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Review

Non-homologous DNA end joining✪

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DNA double-strand breaks (DSBs) are a serious threat for the cell and when not repaired or misrepaired can result in mutations or chromosome rearrangements and eventually in cell death. Therefore, cells have evolved a number of pathways to deal with DSB including homologous recombination (HR), single-strand annealing (SSA) and non-homologous end joining (NHEJ). In mammals DSBs are primarily repaired by NHEJ and HR, while HR repair dominates in yeast, but this depends also on the phase of the cell cycle. NHEJ functions in all kinds of cells, from bacteria to man, and depends on the structure of DSB termini. In this process two DNA ends are joined directly, usually with no sequence homology, although in the case of same polarity of the single stranded overhangs in DSBs, regions of microhomology are utilized. The usage of microhomology is common in DNA end-joining of physiological DSBs, such as at the coding ends in V(D)J (variable(diversity) joining) recombination. The main components of the NHEJ system in eukaryotes are the catalytic subunit of DNA protein kinase (DNA-PK$_{cs}$), which is recruited by DNA Ku protein, a heterodimer of Ku70 and Ku80, as well as XRCC4 protein and DNA ligase IV. A complex of Rad50/Mre11/Xrs2, a family of Sir proteins and probably other yet unidentified proteins can be also involved in this process. NHEJ and HR may play overlapping roles in the repair of DSBs produced in the S phase of the cell cycle or at replication forks. Aside from DNA repair, NHEJ may play a role in many different processes, including

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Abbreviations: DNA-PK$_{cs}$, DNA protein kinase catalytic subunit; DSBs, DNA double-strand breaks; HR, homologous recombination; LigIV, DNA ligase IV; MDS, multiply damaged sites; NHEJ, non-homologous end joining; PNK, polynucleotide kinase; SCID, severe combined immunodeficiency; SSA, single-strand annealing; V(D)J, variable (diversity) joining; WRN, Werner syndrome helicase.

the maintenance of telomeres and integration of HIV-1 genome into a host genome, as well as the insertion of pseudogenes and repetitive sequences into the genome of mammalian cells. Inhibition of NHEJ can be exploited in cancer therapy in radio-sensitizing cancer cells. Identification of all key players and fundamental mechanisms underlying NHEJ still requires further research.

DNA double-strand breaks (DSBs) result from disruption of the phosphodiester backbone on both strands of the DNA double helix. They may be induced by ionizing radiation, some chemicals, including anticancer drugs, or arise spontaneously during DNA replication. Endogenous free radicals – by-products of oxidative metabolism – can also generate DSBs. They may also result from programmed action of endonucleases or can occur as intermediates in DNA excision repair. Furthermore, DSBs arise normally as intermediates in V(D)J recombination, the process that helps to generate the vast range of antigen-binding sites of antibody and T-cell receptor proteins during mammalian lymphoid-cell development. Other sources of DSBs are meiotic recombination, switching of mating types in yeast and transposition of certain mobile elements. A mammalian cell suffers at least 10 spontaneous DSB per day (Haber, 2000).

Although DNA ends generated by DSBs constitute topologically the same category of DNA termini as natural ends of linear chromosomes (telomeres in eukaryotes), they are differently treated by the cell.

REPAIR OF DOUBLE-STRAND DNA BREAKS – THE KEY TO GENOMIC INTEGRITY

DNA double-strand breaks are critical lesions which, if unrepaired or misrepaired, may be lethal for a cell or make it transform to a cancer cell. This is particularly important when associated with abnormal regulation of the cell cycle and may give rise to cancer or hereditary diseases. Cancer is very often associated with the accumulation of genomic aberrations including large deletions, inversion or translocations, which may be consequences of DSBs (Lengauer *et al.,* 1998).

In response to this threat cells have developed a variety of DNA repair mechanisms for restoration of the break site. These include in general two primary pathways involving either homologous or non-homologous recombination (Paques & Haber, 1999). More precisely, three main mechanisms of DSBs repair can be considered: homologous recombination (HR) repair, single-strand annealing (SSA) and non-homologous end joining (NHEJ). These mechanisms are conserved in evolution, but have different contribution to overall DSBs repair in lower and higher eukaryotes. In mammals DSBs are primarily repaired by NHEJ and HR, while HR repair dominates in yeast. The relative contribution varies during development and depends also on the stage of the cell cycle. During early development and in the G_2-phase of the cell cycle, a higher contribution of HR in DSB-repair is observed.

For a long time NHEJ has been regarded as the major mechanism for DSB repair in mammals. But recently it has become clear that also HR has an important role in such repair, particularly in overcoming replication-specific breaks. In mammalian cells, approx. 30–50% of breaks created by the site-specific I-*Sce*I endonuclease can be repaired by HR with the rest repaired by NHEJ (Liang *et al.,* 1998).

Repair by HR involves interaction between the broken DNA molecule and its intact sister chromatid and is mediated by proteins encoded by genes in the *Rad52* epistasis group. An early step in this process involves resection of the site of DSB to produce 3′-extended single-stranded overhang. This newly exposed single strand serves to load recombination proteins that promote homologous recogni-

tion and strand invasion, which leads to DNA resynthesis and crossover resolution (Thompson & Schild, 2001).

SSA can be considered a special form of HR repair and was first reported to explain the results of intramolecular recombination in plasmid substrates introduced in *Xenopus laevis* oocytes or mammalian cells (Lin *et al.,* 1990; Maryon & Carroll, 1991). SSA is dependent on the presence of repeated sequences flanking DSBs. On resection of the 5' termini and exposure of stretches of homology, formation of a joint molecule may take place. After removing of the non-homologous ends and DNA synthesis by a DNA polymerase, DSB repair is completed by a DNA ligase. This mechanism has a non-conservative character, because sequences between regions of the homology are lost. SSA can occur in competition with gene conversion, during which the two resected ends of DSBs invade and copy sequences from a homologous template located on a sister chromatid, a homologous chromosome or at an ectopic location. The molecular mechanism by which gene conversion occurs is not precisely known (Paques & Haber, 1999).

DSBs can also be repaired by several non-homologous repair mechanisms, in which the DNA ends are joined with little or no base pairing at the junction (Haber, 2000). Non-homologous end joining in yeast and in mammals requires the same core set of proteins: the DNA end-binding proteins Ku70 and Ku80, as well as DNA ligase IV and its associated XRCC4 protein. Vertebrate cells also require DNA-PK$_{cs}$, for which no homologue has been found in yeast. In budding yeast there is also a requirement for Rad50, Mre11 and Xrs2, three proteins that have endo- and exonuclease activity, although there is also a Mre11-independent pathway of chromosome rearrangement (Chen & Kolodner, 1999). When the ends of DSBs have short complementary overhangs, yeast cells readily promote their re-ligation by NHEJ, effectively competing with HR repair. However, when the ends are not complementary, NHEJ in budding yeast is much less effective, succeeding on average only in 2 in 1000 cells (Haber, 2000). In contrast, mammalian cells can use NHEJ to join ends of all types. The basis of this difference is not known.

Deficiencies in DSB repair are associated with hereditary diseases such as Nijmegen breakage syndrome, ataxia-telangiectasia, Bloom's syndrome and breast cancer (Venkitaraman, 1999; Karow *et al.,* 2000; Wu *et al.,* 2000).

NON-HOMOLOGOUS END JOINING — FROM MAN TO BACTERIUM

NHEJ in mammalian cells

NHEJ seems to be the primary mechanism of DSBs repair in mammalian cells (Jeggo *et al.,* 1998). In contrast to HR, this pathway does not require homology and can rejoin broken DNA ends directly end-to-end. Genetic studies, using radiosensitive mammalian cell lines defective in DSBs rejoining and with mutations in genes that encode components of NHEJ, have been useful in identifying several proteins involved in the DSB repair process (Jeggo *et al.,* 1991; Lees-Miller *et al.,* 1995; Gu *et al.,* 1997; Riballo *et al.,* 1999). It has been shown that the *XRCC5, XRCC6* and *XRCC7* genes encode the DNA-dependent protein kinase DNA-PK, *XRCC5* and *XRCC6* encode the 80 and 70 kDa subunits of the Ku70/80 heterodimer (the DNA-binding subunit of DNA-PK), and *XRCC7* encodes the 470 kDa DNA-catalytic subunit of protein kinase DNA-PK$_{cs}$ (Weaver *et al.,* 1996; Chu *et al.,* 1997; Critchlow & Jackson, 1998). The XRCC4/DNA ligase IV heterodimer is also required (Critchlow *et al.,* 1997; Grawunder *et al.,* 1997; 1998). Although the exact role of these proteins in the repair process remains unknown, possible three steps have been suggested for the repair of DSBs *via* NHEJ: (i) end-binding and bridging, (ii) terminal pro-

cessing, and (iii) ligation (Fig. 1). In the initial step Ku binds the DNA ends (the end-binding activity of Ku suggests that it may be the primary damage detector in NHEJ), aligns them and thus prepares for ligation and protects from degradation (Cary *et al.,* 1997; Pang *et al.,* 1997; Yaneva *et al.,* 1997; Feldmann *et al.,* 2000). Ku also recruits DNA-PK_{cs} to the DSBs, activating its kinase function. Although the exact target proteins of DNA-PK_{cs} remain to be established, it has been proposed that DNA-PK_{cs} can: (i) phosphorylate XRCC4 and remove or relocate the ligase IV/XRCC4 complex from Ku-bound DNA ends and thus allow necessary processing steps to occur, (ii) regulate accessibility of DNA ends to processing by its inactivation *via* autophosphorylation and/or by allowing translocation of Ku away from the DSB, and (iii) phosphorylate both Ku70 and Ku80 with as yet unknown consequences yet (Chan *et al.,* 1996; Calsou *et al.,* 1999; Chan *et al.,* 1999; Nick McElhinny *et al.,* 2000). Non-homologous end-joining of more complex non-complementary ends requires their terminal processing *via* nucleolysis and polymerization before the final ligation step. Although the precise roles of the polymerases and nucleases in the DNA end processing is not completely clear, it has been suggested that nucleases remove several nucleotides from single-stranded overhangs at the DSB ends termini, especially when a region of microhomology occurs, and DNA polymerases fill in gaps of several nucleotides (Fig. 2). The nuclease complex Mre11/ Rad50/ Nbs1 can also participate in this second step of NHEJ, as well as in damage signaling and protection of the ends from degradation (Paull *et al.,* 1998; Khanna *et al.,* 2001). Other proteins involved in end-processing can be exonuclease FEN-1 and the unwinding enzymes WRN (Werner syndrome helicase) and BLM (Bloom syndrome helicase) (Wu *et al.,* 1999; Wilson *et al.,* 2003). In mammals, DNA polymerases of the PolX family would make NHEJ more efficient by allowing gaps to be filled in during the rejoining of the two non-complementary ends. Indeed, a physical interaction between Ku, the XRCC4 and ligase IV–polymerase μ complex has been reported (Mahajan *et al.*, 2002). Finally, during the third step of NHEJ, the tight complex of DNA ligase IV with XRCC4, which has been recently shown to form a tetramer, is recruited to ligate the DSBs (Junop *et al.,* 2000; Lee *et al.,* 2000; Sibanda *et al.,* 2001).

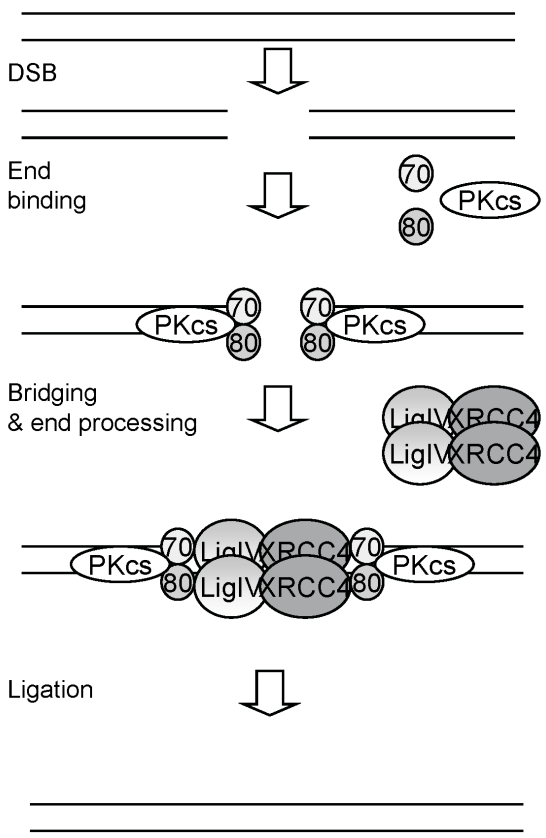

Figure 1. Repair of DNA double strand breaks (DSBs) by non-homologous end joining in mammalian cells.

PK_{cs}, the catalytic subunit of DNA–protein kinase; LigIV, DNA ligase IV; Ku70 and Ku80, Ku70, Ku80 proteins, respectively; XRCC4, X-ray cross complementation 4.

Numerous studies are directed to identify other components involved in NHEJ. Recent research using human radiosensitive SCID (severe combined immunodeficiency) cells identified mutations in a novel DNA DSB repair protein, named Artemis, and showed its

importance in lymphoid differentiation in bone marrow (Moshous *et al.,* 2001, Lieber *et al.,* 2003; Noordzij *et al.,* 2003). Artemis can be involved in DNA end processing due to its 5′ exonuclease activity (Ma *et al.,* 2002). However, a complex of Artemis and DNA-PK_{cs} has a 5′ and 3′ overhang endonuclease cleavage activity. This enzyme can trim 5′ overhangs with a strong preference for the site that blunts the end. In contrast, 3′ overhangs are trimmed with a preference to leave a four- or five-nucleotide single-stranded overhang. The first demonstration of human polynucleotide kinase (PNK) involvement in NHEJ *via* modification of DNA termini was also provided recently (Chappell *et al.,* 2002). When pathological breaks are generated by ionizing radiation or reactive oxygen species, the 5′ and 3′ ends formed are different than the ligatable 5′ phosphate and 3′ hydroxyl. PNK promotes phosphorylation of the 5′ ends, if needed. The efficient repair of non-ligatable ends was dependent on the presence of polynucleotide kinase, but only when other NHEJ proteins were present. It was also found that a non-protein factor, inositol phosphate (IP_6), can specifically interact with human Ku70/80 and stimulate end-joining *in vitro* (Hanakahi *et al.,* 2000; Hanakahi *et al.,* 2002). Involvement of a new factor in NHEJ and V(D)J recombination was reported in SCID a patient with a complete absence of T and B lymphocytes (Dai *et al.,* 2003). This patient's cells were highly radiosensitive and expressed de-

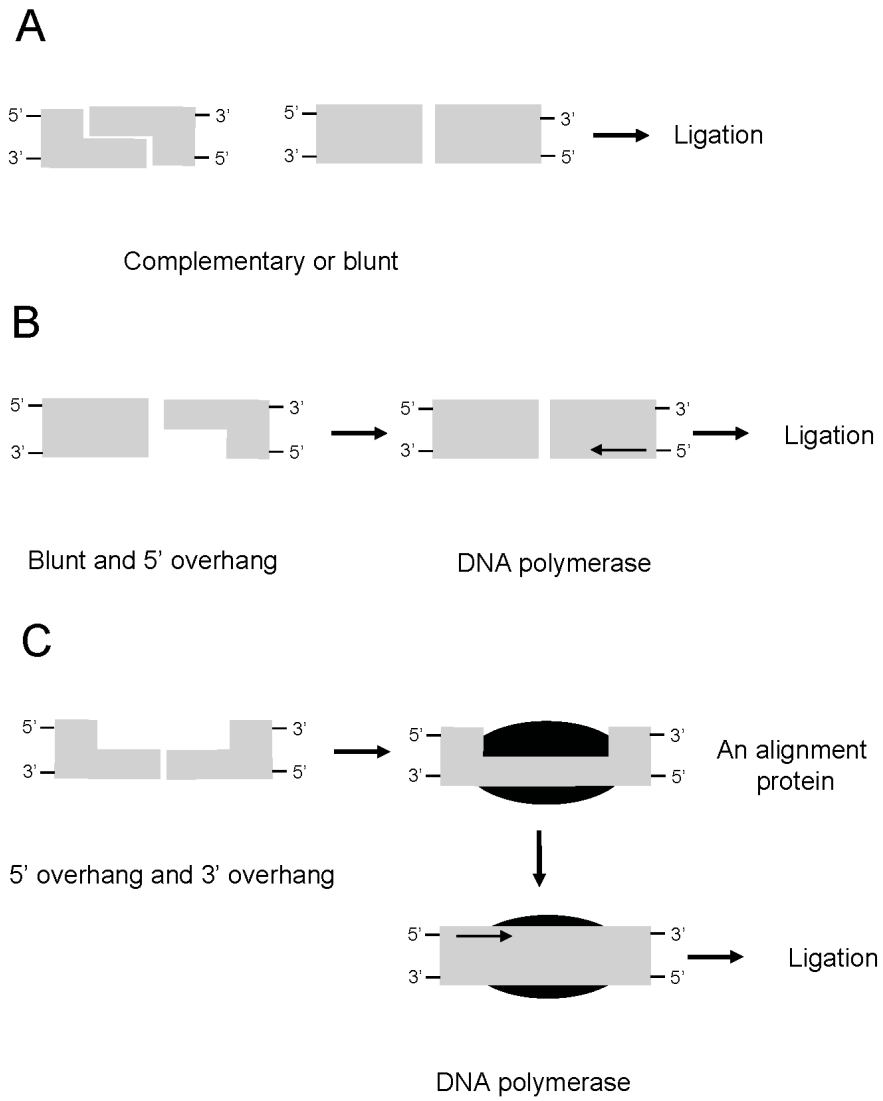

Figure 2. End-processing steps in non-homologous end joining of different structures of DNA ends produced by restriction enzymes.

A. Complementary or blunt ends can be repaired by a single ligation step. **B.** Blunt and 5' overhang ends need an additional step of fill-in synthesis to create two blunt termini, which can be directly ligated. **C.** 5' overhang and 3' overhang ends require the involvement of an alignment protein, which stabilizes the termini and permits DNA synthesis by polymerase and, finally, ligation.

fects in both DSB repair and V(D)J recombination. However, no defects in the known NHEJ components (Ku70/80, DNA-PK$_{cs}$, LigIV, XRCC4 and Artemis) were found, indicating that an additional factor is needed in the NHEJ pathway.

NHEJ in *Saccharomyces cerevisiae*

In yeast, NHEJ is a minor pathway of DSBs repair, with a strong dominance of HR. Gene-knockout studies, using the budding yeast *S. cerevisiae*, have revealed that most components of NHEJ have been conserved between yeast and mammalian cells (Critchlow & Jackson, 1998; Lewis & Resnick, 2000) (Table 1). Yeast homologues of human Ku70 (Yku70) and Ku80 (Yku80), as well as DNA ligase IV (Dnl4) and its associated cofactor XRCC4 (Lif1) have been identified and are required for efficient DNA end joining (Feldmann & Winnacker, 1993; Boulton & Jackson, 1996; Feldmann *et al.,* 1996; Milne *et al.,* 1996; Schar *et al.,* 1997; Teo & Jackson, 1997; Wilson *et al.,* 1997; Herrmann *et al.,* 1998; Teo & Jackson, 2000). So far, a yeast homologue of human DNA-PK$_{cs}$ has not been identified.

In *S. cerevisiae* the Rad50/Mre11/Xrs2 complex is also essential to NHEJ (Boulton & Jackson, 1996; Moore & Haber, 1996). Disruption of the *RAD50, MRE11* or *XRS2* genes impairs NHEJ in the same degree as disruption of *YKU70, YKU80 or DNL4*. However, recent studies, using the fission yeast *Schizosaccharomyces pombe* and vertebrates, suggest that end joining may be independent of this nuclease complex and that these enzymes are not conserved in all eukaryotes (Harfst *et al.,* 2000; Manolis *et al.,* 2001).

Deletions of the *SIR2, SIR3* or *SIR4* genes, which are involved in transcriptional silencing in yeast, may reduce the level of NHEJ (Tsukamoto *et al.,* 1997). The findings that Sir proteins are present at DNA damage sites and interact with Ku indicate that they might influence the accessibility of the broken ends to DNA processing enzymes and/or to the Yku70/80 complex in NHEJ (Martin *et al.,* 1999; Mills *et al.,* 1999).

Table 1. Proteins involved in non-homologous end joining DNA repair pathway in bacterial, yeast and human cells

Bacteria	Yeast	Human
YkoU	Yku70/80	Ku70/80
_____	_____	DNA-PK$_{cs}$
SbcC/SbcD/—	Rad50/Mre11/Xrs2	Rad50/Mre11/Nbs1
_____	Sir2/Sir3/Sir4	Sir2A/—/—
YkoV	Dnl4	LigIV
_____	Lif1, Lif2	XRCC4
_____	Nej1	_____

Recently, a novel haploid-specific gene, *NEJ1,* non-homologous end joining regulator 1 was found to be involved in DSB repair in yeast (Kegel *et al.,* 2001). *nej1* mutants showed an 8-fold reduction in NHEJ efficiency compared with the wild type. Moreover, *dnl4/nej1* double mutants exhibited the same end joining activity as either single mutant. This observation indicates that the Dnl4 and Nej1 proteins are in one epistasis group in yeast. Nej1 was also required for efficient NHEJ even when DNA ligase IV associated cofactor Lif1 was already present in the nucleus at a high level. This suggests that Nej1 does not regulate the transport or stability of Lif1, as had been thought before. Taken to-

gether, these data show that the precise role of Nej1 in NHEJ remains unknown. A new factor, Lif2, interacting with Lif1 in two-hybrid system was identified in *S. cerevisiae* (Frank-Vaillant & Marcand, 2001). Lif2 was required for DSB repair by NHEJ because disruption of the *LIF2* gene decreased the capability of cells to repair to the same extent as *lif1* and *dnl4* mutants. However, in contrast to the genetic evidence, biochemical and physical interactions between Lif2 and the Lif1/Dnl4 complex should be verified by alternative methods.

NHEJ in bacteria

Recently, a DNA non-homologous end joining complex has been identified in bacteria (Weller *et al.*, 2002). Several earlier studies reported that *Bacillus subtilis* has a single homologue of the mammalian heterodimer Ku70/80 YkoU and a protein with very limited homology to DNA ligase IV YkoV (Aravind & Koonin, 2001; Doherty *et al.*, 2001; Weller & Doherty, 2001) (Table 1). Genetic studies, using *ykoU* and *ykoV* mutants showed that they were more sensitive to ionizing radiation than to UV or alkylating agents. That observation suggested that the YkoU and YkoV proteins were involved in a pathway that repairs ionizing radiation-induced DSBs. Moreover, this pathway seems to be different from HR, because recombination-deficient *recA* mutant bacteria and *ykoU* mutants were more radiosensitive than either single mutant. Unlike the Ku70/80 heterodimer in mammalian cells, Ku in *Mycobacterium tuberculosis* is a homodimer, which binds only to linear DNA ends (Weller *et al.*, 2002). It was also shown that bacterial Ku interacts directly with bacterial DNA ligase to promote end joining and exchange for either mammalian Ku or mammalian ligase IV does not decrease repair by NHEJ. These results indicate that although the NHEJ pathway is conserved from bacteria to man, the nature of the proteins involved in this process can be very different. The absence of DNA-PK_{cs} in bacteria indicates that this protein might have developed in a later stadium of evolution (Hiom, 2003). In the future it will be interesting to determine if the *Escherichia coli* ATP-dependent exonucleases SbcC and SbcD, homologues of Rad50 and Mre11 in eukaryotes, respectively, are involved in non-homologous end joining in bacteria (Connelly & Leach, 1996).

OVERLAPPING ROLES OF NON-HOMOLOGOUS END JOINING AND HOMOLOGOUS RECOMBINATION IN REPAIR OF DNA DOUBLE-STRAND BREAKS

NHEJ and HR have been shown to play overlapping roles in the repair of DNA double-strand breaks produced in the S phase of the cell cycle (Takata *et al.*, 1998; Arnaudeau *et al.*, 2001; Saintigny *et al.*, 2001). Both systems can act independently while having complementary roles in repairing DSBs (Boulton & Jackson, 1996; Milne *et al.*, 1996). In mammalian cells it was found that mutants defective in DSB rejoining were sensitive to ionizing radiation, but more resistant in late S/G_2 phase of the cell cycle (Jeggo, 1990; 1997). Taken together, these data suggest the existence of two mechanisms of DSB rejoining, one operating in late S/G_2 and the other operating throughout the cell cycle. To distinguish the roles of the two repair pathways in the cell cycle, cell lines deficient in Rad54 (involved in HR), and Ku (involved in NHEJ), were studied (Takata *et al.*, 1998). In contrast to wild-type cells, *RAD54*$^-$ mutants did not show increased radiation resistance in late S/G_2 phase, as was observed in a mammalian cell line deficient in Xrcc2 (Cheong *et al.*, 1994; Thompson, 1996; Liu *et al.*, 1998). This suggests that the Rad54-dependent recombinational pathway is preferentially used in this phase. On the other hand, *KU70* mutants exhibited elevated sensitivity to IR in G_1/early

S phase, as has been observed in mammalian cell lines deficient in the NHEJ pathway (Stamato *et al.*, 1988; Jeggo, 1990; Lee *et al.*, 1997), which suggests a predominant role of NHEJ at this stage of the cell cycle. Moreover, *RAD54/ KU70* mutant cells were more sensitive to γ-ray irradiation than either single mutant, indicating that these two pathways play a complementary role in the repair of DSBs in DT40 cells. The higher sensitivity of *RAD54* than *Ku70* mutants to radiation indicates that HR is preferentially used to repair the DSBs, while NHEJ can repair them as a back-up for HR, as has been observed in yeast (Feldmann *et al.*, 1993; Teo & Jackson, 1997).

The first genetic evidence that both pathways are involved not only in repair of DSBs, but also in the maintenance of chromosomal DNA during the cell cycle, was provided by Takata *et al.* (1998). This thesis was supported by the observation that spontaneous chromosomal aberrations, as well as spontaneous cell death, were detected in both *RAD54/KU70* and *RAD54* mutant cells, but the double mutant exhibited a much higher level of these DNA lesions. These findings suggest that HR and NHEJ play overlapping roles in chromosomal maintenance.

Restoration of stalled replication forks is essential for proper DNA synthesis. DSBs induced following DNA replication forks arrest in mammalian cells may be recognized by DNA repair proteins from both the HR and NHEJ pathways. HR seems to play a dominant role in such repair (Arnaudeau *et al.*, 2001; Saintigny *et al.*, 2001). It has been proposed that NHEJ, which is thought to be a faster repair pathway in mammals than HR, may be an early response to DSBs at replication forks and HR may be a late response (Saintigny *et al.*, 2001).

Recently, different roles of HR and NHEJ after replication arrest in mammalian cells have been shown (Lundin *et al.*, 2002). Cells deficient in either HR or NHEJ were hypersensitive to the toxic effects of hydroxyurea, while only cells deficient in HR were sensitive

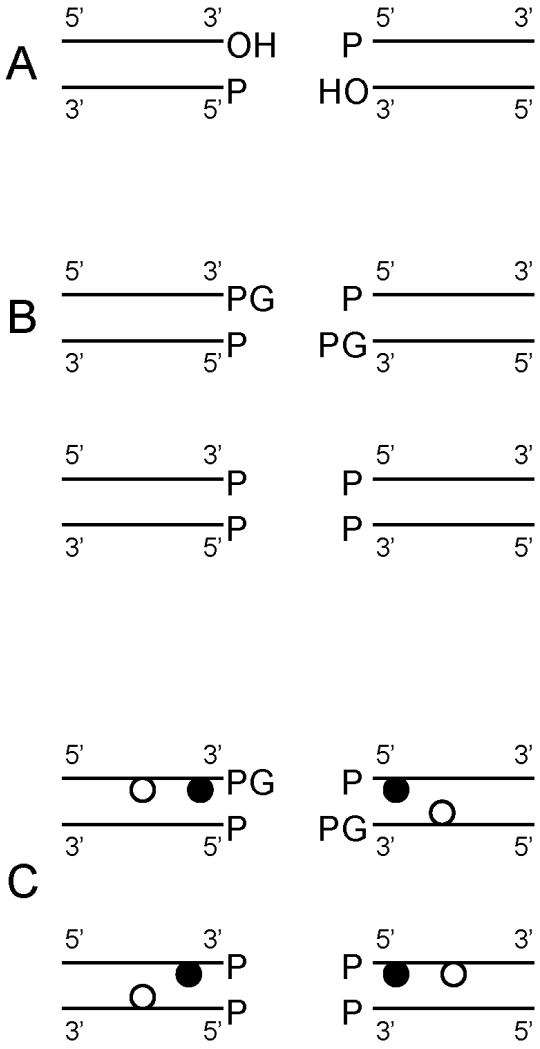

Figure 3. The range of potential DNA double-strand breaks complexity.

A. The simplest type of DSBs produced by restriction enzymes, which are blunt ended with ligatable 3′-hydroxyl (OH) and 5′-phosphate (P) ends. Breaks of this kind can be directly rejoined by a single enzyme, like DNA ligase. **B.** More complex type of DSBs produced by ionizing radiation, damaging agents, e.g. bleomycin or other oxidative processes. Breaks with 3′-phosphoglycolate (PG) or 3′-P blocked ends are non-ligatable and require removal of the 3′-PG or 3′-P moieties prior to ligation. **C.** The most complex type of DSBs known as multiply damaged sites (MDS), produced by high-LET radiation, e.g. Auger emitters like ^{125}I. MDS contain blocked non-ligatable ends, as well as nucleotide damage in the form of base loss (indicated by a filled circle) and/or chemically altered bases (indicated by an open circle).

to thymidine. These data indicate that both HR and NHEJ are required for survival of cells with replication forks arrested by hydroxyurea, but NHEJ was not required for the rescue of DNA replication forks slowed by thymidine. The possible explanation of these data is the involvement of HR and NHEJ in repair at replication forks only when DSBs are produced by hydroxyurea, while HR alone is required for the repair of slowed replication forks, when DSBs are not detectable after thymidine treatment. Finally, the exact nature of the lesions induced at arrested replication forks and determination of the repair proteins involved in this process need further investigations.

DEPENDENCE OF THE NON-HOMOLOGOUS END JOINING REACTION ON THE STATE OF DNA ENDS

The structure of the DSB end groups is an important feature of the mechanism underlying NHEJ because it can directly affect the pathway's ability to repair a break and determine the enzymatic requirements in the process of DNA end-joining. Figure 3 shows a range of potential DSB end structures. To investigate the dependence of the NHEJ reaction on the structure of DSB, restriction enzymes can be employed (Baumann & West, 1998; Feldmann *et al.*, 2000; Sandoval & Labhart, 2002). These enzymes can generate a variety of DSBs with blunt ends or 5′- or 3′-cohesive or non-cohesive (when more than one enzyme is employed) overhangs, the DNA ends being 5′-phosphorylated (5′-P) and 3′-hydroxylated (3′-OH) (Fig. 3A). Such DSBs are efficiently repaired, compared to 3′-phosphoglycolate (3′-PG) blocked ends (Fig. 3B), because they are substrates of DNA polymerases. The easiest type of DSBs for end joining are complementary and blunt ends. They can be directly rejoined by a single enzyme, like T4 DNA ligase (Fig. 2A). More complex, non-complementary 5′-overhangs need to be filled-in by a DNA polymerase before ligation (Fig. 2B) (King *et al.*, 1996). The most complex problem for the NHEJ machinery in studies using restriction enzymes is presented by joining of DNA ends with non-matching 3′-overhangs. This kind of termini should be first aligned by a DNA binding protein to permit ligation *via* fill-in DNA synthesis on the opposite gapped strand (Fig. 2C). What is the puzzling protein alignment factor in NHEJ of restriction endonuclease-induced DSBs? The obvious candidate is the Ku70/80 heterodimer. Support for this hypothesis was provided by recent *in vitro* studies showing that the rejoining between 3′- and 5′-overhanging ends was strongly reduced in Ku-depleted Chinese hamster egg extracts, as well as in Ku80-deficient cell extracts (Labhart, 1999; Feldmann *et al.*, 2000). On the other hand, DNA-PK_{cs}-defective cells displayed a defect in repair of more complex DNA ends while single ligation was not affected (Smith *et al.*, 2001). This can be an evidence for a DNA-PK_{cs} dependent pathway for repair of more complex restriction endonuclease-induced DSBs.

At least two important features of DSBs repair can be considered: efficiency and fidelity. Eukaryotic cells extracts have been reported to be particularly inefficient in end joining of blunt-ended substrates *in vitro* (Ganesh *et al.*, 1993; Baumann & West, 1998; Tomkinson & Mackey, 1998; Chen *et al.*, 2000). However, *in vivo* studies have surprisingly shown that the efficiency was similar for all kinds of DSBs induced by restriction enzymes (Smith *et al.*, 2001). The fidelity of this kind of repair was influenced by the structure of DSB termini. Similar levels of error-free rejoining (without loss or gain of nucleotides) were observed for complementary and blunt ends, while DSBs with non-complementary overhangs displayed a significantly lower level of accurate repair.

Most naturally occurring DSBs are produced by oxidative processes, ionizing radia-

tion and DNA-damaging agents. The oxidatively induced DSBs are formed by loss of at least one nucleotide in each DNA strand at the break site. In contrast to DNA cut by a restriction enzyme, breaks of these types are not directly ligatable and need some form of nucleolytic processing (Chen *et al.*, 1991; Winters *et al.*, 1992; Gu *et al.*, 1996; Suh *et al.*, 1997). To study NHEJ repair using substrates similar to those expected *in vivo*, the radiomimetic drug bleomycin has been used (Chen *et al.*, 2001; Pastwa *et al.*, 2001). Bleomycin produces non-ligatable one-nucleotide gaps flanked by 5′-P and 3′- PG, similar to those produced by ionizing radiation (Dizdaroglu *et al.*, 1975; Henner *et al.*, 1983; Bertoncini & Meneghini, 1995; Błasiak, 2001) (Fig. 3B). The results showed that human cell extracts were able to rejoin bleomycin-induced DSBs, although at a reduced efficiency compared to the ends generated by restriction enzymes.

The most complex DSBs type is a multiply damaged site (MDS) (Fig. 3C) (Ward, 2000; Milligan *et al.*, 2001; Blaisdell & Wallace, 2001). MDS contains blocked non-ligatable ends, as well as nucleotide damage in the form of base loss and/or chemically altered bases. It was found that the efficiency of repair of ^{125}I-induced DSB, an example of MDS, was at least 10-fold lower compared to DNA cut with a restriction enzyme (Odersky *et al.*, 2002, Pastwa *et al.*, 2003). NHEJ was highly mutagenic in cell-free extracts *in vitro* and in mammalian cells transfected with ^{125}I-triplex forming oligonucleotides, generating deletions, base pair substitutions, and insertions at the junction site (Mezhevaya *et al.*, 1999; Odersky *et al.*, 2002).

All these findings indicate that with the increase of the structural complexity of DSBs, the ability of the NHEJ pathway to rejoin the break decreases. In some cases the difficulty with rejoining DNA ends of complex structure may be overcome by capturing a DNA filter at the site of joining, which can be an oligonucleotide or a retrotransposon (Roth & Wilson, 1998). The filter may be blunt ended and provide extra nucleotides, similar to the addition of nucleotides by terminal deoxynucleotidyl transferase during V(D)J recombination in lymphoid cells. DNA from the TyI retrotransposons can also be captured during NHEJ in yeast cells, suggesting that a similar mechanism may function for the insertion of pseudogenes and repetitive sequences into the genome of mammalian cells (Moore & Haber, 1996)

NON-HOMOLOGOUS END JOINING AND TELOMERES

Telomeres are DNA–protein complexes, with short tandem repeats (TTAGGG in humans) and a 3′G-strand overhang, that protect the DNA ends from end-to-end chromosome fusion. Chromosomal fusion can result in genomic instability and cancer. Replicative telomere shortening can impair their function during cellular aging and tumorigenesis. Telomere length is usually maintained by telomerase, and sometimes, when this enzyme is absent, by DSBs repair pathways (Goldman, 2003).

During the last few years it has been of great interest to find a role of DSB repair proteins in telomere maintenance. However, studies using NHEJ proteins gave surprising results. Research with knockout mice and mouse embryonic fibroblasts showed that the Ku70, Ku80, and DNA-PK$_{cs}$ proteins might associate with telomeric DNA, and they were important in telomere capping and protection of chromosome ends from fusion (Bailey *et al.*, 2001; di Fagagna *et al.*, 2001; Gilley *et al.*, 2001; Goytisolo *et al.*, 2001). Unfortunately, the exact role of Ku in this process remains enigmatic. Samper *et al.* (2000) showed the independence of telomere length and G-overhang structure of chromosome fusion and the presence of telomeric DNA at the fusion site, whereas di Fagagna *et al.* (2001) reported telomere shortening in Ku knockout mice and

the absence of telomeric DNA at the fusion site. A recent study using chicken DT40 knockout cell lines confirms the first results related to the lack of correlation between telomere length and the level of Ku70 expression (Wei *et al.*, 2002). However, in contrast to what was observed in Ku-deficient mice, it was found that Ku70 disruption did not result in end-to-end chromosome fusion. Those studies employed Ku-deficient chicken, whose telomeres are structurally very similar to mammalian telomeres in length. Finally, inhibition of the telomere-protective factor TRF2 was used to mimic critically shortened telomeres in mammalian cells (Smagorzewska & Lange, 2002; Karlseder *et al.*, 2002). It was found that these uncapped telomeres are processed by DNA ligase IV, a component of NHEJ, resulting in telomere fusion (Smagorzewska *et al.*, 2002). These controversial results regarding the role of DNA-PK and ligase IV in telomere maintenance show that NHEJ proteins may have a different role at chromosome ends than in within it.

NON-HOMOLOGOUS END JOINING AND ANTICANCER THERAPY

In the last decade combined chemo- and radiotherapy has been extensively studied for cancer treatment (Montana *et al.*, 2000; Santin *et al.*, 2000; Lawrence *et al.*, 2003). Cisplatin is one of the most effective chemotherapeutic agents in cancer therapy with multiple effects on cells, including adduct formation and DNA damage repair inhibition (Cohen & Lippard, 2001). This drug can radiosensitize tumor cells, which promotes its use in combined chemotherapy (Raaphorst *et al.*, 1994; Glass *et al.*, 1997; Geldof *et al.*, 1999). The increased cell killing by cisplatin before and after radiation seems to be mediated by a variety of mechanisms, including inhibition of DNA repair pathways and cell cycle arrest (Amorino *et al.*, 1999; Servidei *et al.*, 2001; Woźniak & Błasiak, 2002). However, the mechanism of the selectivity of cisplatin radiosensitization against cancer cells compared with normal cells has not been determined yet.

The first support of an involvement of an NHEJ protein, Ku80 in the mechanism of cisplatin and radiation interaction have come from murine leukaemic cells studies, showing cross-resistance to ionizing radiation and the drug (Frit *et al.*, 1999). Recently published data showed a relationship between Ku80 and Rad51 activites and the removal of cisplatin-DNA adducts (Turchi *et al.*, 2000; Słupianek *et al.*, 2002). It was found that cisplatin-DNA adducts inhibit translocation of Ku protein complex and decrease interaction between Ku and DNA-PK_{cs}, as well as decrease its kinase activity. These findings that cisplatin can inhibit the NHEJ pathway were helpful in the establishing mechanism of cisplatin radiosensitization (Myint *et al.*, 2002). It has been observed that using Ku80-deficient cell lines and wild type mammalian cells cisplatin treatments result in sensitization of the wild type to ionizing radiation *via* the inhibition of the NHEJ pathway.

An interesting approach to radiosensitization of cancer cells has come from studies using peptide co-therapy for inhibition of DNA-PK_{cs} activity (Kim *et al.*, 2002). It was shown that a peptide-based inhibitor decreased the growth of breast cancer after exposure to ionizing radiation. These data suggest that the peptide is a sensitizer for cancer cells due to blocking of the NHEJ pathway and confirm an important role of DNA-PK_{cs} in radiation and drug resistance of cancer cells.

CONCLUDING REMARKS

Understanding of the many aspects of NHEJ may help in fighting, aside from cancer, several other human diseases. This may also concerns AIDS, since the RNA genome of HIV-1 is reverse transcribed to generate a

cDNA copy, which is integrated into a chromosome of the host cell. Unintegrated viral cDNA is a substrate for the host NHEJ pathway (Li *et al.,* 2001). Therefore, the components of NHEJ may be a target of anti-HIV therapy.

We are at the beginning of understanding how the cell maintains a proper proportion between the competing mechanisms of DSBs repair. Results of studies on DSBs repair, especially NHEJ, may help to establish new approaches to ensure more accurate gene targeting in mammalian cells to prevent and fight severe disorders associated with DSBs repair.

There are still many important unanswered questions in the area of DSBs repair. Some of the central ones are: during the S and G2 phases, what controls whether a DSB is repaired by NHEJ or HR repair? Which proteins directly involved in NHEJ are tumor suppressors? What is the basis of the difference in the effectiveness of DSBs repair by NHEJ in mammals and budding yeast? The Ku heterodimer is required for proper maintenance of telomeres, which are believed not to exist as free DNA ends, but instead are kept in "T-loops", which may be stabilized by specific proteins and repetitive sequences. It will be interesting to determine how Ku interacts with telomeres in the absence of a free DNA end. Maybe it does this in concert with other molecules? The entire set of key players of NHEJ remains to be established.

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