding motif is located within the N-terminal region of RAD50, the Walker B motif within the C-terminus, and the catalytic domain is formed by interaction of the two terminal regions (Dolganov *et al.*, 1996; Hopfner *et al.*, 2000). Whilst the central regions of RAD50 may not exhibit high primary sequence conservation, these domains are predicted to adopt extremely similar coiled-coil structures within both the yeast and human proteins (Dolganov *et al.*, 1996). Moreover, RAD50 and MRE11 from yeast and humans exhibit very similar biochemical properties including the formation of a highly stable RAD50/MRE11 complex (Johzuka and Ogawa, 1995; Dolganov *et al.*, 1996).

Comparatively, the yeast Xrs2 and human NBS1 proteins share very little sequence homology; only 28% identity in the N-terminal 115 amino acids (Carney *et al.*, 1998). As such the two proteins are not true homologues, however the two proteins are the same size (95 kDa) and both are components of the MRE11/RAD50 complex (Carney *et al.*, 1998; Usui *et al.*, 1998). Xrs2 and NBS1 are therefore considered functional analogues. NBS1 contains two identifiable motifs at the N-terminus, a fork-head-associated (FHA) domain and a BRCT domain (Varon *et al.*, 1998). Both motifs are found within other DNA-damage-responsive cell cycle checkpoint proteins, and have been suggested to mediate protein-protein interactions. However, it has been shown that the MRE11-binding domain of NBS1 lies at aa 665 – 693, and the FHA and BRCT domains can be deleted without affecting association with MRE11 (Tauchi *et al.*, 2001). In addition, the radiosensitivity of NBS cells was rescued by expression of *NBS1* constructs lacking either the FHA or BRCT domains. This indicates that these domains are not essential for DSB repair.

The biochemical activities of the MRE11/RAD50/NBS1 complex (referred to in future as the M/R/N complex) have been investigated *in vitro*. Due to the high degree of conservation between the yeast and human MRE11 proteins, it was no surprise when purified MRE11 was found to have 3' – 5' dsDNA exonuclease activity (Paull and Gellert, 1998). The exonuclease activity of MRE11 was stimulated 3- to 4-fold by association with RAD50. However, both the MRE11/RAD50 complex and MRE11 alone showed similar activities in the presence or absence of ATP. Furthermore, the nuclease activity of MRE11 was

shown to permit the joining, by DNA ligase I or the XRCC4/DNA ligase IV complex, of DNA fragments carrying non-complementary termini (Paull and Gellert, 1998; Paull and Gellert, 2000). The mismatched ends were found to undergo deletions of between 13 and 79 nucleotides and junctions were formed when short sequences of microhomology were revealed. In contrast, both wild type and nuclease-defective MRE11 protein stimulated XRCC4/DNA ligase IV-catalysed joining of complementary termini. This suggests that apart from its nuclease activity, MRE11 might also act in end-joining as a DNA end-bridging factor. In support of this theory, MRE11 was also shown to anneal complementary ssDNA molecules (de Jager *et al.*, 2001a).

In addition to the dsDNA exonuclease and annealing activities, MRE11 and the M/R/X complex also exhibited endonuclease activity on ssDNA and hairpin structures (Paull and Gellert, 1998; Trujillo *et al.*, 1998). Briefly, MRE11 alone was able to cleave unpaired hairpin loops, with the majority of cuts made one or two nucleotides 3' of the centre of the hairpin (Paull and Gellert, 1998). In the absence of RAD50 or NBS1, though, MRE11 had little activity on hairpins in which all the bases were paired. Association with NBS1 allowed MRE11 to cleave fully paired hairpins (Paull and Gellert, 1999). Furthermore, in the presence of ATP, the M/R/N complex exhibited limited unwinding of the DNA duplex allowing cleavage of hairpins at a greater variety of sites. One mechanism in which this hairpin cleavage activity of the M/R/N complex could conceivably be required is in the joining of coding sequences during V(D)J recombination.

In support of a role for MRE11, RAD50 and NBS1 in DSB repair in mammalian cells, immunofluorescence studies have found that these three proteins colocalise in distinct nuclear foci following ionising radiation (Maser *et al.*, 1997; Carney *et al.*, 1998). Furthermore, in an innovative piece of work human fibroblasts were exposed to ultrasoft X-rays passed through an irradiation mask consisting of small, evenly spaced strips of gold (Nelms *et al.*, 1998). Consequently the cell nuclei were irradiated in distinctive stripes, and the DNA damage restricted to discrete subnuclear regions of the cells. Immunoflourescent staining of MRE11 was found to correlate with the irradiated regions of the nucleus as early as 30 minutes following irradiation, therefore strengthening the argument that irradiation-induced MRE11 foci correspond to sites of DSB repair.

Further evidence that the M/R/N proteins act in DSB repair has come from the phenotypes of cells in which the complex has been disrupted. Null mutations of *MRE11*, *RAD50* or *NBS1* result either in cell inviability or embryonic lethality, demonstrating the essential role of these gene products in normal cellular processes (Xiao and Weaver, 1997; Luo *et al.*, 1999; Zhu *et al.*, 2001). More informative are non-null mutations which, when located in *MRE11* result in an ataxia telangiectasia-like disorder (ATLD), and within *NBS1* cause Nijmegen breakage syndrome (NBS; Carney *et al.*, 1998; Varon *et al.*, 1998; Stewart *et al.*, 1999). NBS and ATLD patients display many phenotypes seen in ataxia telangiectasia (AT) patients, who carry mutations in the *ATM* gene, and those with mutations in *LIG4* (see Table 1.2, page 57). At the cellular level, deficiency

in *NBS1*, *MRE11* or *ATM* results in chromosomal instability and an increased sensitivity to ionising radiation, which indicates the gene products probably act in DNA repair (Shiloh, 1997; Featherstone and Jackson, 1998; Stewart *et al.*, 1999). Additionally, the cells exhibit radioresistant DNA synthesis brought about by defective G_1/S checkpoint control, which suggests a supplementary role in the signalling of DNA damage. More recently ATM and the M/R/N complex have been shown to act the same pathway (Wu *et al.*, 2000; Zhao *et al.*, 2000). NBS1 is a substrate for ATM kinase activity, and mutation of the phosphorylation sites resulted in a failure to induce radiation-induced foci and restore radiation resistance to NBS cells. This has confirmed the role of the M/R/N complex in the signalling of DNA damage.

V. Development of cell-free systems to permit *in vitro* analysis of NHEJ

In vivo studies

In mammalian cells, exogenous DNA is incorporated into the genome by random integration 100- to 10,000-fold more frequently than by targeted integration. Consequently it was predicted that a) non-homologous repair pathways are highly active in mammals and b) that mammalian cells might represent a rich source of non-homologous repair proteins. Early studies of DNA end-joining used an *in vivo* system in which a linearised SV40 genome was transfected into cultured monkey cells (reviewed in Roth and Wilson, 1988). Recircularisation of the genome was required for viral infectivity, and therefore DNA end-joining could

be scored by the appearance of viral plaques. Moreover, viral DNA isolated from the plaques could be sequenced to give detailed information about the junctions formed. With this extrachromosomal end-joining assay, mammalian cells were shown to efficiently join a number of restriction enzyme-induced DSBs carrying either complementary or non-complementary DNA termini. Analyses of the junctions arising from non-complementary end-joining revealed that around 60% of joining was accurate, conserving the sequences of both termini (Roth and Wilson, 1986). Although the remaining joining events were inaccurate, in general, large alterations to the DNA termini were not observed, just 3% of the junctions displayed deletions of more than 15 nucleotides, and only 14% exhibited deletions of between 6 and 15 nucleotides.

Joining of non-complementary termini was mostly categorised into two different modes of repair (Roth and Wilson, 1986). When a protruding terminus (either 5' or 3' overhang) was joined to a blunt terminus, then the sequence of the overhang was largely preserved, indicating a requirement for gap filling DNA polymerase activity (illustrated in Fig. 1.7A). The same was seen for two protruding termini of opposite polarity (i.e. joining of a 5' overhang to a 3' overhang). When the two termini contained ss extensions of the same polarity (i.e. joining of two 5' overhangs or two 3' overhangs) then joining was mostly mediated by pairing between complementary nucleotides in the extensions (see Fig. 1.7B). A single complementary base pair appeared sufficient to align the two termini, although pairing between such short homologies is expected to be highly unstable. In some cases homologies located further away from the DNA termini

Figure 1.7. Different end-joining mechanisms observed in mammalian cells and ceil-free extracts.

(A) Fill-in repair. Observed when joining occurs between protruding 5' and blunt termini (i); protruding 3' and blunt termini (ii); or protruding 5' and protruding 3' termini (iii). The termini are held in alignment to allow 5' to 3' fill-in DNA synthesis (represented as red triangles) and subsequent ligation.

(B) Microhomology-directed repair. Observed when joining occurs between two non-complementary protruding termini of the same polarity, i.e. 5' to 5' (as shown) or 3' to 3'. The termini are aligned by pairing at microhomologies (represented by thick black line), which may be as little as a single base pair. Any mismatched bases are removed, the single-strand gaps filled by DNA polymerase activity and finally the termini ligated.

(C) Single-strand annealing (SSA)-like repair. Found to be the dominant end-joining activity in certain cell-free extracts, this repair mechanism is independent of the structure of the DNA termini. In all cases, the termini are degraded by exonucleases until small regions of homology are revealed. The termini anneal at these regions of microhomology and repair is completed, presumably by removal of the single-stranded flaps and ligation.

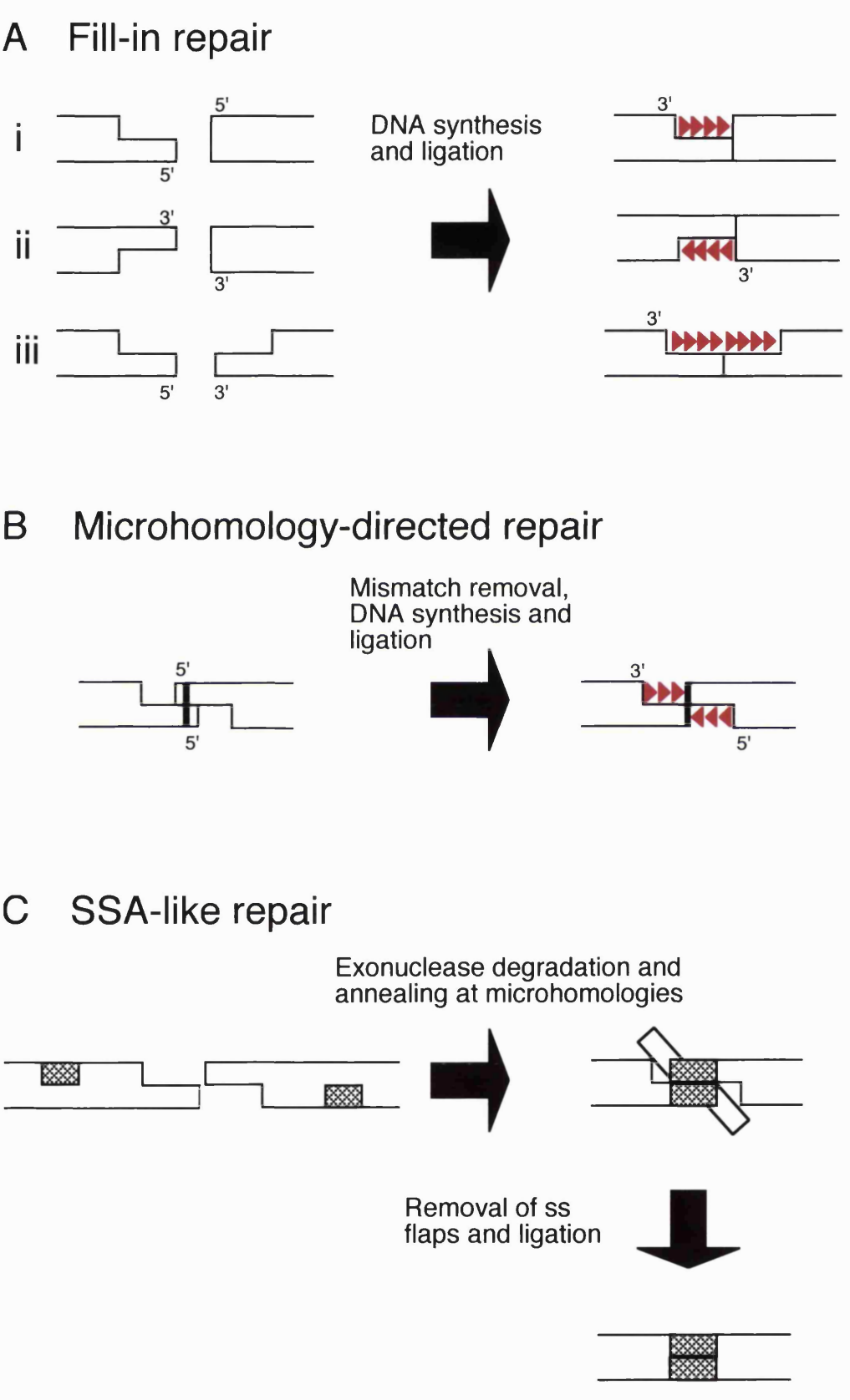

A Fill-in repair
i
ii
iii
5'
3'
DNA synthesis
and ligation
B Microhomology-directed repair
Mismatch removal,
DNA synthesis and
ligation
C SSA-like repair
Exonuclease degradation and
annealing at microhomologies
Removal of ss
flaps and ligation

Figure 1.7

were used during joining (Fig. 1.7C). DNA gap-filling, exonuclease, and possibly flap endonuclease activities would be anticipated to be required in this homology-directed mode of joining.

In vitro studies

Whilst the transfection experiments were very informative as to the type of junctions formed during NHEJ *in vivo*, they gave no indication as to which proteins catalysed repair. For this, *in vitro* assays were developed using cell-free extracts. Unfortunately, although many groups have used extracts as a source of NHEJ activity, there are few similarities between the different studies, as to the source of extracts or the method of extract preparation used. Furthermore, in many of these studies the protein factors required for repair were never identified. Nevertheless, the results give some insight into the DNA end-joining mechanisms that are used by vertebrate cells.

Xenopus egg extracts

The first *in vitro* end-joining assays used extracts prepared from *Xenopus laevis* eggs to promote the joining of linearised plasmid DNA (Pfeiffer and Vielmetter, 1988). Analysis of the end-joining products by gel electrophoresis demonstrated that circular monomers and linear multimers were formed. Transformation of the end-joining products into bacterial strains, selectively propagated the circular products, and permitted subsequent sequencing of the junctions. In brief, the extracts efficiently joined a range of complementary and non-complementary

termini. However, blunt termini were joined with reduced efficiency. The joining of non-complementary termini by *Xenopus* egg extracts was very similar to that seen in the *in vivo* transfection assays, with both fill-in and microhomology-directed repair observed. One consequence of the 5' to 3' direction of DNA polymerases is that during the joining of protruding 3' and blunt termini, the blunt terminus is required to prime DNA synthesis (see Fig. 1.7Aii). Experiments in which dideoxynucleotides were incorporated during joining demonstrated that in *Xenopus* extract-catalysed fill-in repair, DNA synthesis preceded ligation (Thode *et al.*, 1990). Consequently, if ligation does not anchor the two termini together for DNA synthesis, this would need to be carried out by a protein component of the end-joining reaction. Furthermore, during the microhomology-directed repair, a single base pair complementarity (energetically a very weak interaction) was found to be sufficient to align the two termini (see Fig 1.7B). Similar protein factors would therefore be expected to stabilise the aligned termini during this mode of joining.

At the time of these early studies, the role of Ku, DNA-PK_{cs}, XRCC4 and DNA ligase IV in NHEJ had not yet been established. Some years later, however, anti-Ku antibodies were used to assess the requirement for this protein in *Xenopus* egg extract-catalysed end-joining (Labhart, 1999a). Addition of Ku antisera or immunodepletion of Ku from *Xenopus* extracts did not inhibit all end-joining activity, but instead selectively abolished the formation of circular products and not linear multimers. Strangely, the joining of complementary 5' termini was not affected by the absence of Ku, and both circles and linear multimers were

observed as normal. These data indicate that *Xenopus* extracts contain both Ku-dependent and Ku-independent end-joining activities, the former producing circular products, and the latter predominantly generating linear multimers.

Fractionation of *Xenopus* egg extracts also isolated an error-prone rejoining end-joining activity that was Ku-independent and generated only linear multimeric products (Gottlich *et al.*, 1998). All the products generated displayed terminal deletions of between 4 and 206 nucleotides. In addition, the junctions were always formed at sequences of microhomology, a characteristic common to single-strand annealing (SSA)-like repair mechanisms (see Fig. 1.7C).

Mammalian extracts

Incubation of linear plasmid DNA with mammalian cell-free extracts has demonstrated that, similar to the results from *Xenopus* egg extracts, mammalian extracts also contain multiple end-joining activities (reviewed in Labhart, 1999b). In some nuclear extracts the dominant joining activity observed was SSA-like repair (Derbyshire *et al.*, 1994; Nicolas and Young, 1994; Nicolas *et al.*, 1995). With these extracts cohesive and blunt ends were joined with equal efficiency, and all repair was accompanied by deletions from the DNA termini. The detection of this end-joining activity in *Xenopus* egg extracts, as well as nuclear extracts prepared from a variety of monkey, mouse and human cell lines, demonstrated that the SSA-like repair was not specific to just one species. The activity was also seen with nuclear extracts prepared from a SCID cell line,

demonstrating that DNA-PK_{cs} was not required for this end-joining activity (Nicolas and Young, 1994).

Single-strand annealing was not, however, the dominant end-joining activity in all nuclear extracts. Some human nuclear extracts joined complementary protruding 5' termini primarily by an accurate repair mechanism, with SSA only accounting for <1% of joining (North *et al.*, 1990; Fairman *et al.*, 1992; Thacker *et al.*, 1992). Accurate repair was also observed with human cytoplasmic, mouse mitochondrial, and mouse testicular extracts (Bøe *et al.*, 1995; Lakshmipathy and Campbell, 1999; Sathees and Raman, 1999). There were some differences in the end-joining products generated by these extracts, most notably in the ratio of linear and circular products formed. The protein requirements of joining were not determined in any of these studies, however, making any meaningful comparison impossible.

Fractionation of calf thymus extracts led to the isolation of four distinct samples that were able to join protruding 5' termini accurately (Johnson and Fairman, 1996). The NaCl sensitivity of each of the four end-joining activities differed, as did the ratio of linear to circular products generated. Western blotting revealed that only one out of the four fractions contained Ku. Furthermore, another partially purified fraction of calf thymus extracts was shown (when supplemented with T4 DNA ligase) to support joining of non-complementary termini by a SSA-like mechanism (Mason *et al.*, 1996). These data serve to emphasise the fact that mammalian extracts contain multiple end-joining activities.

When the end-joining activities of extracts prepared from *Xenopus* eggs, mouse cells and human cells were directly compared, similar results were obtained from the three extracts (Daza *et al.*, 1996). The human cell-free extracts exhibited end-joining activity that most closely resembled that seen with the *Xenopus* extracts, joining non-complementary termini using the fill-in and microhomology-directed repair mechanisms. In contrast, the mouse extracts contained potent 3' – 5' exonuclease activities. Whilst some fill-in and microhomology-directed repair was observed with mouse extracts, many of the junctions contained deletions. The availability of a Ku80-defective hamster cell line (*xrs-6*) subsequently allowed the role of Ku in rodent extract-catalysed end-joining to be studied (Feldmann *et al.*, 2000). Similar to the results from *Xenopus* extracts, in rodent extracts the generation of circular products was largely dependent on the presence of Ku. Furthermore, the *xrs-6* extract displayed not only lower levels of NHEJ, but also decreased accuracy of joining.

Joining of modified termini

The studies described above all used plasmid DNA that had been linearised by restriction enzymes. Whilst the use of two different enzymes permitted the creation of non-complementary ends, all the termini carried 5'-P and 3'-OH groups. These restriction enzyme-induced breaks do not represent radiation-induced DSBs, which exhibit modified or non-ligatable termini. Bleomycin-cleaved oligonucleotides have been used to construct NHEJ substrates with 3'-phosphoglycolate termini, and therefore mimic IR-induced damage. Joining of

bleomycin-induced breaks was observed in *Xenopus* egg extracts, although repair occurred 30-100 times more slowly than for normal restriction endonuclease-induced breaks (Gu *et al.*, 1996). Addition of the DNA-PK_{cs}-inhibitor wortmannin not only blocked joining but also suppressed phosphoglycolate removal, implying an essential role for DNA-PK early in the repair process. In contrast, a wortmannin-insensitive human nuclear extract was found to join double-strand breaks with normal, but not modified termini (Gu *et al.*, 1998).

In summary, in *Xenopus* and mammalian extracts Ku-dependent joining appears to be largely accurate and preserves the sequences of single strand extensions. With protruding termini of the same polarity, complementary base pairs within the ss overhang are used to align termini. DNA-PK-dependent end-joining could also repair certain modified termini. However, other less accurate end-joining activities were frequently observed alongside the Ku-dependent joining. The existence of multiple end-joining activities in a single extract preparation clearly makes it more difficult to study Ku-dependent end-joining events. The development of an *in vitro* assay using human cell-free extracts in which the end-joining activity was absolutely dependent on DNA-PK, XRCC4 and DNA ligase IV was, therefore, of great significance (Baumann and West, 1998a). Using this system I have; i) investigated the requirement for the MRE11 and NBS1 proteins for Ku-dependent repair; ii) studied the capacity of NHEJ to repair a number of modified (non-ligatable) termini and iii) identified a role for human polynucleotide kinase in the rejoining of 5'-hydroxyl termini by NHEJ.

I. Enzymes and Reagents

2.1 Enzymes

Human polynucleotide kinase was generously provided by Dr M. Weinfield and purified according to published protocols (Mani *et al.*, 2001). Other enzymes were purchased from the following companies:

New England Biolabs Inc.: calf intestinal phosphatase, T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I Klenow fragment (3'-5' exo$^-$), restriction enzymes and NEBuffers.

Sigma-Aldrich Company Ltd.: proteinase K.

2.2 Reagents

Chemical reagents were obtained from Sigma-Aldrich or BDH (Merck Ltd.) unless indicated otherwise below:

Amersham Pharmacia Biotech UK Limited: Phosphorus-32 as orthophosphate and rProtein A sepharose fast flow.

Bio-Rad Laboratories Ltd.: ammonium persulphate, protein assay dye reagent (Bradford reagent), bromophenol blue and xylene cyanol

DAKO A/S, Denmark: horseradish peroxidase-conjugated, anti-rabbit and anti-mouse antibodies.

Duchefa: carbenicillin.

Gibco BRL: agarose.

ICN Biomedicals Inc.: casein hydrochloride.

Medicell International Ltd.: dialysis tubing.

Melford Laboratories Ltd.: DTT.

National Diagnostics Inc.: EcoScintA scintillation fluid.

New England Biolabs Inc.: BSA

Pierce: Slide-A-Lyser dialysis cassettes.

Whatman: phosphocellulose.

2.3 Buffers and solutions

Agarose gel loading buffer (5x): 50% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol.

Deproteinisation buffer (5x): 10 mg/ml proteinase K, 2.5% (w/v) SDS, 100 mM Tris-HCl (pH 7.5), 50 mM EDTA.

E-buffer: 20 mM Tris-HCl (pH 8.0), 100 mM KOAc, 20% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.

EcoPol buffer (NEB): 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT.

***Eco*RI buffer (NEB):** 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 0.025% Triton-X-100.

Formamide gel loading buffer: 80% (v/v) formamide, 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, trace bromophenol blue and xylene cyanol.

High salt buffer (2x): 50 mM Tris-HCl (pH 7.5), 1 M KCl, 2 mM EDTA, 2 mM DTT.

High salt buffer (5x): 125 mM Tris-HCl (pH 7.5), 2.5 M KCl, 5 mM EDTA, 5 mM DTT.

HEPES end joining buffer (5x): 250 mM HEPES (pH 8.0), 200 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM ATP, 5 mM DTT, 0.5 mg/ml BSA.

Hypotonic lysis buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM DTT.

Low phosphate media: 100 mM Tris-HCl (pH 7.5), 8.5 mM NaCl, 108 mM KCl, 20 mM NH_4Cl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM KH_2PO_4, 160 mM Na_2SO_4, 0.01 mg/l $FeCl_3$, 2 mg/l thymine, 0.8 g/l Na pyruvate, 9 g/l casein hydrochloride (vitamin free), 0.4% (w/v) glucose.

Luria broth (LB): 10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 5 g/l NaCl.

LB agar: Luria Broth (as above), 1.5% (w/v) agar.

NEBuffer 1 (NEB): 10 mM Bis Tris Propane-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT.

NEBuffer 2 (NEB): 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT.

NEBuffer 3 (NEB): 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT.

NEBuffer 4 (NEB): 20 mM Tris-acetate (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KOAc, 1 mM DTT.

Phosphate buffered saiine (PBS): 8 g/l NaCl, 0.25 g/l KCl, 1.43 g/l Na_2HPO_4, 0.25 g/l KH_2PO_4.

PBS-Tween: Phosphate buffer saline (as above), 0.025% (v/v) Tween-20.

Protein gel ioading buffer (5x): 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 3% (w/v) SDS, 10% β-mercaptoethanol, 0.2% (w/v) bromophenol blue.

SDS-PAGE gel running buffer: 25 mM Tris base, 190 mM glycine, 0.1% SDS.

SDS-PAGE lower buffer (4x): 1.5 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS.

SDS-PAGE upper buffer (4x): 0.5 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS.

T4 DNA ligase buffer (NEB): 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.025 mg/ml BSA.

T4 PNK buffer (NEB): 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT

TAE buffer: 40 mM Tris base, 1.1% (v/v) glacial acetic acid, 1 mM EDTA.

TBE buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

Tris end-joining buffer (5x): 250 mM Tris-HCl (pH 8.0), 300 mM KOAc, 2.5 mM $Mg(OAc)_2$, 5 mM ATP, 5 mM DTT, 0.5 mg/ml BSA.

Western transfer buffer: 20 mM sodium phosphate (pH 6.7).

2.4 Antibodies

Polyclonal anti-RAD50, anti-MRE11 and anti-NBS1 antibodies were a generous gift from Dr J. Petrini (Dolganov *et al.*, 1996; Carney *et al.*, 1998). Monoclonal and polyclonal anti-human PNK antibodies were kindly provided by Dr M. Weinfeld (Karimi-Busheri *et al.*, 1999). Polyclonal anti-XRCC4 and anti-DNA ligase IV antibodies were a gift from Dr S. E. Critchlow (Critchlow *et al.*, 1997; Riballo *et al.*, 1999). Polyclonal anti-RAD51 antibodies were supplied by Dr F. Benson (Benson *et al.*, 1994). The remaining antibodies used are commercially available:

NeoMarkers: monoclonal anti-Ku80 Ab-2 (111) and monoclonal anti-Ku70 Ab-4 (N3H10).

Serotec UK: polyclonal anti-Ku antigen – 70kDa subunit, polyclonal anti-Ku antigen – 80kDa subunit, polyclonal anti-DNA-PK_{cs} and polyclonal anti-DNA ligase IV.

2.5 Bacterial strains and plasmids

2.5.1 Strains

The *E. coli* strains used during these studies were DH5α (Grant *et al.*, 1990), XL1-Blue (Stratagene) and JM109 (Promega). *E. coli* cells were grown in Luria Broth (LB) or on LB agar plates, and stored at -70°C in 30% (v/v) glycerol.

2.5.2 Plasmids

The following plasmids were used as DNA substrates in the end-joining assay. pDEA-7Z f(+) (3.0 kb) is derived from the plasmids pGEM-7Z f(+) and pBR322 (Shah *et al.*, 1994). pFB585 (7.7 kb) is a derivative of pAcSG2 provided by Dr F.E. Benson (Van Dyck *et al.*, 1998). pAcSG2 (5.5 kb) was supplied by PharMingen and pUC18 (2.7 kb) by Promega.

All plasmids were maintained in *E. coli* by addition of 100 μg/ml carbenicillin to the growth medium.

2.6 Cell lines

Name	Cell type	Mutation	Provided by	Reference
GM00558	Lymphoblast	—	NIGMS Human Genetic Mutant Cell Repository	
HeLa	Epithelial	—	NIGMS Human Genetic Mutant Cell Repository	
M059J	Derived from glioblastoma	20-fold reduction in DNA-PK_{cs} message stability leading to undetectable DNA-PK activity.	Dr S. P. Lees-Miller	(Lees-Miller *et al.*, 1995; Galloway *et al.*, 1999)
M059K	Derived from glioblastoma	Isolated concurrently with M059J, expresses normal levels of DNA-PK_{cs}.	Dr S. P. Lees-Miller	(Lees-Miller *et al.*, 1995; Galloway *et al.*, 1999)
GM7078	Lymphoblast	Homozygous for nonsense mutation in *NBS1* resulting in truncated NBS1	NIGMS Human Genetic Mutant Cell Repository	(Carney *et al.*, 1998; Varon *et al.*, 1998)
GM8036	Lymphoblast	Expresses full length NBS1. Cell line derived from father of patient from whom the GM7078 cell line established.	NIGMS Human Genetic Mutant Cell Repository	(Carney *et al.*, 1998; Varon *et al.*, 1998)
D5576	Lymphoblast	Homozygous for a C -> G transition at nt 1897 of *hMRE11*. This results in a stop codon prematurely truncating the hMRE11 protein at aa 633.	Dr A. M. R. Taylor	Referred to as ATLD1 in (Stewart *et al.*, 1999)

GM00558, and D5576 cells were grown in RPMI 1640 media supplemented with 10% fetal calf serum (FCS). HeLa cells were grown in RPMI 1640 media with 5% FCS, and GM8036 and GM7078 cells were cultured in the same, except with 15 % FCS. M059J and M059K cells were cultured in a 1:1 mix of DMEM E4/Hams F12 supplemented with 10% FCS, 1% non-essential amino acids and 1% pyruvate.

II. Gel electrophoresis and analysis

2.7 SDS-PAGE

SDS-PAGE was conducted using a 20 x 13 cm gel apparatus from Cambridge Biosciences. Separating gels typically contained 7.8 or 10% (w/v) polyacrylamide in SDS-PAGE lower buffer. Stacking gels contained 4.2% (w/v) polyacrylamide in SDS-PAGE upper buffer. The acrylamide was polymerised by the addition of 0.06% (w/v) ammonium persulphate and 0.22% (v/v) TEMED. Protein samples were prepared by the addition of 1/3 – 1/2 vol protein gel loading buffer, and heated to 100°C for 3 min prior to loading. Typically gels were run at 200 V for 2 hrs in SDS-PAGE gel running buffer. Following electrophoresis, proteins were visualised by Western blotting (section 2.29).

2.8 Agarose gel electrophoresis

Gels containing 0.6% (w/v) agarose in TAE buffer were run using the Bio-Rad Wide Mini-Sub and DNA Sub Cell systems. DNA samples were prepared by

addition 1/5 vol agarose gel loading buffer, and separated in TAE buffer at 0.5 V/cm for 2.5 hrs for the single-plasmid assay or at 0.28 V/cm for 16 hrs for the two-plasmid assay. Following electrophoresis, ^{32}P-labelled DNA was detected by autoradiography (section 2.9).

2.9 Autoradiography

After electrophoresis, agarose and polyacrylamide gels were dried onto DE81 anion exchanger paper (Whatman) supported by 3MM filter paper (Whatman). Agarose and polyacrylamide gels were exposed to Kodak Biomax film, typically with an intensifying screen at -70°C. Nitrocellulose membranes from Western blotting were exposed to Kodak X-Omat film at room temperature. Exposed films were developed using a Fuji RGII X-ray film processor.

2.10 Quantitation of results

Developed films were scanned using a Microtek ScanMaker 4 scanner, and the electronic image quantitated using Scion Image 1.62c software.

III. General methods of DNA manipulation

2.11 Determination of DNA concentration

DNA concentrations were determined by measuring the absorbance (A) at 260 nm, using a quartz cuvette and a Pharmacia Ultrospec 200 spectrophotomer. Typically DNA samples were diluted 1:40 – 1:80 in TE buffer and the A_{260}

measured against TE buffer alone. Calculations were based on the assumption that the A_{260} = 1 (measured in a cuvette with a 1 cm pathlength) for a solution of 50 μg/ml dsDNA, 40 μg/ml ssDNA or 33 μg/ml oligonucleotide.

2.12 Solvent extraction

Typically DNA samples were extracted first with phenol/chloroform/iso-amyl alcohol (25:24:1) and then chloroform. Samples were mixed thoroughly with an equal volume of the solvent, the two phases separated by low speed centrifugation and the upper aqueous phase containing the DNA retained.

2.13 Ethanol precipitation

DNA samples were mixed with 1/10 vol 3 M sodium acetate (pH 5.2) and 2.5 vol 100% (v/v) ethanol (prechilled). The DNA was precipitated at -20°C for between 1 to 16 hrs, pelleted by centrifugation (14,000 rpm for 30 min at 4°C in an Eppendorf benchtop centrifuge) and washed with 70% (v/v) ethanol. The DNA was dried using a Savant speed vac concentrator and re-suspended in ddH_2O.

2.14 ^{32}P-end-labelling of DNA

Linear plasmid DNA was 5'-^{32}P-end-labelled using T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP. Typical reactions (50 μl) contained 1 μg *Eco*RI-linearised pUC18 plasmid DNA, 5 U T4 PNK and 5 μCi [γ-^{32}P]ATP in T4 PNK buffer. After incubation for 15 min at 37°C, 0.6 mM cold ATP was added and the reaction

incubated for a further 15 min at 37°C. The reaction was stopped by addition of 50 mM EDTA, and diluted with 2 vol ddH_2O. The DNA was extracted sequentially with phenol/chloroform/iso-amyl alcohol and chloroform as described in section 2.12. Unincorporated label and residual organic solvent were removed on an S-400 Microspin column (Amersham Pharmacia). The column was prepared by centrifugation for 1 min at 3,000 rpm in an Eppendorf benchtop centrifuge. After loading the aqueous phase from the chloroform extraction, the column was centrifuged again for 2 min at 3,000 rpm. The eluate from the column was stored at 4°C until required.

2.15 Transformation of DNA into bacterial strains

Competent *E. coli* XL1-Blue and JM109 cells used for transformation were purchased from Stratagene and Promega respectively. In comparison, DH5α cells were made chemically competent by treatment with $CaCl_2$ (Sambrook *et al.*, 1989). To introduce plasmid DNA into competent cells, 50 ng DNA in TE was mixed with 0.1 – 0.2 ml competent cells. After a 30 min incubation on ice, the cells were heat-shocked at 42°C for 2 min and immediately returned to ice. The cells were supplemented with 0.8 ml LB and incubated at 37°C for 1 hr. Aliquots of the bacterial culture, typically between 0.1 and 0.3 ml, were spread onto LB agar plates containing 100 μg/ml carbenicillin and incubated overnight at 37°C. Larger cultures were inoculated with single colonies.

IV. Preparation of DNA substrates

2.16 Preparation of plasmid DNA

Cultures of XL1-Blue (pUC18; 2 x 400 ml) were grown in LB overnight at 37°C in a shaking incubator. Cells were harvested by centrifugation at 6,000 rpm for 15 min in a Sorvall centrifuge (GSA rotor). The plasmid DNA was isolated using the Qiagen plasmid purification kit (Mega kit), according to the manufacturer's protocol. The DNA obtained from this procedure (typically 2 - 4 mg plasmid DNA) was further purified by caesium chloride gradient. Aliquots of plasmid DNA (typically 100 – 500 μg) were mixed with 743 g/l caesium chloride and 0.6 g/l ethidium bromide and centrifuged at 49,000 rpm overnight at 15°C (Beckman ultracentrifuge, 70.1 Ti rotor). Following centrifugation, covalently closed circular (ccc) DNA accumulates in a band slightly lower than the band corresponding to nicked circular DNA. The ccc DNA was removed from the caesium chloride gradient by use of a hypodermic needle and syringe. The ethidium bromide was removed from the DNA sample by successive extractions with H_2O-saturated butan-1-ol. The DNA was then gently ethanol precipitated (1 hr at 4°C) to remove the caesium chloride. Pelleted DNA was washed with 70% ethanol, dried in a speed vac and re-suspended in TE buffer.

2.17 Preparation of uniformly ^{32}P-labelled plasmid DNA

Uniformly ^{32}P-labelled plasmid DNA was prepared by growth of *E. coli* JM109 or DH5α cells in the presence of [^{32}P]orthophosphate. A 25 ml culture of DH5α

($pAcSG_2$), JM109 (pDEA-7Z) or JM109 (pFB585) was grown overnight with aeration at 37°C in LB supplemented with 100 μg/ml carbenicillin. The next day 6.25 ml of the starter culture was used to inoculate 250 ml of prewarmed low phosphate media containing 50 μg/ml carbenicillin. This culture was incubated for 2.5 hrs at 37°C with aeration before addition of 100 μCi [^{32}P]orthophosphate. The culture was then incubated for a further 5.5 hrs before the cells were harvested by centrifugation (6,000 rpm for 15 min at 4°C in a Sorvall centrifuge, GSA rotor). The dry cell pellet was stored overnight at -20°C.

^{32}P-labelled plasmid DNA was extracted by Qiagen Maxi prep protocol, the DNA pellet re-suspended in 0.5 ml TE and dialysed against 4 litres of TE/10% (v/v) ethanol overnight at 4°C. The DNA concentration was determined by measuring the absorbance of the sample at 260 nm (section 2.11). The procedure typically yielded 100-200 μg ^{32}P-labelled DNA.

2.18 Preparation of DNA with protruding 5' termini

Uniformly ^{32}P-labelled ccc plasmid DNA was linearised with either *Eco*RI, *Nco*I or *Bsa*I to produce DNA with protruding 5'-termini, also referred to as protruding 5'-phosphate (5'-P) termini. Restriction enzyme digests (150 μl) typically contained 40 μg plasmid DNA, and 160 units (U) *Eco*RI in *Eco*RI buffer, 80 U *Nco*I in NEBuffer 4 or 80 U *Bsa*I in NEBuffer 4. All digests were incubated for 3 hrs, at 37°C for *Eco*RI and *Nco*I digests, 50°C for *Bsa*I digests.

For preparation of DNA with 5'-hydroxyl (5'-OH) termini, following linearisation, 2.2 U calf intestinal phosphatase (CIP) was added to an aliquot of

the restriction digest (75 µl, ~ 20 µg DNA) and incubated for 1 hr at 37°C.

Preparation of DNA with recessed 3'-hydrogen (3'-H) termini was achieved by incubation of *Nco*I-linearised, uniformly ^{32}P-labelled plasmid DNA with the Klenow fragment of DNA polymerase I lacking 3'-5' exonuclease activity, in the presence of excess dideoxycytidine 5' triphosphates (ddCTP). Reactions (40 µl) typically contained 4 µg DNA incubated with 20 U Klenow (3'-5' exo$^-$) and 0.1 mM ddCTP in EcoPol buffer. After incubation for 3 hrs at 37°C, the Klenow fragment was heat inactivated by incubation at 75°C for 15 min.

All DNA samples were extracted sequentially with phenol/chloroform/iso-amyl alcohol and chloroform (section 2.12) and precipitated with ethanol (section 2.13). The DNA pellets were re-suspended typically in 50 µl TE and passed over an S-400 microspin column. The concentration of each DNA sample was determined by comparison of counts per second with a known concentration of uniformly ^{32}P-labelled ccc plasmid DNA, as measured in a 1500 Tri-carb liquid scintillation analyzer (Packard).

2.19 Preparation of DNA with recessed 5' termini

Protruding 3' termini (mostly referred to as recessed 5'-phosphate termini) were prepared by incubation of uniformly ^{32}P-labelled, ccc DNA with *Kpn*I. Restriction digests (150 µl) typically contained 40 µg plasmid DNA, 160 U *Kpn*I and 0.1 mg/ml BSA in NEBuffer 1. Digests were incubated for 3 hrs at 37°C.

Removal of the recessed 5'-phosphate to leave a recessed 5'-hydroxyl group was achieved by incubation of *Kpn*I-linearised DNA with calf intestinal

phosphatase. An aliquot of the *Kpn*I restriction digest (75 µl, ~ 20 µg DNA) was initially incubated with 22 U CIP in NEBuffer 2 for 15 min at 37°C. Following the addition of a further 22 U CIP the DNA was then incubated for 45 min at 55°C.

As described for the preparation of DNA with protruding 5' termini (section 2.18), all DNA samples were extracted with solvents, precipitated with ethanol and the concentration determined by scintillation counting.

2.20 Preparation of DNA with blunt termini

Restriction digests (150 µl) typically contained 40 µg uniformly-^{32}P-labelled plasmid DNA and 40 U *Sma*I in NEBuffer 4. Following incubation for 3 hrs at 25°C, the DNA was extracted with solvents (section 2.12) and precipitated with ethanol (section 2.13). The concentration of the re-suspended DNA pellet was determined by scintillation counting (as described in section 2.18).

V. General methods of protein manipulation

2.21 Preparation of cell-free extracts

Whole cell extracts were prepared essentially as described (Baumann and West, 1998a). Typically 6 – 10 litres of GM00558 cells were used per extract. After harvesting, the cells were washed twice in ice-cold PBS and once in hypotonic lysis buffer (HLB). The cell pellet was then re-suspended in 1 vol HLB and incubated on ice for 20 min. The cells were lysed by homogenisation in a douncer ("B" pestle) in the presence of protease inhibitors (PMSF, 1 mg/ml;

aprotinin, 0.01 trypsin inhibitor unit/ml; pepstatin, 1 μg/ml; chymostatin, 1 μg/ml; leupeptin 1 μg/ml). Following a 20 min incubation on ice, 0.5 vol high salt buffer (2x) was added, except when the cell pellets were small when 0.2 vol 5x high salt buffer was used to maintain a high protein concentration in the extract. The cell suspension was centrifuged for 3 hrs at 37,000 rpm in a Beckmann SW41-Ti rotor (at 4°C), and the supernatant was removed carefully to avoid the upper lipid- and lower nucleic acid-containing regions. The supernatant was dialysed for 2 hrs against E-buffer, the protein concentration determined by Bradford assay, and the extract fast-frozen and stored at -70°C.

Cell-free extracts were also prepared from GM8036, GM7078, D5037 and D5576 cells (see section 2.6). M059J and M059K cell extracts were a gift from Dr L. Hanakahi.

2.22 Ammonium sulphate precipitation of extracts

Powdered ammonium sulphate (761 g/l) was added to 0.5 ml GM00558 extract and incubated for 1 hr, at 4°C on a rotary wheel. The precipitated proteins were pelleted by centrifugation (8,000 rpm in a benchtop Eppendorf centrifuge for 30 min at 4°C), and re-suspended in 0.4 ml E-buffer. To remove all salts, the protein sample was dialysed against 1 litre E-buffer for 4 hrs at 4°C. Precipitated extract was fast-frozen and stored at -70°C.

2.23 Immunoprecipitations (IP)

GM00558 extract (~1.8 mg total protein) was incubated with 2-10 μl of various

antibodies in the presence of 100 mM NaCl and 100 μg/ml ethidium bromide. Due to a lack of preimmune serum, an equal volume of the anti-RAD51 antibody FBE-1 was used as a control for all IPs with polyclonal antibodies. The antibodies were incubated with the extract for 2 hrs at 4°C, with mixing. Protein A sepharose fast flow beads were used to recover antibody-bound proteins. The beads (~80 μl/IP) were prewashed in E-buffer/100mM NaCl, then incubated with the extract/antibody mix for 1.5 hrs at 4°C with mixing. After pelleting the protein A beads by low speed centrifugation (3,000 rpm for 2 min in an Eppendorf benchtop centrifuge), the beads were washed twice with 0.5 ml E-buffer/100 mM NaCl. Washed beads were re-suspended in protein gel loading buffer, the antibody-bound proteins separated by SDS-PAGE (section 2.7) and detected by Western blotting (section 2.29).

2.24 Immunodepletions

GM00558 extract (150 – 200 μl) was incubated with polyclonal anti-PNK antibody, at a concentration of 1 μl antibody/10 μl extract, for 2 hrs at 4°C on a rotary wheel. As a control, extract was incubated with the same concentration of the anti-RAD51 antibody FBE-1. Protein A beads were prewashed and re-suspended in E-buffer and aliquots (100 – 120 μl) added to each immunodepletion. Following incubation of protein A beads with the extract for 1.5 hrs at 4°C, the beads were pelleted by low speed centrifugation. The supernatant was carefully removed, stored in aliquots at -70°C and used in end-joining assays (section 2.30.1).

2.25 Fractionation of extract on phosphocellulose

GM00558 extract (10 mg) was loaded onto a phosphocellulose column (1 ml) equilibrated in E-buffer. Proteins were then eluted using E-buffer containing 0.15, 0.6 or 1 M KCl. The peak fractions (0.5 ml) from the flow through (PC-A) and 0.6 M KCl elution (PC-C) were dialysed against E-buffer.

2.26 Gel filtration of PC-C

Gel filtration was carried out on a Superose 12 column (Amersham Pharmacia) at 4°C. The column was equilibrated with E-buffer containing only 10% (v/v) glycerol. HeLa PC-C (a gift from Dr L. Hanakahi) was concentrated using Vivaspin 500 µl concentrators (Vivascience) as described by the manufacturers instructions. Concentrated PC-C (750 µg) was applied directly to the column and run at a speed of 0.04 ml/min. The fractions (50 µl) from four column runs were pooled, the proteins precipitated with TCA and separated by SDS-PAGE. The elution profiles of Ku, DNA-PK_{cs}, DNA Ligase IV, XRCC4 and PNK were analysed by Western blotting (section 2.29). Gel filtration of concentrated PC-C was also repeated in the presence of 500 mM NaCl.

Samples of recombinant PNK (500 ng) were diluted in E-buffer containing only 10% (v/v) glycerol and also applied to the Sepharose 12 column, and the column calibrated with gel filtration standards (Bio-Rad).

2.27 Determination of protein concentration

Protein concentrations of cell-free extracts were determined using the Bradford assay. The protein extract was diluted to a volume of 800 µl in ddH_2O, added to 200 µl of protein assay dye reagent concentrate (Bio-Rad) and incubated for 5 min at room temperature. The absorbance at 595 nm was measured using a Pharmacia Ultrospec 2000 spectrophotomer, and compared against a standard curve obtained in parallel with known concentrations of BSA.

2.28 Molecular weight standards

SeeBlue pre-stained standards (Invitrogen) used for all SDS-PAGE gels were: myosin (250 kDa), bovine serum albumin (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic dehydrogenase (36 kDa), myoglobin (30 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa).

Gel filtration standards from BioRad were: thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B-12 (1.35 kDa).

2.29 Western blotting

Protein samples were separated by SDS-PAGE as described in section 2.7. Gels were aligned next to BA85 nitrocellulose membrane (Schleicher and Schuell) pre-soaked in Western transfer buffer. Western blotting was conducted in transfer buffer at 100 mA overnight and at 4°C, using a Hoefer Transphor

electrophoresis blotting unit. Following transfer, the membrane was blocked in PBS-Tween/5% (w/v) marvel for 1 hr at room temperature. The membrane was incubated with the required primary antibody, typically at 1/500 – 1/5000 dilution, in PBS-Tween/5% (w/v) marvel for 1 hr at room temperature. Four ten-minute washes with PBS-Tween were carried out before incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Secondary antibodies were used at 1/4000 dilution in PBS-Tween/5% (w/v) marvel. The membranes were washed with PBS-Tween, as described above, then incubated with enhanced chemiluminescence (ECL) reagent (NEN) for 1 min, and exposed to X-Omat film.

VI. Biochemical assays

2.30 End-joining assays

2.30.1 Single plasmid end-joining assay

DNA end-joining reactions typically contained 40 ng uniformly ^{32}P-labelled plasmid DNA and 80 μg cell-free extract in HEPES end-joining buffer in a 20 μl reaction volume. Reactions were incubated for 2 hrs at 37°C except where indicated. Where used, aqueous solutions of the DNA-PK inhibitor wortmannin, or antibody dilutions were added directly to the end-joining reaction. To study the cofactor requirements for end-joining, ATP was omitted from the HEPES end-joining buffer, and various cofactors were added directly to the reactions. The end-joining products were deproteinised by addition 0.2 vol deproteinisation

buffer (5x) followed by incubation for 30 min at 37°C. Samples were diluted with 1/4 vol agarose gel loading buffer and the end-joining products separated by electrophoresis through 0.6% agarose gels (section 2.8). ^{32}P-labelled DNA was detected by autoradiography (section 2.9) and quantified by Scion Image 1.62c (section 2.10).

2.30.2 Two-plasmid end-joining assay

End-joining reactions (10 µl) contained uniformly-^{32}P-labelled linear pFB585 plasmid (7.7 kb; 20 ng) and uniformly-^{32}P-labelled linear pDEA-7Z (3.0 kb; 20 ng) incubated with 40 µg GM00558 extract in tris end-joining buffer for 2 hrs at 37°C. Where indicated, reactions were supplemented with 40 µM dATP, 40 µM dGTP, 40 µM dCTP and 40 µM dTTP. The reactions were stopped by addition 0.2 vol deproteinisation buffer (5x) and incubation for 30 min at 37°C. Samples were diluted with 1/4 vol agarose gel loading buffer and the end-joining products separated by electrophoresis through 0.6% agarose gels (section 2.8). ^{32}P-labelled DNA was detected by autoradiography (section 2.9).

2.31 T4 DNA ligation assay

Ligation reactions (10 – 20 µl) contained 40 ng ^{32}P-labelled DNA incubated with 80 U T4 DNA ligase, in T4 DNA ligase buffer at 15°C overnight. Following deproteinisation (incubation with 0.2 vol deproteinisation buffer for 30 min at 37°C), the products were separated by agarose gel electrophoresis and visualised by autoradiography.

2.32 Digestion of end-joining products

Large scale end-joining reactions (5x) were set up, essentially as described in section 2.30.1. Following deproteinisation, the DNA was extracted sequentially with phenol/chloroform/iso-amyl alcohol and chloroform (section 2.12) and ethanol precipitated (section 2.13). The pelleted DNA was dried, re-suspended in 10 µl TE then divided into two equal aliquots, one digested with the required restriction enzyme, the second subject to a mock digestion.

*Eco*RI digestions (10 µl) contained 1.6 U *Eco*RI and 5 µl end-joining products (~50 – 100 ng DNA) in *Eco*RI buffer. Following incubation for 1 hr at 37°C, the reactions were stopped by addition 0.1 vol 0.5 M EDTA and the *Eco*RI restriction enzyme heat denatured by incubation at 65°C for 20 min.

*Kpn*I digestions (10 µl) contained 1.6 U *Kpn*I, 5 µl end-joining products (~50 – 100 ng DNA) and 100 µg/ml BSA in NEBuffer 1. As a control, an equal concentration of ^{32}P-labelled DNA was preincubated with T4 DNA ligase (section 2.31), subject to the same solvent extraction and ethanol precipitation as the end-joining products and digested with an equal amount of *Kpn*I. Furthermore, deproteinised extract was added to an equal concentration of ^{32}P-labelled covalently closed circular plasmid DNA, extracted with solvent and ethanol precipitated as above and digested with an equal concentration of *Kpn*I. All digests were incubated for 3 hrs at 37°C and stopped by addition of 0.1 vol 0.5 M EDTA.

The products of mock- and restriction-digests were analysed by agarose gel electrophoresis (section 2.8) and autoradiography (section 2.9).

2.33 Ligation assay for the detection of PNK activity

End-joining reactions (60 µl) contained uniformly ^{32}P-labelled DNA with 5'-OH termini (120 ng) incubated with M059J extract (240 µg) in HEPES end-joining buffer. Similar reactions (60 µl) contained uniformly ^{32}P-labelled DNA with 5'-OH termini (120 ng) incubated with GM00558 extract (240 µg) in the presence of anti-XRCC4 antibodies (1:200 dilution) in HEPES end-joining buffer. Control reactions (60µl) contained ^{32}P-labelled DNA with 5'-OH termini (120 ng) incubated with E-buffer, or protein extract from GM00558 or M059K cells (240 µg). Identical reactions were set up with ^{32}P-labelled DNA with 5'-P termini. All reactions were incubated for 2 hrs at 37°C, the DNA deproteinised (as described in section 2.30.1) extracted sequentially with phenol/chloroform/iso-amyl alcohol and chloroform (section 2.12) and ethanol precipitated (section 2.13).

Each DNA pellet was re-suspended in 15 µl ddH_2O. A 5 µl aliquot (~ 40 ng DNA) was taken from each tube, and added to a T4 PNK reaction (10 µl) containing 5 U T4 PNK in T4 DNA ligase buffer. Following incubation for 30 min at 37°C, 80 U T4 DNA ligase was added to the reaction mixture and incubated overnight at 15°C. A second 5 µl aliquot was added to a T4 DNA ligation reaction (10µl) containing 80 U T4 DNA ligase in T4 DNA ligase buffer and incubated overnight at 15°C. Following T4 DNA ligation, the DNA was deproteinised (section 2.30.1), diluted in 0.2 vol agarose gel loading buffer and separated by agarose gel electrophoresis (section 2.8). The final 5 µl aliquot was analysed directly by agarose gel electrophoresis.

Further controls included, where indicated in the figures, heat denaturation

of T4 PNK (by incubation at 65°C for 20 min) before the addition of T4 DNA ligase. Also where indicated, 5'-^{32}P-end-labelled linear plasmid DNA was added to each DNA sample either before incubation with T4 DNA ligase, or prior to agarose gel electrophoresis.

Characterisation of DNA end-joining cataiysed by human cell-free extracts

I. Introduction

Studies of NHEJ *in vivo*, in which linear plasmid DNA was transfected into cultured mammalian cells, found that these cells were able to efficiently repair DSBs (reviewed in Roth and Wilson, 1988). Non-complementary termini were joined primarily using fill-in or homology-directed repair mechanisms (illustrated in Fig.1.7, page 83). To permit better biochemical characterisation of this repair process and allow identification of the protein components required for joining, *in vitro* assays using cell-free extracts were subsequently developed. Unfortunately, a number of different end-joining activities were identified, some using microhomologies at the termini for alignment, others resembling single-strand annealing, with greater levels of DNA degradation and alignment at microhomologies located away from the original termini (reviewed in Labhart, 1999b). In addition, many of the early reports were limited either because the protein requirements for end-joining were not established, or because the extracts were shown to contain a mixture of both Ku-dependent and Ku-independent repair activities. The identification of extracts prepared from human cells that catalysed only Ku-dependent end-joining was therefore of great importance (Baumann and West, 1998a).

The requirements for Ku, DNA-PK_{cs}, XRCC4 and DNA ligase IV in the end-joining reactions promoted by human cell-free extracts was extensively investigated using chemical inhibitors such as wortmannin, and antibodies raised

against the individual NHEJ proteins (Baumann and West, 1998a). Wortmannin is a fungal metabolite that inhibits all PI 3-kinases as well as the PI 3-kinase-related kinases DNA-PK_{cs}, ATM and ATR (Ui *et al.*, 1995; Sarkaria *et al.*, 1998). The individual kinases of the PI 3-kinase superfamily can be distinguished, however, because they exhibit different sensitivities to wortmannin. A concentration of 2 – 4 nM wortmannin is required to give 50% inhibition of the PI 3-lipid-kinases (Powis *et al.*, 1994). Comparatively, the PI 3-kinase-related protein kinases are at least 25- to 100-fold less sensitive to wortmannin. A concentration of 100 nM is required for half-maximal inhibition of ATM immunoprecipitates; 250 nM for purified DNA-PK_{cs} and 1.8 μM for purified ATR (Hartley *et al.*, 1995; Banin *et al.*, 1998; Sarkaria *et al.*, 1998). As human cell-free extract-catalysed DNA end-joining was unaffected by addition of 100 nM wortmannin but abolished by 1 μM wortmannin, this effect is most probably due to inhibition of DNA-PK_{cs} (Baumann and West, 1998a).

Further evidence that DNA-PK is required for the extract-catalysed end-joining came from experiments using anti-Ku or anti-DNA-PK_{cs} antibodies. Addition of these antibodies caused a substantial reduction in end-joining activity, although inhibition was incomplete due to the high levels of these proteins in the extracts (Baumann and West, 1998a). In comparison, addition of anti-XRCC4 and anti-DNA ligase IV antibodies completely abolished the end-joining activity of human extracts. Together these data demonstrated that the extracts prepared from human cells catalysed only DNA-PK-, XRCC4- and DNA ligase IV-dependent end-joining.

With the identification of extracts that selectively catalysed Ku-dependent end-joining, for the first time, a system has been established that allows the complete protein requirements of NHEJ to be investigated. One set of proteins shown to act in NHEJ in yeast but, as yet, have less clearly defined roles in mammalian NHEJ are Mre11, Rad50 and Xrs2 (NBS1 in mammals). In yeast, *rad50*, *mre11* or *xrs2* mutants are severely impaired in the joining of linearised plasmid DNA with complementary termini (Boulton and Jackson, 1998). Moreover, disruption of *RAD50*, *MRE11* or *XRS2* in *yku70* or *lig4* mutant backgrounds demonstrated that all these proteins function in the same repair pathway. In mammalian cells, the MRE11, RAD50 and NBS1 proteins colocalise at distinct radiation-induced foci, which are thought to represent sites of DNA repair (Maser *et al.*, 1997; Carney *et al.*, 1998; Nelms *et al.*, 1998). MRE11 has been shown to interact with Ku70 by coimmunoprecipitation and yeast two-hybrid analysis (Goedecke *et al.*, 1999). Furthermore, the phenotypes of human disorders in which members of the MRE11/RAD50/NBS1 (M/R/N) complex are mutated implicate these proteins in DNA damage signalling and repair. NBS1 is mutated in patients suffering from Nijmegen breakage syndrome (NBS), resulting in chromosomal instability, hypersensitivity to ionising radiation, immuno-deficiency and a predisposition to cancer at an early age (Featherstone and Jackson, 1998). MRE11 is mutated in patients with an ataxia-telangiectasia-like disorder (ATLD; Stewart *et al.*, 1999). Affected individuals display a similar phenotype to that of NBS patients, including chromosomal instability, ionising radiation sensitivity and a failure to suppress DNA synthesis upon IR-treatment.

Analysis of the repair of IR-induced DNA damage, using pulsed-field gel electrophoresis, demonstrated that cells derived from ATLD or NBS patients had no gross deficiency in DSB repair (Kraakman-van der Zwet *et al.*, 1999; Stewart *et al.*, 1999). With the establishment of the Ku-dependent, plasmid-based end-joining assay, however, the requirement for NBS1 and MRE11 in the repair of defined DNA lesions can be studied.

In this chapter, the protein and cofactor requirements for human extract-catalysed end-joining are investigated.

II. Joining of protruding 5' termini

Previously, it was shown that human cell-free extracts catalysed the joining of linear plasmid DNA with protruding 3', protruding 5', or blunt termini (Baumann and West, 1998a). The products of the reactions were linear multimers rather than circular products. As shown in Fig. 3.1, incubation of uniformly-^{32}P-labelled, linear plasmid DNA with protruding 5' termini with extracts from the human lymphoblastoid cell line GM00558, resulted in the production of linear multimers. Titration of the extracts demonstrated that the efficiency of end-joining correlated with the protein concentration in the reaction.

III. Requirements for XRCC4 and DNA-PK$_{cs}$

Antibodies raised against XRCC4 and DNA ligase IV were previously found to be very effective in inhibiting extract-catalysed end-joining (Baumann and West, 1998a). To verify that the end-joining activity observed in Fig. 3.1 was catalysed

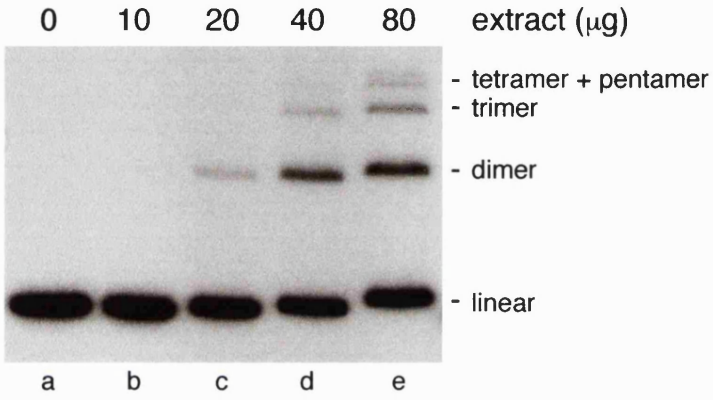


Figure 3.1. Joining of protruding 5' termini by human cell-free extracts. End-joining reactions (20 μl) were carried out as described in Materials and Methods (section 2.30.1), and contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with protruding 5' termini (40 ng) and the indicated amount of GM00558 cell-free extracts. Following deproteinisation, the products were separated by agarose gel electrophoresis and visualised by autoradiography. Mobilities of linear and multimeric DNA species are indicated.

by the NHEJ proteins, reactions were supplemented with serial dilutions of XRCC4 antisera (Fig. 3.2). DNA end-joining by the GM00558 extracts was highly sensitive to the addition of anti-XRCC4 antibodies with complete inhibition observed when XRCC4 antisera was used at a final dilution of 1 in 400 (lane f).

Previously, the chemical inhibitor wortmannin was used to demonstrate the involvement of DNA-PK_{cs} in extract-catalysed end-joining (Baumann and West, 1998a). As discussed above, wortmannin is a general inhibitor of all PI 3-kinases and PI 3-kinase-related kinases. The concentration of wortmannin required for inhibition of extract-catalysed end-joining, however, suggested that this effect was due to inactivation of DNA-PK_{cs}. To confirm the requirement for DNA-PK_{cs} in extract-catalysed end-joining, extracts were prepared from the human M059J and M059K cell lines. Isolated from a malignant glioma, the M059J cell line is radiation sensitive, defective in the rejoining of double-strand breaks and lacks DNA-PK_{cs} activity (Lees-Miller *et al.*, 1995). The underlying mutation in the M059J cell line has been determined to be a single nucleotide deletion from codon 1351 in exon 32 (Anderson *et al.*, 2001a). This frameshift mutation causes termination of DNA-PK_{cs} protein synthesis early in exon 33, which would result in a truncated protein lacking the kinase domain. The defective DNA-PK_{cs} mRNA in M059J cells is thought to be degraded, however, so that the truncated protein is not expressed. A control cell line, M059K, which exhibits normal levels of DNA-PK_{cs} activity, was derived from the same malignant glioma biopsy specimen (Lees-Miller *et al.*, 1995).

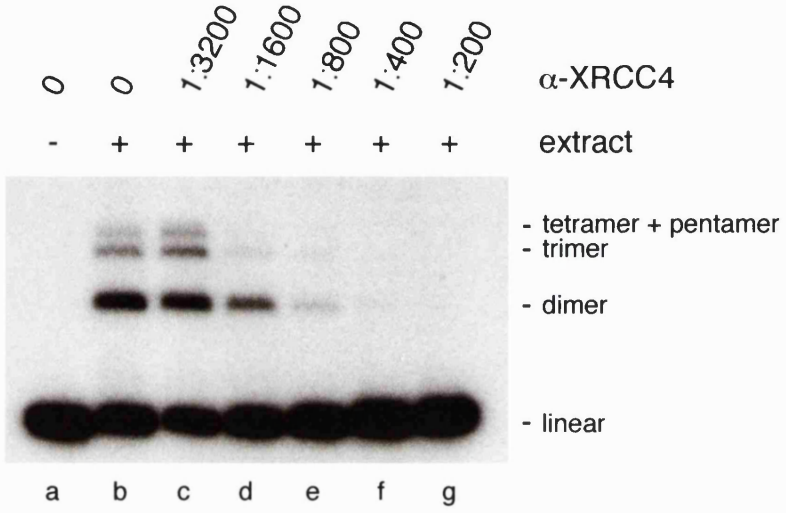


Figure 3.2. XRCC4 requirement for joining of protruding 5' termini by cell-free extracts.

End-joining reactions (10 μl), carried out as described in Materials and Methods (section 2.30.1), contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (20 ng) and, where indicated, GM00558 extracts (40 μg). Polyclonal anti-XRCC4 antibodies (α-XRCC4) were added to the final dilution indicated. Following deproteinisation, the products were analysed by agarose gel electrophoresis and autoradiography.

In agreement with a requirement for DNA-PK_{cs} in the extract-catalysed end-joining reaction, M059K extracts showed high levels of end-joining activity, whereas end-joining was not observed with M059J extracts (Fig. 3.3, compare lanes b – e with lanes g – j).

IV. DNA end-joining catalysed by NBS1- and MRE11-defective extracts

To investigate the role of the M/R/N complex in NHEJ, extracts were prepared from the GM7078 cell line established from an NBS patient. The patient was homozygous for a nonsense mutation in *NBS1*, and produced a truncated protein that contained the N-terminal FHA and BRCT domains but lacked the C-terminal half of the protein (Carney *et al.*, 1998; Varon *et al.*, 1998). To confirm that full length NBS1 was lacking in GM7078 cells, extracts were analysed by Western blotting (Fig. 3.4A). The anti-NBS1 antibodies used were raised against the C-terminal 352 amino acids of the protein, residues that are not present in the truncated protein. NBS1 was, therefore, not detected in GM7078 extracts (lane b), but clearly observed in extracts from a control cell line GM8036, established from the father of the NBS patient (lane a). The levels of RAD50 and MRE11 were unaltered in the NBS1-defective extracts.

Joining of protruding 5' termini by the control and NBS1-defective extracts was investigated (Fig. 3.4B). No significant difference in end-joining activity was observed over the range of protein concentrations tested. To ensure that the end-joining activity observed was catalysed by the NHEJ proteins, reactions were

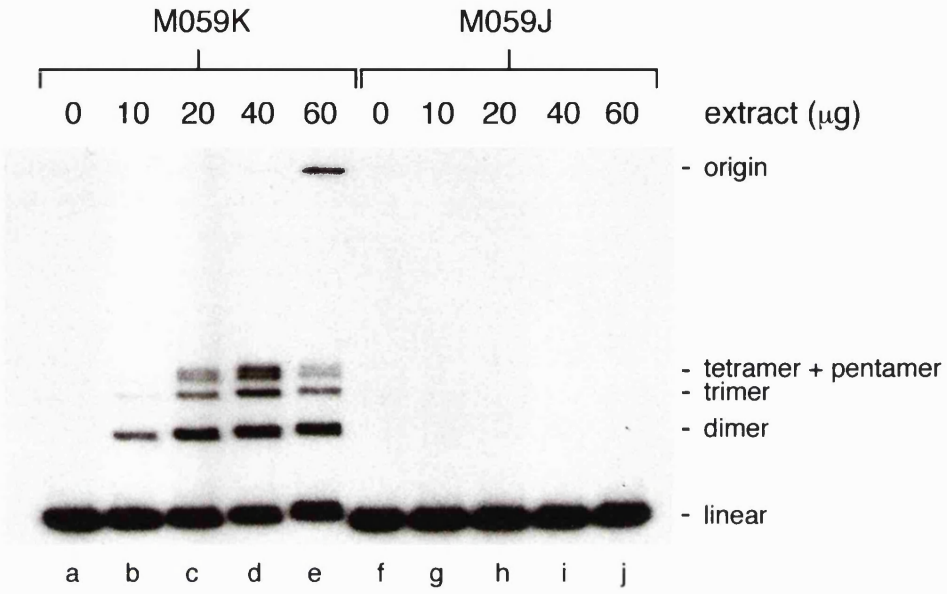


Figure 3.3. Extract-catalysed joining of protruding 5' termini requires DNA-PK$_{cs}$.

End-joining reactions were performed as described in Materials and Methods (section 2.30.1). Reactions (10 μl) contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (20 ng) incubated with the indicated amount of protein extract from DNA-PK$_{cs}$-deficient cells (M059J) or control cells (M059K). Products were separated by agarose gel electrophoresis and detected by autoradiography.

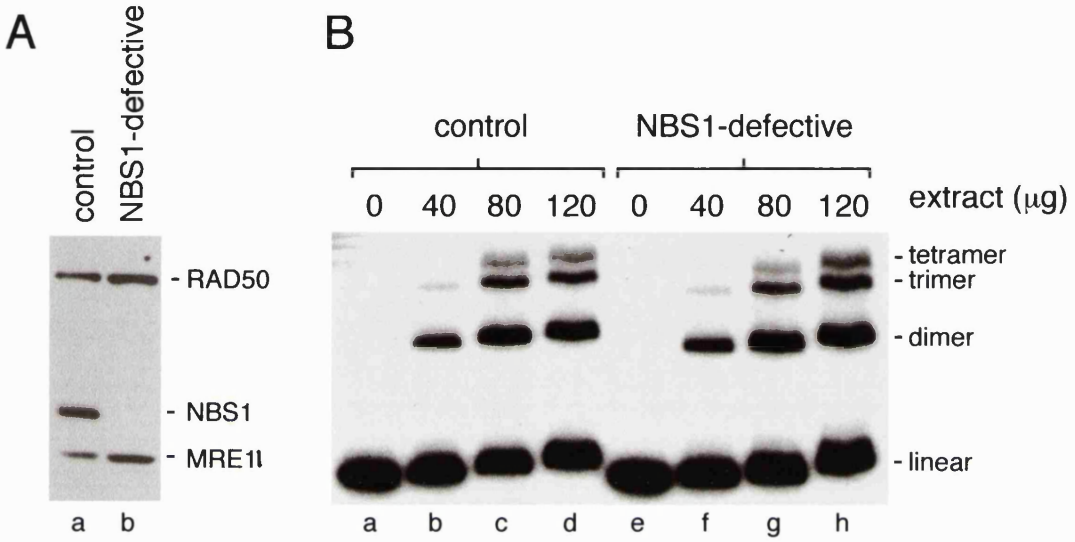


Figure 3.4. Effect of defect in NBS1 on extract-catalysed joining of protruding 5' termini.

(A) Samples of extracts (~20 μg total protein) prepared from NBS1-defective cells (GM7078) and a parental control cell line (GM8036) were separated by SDS-PAGE, and subject to Western blotting analysis with RAD50, MRE11 and NBS1 antisera (as described in Materials and Methods, sections 2.7 and 2.29).

(B) End-joining reactions (20 μl) were set up as described in Materials and Methods (section 2.30.1), containing uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (40 ng) incubated with the indicated amount of control or NBS1-defective extract. Following deproteinisation, the end-joining products were separated by agarose gel electophoresis and visualised by autoradiography.

supplemented either with anti-XRCC4 antibodies or the DNA-PK_{cs} inhibitor wortmannin. Both the control and the NBS1-defective extracts exhibited similar sensitivity to these inhibitors as that observed with the GM00558 extracts (data not shown). Complementary protruding 5' termini can, therefore, be joined by NBS1-defective extracts in an XRCC4- and DNA-PK_{cs}-dependent manner.

Within NBS1-defective cells MRE11 still associates with RAD50, but radiation-induced MRE11/RAD50 foci are not formed, suggesting that NBS1 is required for the targeting of these proteins to sites of DNA damage (Carney *et al.*, 1998). In addition, biochemical characterisation of the M/R/N complex *in vitro*, has determined that interaction of NBS1 with MRE11 and RAD50 permits the opening of fully paired DNA hairpins and limited unwinding of DNA at ds/ss transitions (Paull and Gellert, 1999). In the extract-catalysed end-joining assay, there is no requirement for hairpin opening, and probably no need for NBS1 to direct MRE11/RAD50 to the DNA ends. Consequently, there may be no role for NBS1 in the *in vitro* repair reactions. Further investigations into the role of the M/R/N complex in extract-catalysed end-joining, therefore, included preparation of extracts from a MRE11-defective cell line, D5576.

Derived from an ATLD patient, D5576 cells contain truncated MRE11 protein containing only the first 633 amino acids (Stewart *et al.*, 1999). Western blotting demonstrated that, in contrast to the GM00558 extracts (control), MRE11 was not detected in D5576 extracts (Fig. 3.5A). Lower levels of RAD50 and NBS1 were also observed. Comparison of the end-joining activities of the MRE11-deficient and normal GM00558 extracts showed that there was no

Figure 3.5. Effect of defect in MRE11 on extract-catalysed joining of protruding 5' termini.

(A) Extracts were prepared from control cells (GM00558) and MRE11-defective cells (D5576) as described in Materials and Methods (section 2.21). Samples (~40 μg total protein) were separated by SDS-PAGE and analysed by Western blotting with antisera raised against RAD50, NBS1 and MRE11.

(B) End-joining reactions were carried out as described in Materials and Methods, section 2.30.1. Reactions contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (40 ng) incubated with the indicated amounts of control or MRE11-defective extracts. End-joining products were analysed by agarose gel electrophoresis and autoradiography.

(C) Time course of joining of protruding 5' termini by control (■), or MRE11-defective extracts (◆). Large scale end-joining reactions (160 μl) were set up as described in Materials and Methods, section 2.30.1. Samples (20 μl) were removed at the indicated times, deproteinised and analysed by agarose gel electrophoresis. Results shown are an average of three independent experiments.

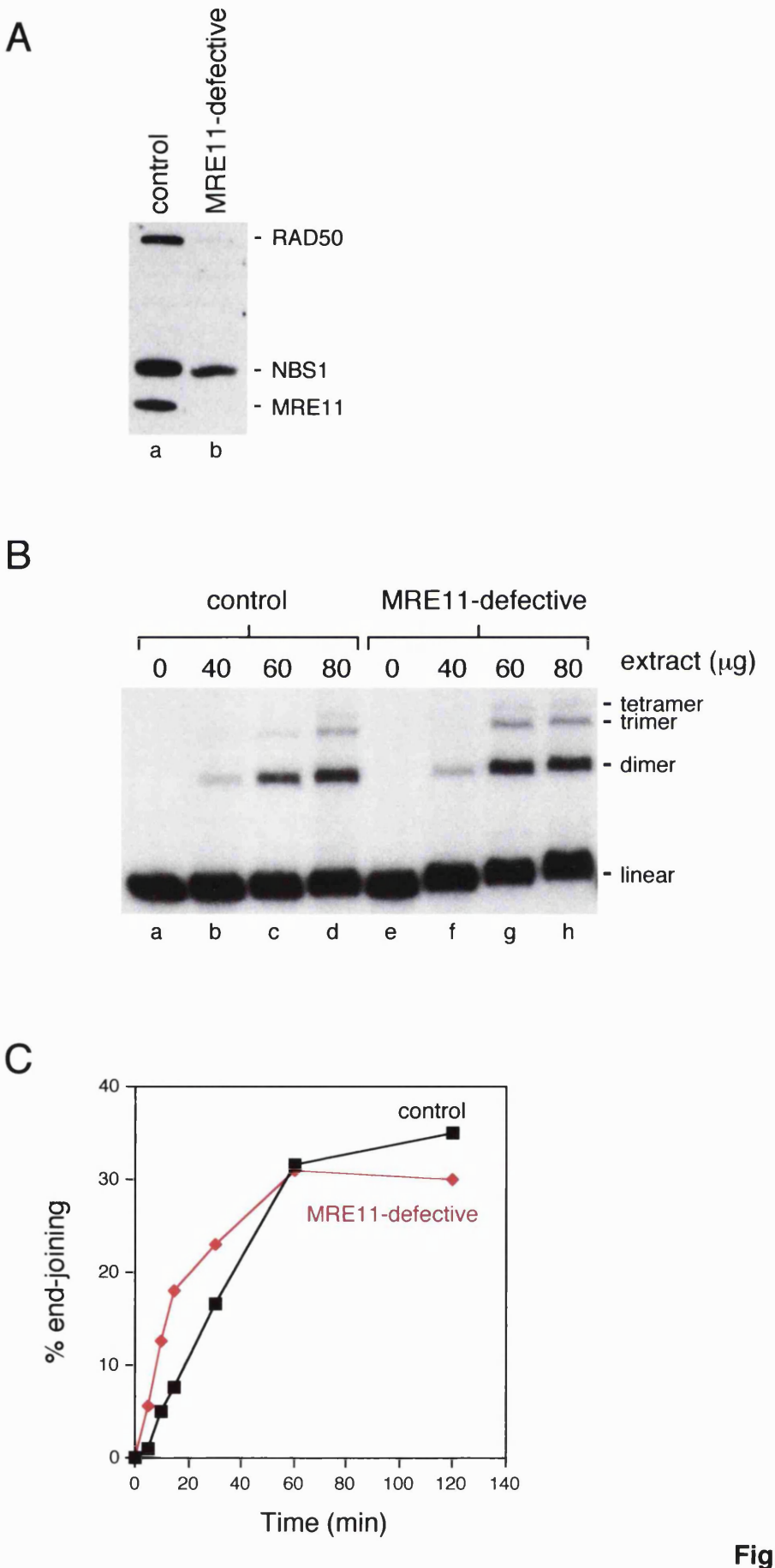

A
control
MRE11-defective
RAD50
NBS1
MRE11
a
b
B
control
MRE11-defective
0
40
60
80
0
40
60
80
extract (μg)
tetramer
trimer
dimer
linear
a
b
c
d
e
f
g
h
C
% end-joining
40
30
20
10
0
control
MRE11-defective
0
20
40
60
80
100
120
140
Time (min)

Figure 3.5

significant difference in the ability of the two extracts to join uniformly-^{32}P-labelled, linear plasmid DNA with protruding 5' termini (Fig. 3.5B). Furthermore, when the efficiency of end-joining was studied over a two-hour time course, although some differences were observed in the kinetics of joining, both extracts had generated comparable amounts of ligation products after two hours (Fig. 3.5C).

Addition of anti-XRCC4 antibodies or wortmannin inhibited the end-joining activity of MRE11-defective extracts, demonstrating that the observed joining was catalysed by the XRCC4 and DNA-PK$_{cs}$ proteins (data not shown). Moreover, re-digestion experiments to determine the fidelity of end-joining established that both the control and MRE11-defective extracts joined protruding 5' termini accurately (data not shown). In summary, MRE11-defective extracts catalysed accurate, XRCC4- and DNA-PK$_{cs}$-dependent joining of protruding 5' termini, which was comparable to that observed with control GM00558 extracts in all aspects tested.

V. Cofactor requirements

End-joining reactions are typically carried out in HEPES end-joining buffer containing 1 mM ATP and 1 mM magnesium acetate. When Mg^{2+} was omitted, no end-joining was observed (Fig. 3.6A, lanes c). In contrast, however, some residual joining could be seen in the absence of exogenously added ATP (lane d). This residual end-joining could result from either the presence of small amounts of free ATP in the extract, or preformed DNA ligase IV-AMP complexes.

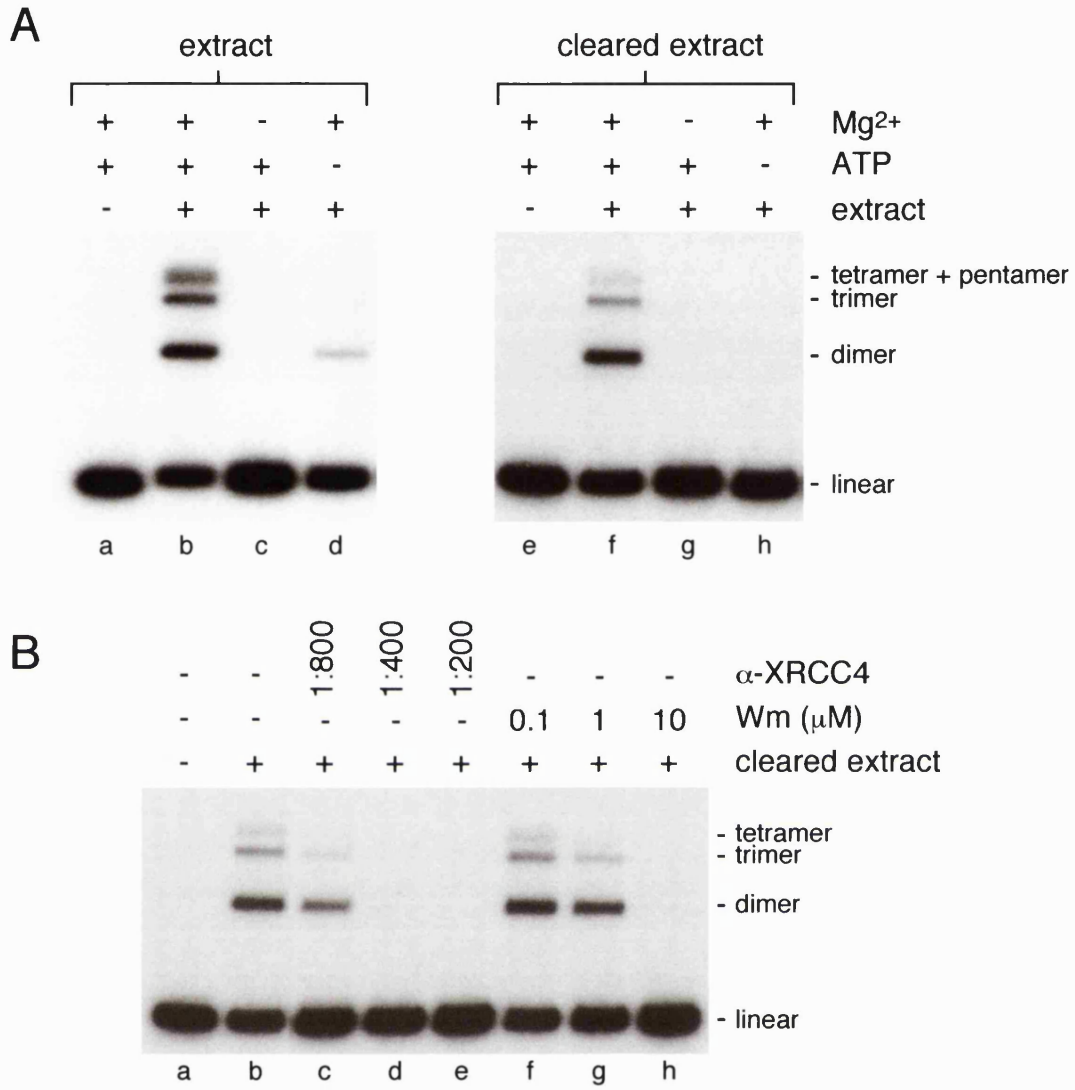


Figure 3.6. ATP and magnesium requirements for joining of protruding 5' termini.

(A) End-joining reactions (20 μl) containing uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (40 ng) were conducted as described in Materials and Methods (section 2.30.1). Where indicated, reactions contained GM00558 extracts or cleared GM00558 extracts (80 μg). Cleared extracts were prepared as described in Materials and Methods, section 2.22. Reactions contained MgOAc (Mg^{2+}; 1 mM) and ATP (1 mM) as indicated. The products were separated by agarose gel electrophoresis and detected by autoradiography.

(B) Protein requirements for joining of protruding 5' termini by cleared extracts. End-joining reactions, carried out as described in (A), contained 80 μg cleared extract. Anti-XRCC4 antibodies (α-XRCC4) or wortmannin (Wm) were added to the end-joining reaction at the indicated concentrations, and the products analysed by agarose gel electrophoresis.

Mammalian DNA ligases join 5'-P and 3'-OH termini in a reaction that requires ATP. The first step of the reaction is the formation of a DNA ligase-AMP intermediate also referred to as a DNA ligase-adenylate (Tomkinson and Mackey, 1998). This is formed by nucleophilic attack of ATP by the DNA ligase resulting in covalent linkage of AMP to the active site of the ligase and release of pyrophosphate. After formation of the adenylated ligase intermediate, ATP is no longer required for ligation. Early studies of DNA ligase IV found that it purified as a DNA ligase IV-AMP complex, indicating that this DNA ligase is naturally pre-adenylated in mammalian cells (Robins and Lindahl, 1996).

To determine if the low level of residual end-joining observed in the absence of ATP was caused by small amounts of free ATP, or pre-adenylated DNA ligase IV, samples of extract were precipitated with saturating amounts of ammonium sulphate. Following re-suspension of the precipitated proteins, the samples were dialysed extensively against E-buffer to lower the salt concentration, as described in Materials and Methods, section 2.22. In this way, the precipitated extract would be cleared of any free ATP (hence termed cleared extract), but would still retain preadenylated ligase complexes, which are only disrupted by treatment with inorganic pyrophosphate (Grawunder *et al.*, 1997). Cleared extract was found to be competent for joining of DNA with protruding 5'-termini, although at a slightly lower efficiency (Fig. 3.6A, compare lanes b and f). End-joining by the cleared extract, however, displayed an absolute requirement for Mg^{2+} and ATP (lanes g and h). These results show that the human cell-free extracts contain free ATP resulting in some end-joining activity in the absence of

added ATP (lane d). As expected, end-joining of protruding 5' termini by the cleared extract was inhibited by addition of anti-XRCC4 antibodies and by 1 – 10 μM wortmannin (Fig. 3.6B), verifying that end-joining was still dependent on XRCC4 and DNA-PK_{cs}.

The cofactor requirements for extract-catalysed joining of protruding 5' termini were analysed further by replacement of ATP with a variety of cofactors. Each ribo-, deoxy- and dideoxynucleotide was tested to determine which could serve as ATP analogues in the end-joining reaction and to analyse the stringency of ATP requirement. Surprisingly, all ribo- and deoxynucleotides were able to support end-joining with varying efficiencies (Fig. 3.7). As expected, ATP gave the highest end-joining activity although this was equalled by dATP and ddATP. Lower end-joining activity was observed when nucleoside triphosphate molecules with other bases (GTP, CTP and UTP) were used as cofactors. These ribonucleotides, however supported more end-joining activity than the corresponding deoxynucleotides (dGTP, dCTP and dTTP) and very little, if any, end-joining activity was observed with the dideoxynucleotides ddGTP, ddCTP and ddTTP. Whilst these results indicate a low stringency for the type of nucleotide cofactor required for end-joining activity, they also demonstrate that slight differences in the cofactor structure can have a significant effect on its ability to stimulate NHEJ *in vitro*. For example the attachment of a hydroxyl or hydrogen group to carbon-3 of the ribose ring which determines whether it is a deoxy- or dideoxynucleotide (see Fig. 3.9) affects end-joining activity. However

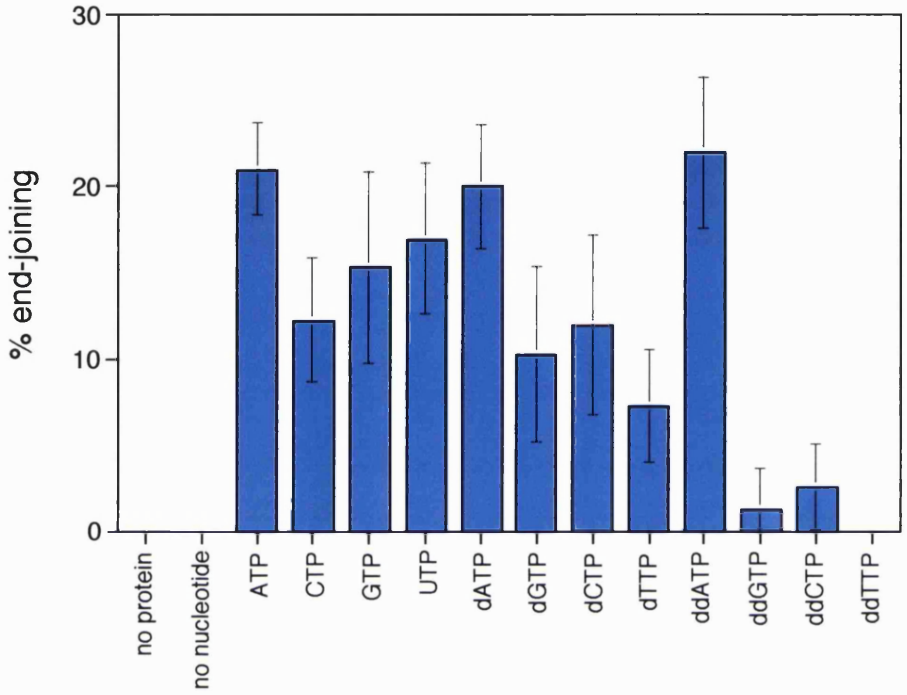


Figure 3.7. Nucleotide requirements for joining of protruding 5' termini.

End-joining reactions were carried out as described in Fig. 3.6A, but contained cleared extracts and end-joining buffer without ATP. Reactions were supplemented with the indicated nucleotides (1 mM). End-joining products were analysed by agarose gel electrophoresis and autoradiography and quantified. The results are an average of three separate experiments, with the percentage end-joining representing the proportion of multimeric DNA out of the total DNA. The standard deviation is represented by the error bars.

these differences appear to be overridden if an adenine base is attached to the sugar ring.

Extending the study it was important to determine if the cofactor required a triphosphate moiety, or if nucleoside mono- or diphosphates could be used. Furthermore did end-joining exhibit a requirement for a nucleotide-like cofactor or were inorganic phosphate chains sufficient to support end-joining activity? Finally, because inositol hexakisphosphate (IP_6) has been shown to stimulate end-joining (Hanakahi *et al.*, 2000), it was necessary to establish if it was acting in the reaction as an ATP analogue. The results showed that the nucleoside diphosphate ADP was able to replace ATP with little change to the end-joining activity (Fig. 3.8). However, the nucleoside monophosphates AMP and dGMP did not support end-joining. Addition of the slowly hydrolysable analogue of ATP, ATPγS, allowed reduced end-joining activity, whilst no activity was observed in the presence of the non-hydrolysable ATP analogue AMP-PNP. The nucleotide-like cofactors NAD and diADE (P^1,P^4-di(adenosine-5')tetraphosphate; see Fig. 3.9) also supported end-joining to some extent. End-joining did not occur, however, when reactions were supplemented with inorganic phosphates [sodium phosphate (NaP), sodium pyrophosphate (NaPPi), or pentasodium tripolyphosphate (NaTriP)] nor when ATP was replaced by IP_6. In summary, all cofactors that supported end-joining activity resembled nucleotides in that they contained a base, a pentose ring and either a di- or triphosphate motif (see Fig. 3.9). Whilst the phosphate chain was essential to end-joining activity, inorganic polyphosphates were not sufficient to support end-joining. Finally, it was

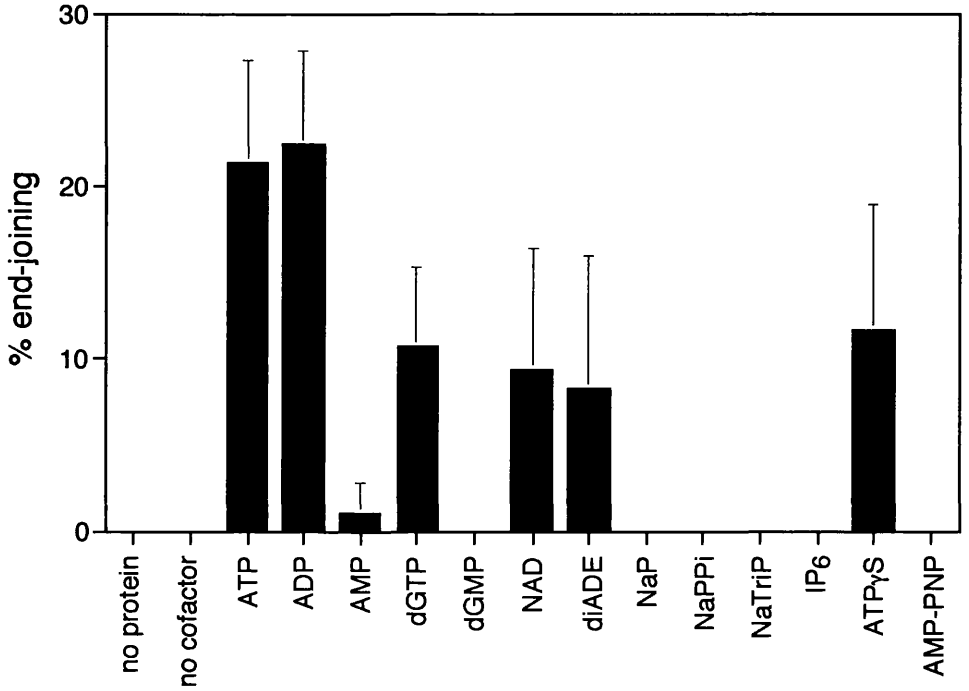
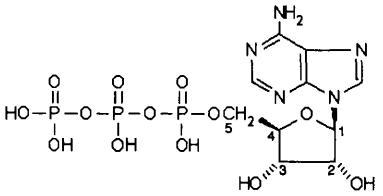


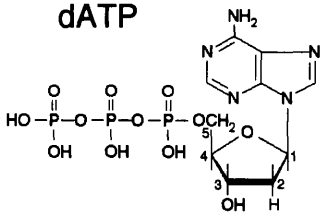
Figure 3.8. Cofactor requirements for joining of protruding 5' termini. End-joining reactions were carried out as described in Fig. 3.7, using 1 mM of the indicated cofactors. Products were analysed by agarose gel electrophoresis and autoradiography and the percentage end-joining quantified. Results shown are an average of three separate experiments with the standard deviation represented by error bars.

Figure 3.9. Cofactor structures.
Schematic representation of the cofactors tested in Figures 3.7 and 3.8 (adapted from the Sigma web site). Left-hand side, cofactors supporting high levels of extract-catalysed end-joining activity; middle, cofactors supporting intermediate levels of end-joining and right-hand side, those which did not support end-joining. The hydrogen groups marked in red attached to carbons-2 and -3 of the pentose ring in the dATP and ddATP structures highlight the differences between ribo-, deoxy- and dideoxynucleotides (compare with ATP).

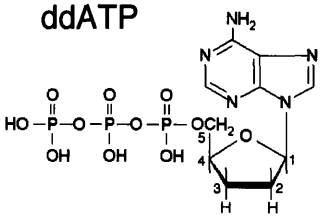
ATP



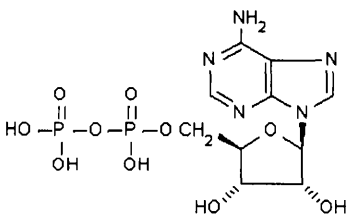
dATP



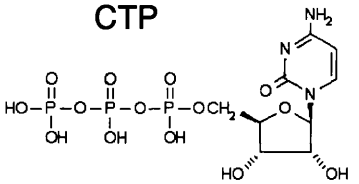
ddATP



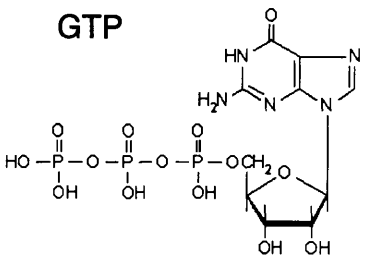
ADP



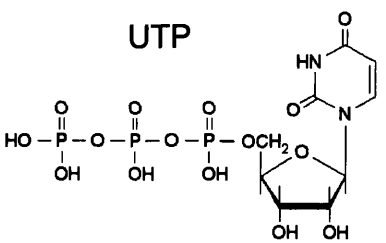
CTP



GTP

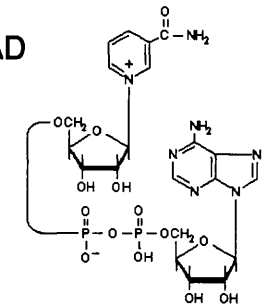


UTP

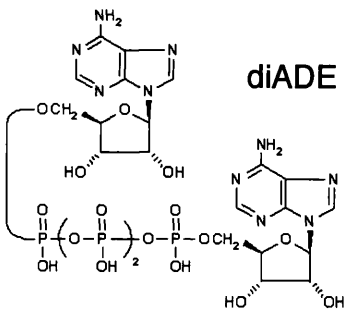


also dGTP
dCTP +
dTTP

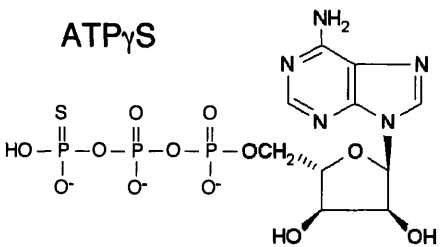
NAD



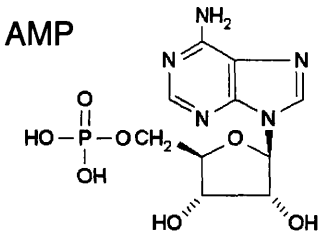
diADE



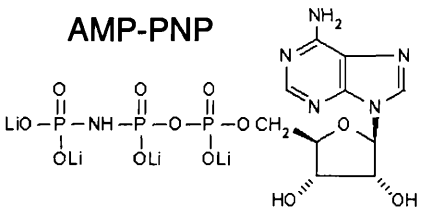
ATPγS



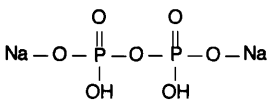
AMP



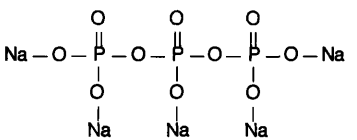
AMP-PNP



NaPPi



NaTriP



also NaP
ddGTP
ddCTP
ddTTP
+ dGMP

IP6

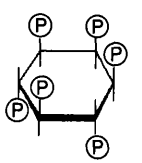


Figure 3.9

demonstrated that IP_6 does not stimulate end-joining by acting as an ATP analogue.

VI. Summary and discussion

Multiple end-joining activities have been observed in mammalian extracts, with the type of end-joining (accurate fill-in and microhomology-directed repair or inaccurate SSA) apparently altering with the method of extract preparation (reviewed in Labhart, 1999b). Furthermore, preparation of extracts by the same method but from different organisms can lead to variation in the predominance of Ku-dependent or Ku-independent repair (Baumann and West, 1998a). There is a need, therefore, to identify the protein factors required for joining in any given system. DNA end-joining catalysed by human cell-free extracts was previously shown to be abolished by antibodies against XRCC4 and DNA ligase IV (Baumann and West, 1998a). In addition, end-joining activity was absent from extracts immunodepleted with XRCC4 antisera, but could be reconstituted by addition of purified XRCC4-DNA ligase IV. Complete inhibition of end-joining by anti-XRCC4 antibodies was also observed in the experiments described in this chapter (Fig. 3.2).

Human extract-catalysed DNA end-joining was also abolished by the PI 3-kinase inhibitor wortmannin, at concentrations that implicated a requirement for DNA-PK_{cs} activity in the *in vitro* reaction (Baumann and West, 1998a). To confirm the role of this protein in extract-catalysed end-joining, extracts were prepared from DNA-PK_{cs}-defective M059J cells. As expected, end-joining was

observed with extract prepared from the control cell line M059K, which is DNA-PK_{cs}-proficient, whereas the DNA-PK_{cs}-defective M059J extracts failed to support DNA end-joining (Fig. 3.3). Furthermore, the end-joining activity of M059J extracts could be restored by addition of purified DNA-PK_{cs} (Hanakahi *et al.*, 2000). The joining of protruding 5' termini by human cell-free extracts, therefore, shows an absolute requirement for the NHEJ proteins, and provides a system to evaluate the further protein and cofactor requirements of DNA-PK-dependent end-joining.

In addition to reactions using human cell-free extracts being the first established *in vitro* assay in which only DNA-PK-dependent end-joining is observed, the repair activity also differs from that observed in earlier studies, because only linear products are generated (Fig. 3.1). In contrast to the Ku-dependent end-joining activity of human extracts, Ku-dependent end-joining in *Xenopus* and hamster extracts generated circular products (Labhart, 1999a; Feldmann *et al.*, 2000). This suggests that, although essential for NHEJ, Ku does not appear to influence whether the products generated are linear or circular. *In vitro* reconstitution experiments using purified XRCC4, DNA ligase IV and DNA-PK_{cs}, showed that the formation of linear multimers was favoured when DNA-PK_{cs} was added to XRCC4-DNA ligase IV catalysed end-joining reactions (Chen *et al.*, 2000). Protein-protein interactions between DNA-PK_{cs}-containing complexes at DNA termini were suggested to promote the formation of linear multimers. DNA-PK_{cs} may therefore, have a similar role in the cell-free system. Alternatively, it is possible that the factor(s) determining the formation of linear

versus circular products are not components of the NHEJ pathway, but instead non-specific DNA binding proteins, or crowding agents which differ in the vertebrate extracts tested.

To facilitate an investigation into the cofactor requirements for efficient end-joining, GM00558 extracts were precipitated with ammonium sulphate to remove free ATP. No end-joining activity was observed with these cleared extracts without addition of exogenous ATP (Fig. 3.6). A variety of ribo- and deoxynucleotides as well as other nucleotide-like cofactors could, however, substitute for ATP with relatively high efficiency (Figs. 3.7 and 3.8). End-joining activity was highest in the presence of nucleotides containing an adenine base, and absent when nucleoside monophosphates or inorganic polyphosphates were used. In general, a nucleotide-like cofactor was required to support end-joining. It is important to note, however, that because the *in vitro* end-joining assay uses a crude extract preparation, the cofactors added to the reaction might possibly be converted to an alternative form. End-joining by purified NHEJ proteins may not, therefore, use the same range of cofactors as the extract-catalysed reaction.

The ability of certain enzymes to use a variety of nucleotide cofactors has been observed previously. For example, T4 polynucleotide kinase (PNK) was found to have a low stringency of ATP requirement, with all ribo- and deoxynucleoside triphosphates as well as inorganic polyphosphates serving as phosphate donors (Kinoshita and Nishigaki, 1997). T4 DNA ligase and T4 RNA ligase in comparison, exhibited moderate stringency in regard to ATP replaceablilty. Both enzymes were able to utilise dATP in addition to ATP, albeit

at a lower efficiency. T4 RNA ligase was, however, unable to use ddATP, nor nucleoside triphosphates with cytosine, guanine or thymine bases. In addition, neither enzyme was able to use inorganic triphosphates. These results demonstrate that some enzymes can use more than one nucleotide cofactor, but that proteins vary in their accommodation of different cofactor structures.

With regard to the nucleotide cofactor requirement of *in vitro* DNA end-joining, it is not presently known which of the NHEJ proteins binds the cofactor. As already mentioned, DNA ligase IV purified from HeLa cells was reported to be isolated in a preadenylated form (Robins and Lindahl, 1996). Consequently, ligation by the purified XRCC4-DNA ligase IV complex has been observed in the absence of ATP (data not shown). Making the assumption that DNA ligase IV remains preadenylated in cell-free extracts, then this protein is unlikely to be responsible for the nucleotide cofactor requirements reported in this chapter. Furthermore, if preadenylated ligase is present in the cleared extract, the lack of end-joining in the absence of ATP indicates that adenylation of DNA ligase IV is not sufficient for joining of ligatable termini in this assay, and that binding of a nucleotide cofactor by another protein is required. Of the other known end-joining components, XRCC4 does not contain a conserved nucleotide binding motif. The individual subunits of DNA-PK do, however, bind ATP. The kinase activity of DNA-PK requires binding of ATP to the active site within the catalytic subunit. DNA-PK_{cs} was, however, found not to bind GTP, nor use it as a phosphate donor (Lees-Miller *et al.*, 1990), suggesting that DNA-PK_{cs} is not responsible for the low stringency cofactor requirement exhibited in the *in vitro*

reactions. Both the Ku70 and Ku80 subunits have weak ATP binding site homologies, and the heterodimer has been shown to exhibit DNA-dependent ATPase activity (Cao *et al.*, 1994). However *Ku70*$^{-/-}$ embryonic stem cells reconstituted with Ku70 protein carrying a mutation in the putative nucleotide binding site, showed levels of IR-induced repair and DNA end-binding equivalent to that seen with cells reconstituted with wild-type Ku70 protein (Jin and Weaver, 1997). This indicates that the putative nucleotide binding motif of Ku70 is not essential for DNA repair and suggests that Ku is unlikely to be responsible for the cofactor requirement exhibited in the *in vitro* assay.

The role of MRE11, RAD50 and Xrs2/NBS1 in NHEJ has been the subject of many debates. In *S. cerevisiae*, the M/R/X complex is required for Ku-dependent joining of ligatable termini *in vivo* (Boulton and Jackson, 1998). Moreover, using purified proteins, the M/R/X complex was shown to selectively stimulate yKu70/yKu80- and Lif1/Lig4-catalysed DNA end-joining (Chen *et al.*, 2001a). In contrast to the essential role for Mre11 and Rad50 in NHEJ in *S. cerevisiae*, the *Schizosaccharomyces pombe* homologues, Rad32 and Rad50, appear to have little or no role in DNA end-joining (Wilson *et al.*, 1999; Manolis *et al.*, 2001). Furthermore, *MRE11* is not epistatic to *KU70* in the chicken DT40 cell line (Yamaguchi-Iwai *et al.*, 1999). This was shown by assessment of the ionising radiation sensitivity of DT40 cells synchronised at G_1 and early S phase. During this stage of the cell cycle Ku-dependent end-joining is the dominant DSB repair mechanism, however, *MRE11*$^{-/-}$ cells displayed very little radiation sensitivity in comparison to the significant radiosensitivity exhibited by *KU70*$^{-/-}$

cells. Moreover, the increase in radiation-induced chromosome aberrations observed between *RAD54*$^{-/-}$ single and *RAD54*$^{-/-}$/*KU70*$^{-/-}$ double mutants was comparable to that seen between *MRE11*$^{-/-}$ and *MRE11*$^{-/-}$/*KU70*$^{-/-}$ cells, indicating that MRE11 deficiency caused a defect in HR and not in NHEJ.

Investigations into the role of the M/R/N complex in human cells have included biochemical characterisation of cell lines derived from NBS and ATLD patients. Initial analyses of DSBR by pulsed field gel electrophoresis, showed that ATLD2, ATLD3 and NBS-1LB cells had comparable rates of repair to those observed in wild type cells (Kraakman-van der Zwet *et al.*, 1999; Stewart *et al.*, 1999). The IR sensitivity displayed by these cells was therefore attributed to impaired cell cycle arrest following DNA damage. In contrast, the 347BR NBS cell line differed because IR-induced S-phase arrest was only slightly impaired, and the ionising radiation sensitivity of this cell line was assumed to be a result of defective DSB repair rather than a cell cycle checkpoint defect (Girard *et al.*, 2000). Further evidence suggesting a requirement for the M/R/N complex in human NHEJ came from the stimulation of Ku- and XRCC4/DNA ligase IV-catalysed DNA end-joining by partially purified fractions containing MRE11, RAD50 and NBS1 (Huang and Dynan, 2002).

The data presented in this chapter, however, show that the M/R/N complex does not have a role in Ku-dependent joining of ligatable termini in human cells. The plasmid-based repair assay allows both the efficiency and the fidelity of repair of a defined DSB to be investigated, and can therefore give a more detailed picture of DSBR than pulsed field gel electrophoresis. The mild DSBR

defect observed in 347BR cells is mainly characterised by an increased fraction of unrepaired DSBs at prolonged incubation times (Girard *et al.*, 2000). Comparatively, an elevated rate of repair was observed at early time points post irradiation. In agreement with the data presented in this chapter, this would indicate that MRE11 and NBS1 do not to have a role in the repair of ligatable DSBs. However, a requirement for the M/R/N complex during the repair of damaged DNA termini that require processing before rejoining or chromatin-bound DNA DSBs cannot be excluded.

Joining of DNA molecules with non-ligatable termini

I. Introduction

DNA double-strand breaks are induced by ionising radiation and radiomimetic drugs, but also occur naturally during T-cell receptor and immunoglobulin gene rearrangements in lymphoid development. In V(D)J recombination, RAG-mediated cleavage of DNA produces blunt termini at signal sequences, and hairpins at coding sequences (Van Gent *et al.*, 1995). Additionally, DSBs induced by ionising radiation exhibit modified DNA termini that cannot be rejoined simply by ligation (Obe *et al.*, 1992). Nucleotide loss from the break site as well as alterations to the pentose ring of terminal nucleotides have been observed. DNA fragments, either irradiated in aqueous solutions or extracted from irradiated cells, display termini containing 5'-phosphate or hydroxyl groups and 3'-phosphate or phosphoglycolate groups (Coquerelle *et al.*, 1973; Lennartz *et al.*, 1975; Obe *et al.*, 1992).

Since NHEJ is implicated in the repair of both types of DSBs discussed above it seems probable that this DNA repair mechanism incorporates end-processing activities that act on such modified termini, allowing repair to be completed. Enzymatic activities that could be envisaged to play a role in the processing of modified termini include nuclease activities, either exo-, endo-, or flap endonucleases, polynucleotide kinase and DNA polymerase activities. Genetic studies in yeast have, however, implicated only two such factors as acting in NHEJ - Mre11 and Pol4, a Polβ-related DNA polymerase. Whereas the

Mre11/Rad50/Xrs2 (M/R/X) complex is essential for the joining of ligatable termini in *S. cerevisiae*, the nuclease activity of Mre11 appears to be dispensable, suggesting that Mre11 does not act as a nuclease in NHEJ (Moreau *et al.*, 1999; Chen *et al.*, 2001a). However, as discussed in the previous chapter the role of MRE11, RAD50 and NBS1 in mammalian NHEJ remains unclear.

Mutation of *POL4* in *S. cerevisiae* has implicated this DNA polymerase as acting in a subset of end-joining reactions (Wilson and Lieber, 1999). Simple ligation reactions catalysed by the NHEJ proteins were not impaired in *pol4* yeast. In contrast, a two-fold decrease was observed in the joining of mismatched termini in which gap filling DNA synthesis was required.

Biochemical approaches to identify end-processing activities in cell-free extracts have been limited by the use of restriction endonuclease-generated DNA fragments, many of which can be rejoined by simple ligation. In one of the few studies in which the joining of non-ligatable termini was investigated, *Xenopus* egg extracts were shown to promote the joining of non-complementary termini by a reaction that required DNA polymerase activity (Thode *et al.*, 1990). Using a variety of modified restriction enzyme-generated termini and the DNA-PK-dependent *in vitro* end-joining assay characterised in the previous chapter, the processing of non-ligatable termini by NHEJ has been investigated.

II. Joining of protruding 5'-hydroxyl termini

Strand breaks with terminal 5'-hydroxyl groups have been detected in DNA recovered from irradiated thymocytes (Coquerelle *et al.*, 1973; Lennartz *et al.*,

1975). To mimic this type of DNA damage and to determine if human cell-free extracts could join non-ligatable termini, uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA was treated with calf intestinal phosphatase (CIP) to generate protruding 5'-hydroxyl (5'-OH) termini (see Fig. 4.1A). Joining of DNA with protruding 5'-OH termini was compared with the joining of DNA with protruding 5'-phosphate (5'-P) termini (Fig. 4.1B). T4 DNA ligase incubated with plasmid DNA with protruding 5'-P termini under favourable ligation conditions (relatively high concentration of T4 DNA ligase and overnight incubation at 15°C) resulted in the generation of predominantly circular products (lane b). Ligation products were not observed, however, when plasmid DNA with protruding 5'-OH termini was incubated with T4 DNA ligase under the same conditions (lane e). This demonstrates that the CIP treatment of linear DNA was complete, and each terminus carried a 5'-OH group.

In contrast to T4 DNA ligase, GM00558 cell-free extracts were able to join both protruding 5'-P and 5'-OH termini with apparently similar efficiencies (Fig. 4.1, compare lanes c and f). Indeed, time course studies showed that there were no significant differences in the joining efficiency of protruding 5'-P and 5'-OH termini (Fig. 4.2). Furthermore, analyses of the end-joining products from both time courses by alkaline agarose gel electrophoresis showed that these products were ligated and not just annealed (data not shown). This demonstrates that extracts from human cells are capable of joining non-ligatable DNA with 5'-hydroxyl termini. Moreover, during this joining reaction the end-processing

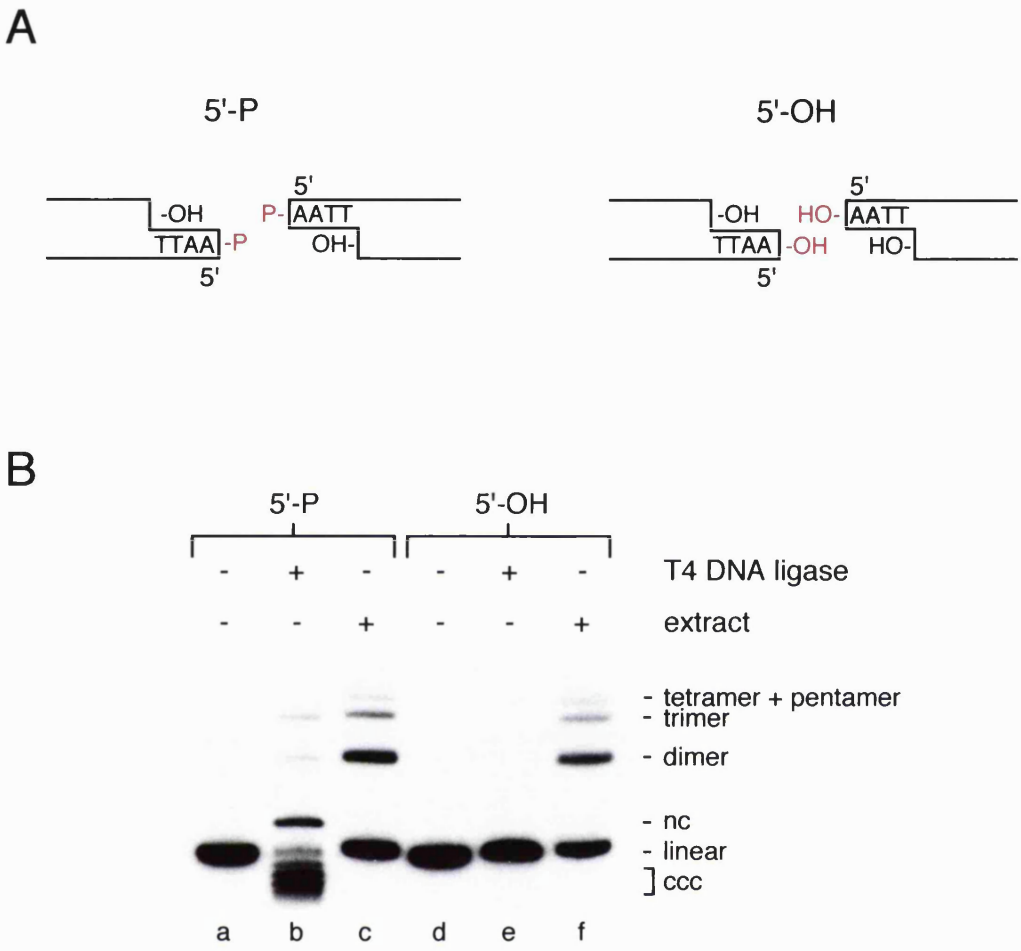


Figure 4.1. Comparison of joining of protruding 5'-phosphate and 5'-hydroxyl termini by T4 DNA ligase or human cell-free extracts.

(A) Schematic representation of termini used in end-joining reactions. Uniformly-^{32}P-labelled plasmid DNA was linearised with *Eco*RI to give protruding 5'-phosphate (5'-P) termini. Further treatment with calf intestinal phosphatase, as described in Materials and Methods section 2.18, resulted in protruding 5'-hydroxyl (5'-OH) termini.

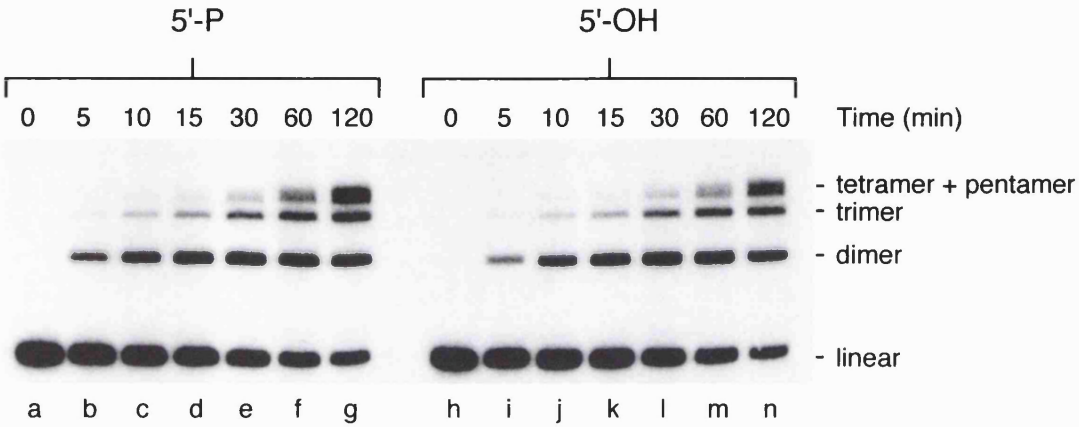
(B) End-joining and T4 DNA ligation reactions were carried out as described in Materials and Methods (section 2.30.1 and 2.31). Where indicated, reactions (20 μl) contained uniformly-^{32}P-labelled plasmid DNA (40 ng) with protruding 5'-phosphate or 5'-hydroxyl termini, incubated with T4 DNA ligase (80 U) or GM00558 extracts (80 μg). Following deproteinisation, the products were separated by agarose gel electrophoresis and detected by autoradiography.

Figure 4.2. Comparison of extract-catalysed end-joining of protruding 5'-phosphate and 5'-hydroxyl termini.

(A) Large scale end-joining reactions (160 μl), performed as described in Materials and Methods section 2.30.1, contained GM00558 extracts (640 μg) and where indicated, uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (320 ng) with 5'-phosphate or 5'-hydroxyl termini. Reactions were incubated at 37°C, and at the indicated times samples (20 μl) removed and deproteinised. All products were separated by agarose gel electrophoresis and visualised by autoradiography.

(B) Quantification of time course shown in (A). (■) 5'-P, (●) 5'-OH. End-joining products are expressed as a percentage of multimeric DNA out of total DNA.

A


5'-P
5'-OH
0 5 10 15 30 60 120
0 5 10 15 30 60 120
Time (min)
- tetramer + pentamer
- trimer
- dimer
- linear
a b c d e f g
h i j k l m n

B

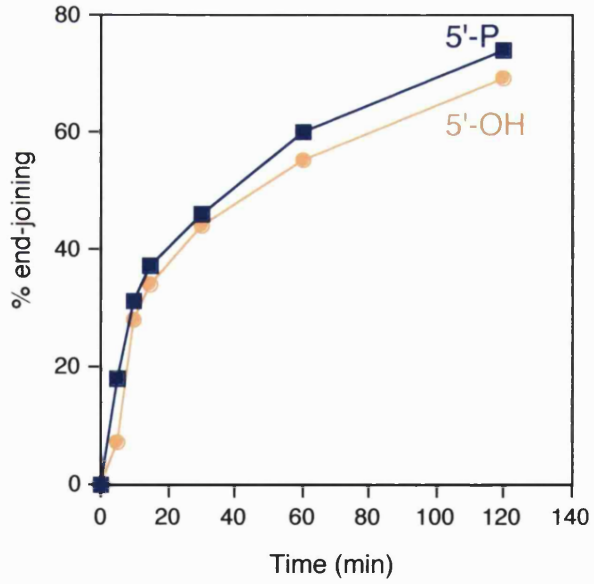

80
60
40
20
0
% end-joining
5'-P
5'-OH
0 20 40 60 80 100 120 140
Time (min)

Figure 4.2

mechanism that restores the terminal 5'-phosphate is highly efficient and does not appear to be the rate-limiting step.

In previous studies characterising extract-catalysed DSB repair, reactions containing different termini exhibited variations in the requirement for the NHEJ factors (Labhart, 1999a; Feldmann *et al.*, 2000). To establish that the extract-catalysed repair of 5'-OH termini observed above was dependent on the NHEJ proteins, end-joining was studied in the presence of XRCC4 antisera or using M059K and M059J extracts (Fig. 4.3). Ligation of DNA with 5'-OH termini was severely inhibited by addition of anti-XRCC4 antibodies at a final dilution of 1 in 1250, and completely abolished at 1 in 250 dilution (lanes e and f). This is consistent with the inhibition of joining of 5'-P termini by anti-XRCC4 antibodies (Fig. 3.2). Also, as seen with 5'-P termini (Fig. 3.3), ligation of 5'-OH termini was supported by DNA-PK_{cs}-proficient M059K extracts, but not by DNA-PK_{cs}-defective M059J extracts (Fig. 4.3, lanes g and h). In conclusion the joining of DNA with either protruding 5'-P or 5'-OH termini by GM00558 extracts is dependent on the presence of the NHEJ proteins.

Non-homologous repair of protruding 5'-phosphate termini by human cell-free extracts is known to be accurate (Baumann and West, 1998a). To determine if protruding 5'-hydroxyl termini were also joined accurately, the reaction products were subjected to digestion with *Eco*RI - the same restriction enzyme used to initially linearise the plasmid DNA. For both protruding 5'-P and 5'-OH termini, all products were re-cut with *Eco*RI (Fig. 4.4 compare lanes b and c, d and e). This indicates that the extract-catalysed joining of protruding 5'

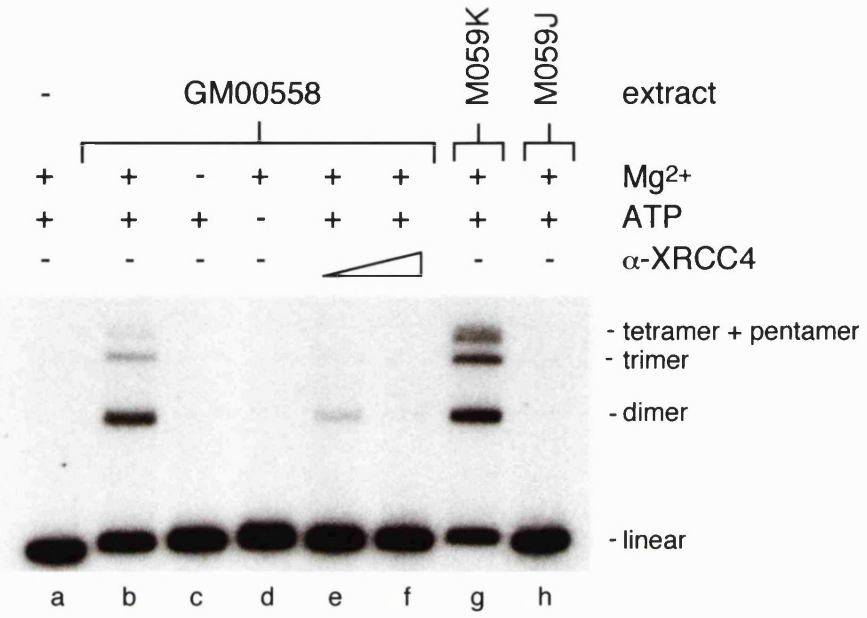


Figure 4.3. Protein and cofactor requirements for extract-catalysed joining of protruding 5'-hydroxyl termini.

End-joining reactions (20 μl) were set up as described (Materials and Methods, section 2.30.1), containing uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-OH termini (40 ng). Where indicated, reactions were supplemented with E-buffer (-) or protein extracts (80 μg) from GM00558, M059K or M059J cells. Where indicated, $Mg(OAc)_2$ (Mg^{2+}, 1mM) or ATP (1mM) were omitted from the end-joining buffer, and anti-XRCC4 antibodies added to a final dilution of 1:1250 or 1:250 (α-XRCC4).

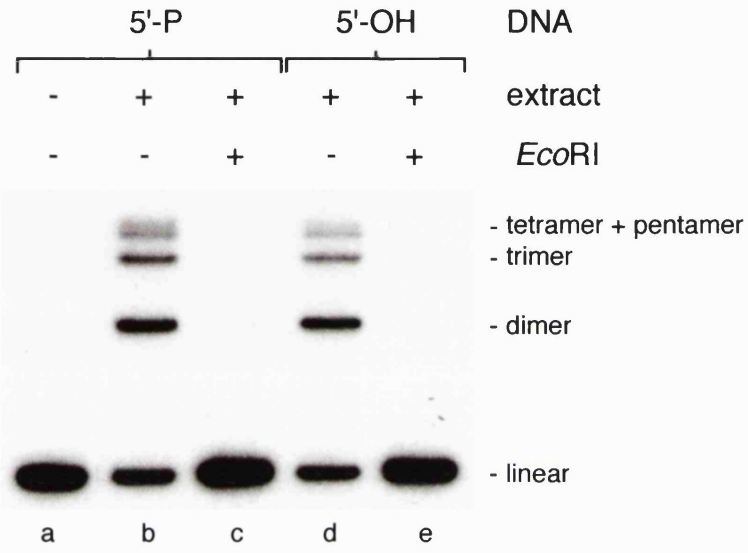


Figure 4.4. Accurate joining of protruding 5' termini.

Large scale end-joining reactions (100 μl) contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (200 ng) (with 5'-phosphate or 5'-hydroxyl termini as indicated) incubated with (+) or without (-) GM00558 extracts (400 μg) as described in Materials and Methods (section 2.30.1). Following incubation for 2 hrs at 37°C, the DNA was deproteinised and aliquots (40 ng) incubated with (+) or without (-) *Eco*RI as described in Materials and Methods (section 2.32). All products were separated by agarose gel electrophoresis and visualised by autoradiography.

termini, either with phosphate or hydroxyl groups is accurate. Therefore, during joining of protruding 5'-OH termini, the repair occurs without mutations arising at the break site.

III. Joining of recessed 5'-hydroxyl termini

To establish that cell-free extracts were competent to repair modified recessed termini in addition to the modified protruding termini discussed above, uniformly-^{32}P-labelled plasmid DNA was linearised with *Kpn*I and an aliquot treated with CIP to produce recessed 5'-P and 5'-OH termini (Fig. 4.5A). Joining of protruding 3' termini (referred to here as recessed 5'-P termini) by human cell-free extracts has been demonstrated (Baumann and West, 1998a). In these experiments, however, a small percentage of the products were joined inaccurately. Given the ability of human cell-free extracts to join protruding 5'-OH termini, we would expect that the extracts would also be capable of joining recessed 5'-OH termini. If the same enzymatic activities which act on protruding 5'-OH termini also catalyse the processing of recessed 5'-OH groups then, consequently, the joining of recessed 5'-OH termini would expected to be mainly accurate.

Comparison of ligation of recessed 5'-P and 5'-OH termini by T4 DNA ligase showed that whilst recessed 5'-P termini were joined efficiently, 5'-OH termini were not (Fig. 4.5B, lanes b and e). The inability of T4 DNA ligase to form any joined products with DNA carrying recessed 5'-OH termini, demonstrated the lack of the necessary 5'-P. In contrast, both recessed 5'-P and 5'-OH termini were joined by human cell-free extracts (lanes c and f). Fewer multimers were

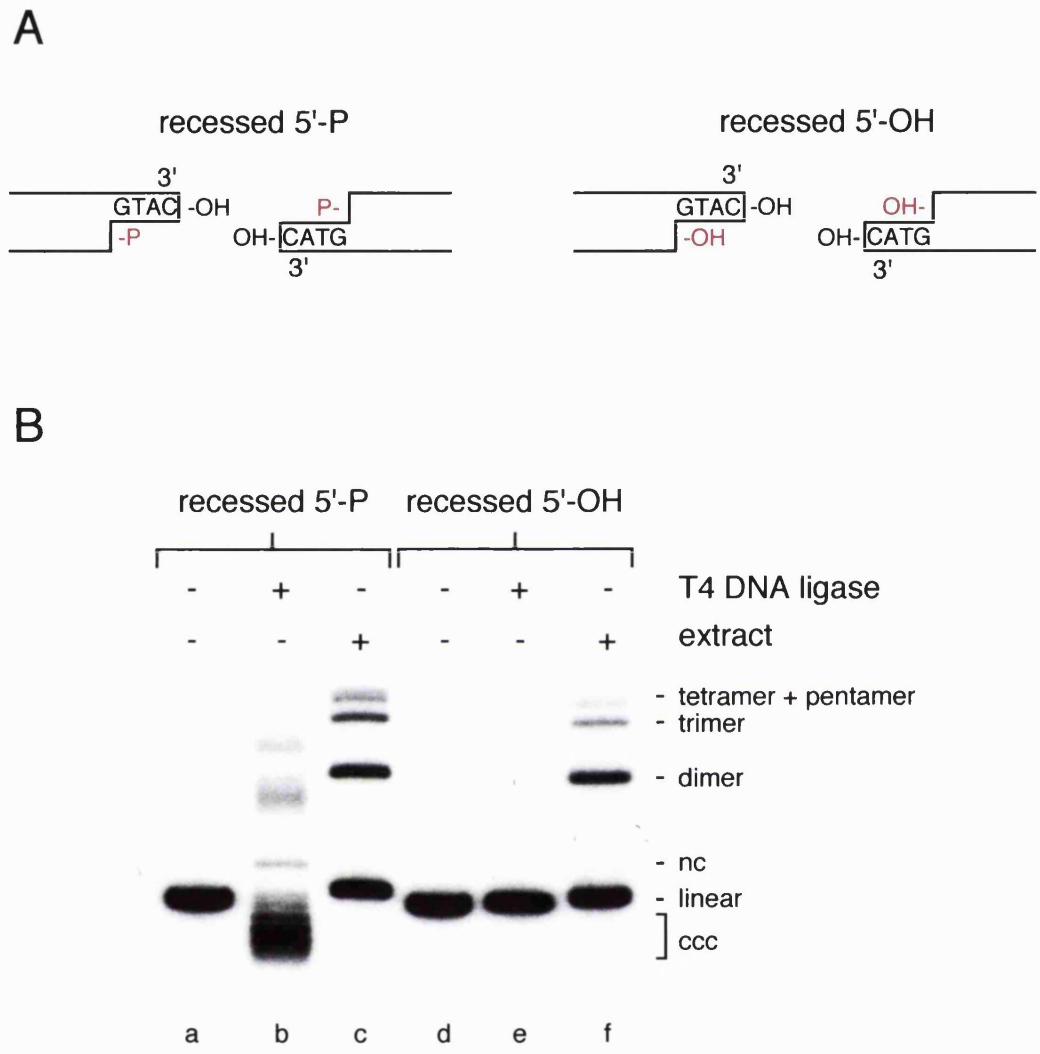


Figure 4.5. Comparison of joining of recessed 5' termini by T4 DNA ligase or human cell-free extracts.

(A) Schematic representation of termini used in end-joining reactions. Uniformly-^{32}P-labelled plasmid DNA was linearised with *Kpn*I and modified as described in Materials and Methods (section 2.19) to produce recessed 5'-phosphate and 5'-hydroxyl termini.

(B) Reactions (20 μl) contained uniformly-^{32}P-labelled, linear DNA (with recessed 5'-phosphate or 5'-hydroxyl termini as indicated, 40 ng), incubated with T4 DNA ligase (80 U), or with GM00558 extracts (80 μg), as described in Materials and Methods (sections 2.30.1 and 2.31). Products were analysed by agarose gel electrophoresis and autoradiography.

observed, however, when the DNA had recessed 5'-OH termini. To investigate this further, time course experiments were set up to directly compare the extract-catalysed repair of recessed 5'-P and 5'-OH termini (Fig. 4.6). The results confirmed that recessed 5'-P termini were joined somewhat more efficiently than recessed 5'-OH termini. There was an initial lag in joining of recessed 5'-OH termini, such that after 15 minutes only 4% of the linear DNA with these termini had been ligated compared with 10% for recessed 5'-P termini. The difference in joining efficiency became more pronounced after 2 hours, when 44% of the 5'-P termini were joined compared with 33% of 5'-OH termini.

In contrast to the results for protruding 5'-P termini in which all repair was catalysed by the NHEJ proteins, for recessed 5'-P termini a small percentage of end-joining was found to be XRCC4- and DNA-PK_{cs}-independent (Fig. 4.7A, lanes d and f). In terms of studying DNA- PK_{cs}-dependent NHEJ, this secondary end-joining activity was fortunately very inefficient constituting only 2 – 6% of the total repair. Furthermore, the DNA-PK_{cs}-independent activity was not observed with the recessed 5'-OH termini (Fig. 4.7B, lanes d and f).

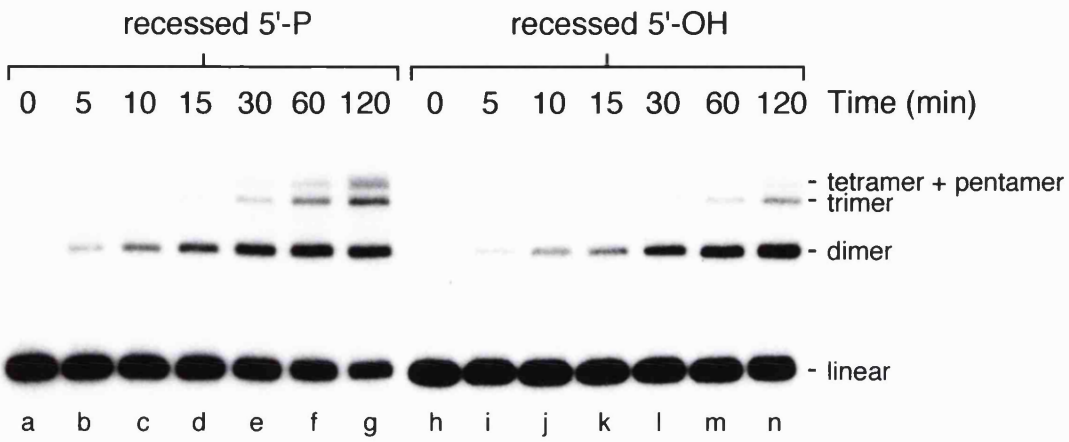
The accuracy of extract-catalysed joining of recessed 5'-OH termini was compared with that observed for 5'-P termini (Fig. 4.8). In agreement with earlier observations (Baumann and West, 1998a), ligation of recessed 5'-P termini was found to be predominantly accurate (compare lanes d and e). However, a few of the repair products (~6%) were resistant to digestion with *Kpn*I, indicating some alteration to the DNA sequences at these termini. When the end-joining reaction contained DNA with recessed 5'-OH termini, 18% of the products were resistant

Figure 4.6. Comparison of extract-catalysed joining of recessed 5'-phosphate and recessed 5'-hydroxyl termini.

(A) Large scale end-joining reactions (160 μl) were set up as described in Fig. 4.2 except they contained uniformly-^{32}P-labelled, *Kpn*I-linearised plasmid DNA (320 ng) with recessed 5'-P or 5'-OH termini, as indicated. Reactions were incubated at 37°C, and at the indicated times samples (20 μl) were removed and deproteinised. All products were separated by agarose gel electrophoresis and visualised by autoradiography.

(B) Quantification of gel shown in (A). Recessed 5'-P termini (■), recessed 5'-OH termini (●). End-joining products are expressed as the percentage of multimeric DNA compared with total DNA.

A


recessed 5'-P
recessed 5'-OH
0 5 10 15 30 60 120 0 5 10 15 30 60 120 Time (min)
- tetramer + pentamer
- trimer
- dimer
- linear
a b c d e f g h i j k l m n

B

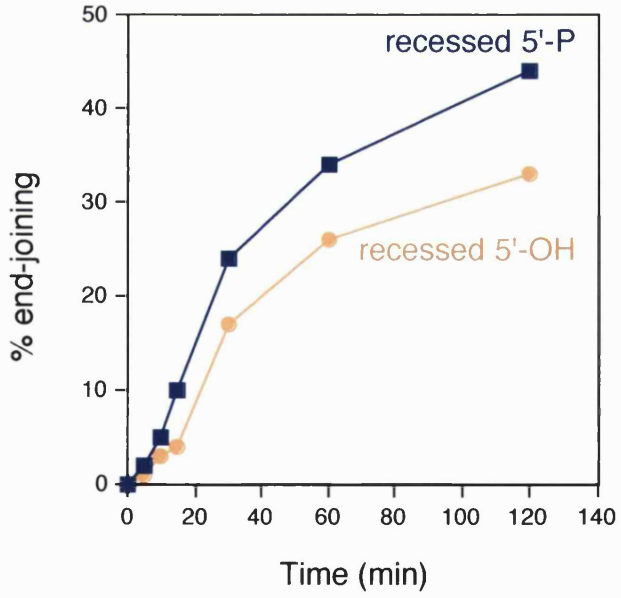

recessed 5'-P
recessed 5'-OH
% end-joining
Time (min)
0
10
20
30
40
50
0 20 40 60 80 100 120 140

Figure 4.6

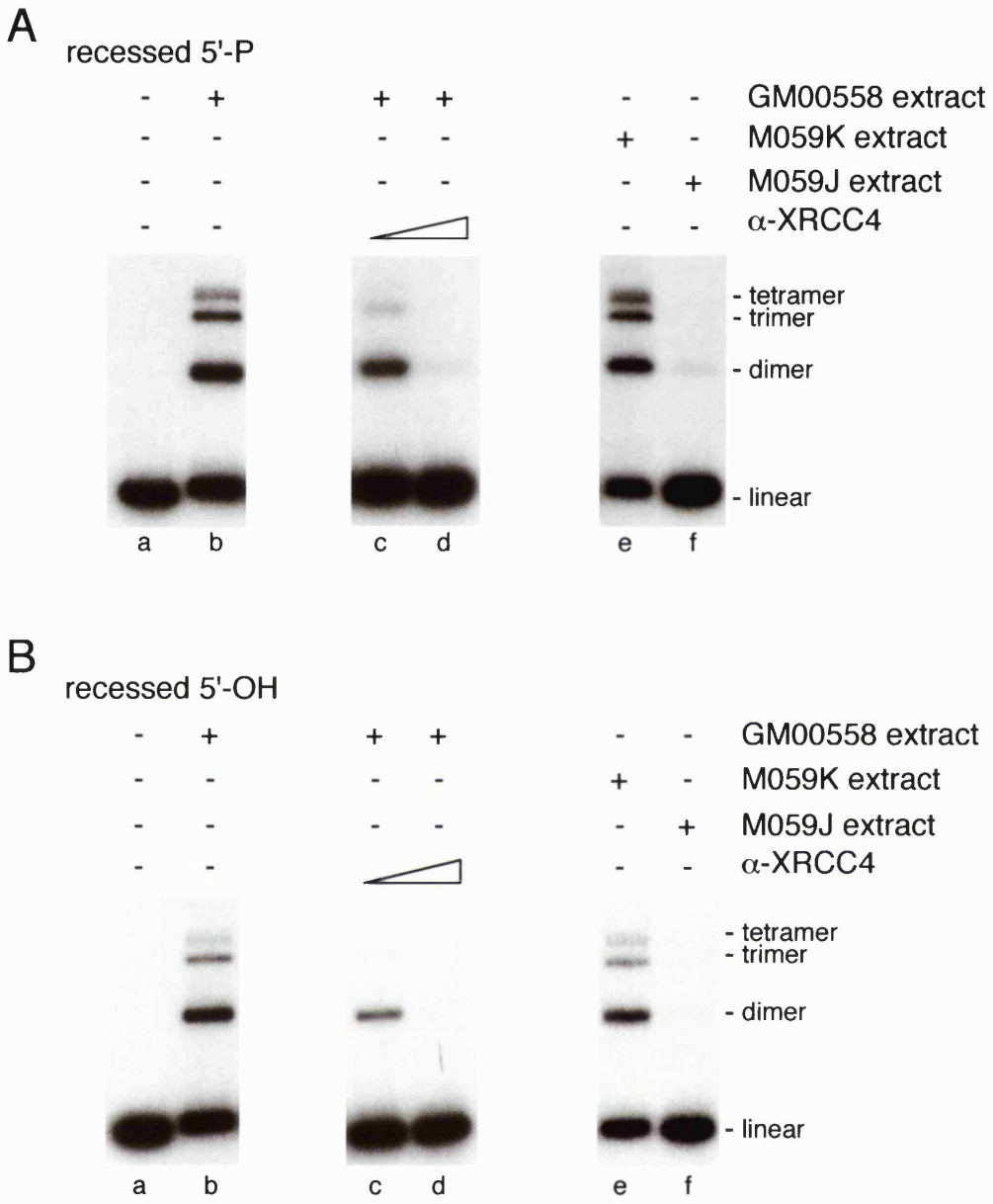


Figure 4.7. Protein requirements for extract-catalysed joining of recessed 5' termini.

(A) End-joining reactions were performed as described in Materials and Methods (section 2.30.1). Reactions (20 μl) contained uniformly-^{32}P-labelled, *Kpn*I-linearised plasmid DNA with recessed 5'-P termini (40 ng) incubated with extracts prepared from GM00558, M059K or M059J cells (80 μg) and XRCC4 antisera (α-XRCC4, 1:1250 and 1:250 dilution), where indicated. Products were separated by gel electrophoresis and visualised by autoradiography.

(B) End-joining reactions were set up as described in (A) except they contained uniformly-^{32}P-labelled, *Kpn*I-linearised plasmid DNA with recessed 5'-OH termini.

to *Kpn*I digestion (lanes f and g). Repetition of this experiment showed that although the percentage of inaccurate repair products varied between experiments, there was always a larger percentage of recessed 5'-OH termini joined inaccurately than recessed 5'-P termini.

Further controls were set up to ensure that the restriction enzyme was not inactivated during the digests. An equal concentration of linear plasmid DNA with recessed 5'-P termini was joined by T4 DNA ligase, and the products deproteinised, precipitated and digested with *Kpn*I in the same manner as end-joining products (lanes b and c). Furthermore, to eliminate the possibility that an inhibitor from the extract was transferred to the *Kpn*I restriction digest, an equal concentration of covalently closed circular (ccc) plasmid DNA was mixed with deproteinised extract, then precipitated and digested by *Kpn*I (lane j). The complete digestion of these control samples demonstrates that the incomplete re-digestion of end-joining products was due to inaccurate repair and not due to non-specific inhibition of *Kpn*I.

IV. Joining of recessed 3'-hydrogen termini

To investigate whether other modified termini could be joined by human cell-free extracts, a dideoxynucleotide was added to a recessed 3' termini using the Klenow fragment of DNA polymerase I (see Materials and Methods section 2.20, and Fig. 4.9A). Joining of DNA with this recessed 3'-hydrogen (3'-H) termini would involve excision of the terminal nucleotide harbouring the 3'-hydrogen and restoration of a ligatable 3'-OH group. The ligation of recessed 3'-OH and 3'-H

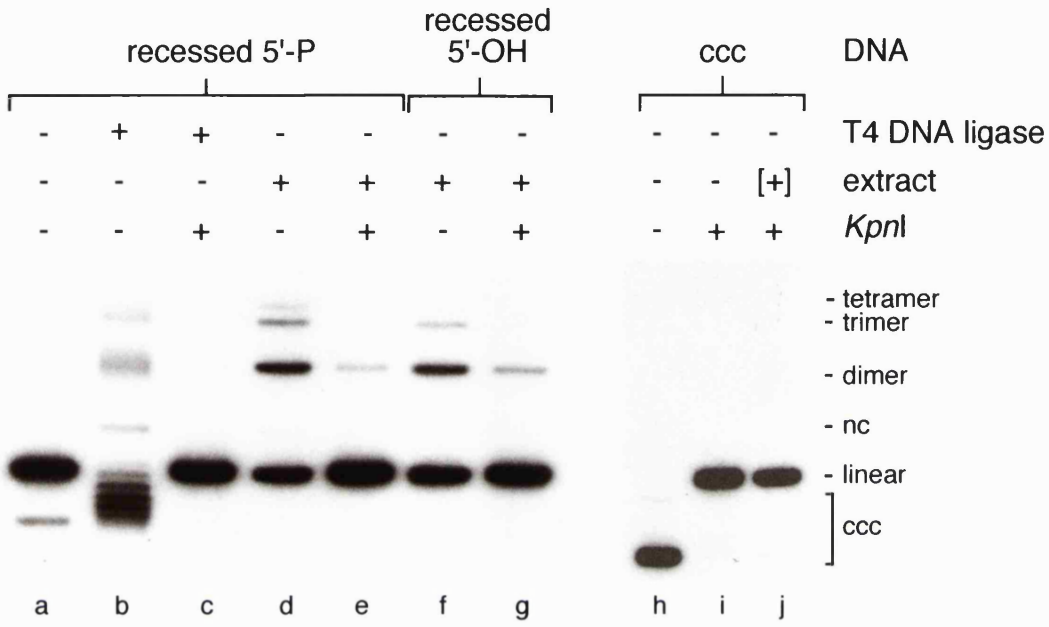


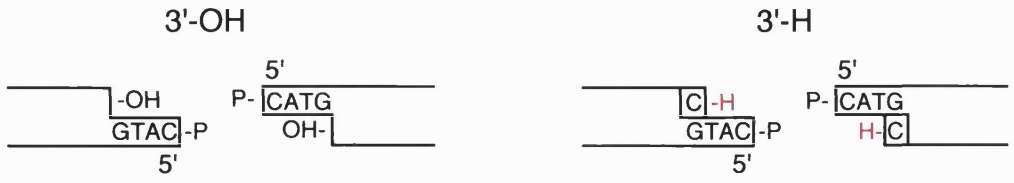
Figure 4.8. Fidelity of extract-catalysed joining of recessed 5' termini. Large scale end-joining and T4 DNA ligation reactions were set up as described in Materials and Methods (sections 2.30.1 and 2.31). Where indicated, reactions (100 μl) contained uniformly-^{32}P-labelled, *Kpn*I-linearised plasmid DNA (200 ng) with recessed 5'-P or 5'-OH termini, incubated with T4 DNA ligase (400 U) or GM00558 extracts (400 μg). All DNA samples were deproteinised and aliquots incubated with (+) or without (-) *Kpn*I as described in Materials and Methods (section 2.32). Samples of uniformly-^{32}P-labelled, covalently closed circular (ccc) plasmid DNA (40 ng) were also subject to *Kpn*I digestion and were premixed with deproteinised extract where indicated ([+]). All products were separated by agarose gel electrophoresis and visualised by autoradiography.

termini by T4 DNA ligase and human cell-free extracts was compared (Fig. 4.9B). T4 DNA ligase joined 3'-OH termini efficiently but was unable to join 3'-H termini, showing that modification of the recessed 3' termini in this sample was complete (lanes b and e). Similarly, after a two-hour incubation, GM00558 extracts had joined 3'-OH termini with high efficiency, but there was little joining of 3'-H termini (compare lanes c and f). Comparison of extract-catalysed joining of the recessed 3'-OH and 3'-P termini over a three-hour time course highlighted the differences in end-joining efficiencies (Fig. 4.10). After 30 minutes 40% of the DNA with 3'-OH termini had been rejoined, whereas essentially none of the 3'-H termini were ligated. After 3 hours, over 80% of the DNA with 3'-OH termini was found in multimeric forms compared with only 10% for DNA with 3'-H termini. This data demonstrates that human cell-free extract can catalyse the removal of a recessed 3' dideoxynucleotide (3'-H) and restore a 3'-OH group at the terminus, albeit reasonably slowly. The simplest explanation for this joining is the presence of a 3' – 5' exonuclease activity in the extract. Further experiments are required, however, to investigate if the nuclease activity only removes the terminal dideoxynucleotide or if nucleolytic degradation extends further and causes the subsequent repair to be inaccurate.

V. Joining of non-complementary termini

In previous studies, plasmid DNA cut with two different restriction enzymes at sites situated close together was used to examine joining of non-complementary termini (Pfeiffer and Vielmetter, 1988). Production of linear multimers in these

A



B

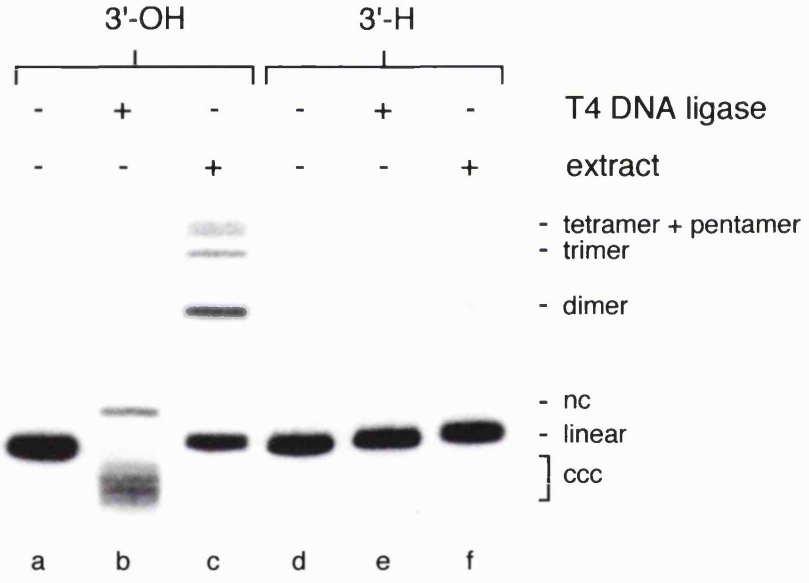


Figure 4.9. Comparison of joining of recessed 3' termini by human cell-free extracts or T4 DNA ligase.

(A) Uniformly-^{32}P-labelled plasmid DNA was linearised with *Nco*I to produce recessed 3'-OH termini. Addition of a dideoxyribonucleotide by the Klenow fragment of DNA polymerase I (see Materials and Methods section 2.18) resulted in a recessed 3'-H termini.

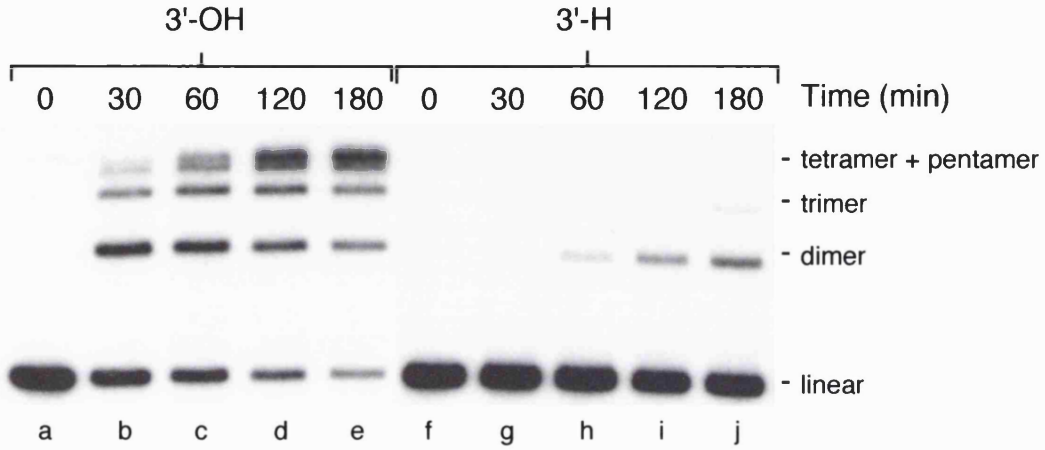
(B) End-joining and T4 DNA ligation reactions (10 μl) were set up (as described in Materials and Methods, sections 2.30.1 and 2.31) containing, where indicated, uniformly-^{32}P-labelled plasmid DNA with either 3'-OH or 3'-H termini (20 ng), T4 DNA ligase (40 U) and GM00558 extracts (80 μg). Following deproteinisation, the products were separated by agarose gel electrophoresis and visualised by autoradiography.

Figure 4.10. Extract-catalysed joining of recessed 3'-OH and 3'-H termini.

(A) Large scale end-joining reactions were set up as described in Materials and Methods (section 2.30.1). Reactions (120 μl) contained GM00558 extracts (480 μg) incubated with uniformly-^{32}P-labelled *Nco*I-linearised plasmid DNA with 3'-OH or 3'-H termini (240 ng) as indicated. The reactions were incubated at 37°C, and at the indicated times samples (20 μl) were removed and deproteinised. All products were separated by agarose gel electrophoresis and visualised by autoradiography.

(B) Quantification of time course shown in (A). 3'-OH (●), 3'-H (■). End-joining is expressed as the percentage of multimeric DNA compared with total DNA.

A



B

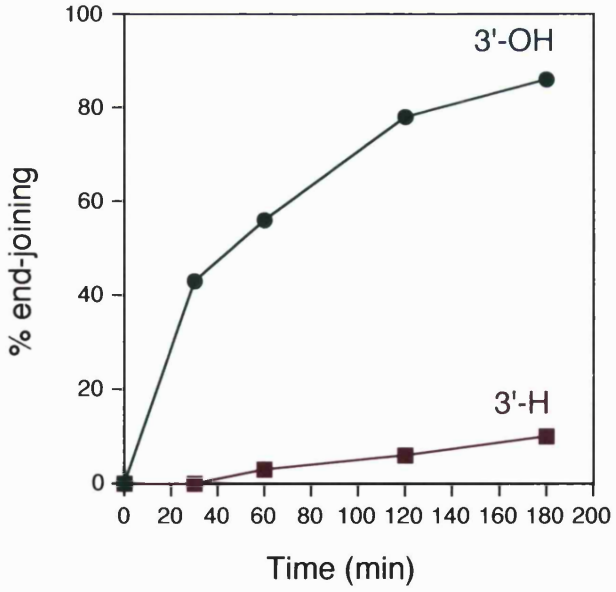


Figure 4.10

experiments could result from joining of non-complementary termini, but are more likely to be generated by joining of complementary termini in a head-to-head or tail-to-tail configuration. In comparison, circular monomeric products could only be generated by the joining of non-complementary termini (head-to-tail). Because end-joining catalysed by human cell-free extracts does not generate circular products, a two-plasmid assay was devised to study the joining of non-complementary termini (Fig. 4.11).

For this assay two uniformly-^{32}P-labelled plasmids of different sizes were linearised with different restriction enzymes. Incubation of the plasmids separately with cell free extracts results only in the production of multimers of plasmid 1 or multimers of plasmid 2. These products exhibit different mobilities upon agarose gel electrophoresis (see schematic in Fig. 4.11, lanes a and b). When both plasmids are incubated together with extracts, the products generated might include multimers of plasmid 1 or plasmid 2 and mixed multimers, resulting from the joining of plasmid 1 to plasmid 2. The mixed multimers are the products of non-complementary end-joining. Due to the difference in sizes of plasmids 1 and 2, any mixed multimers formed from the ligation of the two different plasmids (represented as green bands in lane c), will have a size distinct from the multimers of plasmid 1 or multimers of plasmid 2.

Joining of protruding 3' termini to blunt termini, protruding 5' termini to blunt termini and protruding 3' termini to protruding 5' termini was analysed using the two-plasmid assay (Fig. 4.12A, B and C). Reactions contained GM00558 extracts and plasmid 1 alone (lane a), plasmid 2 alone (lane b) or both plasmids

Figure 4.11. Two-plasmid end-joining assay.
End-joining reactions, containing linearised plasmid DNA of two different sizes, not only generates multimers of plasmid 1 and multimers of plasmid 2, but also mixed multimers resulting from the joining of plasmids 1 and 2. Agarose gel electrophoresis of the reaction products separates the mixed multimers (shown in green in lane c) from multimers of plasmids 1 and 2.

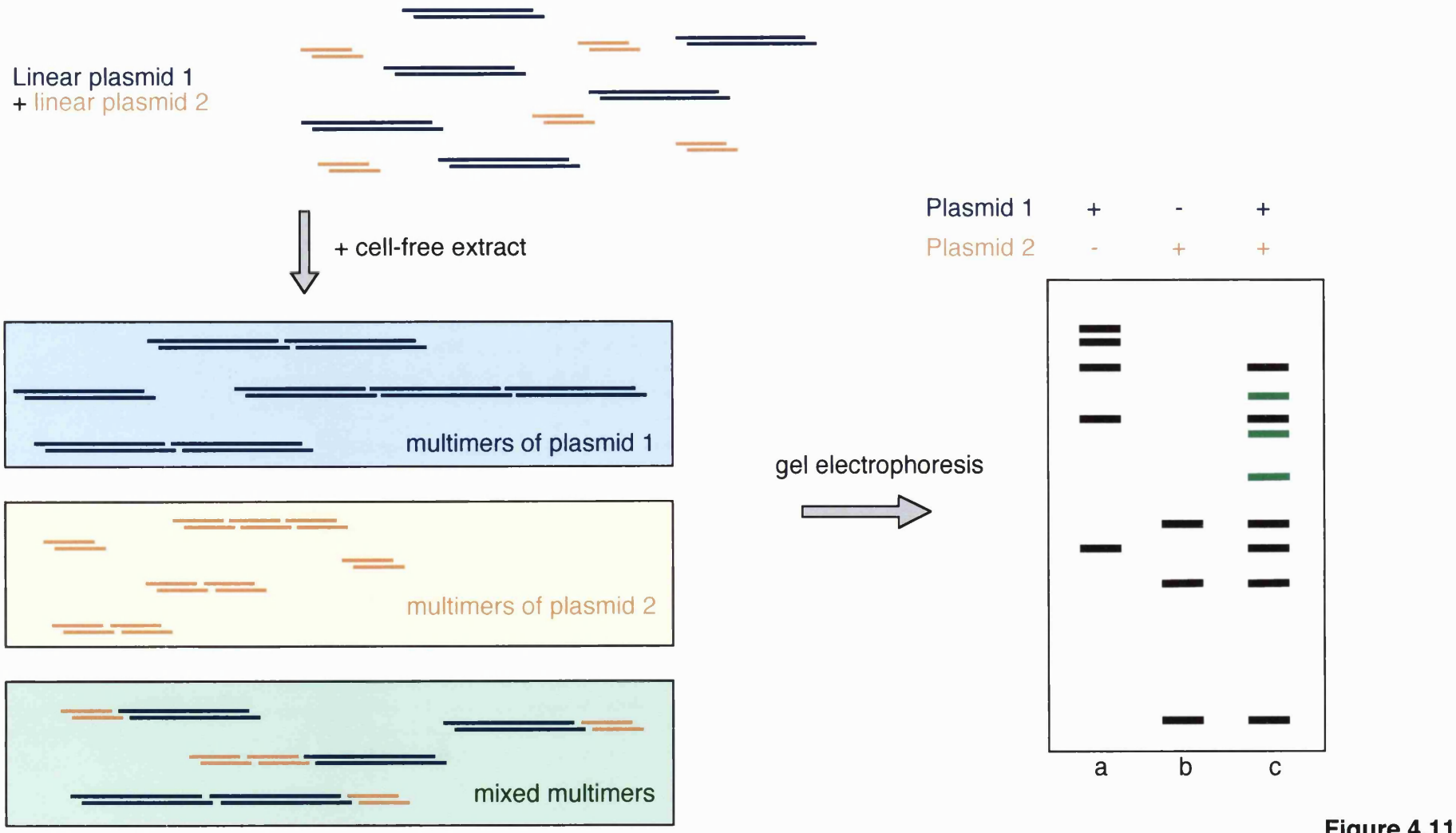

Linear plasmid 1
+ linear plasmid 2
+ cell-free extract
multimers of plasmid 1
multimers of plasmid 2
mixed multimers
gel electrophoresis
Plasmid 1 + - +
Plasmid 2 - + +
a
b
c

Figure 4.11

together (lanes c and d). dNTPs were added to some reactions (lane d) to stimulate any potential polymerase activity. Mixed multimers, the products of non-complementary end-joining, were observed when protruding 3' termini were incubated with blunt termini (Fig. 4.12A, lane c). Addition of 40 μM dNTPs stimulated the joining of non-complementary termini, whilst having no effect on the joining of complementary termini (compare lanes c and d). Joining of protruding 5' termini to blunt termini was observed in the presence of 40 μM dNTPs (Fig. 4.12B, lane d). In contrast, joining was not observed between protruding 3' termini and protruding 5' termini under these conditions (Fig. 4.12C, lane d). All end-joining observed was inhibited by a 1 in 250 dilution of anti-XRCC4 antibodies (data not shown), demonstrating that the ligation of non-complementary termini involved enzymes of the NHEJ pathway.

These data, especially the selective stimulation of non-complementary end-joining by addition of dNTPs, implicate polymerase fill-in activity in the joining of protruding 3' termini to blunt termini and protruding 5' termini to blunt termini. However, sequencing of the junctions would be required to confirm this.

VI. Summary and discussion

The data presented in this chapter demonstrate that a range of non-ligatable and non-complementary termini can be joined by the NHEJ pathway, albeit with widely differing efficiencies. Human cell-free extracts, therefore, not only contain the core NHEJ proteins, but also the supplementary factors required for processing of non-ligatable termini, of which little is currently known.

Figure 4.12. Extract-catalysed joining of non-complementary DNA termini.

Two-plasmid end-joining reactions were set up as described in Materials and Methods (section 2.30.2). Reactions (10 μl) contained, where indicated, uniformly-^{32}P-labelled linear pFB585 plasmid DNA (plasmid 1; 7.7 kb; 20 ng) and uniformly-^{32}P-labelled linear pDEA-7Z DNA (plasmid 2; 3.0kb; 20 ng), incubated with GM00558 extracts (40 μg) in tris end-joining buffer. Where indicated, reactions were supplemented with 40 μM dATP, 40 μM dGTP, 40 μM dCTP and 40 μM dTTP (dNTPs). Following deproteinisation, the products were separated by agarose gel electophoresis and visualised by autoradiography. The mobilities of linear, multimeric and mixed multimeric (mm) DNA species are marked.

(A) Joining of *Kpn*I-linearised plasmid 1 and *Sma*I-linearised plasmid 2.

(B) Joining of *Bsa*I-linearised plasmid 1 and *Sma*I-linearised plasmid 2.

(C) Joining of *Kpn*I-linearised plasmid 1 and *Bsa*I-linearised plasmid 2.

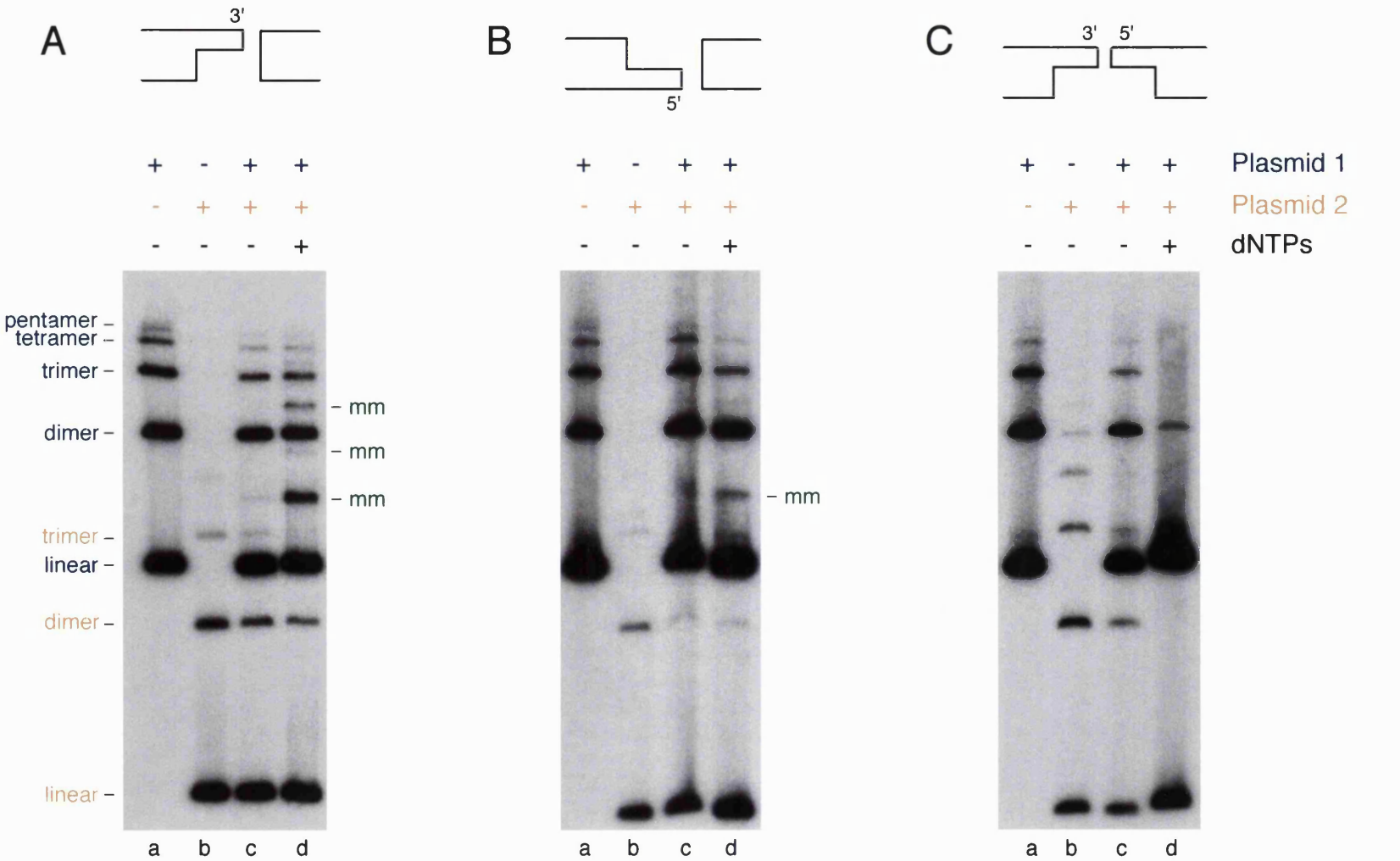

A
3'
B
5'
C
3'
5'
+ - + +
- + + +
- - - +
Plasmid 1
Plasmid 2
dNTPs
pentamer
tetramer
trimer
dimer
trimer
linear
dimer
linear
mm
mm
mm
mm
a b c d
a b c d
a b c d

Figure 4.12

The joining of protruding 5'-OH termini by human cell-free extracts was shown to be highly efficient and accurate, without loss or gain of nucleotides at the junction (Figs. 4.2 and 4.4). In previous *in vitro* end-joining studies, using extracts from various human cells, accurate joining of protruding 5' termini was also observed (Fairman *et al.*, 1992; Bøe *et al.*, 1995; Baumann and West, 1998a). This is the first time, however, that joining of both protruding 5'-P and 5'-OH termini has been shown to be dependent upon the NHEJ proteins. Two possible mechanisms could be used by the cell to accurately replace the hydroxyl groups with phosphate groups, thus permitting subsequent ligation. The first simply involves the activity of a polynucleotide kinase (PNK). The 5'-DNA kinase activity of this enzyme can phosphorylate 5'-OH termini restoring a 5'-P group. Alternatively, the terminal nucleotide carrying the 5'-hydroxyl could be removed and replaced by a nucleotide with a 5'-phosphate. This method would require both exonuclease and DNA polymerase activities. For the joining to remain accurate, it is unlikely that the nucleolytic degradation would extend further than the protruding four nucleotides. Furthermore, due to the 5' to 3' directionality of DNA polymerases, the gap-filling DNA synthesis would have to proceed from an aligned second terminus. Additional investigations into the mechanism for joining of protruding 5'-OH termini are presented in Chapter 5.

Extract-catalysed joining of recessed 5' termini (both 5'-P and 5'-OH termini) exhibited some notable differences from the joining of protruding 5'-P and 5'-OH termini. In comparison to the completely accurate joining of protruding 5' termini, a percentage of recessed 5' termini were joined inaccurately (Figs. 4.4 and 4.8).

Similar observations have been made during the joining of non-complementary termini promoted by extracts prepared from *Xenopus* eggs, mouse cells and human cells (Daza *et al.*, 1996). In these experiments, protruding 3' termini showed significantly higher levels of nucleotide loss than protruding 5' termini. These data either indicate that 3'-5' exonucleases are more prevalent than 5'-3' exonucleases in cell-free extracts, or that there is less efficient protection of protruding 3' termini from nucleolytic degradation. Nevertheless, there appears to be precedence for inaccurate joining of recessed 5' termini whilst protruding 5' termini are accurately rejoined.

Recessed 5'-OH termini were joined significantly slower than recessed 5'-P termini (Fig. 4.6). This is in contrast to protruding 5'-P and 5'-OH termini, which were ligated with similar efficiencies (Fig. 4.2). This could indicate that the recessed termini are less accessible for the 5'-OH modifying enzyme(s). The reduced efficiency of joining of recessed 5'-OH termini may also explain the greater number of inaccurate joints observed. The delay in repair caused by less efficient processing of the recessed 5'-OH group, might permit more exonucleolytic degradation of the termini, resulting in less accurate joining.

Joining of 3'-H and non-complementary termini, which require more complex processing than replacement of a 5'-phosphate, suggests that NHEJ in human cell-free extracts can employ both DNA polymerase and exonuclease activities (Figs. 4.10 and 4.12). The joining of 3'-H termini which requires 3'-5' exonuclease activity appeared to be relatively inefficient, especially in comparison to the rapid repair of 5'-OH termini (Figs. 4.2 and 4.10). Similar rates

of end-joining have been observed, however, with bleomycin-cleaved substrates (Chen *et al.*, 2001b; Pastwa *et al.*, 2001). Strand breaks induced by bleomycin have modified 3'-phosphoglycolate (PG) termini, making them comparable substrates to the 3'-H termini used above. Exonuclease activity is thought to be responsible for removal of the terminal nucleotide containing the 3'-PG group during joining of these substrates (Chen *et al.*, 2001b). Joining of 3'-phosphoglycolate termini by human extracts was found to be considerably slower than the joining of corresponding 3'-OH termini, with only 5% of the 3'-PG substrate joined after 3 hrs (Pastwa *et al.*, 2001).

The stimulation of joining of protruding 3' to blunt termini, and protruding 5' to blunt termini by addition of dNTPs (shown in Fig. 4.12), indicates that DNA synthesis is used in the joining of these non-complementary ends by human extracts. Without additional experiments however, I was not able to determine whether the inability of human extracts to join protruding 3' termini to protruding 5' termini (Fig. 4.12C) was due to limited processivity of the polymerase, or was an artifact of the assay system. Joining of non-complementary termini similar to those used in Fig. 4.12, has been studied previously in extracts from various organisms (Pfeiffer *et al.*, 1994; Wilson and Lieber, 1999; Pospiech *et al.*, 2001). There are conflicting reports, however, as to which DNA polymerase catalyses the fill-in synthesis. Loss of the polymerase β-related Pol4 in budding yeast, led to a 2-fold reduction in the joining of non-complementary termini (Wilson and Lieber, 1999). Moreover, non-complementary end-joining by *Xenopus* egg extracts, was inhibited by ddNTPs, but not aphidicolin, suggesting an

involvement of Polβ in vertebrate end-joining (Pfeiffer *et al.*, 1994). In contrast, V(D)J recombination proceeds normally in Polβ-deficient mouse cells, arguing against the involvement of this polymerase in this specialised form of NHEJ (Esposito *et al.*, 2000).

More recently, it has been suggested that DNA polymerase α acts in NHEJ in human cells (Pospiech *et al.*, 2001). HeLa cell-free extracts were shown to catalyse joining of non-complementary DNA termini in a Ku-, DNA-PK_{cs}- and ATP-dependent manner. Indicative of a requirement for DNA polymerase activity, addition of dNTPs stimulated the joining of non-complementary, but not complementary termini. The joining of mismatched protruding 5' termini was inhibited by addition of aphidicolin and anti-DNA polymerase α antibodies, but not antibodies against DNA polymerases β or ε. In contrast, mismatched protruding 3' termini were joined by an aphidicolin-insensitive activity. The current information as to which DNA polymerase acts during DNA end-joining therefore remains ambiguous. Indeed, it might be possible that different polymerases are used in different organisms, or even for the joining of different substrates.

Requirement for polynucleotide kinase activity during joinlng of protruding 5'-hydroxyl termini

I. Introduction

The preliminary characterisation of end-joining reactions that take place between linear plasmid DNA molecules with 5'-OH termini has been described in the previous chapter. Repair of these termini requires two enzymatic activities. First, the hydroxyl groups at the 5' termini need to be replaced by phosphate groups (end-processing). Second, adjoining 5'-P and 3'-OH termini are ligated (end-joining). Extract-catalysed joining of protruding 5'-OH termini was shown to be efficient, accurate and dependent on the XRCC4 and DNA-PK_{cs} proteins. This indicates that the processing of these termini occurs without alteration to the sequence at the break point, and that the subsequent joining reaction is catalysed by NHEJ proteins. Two mechanisms might account for the processing of 5'-OH termini; either polynucleotide kinase (PNK) activity is responsible for phosphorylation of the termini, or the terminal nucleotide is removed by nuclease activity and replaced by DNA synthesis. Due to the 5' – 3' directionality of DNA polymerases, however, the gap-filling DNA synthesis would catalyse the addition of the replacement nucleotide at the recessed 3'-OH of an aligned terminus.

The experiments described in this chapter were carried out to determine if the two steps of end-processing and end-joining were co-ordinated during repair, or occurred as separate reactions. To do this, plasmid DNA with 5'-OH termini was incubated with human extracts under conditions in which end-joining activity

was blocked, either by use of DNA-PK_{cs}-defective extracts or XRCC4 antisera. The termini were subsequently analysed to establish whether, in the absence of end-joining, phosphate groups were restored to the 5' termini. Additionally, experiments were designed to elucidate whether polynucleotide kinase or nuclease and DNA polymerase activities were responsible for the restoration of 5'-P termini.

The most extensively characterised polynucleotide kinase is from bacteriophage T4. T4 PNK contains two distinct catalytic activities; it is able to phosphorylate 5'-OH termini and dephosphorylate 3'-P termini (Midgley and Murray, 1985). The 5'-kinase activity of T4 PNK phosphorylates DNA and RNA. In comparison, eukaryotic PNKs are divided into those that preferentially phosphorylate DNA or those that act on RNA. The enzymes with 5'-DNA kinase activity are more closely related to T4 PNK, because they also display 3'-phosphatase activity (Karimi-Busheri and Weinfeld, 1997). Mammalian DNA kinases have been isolated from a variety of sources including rat liver, testes and calf thymus (Prinos *et al.*, 1995; Karimi-Busheri and Weinfeld, 1997; Karimi-Busheri *et al.*, 1998; Jilani *et al.*, 1999). In general, most purified mammalian PNKs display similar properties. Optimal kinase activity was observed at pH 5.5 – 6.0, and phosphorylation was seen only with oligonucleotides greater than 8 nucleotides in length. (Karimi-Busheri and Weinfeld, 1997; Jilani *et al.*, 1999). The human *PNK* gene has been cloned and encodes a 521-aa protein with defined ATP binding and 3'-phosphatase domains (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). Following the cloning of *hPNK*, antibodies have been

raised against the human protein. This has enabled the role of human PNK in the joining of 5'-OH termini to be investigated using extracts immunodepleted for PNK.

II. Processing of 5'-OH termini in DNA-PK_{cs}-defective extracts

In vitro assay for detection of repaired 5'-OH termini

To determine if the processing of 5'-OH termini could occur in the absence of end-joining, a new multi-stage assay was established (see Materials and Methods section 2.33 and Fig. 5.1). Essentially, uniformly-^{32}P-labelled linear plasmid DNA with 5'-OH termini was incubated with extracts under conditions where end-joining was inhibited, either by use of anti-XRCC4 antibodies, or DNA-PK_{cs}-defective extracts (Fig. 5.1, column A). The proteins were subsequently removed and the DNA concentrated by ethanol precipitation. To assess the extent of processing of the 5' termini an aliquot of the DNA was incubated with T4 DNA ligase and analysed by agarose gel electrophoresis (Aii). If end-joining-inhibited extracts were still competent for replacement of the 5'-hydroxyl groups with phosphate groups, then the resulting 5'-P termini would be joined by T4 DNA ligase. If the processing of the 5'-OH termini was, however, co-ordinated with end-joining, then ligation products would not be observed.

Several controls were required to validate the results of the T4 ligase reaction. First, after incubation with extracts under conditions of blocked end-joining, the DNA was analysed directly by agarose gel electrophoresis (Ai). This was necessary to establish that all end-joining activity had indeed been inhibited.

Figure 5.1. Analysis of the processing of 5'-hydroxyl termini in the absence of DNA-PK$_{cs}$ or XRCC4.
Schematic representation of experimental procedure used in Figures 5.2 – 5.5.

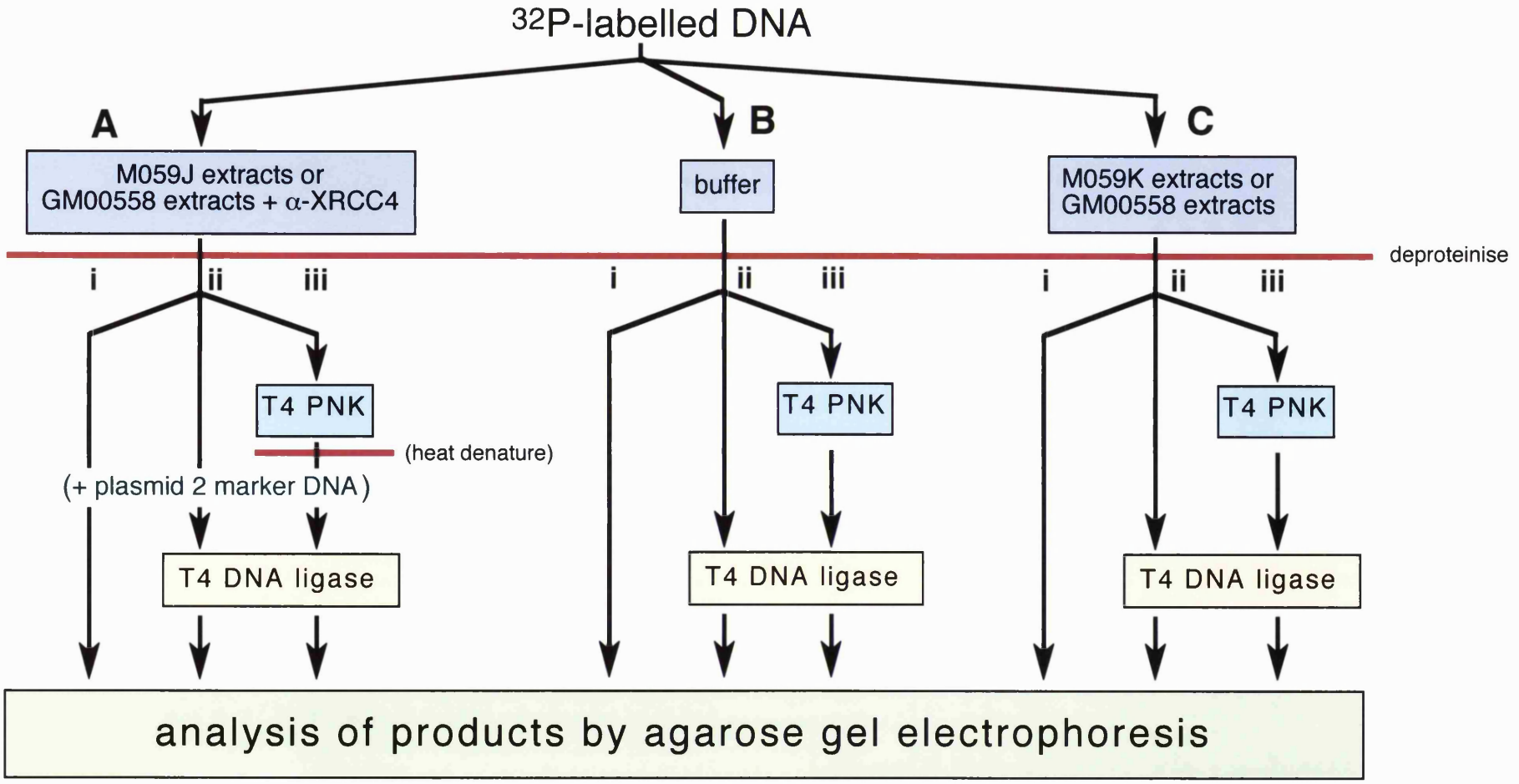

32P-labelled DNA
A
B
C
M059J extracts or
GM00558 extracts + α-XRCC4
buffer
M059K extracts or
GM00558 extracts
deproteinise
i
ii
iii
i
ii
iii
i
ii
iii
T4 PNK
T4 PNK
T4 PNK
(heat denature)
(+ plasmid 2 marker DNA)
T4 DNA ligase
T4 DNA ligase
T4 DNA ligase
analysis of products by agarose gel electrophoresis

Figure 5.1

Second, to determine that no other alteration to the 5'-OH termini had occurred during incubation with the extracts, an aliquot of DNA was first incubated with T4 PNK before being added to the T4 ligation reaction (Aiii). Ordinarily, the DNA was incubated with T4 PNK in the presence of T4 DNA ligase buffer at 37°C. The T4 ligation assay was then simply started by supplementing the reaction with T4 DNA ligase and cooling the reactions to 15°C. Where indicated in the following figures, however, the T4 PNK was heat denatured before the ligation reaction. Finally, in later repetitions of the experiment, smaller, 5'-^{32}P-end-labelled, linear plasmid DNA (plasmid 2 marker DNA) was added to all DNA aliquots to control for non-specific inhibition of the T4 DNA ligase. Such inhibition might occur due to the extensive manipulations of the DNA (especially solvent extraction) required prior to the ligation reaction.

To serve as further controls, the DNA was incubated with buffer alone and aliquots analysed by agarose gel electrophoresis with or without prior incubation with T4 PNK and T4 DNA ligase (see column B). Similar reactions were also carried out with DNA which had been incubated with end-joining proficient GM00558 or M059K extracts (column C).

Analysis of processing of 5'-OH termini in absence of DNA-PK$_{cs}$

As indicated in the schematic diagram (Fig. 5.1, column A), uniformly-^{32}P-labelled linear plasmid DNA with 5'-OH termini was incubated with DNA-PK$_{cs}$-defective M059J extracts and then deproteinised (Fig. 5.2, lanes a – c). Analysis of this DNA by agarose gel electrophoresis, confirmed that M059J extracts are defective

in end-joining, with only linear monomers observed (lane a). Incubation of the DNA with T4 DNA ligase resulted in the production of a few dimers and a few nicked circular products (lane b). The majority of the DNA remained as monomers, however, suggesting that during incubation with M059J extracts, very few hydroxyl groups at the 5' termini were replaced by phosphate groups. In comparison, treatment of the DNA with T4 PNK before the T4 ligation reaction led to the production of many more ligated products (including nicked circular and covalently closed circular products as well as linear dimers and trimers – lane c). These results indicated that very little processing of 5'-OH termini occurred during a two-hour incubation of the DNA with DNA-PK_{cs}-defective extracts. In fact, many of the 5'-OH termini remained unaltered by exposure to M059J extracts, as demonstrated by joining of the DNA by T4 DNA ligase after directed phosphorylation of the termini with T4 PNK. The absence of DNA-PK_{cs} therefore results not only in the inhibition of the NHEJ ligation reaction, but also the earlier end-processing events. This is the first evidence to suggest that during the joining of 5'-OH termini the two stages of end-processing and end-joining are co-ordinated.

The controls using buffer-treated DNA (equivalent to column B in Fig. 5.1) showed that, as expected, 5'-OH termini exposed only to buffer could not be joined by T4 DNA ligase (Fig. 5.2, lane e). However incubation with T4 PNK before the T4 ligation reaction, led to most of the DNA molecules being ligated (lane f). The DNA manipulations required to clean up the DNA following

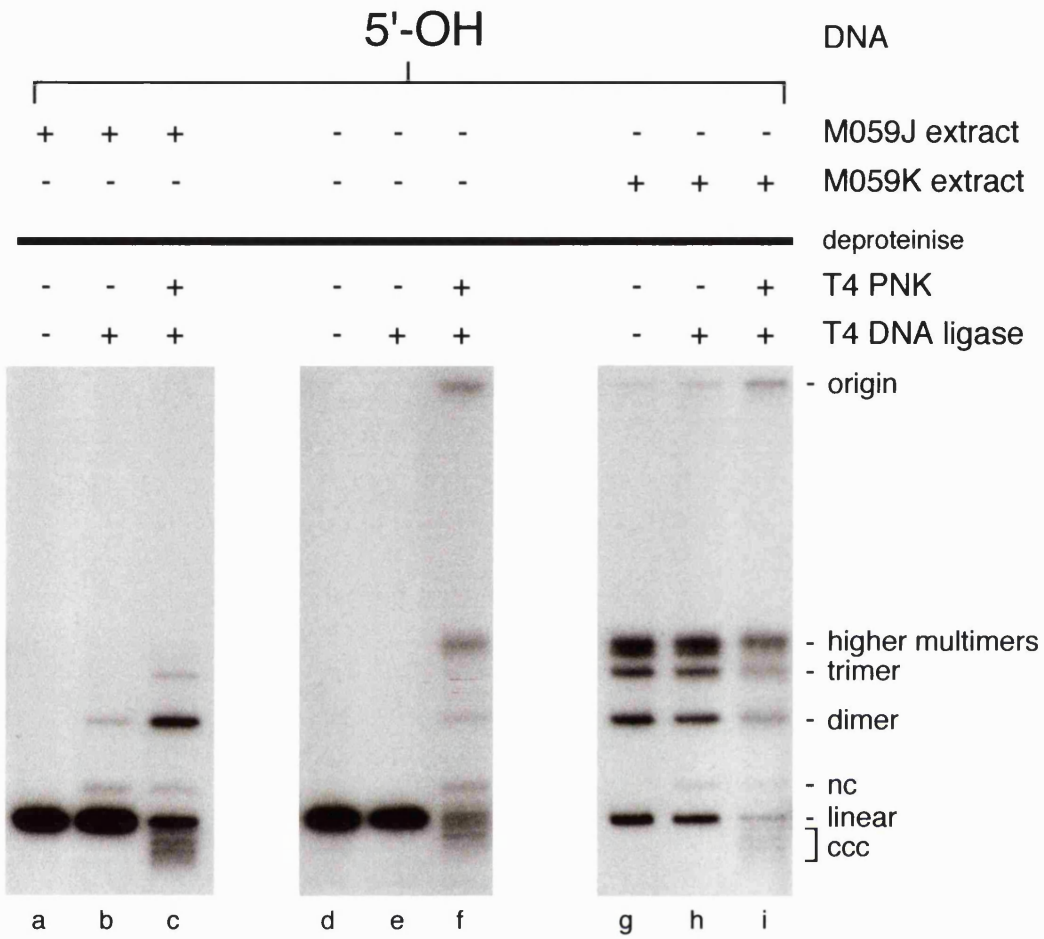


Figure 5.2. Role for DNA-PK$_{cs}$-dependent NHEJ in processing of 5'-hydroxyl termini.

Experiments to determine the extent of processing of 5'-OH termini during various end-joining reactions were carried out as described in Materials and Methods, section 2.33, and in Fig. 5.1. As shown in Fig. 5.1A i - iii, uniformly-^{32}P-labelled *Eco*RI-linearised plasmid DNA with 5'-OH termini (120 ng) was incubated with M059J extracts (240 μg) (lanes a - c). Following deproteinisation, where indicated, aliquots of DNA (40 ng) were incubated with T4 polynucleotide kinase (T4 PNK, 5 U) and T4 DNA ligase (80 U). Uniformly-^{32}P-labelled *Eco*RI-linearised plasmid DNA with 5'-OH termini was also incubated with buffer alone (lanes d - f, corresponding to Fig. 5.1B i - iii) and M059K extracts (lanes g - h, corresponding to Fig 5.1C i - iii). All products were separated by agarose gel electrophoresis and visualised by autoradiography.

incubation with human extracts therefore do not inhibit any subsequent polynucleotide kinase or ligase activities.

As expected, incubation of DNA with 5'-OH termini with the DNA-PK_{cs}-proficient M059K extracts resulted in the production of many linear multimeric products (lane g). Incubation of the products of end-joining with T4 DNA ligase did not result in the generation of many more products (lane h), although a few nicked circular products were observed. These resulted from ligation by T4 DNA ligase, as extract-catalysed end-joining generates only linear products. Incubation of the end-joining products with first T4 PNK, and then T4 DNA ligase resulted in the formation of a few additional products, including covalently closed DNA molecules and some large DNA fragments that remained at the origin of the gel (lane i). The results shown in lanes g and h indicate that when the end-joining reactions are terminated, few DNA molecules are found with non-ligated 5'-P termini. This provides further evidence that the processing and joining of 5'-OH termini occur by a coupled reaction.

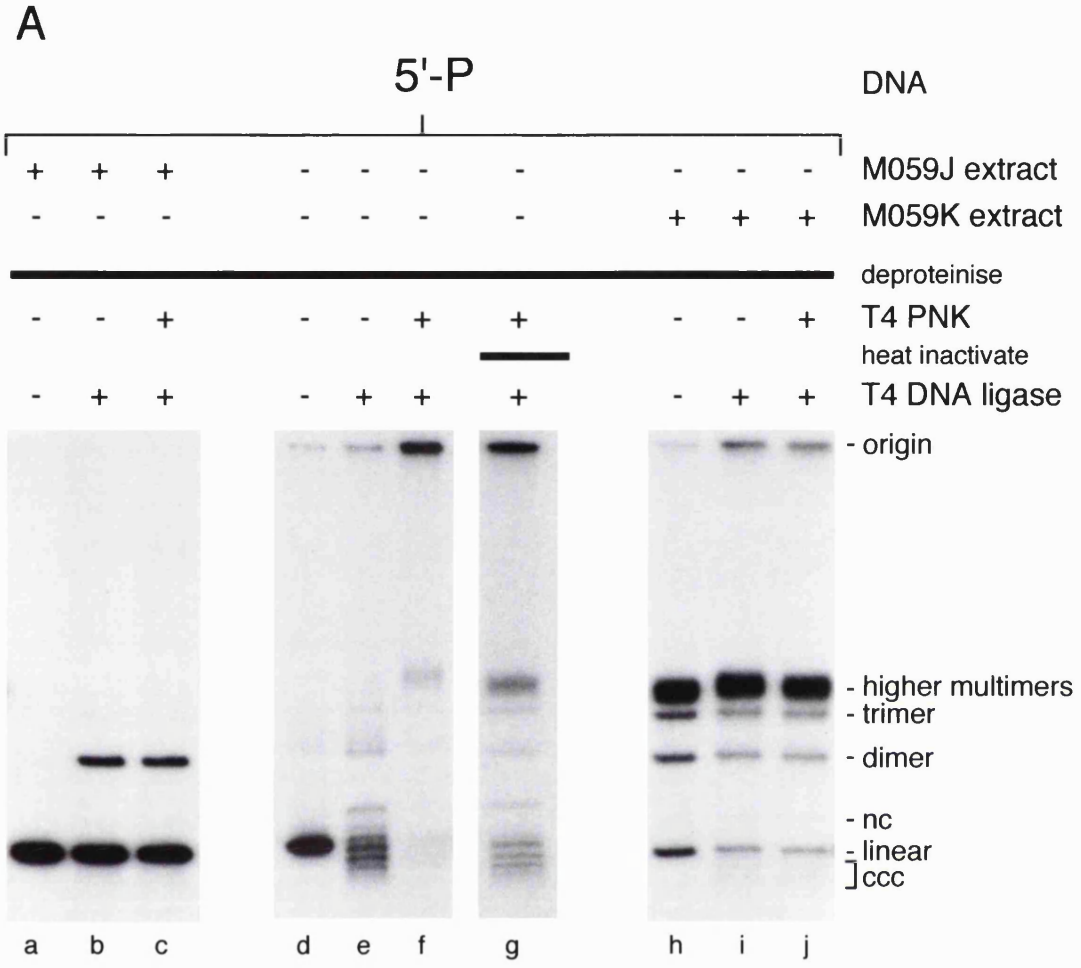
Analysis of processing of 5'-P termini in absence of DNA-PK_{cs}

Similar experiments were conducted with linear plasmid DNA with 5'-P termini (Fig. 5.3A). The results were comparable to those observed for DNA molecules with 5'-OH termini, with a few exceptions. Primarily, DNA with 5'-P termini did not require phosphorylation by T4 PNK to permit ligation by T4 DNA ligase. Indeed, DNA samples incubated with T4 DNA ligase with or without prior incubation with T4 PNK showed the same ligation products (compare lanes b

Figure 5.3. Analysis of integrity of DNA termini following incubation with DNA-PK$_{cs}$-defective extracts.

(A) Experiments similar to those described in Fig. 5.2 were carried out with uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-P termini. Lanes a - c correspond to Fig. 5.1A i - iii, and lanes h - j correspond to Fig. 5.1C i - iii. Lanes d - f equate to Fig. 5.1B i - iii and lane g is a repetition of lane f, with the exception that the T4 PNK was heat inactivated prior to the T4 DNA ligation reaction. All samples were analysed by agarose gel electrophoresis.

(B) Quantification of results shown in Fig. 5.2 and 5.3(A). (■) 5'-OH termini, (□) 5'-P termini. Joined products are expressed as the percentage of DNA found in circular and/or multimeric forms out of the total DNA, following incubation with M059J extracts, M059K extracts, buffer alone (mock ej), T4 DNA ligase (lig) or T4 PNK (PNK). Results are an average of at least three separate experiments, with the standard deviation represented by error bars.


A
5'-P
DNA
M059J extract
M059K extract
deproteinise
T4 PNK
heat inactivate
T4 DNA ligase
origin
higher multimers
trimer
dimer
nc
linear
ccc
a b c d e f g h i j

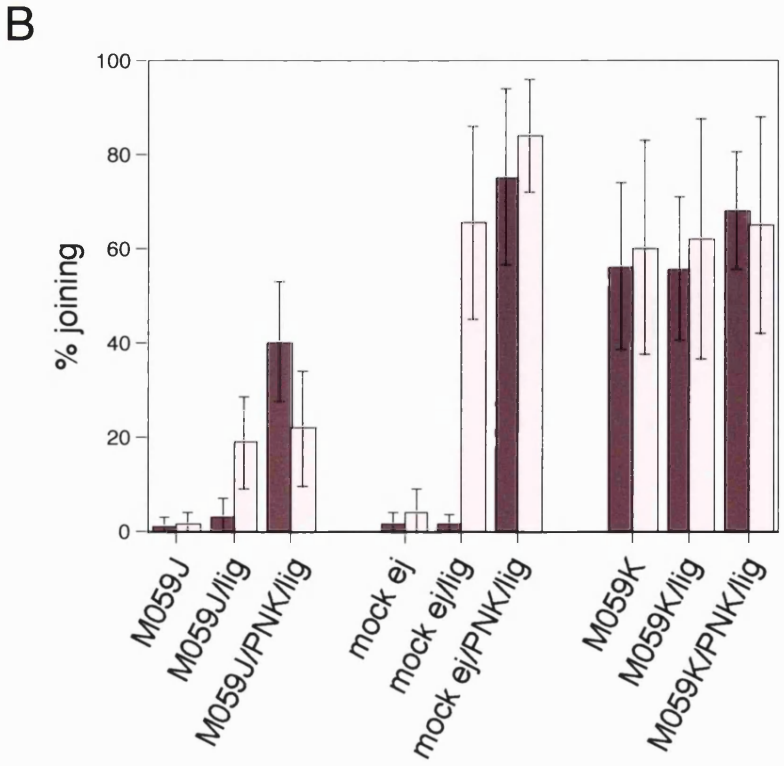

B
% joining
100
80
60
40
20
0
M059J
M059J/lig
M059J/PNK/lig
mock ej
mock ej/lig
mock ej/PNK/lig
M059K
M059K/lig
M059K/PNK/lig

Figure 5.3

and c, and i and j). When linear DNA with 5'-P termini was incubated first with buffer alone and then T4 PNK and T4 DNA ligase, a large percentage of the DNA was observed in very large multimeric complexes that failed to enter the gel (lane f). This was, however, shown to be an artifact of the presence of T4 PNK in the ligase reaction, because heat denaturation of T4 PNK, restored the normal pattern of ligation products (lane g).

Modification of DNA termini in DNA-PK_{cs}-defective extracts

The results of three independent repetitions of the above experiments were quantified and are represented as a bar chart in Fig. 5.3B. Graphical representation of the data serves to emphasis a number of points: -

i) In the absence of DNA-PK_{cs} very few 5'-OH termini were converted to 5'-P termini (maroon bar, M059J/lig).

ii) Many of the 5'-OH termini remain unaltered after incubation with M059J extracts (40% of the termini are joined by sequential incubation with T4 PNK and T4 DNA ligase – maroon bar, M059J/PNK/lig).

iii) Fewer DNA molecules are joined after incubation with T4 PNK and T4 DNA ligase when the DNA is initially incubated with M059J extracts (maroon and pink bars, M059J/PNK/lig) in comparison to when the DNA is incubated with buffer alone (mock ej/PNK/lig).

The results described in point iii) may suggest that a percentage of the DNA termini, both 5'-OH and 5'-P, are modified in some way during incubation with the DNA-PK_{cs}-defective extract, such that they become non-ligatable. Alternatively,

the T4 DNA ligase may be inhibited by a molecule carried over from the extracts that is not present in the buffer control. To investigate further, the experiments were repeated with addition of 5'-^{32}P-end-labelled marker DNA with 5'-P termini (Fig. 5.4). The positions of linear marker DNA (plasmid 2) and the linear M059J-treated DNA (plasmid 1) are shown in lanes a and e. In brief, the linear M059J-treated DNA shows the same ligation pattern as seen previously (compare Fig. 5.2, lanes a – c with Fig. 5.4, lanes a – c and Fig. 5.3, lanes a – c with Fig. 5.4, lanes e – g). In contrast, incubation of linear plasmid 2 marker DNA with T4 DNA ligase always resulted in efficient joining of this smaller plasmid DNA (Fig. 5.4, lanes b, c, f and g). This demonstrates that the inefficient joining of the M059J-treated DNA can not be attributed to inhibition of T4 DNA ligase activity.

In summary, after incubation with DNA-PK$_{cs}$-defective extracts, of the original 5'-OH termini, a few percent are converted to 5'-P termini, around 40% remain as 5'-OH termini, and the rest are modified, such that they contain neither 5'-OH or 5'-P terminal groups. Most importantly these results show that the absence of DNA-PK$_{cs}$ prevents both the processing of 5'-OH termini and the ligation reaction.

III. Processing of 5'-OH termini inhibited by anti-XRCC4 antibodies

DNA-PK$_{cs}$ has been implicated in acting in the early stages of NHEJ, helping to keep broken DNA ends in close proximity. In contrast, the XRCC4/DNA ligase IV complex is required later for joining of the termini. Since the processing of 5'-OH

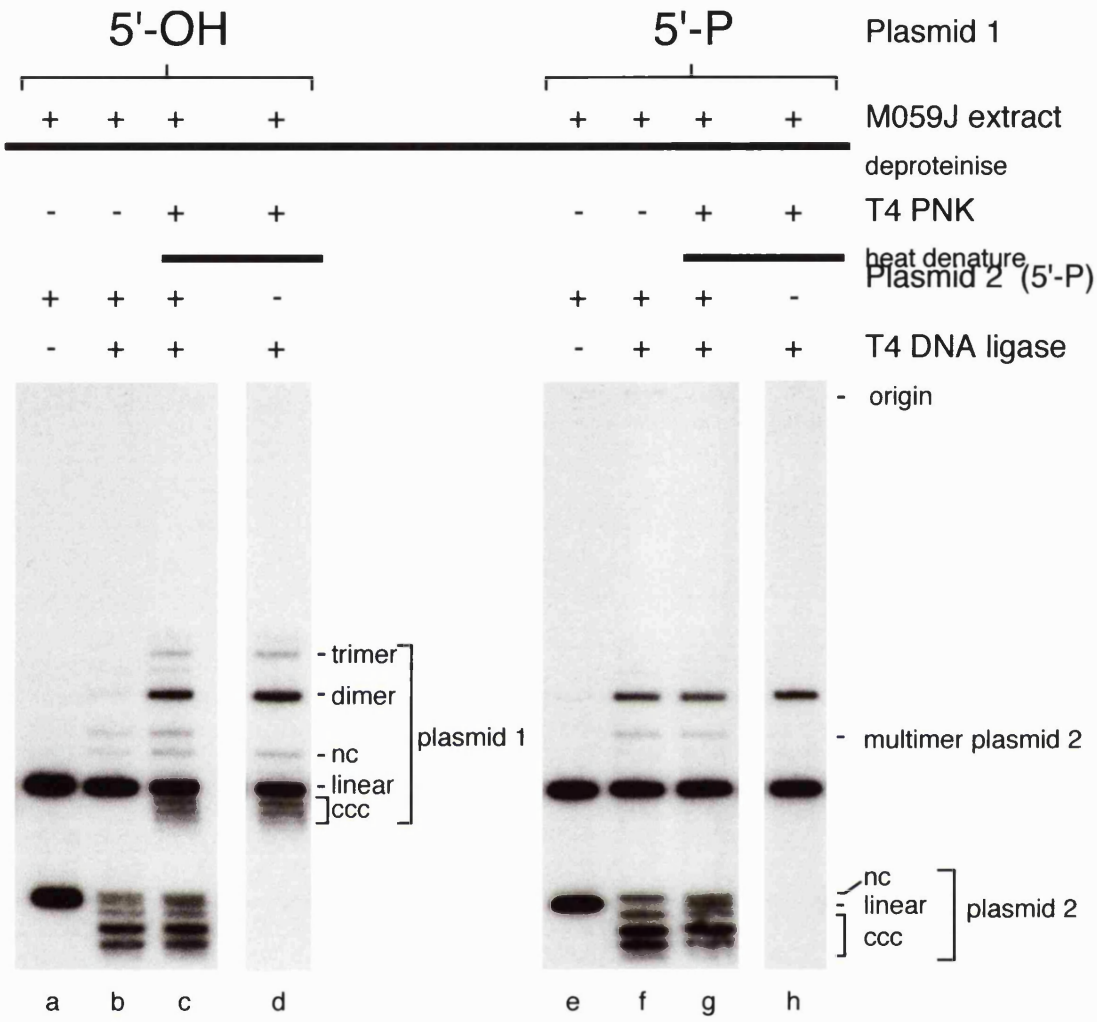


Figure 5.4. Investigation of non-specific inhibition of T4 DNA ligase. End-joining reactions were performed as described in Materials and Methods, section 2.33, and Fig. 5.1A. Uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA (160 ng) either with 5'-P or 5'-OH termini as indicated, was incubated with M059J extracts (320 μg). Following deproteinisation, where indicated, aliquots of DNA (40 ng) were incubated with T4 PNK (5 U), supplemented with 5'-^{32}P-end-labelled linear plasmid DNA (plasmid 2) and/or incubated with T4 DNA ligase (80 U). All products were visualised by agarose gel electrophoresis and autoradiography.

termini was found to be dependent on DNA-PK_{cs}, it was of interest to determine whether this processing could occur in the absence of the XRCC4/DNA ligase IV complex. To do this, similar reactions were carried out except the linear plasmid DNA was initially incubated with GM00558 extracts, either in the presence or absence of anti-XRCC4 antibodies. In general, the same results were seen as for the M059K and M059J extracts, however only the results for the plasmid DNA with 5'-OH termini are shown (Fig. 5.5). Incubation of linear plasmid DNA with 5'-OH termini with extract in the presence of anti-XRCC4 antibodies, resulted in the DNA remaining as monomers (Fig. 5.5A, lane a). If this DNA was then incubated with T4 DNA ligase, a few ligated products were observed (lane b), whereas prior phosphorylation of the DNA by T4 PNK resulted in substantially more ligation products (lane c). When the linear plasmid DNA with 5'-OH termini was incubated with the extract without XRCC4 antisera, then efficient end-joining was observed and no further products were formed by subsequent treatment of the DNA with T4 PNK or T4 DNA ligase (compare lanes d – f).

To control for non-specific inhibition of the T4 DNA ligase, the same reactions as seen in Fig 5.5A (lanes a – c) were carried out in the presence of the smaller, plasmid 2 marker DNA (Fig 5.5B, lanes a – c). In all reactions containing T4 DNA ligase, the smaller marker DNA was efficiently joined irrespective of the efficiency of joining of the larger, plasmid 1 DNA (lanes b and c). Lane c has been duplicated without the plasmid 2 marker DNA to allow identification of each band (lane d).

Figure 5.5. Effect of XRCC4-inhibition on processing of 5'-hydroxyi termini.

(A) End-joining reactions were carried out as described in Materials and Methods, section 2.33 and Fig. 5.1. Corresponding to Fig. 5.1Ai – iii, uniformly-^{32}P-labelled, *Eco*RI-linearised DNA with 5'-OH termini (120 ng) was incubated with GM00558 extracts (240 μg) in the presence of XRCC4 antisera (α-XRCC4; 1:200 dilution; lanes a – c). Following deproteinisation, where indicated, aliquots of DNA (40 ng) were incubated with T4 PNK (5 U) and T4 DNA ligase (80 U). Lanes d – e, corresponding to Fig. 5.1Ci – iii, contained uniformly-^{32}P-labelled, *Eco*RI-linearised DNA with 5'-OH termini incubated with GM00558 extracts, and where indicated, T4 PNK and T4 DNA ligase. All samples were analysed by agarose gel electrophoresis.

(B) A large scale end-joining reaction was carried out, essentially as described in (A), containing uniformly-^{32}P-labelled, *Eco*RI-linearised DNA with 5'-OH termini (160 ng), GM00558 extracts (320 μg) and XRCC4 antisera (1:200 dilution). Following deproteinisation, where indicated, aliquots of DNA (40 ng) were incubated with T4 PNK (5 U) and T4 DNA ligase (80 U) in the presence (+) or absence (-) of 5'-^{32}P-end-labelled linear plasmid DNA (plasmid 2). All products were separated by agarose gel electrophoresis and visualised by autoradiography.

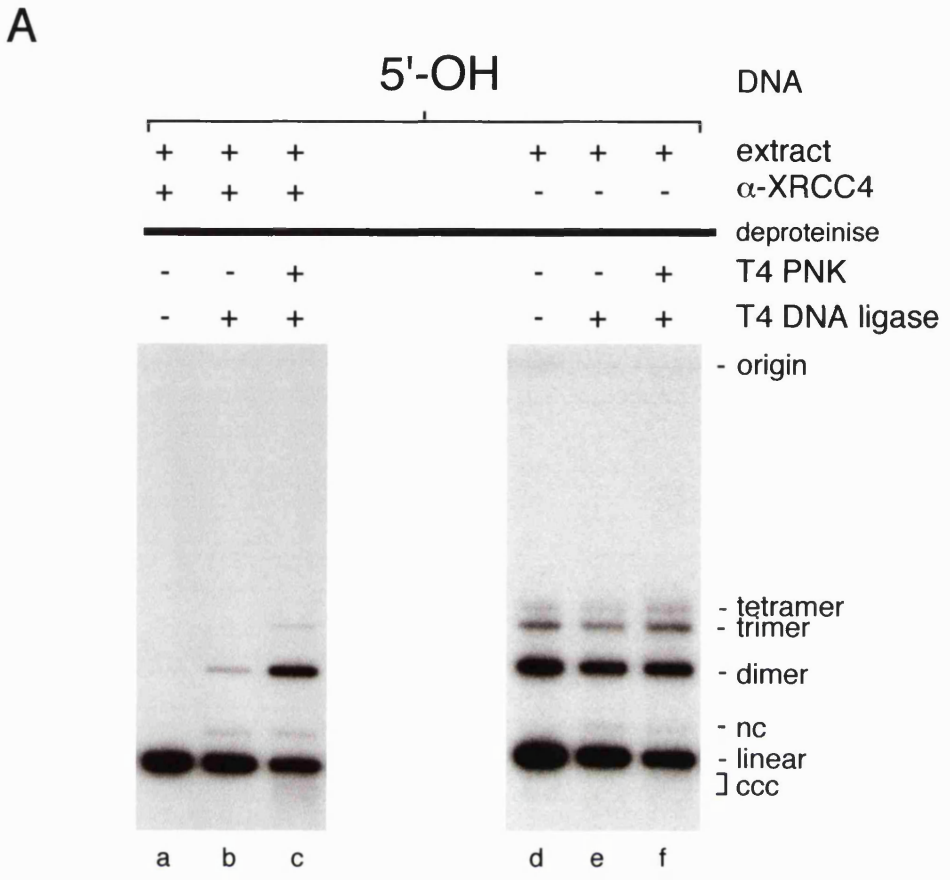

A
5'-OH
DNA
+ + + + + + extract
+ + + - - - α-XRCC4
deproteinise
- - + - - + T4 PNK
- + + - + + T4 DNA ligase
- origin
- tetramer
- trimer
- dimer
- nc
- linear
] ccc
a b c d e f
B
- - + + T4 PNK
heat denature
+ + + - Plasmid 2 (5'-P)
- + + + T4 DNA ligase
- origin
higher multimers
plasmid 2
linear -
ccc
- trimer
- dimer
- nc
- linear
] ccc
plasmid 1
a b c d

Figure 5.5

In conclusion, very little processing of the 5'-OH termini occurs in the absence of either DNA-PK_{cs} or XRCC4. When NHEJ is inhibited some termini are subject to modifications that leave them refractory to joining by T4 DNA ligase. Many DNA molecules simply remain unaltered, such that they can be phosphorylated by T4 PNK and subsequently joined by T4 DNA ligase. These results are highly significant because they indicate that enzymatic processing of 5'-OH termini is co-ordinated with XRCC4- and DNA-PK_{cs}-catalysed end-joining. The end-processing mechanisms are, therefore, likely to be an integral part of the end-joining mechanism.

IV. Requirement for PNK activity for joining of 5'-OH termini by the NHEJ proteins

Immunodepletion of PNK

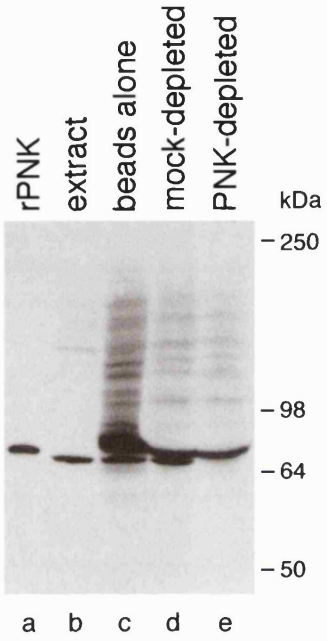
Human polynucleotide kinase phosphorylates 5'-OH DNA termini and dephosphorylates 3'-P termini. (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). To determine if PNK activity is required for extract-catalysed joining of 5'-OH termini, antibodies raised against human PNK (Karimi-Busheri *et al.*, 1999) were used to immunodeplete PNK from cell-free extracts. As shown in Fig. 5.6A, PNK is easily identifiable by Western analysis of human cell-free extracts (lane b). PNK is no longer observed, however, after incubation of extracts with PNK antisera and protein A sepharose beads (lane e). The higher molecular weight band seen in lane e, is caused by non-specific cross reaction of the blotting antibody with the protein A beads, as verified in lanes c and d, in which the

Figure 5.6. Effect of immunodepletion of PNK on efficiency of joining of 5'-hydroxyl termini.

(A) Western analysis of PNK- and mock-depleted extracts. Samples of GM00558 extracts (100 μg) cleared with, where indicated, beads alone; anti-RAD51 antibodies (mock-depleted); or anti-PNK antibodies (PNK-depleted) were analysed by SDS-PAGE and Western blotting with monoclonal anti-PNK antibodies. Also shown, a sample of recombinant, his-tagged PNK (rPNK, 15 ng) and untreated GM00558 extracts (85 μg).

(B) Joining of 5'-hydroxyl termini by PNK- and mock-depleted extracts. DNA end-joining reactions containing uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-OH termini (20 ng) incubated with the indicated amount of PNK- or mock-depleted extracts, were performed as described in Materials and Methods (section 2.30.1). The reaction products were separated by agarose gel electrophoresis and the ^{32}P-labelled DNA detected by autoradiography.

A


rPNK
extract
beads alone
mock-depleted
PNK-depleted
kDa
250
98
64
50
a b c d e

B

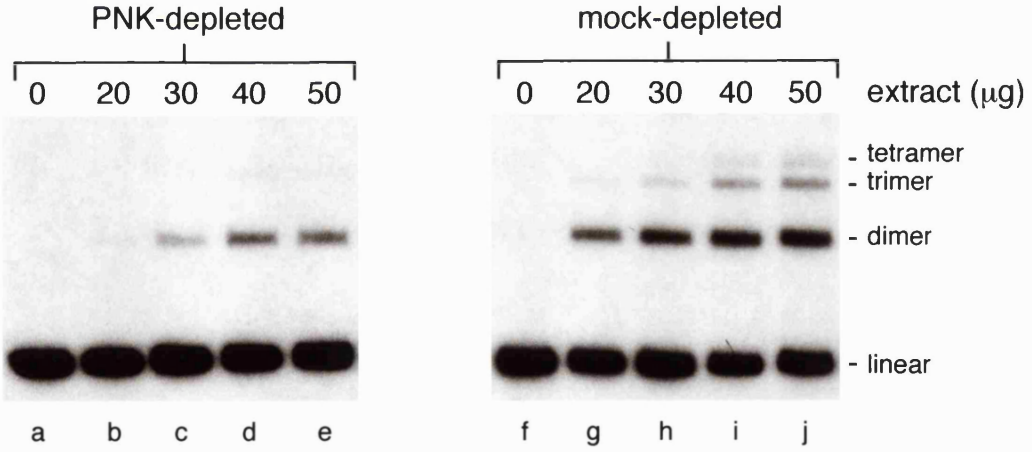

PNK-depleted
0 20 30 40 50
mock-depleted
0 20 30 40 50
extract (μg)
tetramer
trimer
dimer
linear
a b c d e
f g h i j

Figure 5.6

extracts have been immunodepleted with beads alone, or anti-RAD51 antibodies (mock-depleted), respectively.

The PNK-depleted extracts were tested for their ability to join 5'-OH termini (Fig. 5.6B). Using increasing concentrations of extract, few products were observed (lanes b – e). At the highest point of the titration (50 μg), only 7% of the termini were joined. In comparison, mock-depleted extracts efficiently joined 5'-OH termini (lanes g – j), with 30% joining observed in the presence of 50 μg of extract. To determine if the PNK-depleted extracts were defective in end-joining in general, or specifically the joining of 5'-OH termini, end-joining reactions were carried out using DNA with 5'-P termini (Fig. 5.7A). In contrast to the results for 5'-OH termini, PNK-depleted extracts were competent for joining 5'-P termini (lanes c – e). The end-joining activity was, however, somewhat reduced in comparison to that observed with mock-depleted extracts (compare lanes b – d and g – j).

The results of three independent repetitions of the above experiments were quantified and are shown in Fig. 5.7B. Graphical representation of the results highlights the deficiency in joining of 5'-OH termini by PNK-depleted extracts. Across the titration range, a 2.7- to 5.5-fold decrease in end-joining was observed with the PNK-depleted extracts compared to the mock-depleted extracts. The reduction in the efficiency of joining of 5'-P termini by the PNK-depleted extract was less pronounced. The PNK-depleted extracts generally displayed a 2-fold reduction in joining of 5'-P termini in comparison to the mock-depleted extracts. In conclusion, the PNK-depleted extracts displayed a reduced

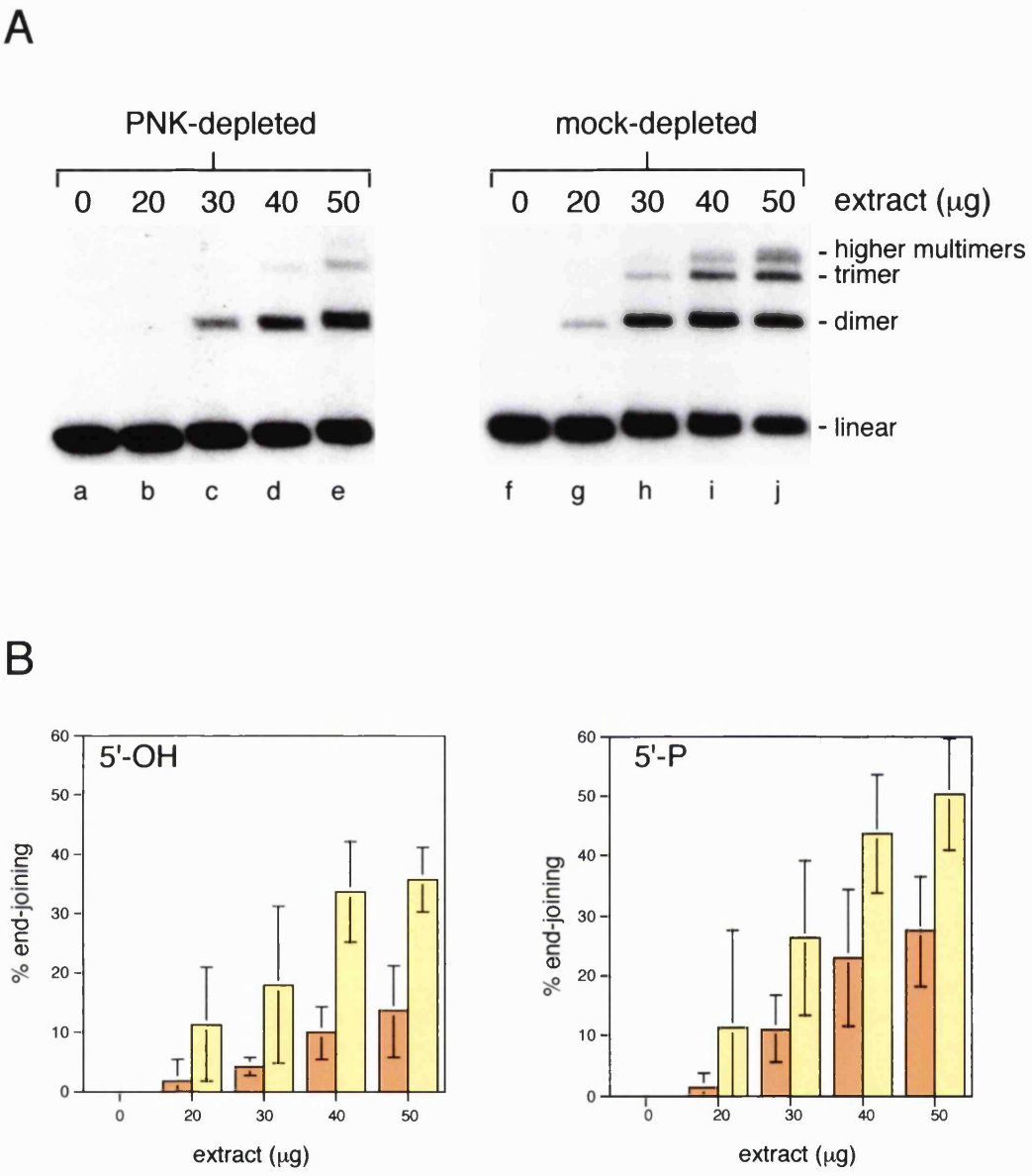


Figure 5.7. End-joining activity of PNK-depleted extracts.
(A) Joining of 5'-P termini. End-joining reactions carried out as described in Fig. 5.6B, except the reactions contained uniformly-^{32}P-labelled, *Eco*RI-linearised DNA with 5'-P termini. Products were separated by agarose gel electrophoresis and visualised by autoradiography.
(B) Quantification of end-joining with PNK-depleted (■) and mock-depleted (□) extracts. The results are an average of at least three independent experiments with the standard deviation represented as error bars.

ability to join 5'-OH termini. This indicates that during end-joining, PNK is responsible for phosphorylation of these termini. It was also interesting to note that PNK-depleted extracts were proficient for joining 5'-P termini (for which there is no requirement for PNK catalytic activity), but still displayed lower end-joining activity than the mock-depleted extracts. This may indicate that PNK has an additional role in the normal DNA end-joining reaction.

Complementation with recombinant PNK

To verify that the deficiency in end-joining described above was caused by the absence of PNK, PNK-depleted extracts were complemented with recombinant PNK protein (Karimi-Busheri *et al.*, 1999). End-joining reactions were carried out using 50 μg of depleted extracts, supplemented with the indicated amount of PNK protein (Fig. 5.8). Addition of between 5 and 25 ng of recombinant PNK protein resulted in stimulation of joining of 5'-OH termini (lanes a – d). Significantly, estimation of the amount of PNK protein found in normal cell-free extracts (based on the Western blot shown in Fig. 5.6A) suggested that 50 μg of cell-free extract would contain around 9 ng of PNK. It would appear, therefore, that addition of physiological amounts of PNK to PNK-depleted extracts complemented the deficiency in joining of 5'-OH termini. Addition of corresponding amounts of PNK protein to reactions containing mock-depleted extracts had no stimulatory affect (lanes g – j). The end-joining activities of both PNK- and mock-depleted extracts were stimulated, however, by addition of excessive amounts of PNK protein (lanes e, f, k and l).

Figure 5.8. Role of PNK in extract-catalysed DNA end-joining.

(A) End-joining reactions were carried out as described in Materials and Methods (section 2.30.1). Reactions (10 μl) contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-OH termini (20 ng) incubated with PNK- or mock-depleted extracts (50 μg) and supplemented with the indicated amount of recombinant human PNK. Products were analysed by agarose gel electrophoresis and autoradiography.

(B) End-joining reactions were set up as in (A) except uniformly-^{32}P-labelled plasmid DNA with 5'-P termini (20 ng) was used.

(C) Quantification of results for (■) PNK-depleted and (□) mock-depleted extracts.

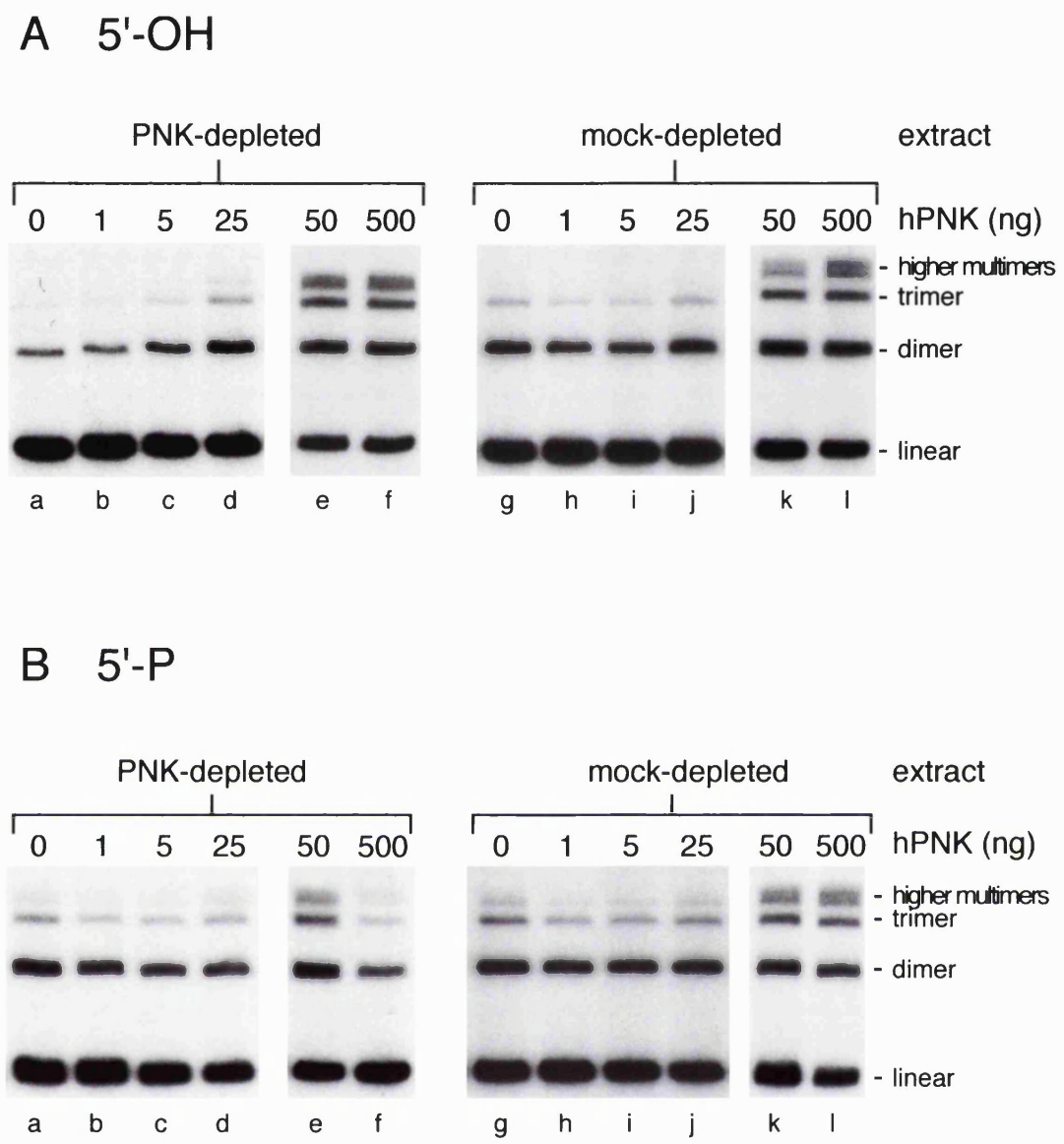

A 5'-OH
PNK-depleted
mock-depleted
extract
0 1 5 25 50 500
0 1 5 25 50 500
hPNK (ng)
- higher multimers
- trimer
- dimer
- linear
a b c d e f
g h i j k l
B 5'-P
PNK-depleted
mock-depleted
extract
0 1 5 25 50 500
0 1 5 25 50 500
hPNK (ng)
- higher multimers
- trimer
- dimer
- linear
a b c d e f
g h i j k l
C
5'-OH
% end-joining
60
50
40
30
20
10
0
0 1 5 25 50 500
PNK (ng)
5'-P
% end-joining
60
50
40
30
20
10
0
0 1 5 25 50 500
PNK (ng)

Figure 5.8

End-joining reactions containing 5'-P termini were also supplemented with recombinant PNK (Fig. 5.8B). In these reactions PNK activity is not required for joining, and consequently addition of between 1 – 25 ng of PNK had no effect on the end-joining activity of either the PNK- or mock- depleted extracts (lanes a – d and g – h). As described above for 5'-OH termini, high amounts of PNK (50 or 500 ng) stimulated the joining of 5'-P termini by both extracts (lanes e, f, k and l). Inexplicably, when the amount of PNK protein added to 5'-P end-joining reactions catalysed by PNK-depleted extracts was increased from 50 ng to 500 ng, this reproducibly led to reduced end-joining activity (see lanes e and f and section C).

In summary, addition of physiological amounts of recombinant PNK protein to end-joining reactions catalysed by PNK-depleted extracts, stimulated the joining of 5'-OH termini but not 5'-P termini (Fig. 5.8C – orange bars). Addition of the same amounts of PNK protein to end-joining reactions catalysed by mock-depleted extracts, had no significant effect, irrespective of the structure of the 5' termini (yellow bars). Most importantly, addition of 25 ng of PNK protein to 5'-OH end-joining reactions restored the end-joining activity of the PNK-depleted extracts to a similar level seen with the mock-depleted extracts. This demonstrates that the inefficient joining of DNA molecules with 5'-OH termini in PNK-depleted extracts is due to a lack of PNK activity. Addition of high amounts of PNK protein stimulated all end-joining reactions. The stimulation of joining of 5'-OH termini by high levels of PNK protein is understandable even in mock-depleted extracts, but why high levels of PNK should stimulate the joining of 5'-P termini is less obvious.

Can T4 PNK substitute for hPNK in DNA end-joining reactions?

To test the specificity of the end-joining reaction, PNK-depleted extracts were supplemented with T4 PNK. Both the human and T4 PNK proteins exhibit 5'-DNA kinase and 3'-phosphatase activities, however they share little homology outside the catalytic domains (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). Addition of T4 PNK to PNK-depleted extracts failed to stimulate the joining of 5'-OH termini (Fig. 5.9A, lanes a – e). In fact, addition of T4 PNK caused a slight decrease in the end-joining activity. This was tested further by studying the effect of T4 PNK on the joining of DNA with 5'-P termini by PNK-depleted extracts (lanes f – j). Here the addition of T4 PNK clearly inhibited NHEJ. Comparison of the end-joining activity of PNK-depleted extracts supplemented with human or T4 PNK is shown in Fig. 5.9B. Whilst human PNK corrected the deficiency of PNK-depleted extracts in joining 5'-OH termini, T4 PNK did not. For joining of 5'-P termini, as described previously, the effect of recombinant human PNK on the activity of PNK-depleted extracts was less pronounced, although some stimulation was seen at high levels. These results indicate that the co-operation between PNK and the NHEJ proteins to achieve efficient joining of 5'-OH termini is species specific, and that T4 PNK is unable to process blocked 5'-OH termini.

V. Potential interactions between PNK and the NHEJ proteins

The results presented in this chapter demonstrate that joining of 5'-OH termini by NHEJ proteins is stimulated by human PNK. Furthermore, the results indicate a

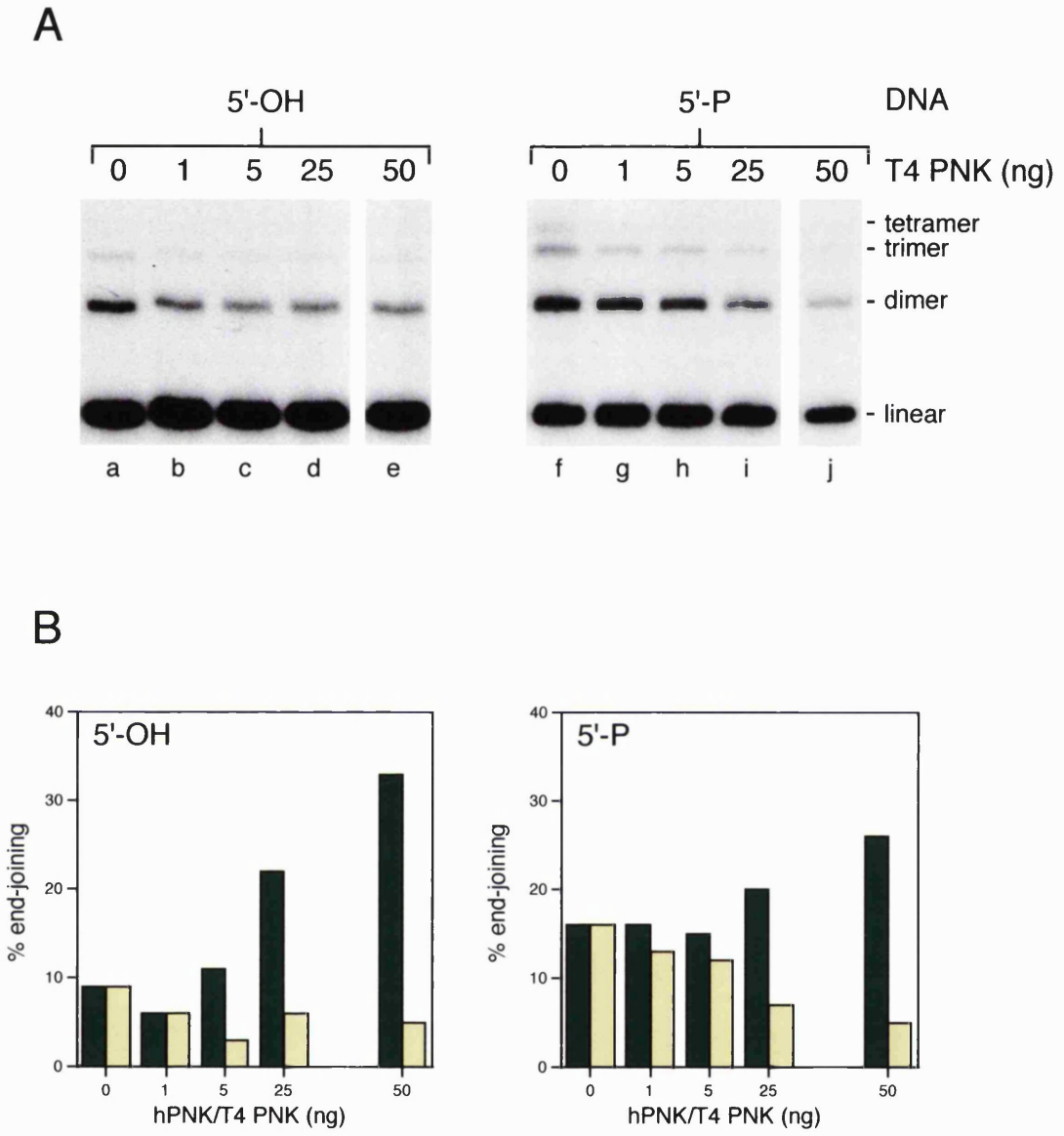


Figure 5.9. Addition T4 PNK to PNK-depleted extracts.

(A) DNA end-joining reactions (10 μl) contained where indicated, uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-P or 5'-OH termini, incubated with PNK-depleted extracts (50 μg) and supplemented with the indicated amount of T4 PNK. Reactions were performed as described in Materials and Methods (sections 2.30.1) and the products analysed by agarose gel electrophoresis and autoradiography.

(B) Comparison of end-joining activity of PNK-depleted extracts supplemented with hPNK (■) or T4 PNK (□).

physical interaction between PNK and the NHEJ proteins. For example, phosphorylation of 5'-OH termini did not occur in the absence of DNA-PK_{cs} or XRCC4, implicating a requirement for one of these NHEJ proteins to target the PNK protein to DNA termini. Additionally, the highly inefficient joining of DNA with 5'-OH termini by PNK-depleted extracts can be stimulated by addition of human PNK, but not T4 PNK. The inability of the DNA kinase activity of T4 PNK to complement PNK-depleted extracts may signify that the bacteriophage protein lacks protein:protein interaction domains, which are required for direction of PNK to DNA termini by the NHEJ proteins.

Coimmunoprecipitations

To investigate the possible interaction of PNK with one of the NHEJ proteins, a series of immunoprecipitations were carried out using normal cell-free extracts. The only NHEJ protein that was precipitated selectively by anti-PNK antibodies and not the beads alone control, was DNA ligase IV (Fig. 5.10A, lanes a and b). The DNA ligase IV immunoprecipitation is included as a positive control (lane c). The presence of ethidium bromide did not disrupt the comimmunoprecipitation of PNK and DNA ligase IV, indicating that the putative association of these two proteins is not mediated by DNA binding. Furthermore DNA ligase IV was not precipitated by antibodies raised against RAD51, another DNA binding protein (lane d). In the reciprocal experiments, however, the PNK protein was not detected in samples immunoprecipitated by anti-DNA ligase IV antibodies (Fig.

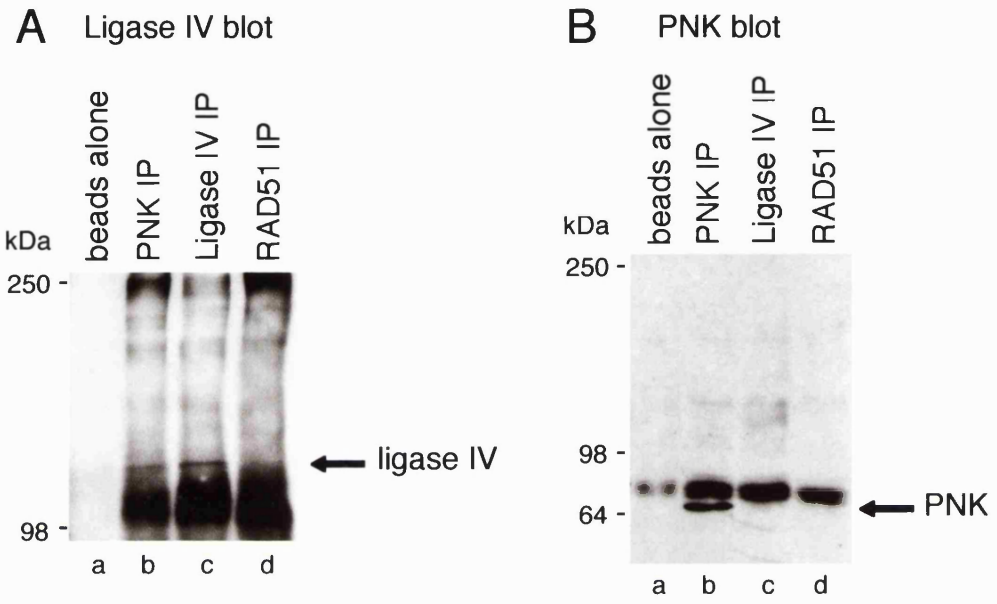


Figure 5.10. Coimmunoprecipitation of PNK and DNA Ligase IV from cell-free extracts.

PNK and DNA ligase IV were immunoprecipitated from cell-free extracts using polyclonal antibodies (PNK IP and Ligase IV IP, respectively) as described in Materials and Methods (section 2.23). Samples of the immunoprecipitates were separated by SDS-PAGE and analysed by Western blotting. Also shown, extracts incubated with Protein A beads in the absence of any antibody (beads alone) or with a polyclonal anti-RAD51 antibody (RAD51 IP).

(A) Western blotting using anti-DNA Ligase IV antibody.

(B) Western blotting of same samples using anti-PNK antibody.

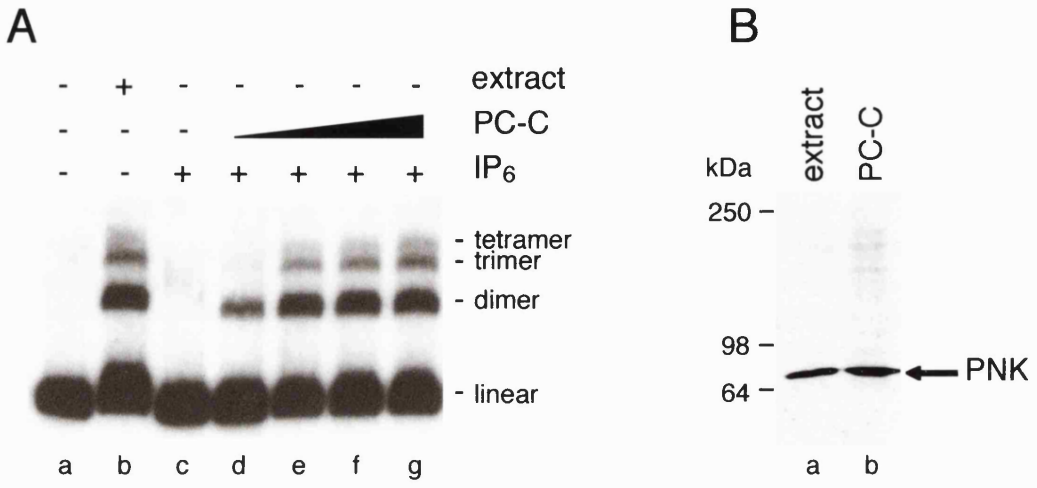
5.10B, lane c), although this could result from the DNA ligase IV antibodies masking the PNK-interaction domain.

The immunoprecipitation of DNA ligase IV by PNK antibodies is suggestive of an association of the two proteins. However, the results of coimmunoprecipitations with other NHEJ proteins proved inconclusive. Additional experiments were therefore required, and gel filtration chromatography was used to investigate whether PNK and the NHEJ proteins formed a multiprotein complex in human extracts.

Gel filtration

Previously, following fractionation of human cell-free extracts over a phosphocellulose column, end-joining activity was reconstituted by mixing samples of the unbound proteins (PC-A) and the proteins eluted at 0.5 M KCl (PC-C; Baumann and West, 1998a). PC-C contained the known NHEJ proteins, and PC-A was shown to contain the stimulatory factor inositol hexakisphosphate (IP_6; Hanakahi *et al.*, 2000). Since PC-C represents a partially purified sample of NHEJ proteins, it was a preferential source of proteins for the gel filtration experiments. First, however, it was important to establish that the PC-C fraction contained all the proteins required for joining 5'-OH termini. As shown in Fig. 5.11A, DNA with 5'-OH termini was joined by PC-C supplemented with IP_6 (lanes d – g). Furthermore, western blotting confirmed the presence of PNK in PC-C (Fig. 5.11B). Concentrated samples of PC-C were therefore separated by gel

Figure 5.11. Gel filtration of phosphocellulose fraction PC-C.
(A) End-joining reactions catalysed by PC-C. Cell-free extracts were fractionated on phosphocellulose as described in Materials and Methods (section 2.25) to generate fraction PC-C. End-joining reactions (10 μl) contained uniformly-^{32}P-labelled, *Eco*RI-linearised plasmid DNA with 5'-OH termini (20 ng) incubated with, where indicated, GM00558 extracts (40 μg), or PC-C (3.6, 5.4, 7.2 or 9 μg) supplemented with inositol hexakisphosphate (IP_6, 1 μM). Following deproteinisation products were separated by agarose gel electrophoresis and visualised by autoradiography.
(B) Western blotting of GM00558 extracts (85 μg total protein) and PC-C (30 μg total protein) with polyclonal PNK antibody.
(C) Gel filtration analysis of recombinant and cellular PNK. Recombinant hPNK (rPNK) was separated on a Superose 12 column as described in Materials and Methods (section 2.26). Fractions were analysed by SDS-PAGE and Western blotting with PNK antisera. Identical gel filtration runs were performed with PC-C under low salt conditions (100 mM KCl, see Materials and Methods section 2.26). The fractions were analysed by blotting with antibodies specific for PNK, DNA ligase IV, Ku70, Ku80 and XRCC4 (Materials and Methods section 2.29). L represents the load sample. The molecular weight standards used were thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa).
(D) Gel filtration of PC-C under moderate salt conditions. Gel filtration of PC-C was also performed as described in (C), with the exception that NaCl was added to the load sample and all buffers to a final concentration of 0.5 M.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PC-C
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trimer
dimer
linear
a b c d e f g
B
kDa
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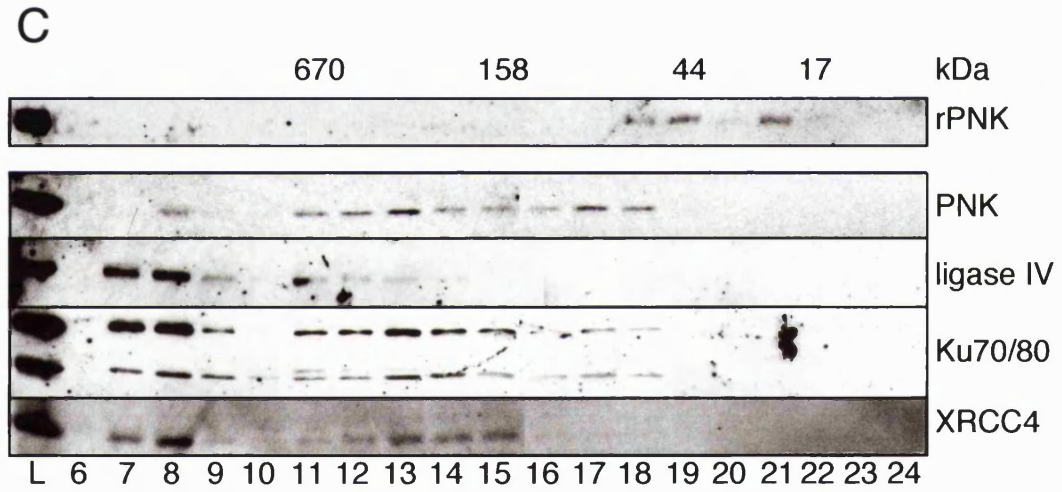
98
64
PNK
a b


C
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rPNK
PNK
ligase IV
Ku70/80
XRCC4
L 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

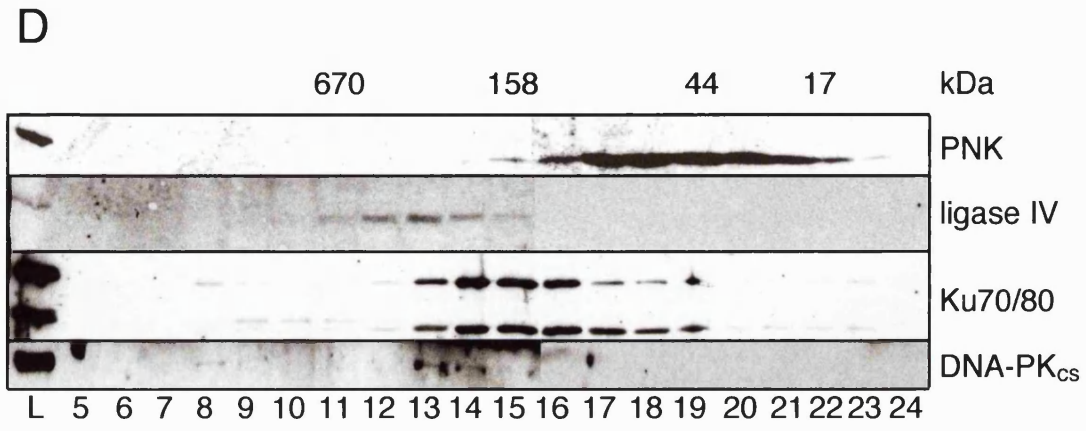
D
670 158 44 17 kDa
PNK
ligase IV
Ku70/80
DNA-PKcs
L 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 5.11

filtration, and the elution profiles of PNK, DNA ligase IV, Ku, DNA-PK_{cs} and XRCC4 determined by Western analysis (Fig. 5.11C and D).

Gel filtration chromatography of recombinant human PNK (rPNK) demonstrated that the purified protein elutes as a monomer (Fig. 5.11 C). In comparison, gel filtration analysis of PC-C revealed that the majority of the endogenous PNK eluted much earlier. This is indicative of the protein being in larger protein complexes. Although it is possible that these complexes might result from self association of the PNK protein, the elution profiles of DNA ligase IV, XRCC4, Ku70 and Ku80 were strikingly similar to that of PNK. All the proteins appeared to elute in two peaks, the first representing a very large complex eluting in the void (> 2 MDa), and the second in complexes with a molecular weight of between 158 and 670 kDa.

The gel filtration of PC-C was repeated under more stringent conditions (0.5 M NaCl) to establish the strength of the protein-protein interactions. Under these conditions, PNK eluted mainly as monomers (Fig. 5.11D). Some of the protein did, however, remain in protein complexes exhibiting a molecular weight of approximately 158 kDa. Western analysis of DNA ligase IV and Ku70 and Ku80, revealed that these proteins also eluted later in the presence of 0.5 M NaCl (Fig. 5.11D). The majority of Ku70 and Ku80 were found as heterodimers (~150 kDa). The DNA ligase IV eluted earlier than the predicted molecular weight of 96 kDa, presumably due to interactions with XRCC4. The DNA ligase IV/XRCC4 complex has been shown to be stable at 1 M salt (Critchlow *et al.*, 1997). Most notably under higher salt conditions the PNK and NHEJ proteins were no longer

observed in a very large complex of around 2 MDa. These results indicate that the postulated interaction of PNK and the NHEJ proteins is sensitive to salt.

IV. Summary and discussion

The data presented in this chapter indicate an important role for PNK in the repair of double strand breaks with 5'-OH termini. In comparison to mock-depleted extracts, PNK-depleted extracts were defective in the joining of linear plasmid DNA with 5'-OH termini (Fig. 5.6). Furthermore, this defect was complemented by addition of physiological amounts of purified PNK (Fig. 5.8). During NHEJ the PNK protein is required to phosphorylate 5'-OH termini, thus permitting subsequent rejoining.

The human PNK protein has recently been shown to act in another DNA repair pathway, single-strand break repair (SSBR; Whitehouse *et al.*, 2001). Key proteins identified as mediating SSBR in mammals include XRCC1, poly (ADP-ribose) polymerase (PARP), DNA polymerase-β (Pol β) and DNA ligase IIIα (reviewed in Thompson and West, 2000; Caldecott, 2001). PARP, an abundant nuclear protein with high affinity for SSBs, is thought to act as an initiator and regulator of SSBR. Pol β and Ligase III are required for filling small gaps in the DNA and ligating nicks, respectively. XRCC1 interacts with all the SSBR proteins, as well as binding directly to gapped and nicked DNA and appears to act as a facilitator or co-ordinator of SSBR.

SSBs caused by ionising radiation display modified DNA termini comparable to those seen in IR-induced DSBs, including 5'-OH and 3'-P termini. Moreover,

analogous to the NHEJ proteins in DSBR, the four established SSBR proteins can repair breaks with normal 5'-P and 3'-OH termini, but are unable to join modified termini (Whitehouse *et al.*, 2001). PNK was found to interact with XRCC1, Polβ and Ligase III in coimmunoprecipitation and yeast two-hybrid analyses (Whitehouse *et al.*, 2001). Furthermore association with XRCC1 stimulated the enzymatic activity of PNK. During *in vitro* reconstitution experiments, the 3'-phosphatase and 5'-kinase activities of PNK catalysed the processing of abnormal 3'-P and 5'-OH termini, permitting subsequent repair by the SSBR proteins. It has been proposed that XRCC1, Ligase III, Pol β and PNK form a multiprotein complex at sites of SSBs allowing the different stages of repair, including processing of damaged termini, DNA polymerisation and ligation, to be co-ordinated.

In view of the precedence set for the involvement of PNK in DNA repair, and the functional interaction between PNK and XRCC1, it was of interest to determine whether PNK associated with any of the NHEJ proteins. Several pieces of indirect evidence suggested an interaction between the NHEJ factors and the PNK protein. First, phosphorylation of 5'-OH termini by PNK did not occur in the absence of either DNA-PK_{cs} or XRCC4 (Figs. 5.2 – 5.5), indicating that the NHEJ proteins might be required for targeting of PNK to DNA termini. Second, the two stages of DNA processing and DNA end-joining appeared to be co-ordinated. During joining of 5'-OH termini by wild type extracts, after a two-hour reaction, very few termini were left with unligated 5'-P termini, even though 5'-OH termini remained (Fig. 5.3B). To confirm the co-ordination of end

processing with end-joining, however, it will be necessary to determine the ratio of 5'-OH to 5'-P termini at a range of earlier time points in the end-joining reaction. Finally, T4 PNK was unable to substitute for human PNK in the end-joining reactions (Fig. 5.9). Although, T4 PNK shares the same enzymatic activities as human PNK, the two proteins display little homology outside of the catalytic domains. T4 PNK may lack a domain critical for interaction with the NHEJ proteins, and is, therefore, not targeted the 5'-OH termini.

To establish direct evidence for an interaction between PNK and the NHEJ proteins, coimmunoprecipitations and gel filtration chromatography were used. DNA ligase IV was precipitated from human extracts with anti-PNK antibodies, suggesting an association between these two proteins (Fig. 5.10A). This interaction was not mediated by DNA binding, because the co-immunoprecipitations were performed in the presence of ethidium bromide. Considering that crude cell-free extracts were used for the immunoprecipitations, however, the association of PNK and DNA ligase IV may potentially be mediated by a third protein, possibly another NHEJ protein. Due to technical difficulties, immunoprecipitation of other NHEJ proteins by PNK antibodies proved inconclusive. Furthermore, in the converse experiments, PNK was not precipitated by anti-DNA ligase IV antibodies (Fig. 5.10B), although the antibodies may mask the PNK-binding domain of DNA ligase IV.

Gel filtration chromatography of concentrated samples of PC-C (a partially purified sample of NHEJ proteins) demonstrated that PNK was found in a very large complex of around 2 MDa as well as in smaller complexes ranging from

below 150 kDa to 670 kDa (Fig. 5.11C). This elution profile was mirrored almost exactly by the Ku heterodimer, and at the higher molecular weights by XRCC4 and DNA ligase IV. Whilst this in itself is not proof of an interaction of PNK with the NHEJ proteins, it does suggest that the majority of PNK is found in large molecular weight complexes. Furthermore, the PNK cannot be complexed with XRCC1 because Western analysis demonstrated that this protein is not present in PC-C (data not shown).

Coelution of PNK and the NHEJ proteins during gel filtration was not observed at 0.5 M NaCl (Fig. 6.11D). In fact, under these conditions the majority of the PNK appeared as monomers. Correspondingly, the immunoprecipitation of DNA ligase IV by PNK was not observed at 0.5 M NaCl (data not shown). These data indicate that the PNK protein weakly associates with DNA ligase IV in cell-free extracts.

Joining of 5'-OH termini was severely inhibited but not completely abolished in PNK-depleted extracts. Several explanations may account for the observed residual end-joining activity. The activity may result from a small amount of PNK remaining in the extract, which is undetectable by Western analysis. Alternatively, although currently only one human *PNK* gene has been cloned, early studies identifying PNK activity in mammalian extracts suggested that a second PNK, distinguished by a neutral pH optimum, may be present in some mammalian tissues (Prinos *et al.*, 1995). The joining may, therefore, be caused by a second PNK present in human extracts. Finally, the residual end-joining might be the consequence of an inefficient and secondary repair activity

observed only in the absence of PNK. This alternative repair mechanism may be catalysed by the NHEJ proteins, but use exonuclease activity to remove the terminal 5'-OH nucleotide. Further experiments to determine the cause of the residual end-joining activity would, therefore, include investigations into the requirement for NHEJ proteins and the accuracy of joining in the absence of PNK.

The requirement for PNK in end-joining reactions containing DNA molecules with 5'-OH termini was anticipated. The reduced joining of 5'-P termini by PNK-depleted extracts was, however, unexpected because there is no necessity for PNK activity in these reactions (Fig. 5.7). Nevertheless, the observed association of PNK and DNA ligase IV may explain these results. If depletion of PNK from human extracts leads to a concomitant reduction in the DNA ligase IV levels, this may cause a reduction in normal end-joining activity. In support of this theory, the end-joining defect for 5'-P termini was not complemented by addition of physiological amounts of PNK (Fig. 5.8). The complementation of this end-joining defect by addition of purified DNA ligase IV has not been investigated and would be required to confirm this hypothesis.

Addition of excessive amounts of PNK stimulated the joining of both 5'-OH and 5'-P termini by either PNK- or mock-depleted extracts (Fig. 5.8). The end-joining reactions were set up on ice, with the components added in the order - buffer, DNA, purified PNK and finally depleted extract. In this respect, the PNK protein may well bind to the DNA first. In view of the association of PNK and DNA ligase IV, the pre-binding of DNA termini by the PNK protein may accelerate

repair by helping the NHEJ proteins to assemble at the DNA termini. This general stimulation of all end-joining reactions irrespective of the structure of the DNA termini, is only observed when high amounts of PNK are used, presumably when most of the DNA termini are bound by PNK. When low amounts of PNK are added, however, only the joining of 5'-OH termini is stimulated for which the PNK kinase activity is required. To confirm that the general stimulation of end-joining activity by PNK is due to targeting of the NHEJ proteins to the DNA termini, a series of experiments would be required in which the order of addition of the end-joining components is varied.

In conclusion, the defective joining of 5'-OH termini exhibited by PNK-depleted extracts and complementation of this defect by addition of recombinant PNK has demonstrated a requirement for PNK 5' kinase activity in the *in vitro* repair of double-strand breaks with 5'-OH termini. Furthermore, immuno-precipitations and gel filtration chromatography have implicated a weak association between PNK and the NHEJ proteins, specifically DNA ligase IV. To determine whether PNK interacts directly with DNA ligase IV or other NHEJ proteins, future investigations might include either yeast two-hybrid analysis or coimmunoprecipitations using purified proteins. Additionally, it would be of interest to see if the purified NHEJ proteins can stimulate the *in vitro* activity of recombinant PNK, similar to that observed with the SSBR proteins.

Chapter 6: General Discussion

The principal aim of this thesis has been to investigate the mechanisms by which cells process modified or non-ligatable DNA termini during non-homologous end-joining.

I. Identification of auxiliary factors which act during NHEJ

Human and rodent cells that contain defective Ku, DNA-PK_{cs}, XRCC4 or DNA ligase IV proteins exhibit hypersensitivity to ionising radiation (Blunt *et al.*, 1995; Lees-Miller *et al.*, 1995; Li *et al.*, 1995; Gu *et al.*, 1997; Singleton *et al.*, 1997; Riballo *et al.*, 1999). Thus in mammalian cells NHEJ is an important mechanism for repairing IR-induced DSBs. Strand breaks resulting from exposure to ionising radiation have modified DNA termini, however, and cannot be joined by a DNA ligase (Friedberg *et al.*, 1995). Nevertheless, in the past much of the research into NHEJ has focused on the four core proteins and it is presently unclear what mechanisms process damaged DNA termini.

To identify the auxiliary factors that act during the joining of altered DNA termini, plasmid DNA was linearised by restriction enzymes and the termini then modified enzymatically such that they were no longer substrates for DNA ligase activity. When tested, human cell-free extracts were able to join DNA molecules carrying terminal dideoxynucleotides. In this repair reaction, for ligation to occur the terminal ddNTP must be removed, presumably through exonuclease activity. Human cell-free extracts also catalysed the repair of bleomycin-induced DSBs (Chen *et al.*, 2001b; Pastwa *et al.*, 2001). These substrates have terminal 3'-

phosphoglycolate groups that must similarly be removed by exonuclease activity before ligation.

Using a two-plasmid assay, human cell-free extracts were also found to join non-complementary termini, which when aligned retained single-stranded gaps. Addition of dNTPs selectively stimulated non-complementary end-joining whilst having no effect on the joining of complementary termini. These results indicate that DNA polymerase activity is used in a subsection of DNA end-joining reactions. Other studies have found similar requirements for DNA synthesis during some end-joining reactions, but there appears to be little agreement as to which DNA polymerase is used in NHEJ (Pfeiffer *et al.*, 1994; Wilson and Lieber, 1999; Pospiech *et al.*, 2001). In *S. cerevisiae,* polymerase β-related Pol4 has been reported to act during the joining of non-complementary termini (Wilson and Lieber, 1999). These results have been strengthened by the recent discovery of a physical interaction between Pol4 and the Lig4/Lif1 complex (A. Tomkinson, unpublished data). Furthermore, polymerase β is thought to be required for similar end-joining reactions catalysed by *Xenopus* egg extracts (Pfeiffer *et al.*, 1994). However, the results obtained using human cell-free extracts are somewhat contradictory. For example, DNA polymerase α was required for the joining of mismatched protruding 5' termini, yet dispensable for the joining of mismatched protruding 3' termini (Pospiech *et al.*, 2001). Further studies are evidently required to resolve this question conclusively.

Above I have argued that my results, taken together with others, indicate a putative role for DNA polymerases and exonucleases during certain NHEJ

reactions. Meanwhile, in a second study I demonstrated that PNK activity is required during end-joining reactions between DNA molecules with 5'-hydroxyl termini. The phosphorylation of 5'-OH termini was severely compromised in the absence of PNK, DNA-PK_{cs} or XRCC4. These results indicate that not only is PNK required to phosphorylate 5'-OH termini pior to rejoining by the DNA-PK, XRCC4 and DNA ligase IV proteins, but that the PNK activity is tightly regulated by the presence of the NHEJ proteins. Indeed, the end-joining defect of PNK-depleted extracts was rescued by addition of human PNK but not T4 PNK, indicating a species-specific requirement for PNK. These results have recently been published (Chappell *et al.*, 2002).

II. Role for PNK in both single-strand and double-strand break repair

In addition to the evidence presented in this thesis which indicates that PNK activity is required for the joining of double-strand breaks with 5'-OH termini, PNK has also been shown to act at single-strand breaks with 5'-OH and 3'-P termini (Whitehouse *et al.*, 2001). The interaction of PNK with the other SSBR proteins XRCC1, DNA polymerase β and DNA ligase III may serve as a method of targeting PNK to sites of ssDNA damage. Moreover, the association of all the SSBR proteins in one multiprotein complex would allow the individual steps of DNA phosphorylation/dephosphorylation, DNA synthesis and ligation to be co-ordinated. I propose that a similar system is used during NHEJ. Purified PNK preferentially acts at ssDNA nicks and phosphorylates ds breaks with low

efficiency (Karimi-Busheri and Weinfeld, 1997). Consequently, there is a need for PNK to be targeted to sites of DSBs. This may be achieved through the putative association between PNK and DNA ligase IV. Furthermore, the different phases of NHEJ have been shown to be tightly co-ordinated, phosphorylation of 5'-OH termini did not occur if the later stages of end-joining were blocked and vice versa. There appears therefore to be a requirement for a functional multiprotein complex at DSBs with 5'-OH termini, which contains PNK and the NHEJ proteins.

The use of the same enzyme by two different DNA repair pathways to process damaged DNA termini is not entirely surprising, especially considering that ionising radiation induces both ss and ds breaks and therefore similar types of DNA damage can occur at nicks and breaks. In addition to roles in SSBR and DSBR, PNK activity has been reported to act during DNA replication, phosphorylating the 5'-termini of Okazaki fragments (Pohjanpelto and Holtta, 1996). Indeed, all the auxiliary factors that are proposed to process damaged DNA termini during NHEJ also function in general DNA metabolism. Consequently, it seems unlikely that these auxiliary factors are constantly associated with the NHEJ proteins. Conceivably, a certain percentage of the total cellular PNK may always be complexed with the NHEJ proteins, whilst another proportion is associated with the SSBR proteins and a third is used for DNA replication. It is more probable, however, that PNK and the other auxiliary enzymes only associate with the repair proteins at sites of DNA damage. Indeed it has been proposed that the core NHEJ and SSBR proteins only assemble into

large repair complexes when bound to DNA (Featherstone and Jackson, 1999a; Caldecott, 2001). Ku and PARP appear to serve as molecular sensors for NHEJ and SSBR respectively, binding rapidly to free DNA ends or nicks (de Murcia and Menissier de Murcia, 1994; Featherstone and Jackson, 1999b). The other repair proteins are then targeted to the DNA by association with Ku or PARP.

III. Limits of *S. cerevisiae* as a model for mammalian NHEJ

There have always been some clear differences between NHEJ in yeast and NHEJ in mammals. Despite the Ku, XRCC4 and DNA ligase IV proteins being conserved amongst eukaryotes, no obvious DNA-PK_{cs} homologue has ever been identified in *S. cerevisiae* (Gottlieb and Jackson, 1993). Furthermore, whilst homologous recombination appears to be the predominant DSB repair mechanism in yeast, in mammals NHEJ is important for repairing IR-induced DNA damage. Nevertheless, results obtained in *S. cerevisiae* have frequently been incorporated into models for mammalian NHEJ. However, I believe that more recent observations only serve to emphasis the divergence between NHEJ in budding yeast and in humans.

In *S. cerevisiae* the Mre11, Rad50 and Xrs2 proteins are required for the *in vivo* joining of naked, linear plasmid substrates with complementary termini (Boulton and Jackson, 1998). The M/R/X complex has also been shown to associate with the Lig4/Lif1 complex *in vitro*, through a direct interaction between Xrs2 and Lif1 (Chen *et al.*, 2001a). Moreover, the M/R/X complex selectively stimulated intermolecular ligation by Lig4/Lif1, and promoted end-to-end

association of linear DNA fragments to form oligomers. These data indicate that the M/R/X complex may act as a bridging factor bringing the ends of linear DNA molecules into close proximity during NHEJ in yeast.

In chicken cells, however, disruption of *MRE11* was found to impair HR but not Ku-dependent end-joining (Yamaguchi-Iwai *et al.*, 1999). Similarly, studies in *S. pombe* have shown that the Mre11 and Rad50 homologues, Rad32 and Rad50, do not act in NHEJ (Manolis *et al.*, 2001). Furthermore, I have shown that in direct opposition to the results from *S. cerevisiae*, in human cell-free extracts the M/R/N complex is not required for the joining of linear plasmid DNA with complementary termini. Although, a more recent study has reported that a fraction containing MRE11, RAD50 and NBS1 was required to stimulate the joining of cohesive ends by purified Ku and XRCC4/DNA ligase IV (Huang and Dynan, 2002). This stimulatory activity copurified with the M/R/N complex during fractionation of HeLa cell-free extracts, and was inhibited somewhat by antibodies raised against MRE11. However, the stimulatory activity of each fraction also correlated with a loss of DNA-PK_{cs}. Consequently, one major difference between my experiments and those of Huang and Dynan is that whereas extract-catalysed end-joining displayed an absolute requirement for DNA-PK_{cs}, this protein was absent from the partially purified stimulatory fractions.

Human MRE11 alone mediates the annealing of complementary ssDNA molecules (de Jager *et al.*, 2001a), and scanning force microscopy has shown that the MRE11/RAD50 complex can promote the association of linear DNA molecules (de Jager *et al.*, 2001b). Thus the end-bridging properties of the

M/R/N complex appear to be conserved between yeast and humans. I propose therefore that in human cells the M/R/N complex stimulates end-joining by acting as an end-bridging factor only in the absence of DNA-PK_{cs}.

One question that then remains is whether the M/R/N complex has a role in DNA-PK_{cs}-dependent NHEJ? Given the exonuclease activity exhibited by MRE11, the M/R/N complex may be involved in the processing of damaged or modified DNA termini. In support of this hypothesis, the exonuclease activity of purified MRE11 was stimulated by mismatched but not complementary termini (Paull and Gellert, 2000). MRE11 is therefore proposed to facilitate the joining of mismatched ends by degrading the DNA until a site of microhomology is revealed. I believe that the use of either bleomycin-cleaved or 3'-H DNA substrates could help determine if MRE11, RAD50 and NBS1 are required for the processing of damaged DNA termini.

There is also an apparent difference between *S. cerevisiae* and humans in the use of polynucleotide kinase during the repair of DNA strand breaks with 5'-OH termini. In human cells there is strong evidence to support a role for PNK in the joining of both ss and ds breaks with 5'-OH termini (Whitehouse *et al.*, 2001; Chappell *et al.*, 2002). Homology searches and biochemical assays have, however, failed to identify an enzyme with 5' DNA kinase activity in *S. cerevisiae*, even though yeast cells competent for joining linear DNA molecules with 5'-OH termini (Vance and Wilson, 2001). Polynucleotide kinases from bacteriophage T4 and humans contain conserved 5'-kinase and 3'-phosphatase domains (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). The coupling of these two domains

allows both the 5'-OH and 3'-P lesions which result from IR-induced damage to be reversed by a single enzyme. Whilst a gene encoding a 3'-phosphatase has been identified in *S. cerevisiae* (designated three prime phosphatase 1 or *TPP1*), the gene does not appear to contain an accompanying 5'-kinase domain (Vance and Wilson, 2001). Furthermore, extracts prepared from budding yeast fail to exhibit 5'-kinase activity. In this respect, the 3'-phosphatase and 5'-kinase activities of PNK appear to be uncoupled in *S. cerevisiae*. Consequently, it has been proposed that the observed repair of plasmids with 5'-OH termini in budding yeast must occur by base removal and resynthesis (Vance and Wilson, 2001).

A homologue of the human polynucleotide kinase, called Pnk1, has been identified in *S. pombe* (Meijer *et al.*, 2002). In contrast to the *S. cerevisiae* Tpp1 protein, recombinant Pnk1 exhibits both 5'-kinase and 3'-phosphatase activities. Moreover, deletion of *PNK1* resulted in significant hypersensitivity to γ-irradiation and the topoisomerase I inhibitor camptothecin, indicating that Pnk1 is required for the repair of strand breaks induced by these DNA damaging agents. Finally, expression of both Pnk1 and human PNK was found to complement the IR-sensitivity of Pnk1-deficient *S. pombe* cells, suggesting that the two proteins have been functionally as well as structurally conserved. With respect to the requirement for PNK activity during the joining of 5'-OH termini, *S. pombe* appears to provide a better model system for human NHEJ than *S. cerevisiae*.

In conclusion, the differences observed between the role of the M/R/N(X) complex and PNK in *S. cerevisiae* and human cells should serve as a caution to

those wishing to extrapolate results obtained in budding yeast onto models for human NHEJ.

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Binding of Inositol Phosphate to DNA-PK and Stimulation of Double-Strand Break Repair

Les A. Hanakahi,* Michael Bartlet-Jones,†
Claire Chappell,* Darryl Pappin,†
and Stephen C. West,*‡
*Imperial Cancer Research Fund
Clare Hall Laboratories
South Mimms
Hertfordshire EN6 3LD
United Kingdom
†Imperial Cancer Research Fund
44 Lincoln's Inn Fields
London WC2A 3PX
United Kingdom

Summary

In mammalian cells, double-strand breaks in DNA can be repaired by *non*homologous end-*joining* (NHEJ), a process dependent upon Ku70/80, DNA-PK$_{cs}$, XRCC4, and DNA ligase IV. Starting with HeLa cell-free extracts, which promote NHEJ in a reaction dependent upon all of these proteins, we have purified a novel factor that stimulates DNA end-joining in vitro. Using a combination of phosphorus NMR, mass spectroscopy, and strong anion exchange chromatography, we identify this factor as inositol hexakisphosphate (IP$_6$). Purified IP$_6$ is bound by DNA-PK and specifically stimulates DNA-PK-dependent end-joining in vitro. The involvement of inositol phosphate in DNA-PK-dependent NHEJ is of particular interest since the catalytic domain of DNA-PK$_{cs}$ is similar to that found in the phosphatidylinositol 3 (PI 3)-kinase family.

Introduction

The repair of double-strand breaks (DSBs) in DNA is essential for the maintenance of genomic stability. Failure to repair DSBs can result in the loss of genetic information, chromosomal translocations, and cell death. Two mechanisms for the repair of DSBs have been described, involving either homologous recombination (HR) or nonhomologous end-joining (NHEJ). Homologous recombination is particularly effective in S phase when the break can be repaired using genetic information from a sister chromatid, whereas NHEJ is thought to be effective at all times in the cell cycle (Takata et al., 1998; Essers et al., 2000). NHEJ also plays an important role in DSB repair during V(D)J recombination (Taccioli et al., 1993; Blunt et al., 1995).

The repair of double-strand breaks by nonhomologous end-joining requires the products of the *XRCC4*, *XRCC5*, *XRCC6*, and *XRCC7* genes (reviewed by Weaver, 1996; Chu, 1997; Critchlow and Jackson, 1998). *XRCC4* encodes a protein (XRCC4) that forms a heterodimer with DNA ligase IV, *XRCC5* and *XRCC6* encode the 70 and 80 kDa subunits of the DNA end binding protein Ku, and *XRCC7* encodes the catalytic subunit of the DNA-dependent protein kinase DNA-PK$_{cs}$. Although the precise targets of DNA-PK$_{cs}$ are unknown, it has been shown to phosphorylate XRCC4 in vitro and to modulate its DNA binding activity (Critchlow et al., 1997; Leber et al., 1998; Modesti et al., 1999).

DNA-PK$_{cs}$ is a large protein (~3500 amino acids, M_w ~465 kDa) (Smith and Jackson, 1999), the carboxyl terminus of which contains a catalytic domain that is related to that found in the *p*hosphatidyl*i*nositol *3* (PI 3)-kinase family (Hartley et al., 1995). This similarity initially suggested that DNA-PK$_{cs}$ might be capable of phosphorylating inositol phospholipids, but no such activity has been detected. Instead, DNA-PK$_{cs}$ was shown to be a serine/threonine protein kinase. Other members of the PI 3-kinase related family include ATM, a protein *d*eficient in *A*taxia *t*elangiectasia, and ATR, *d*efects in which lead to an AT-related disorder (Keith and Schreiber, 1995; Smith and Jackson, 1999). Why these proteins should have retained the protein motifs characteristic of a phosphatidylinositol kinase remains a mystery.

A scheme for nonhomologous end-joining is shown in Figure 1. It is thought that broken termini are recognized by the Ku heterodimer, which then recruits DNA-PK$_{cs}$, thereby activating its kinase activity. This large complex serves to protect the DNA ends from nuclease attack, while also facilitating the recruitment of the XRCC4/DNA ligase IV heterodimer. Although it is not at present clear how end-bridging is achieved, these reactions result in the religation of the DSB, restoring the integrity of the DNA.

In an attempt to define in molecular detail the mechanism of NHEJ, an in vitro system for end-joining was recently developed (Baumann and West, 1998). The reactions exhibited a requirement for DNA-PK$_{cs}$, Ku70/80, XRCC4, and DNA ligase IV, consistent with the in vivo requirements. Preliminary fractionation and complementation assays, however, revealed that these factors were not sufficient for efficient end-joining, and that other components of the reaction remained to be identified. In the work described here, the complementation assay has been used to purify an additional component of the in vitro NHEJ reaction. We identify it as an inositol phosphate (IP$_6$) and show that purified IP$_6$ is bound by DNA-PK.

Results

DNA End-Joining In Vitro Is Dependent upon DNA-PK$_{cs}$

Previous studies have shown that in vitro DNA end-joining is sensitive to the DNA-PK inhibitors wortmannin and LY294002 (Baumann and West, 1998). These data suggest that DNA-PK$_{cs}$ plays an important role in the end-joining mechanism. To further examine the participation of DNA-PK$_{cs}$ in these in vitro reactions, we compared the end-joining activity of human glial cell lines MO59K (normal for DNA-PK$_{cs}$ expression) and MO59J (defective for DNA-PK$_{cs}$ expression) (Lees-Miller et al., 1995). As shown in Figure 2, whole cell extracts from MO59K cells promote efficient end-joining, while extracts from MO59J cells do not. Addition of purified

‡To whom correspondence should be addressed (e-mail: s.west@icrf.icnet.uk).

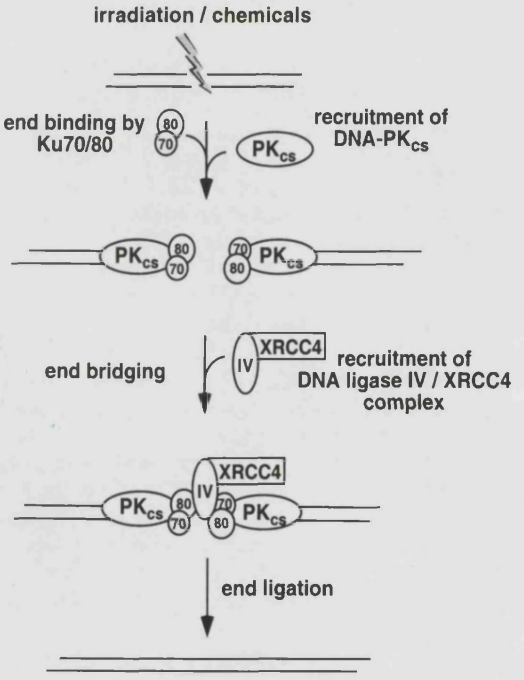

Figure 1. Repair of Double-Strand Breaks by Nonhomologous End-Joining

In this schematic model DSBs, caused by either irradiation or chemical assault, are bound by the Ku heterodimer (Ku70/80) and the catalytic subunit of the DNA-dependent protein kinase, DNA-PK_{cs}. Binding protects the free ends from nuclease attack while simultaneously initiating the assembly of the NHEJ apparatus. Through an as yet undefined process, DNA ends are bridged, and the XRCC4/DNA ligase IV complex is recruited to the DSB where it effects repair.

DNA-PK_{cs} to MO59J extracts, however, restored the ability to join DNA ends in vitro. These results demonstrate the dependence of the in vitro end-joining reaction upon DNA-PK_{cs}.

Purification of a Factor that Stimulates DNA End-Joining In Vitro

In previous studies, cell-free extracts capable of promoting DNA end-joining were fractionated by phosphocellulose chromatography (Baumann and West, 1998). One fraction (designated PC-C), which contained all components known to be required for NHEJ in vivo (Ku70/80, DNA-PK_{cs}, XRCC4, DNA ligase IV), showed only limited ability to join DNA ends in vitro. The ability to promote end-joining, however, could be restored by addition of a second fraction (designated PC-A). This complementation assay provided the basis for the purification of the stimulatory factor in PC-A, which we now designate *S*timulatory *F*actor *A* (SFA).

Hypotonic lysis and subcellular fractionation of HeLa cells into nuclear and cytoplasmic fractions showed the cytoplasm to be rich in SFA (data not shown). By comparison, relatively low levels of SFA were detected in nuclear extracts. The presence of sheared chromosomal DNA in the nuclear extracts, which could compete for the factors involved in NHEJ, however, made it difficult to accurately assess the relative levels of SFA in these two subcellular compartments. Given that the removal of nuclei would minimize the amount of contaminating DNA, we chose to prepare SFA from the cytoplasmic fraction of 300 liters of HeLa cells.

Preliminary binding trials showed that cation-exchange resins such as Mono S or SP-sepharose failed to bind SFA. Therefore, anion-exchange resins of various strengths were used throughout the purification scheme (Figure 3). During purification, we were somewhat surprised to discover that SFA was heat stable, insensitive to treatment with phenol, and insoluble in $CHCl_3$. UV absorbance spectroscopy demonstrated that a sample of concentrated SFA did not absorb at 280 nm, indicating a lack of aromatic amino acids (data not shown). All attempts to degrade SFA using proteases (trypsin, V8 protease, and proteinase K + SDS) failed (data not shown). Taken together, these observations suggest that SFA, previously assumed to be a protein participant in NHEJ, is not a polypeptide.

To investigate the possibility that SFA might be a nucleic acid (RNA or DNA), SFA was treated with either RNase A, NaOH (0.1–1.0 M at 60°C), DNase I, or micrococcal nuclease. These treatments had no effect on the ability of SFA to stimulate end-joining (data not shown). UV absorbance spectroscopy demonstrated that a sample of concentrated SFA did not absorb at 260 nm, indicating a lack of purine or pyrimidine moieties in the sample (data not shown). These data demonstrate that SFA is not a nucleic acid.

Chemical Analysis of SFA

To identify the active component in SFA, the sample was subjected to NMR and mass spectroscopy. Proton decoupled phosphorus NMR spectra (Figure 4A) revealed four peaks (ratio 1:2:2:1) close to the phosphoric acid standard suggesting six phosphate groups. The 1:2:2:1 ratio of peak intensities suggests two independent sets of equivalent phosphates as well as two nonequivalent individual phosphates. Proton phosphorus coupled spectra (Figure 4B) revealed phosphorus proton doublets, consistent with each of the phosphate

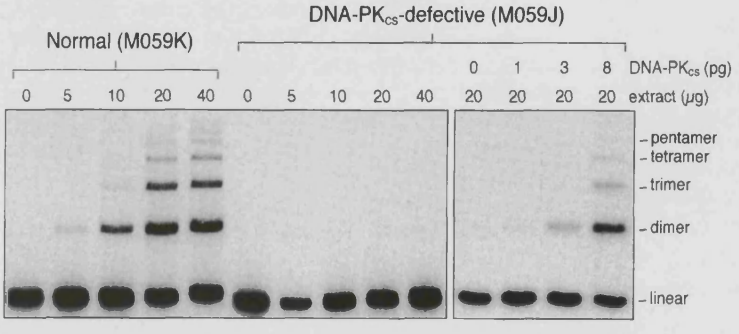

Figure 2. DNA End-Joining by Human Cell Free Extracts Requires DNA-PK_{cs}

Whole cell extracts from M059K and M059J were incubated with 5′-^{32}P-end-labeled HindIII-linearized pDEA-7Z DNA as described in Experimental Procedures. Purified DNA-PK_{cs} was added directly to the reaction in the amounts indicated. Samples were analyzed by gel electrophoresis and ^{32}P-labeled DNA visualized by autoradiography. Mobilities of linear and multimeric DNA species are indicated.

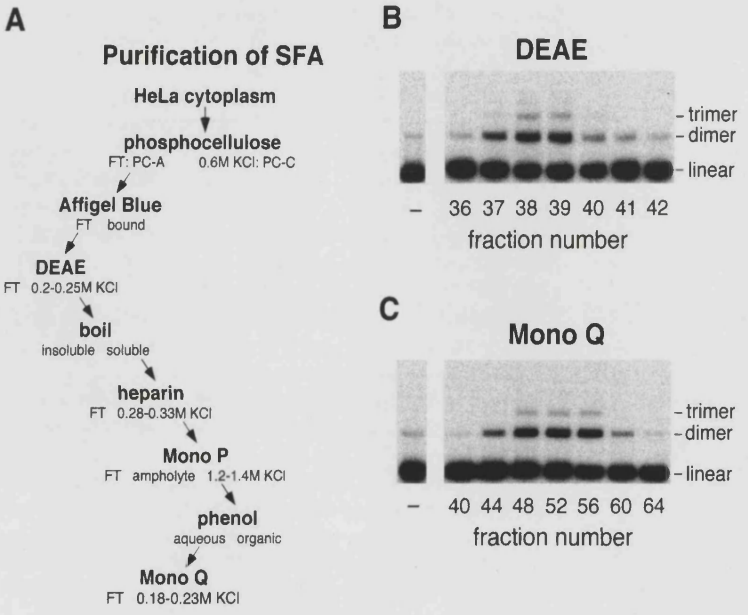

Figure 3. Purification of SFA

(A) Schematic representation of the chromatographic steps taken to purify SFA from HeLa cytoplasmic extracts.

(B) Complementation of DNA end-joining by the addition of undiluted DEAE fractions to PC-C. End-joining by PC-C alone (−) and a selected region of the column elution profile are shown.

(C) Fractions eluting from mono Q were diluted 1:50 in L buffer and assayed for the ability to complement end-joining by PC-C. End-joining by PC-C alone (−) and a selected region of the column elution profile are shown.

groups being linked to a carbon participating in a carbon-hydrogen bond. These data suggest that the SFA sample contains an organophosphorus compound containing 6 phosphates, each directly linked to a (−CH) group.

The molecular mass of SFA was determined by mass spectroscopy (Figure 4C). While no significant signal was observed in the range commonly associated with macromolecules, polypeptides, or polymeric nucleic acids, the SFA sample was found to contain a number of species of low molecular mass. Although the SFA sample was found to be heterogeneous, a clear peak was detected at a mass of 660.9 Da. Additionally, an array of peaks that differed from the original 660.9 Da peak by 22 Da (the mass of sodium Na^+) were observed downstream of the 660.9 Da peak. These masses appear to correspond to the +1 (Na^+), +2 (Na^+), and +3 (Na^+) salts of the 660.9 Da species.

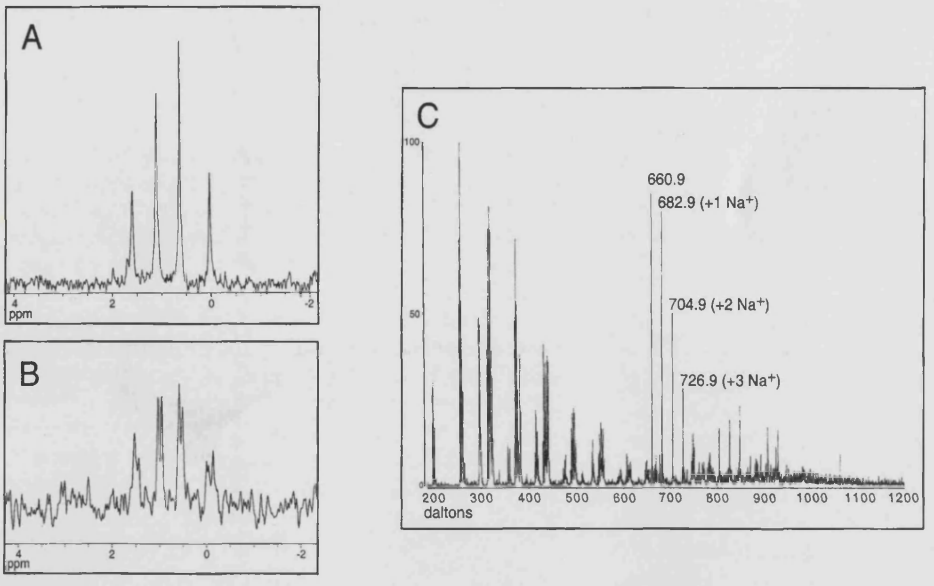

Figure 4. Physical Characteristics of SFA

(A) Proton decoupled phosphorus spectra revealed four peaks (ratio 1:2:2:1) close to the phosphoric acid reference at 1.4909 ppm (intensity 2.582), 1.0003 ppm (intensity 5.465), 0.5554 ppm (intensity 6.002), and −0.0850 ppm (intensity 2.927), suggesting phosphate groups and showing no evidence of phosphorus–phosphorus coupling.

(B) Proton phosphorus coupled spectrum revealed phosphorus proton doublets consistent with the phosphorus being linked to a carbon hydrogen bond.

(C) Ion-trap spectrum revealed a mass of 660.9 Da, which represents the [mass $+1]^{+1}$-ion or [659.9 $+1]^{+1}$-ion followed by a series of related sodium salts at 682.9 Da (+1 Na^+), 704.9 Da (+2 Na^+), 726.9 Da (+3 Na^+).

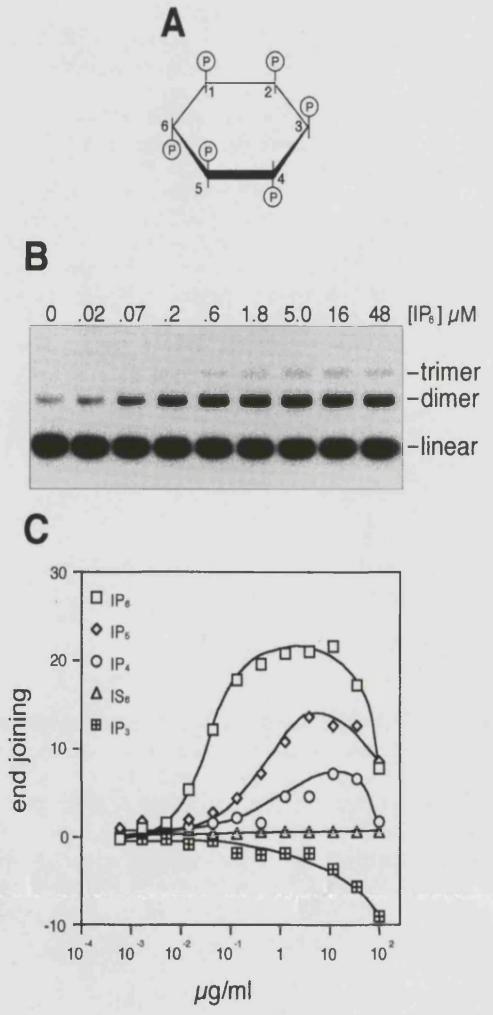

Figure 5. Stimulation of DNA-PK-Dependent End-Joining by Inositol Phosphates

(A) Schematic representation of IP_6.

(B) Complementation of PC-C by IP_6. End-joining assays were carried out using PC-C complemented with increasing amounts of IP_6.

(C) Effect of inositol phosphates on DNA-PK-dependent NHEJ. Inositol hexakisphosphate (IP_6), inositol pentakisphosphate (IP_5), inositol tetrakisphosphate (IP_4), inositol trisphosphate (IP_3), or inositol hexasulphate (IS_6) were assayed for their ability to stimulate DNA end-joining by PC-C.

Identification of the Active Component of SFA as IP_6

Inositol is a fully hydroxylated six-carbon ring which is found in a number of phosphorylation states ranging from mono- through hexakisphosphate. Inositol hexakisphosphate (IP_6) (Figure 5A) shares the same molecular weight as SFA (659.9 Da) and has the same phosphorus content. The presence of six phosphates in IP_6 and their conformation in aqueous solution (Costello et al., 1976; Emsley and Niazi, 1981; Barrientos and Murthy, 1996) is in keeping with the proton-decoupled phosphorus NMR spectra of SFA (Figure 4A), which matches the proton-decoupled phosphorus NMR spectrum for the mono-ionic form of IP_6. Furthermore, each phosphate group of IP_6 is linked to a carbon participating in a carbon-hydrogen bond, which is in accord with the proton-coupled phosphorus NMR spectrum (Figure 4B). These results indicate that the 660.9 Da species detected by mass spectroscopy is the protonated form of IP_6 ($[659.9 + 1]^{+1}$ Da), and that the array of peaks described above represent the sodium salts that would readily form with IP_6.

To confirm that the active component in SFA is indeed IP_6, commercially available IP_6 was assayed for its ability to stimulate end-joining by PC-C. As shown in Figure 5B, IP_6 stimulated end-joining at concentrations in the region of 100 nM and stimulation was maximal at 1 μM. End-joining by PC-C in the presence of 1 μM IP_6 was found to be sensitive to the DNA-PK inhibitor LY294002 (data not shown). These results indicate that the end-joining reaction, which is stimulated by purified IP_6, proceeds via the same DNA-PK-dependent pathway as that observed in whole cell extracts (Figure 2).

To assess the specificity of NHEJ for IP_6, we compared the ability of IP_6 to stimulate end-joining with other inositol phosphates (IP_5, IP_4, and IP_3). In addition, inositol hexasulphate (IS_6—an inositol compound that would provide a charge distribution similar to that of IP_6 while presenting sulfate rather than phosphate groups) was also assayed. It was found that IS_6 was unable to stimulate end-joining, demonstrating a clear requirement for phosphate groups (Figure 5C). Indeed, we found that IP_6 proved to be the most effective inositol phosphate compound of those tested. IP_5 and IP_4 were also able to stimulate end-joining, but the efficiency of this stimulation was reduced relative to IP_6. These data show that end-joining requires a phosphorylated inositol species, and that the stimulation of NHEJ is directly related to the extent of phosphorylation.

Further evidence that IP_6 is the active component in SFA was obtained by strong anion exchange (SAX) chromatography using a resin that is commonly utilized to separate highly charged molecules such as the inositol phosphates. To determine whether the NHEJ stimulating activity in SFA cofractionated with IP_6, an aliquot of SFA was spiked with a trace amount of ^3H-IP_6 (4 nM), and the mixture was chromatographed on AG 1-X8 resin. Complementation assays were performed to detect the presence of SFA, and the ^3H-IP_6 content was assessed by scintillation counting. As shown in Figure 6, the peaks of SFA and ^3H-IP_6 were coincident.

Although IP_6 is of small molecular size (660 Da), its high charge to mass ratio and the hydrogen bonding observed between phosphate groups (Emsley and Niazi, 1981) result in a larger apparent molecular size in aqueous solutions at low ionic strength. This has been observed by the retention of IP_6 by dialysis membranes at low ionic strength, and the passage of IP_6 through the same membrane at high ionic strength (data not shown). Equilibrium dialysis trials were performed to compare the movement of SFA and ^3H-IP_6 across a dialysis membrane (12–14 kDa cutoff). Both SFA and ^3H-IP_6 were retained during equilibrium dialysis at low ionic strength, and at high ionic strength both SFA and ^3H-IP_6 passed through the dialysis membrane (data not shown). These observations add to the list of physical properties shared by SFA and IP_6.

Specificity of IP_6 for DNA-PK-Dependent End-Joining

The data presented above show that the stimulatory factor purified from HeLa cells is inositol hexakisphosphate (IP_6). Because many substances (e.g., PEG, PVA) have nonspecific stimulatory effects on the efficiency of DNA ligases in vitro, we next determined whether IP_6 was specific for NHEJ mediated by DNA-PK. To do this,

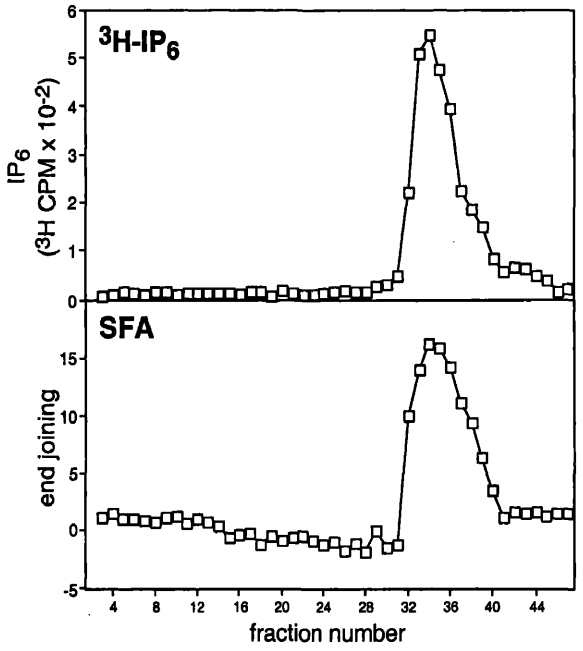

Figure 6. SFA and IP_6 Cofractionate by Strong Anion Exchange Chromatography

A trace amount (4 nM) of ^{3}H-IP_6 was added to a 1 ml aliquot of SFA. The resulting sample was applied to AG 1-X8 resin and eluted as described in Experimental Procedures. Top, elution profile of ^{3}H-IP_6 as measured by scintillation counting. Bottom, elution profile of SFA, determined by the complementation of PC-C-mediated end-joining. End-joining has been calculated as % total ends joined.

we compared the ligation efficiencies of PC-C and T4 DNA ligase in the presence or absence of IP_6. It was found that IP_6 stimulated end-joining by PC-C whereas the efficiency of ligation by T4 DNA ligase was unaffected (Figure 7).

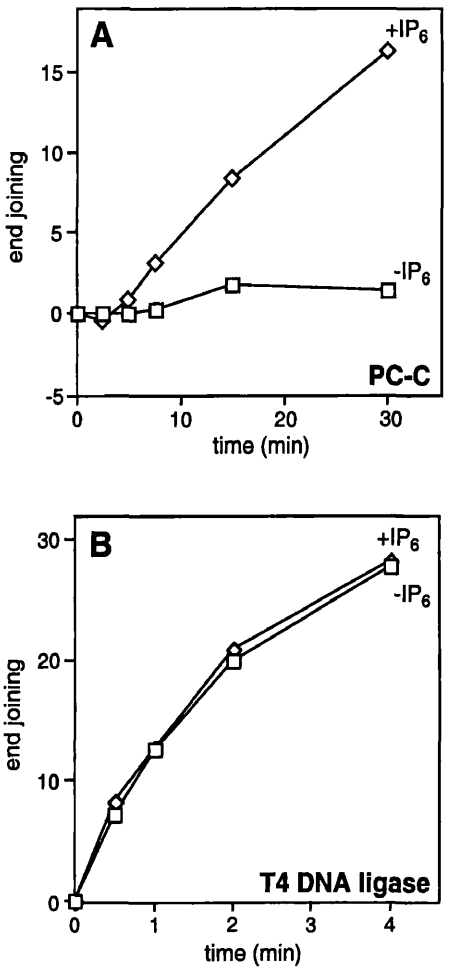

Figure 7. Specificity of IP_6 for DNA-PK-Mediated End-to-End Ligation

(A) DNA end-joining reactions catalyzed by PC-C were analyzed in the presence or absence of 2 μM IP_6.

(B) Similar reactions carried out using T4 DNA ligase (0.03 u/μl) in place of PC-C.

Binding of ^{3}H-IP_6 by DNA-PK

The catalytic domain of DNA-PK_{cs} is related to that found in the PI 3-kinases, which phosphorylate inositol phospholipids. Previous studies have shown that recognition of the inositol phosphate headgroup by the PI 3-kinases is mediated by defined sequences within this conserved domain (Figure 8A) (Bondeva et al., 1998). Given that NHEJ is dependent upon DNA-PK_{cs} (Figure 2) and is stimulated by the addition of IP_6, it was plausible that this stimulatory effect was due to a physical interaction between IP_6 and DNA-PK.

Gel filtration analysis using ^{3}H-IP_6 and purified DNA-PK was carried out to assess the ability of DNA-PK to interact with IP_6. As shown in Figure 8B, ^{3}H-IP_6 was retained by the gel filtration media in the absence of DNA-PK and eluted late from the column. In this experiment, the retention of ^{3}H-IP_6 was observed in the presence of nonspecific proteins that have no effect on the mobility of ^{3}H-IP_6. In the presence of DNA-PK, however, an additional peak of ^{3}H-IP_6 was observed in early fractions of the elution profile. This novel DNA-PK-dependent peak of ^{3}H-IP_6 was found to correlate with the peak of DNA-PK activity. DNA-PK-dependent retention of ^{3}H-IP_6 was not observed in the presence of a 9-fold molar excess of unlabeled IP_6 (data not shown). The altered retention of ^{3}H-IP_6 by gel filtration media in the presence of DNA-PK, the competition of ^{3}H-IP_6 retention by unlabeled IP_6, and the cofractionation of ^{3}H-IP_6 and DNA-PK activity indicate that DNA-PK is capable of binding IP_6.

To assess the specificity of DNA-PK for IP_6, we examined the ability of DNA-PK to bind another inositol phosphate. Given that IP_3 does not stimulate NHEJ (Figure 5C), we selected ^{3}H-IP_3 for this comparison. In contrast with ^{3}H-IP_6, the retention of ^{3}H-IP_3 by the gel filtration matrix was not altered by the presence of DNA-PK (Figure 8C). Furthermore, we failed to observe the coelution of ^{3}H-IP_3 with DNA-PK kinase activity. This argues against the possibility that the observed binding of IP_6 by DNA-PK might be due to nonspecific interactions between DNA-PK and the multiple phosphate groups of IP_6. Taken together, the data presented in Figure 8 demonstrate a physical interaction between DNA-PK and IP_6 that does not extend to all of the inositol phosphates.

Discussion

We have isolated a factor from HeLa cell extracts that stimulates NHEJ in vitro, and have identified it as inositol

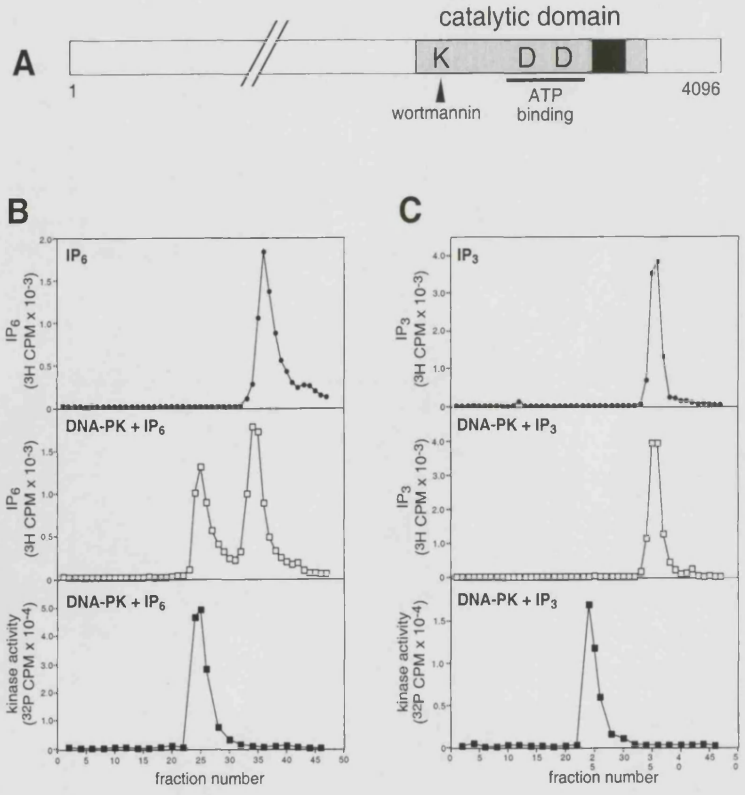

Figure 8. Binding of IP_6 by DNA-PK

(A) Schematic representation of human DNA-PKcs. The gray area represents the C-terminal 380 aa which shares sequence similarity to the catalytic domain of the phosphatidylinositol 3-kinases (Hartley et al., 1995). The black box indicates the location of the putative inositol phosphate headgroup binding domain of the phosphatidylinositol 3-kinases (Wymann and Pirola, 1998). The lysine (K) residue believed to be the target of wortmannin interaction is shown as are the two aspartate (D) residues believed to be located in the ATP binding active site of the protein kinase.

(B) Gel filtration analyses of ^3H-IP_6 binding by DNA-PK. Filled circles, elution profile of ^3H-IP_6 in the presence of nonspecific proteins. Open squares, elution profile of ^3H-IP_6 in the presence of DNA-PK. Filled squares, profile of DNA-PK kinase activity of fractions represented by open squares.

(C) Gel filtration analysis of ^3H-IP_3 in the presence or absence of DNA-PK. Experimental details are as described in (B).

hexakisphosphate (IP_6). The concentration of IP_6 in mammalian cells has been estimated to be 10 μM (Szwergold et al., 1987). Much of this is likely to be bound by proteins and by monovalent and divalent cations, such that the available pool of unbound IP_6 may be significantly lower (Theibert et al., 1992; Hawkins et al., 1993; Fukuda and Mikoshiba, 1997). Our observation that IP_6 stimulates end-joining at a concentration of 100 nM indicates that the intracellular concentrations of unbound IP_6 will be more than sufficient to facilitate DNA-PK-dependent end-joining in vivo.

Recent studies on the metabolism of IP_6 in yeast have identified a factor, ArgRIII, which catalyzes the ATP-dependent phosphorylation of IP_3 and IP_4. Mutation of the *ARGRIII* gene leads to a dramatic decrease in intracellular IP_6 and results in defects in mRNA export (York et al., 1999; Odom et al., 2000; Saiardi et al., 2000). Here we have described biochemical studies linking IP_6 with a second nuclear function, NHEJ.

What is the role of inositol phosphate in NHEJ? IP_6 has been shown to function as an inhibitor of serine/threonine phosphatases at concentrations of 10 μM or greater (Larsson et al., 1997). Given that IP_6 is capable of stimulating NHEJ at 1% of this concentration, it is unlikely that IP_6 functions in NHEJ as a phosphatase inhibitor. Also, the inability of IP_6 to enhance end-joining by T4 DNA ligase argues against the possibility that IP_6 stimulates NHEJ via a nonspecific mechanism such as macromolecular crowding by volume exclusion.

The ability to stimulate NHEJ does not appear to be restricted to IP_6 because we found that IP_5 and IP_4 were also able to stimulate end-joining, though to a lesser extent. Inositol polyphosphate kinases, however, are known to act on IP_6 and convert it to IP_7 (Saiardi et al., 1999), and at the present time we cannot rule out the possibility that IP_6 may be phosphorylated by the extract and that the true effector of NHEJ may be IP_7 or IP_8. The correlation between the degree of phosphorylation and end-joining efficiency fits a model in which inositol phosphate functions as a ligand and is bound by a ligand binding species. In such a model, small alterations in the structure of the ligand (in this case changes from IP_6 to IP_5 or to IP_4) would be predicted to result in incremental decreases in the affinity or stability of the ligand binding interaction. If ligand binding is essential for end-joining, then these small alterations in ligand structure would be apparent as incremental decreases in end-joining efficiency. An important prediction made by this model is that a species which both binds IP_6 and participates in NHEJ is present in PC-C. We have shown that DNA-PK can bind IP_6, leading us to suggest that the IP_6 binding component in PC-C is indeed DNA-PK. At the present time, however, we have not identified which component of DNA-PK (Ku70, Ku80, or $DNA\text{-}PK_{cs}$) makes specific contact with IP_6.

The apparent requirement for IP_6 in efficient NHEJ suggests that DNA-PK is in some way NHEJ-inactive in the unbound state, and that there is a transition upon IP_6 binding resulting in an NHEJ-active species. Preliminary studies indicate that IP_6 has no significant effect on the ability of DNA-PK to phosphorylate a synthetic p53 peptide substrate in the presence of DNA (data not shown). However, we do not rule out the possibility that specific protein phosphorylation of one or more of the in vivo substrates of DNA-PK may be altered upon IP_6 binding. Additionally, binding of IP_6 may influence the ability of DNA-PK to interact with other components of the NHEJ apparatus.

The consequences of IP_6 binding by DNA-PK might be structural in nature, possibly due to an allosteric shift upon association with IP_6. Alternatively, binding of IP_6 could simply alter the surface charge distribution of

DNA-PK. Such an alteration of local electrostatic potential has been observed in the binding of IP_6 to phosphoglycerate mutase (Rigden et al., 1999). In this case, ligand binding was mediated by both hydrogen bonding interactions and by the strong positive electrostatic potential of the active site cleft. Occupancy of this highly charged cleft by IP_6 exposes several phosphates to the solvent, which has a pronounced effect on the local electrostatic potential relative to the unbound state. An alteration of this kind would result in a more passive transition from the NHEJ-inactive to the NHEJ-active state of DNA-PK. Of course these two possibilities are not mutually exclusive and both mechanisms might influence the substrate specificity of DNA-PK as well as its potential to participate in extensive protein complexes.

Previous studies have demonstrated the ability of the XRCC4/DNA ligase IV complex to promote DNA end-joining in vitro (Grawunder et al., 1997). The efficiency of this reaction is stimulated by the addition of Ku (Ramsden and Gellert, 1998) and further enhanced by the presence of DNA-PK (Chen et al., 2000). Under our assay conditions we observed low-efficiency end-joining by PC-C (demonstrated to contain DNA-PK and XRCC4/DNA ligase IV) (Baumann and West, 1998), and that this reaction is stimulated by IP_6. The requirement for IP_6 by PC-C may relate to the fact that fractionation was carried out using a phosphocellulose resin that most likely depletes DNA-PK of IP_6. Alternatively, it is possible that PC-C contains an inhibitor of end-joining which must be modified by an IP_6-dependent process to permit efficient end-joining.

DNA-PK is the only factor known to participate in NHEJ that has been demonstrated to bind IP_6. DNA-PK_{cs} is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase family, as are ATM and ATR. All three proteins exhibit a strong sequence homology to the PI 3-kinases, especially in the catalytic core domain that binds and phosphorylates the phosphoinositol headgroup of phosphatidylinositol. However, no phosphorylation of lipid substrates has been observed by DNA-PK_{cs} or by other members of this PI3K-related family of kinases (Hunter, 1995; Keith and Schreiber, 1995; Carpenter and Cantley, 1996; Wymann and Pirola, 1998).

It is tempting to postulate that the PI3K-related kinases are derived from a common ancestor that possessed both protein kinase and lipid kinase functions. Mutation of the PI3K catalytic domain could result in loss of lipid kinase activity, while retaining an affinity for the phosphoinositol headgroup. This hypothesis is supported by observations demonstrating that substitution of the putative phosphoinositol headgroup interaction site of PI3Kγ can alter or abort lipid kinase activity in vitro, while leaving protein kinase function unaffected (Bondeva et al., 1998). Clearly an important next step in the investigation of both NHEJ and the PI3K-related kinases will be to characterize the interaction between DNA-PK and IP_6 and to examine the consequences of this binding event. It will also be of interest to determine whether the activities of other PI3K-related kinases are modified through interactions with inositol phosphates.

Experimental Procedures

Materials

The reagents *myo*-inositol 1,4,5-trisphosphate (IP_3), D-*myo*-inositol 1,3,4,5-tetrakisphosphate (IP_4), *myo*-inositol 1,3,4,5,6-pentakisphosphate (IP_5), *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP_6), and inositol hexasulphate (IS_6) were purchased from Sigma. IP_6 was also purchased from Calbiochem. ^{3}H-IP_6 and ^{3}H-IP_3 (10–30 Ci/mmol) were purchased from NEN. Human glial cell lines MO59J and MO59K were generous gifts from Dr. Susan Lees-Miller (Univ. of Calgary, Canada).

Proteins

Whole cell extracts were prepared and fractionated on phosphocellulose as described (Baumann and West, 1998). Fraction PC-C was dialyzed for 2 hr against L buffer (20 mM Tris-HCl pH 8.0, 25 mM KOAc, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) and stored at −80°C. DNA-PK_{cs} was purified essentially as described (Dvir et al., 1993).

Purification of SFA

HeLa cells (300 liters) were cultured in suspension to a density of 5×10^5 cells/ml, harvested by centrifugation, washed twice with PBS, flash-frozen on liquid nitrogen, and stored at −80°C. When required, two packed cell volumes of hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT) was added and the cells were held on ice for 20 min. The cells were opened by dounce homogenization (35 strokes with a "B" pestle) in the presence of protease inhibitors (1 mM PMSF, 2.2 ng/ml aprotinin, 1 ng/ml leupeptin, 1 ng/ml pepstatin A, and 1 ng/ml chymostatin). The resulting homogenate was centrifuged for 30 min at 10,000 rpm to pellet membraneous cellular debris and intact nuclei. The resulting cytoplasmic fraction was dialyzed for 2 hr at 4°C against L buffer and then centrifuged for 45 min at 45,000 rpm at 4°C in a Beckman Ti45 rotor. All dialysis steps were carried out in tubing with a molecular weight cutoff of 12–14 kDa.

Cytoplasmic extracts (from the equivalent of 100 liter cultured cells) were first batch fractionated by absorption to 1/3 vol phosphocellulose (Whatman P-11) by gently rocking for 30 min at 4°C. Following centrifugation, the unbound fraction was reserved. The resin was washed twice with an equal volume of L buffer. The unbound fraction and the two washes were pooled, and spun for 45 min at 45,000 rpm at 4°C in a Beckman Ti45 rotor. The supernatant was then loaded onto a 5×10 cm phosphocellulose column equilibrated in L buffer. The flowthrough passed directly onto a 5×10 cm affigel blue column (Bio-Rad) equilibrated in the same buffer. The flowthrough was loaded onto a 1.6×39 cm DEAE Fast Flow column (Pharmacia) equilibrated in L buffer, which was eluted with 15 column volumes of a 0–0.5 M KCl linear gradient in L buffer. Active fractions eluting between 0.2–0.25 M KCl were pooled (designated DEAE SFA) and stored at −80°C until all 300 liter equivalent of HeLa cell cytoplasm had been fractionated.

DEAE-SFA fractions were heat denatured by boiling for 15–20 min, then centrifuged for 45 min at 45,000 rpm at 4°C in a Beckman Ti45 rotor to remove insoluble aggregates. The supernatant was dialyzed for 6 hr against L buffer, applied to a 2.6×37 cm affigel heparin column (Bio-Rad), equilibrated in the same buffer, then eluted with a 15 column volume 0–1.0 M KCl linear gradient in L buffer. Active fractions eluting between 0.28–0.33 M KCl were pooled, dialyzed for 6 hr against L buffer, then loaded onto a Mono P HR 5/20 column (Pharmacia). The column was washed with 5 column volumes of L buffer, then with 14 volumes of polybuffer 74-HCl (pH 4.0), and finally eluted with a 50 column volume 0–2.0 M KCl linear gradient. Active fractions eluting between 1.2–1.35 M KCl were pooled, extracted with phenol/$CHCl_3$, dialyzed for 6 hr against L buffer, loaded onto a Mono Q HR 5/5 column (Pharmacia), equilibrated in L buffer, and then eluted with a 30 column volume 0–1.0 M KCl linear gradient in L buffer. Active fractions eluting from the column between 0.18–0.23 M KCl were pooled, dialyzed overnight at 4°C against dH_2O, and stored at −20°C.

End-Joining Reactions

Reactions (10 μl) were carried out in 50 mM HEPES pH 8.0, 40 mM KOAc, 0.5 mM $Mg(OAc)_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, and contained 2–3 μl (3–5 μg) of PC-C and HindIII-linearized 5′-^{32}P-labeled pDEA-7Z DNA (10 ng). Incubation was for 1 hr at 37°C. ^{32}P-labeled DNA products were deproteinized and analyzed by electrophoresis through 0.6% agarose gels followed by autoradiography. Quantification of joining efficiency was carried out by phosphorimaging and the data are presented as (% total ends-joined$_{sample}$) − (% total ends-joined$_{PC\text{-}C}$) unless otherwise stated. For assays of LY204002 and inositol phosphates, aqueous solutions were added

directly to the end-joining reactions. For the screening of column fractions, 0.5–1 μl of diluted (in L buffer) or undiluted column fractions were added.

Strong Anion Exchange Chromatography

AG 1-X8 resin (200–400 mesh, formate form, Bio-Rad) was washed with 5 volumes of 1.0 M formic acid, then with 15 column volumes of dH_2O. SFA was dialyzed against dH_2O overnight at 4°C. A trace (4 nM) amount of ^3H-IP_6 was added, and the sample was applied to a 1.0 × 1.3 cm AG 1-X8 column equilibrated with dH_2O. The column was washed with 10 column volumes of dH_2O, then eluted with a 30 column volume 0–2.75 M ammonium formate linear gradient in 0.1 M formic acid.

Mass Spectrometry

SFA sodium salt was acidified with 1 M HCl to approximately pH 2.0, diluted with one equivalent of 70% methanol/30% formic acid, and a 10 μl sample was loaded into a carbon-coated nano-spray capillary needle (Protana) and the molecular weight determined by mass spectrometry using an ion-trap (LCQ Thermoquest).

NMR

SFA was lyophilized and resuspended in D_2O. Measurements were carried out using a Bruker AC-300 pulse Fourier transform NMR spectrometer operating at 300.13 MHz for protons ^{31}P spectroscopy (5 mm sample in 10 mm broad band probe), and at 121.497 MHz with broad band proton decoupling (composite pulse). Reference ^{31}P signals were detected in a ± 50 ppm window.

Equilibrium Dialysis

For analysis at low ionic strength, samples (1 ml) were dialyzed against 0.5 liter dH_20 for 21 hr at 4°C with stirring. For high ionic strength, samples were dialyzed against 0.25 liter of 2.5 M NaCl for 48 hr at 4°C. Dialysis tubing with a molecular weight cutoff of 12–14 kDa was used.

T4 DNA Ligation Assay

Reactions (10 μl) containing T4 DNA ligase (NEB) were carried out in T4 DNA ligase buffer (NEB) at 16°C in the presence and absence of inositol phosphates. Reactions were stopped, deproteinized, and the products analyzed by agarose gel electrophoresis.

Binding of ^3H-IP_6 by DNA-PK

Binding reactions (55 μl) were carried out in 25 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.1% NP-40 with 5000 units of DNA-PK (Promega) or 1.8 mg/ml of protein size standards for gel filtration (BioRad) and 100 nM ^3H-IP_6 or ^3H-IP_3 at 4°C for 30 min. Complexes were resolved on a Superose 12 PC3.2/30 column run in 50 mM HEPES pH 8.0, 40 mM KOAc, 0.1M KCi, 10% glycerol, and 1 mM DTT at 40 μl/min. 50 μl fractions were collected, and aliquots of each fraction were assayed for ^3H-IP by scintillation counting and for DNA-PK kinase activity. DNA-PK kinase activity was assessed using the SignaTECT DNA-PK assay system (Promega), which utilizes a p53 peptide substrate of DNA-PK.

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Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining

Claire Chappell, Les A.Hanakahi, Feridoun Karimi-Busheri[1], Michael Weinfeld[1] and Stephen C.West[2]

Cancer Research UK, London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK and [1]Cross Cancer Institute, Edmonton, Alberta, Canada T6G 1Z2

[2]Corresponding author
e-mail: stephen.west@cancer.org.uk

The efficient repair of double-strand breaks (DSBs) in DNA is critical for the maintenance of genome stability. In mammalian cells, repair can occur by homologous recombination or by non-homologous end joining (NHEJ). DNA breaks caused by reactive oxygen or ionizing radiation often contain non-conventional end groups that must be processed to restore the ligatable 3′-OH and 5′-phosphate moieties which are necessary for efficient repair by NHEJ. Here, using cell-free extracts that efficiently catalyse NHEJ *in vitro*, we show that human polynucleotide kinase (PNK) promotes phosphate replacement at damaged termini, but only within the context of the NHEJ apparatus. Phosphorylation of terminal 5′-OH groups by PNK was blocked by depletion of the NHEJ factor XRCC4, or by an inactivating mutation in DNA-PK$_{cs}$, indicating that the DNA kinase activity in the extract is coupled with active NHEJ processes. Moreover, we find that end-joining activity can be restored to PNK-depleted extracts by addition of human PNK, but not bacteriophage T4 PNK. This work provides the first demonstration of a direct, specific role for human PNK in DSB repair.

Keywords: DNA repair/double-strand break repair/genomic instability/Ku/recombination

Introduction

Breakage of the sugar-phosphate backbone of DNA is a common event in cells that are irradiated or suffer the effects of genotoxic agents. Major forms of breakage include the introduction of single- or double-strand DNA breaks (DSBs), which must be efficiently repaired to restore the integrity and functionality of the genome. In mammalian cells, DSBs are repaired by non-homologous end joining (NHEJ), a process that depends on the DNA end-binding protein Ku70/80, a protein kinase DNA-PK$_{cs}$ and the XRCC4/ligase IV heterodimer (reviewed by Chu, 1997; Critchlow and Jackson, 1998; Kanaar *et al.*, 1998).

Whereas DNA ligation requires the presence of 5′-phosphate and 3′-OH termini, many chemically distinct groups are found at break points. For example, ionizing radiation, alkylating agents such as BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea] and camptothecin promote the formation of non-ligatable 5′-OH and/or 3′-phosphate terminal groups (Coquerelle *et al.*, 1973; Henner *et al.*, 1983; Eisenbrand *et al.*, 1986; Pouliot *et al.*, 1999). Such groups need to be removed or modified to enable efficient repair.

Polynucleotide kinase (PNK), which catalyses the phosphorylation of 5′-OH termini and removes 3′-phosphate groups, is thought to play an important role in the modification of damage-induced DNA termini in human cells (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). Consistent with this, inactivation of the *Schizosaccharomyces pombe* homologue of PNK, *PNK1*, results in hypersensitivity to ionizing radiation without affecting growth under normal conditions (Meijer *et al.*, 2002).

A role for PNK in single-strand break repair has recently been established. First, it was shown that human PNK, DNA polymerase β and DNA ligase I can repair nicks and short gaps bounded by 3′-phosphate and 5′-OH termini (Karimi-Busheri *et al.*, 1998). Secondly, direct interactions between PNK and polymerase β, DNA ligase III and XRCC1 have been demonstrated, and the four proteins appear to associate in a multiprotein complex that can promote the repair of single-strand breaks typical of those produced by ionizing radiation or reactive oxygen species (Whitehouse *et al.*, 2001). Moreover, XRCC1 was shown to stimulate the DNA kinase/phosphatase activities of PNK, suggestive of a concerted role for XRCC1 and PNK in the processing of damaged termini at single-strand breaks in duplex DNA. In contrast to its role in the repair of single-strand DNA breaks, a direct involvement of PNK in DSB repair has not been demonstrated.

Recently, using human cell-free extracts, an *in vitro* system for the repair of DSBs by NHEJ was described (Baumann and West, 1998; Hanakahi *et al.*, 2000). Using linearized plasmid DNA molecules, end joining was shown to be dependent upon Ku70/80, DNA-PK$_{cs}$, DNA ligase IV and XRCC4. However, in this assay, end joining was demonstrated only with simple restriction endonuclease termini that contain easily ligatable 5′-phosphate and 3′-OH termini. In the present work, we have used this *in vitro* system to analyse the repair of DNA termini containing 5′-OH groups. We find that repair occurs efficiently in human cell-free extracts, and is dependent upon the presence of PNK. Surprisingly, phosphorylation of the terminal 5′-OH group by PNK failed to occur in extracts from a DNA-PK$_{cs}$-defective cell line, or when NHEJ was blocked by addition of XRCC4 antibodies. These results indicate that the DNA kinase activity in the extract is coupled with NHEJ. Extracts that were immunodepleted for PNK exhibited a reduced end-joining efficiency, but activity could be restored by addition of purified human PNK, but not bacteriophage T4 PNK. These studies indicate that human PNK is an integral

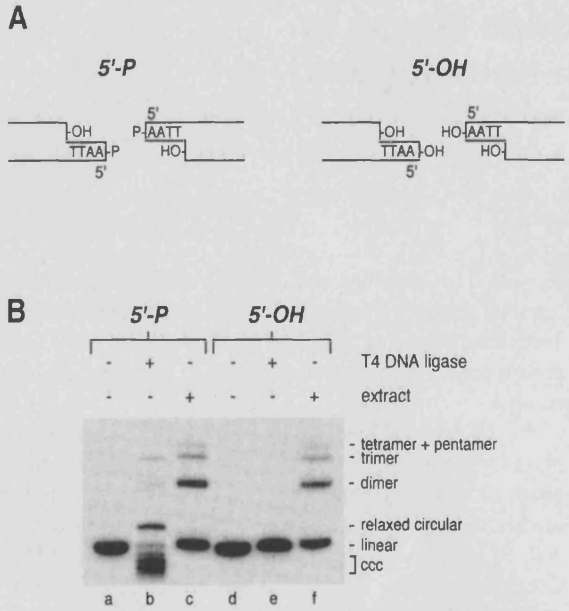

Fig. 1. End joining of 5′-OH termini by human cell-free extract. (**A**) Schematic representation of the DNA termini used in end-joining reactions. Plasmid DNA was linearized with *Eco*RI to produce protruding 5′-phosphate (5′-P) termini (left). Further treatment with CIP resulted in the formation of protruding 5′-OH termini (right). (**B**) Uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNAs (40 ng), with protruding 5′-phosphate or 5′-OH termini as indicated, were incubated with T4 DNA ligase (80 U) or GM00558 cell-free extract (80 µg). After incubation, the products were deproteinized and analysed by agarose gel electrophoresis.

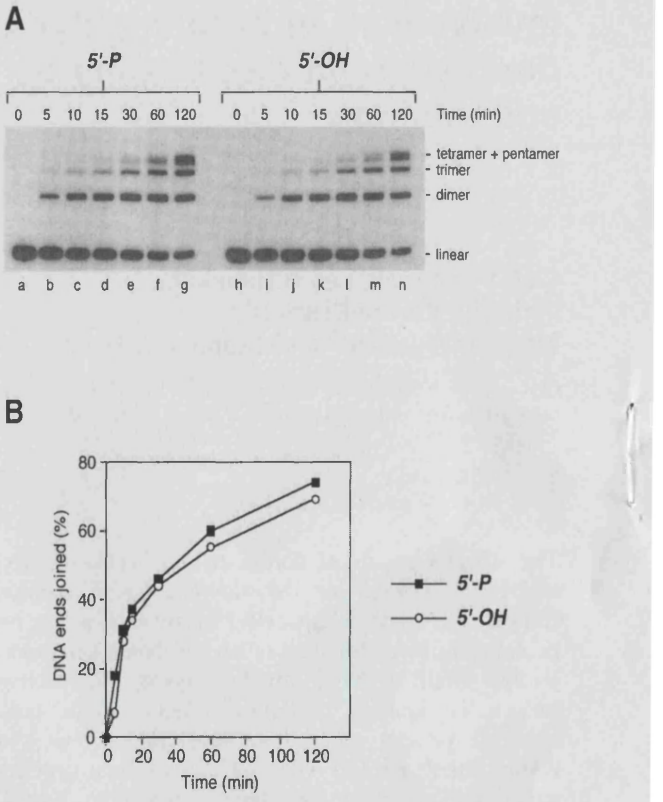

Fig. 2. Comparison of the rate of joining of 5′-phosphate and 5′-OH termini. (**A**) Large scale end-joining reactions (160 µl) contained uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNA (320 ng) with 5′-phosphate or 5′-OH termini, and GM00558 extract (640 µg). Reactions were incubated at 37°C and at the indicated times 20-µl samples were removed, deproteinized and the DNA products analysed by agarose gel electrophoresis. (**B**) Quantification of the time course shown in (A). The DNA products were quantified by phosphoimaging, and end joining is expressed as the percentage of multimeric DNA species out of total DNA. Squares, 5′-phosphate termini; circles, 5′-OH termini.

partner of the end joining machinery that is active on modified DNA termini at DSBs.

Results

Joining of DNA termini with 5′-OH groups by cell-free extracts

Plasmid DNA was linearized with *Eco*RI and then dephosphorylated with calf intestinal phosphatase (CIP) to produce 5′-OH termini. The ability of cell-free extracts, prepared from the human lymphoblastoid cell-line GM00558, to join these termini was compared with ligatable 5′-phosphate termini. The end structures are shown in Figure 1A. We found that the human cell-free extracts promoted efficient end joining of DNAs with 5′-phosphate or 5′-OH termini to form linear multimeric DNA species (Figure 1B, lanes c and f). Denaturing gel electrophoresis revealed that both strands were fully religated (data not shown). In contrast, T4 DNA ligase could only rejoin DNA molecules with 5′-phosphate termini (Figure 1B, compare lanes b and e). Time course experiments indicated that the human extracts catalysed comparable rates of rejoining with the 5′-phosphate or 5′-OH termini (Figure 2A and B), indicating that the phosphorylation of 5′-OH termini is not a rate-limiting step in this reaction.

Previously, it was shown that the rejoining of linear duplex DNA by human cell-free extracts was dependent upon the NHEJ factors, indicating the value of this *in vitro* system as a model for DSB repair (Baumann and West, 1998). To establish that the joining of 5′-OH termini was also dependent on the NHEJ proteins, we compared the end joining activity of extracts prepared from M059J (DNA-PK_{cs} defective) and M059K (control) cell lines, both of which derive from human malignant glioma biopsies (Lees-Miller *et al.*, 1995). In contrast to the M059K extract, which catalysed efficient end joining (Figure 3A, lane g), the DNA-PK_{cs}-deficient extract failed to promote multimer formation (lane h). Similarly, end joining by the GM00558 extract was inhibited by polyclonal antibodies raised against XRCC4 (Figure 3A, compare lanes e and f with lane b). All reactions were dependent upon the presence of Mg^{2+} and ATP co-factors (Figure 3A, lanes c and d). These results show that the rejoining of 5′-OH termini is dependent upon those factors previously shown to be involved in the NHEJ reaction *in vitro*.

The joining of protruding 5′-phosphate termini by human extracts is known to be accurate (Baumann and West, 1998). To determine whether 5′-OH termini were also joined accurately, the products of end joining were subjected to digestion with *Eco*RI, the same restriction enzyme that was used to initially linearize the plasmid DNA. For both 5′-phosphate and 5′-OH termini, we found that all end joining products were recut with *Eco*RI (Figure 3B, compare lanes b and c, and d and e). Thus, the

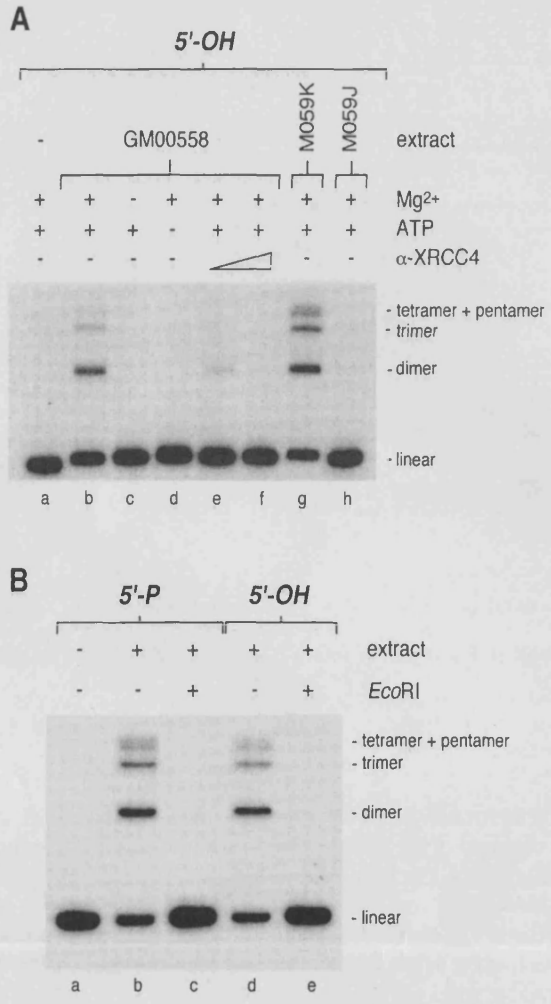

Fig. 3. Requirements and accuracy of extract-catalysed joining of 5′-OH termini. (**A**) Protein and co-factor requirements. *Eco*RI-linearized plasmid DNA with 5′-OH termini was incubated with cell-free extracts prepared from GM00558, M059K and M059J (DNA-PK_{cs} defective) cell lines, as described in the legend to Figure 1. Where indicated, Mg^{2+} or ATP was omitted from the end-joining buffer, or XRCC4 polyclonal antibodies (α-XRCC4) were added to a final dilution of 1:1250 or 1:250. (**B**) Accuracy of end joining. Large scale end-joining reactions (100 µl) containing *Eco*RI-linearized plasmid DNA (200 ng) [with 5′-phosphate (5′-P) or 5′-OH termini as indicated] and GM00558 extract (400 µg) were incubated for 2 h at 37°C. The DNA was deproteinized and aliquots (40 ng) incubated, with (+) or without (–) *Eco*RI as indicated, to recut the joint site. All products were separated by agarose gel electrophoresis and visualized by autoradiography.

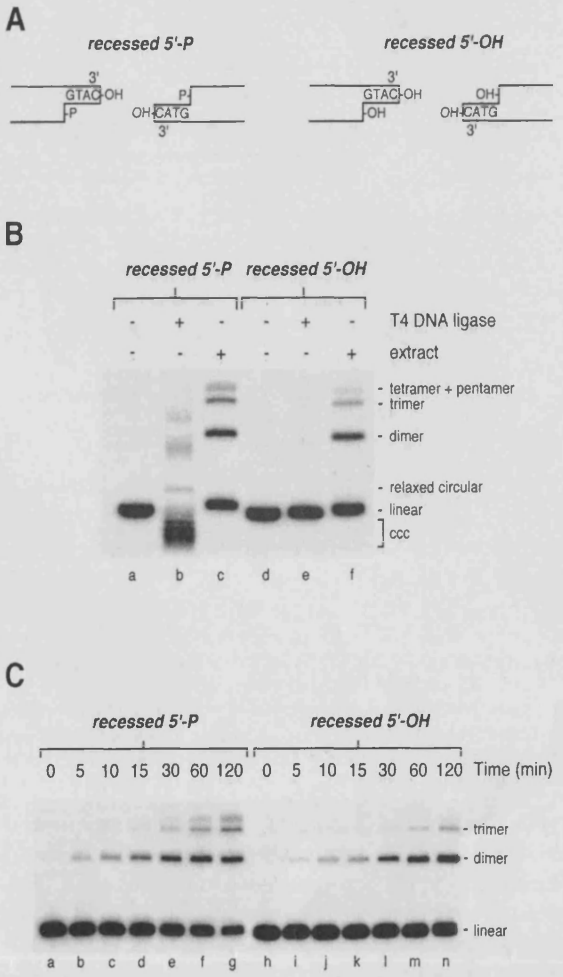

Fig. 4. End joining of recessed 5′-OH termini by human cell-free extract. (**A**) Schematic representation of termini. Plasmid DNA was linearized with *Kpn*I to produce recessed 5′-phosphate (5′-P) termini (left) and dephosphorylated with CIP giving 5′-OH termini (right). (**B**) End-joining reactions were carried out as described in the legend to Figure 1 using the recessed 5′-termini (5′-P) shown in (A). (**C**) Time course of end joining. Large scale reactions (160 µl) containing DNA with recessed 5′-phosphate (5′-P) or 5′-OH termini (320 ng) were incubated with cell-free extract and at the indicated times 20 µl samples were removed, deproteinized and analysed by agarose gel electrophoresis.

end joining reactions are accurate and there are no base losses or additions at the joint sites.

The experiments described above were carried out with protruding 5′-phosphate and 5′-OH termini produced by *Eco*RI digestion. Similar experiments were therefore carried out using *Kpn*I, which gives rise to 5′-recessed termini (Figure 4A). We found that human extracts, in contrast to the T4 DNA ligase control, were again capable of promoting the joining of both 5′-phosphate and 5′-OH termini (Figure 4B). However, the rate of end joining with the recessed 5′-OH termini was reduced slightly in comparison with the 5′-phosphate termini (Figure 4C). Moreover, the accuracy of end joining was reduced, with 18% of the joined 5′-OH termini resistant to restriction digestion with *Kpn*I compared with only 6% of the 5′-phosphate termini (data not shown). These results lead us to suggest that phosphate replacement at protruding termini occurs more efficiently than when the 5′-OH residue is recessed and the terminal base is paired with its complementary strand.

Replacement of the 5′-phosphate group is dependent upon active NHEJ

To determine whether the processing of 5′-OH termini could occur in the absence of end joining, a multistage assay was developed. *Eco*RI-linearized CIP-treated DNA with protruding 5′-OH termini was incubated with extracts, under conditions where NHEJ was inhibited, either by anti-XRCC4 antibodies or by the use of DNA-PK_{cs}-defective extracts (stage 1). The products of the reaction were then deproteinized and analysed for the presence of ligatable 5′-phosphate termini by incubation

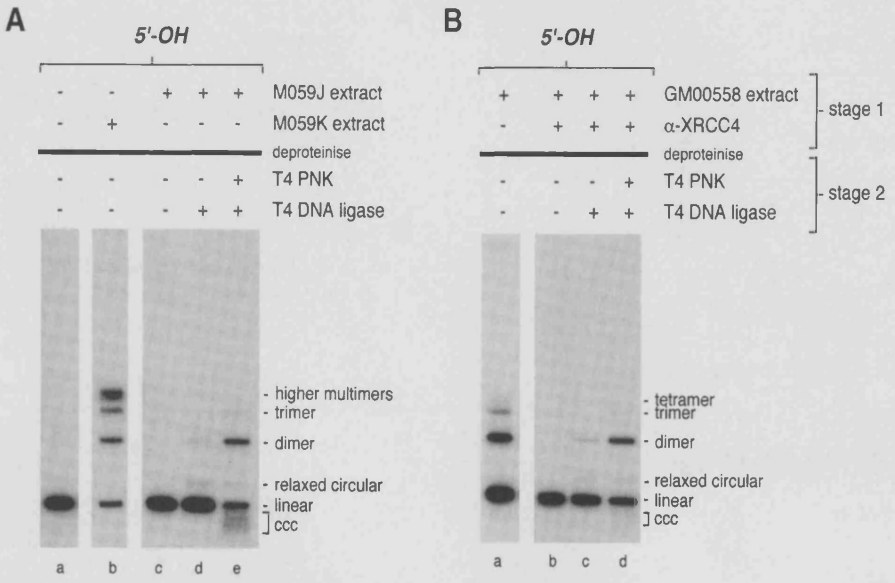

Fig. 5. Role for DNA-PK-dependent NHEJ in the processing of 5′-OH termini. (**A**) Requirement for DNA-PK_{cs} in the processing of 5′-OH termini. Uniformly ^{32}P-labelled *Eco*RI-linearized plasmid DNA with 5′-OH termini (120 ng) was incubated with M059K or M059J (DNA-PK_{cs} defective) extracts (240 μg) for 2 h at 37°C as indicated. The products were then deproteinized and aliquots of DNA (40 ng) were incubated alone, or with T4 DNA polynucleotide kinase (5 U) and/or T4 DNA ligase (80 U). (**B**) Requirement for XRCC4 in the processing of 5′-OH termini. End-joining reactions were carried out as described in (A) using GM00558 extracts, except that XRCC4 antiserum (α-XRCC4, 1:200 dilution) was added where indicated. Following deproteinization, aliquots of DNA (40 ng) were incubated with T4 PNK (5 U) and T4 DNA ligase (80 U) as shown. All DNA products were analysed by agarose gel electrophoresis and visualized by autoradiography.

with T4 DNA ligase (stage 2). The results are shown in Figure 5A and B. We found that DNA which had been incubated in extracts prepared from the DNA-PK_{cs}-defective cell line M059J served as a poor substrate for T4 DNA ligase (Figure 5A, lane d), but that ligation efficiency could be restored by inclusion of T4 polynucleotide kinase in the stage 2 reaction. These results show that conversion of the 5′-OH to a ligatable 5′-phosphate requires the NHEJ factor DNA-PK_{cs}. Similarly, we found that the addition of anti-XRCC4 antibodies to reactions catalysed by GM00558 extracts blocked phosphate addition to the 5′-OH termini, because we again found that the products of the stage 1 reaction were rejoined inefficiently by T4 DNA ligase (Figure 5B, lane c).

These results show that the inhibition of NHEJ, by inactivation of either DNA-PK_{cs} or XRCC4, affects the ability of the extract to promote the conversion of terminal 5′-OH groups to 5′-phosphates. Our interpretation of these data is that phosphate addition occurs by a mechanism that is dependent upon NHEJ, and that DNA kinase activity is coupled with DNA end joining.

Involvement of human PNK in NHEJ

Reasoning that human polynucleotide kinase is likely to be responsible for the observed kinase activity in NHEJ-competent cell-free extracts, antibodies raised against human PNK were used to immunodeplete PNK from GM00558 extracts. Western blotting confirmed that the majority of the PNK was depleted (data not shown). The PNK-depleted extracts were then assayed for their ability to promote the end joining of linear DNA molecules with 5′-OH termini. We found that the PNK-depleted extracts were severely compromised in their ability to promote multimer formation compared with a mock RAD51-depleted control (Figure 6A). Indeed, at the highest extract concentration used we observed only 7% end joining compared with >30% with the mock-depleted extract (Figure 6A, compare lanes e and j). Control experiments showed that the PNK-depleted extracts were capable of joining DNA molecules with 5′-phosphate termini, albeit with an efficiency that was less than the mock-treated control (data not shown).

To verify that the defect in end joining was caused by the absence of PNK, reactions containing the PNK-depleted extracts (50 μg) were supplemented with purified human PNK (Figure 6B, lanes a–e). We found that ~25 ng purified PNK restored normal end-joining efficiency. An analysis of the amount of PNK in an extract by quantitative western blotting showed that 50 μg of cell-free extract contained ~9 ng of PNK (data not shown), indicating that the levels of PNK required for complementation were in the physiological range. In addition, we found that the efficiency of end joining could be stimulated significantly by the presence of excess PNK (Figure 6B, lane e).

The data presented in Figure 5 led us to suggest that the kinase activity on 5′-OH termini was, in some way, coupled with the process of NHEJ. Since addition of human PNK to a PNK-depleted extract restored end-joining activity, we next tested whether bacteriophage T4 PNK could also complement the reaction. In contrast to human PNK, however, we found that T4 PNK failed to restore end-joining activity (Figure 6B, lanes f–j). These results show that the co-operation between PNK and the NHEJ machinery is species specific, and imply that a non-specific kinase such as T4 PNK is unable to access DNA termini bound by the human NHEJ factors.

Discussion

The primary conclusions of the work presented here are as follows: (i) DNA molecules with non-ligatable 5′-OH

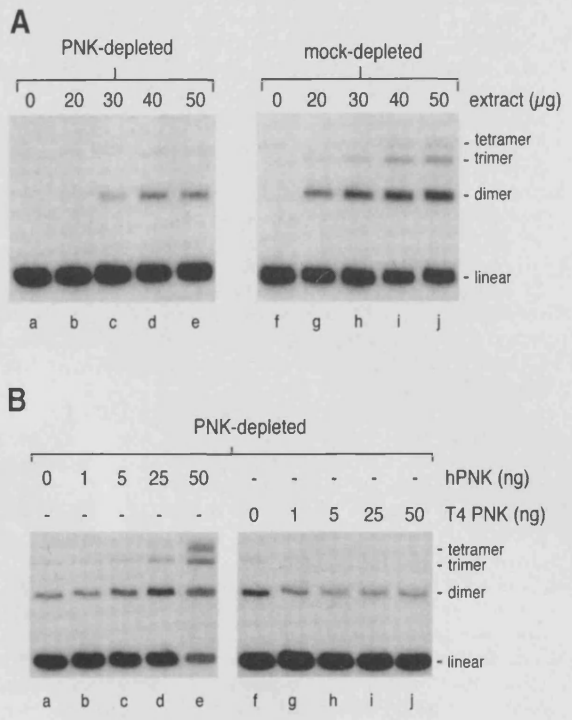

Fig. 6. End joining of 5′-OH termini exhibits a specific requirement for human polynucleotide kinase. (**A**) Immunodepletion of PNK. GM00558 extracts were immunodepleted for PNK or mock (RAD51) depleted using polyclonal antibodies. The depleted extracts were then analysed for their ability to promote end joining using *Eco*RI-linearized plasmid DNA with 5′-OH termini (20 ng). (**B**) Reconstitution of end-joining activity by complementation with human PNK. *Eco*RI-linearized plasmid DNA with 5′-OH termini (20 ng) was incubated with PNK-depleted extracts (50 μg) supplemented with the indicated amounts of purified human or T4 PNK.

termini can be efficiently processed by human cell-free extracts to render them ligatable; (ii) conversion of the 5′-OH moiety to 5′-phosphate is catalysed by polynucleotide kinase; (iii) conversion fails to occur when NHEJ is inhibited by inactivation of either XRCC4 or DNA-PK_{cs}; and (iv) T4 PNK cannot substitute for human PNK in reconstitution experiments with PNK-depleted extracts. Taken together, these data lead us to suggest that the processes of phosphate addition and DNA end joining occur by a mechanism that is inter-dependent, i.e. NHEJ cannot occur without phosphorylation, and phosphorylation cannot occur without proper assembly of the NHEJ end-binding proteins. While these studies provide the first direct evidence for a role of PNK in NHEJ, the results are not entirely unexpected in light of observations showing that *S.pombe* mutants carrying *pnk1* mutations are sensitive to ionizing radiation and yet grow normally in the absence of DNA damage (Meijer *et al.*, 2002).

A role for PNK in the repair of 5′-OH groups at single-strand DNA breaks has already been established (Jilani *et al.*, 1999; Whitehouse *et al.*, 2001). Of particular interest are observations that human PNK interacts with XRCC1, a protein which associates with DNA ligase III and is required for single-strand break repair and genome stability (Thompson *et al.*, 1990; Thompson and West, 2000). This interaction is likely to facilitate the targeting of PNK to single-strand break sites, where PNK, DNA polymerase β, XRCC1 and DNA ligase III promote gap filling and repair. In addition, XRCC1 has been shown to stimulate both the 5′-kinase and 3′-phosphatase activities of PNK (Whitehouse *et al.*, 2001).

The present study indicates that PNK is also required for double-strand break repair. Moreover, the evidence provided here indicates that the NHEJ apparatus not only accommodates PNK, but that there may be specific interactions between the factors involved in this repair process. Indeed, in preliminary studies that will require further analysis, we note that PNK (mol. wt 57.1 kDa), co-elutes during phosphocellulose and gel filtration chromatography with Ku70/80 and DNA ligase IV in a high molecular weight complex of >500 kDa (data not shown). This association, which was observed during gel filtration at 100 mM KCl, may be quite weak since it is lost at high salt conditions in which Ku70/80 elutes in the position expected of a heterodimer and PNK elutes in its monomeric form (data not shown).

At the present time, little is known about the detailed mechanism of NHEJ in mammalian cells. The reaction involves DNA-PK (consisting of the protein kinase DNA-PK_{cs} and end-binding protein Ku70/80) and the DNA ligase IV/XRCC4 heterodimer (Chu, 1997). The Ku70/80 heterodimer associates with DNA termini and facilitates DNA–DNA interactions, leading to end-bridging (Bliss and Lane, 1997; Ramsden and Gellert, 1998). At this stage, Ku70/80 is thought to cradle the DNA and confine its movement to a helical path that leads to alignment of the two DNA ends (Walker *et al.*, 2001). The fit to major and minor groove contours is steric, and few contacts are made either to the DNA bases or to the sugar phosphate backbone. Thus, the shape of the DNA binding site on Ku appears well designed to structurally support but not obscure DNA ends. Upon DNA-PK_{cs} binding, Ku shifts inwards ~10 bp and DNA-PK_{cs} itself adopts a position at the DNA end (Yoo and Dynan, 1999). We therefore suggest that the end-binding complex containing Ku70/80 and DNA-PK_{cs} can recruit and accommodate both DNA ligase IV/XRCC4 complex (Chen *et al.*, 2000; McElhinny *et al.*, 2000) and end-modifying activities such as PNK. Access to the DNA may be facilitated by the relatively small size of PNK and/or associations with the other factors of the NHEJ apparatus.

Materials and methods

Cell lines

The lymphoblastoid cell line GM00558 was obtained from the NIGMS human genetic mutant cell repository, and the glioblastoma lines M059J and M059K were generous gifts from Dr Susan Lees-Miller (University of Calgary, Canada).

Cell-free extracts

Whole-cell extracts were prepared essentially as described previously (Baumann and West, 1998). Typically, 6–10 l of cells were harvested, washed twice in iced phosphate-buffered saline and once in hypotonic lysis buffer [HLB; 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT)]. The pellet was resuspended in 1 vol. HLB and incubated on ice for 20 min. The cells were lysed by homogenization using a B dounce pestle in the presence of a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, pepstatin, chymostatin and leupeptin). After 20 min, 0.5 vol. hypertonic lysis buffer (50 mM Tris–HCl pH 7.5, 1 M KCl, 2 mM EDTA, 2 mM DTT) was added and the suspension was centrifuged for 3 h at 37 000 r.p.m. in a Beckman SW41-Ti rotor (at 4°C). The supernatant was removed carefully to avoid the upper lipid and lower nucleic acid containing regions, and dialysed for 2 h

against buffer E (20 mM Tris–HCl pH 8.0, 100 mM KOAc, 20% glycerol, 0.5 mM EDTA, 1 mM DTT). Protein concentrations were determined by Bradford assays, and the extract fast-frozen and stored at –70°C.

Proteins and DNA

T4 polynucleotide kinase and T4 DNA ligase were purchased from NEB. Human PNK was purified as described previously (Mani *et al.*, 2001). The 5.5 kbp plasmid $pACSG_2$ (PharMingen) was amplified in DH5α in the presence of [^{32}P]orthophosphate and the DNA prepared using Qiagen Maxi plasmid purification kits. Uniformly ^{32}P-labelled form I DNA was linearized with *Eco*RI (to produce protruding 5′-termini) or *Kpn*I (to produce recessed 5′-termini). Dephosphorylation was carried out by treatment with CIP.

End-joining assay

DNA end-joining reactions (20 µl) typically contained 40 ng of uniformly ^{32}P-labelled plasmid DNA and 80 µg of cell-free extract in 50 mM HEPES pH 8.0, 40 mM KOAc, 1 mM $Mg(OAC)_2$, 1 mM ATP, 1 mM DTT, 100 µg/ml bovine serum albumin. Reactions were incubated for 2 h at 37°C, except where indicated. ^{32}P-labelled products were deproteinized and analysed by electrophoresis through 0.6% agarose gels followed by autoradiography or phosphoimaging.

Antibodies and immunodepletions

Polyclonal antibodies against human PNK (Karimi-Busheri *et al.*, 1999), XRCC4 (Critchlow *et al.*, 1997) and RAD51 (Davies *et al.*, 2001) have been described elsewhere. For immunodepletions, cell-free extracts (150–200 µl) were incubated with polyclonal antibodies raised against PNK or RAD51 at a concentration of 1 µl antibody/10 µl extract for 2 h at 4°C on a rotary wheel. Protein A beads (Pharmacia) were pre-washed and resuspended in buffer E and aliquots (100–120 µl) added to the extract. After 1.5 h at 4°C, the beads were pelleted by low-speed centrifugation, and the supernatant was carefully removed, stored in aliquots at –70°C and used in end-joining assays.

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