DNA-Dependent Protein Kinase in Non-Homologous End-Joining: Guarding Strategic Positions

Eric Weterings

Cover:
The caduceus symbolizes an interplay between the alchemistic principles Sol and Luna, as well as the divine unity between heavens and earth. The combination of this ancient symbol with a DNA helix represents the progress of human knowledge from alchemy to modern molecular research. For the author of this thesis it also contains a distinct warning not to abandon spiritual appreciation of nature for an intellectual understanding of its manifestations, but rather to combine spirituality and intellectual curiosity.

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DNA-Dependent Protein Kinase in Non-Homologous End-Joining: Guarding Strategic Positions

DNA-Afhankelijk Proteïne Kinase in Niet-Homologe End-Joining: Bewaker van Strategische Posities

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Wat de mensen echt willen,
is geen kennis, maar zekerheid.

Bertrand Russell

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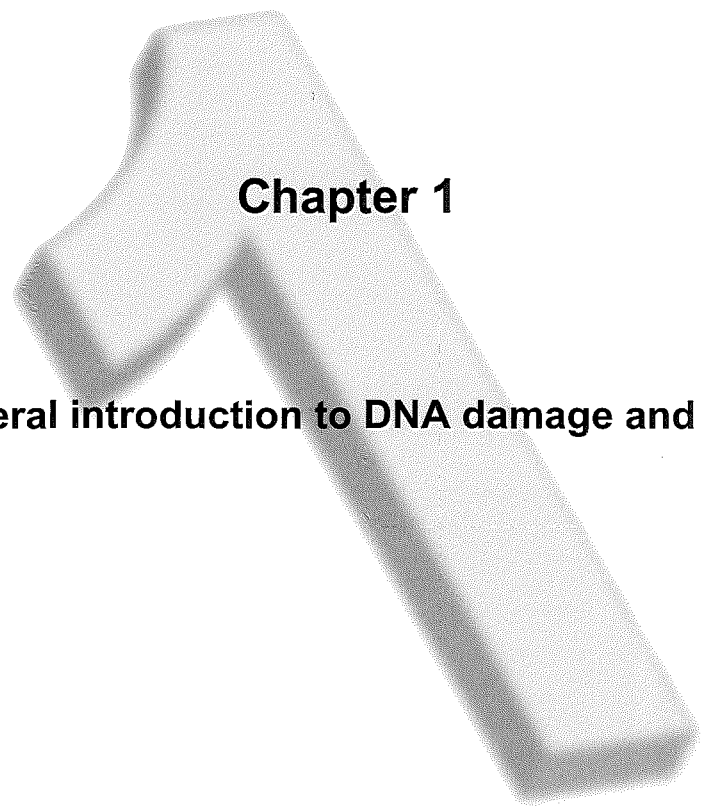
Aim and scope of the thesis

Careful maintenance of genetic information throughout generations is of vital importance to all living creatures. A battery of both endogenous and exogenous factors continuously threatens genetic integrity by altering the DNA chemistry. As a consequence, DNA damage types are as diverse as their causes. DNA double-strand breaks (DSBs) are among the most deleterious lesions, since they introduce chromosomal breakage or translocation and are able to trigger carcinogenesis. Perhaps even more importantly, DSBs may cause either cell death or permanent growth arrest. Fortunately, the mammalian cell has two effective DSB repair mechanisms at its disposal: homologous recombination (HR) and non-homologous end-joining (NHEJ). The research efforts presented in this thesis contribute to the understanding of the molecular mechanism of NHEJ in general, and the function of one of its core enzyme complexes, DNA-dependent protein kinase (DNA-PK), in particular.

Chapter 1 surveys the field of DNA damage and repair, briefly discusses the different types of DNA lesions, their impact on cellular function and the enzymatic machineries that have evolved in order to repair these lesions. Aim of this chapter is to place the subject of NHEJ, the theme of this thesis, in its context. **Chapter 2** is an in-depth review of relevant and recent knowledge about the mechanism of NHEJ, the enzymes that are involved in this process and the interplay between these enzymes. The next chapters present the experimental data that supports the conclusions of this thesis. **Chapter 3** describes the techniques that were used to isolate DNA-PK from human placenta or cultured cells and reports several improvements to the conventional extraction protocol. **Chapter 4** deals with the biochemical characteristics of DNA-PK when it is associated with DNA termini and reports a novel model in which DNA-PK autophosphorylation influences access of ligases or nucleases to the DNA termini. **Chapter 5** describes several biochemical assays that mimic *in vivo* NHEJ. These experiments show that DNA-PK phosphorylation status regulates *in vitro* end-joining by the NHEJ ligase complex, ligase IV/XRCC4. The chapter also includes experimental data on interplay between separate DNA-PK components and ligase IV/XRCC4. **Chapter 6** summarizes the conclusions of the experimental work and discusses their relevance for the NHEJ field.



Chapter 1

A general introduction to DNA damage and repair

Introduction

When Watson and Crick first postulated their model for the molecular structure of DNA in 1953, they immediately realized that the specific pairing of bases provides the DNA molecule with an excellent means of replication [1]. It is a major implication of this duplication mechanism, that the integrity of genetic material throughout generations depends on a faithful maintenance of base sequence and chromosome stability.

Since the 1960's an increasing amount of research has been done on the physical and chemical processes that lead to alteration of DNA [2]. Early work concentrated on the formation of pyrimidine dimers under the influence of UV radiation. Studies on UV sensitive and resistant *E. coli* strains revealed that pyrimidine dimers disappeared readily from the DNA in the resistant strains, whereas they remained present in the DNA of the sensitive strains. These experiments showed that DNA modifications can easily result in decreased cell viability. Even more importantly, they suggested an 'error-correcting' system that supported removal of these dimers in the resistant bacteria [2]. Apparently, this correcting system was not functioning efficiently in the UV sensitive bacterial strains. It was the first example of a DNA repair mechanism.

Pyrimidine dimers were the first DNA lesions studied in great detail, but many other damage types have been identified since that time. The molecular mechanisms of their formation and removal are subject to continuing investigation. DNA damages are generally classified as either spontaneous (endogenous) or environmentally induced, with the restriction that the cause of 'spontaneously' arising damages may well be environmental but simply not understood [3].

Endogenous DNA lesions arise from chemical alterations to the DNA, mediated by factors that are present or generated inside the cell. They include e.g. the lesions that can arise from the mispairing of bases or incorrect DNA repair. Metabolic products, such as reactive oxygen species and radicals, are important causes of DNA damage, since they can react with either the bases or the sugar-phosphate backbone of the DNA. These alterations are influenced by the pH, temperature and ionic strength of the intracellular or intracompartmental environment.

Environmental types of DNA lesions, in contrast, are caused by exogenous factors such as ionizing or ultraviolet (UV) radiation or genotoxic chemicals. These kinds of lesions may occur with a frequency that is several orders of magnitude lower than the frequency of damages caused by endogenous factors [3], but they include especially genotoxic types of damages such as interstrand cross-links and DNA strand breaks.

Cellular effects of DNA damage

The consequences of DNA lesions and mutations are as diverse as their causes. In general, a distinction can be made between acute and long-term responses [4].

Acute effects include induction of programmed cell death (also called apoptosis) and arresting progression of the affected cell through the cell cycle. Several types of lesions, especially pyrimidine dimers, stall the enzymatic machineries that perform DNA replication or transcription. Other damage types, DNA double-strand breaks, thwart segregation of chromosomes during cell division. Such genomic injuries disrupt normal DNA replication and cell division and necessitate cell cycle arrest until repair or removal of the lesion has been completed. When the damage cannot be effectively repaired, suicide of the cell may be the only safe option [5, 6].

Long-term effects include accumulation of unrepaired lesions, which can lead to mutations and attribute to the onset of cancer. A distinct link between a failure in DNA damage repair and carcinogenesis was made in the 1970's and 1980's. For example, fish thyroid cells that were irradiated with UV light to introduce pyrimidine dimers, gave rise to thyroid tumors when injected back into the animals [7]. In contrast, when repair of pyrimidine dimers was induced by photoreactivating illumination, the number of animals that developed a tumor dropped dramatically from 95% to 5%. These experiments, and numerous others performed since that time, show a close relationship between unrepaired DNA damage and the onset of cancer.

These days, cancer is envisioned as a disease that is caused by accumulation of mutations or lesions in the genome. Not every alteration in the genetic sequence will have a profound effect on cell function, since less than 5% of the genome codes for protein sequences. On the other hand, even small mutations in an essential gene can lead to changes in the activity of the protein it encodes. When such a gene is essential for regulation of cell cycle progression or differentiation (in the case of proto-oncogenes or tumor-suppressor genes), impairment or alteration of its function possibly induces tumorigenesis.

In summary, the prognosis for cells that suffer from corrupted genomic integrity is rather poor. Inability to remove transcription or replication stalling lesions leads to apoptosis. Accumulation of mutations may have long-term effects on cellular function and attributes to the formation of cancer. Fortunately, all known cells have a set of countermeasures available, each one specifically equipped to remove a certain type of lesion. These repair mechanisms in general use a set of enzymes that are able to function as molecular machines, performing specific tasks like placing incisions in the DNA or adding or removing DNA fragments. DNA damage repair mechanisms are usually conserved from prokaryotes to eukaryotes, clearly showing the universal importance of genome integrity maintenance. This importance is further stipulated by the fact that defects in these DNA repair pathways, caused by deficiency of a repair enzyme, usually predispose an organism to various cancers [4].

Types of DNA lesions and their repair

Repair of base alterations: base-excision repair

Base-excision repair (BER) is specialized in the repair of small chemical alterations

to the nucleotide bases, which are usually caused by endogenous factors, such as the reactive oxygen species that result from normal cellular metabolism. Frequently occuring lesions include methylated and deaminated bases and abasic sites that are formed upon naturally occuring loss of base residues. These lesions do not always lead to impairment of replication or transcription, but they may induce miscoding of the affected base. BER is therefore an important countermeasure against mutagenesis. The core BER reaction constitutes removal of the affected nucleotide by the action of damage specific glycosylases, followed by nuclease mediated strand incision at the created abasic site and a gap-filling reaction of one or a few nucleotides.

Repair of mismatched bases: mismatch repair

During normal replication, nucleotides are not always correctly matched to the template nucleotide by DNA polymerases. In addition, short fragments can be either added or deleted during replication of repetitive sequences, due to slippage of the DNA polymerase. These mispairings are dealt with by the mismatch repair (MMR) machinery, which probably includes incision in the newly formed DNA strand, 5' of the mismatch site, removal of the incised DNA strand and resumption of DNA replication from the point where the incision was made [8]. Impaired MMR necessarily results in dramatically increased mutation frequencies. This is demonstrated by the human disease hereditary non-polyposis colorectal cancer (HNPCC), which is characterized by an approximately 100-fold increase in mutation rate [9].

Repair of helix-distorting lesions: nucleotide excision repair

Nucleotide excision repair (NER) facilitates the removal of lesions that in general cause obstruction of replication and transcription. NER has a wide range of target lesions, including those that arise from UV light (e.g. pyrimidine dimers and 6-4 photoproducts). Two subpathways of NER have been identified: global genome NER (GG-NER) and transcription coupled repair (TCR). The GG-NER enzymatic machinery scans the entire genome for lesions that in general distort the DNA helix structure, whereas TCR deals with a subset of damages that interferes with the action of RNA polymerases. In essence, both pathways initiate repair by helicase mediated DNA opening around the lesion. Subsequently, the damaged strand is excised by the action of endonucleases, making incisions at the 3' and 5' borders of the opened DNA fragment. Finally, gap-filling is performed by the normal replication machinery, using the undamaged strand as a template [10]. Clearly, such indiscriminate removal of damaged DNA fragments allows for a broad range of lesions to be covered by NER. Three rare human syndromes are associated with defects in NER: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The symptoms of all three diseases include UV sensitivity [11].

Repair avoiding mechanisms: translesion synthesis

DNA lesions are not always repaired. A mechanism known as translesion synthesis (TLS) enables replicational bypass of lesions, carried out by specific translesion polymerases that replace the regular DNA polymerase and are able to continue replication over the damaged region [12]. After passing the lesion, the regular DNA polymerase takes over again. There are several different TLS polymerases, each having its own characteristic activity. For example, pol η is able to insert nucleotides opposite a T-T dimer. TLS may be especially beneficial to cellular survival after the introduction of DNA damage, since it can enable the cell to avoid apoptosis when the characteristics of the damage would make this measure otherwise imminent. On the other hand, TLS allows accumulation of mutations and must therefore distinctly contribute to naturally occuring mutagenesis. The factors that play a role in the decision whether a lesion is repaired or bypassed are not well understood at the moment, but bypass of lesions appears to be specific for the S phase of the cell cycle.

DNA double-strand break repair

All types of DNA damage that were discussed in the previous section affect only one of the two DNA strands, thereby interfering with regular replication or transcription processes. Repair of these lesions is in general performed by removal of the damaged strand, after which the undamaged complementary strand is used as a template to guide resumed replication or transcription. But what happens in de case of a DNA double-strand break?

DNA double-strand breaks (DSBs) are introduced by ionizing radiation and by the action of reactive radicals that emerge from normal metabolism. They are also introduced by nucleolytic enzymes during V(D)J recombination, a process that is critical for normal development of the immune system (discussed in chapter 2). These DSBs are considered to be very deleterious, since they can cause cell death or give rise to chromosomal breaks and translocation of DNA fragments, thereby possibly triggering carcinogenesis. It is therefore not surprising that two distinctly different and evolutionarily well conserved pathways have arisen to deal with the repair of DSBs. These pathways are known as homologous recombination (HR) and non-homologous end-joining (NHEJ) [13, 14]. Here, an outline of these two pathways will be discussed briefly.

DSB repair via homologous recombination

HR is a highly accurate mechanism that repairs DSBs by using a homologous template (most frequently the sister chromatid) to guide repair (chapter 2, figure 1A). Therefore, HR mediated DSB repair is probably restricted to the S and G2 phases of the mammalian cell cycle, when such a template is available. DSB repair by HR

is initiated by the recognition of a DSB, possibly by the DNA binding protein RAD52, and subsequent nucleolytic processing of the DNA terminus, which may involve the RAD50-MRE11-NBS1 complex. This processing yields DNA termini with 3' single-stranded overhangs. The DNA termini then form nucleoprotein filaments with RAD51, the single-stranded DNA binding protein RPA and RAD52, which search for a homologous DNA fragment. Subsequently, strand exchange facilitates the formation of a heteroduplex: a joint molecule between the damaged and undamaged, homologous strand. RAD54 and possibly the RAD51 paralogs are required for the formation of this heteroduplex structure. DNA polymerase can subsequently use the undamaged strand as a template to resynthesize the affected part of the damaged strand. Finally, the newly synthesized DNA is ligated to the other end of the broken DNA molecule.

In addition to DSB repair, HR is also involved in the repair of interstrand cross-links (ICLs), a lesion type that is introduced by the action of psoralens (combined with UV radiation), mitomycin C and chemotherapeutic agents such as platinum compounds [15]. Such crosslinks prevent separation of the two strands of a double-stranded DNA molecule and are therefore very potent disruptors of DNA replication machineries. Although poorly understood, it is known that the repair of ICLs involves removal of a DNA fragment containing the lesion - which requires a NER endonuclease complex (ERCC1/XPF) - and DNA resynthesis in a template guided, HR-like fashion. In addition, the translesion repair genes RAD6 and RAD18 may also play a role in ICL repair, since mutations in these genes results in ICL agent sensitivity.

DSB repair via non-homologous end-joining

Although complicated in molecular detail, the repair of DSBs via NHEJ is very simple in outline. It most likely involves the formation of a molecular bridge that tethers the two ends of a broken DNA fragment and subsequently mediates direct ligation (chapter 2, figure 1B). Prior to ligation, non-complementary or damaged DNA strands need to be processed in order to form a ligatable substrate. Since small deletions can occur at the DNA termini during this process, DSB repair via NHEJ is imprecise in nature. Although inaccurate, NHEJ has the advantage over HR that it does not need the presence of a homologous template and is thus not restricted to the S and G2 phases of mammalian cell cycle. DSB repair by NHEJ is initiated by the association of DNA ends with the DNA binding enzyme Ku70/80, a heterodimer that has a ring structure with high affinity for DNA ends. Ku70/80 subsequently attracts the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) towards the DNA termini. DNA-PK_{cs} is a serine/threonine kinase that needs association to Ku and DNA in order to support its kinase activity. Both Ku and DNA-PK_{cs} may be involved in tethering of DNA ends, binding and activation of processing enzymes and recruitment of the functional ligase that joins the DNA termini: the ligase IV/XRCC4 complex. For an in-depth overview of the molecular mechanisms, NHEJ enzymes and contemporary issues of debate, we refer to chapter 2.

Human diseases associated with deficiencies in DSB repair pathways

Deficiencies in DSB repair enzymes, either belonging to the HR pathway or to the NHEJ pathway, are closely associated with ionizing radiation sensitivity and cancer predisposition [13]. Unrepaired DSBs introduce chromosomal breakage, which will most likely cause a cell to go into apoptosis, unless the damage is not severe enough. Surviving cells, however, normally display chromosomal aberrations that may trigger carcinogenesis in several ways [14]:

1. Loss or gain of whole chromosomes or fragments of chromosomes. This type of aberration may lead to carcinogenesis by loss of heterozygosity, when a lost chromosome fragment containes a tumor suppressor gene and the retained homolog carries mutations.
2. Translocation of chromosome fragments. A common aberration of this type is the exchange of chromosome arms, which possibly triggers carcinogenesis by deregulation of the expression of tumor suppressor genes or proto-oncogenes.

These chromosomal aberrations have been found in many different types of tumors, but most frequently in lymphoid tumors [16]. This bias is caused by the fact that DSBs are naturally generated within developing B- and T-cells in order to initiate V(D)J recombination (as discussed in chapter 2). In addition, DSBs are also created during immunoglobulin heavy chain class switching. Inability to repair these endogenously introduced breaks greatly increases the likelihood of lymphoid neoplasia and may also interfere with the development of a functional immune system.

Several rare human syndromes are associated with DSB repair failure, all characterised by a marked predisposition to cancer and the appearance of chromosomal instability in (cultured) patient cells [17]. These syndromes are caused by deficiencies in enzymes that are either directly involved in the DSB repair process or in the upstream signal transduction cascade that regulates cell cycle arrest or coordinates the onset of repair. It may be important to notice that these syndromes, although severe, represent deficiencies with a relatively mild phenotype. More deleterious DSB repair deficiencies are not compatible with life and result in embryonic lethality, exemplified by RAD51 or DNA ligase IV knock-out mice.

Severe Combined Immunodeficiency Syndrome (SCID)

The ability to complete V(D)J recombination, essential for the development of normal B- and T-cells, requires a functional NHEJ machinery. Therefore, defects in NHEJ enzymes induce severe immunodeficiency (SCID), characterised by the absence of mature B- and T-cells. Several patients with mutations in ligase IV suffer from growth retardation and immunodeficiency [18]. Defects in the Artemis protein, which is responsible for proper processing of hairpin structures that arise during V(D)J recombination, result in ionizing radiation sensitivity and a SCID phenotype [19, 20].

Deficiencies in DNA-PK_{CS}, Ku70/80 and XRCC4 have not been found in humans, but induce SCID in mice. At least two other, still unidentified factors are associated with the onset of radiosensitive SCID in humans [21] (M. van der Burg and D.C. van Gent, personal communication).

Ataxia telangiectasia (AT)

This syndrome (also known as Louis-Bar) is characterized by childhood neurological deterioration, the appearance of telangiectasia (dilated blood vessels), premature aging, chromosomal instability and immunodeficiencies that result in frequent pulmonary infection. The most common tumors that are associated with this disease are non-Hodgkin lymphomas and leukemias. Patients (and cells derived from them) show severe ionizing radiation sensitivity. All AT cases can be attributed to a mutation in a gene that encodes one of the most important signal transduction enzymes upstream of DSB repair: ATM (Ataxia Telangiectasia Mutated). The ATM enzyme is a protein kinase that phosphorylates p53, which in turn mediates cell cycle arrest and apoptosis [4]. Perhaps related to this ATM function, AT cells display defective G1/S and intra S checkpoint arrest, leading to replication on a damaged DNA template [22]. ATM, however, is also known to phosphorylate the NBS1 component of the MRE11/ RAD50/NBS1 (MRN) complex, which is involved in HR and possibly in NHEJ [23]. This indicates that the observed chromosomal instability in AT cells may not only be caused by failure of cell cycle arrest, but also by impaired HR or NHEJ.

AT-like disorder (ATLD)

Two families have been reported in which several individuals displayed features that were similar to AT symptoms, but did not include ocular telangiectasia. Patient cells show defective intra S checkpoint arrest. The disorder was found to be caused by hypomorphic mutations in the MRE11 gene, a genetic defect that is dissimilar from the cause of AT [24]. MRE11 is part of the MRN complex, which plays a role in HR and possibly in NHEJ mediated DSB repair.

Nijmegen breakage syndrome

This syndrome is very similar to AT, but the symptoms do not include telangiectasia and cerebellar ataxia. Patients display microcephaly and growth retardation, chromosomal instability, ionizing radiation sensitivity, immunodeficiency and predisposition to lymphomas. Cells derived from patients display impaired intra S checkpoint arrest. The affected gene in this syndrome encodes the NBS1 protein, part of the MRN complex. Nijmegen breakage syndrome is therefore, after AT and ATLD, the third DSB repair deficiency syndrome that results from impaired MRN function [25].

Bloom syndrome

A rare syndrome that predisposes patients to lymphomas and leukemias and causes immunodeficiencies. Cultured patient cells display hyperrecombination, resulting in abnormal exchange between sister chromatids. The affected protein in Bloom syndrome, BLM, is a DNA helicase that belongs to the RecQ DNA helicase family. These helicases are involved in recognition and binding of heteroduplex molecules and branch migration during HR.

Werner syndrome (WS)

WS is characterised by premature aging, growth retardation and high tumor incidence. WS cells display chromosomal translocations and deletions. The disease is caused by mutation of the WRN protein, a RecQ helicase that displays exonuclease activity [26, 27]. Impairment of the WRN helicase activity may interfere with DSB repair by recombination [17]. However, the WRN exonuclease is stimulated by interaction with Ku, which suggests a link between NHEJ and WS [28]. Therefore, the incidence of chromosomal aberrations in WS may also be caused by failure to process DNA termini during NHEJ.

Rothmund-Thompson syndrome

Symptoms of Rothmund-Thompson syndrome include skeletal abnormalities and a high incidence of osteosarcomas [17, 29]. Strikingly, this syndrome is also caused by deficiency of a RecQ DNA helicase (RTS) [30].

BRCA1 and BRCA2 mutations

Mutations in the tumor supressor genes BRCA1 and BRCA2 are associated with high incidence of breast carcinomas. The proteins encoded by these genes are involved in a pathway that mediates DSB repair via HR: both BRCA1 and BRCA2 form complexes with RAD51[31]. It is, however, unclear which part of the cancer predisposition phenotype is caused by DSB repair failure, since BRCA1 is a ubiquitin ligase which may regulate several proteins and processes. In addition, BRCA1 forms a complex with MRN and it may play a role in regulation of G2/M checkpoint control and TCR of oxidative damage [31].

Fanconi anemi (FA)

Fanconi anemia patients suffer from growth retardation, abnormalities in cardiovascular, gastrointestinal, musculoskeletal and central nervous systems, chromosomal instability, pancytopenia and a high cancer incidence. Patient cells are very sensitive for ICL inducing agents. These agents introduce quadriradial

chromosomes in FA cells [32]. Although the causes of Fanconi anemia are unclear, at least 11 complementation groups have been identified. One of the genes affected in Fanconi anemia (FANCD2) encodes a protein that colocalizes with BRCA1 [33]. In addition, the FANCD1 gene was identified as BRCA2 [34], indicating that Fanconi anemia may be caused by deficiency in the signal transduction cascade upstream of the HR process [35].

Overlapping functions of repair enzymes

Looking at the diversity of pathways that have evolved to facilitate repair of lesions that threaten genomic integrity, it may appear as if each type of lesion has its own set of specific repair enzymes, entirely dedicated to the removal of this lesion. In reality, certain enzymes may play a role in more than one repair process. This suggests that the enzymes that are used in repair processes are not always specifically designed to facilitate repair, but are rather recruited from a general pool of available molecular 'tools'.

For instance, the basal transcription initiation factor TFIIH, that harbours (among others) a kinase and two helicases, is an indispensable factor during NER. The two helicase subunits of TFIIH are required for opening of the DNA helix around the lesion in both GG-NER and TCR. In addition, the TFIIH holo-enzyme is also suggested to be involved in positioning of other repair factors during the NER process [10].

Most repair pathways, including BER, MMR, NER, HR and TLS, need polymerases to resynthesize short DNA patches after removal of damaged DNA fragments. The regular leading-strand synthesis polymerase pol δ/ε is shared by MMR, NER and TLS [4]. This example highlights the idea that repair processes are highly versatile and flexible in the use and allocation of available enzymes.

The excellent DNA binding ability of the Ku70/80 heterodimer is not only utilized for DNA damage recognition during NHEJ, but plays an additional role in the maintenance of naturally occuring DNA termini: the telomeres [36-38]. The telomeric DNA is composed of simple tandem repeat sequences, the length of which must be carefully maintained in order to prevent telomere erosion after a limited number of cell divisions, which possibly causes chromosomal end-to-end fusions. Yeast strains that are defective in the Ku70 or Ku80 homologs (YKU70 and YKU80) display shortening of telomeres, indicating that both proteins are involved in telomere length maintenance. This hypothesis is further supported by the finding that Ku70 and Ku80 are present at mammalian telomers. Recently, it has been shown that other NHEJ components, DNA-PK_{CS} and XRCC4, are required for efficient telomere end-protection, as well [38].

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Chapter 2

The mechanism of non-homologous end-joining: A synopsis of synapsis

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Abstract

Repair of DNA double-strand breaks by non-homologous end-joining is required for resistance to genotoxic agents, such as ionizing radiation, but also for proper development of the vertebrate immune system. Much progress has been made in identifying the factors that are involved in this repair pathway. We are now entering the phase in which we begin to understand basic concepts of the reaction mechanism and regulation of non-homologous end-joining. This review concentrates on novel insights into damage recognition and subsequent tethering, processing and joining of DNA ends.

Introduction

DNA Double-strand breaks and non-homologous end-joining

Although all DNA lesions pose their own threat to genetic integrity, DNA double-strand breaks (DSBs) are considered especially deleterious. When they are not repaired correctly, they can give rise to chromosomal breakage, fragmentation and translocation. The most likely fate of any cell that is victim of such havoc is cell death, but in the worst case DSBs are able to trigger carcinogenesis by inactivation of tumour suppressor genes (e.g. by deletion) or activation of oncogenes (e.g. by translocation). DSBs may be induced by exogenous causes such as ionizing radiation (IR) and mutagenic chemicals, but also by radicals that emerge from normal metabolism. Therefore, it is not surprising that well conserved repair mechanisms for DSBs are present in all living organisms.

Two fundamentally different DSB repair pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), have been identified both in mammalian cells and in yeast (reviews: [1-3]). Whereas HR depends on the use of a template - as can be found on a sister chromatid during S and G2 cell cycle phase - NHEJ brings the DNA termini together in a protein-DNA complex and joins them without the need for homology (figure 1).

Both HR and NHEJ play a role in DSB repair in mammalian cells. The most important factor determining the choice of repair pathway is probably the cell cycle stage ([4], reviews: [1, 3]), although other variables may have some influence, as well. For instance, it has been suggested that competition for DNA ends between two key enzymes of NHEJ and HR - the Ku70/80 and Rad52 proteins respectively - may determine which repair pathway is utilized [5]. However, careful analysis of the DNA binding properties of these proteins revealed that Rad52 binds preferentially to DNA species with long single-stranded overhangs, whereas Ku70/80 associates with all DNA ends [6], suggesting that the choice of pathway depends more on the nature of the DNA ends that are present at the DSB site, than on the choice made by a DNA end-binding protein. It is nevertheless conceivable that some form of competition between HR and NHEJ exists in the living cell, provided that a template is present

that allows HR mediated repair. The absence of NHEJ enzymes in an *in vivo* DSB repair assay led to increased homology-directed repair [7], suggesting that cells may be relatively flexible in their choice of repair pathway.

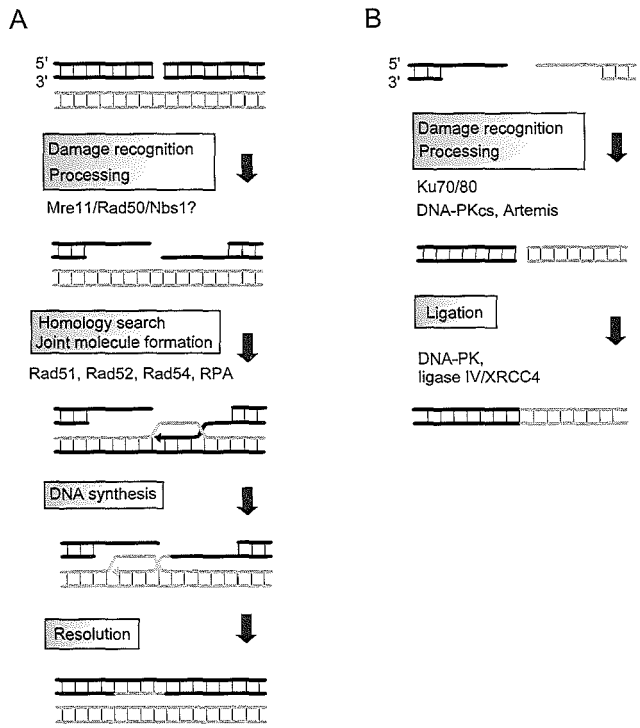

Figure 1 The two DNA double strand break repair pathways. (A) Homologous recombination is a highly accurate repair mechanism, relying on the presence of a homologous DNA fragment that can be used as a template. HR is utilized by both prokaryotic and eukaryotic cells. Free DNA ends that are formed at the site of a DSB are first processed, possibly involving the action of the Mre11/Rad50/Nbs1 complex. Rad51, Rad52 and RPA associate with single-strand overhangs. This nucleoprotein filament searches for homologous DNA. Subsequently, a joint molecule is formed between the damaged and undamaged strands. Template guided DNA synthesis then provides the damaged molecule with a copy of the undamaged strand. **(B)** Non homologous end-joining does not require the presence of a homologous template, but mediates repair by directly re-joining DNA strands. Although complicated in molecular detail, the general outline of the process is simple. It involves recognition of a DSB, processing of non-complementary or damaged DNA ends and the subsequent ligation of DNA termini. Processing of DNA ends can lead to loss or gain of nucleotides, rendering NHEJ less accurate than HR.

Although the molecular mechanism of the NHEJ pathway is still not resolved in detail, it is to be expected that the direct joining of two DNA termini requires at least four steps, namely (1) detection of a DSB, (2) formation of a molecular bridge that holds the DNA ends together, (3) a processing procedure that modifies non-matching and/ or damaged DNA ends into compatible and ligatable ends and (4) the final ligation.

The involvement of several genes in this process has been firmly established by characterization of IR sensitive cell lines (review: [8]). The core NHEJ machinery

known to date consists of XRCC4, DNA ligase IV, the Ku70/80 heterodimer and the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}). Each one of these key enzymes has affinity for DNA ends, which is consistent with a role in the end-joining process. The literature on NHEJ suggests an intimate interplay between the central proteins, presenting us with a complicated and confusing image of activities that take place at the DNA ends during repair. In this review, we will focus on the molecular mechanism of DNA end-joining, in particular on new insights into the biochemistry of protein-DNA complexes that exist at DNA termini, prior to ligation.

Non-homologous end-joining and V(D)J recombination

DNA breaks are not only made by exogenous causes, they are also generated in developing B- and T-cells to initiate V(D)J recombination, which is an essential process in the development of a functional immune system. DSBs are introduced at specific sites (recombination signal sequences) in the immunoglobulin and T-cell receptor loci (review: [9]). These loci do not contain the mature Ig or Tcr genes, but an array of gene segments, classified in three groups: variable (V), diversity (D) and joining (J) segments. Unique Ig or Tcr genes are assembled in each B- or T-cell, by combination of one segment of each class (V, D and J or V and J) and removal of the intervening DNA. This process requires incisions in the DNA at the borders of the segments that will be included in the final assembly. Such incisions, leading to DSBs, are made by RAG1 and RAG2 proteins, yielding blunt signal ends and coding ends with a hairpin structure (figure 2). After arrangement of the three segments, the hairpin coding ends are processed and joined by a mechanism that requires the key enzymes of NHEJ: XRCC4, DNA ligase IV, Ku70/80 and DNA-PK_{cs} and a processing enzyme called Artemis. Deficiency in any of these enzymes leads to severe combined immunodeficiency syndrome (SCID) in mutant mice [2, 10]). SCID symptoms include strongly reduced B- and T-cell levels as a result of reduced coding joint formation.

Non-homologous end-joining, the central enzymes

DNA-dependent protein kinase

DNA-dependent protein kinase (DNA-PK) is a holo-enzyme that is composed of the Ku70/80 heterodimer and a 469 kDa catalytic subunit, DNA-PK_{cs} [11, 12]. DNA-PK_{cs} exerts a serine/threonine protein kinase activity that is greatly enhanced by a simultaneous association with both Ku70/80 and DNA.

The Ku70/80 heterodimer displays high affinity for DNA ends and consists of two subunits, the human variants having a molecular mass of 73 kDa and 86 kDa respectively. The three-dimensional structure of Ku70/80 reveals that the dimer forms a hollow ring around the DNA helix, with Ku70 and Ku80 each making up half of the ring [13]. This structure allows a firm interaction with the DNA molecule and provides an explanation for the preferential binding of Ku70/80 to DNA termini. Once

associated with DNA ends, the Ku ring can slide to inward positions on the DNA in an ATP independent manner [11].

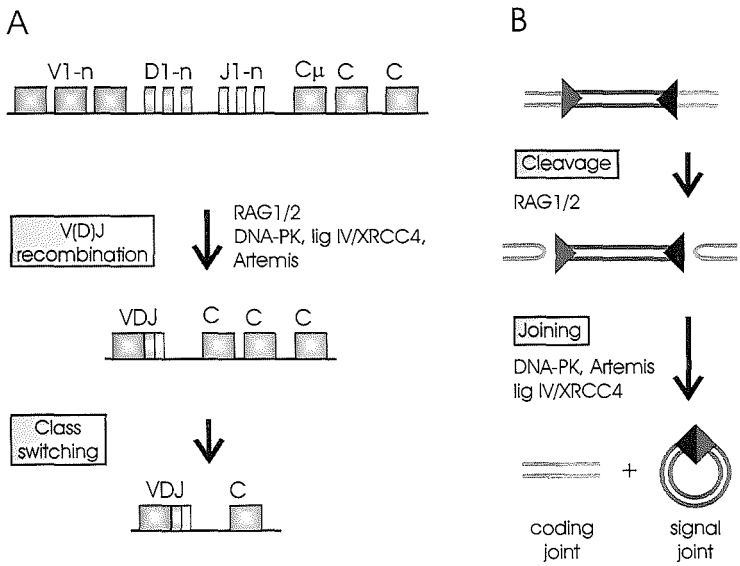

Figure 2 DNA recombination in the immune system. (A) V(D)J recombination is a process that takes place in developing B- and T-cells. The genes that encode immunoglobulins (Ig) or T-cell receptors (Tcr) are not present in an active form in these cell. In fact, the loci contain gene fragments that have to be combined to form a mature Ig or Tcr gene. The great number of combinations that can be made with these building blocks, forms the basis for diversity in Ig and Tcr gene products. This assembly process is called V(D)J recombination. Segments are classified in several groups, referred to as variable (V), diversity (D) and joining (J) segments. The situation at the IgH locus is schematically depicted in the figure. D and J segments are first coupled. Subsequently, the DJ assembly is joined with a V segment, to form the mature IgH gene. B-cells can change the Ig isotype via a process called class switching, which selects for a constant (C) segment. **(B)** Ig or Tcr gene segments are assembled by introduction of DNA breaks at the edges of gene segments, removal of intervening DNA and ligation of selected segments. DSBs are made by the RAG1 (recombination-activating gene) and RAG2 proteins at specific sites, the recombination signal sequences (depicted as triangles). Ends of the fragments that contain the recombination signal sequences are called signal ends, whereas ends of the fragments that will form the V(D)J exon are referred to as coding ends. Coding ends have a hairpin structure, whereas signal ends are blunt. Joining of coding ends requires opening and processing of hairpin structures by the Artemis protein, as well as the action of NHEJ enzymes: DNA-PK_{cs}, Ku70/80, XRCC4 and DNA ligase IV.

Formation of a kinase competent complex by Ku70/80 and DNA-PK_{cs} requires a DNA terminus. Although DNA-PK_{cs} can bind to DNA ends in the absence of Ku70/80 [14], its dissociation constant decreases 100-fold in the presence of DNA bound Ku [15]. Therefore, it is questionable whether DNA-PK_{cs} binding to DNA will take place *in vivo* without the stabilizing influence of Ku. It seems likely that the Ku70/80 dimer is the first to bind DNA ends and that it subsequently attracts DNA-PK_{cs}. Upon binding of DNA-PK_{cs}, the Ku dimer moves approximately 10 bp inward, but remains closely associated with the catalytic subunit [16, 17].

Binding of the Ku heterodimer to DNA is mediated by the central regions

of both Ku70 (amino acids 261-505) and Ku80 (amino acids 251-509) (review: [12]). Dimerization of Ku70 and Ku80 is facilitated by von Willebrand domains that are present in both proteins (amino acids 37-260 in Ku70 and 9-235 in Ku80). The central and von Willebrand domains of Ku70 and Ku80 show similarity in amino acid sequence, wheras the other parts of these polypeptides differ from each other. The carboxy-terminus of Ku70 consists of a SAP domain with DNA binding ability, although it is not situated close to the ring that surrounds the DNA end. The Ku80 carboxy-terminus is involved in both binding of Ku to DNA-PK_{cs} and activation of its kinase activity [18-20].

DNA-PK_{cs} is classified among the family of phosphatidylinositol-3 kinase related protein kinases (PIKK), which also includes ATM (ataxia telangiectasia mutated) and ATR (ATM- and RAD3-related), two proteins that function in DNA damage induced signaling [12, 21]. DNA-PK_{cs} is a large protein: the human variant consists of 4129 amino acid residues. The carboxy-terminal part of DNA-PK_{cs} contains both its kinase domain (amino acids 3645 – 4029, review: [12]) and a region that is reported to mediate interaction with Ku (amino acids 3002-3850) [22]. A point mutation in the extreme C-terminus of DNA-PK_{cs} leads to increased radiosensitivity in CHO cells, indicating the importance of the amino acid residues C-terminal of the kinase domain for DSB repair [23, 24]. The N-terminal 3000 amino acids contain both a leucine rich region (1500-1550) that may be involved in yet unidentified protein-protein interaction and a cluster of autophosphorylation sites between amino acids 2609 and 3205 [12]. Electron microscopy studies show a resemblance between the structures of DNA-PK_{cs} and ATM, both having cavities that fit a DNA molecule [25, 26]. DNA-PK_{cs} assumes a 'handpalm-like' structure that may be involved in binding to DNA and subsequent activation of kinase activity [27].

The DNA ligase IV/XRCC4 complex

The DNA ligase IV/XRCC4 complex is the functional enzyme machinery that ligates two juxtaposed DNA ends during NHEJ. Both constituents are of vital importance to organisms, since deficiency of either ligase IV or XRCC4 leads to embryonic lethality in knock-out mice [28, 29 , 30]. Their role in NHEJ is highlighted by cells that are defective in either XRCC4 or DNA ligase IV. They display radiosensitivity and impaired V(D)J recombination (review: [2]).

DNA ligase IV is an approximately 100 kDa, ATP dependent enzyme that shares homology with ligases I and III in the N-terminal region, which includes the ligase active site, but differs from them in the C-terminal part that contains two BRCT motifs (review: [31]). Interaction with XRCC4 requires the C-terminal part of ligase IV and is probably mediated by the region in between the BRCT motifs [32, 33]. The purified ligase IV/XRCC4 complex has a molecular mass of approximately 300 kDa, which would fit a tetramer that contains two molecules of each protein [34]. Crystallography and biochemical studies, however, have shown that a combination

of one ligase IV molecule and two XRCC4 molecules is the most likely structure to occur [35, 36].

The three-dimensional structure of the N-terminal 200 amino acids of the XRCC4 protein has been found to consist of a more or less globular N-terminal head that may interact with DNA, and a long C-terminal α-helical stalk that binds to ligase IV [35, 37]. The C-terminal amino acids 204-265 were disordered in the crystal, suggesting that they may not have a fixed position relative to the remainder of the protein in the absence of ligase IV. Ligase IV is both stabilized and activated by the formation of a complex with XRCC4 [32, 38]. However, the ability of XRCC4 to bind DNA structures with nicks or ends (an activity that is not required for ligase IV stimulation), as well as the severity of the knock-out phenotype, suggest an additional role for this protein [39]. XRCC4 possibly exerts this unknown activity in a tetramerised form, which it can only assume in the absence of ligase IV [36].

The XRCC4 protein can be phosphorylated by DNA-PK_{CS} *in vitro* [40], which decreases its affinity for DNA, suggesting that protein-DNA interactions of either XRCC4 alone or the ligase IV/XRCC4 complex may be regulated by DNA-PK [39]. On the other hand, mutation of XRCC4 serine residues that are phosphorylated by the action of DNA-PK_{CS} does not impair the ability of the mutant protein to complement the radiosensitive phenotype or V(D)J recombination defects of XRCC4 deficient cells [41]. This finding does, however, not exclude the possibility that DNA-PK regulates an activity of the XRCC4 protein that is not measured in these assays.

Processing enzymes

The NHEJ process appears to be a rather imprecise pathway, that often causes nucleotide loss at the DSB site. It is most likely that these errors are introduced during processing of the DNA termini, a step that is necessary in the case of a DSB with non-complementary or damaged ends that cannot be ligated directly. This idea is supported by the findings that *in vivo* repair of linearized plasmids with non-complementary ends is more error prone than repair of complementary or blunt ends [42], and that blunt DSBs, induced by transposon excision, are almost exclusively repaired in a precise fashion [43].

Knowledge about the enzymes that process DNA ends prior to ligation is limited, but it is to be expected that processing activities will include the removal or addition of phosphate groups by phosphatases or nucleotide kinases respectively, as well as extension or resection of single-stranded overhangs by the action of polymerases and nucleases. Studies on radiation sensitive mutants have rarely led to the discovery of processing enzymes, suggesting that these enzymes either have essential functions in other cellular processes or that several proteins may be able to take care of the same subset of DNA ends.

The recently discovered Artemis protein is the clearest example of a processing enzyme that is associated with NHEJ. Artemis is essential for successful

V(D)J recombination and defects in the protein result in radiosensitivity and a SCID phenotype [10, 44-46]. The Artemis protein is able to form a complex with DNA-PK_{CS}, which activates Artemis endonuclease activity in an ATP dependent manner [47], suggesting that DNA-PK_{CS} mediated phosphorylation of Artemis is required for its activity. The DNA-PK_{CS} – Artemis complex is able to open hairpin structures of coding ends that arise during V(D)J recombination, which explains the similarity between DNA-PK_{CS} and Artemis mutants: both mutants display impaired V(D)J recombination due to corrupted processing of hairpin coding ends. In addition, the rare coding joints that are formed in Artemis deficient cells display longer p-nucleotide tracts than the ones that are formed in wild-type cells, which can be explained by aberrant hairpin processing [10]. The N-terminal 385 amino acids of the 692 amino acid Artemis protein have been shown to constitute the catalytic core that is responsible for hairpin opening activity [48].

In addition to its well established function in V(D)J recombination, the Artemis protein may also be involved in NHEJ of IR induced DSBs. Complementation of the radiosensitive phenotype of RS-SCID cells by Artemis requires also the C-terminal domain (amino acids 385-692) of Artemis [48]. In their review, Lieber et al. [3] propose a model in which the DNA-PK_{CS} – Artemis complex processes single-strand DNA termini at the site of the break. In this model, the nuclease activity of Artemis would be specifically recruited to a DSB site by the action of DNA-PK_{CS}.

Several other processing enzymes have been found to have properties that are consistent with a role in NHEJ as well. One of them is the mammalian polynucleotide kinase (PNK). PNK displays both 5' DNA kinase and 3' DNA phosphatase activities and requires functional XRCC4 and DNA-PK_{CS} for efficient phophorylation of 5'-OH groups in a cell free end-joining assay. Moreover, end-joining activity is enhanced by addition of PNK to a PNK depleted extract [49]. These findings still await genetic confirmation.

Several enzymes that are able to add nucleotides to DNA termini, are also considered interesting candidates for a role in NHEJ. Human terminal deoxynucleotid yltransferase (TdT) adds nucleotides to DNA ends, interacts with Ku or DNA-PK and colocalizes with Ku at DSB sites [50-52]. TdT is a lymphoid specific enzyme, indicating that its processing activities may be specifically required for V(D)J recombination but not for general DSB repair. DNA polymerase μ may also function in NHEJ associated processing, since it interacts with Ku, stimulates end-joining *in vitro* and possibly fills single-stranded gaps that arise at the site of juxtaposed DNA ends [53]. Polymerase μ deficient mice display impaired Ig light chain gene rearrangement due to deletions at the Vκ-Jκ and Vλ-Jλ coding joints [54], showing that this DNA polymerase may be involved in NHEJ, although others can obviously take over most of its functions. One such candidate is polymerase λ: depletion of this enzyme from a nuclear HeLa extract rendered this extract inefficient in both gap-filling and joining in an *in vitro* assay [55].

Protein-DNA interactions at the DNA termini during NHEJ

Recognition of DNA damage and tethering of DNA ends

The first step of NHEJ is generally considered to be recognition of a DSB, which requires association of DNA termini with the Ku70/80 heterodimer. Once the Ku ring is bound to DNA ends, it facilitates recruitment of DNA-PK_{cs} to the DSB. The simultaneous and specific binding of both Ku and DNA-PK_{cs} to DNA ends activates the serine/threonine kinase activity of DNA-PK_{cs}. This activity is important for successful NHEJ: expression of a mutant protein with an inactivated kinase domain does not rescue the defects in DSB rejoining and V(D)J recombination of DNA-PK_{cs} deficient cells [56]. Furthermore, inhibition of DNA-PK_{cs} kinase activity by addition of wortmannin results in impaired V(D)J recombination and deficient repair of radiation induced damage [57].

How DNA-PK is exactly involved in the NHEJ process is a current matter of debate. Many *in vitro* substrates for its kinase activity have been described, including p53, Ku, XRCC4, Artemis and DNA-PK_{cs} itself [11]. However, in most cases it is not clear whether these phopshorylation events also take place *in vivo* or have functional significance. It is luring to think of DNA-PK, having protein kinase activity, as a component of a signal transduction cascade which may be involved in regulation of either the end-joining process or cell cycle checkpoints. Relatively little is known about regulatory mechanisms and the possible existence of signaling pathways that feedback information from DNA-PK to cell cycle regulators, although DNA-PK dependent phosphorylation of p53 has been observed *in vitro* (review [58]). The *in vivo* relevance of these observations, however, remains rather doubtful, since cell lines that are deficient or mutated in any of the DNA-PK components do not display abnormalities in p53 regulation or induction of cell cycle checkpoints [58]. Possibly, the role of DNA-PK_{cs} in signaling is obscured by redundancy with ATM, since simultaneous deletion of both DNA-PK_{cs} and ATM is not viable in mice [59, 60].

An increasing amount of data argues for a direct role of DNA-PK in the repair process itself. Atomic force microscopy and biochemical studies clearly show the presence of DNA-PK or one of its components at juxtaposed DNA ends (synapses) [17, 61-63]. A role for Ku in bridging DNA ends was deduced from the observation that this protein stimulates ligation by mammalian DNA ligases [64, 65] and that it is able to transfer between matching and non-matching DNA ends [66, 67]. These observations suggest that DNA-PK may act at the interface between the two ends of a broken chromosome.

The lack of a detailed three-dimensional structure of DNA-PK_{cs} makes it difficult to speculate on the exact nature of physical tethering of DNA ends. The simplest model one can imagine, is a direct head-to-head interaction of DNA ends with DNA-PK situated in the middle [62, 68, 69]. However, such a conformation would strongly reduce available space for other enzymes at the DNA synapse.

Another model, that leaves DNA termini available for processing factors,

can be deduced from the observation that DNA-PK alters the pathway via which DNA molecules are ligated. This characteristic has been described many times by investigators that use artificial DNA substrates in *in vitro* end-joining systems, utilizing either purified DNA-PK or partially purified cell extracts [70-73]. The presence of DNA-PK favors ligation of artificial DNA substrates via an intermolecular route, as evidenced by the formation of oligomeric DNA species after ligation. Intramolecular ligation products are not formed, even when the DNA is present at low concentrations that would normally favor intramolecular ligation [73]. This feature has, to our knowledge, not been subject to any detailed study, but it suggests a DNA end synapsis model in which the two termini of the same DNA molecule are juxtaposed in a protein-DNA complex, in such a way that they cannot be ligated to each other. The only available partner for joining would then be one of the termini of another DNA molecule. This configuration ensures synapsis of DNA termini in tight proximity without blocking access to the extreme DNA termini. There is, however, a limitation to this model: NHEJ *in vivo* would be disabled completely when synapsed DNA ends are not allowed to ligate. It is possible that as yet unidentified NHEJ enzymes relieve the block prior to ligation by modulating the protein-DNA structure.

Processing of DNA ends

A major problem in DSB repair is the presence of non-matching or damaged DNA ends in a large fraction of DSBs. As discussed above, several processing enzymes have been found in higher eukaryotes and it is to be expected that more of them will be found in the (near) future. Interestingly, the majority of these processing factors has not been found in yeast or prokaryotes, although such organisms do contain the core NHEJ factors Ku and ATP-dependent ligase [74-77]. Therefore, yeasts and prokaryotes probably rely on homologous recombination for the majority of the DSBs they sustain. The lack of processing factors correlates with absence of DNA-PK_{cs}, suggesting that this enzyme may be important for coordination of processing activities.

In this context it may be useful to stipulate that DNA-PK_{cs} deficient mice are viable, in contrast to ligase IV $^{-/-}$ and XRCC4 $^{-/-}$ mice (review: [2]). It therefore appears that the phenotype of DNA-PK_{cs} deficiency is less severe than that of mutations in other NHEJ factors. Such a relatively mild phenotype is difficult to reconcile with a central role of DNA-PK_{cs} in end-joining and would rather plea for a function of DNA-PK_{cs} in stimulating repair of a subset of DSBs.

Signal joint formation is relatively normal in DNA-$PK^{-/-}$ mice and mutant cell lines (reviewed by [11, 78]). In addition, blunt ended DSBs that are artificially introduced in DNA-PK_{cs} deficient cell lines, are rejoined with an efficiency that is only moderately less than that observed in wild-type cells [43]. Those findings favor a model in which DNA-PK_{cs} stimulates, but is not absolutely required for joining of blunt ends or, in general, ends that do not require processing.

Repair of complicated breaks, however, would depend on the presence of DNA-PK_{cs}.

DNA-PK autophosphorylation and accessibility of DNA ends

A plethora of observations points in the direction of roles for DNA-PK in addition to facilitation of processing. Unphosphorylated DNA-PK occupies DNA ends, thereby possibly offering protection against premature nucleolytic degradation [11, 70], as well as regulation of DNA end accessibility. It is reasonable to assume that such regulation necessitates the introduction of conformational changes in end-bound protein-DNA complexes, possibly by phosphorylation events.

In addition to phosphorylating XRCC4, p53 and Artemis, DNA-PK also (auto)phosphorylates the Ku70, Ku80 and DNA-PK_{cs} polypeptides of the DNA-PK holo-enzyme itself [79, 80]. Seven clustered autophosphorylation sites have been documented on DNA-PK_{cs}, one phosphorylation site has been identified in Ku70 (serine 6) and three were described for Ku80 (amino acids 577, 580 and 715) [12].

Autophosphorylation of DNA-PK_{cs} leads to loss of kinase activity [79, 80], which can be restored by protein phosphatase mediated dephosphorylation [81]. In addition, dissociation of the catalytic subunit from the DNA molecule upon autophosphorylation has been reported [79, 80]. Several authors, however, were unable to observe a (complete) dissociation of phosphorylated DNA-PK_{cs} [16, 73, 82]. It is possible that those latter results were obtained under conditions that did not allow for complete phosphorylation of DNA-PK_{cs}, or that there is a dynamic equilibrium between dissociation of phosphorylated proteins and re-association of unphosphorylated ones. In any case, the fate of phosphorylated DNA-PK_{cs} at the DNA termini remains open to debate. Certainly, the situation *in vivo* may even be quite different from the *in vitro* reactions, because more proteins will probably be present in end-joining complexes and the chromatin status may also influence the course of the joining reaction.

The importance of DNA-PK_{cs} autophosphorylation for NHEJ has not only been shown *in vitro*, but it is also an integral part of the NHEJ process *in vivo*. DNA-PK_{cs} autophosphorylation takes place in cells upon IR treatment and phosphorylated DNA-PK_{cs} colocalizes with sites of DNA damage [83]. Furthermore, rejoining of DSBs is inhibited when DNA-PK_{cs} lacks one or more of its autophosphorylation sites [82-84], suggesting that autophosphorylation is required for rejoining of DNA ends. This hypothesis is further supported by the observation that dephosphorylation of two DNA-PK_{cs} autophosphorylation sites by overexpressed protein phosphatase 5 (PP5) coincides with increased radiosensitivity [85]. However, the actual *in vivo* target of PP5 may also be the ATM protein instead of DNA-PK_{cs} [86], since dephosphorylation of ATM is equally likely to influence radiosensitivity.

In which manner does DNA-PK_{cs} autophosphorylation influence the end-joining process? Sequence analysis of V(D)J recombination substrates that were

isolated from a cell line expressing DNA-PK_{cs} mutated at autophosphorylation sites, revealed a decreased loss of nucleotides at the (rare) coding joints, compared to the wild-type situation [82]. This finding can be attributed to altered end-processing, but is not a result of failure to form an active DNA-PK_{cs} - Artemis complex [82]. As a consequence, it can be speculated that inhibition of DNA-PK_{cs} autophosphorylation renders the DNA termini less available for processing.

This conclusion agrees well with the observation that access of nucleases or ligases to the DNA termini is facilitated by DNA-PK_{cs} kinase activity [70, 73, 82]. Since DNA-PK is known to be present at juxtaposed DNA ends and DNA-PK_{cs} autophosphorylation is stimulated by DNA synapsis [73], it is likely that autophosphorylation takes place at the moment when two DNA termini with bound DNA-PK are juxtaposed. This introduces a conformational change in the DNA-protein complex, rendering DNA termini more accessible to enzymes that mediate subsequent steps in the NHEJ process.

Ligation of DNA ends

End-joining *in vivo* depends on the presence of ligase IV/XRCC4 , although ligase I and III can mediate ligation of DNA ends *in vitro*. Ligase IV/XRCC4 is not very active in end-to-end ligation by itself, but requires interaction with Ku [87]. The ligation activity of purified ligase IV/XRCC4 in a cell free end-joining system is enhanced by addition of whole cell extracts and reduced again when either Ku or DNA-PK_{cs} is removed from the reaction system by immuno-depletion [34].

Mobility shift assays have clearly shown a physical interaction between Ku70/80 and ligaseIV/XRCC4 on DNA fragments, accelerating the initial ligation rate 20-fold [87]. In contrast, another paper states that the presence of Ku at DNA ends inhibits ligation by ligase IV/XRCC4, although Ku and ligase IV/XRCC4 loaded simultaneously at DNA ends [72]. This discrepancy may be explained by slightly different reaction conditions used in the various laboratories. Depending on the concentrations of the components, a stimulation or inhibition of ligation by ligase IV/XRCC4 can be observed [65]. Yeast lacks a DNA-PK_{cs} homolog, but displays a similar requirement for interaction between ligase IV and Ku. lif1, the yeast XRCC4 homolog, needs interaction with yKu70p for DNA end binding [88].

Although the influence of Ku70/80 on ligation is controversial, it is generally recognized that the complete DNA-PK holo-enzyme has a profound effect on ligation by altering the ligation route from intramolecular to intermolecular (as described above). This suggests that all components of both complexes must be present to maintain a stable interaction. Indeed, association of DNA-PK and ligase IV in a DNA independent manner has been reported, resulting from interactions between ligase IV and Ku and between XRCC4 and DNA-PK_{cs} [89].

Missing links in the NHEJ process

Although DNA-PK and ligase IV/XRCC4 undoubtedly form the core of the NHEJ process, other factors have been reported either to enhance end-joining or to have interactions with NHEJ enzymes. We briefly discuss a selection of these factors.

Werner syndrome protein (WRN)

Werner syndrome (WS) is associated with premature aging (progeria) and high tumour incidence. WS cells show increased chromosomal translocations and deletions, indicating corrupted DNA repair. The syndrome is caused by mutations in the Werner syndrome protein (WRN), an enzyme with both helicase and exonuclease activity [90, 91]. WRN can interact with Ku, which disrupts binding of DNA-PK_{cs} to the DNA-bound Ku complex [92]. Furthermore, the WRN exonuclease activity is stimulated by interaction with Ku [92-96], suggesting a link between WS and NHEJ. Both activities of WRN may be involved in end-joining: its exonuclease activity possibly enables it to function as a processing enzyme, whereas its helicase activity may assist the repair process by partially unwinding DNA at the DSB site. It should, however, be noticed that WS mice are not hypersensitive for IR [97], which shows that WRN is not absolutely required for NHEJ, but possibly has a redundant function.

Factors with unknown activities, associated with end-joining

The Mre11/Rad50/Nbs1 complex (MRN) is considered to be one of the players in HR, possibly functioning in recognition of DNA damage upon activation by ATM downstream signalling [2]. All three components are of vital importance, since disruption of their genes results in embryonic lethality. Moreover, cancer predisposition is caused by mutations in Nbs1 [98]. Next to a role in HR, the complex may be involved in NHEJ, since an *in vitro* end-joining reaction that depends on Ku and ligase IV/XRCC4, is stimulated by addition of a HeLa cell fraction containing MRN [99]. In addition, the yeast equivalent of this complex, Mre11/Rad50/Xrs2, functions in a Ku dependent end-joining process *in vivo* [100, 101]. Possibly, MRN functions in NHEJ by tethering DNA ends [102] or by exerting exonuclease activity (review: [98]).

In yeast, regulation of NHEJ is mediated by a recently discovered factor, Nej1p (also called Lif2p). Mating type dependent down regulation of NHEJ in *MAT*a/*MAT*α diploid cells, in which Nej1p levels are reduced, can be rescued by overexpression of this protein [103-105]. Nej1p interacts with the yeast ortholog of XRCC4, Lif1p, and facilitates, most likely by direct interaction, transport of Lif1p into the nucleus [106]. This regulation mechanism may be yeast specific, since no mammalian homolog has been discovered yet.

In addition to known gene products, some unidentified novel factors have been reported. One paper describes a SCID patient cell line that is not defective in

any of the known components of NHEJ, but still displays radiosensitivity, decreased DSB rejoining and corrupted V(D)J recombination [107]. The missing factor was not identified, but these data provide evidence that at least one unknown factor in NHEJ still awaits discovery. Furthermore, a recent biochemical study identified a novel 200 kDa protein which is required for DNA-PK dependent end-joining [108].

A small, non-protein factor appears to be involved in NHEJ as well. Inositol hexaphosphate (IP_6) stimulates DNA-PK dependent end-joining *in vitro*, but does not influence DNA-PK activity [71]. It is reported to bind to the Ku component of the DNA-PK holo-enzyme [109, 110].

Conclusions and future prospects

Taken the available information on the biochemistry of NHEJ together, it is becoming more and more apparent that both key players, DNA-PK and the ligase IV/XRCC4 complex, are present at the juxtaposition of DNA ends during repair (figure 3). Binding of Ku70/80 to DNA termini facilitates association and activation of $DNA\text{-}PK_{CS}$, an enzyme that may play several roles in the NHEJ process: enhancing juxtaposition of broken DNA ends by physical tethering, regulating accessibility of DNA ends, assisting processing and recruiting DNA ligase IV/XRCC4.

Although the general outline of the NHEJ mechanism begins to emerge from all the data that have been reviewed here, several issues will have to be tackled in the next few years. First, most structural information regarding NHEJ proteins and complexes is still lacking. We need three-dimensional structures of the proteins involved and a good understanding of how they interact with each other in the various complexes at DNA ends. In addition to the classical X-ray crystallography and microscopic techniques, other mehods will have to be developed that can deal with the large multi-protein complexes involved in NHEJ.

Secondly, several novel factors involved in NHEJ are bound to be discovered. As indicated above, biochemical and genetic methods have already revealed the existence of some of these factors, but more participants in (a subset of) NHEJ reactions, especially DNA end processing factors, are likely to emerge.

A third type of development that can be expected, is a better understanding of the role of chromatin in the NHEJ process. Most biochemical data have been derived from studies using naked DNA substrates, but the presence of nucleosomes will pose different constraints on the repair process *in vivo*. This may explain discrepancies between *in vivo* and *in vitro* studies. The availability of well-defined chromatinized substrates will be of great help to address these issues.

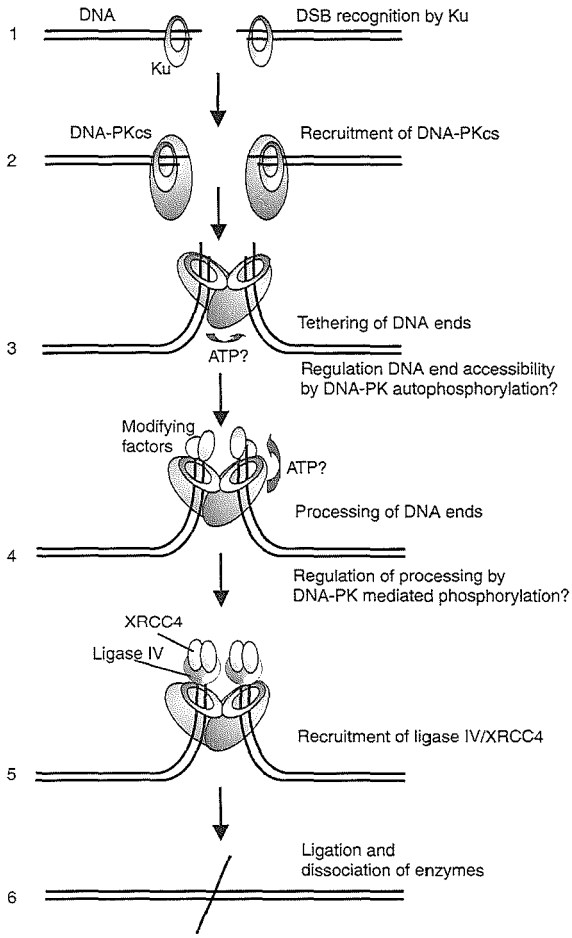

Figure 3 A contemporary model for NHEJ. Taken all biochemical data on the core enzymatic machinery of NHEJ together, an increasingly more transparent picture of NHEJ mechanistics is arising. Most data can be reconciled in a model along the following lines: Recognition of a DSB by the Ku 70/80 heterodimer **(1)**, which recruits DNA-PK_{cs} **(2)**. Subsequently, the ends are tethered **(3)** and complex, non-complementary breaks are processed **(4)**. Ligase IV/XRCC4 is recruited to the DSB **(5)**. The presence of Ku and possibly the complete DNA-PK holo-enzyme facilitates the binding of ligase IV/XRCC4 to the DNA ends. Note that the configuration with Ku rings at a more inward position in the DNA would allow for a strong tether between the DNA ends without covering the extreme termini. Finally, the processed DSB is ligated **(6)**.

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Chapter 3

Purification of the DNA-dependent protein kinase:

A novel approach

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Introduction

DNA-dependent protein kinase (DNA-PK) is a holo-enzyme that is composed of the Ku70/80 heterodimer and a 469 kDa catalytic subunit, DNA-PK_{cs} [1, 2]. DNA-PK_{cs} exerts a serine/threonine protein kinase activity that requires simultaneous association with both Ku70/80 and DNA. Deficiency in any of the three components of DNA-PK results in ionizing radiation sensitivity and impaired V(D)J recombination [1], evidencing the involvement of this enzyme in repair of DNA double-strand breaks (DSBs) via the non-homologous end-joining (NHEJ) pathway.

DNA-PK was first isolated and characterised from immortalized human cervical carcinoma (HeLa) cells [3, 4]. These studies led to the identification of DNA-PK_{cs} and revealed a distinct requirement for double-stranded DNA in order to stimulate its kinase activity. Later, significant DNA-PK activity was also found in normal human tissues, such as placenta, from which the protein can be extracted and isolated [5].

Since DNA-PK_{cs} is such a large protein, overexpression in a bacterial system is thwarted by both recombination of DNA-PK_{cs} gene segments after transformation of the bacterial host (unpublished results, Hennie Brüggenwirth) and low expression levels. Attempts to overexpress the protein in a baculovirus system also failed to yield workable amounts of DNA-PK_{cs}, thus necessitating its extraction by conventional, labour-intensive biochemical methods from either HeLa cells or readily obtainable human tissue such as placenta. These procedures generally include mechanical disruption of tissues or cells, resulting in shearing of genomic DNA into small fragments. Since DNA-PK has strong affinity for DNA ends, it is to be expected that shearing of DNA will result in increased binding of the protein to DNA fragments and possibly to significant reduction of DNA-PK yield during subsequent removal of DNA from the preparation.

We here present a procedure for the extraction and purification of DNA-PK_{cs} from HeLa cells, which differs from the down-stream processing schedules that have been described so far [3-5]. Our method is based on the standard Manley procedure for extraction of nuclear proteins [6]. This method has the advantage of efficient removal of DNA in an early stage of the procedure, without the risk of shearing the genome into small fragments during homogenization. We compare this novel method with the conventional DNA-PK isolation from human placenta.

Material and methods

Conventional purification of DNA-PK from human placenta

DNA-PK was purified from human placenta, according to a protocol adapted from Chan et al [5]. Human placentas were obtained directly after birth, cleaned (washed in phosphate buffered saline (PBS), followed by removal of the umbilical cord), cut into small pieces of approximately 2 x 2 x 2 cm, frozen in liquid nitrogen and stored at –80°C for further use.

Prior to homogenization, placenta pieces (900 g) were thawed at 4°C and completely submerged in 1500 ml extraction buffer (50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 500 mM KCl, 20 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM PMSF and 1 μg/ml proteinase inhibitors chymostatin, leupeptin, antipain and pepstatin A (CLAP)). Homogenization took place in a Waring blender, performing 10 x 5 second bursts at maximal speed. This procedure was performed in a fumehood, in order to prevent spreading of potentially infected aerosoles. Remaining large debris was removed by filtration of the homogenate over a piece of cheese cloth, followed by centrifugation at 8000 rpm (Sorvall GSA rotor) for 30 minutes and subsequent separation of pellet and supernatant. The pellet was extracted with 200 ml extraction buffer and centrifuged, after which the supernatant was combined with the first supernatant.

Downstream processing of the combined supernatants took place by ammoniumsulphate precipitation. First, protein contaminants were removed by adding ammoniumsulphate to 20% saturation, followed by stirring for 1 hour and centrifugation for 30 minutes at 8000 rpm. The supernatant was separated from the pellet and ammoniumsulphate was added to 60% saturation, followed by stirring for 1 hour and centrifugation for 30 minutes at 8000 rpm. The pellet, which contained the bulk of DNA-PK, was subsequently resuspended in 500 ml buffer B (50 mM Tris-HCl pH 7.5, 100 mM KCl, 5% glycerol, CLAP, 2 mM DTT). Salts were removed by dialysis (3x, once overnight and twice during a period of several hours) against 10 volumes buffer B (with no CLAP added after this point).

The dialysed preparation was centrifuged at 8000 rpm (Sorvall GSA rotor) for 30 minutes, followed by low pressure column chromatography on DEAE sepaharose fast flow (Amersham Pharmacia, bed volume approximately 100 ml). After complete loading of the preparation onto the column, the flow through was again loaded onto the same column, in order to increase yields ('repeated loading'). The DEAE column was washed with buffer B and eluted with elution buffer (50 mM Tris-HCl pH 7.5, 1000 mM KCl, 5% glycerol, 1 mM DTT). The eluate was 3x dialysed against 10 volumes loading buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 5% glycerol, 2 mM DTT), centrifuged at 8000 rpm (Sorvall GSA rotor) for 30 minutes and the supernatant was loaded repeatedly onto an SP sepaharose fast flow column (Amersham Pharmacia, bed volume approximately 40 ml), followed by washing and subsequent elution with elution buffer (50 mM Tris-HCl pH 7.5, 1000 mM KCl, 5% glycerol, 1 mM DTT). The eluate was 3x dialysed against 10 volumes loading buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 5% glycerol, 1 mM DTT) and passed over a second DEAE sepaharose fast flow column (bed volume approximately 20 ml), which does not bind DNA-PK under the chosen conditions.

After these low pressure chromatography steps, the preparation was further purified by FPLC, on either a resource S or resource Q column (Amersham Pharmacia). In contrast to the procedure of Chan et al., no double-stranded DNA cellulose column was used. DNA-PK was loaded onto FPLC columns in buffer containing 50 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM DTT and was eluted with a linear gradient from 50

- 1000 mM KCl. Alternatively, small scale purification of DNA-PK_{cs} took place on a 100 µl monoS column (Smart system, Amersham Pharmacia), using the same 50 – 1000 mM KCl gradient. The final preparation was 2x dialysed against 100 volumes 25 mM Hepes-KOH pH 7.5, 50 mM KCl, 5% glycerol, 2 mM DTT and stored in small aliquots at –80°C.

Purification of DNA-PK_{cs} from HeLa cells

Pellets of frozen HeLa cells were commercially obtained from Cilbiotech s.a. (Belgium). 3 x 10^{10} cells (approximately 120 ml) were resuspended in 300 ml hypotonic lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 µg/ml proteinase inhibitors chymostatin, leupeptin, antipain and pepstatin A (CLAP)). Cells were lysed with 25 strokes in a Dounce homogenizer, using a type B pestle, after which 300 ml of sucrose-glycerol buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 25% sucrose, 50% glycerol, CLAP, 1 mM DTT) was added. This was very carefully mixed with 40 ml saturated ammoniumsulphate pH 7.4 for 30 minutes, yielding a very viscous fluid. Removal of genomic DNA and cellular debris took place by ultracentrifugation at 160.000 x g (36.000 rpm, 45Ti rotor, Beckmann L-80). During separation of the supernatant from the pellet, care was taken not to disturb the pellet, by allowing approximately 1.5 cm of supernatant to remain above the pellet.

Downstream processing of the supernatant was performed by ammoniumsulphate precipitation at 60% saturation. Salts were removed by resuspension of the ammoniumsulphate pellet in loading buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 5% glycerol, 1 mM DTT) and 3x dialysis against 10 volumes of the same buffer. Low pressure column chromatography on DEAE sepharose fast flow (eluted with 300 mM KCl instead of 1000 mM as described by the conventional protocol) and SP sepharose fast flow, took place according to the method described in the previous section. A second DEAE column was not considered necessary.

Subsequently, the preparation was dialysed against loading buffer and repeatedly loaded onto a ds DNA-cellulose column (Sigma D-8515), containing approximately 1 g of DNA-cellulose. After washing, elution took place with a linear gradient from 50 – 1000 mM KCl. Peak fractions were pooled, 3x dialysed against 10 volumes loading buffer and further purified by FPLC, using a 1 ml mono S column (Amersham Pharmacia). Elution was performed with a linear gradient from 50 – 1000 mM KCl. The final preparation was 2x dialysed against 100 volumes 25 mM Hepes-KOH pH 7.5, 50 mM KCl, 5% glycerol and 2 mM DTT, using Spectra/Por cellulose ester dispodialyzers (Spectrum Laboratories Inc.), and stored in small aliquots at –80°C.

Determination of specific kinase activity

Specific kinase activity was determined using the SignaTECT DNA-dependent Protein Kinase Assay System (Promega), according to the protocol of the manufacturer.

DNA-PK$_{cs}$ specific antibodies

Antibodies for immunoblot analysis (Western blot analysis) were raised in rabbits. Animals were immunized with polypeptides derived from either the N-terminal part (amino-acids 356-570) or the C-terminal part (amino-acids 3651-3841) of the DNA-PK$_{cs}$ protein, which were produced in *E. coli* as histidine-tagged fusion-proteins.

Results and discussion

Isolation of DNA-PK from human placenta

We isolated the DNA-PK holo-enzyme from human placenta. In order to obtain a preparation that contained the three components of DNA-PK (Ku70, Ku80 and DNA-PK$_{cs}$), we did not include a ds DNA-cellulose column, as was described in the original protocol [5] and which would separate Ku70/80 from DNA-PK$_{cs}$.

After homogenization of human placenta and bulk purification by ammoniumsulphate precipitation, low-pressure DEAE sepharose column chromatography was performed. We observed that the characteristics of DNA-PK$_{cs}$ retention on this column matrix are highly unpredictable and differ between extracts obtained from different placentas and even between batches of the same extract. At 100 mM KCl, DNA-PK$_{cs}$ did bind effectively in approximately 50% of the attempts, whereas on other occasions the bulk of DNA-PK$_{cs}$ was found in the flow through.

We suspect that this phenomenon is due to the presence of short DNA fragments in the homogenate, which associate with both Ku70/80 and DNA-PK$_{cs}$ and thereby alter the affinity of these proteins for the column matrix. Obviously, the amount of contaminating DNA and the extent of shearing differ among batches of extracts. In addition, the crude extract still contains massive amounts of other contaminants, such as hemoglobin and lipids, after ammoniumsulphate precipitation. These contaminants possibly facilitate formation of micelles or alteration of ionic strength or pH values, causing unpredictable behaviour of proteins during column chromatography. We infer that the downstream processing procedure would greatly gain in reproducibility, protein yield and labour intensity, if initial extracts were reduced in contaminating DNA and tissue remnants before commencing column chromatography.

This first DEAE column chromatography step was effective in removal of blood components and lipids, although some contaminating lipids were still present in the eluate, visible by eye. DNA-PK was clearly enriched during this step, as judged from coomassie stained gel analysis (figure 1A) and Western blot analysis.

Further purification of the preparation took place by low-pressure chromatography on SP sepharose. Initially, we experienced again that DNA-PK$_{cs}$ binding to the column matrix is unpredictable at the salt conditions that are described by Chan et al. However, by lowering the KCl concentration from 100 to 50 mM, we found that DNA-PK$_{cs}$ bound to the column reproducibly.

Based on coomassie stained gel analysis, we estimate a further DNA-PK enrichment by a factor 5 upon chromatography on SP sepharose.

After passing a second DEAE sepharose column in the presence of Mg^{2+} ions, a step that was probably introduced by Chan et al. to remove remaining DNA, DNA-PK was further purified and concentrated by FPLC. This chromatography step notably removed the last remaining major contaminant, a protein with an apparent molecular mass of 170 kDa. Both mono Q and mono S columns can be used, although mono S yielded, in our hands, more reproducible results. For small scale purification, we preferred the use of a 100 µl mono S column (Amersham Pharmacia Smart system), which concentrated DNA-PK approximately 40 fold. The elution profile on this column was reproducible and DNA-PK eluted at 300-350 mM KCl.

The final preparation (figure 1B), obtained after chromatography on the Smart mono S column, was estimated to be 70% pure and contained both DNA-PK_{CS} and Ku70/80 in a molar ratio of 1:3, as confirmed by coomassie stained gel analysis and Western blot analysis (figure 1C). DNA-PK_{CS} concentration was approximately 100 ng/µl and the preparation displayed a specific kinase activity of 1.4 nmol ATP/ min/µg. Several batches of purified DNA-PK, obtained from different placentas and processed separately, yielded comparable amounts of DNA-PK with comparable purity and specific activity. Total yield of DNA-PK was typically in the order of 1 µg per 10 g placenta. DNA binding ability of the purified DNA-PK was demonstrated by exonuclease protection assays and footprinting analysis, and its autophosphorylation activity by measurement of radioactive phosphate incorporation, as described in chapter 4 [7].

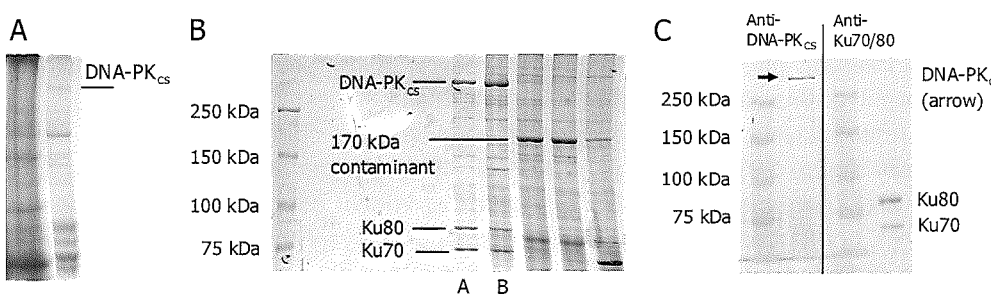

Figure 1 Isolation of DNA-PK from human placenta. (A) An example of the enrichment in DNA-PK_{CS} that is obtained by separation of the crude extract on DEAE sepharose (coomassie staining). The flow through of this column (left lane) contains the bulk of contaminating proteins, resulting in coomassie staining of the entire lane in smear-like pattern, whereas the 1 M KCl eluate (right lane) is already clean enough to allow resolution of DNA-PK_{CS}. **(B)** Final purification of the DNA-PK holo-enzyme by FPLC on a mono S column. Each lane in the coomassie stained gel represents a fraction that was collected during gradient elution. Purification on a mono S column effectively separates DNA-PK from its major contaminant: an unidentified 170 kDa protein. Fractions A and B were pooled in order to obtain the final DNA-PK preparation. **(C)** Western blotting analysis of the final preparation, using antibodies against either DNA-PK_{CS} (left lane) or Ku70/80 (right lane).

Isolation of DNA-PK$_{cs}$ from HeLa cells

Starting with commercially obtainable HeLa cell pellets, we efficiently isolated the catalytic subunit of DNA-PK. As discussed in the previous section, the conventional purification procedure would, with respect to protein yield and reproducibility, benefit from an extraction procedure that results in a crude preparation which is already devoid of DNA and lipids. By its nature, extraction from cultured human cells results in relatively clean extracts, since these cultures do not contain hemoglobin, excessive amounts of lipids or other remnants that are associated with organ tissue. In addition, we further purified this crude extract by introducing a novel approach, namely the removal of both DNA and protein contaminants via standard Manley extraction.

In contrast to the crude placenta preparation, this protocol yielded an extract from which DNA-PK$_{cs}$ bound effectively and reproducibly to the first DEAE sepaharose column. We attribute this effect to the absence of large quantities of sheared DNA fragments and tissue remnants and found that the used extraction protocol enhances downstream processing by increasing protein yield and decreasing labour intensity.

Further purification was performed by low pressure column chromatography on SP sepharose and ds DNA-cellulose. DNA-PK$_{cs}$ eluted from the ds DNA cellulose column at 200 – 300 mM KCl. This chromatography step proved to be most efficient in removal of contaminating proteins, yielding an approximately 40% pure preparation of DNA-PK$_{cs}$, in which little or no Ku70/80 could be detected by coomassie staining. Most (estimated 90%) of the contaminating protein mass, consisted of 120 kDa, 150 kDa or 200 kDa polypeptides, the latter two of which were readily removed by FPLC chromatography on a mono S column (figure 2A).

After final FPLC purification, yielding a 95% pure preparation, the eluate was dialysed into reaction buffer, which resulted in precipitation of unidentified material. Interestingly, we observed that this precipitation removed the last remaining contaminant, a small amount of the 120 kDa protein.

After dialysis, a preparation was obtained that contained DNA-PK$_{cs}$ at > 95% purity (figure 2B), in a concentration of 400 ng/µl, as determined by Bradford staining and comparison with BSA standards of known concentration. The identity of DNA-PK$_{cs}$ was confirmed by Western blotting analysis (figure 2C). No Ku70/80 could be detected by coomassie staining, but minor amounts were detectable by Western blotting. From approximately 120 ml of HeLa cell pellet, we obtained 400 µg DNA-PK$_{cs}$, which is the equivalent of what would have been yielded from 2 - 4 kg of placenta via conventional purification. When supplemented with purified Ku70/80 (obtained from HeLa cells) in a molar ratio of approximately DNA-PK$_{cs}$: Ku70/80 = 1:2, the DNA-PK$_{cs}$ preparation proved to be able to perform autophosphorylation, as measured by radioactive phosphate incorporation, and displayed a specific kinase activity of 1.7 nmol ATP/min/µg, determined by measuring peptide phosphorylation. This specific activity corresponds well with the observed average of placenta purified DNA-PK (approximately 1.4 nmol ATP/min/µg).

In conclusion, our method of DNA-PK_{cs} isolation from HeLa cell pellets yields relatively high amounts of pure catalytic subunit. Next to high yields, it offers a simpler, faster and less labour intensive purification scheme, compared to conventional methods. We would like to stipulate that commercially available HeLa cell pellets are not only affordable, but also more readily obtainable in workable amounts than human placenta. In addition, the use of HeLa cells does not necessitate extensive safety precautions.

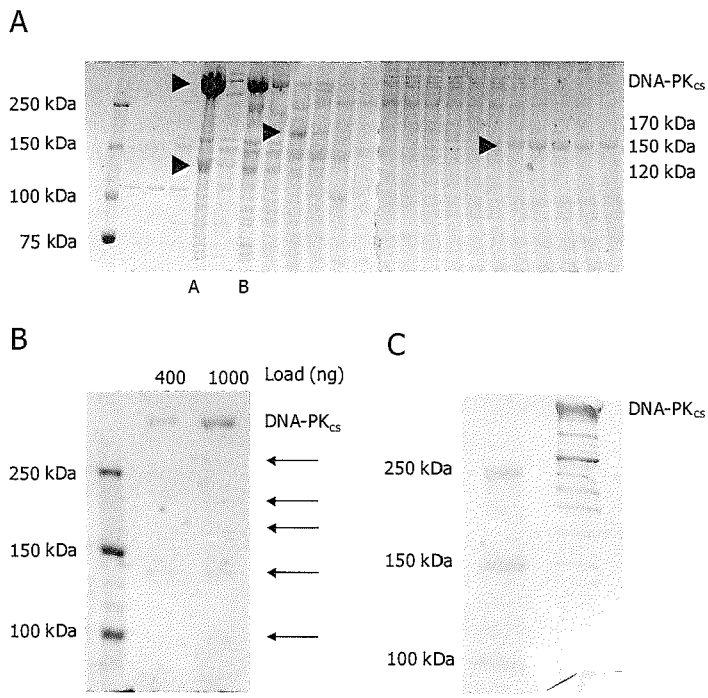

Figure 2 Isolation of DNA-PK_{cs} from HeLa cells. (A) FPLC purification of DNA-PK_{cs} on a mono S column. Each lane in the coomassie stained gel represents a fraction that was collected during gradient elution. This chromatography step effectively separates DNA-PK_{cs} from its major contaminants: unidentified proteins with apparent molecular masses of 150 kDa and 200 kDa. Fractions A and B were pooled in order to obtain the final DNA-PK preparation. **(B)** The final preparation (coomassie staining). Although a few minor contaminants are present (indicated by arrows), DNA-PK_{cs} is isolated to approximately 95% purity. No Ku70/80 is detectable by coomassie staining. **(C)** Western blotting analysis of the final preparation.

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Chapter 4

The role of DNA-dependent protein kinase in synapsis of DNA ends

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Abstract

DNA-dependent protein kinase (DNA-PK) plays a central role in the non-homologous end-joining pathway of DNA double strand break repair. Its catalytic subunit (DNA-PK_{CS}) functions as a serine/threonine protein kinase. We show that DNA-PK forms a stable complex at DNA termini that blocks the action of exonucleases and ligases. The DNA termini become accessible after autophosphorylation of DNA-PK_{CS}, which we demonstrate to require synapsis of DNA ends. Interestingly, the presence of DNA-PK prevents ligation of the two synapsed termini, but allows ligation to another DNA molecule. This alteration of the ligation route is independent of the type of ligase that we used, indicating that the intrinsic architecture of the DNA-PK complex itself is not able to support ligation of the synapsed DNA termini. We present a working model in which DNA-PK creates a stable molecular bridge between two DNA ends, that is remodeled after DNA-PK autophosphorylation in such a way that the extreme termini become accessible without disrupting synapsis. We infer that joining of synapsed DNA termini would require an additional protein factor.

Introduction

DNA double strand breaks (DSBs), which can be introduced by the action of ionizing radiation or mutagenic agents, are repaired either by homologous recombination or non-homologous end-joining (NHEJ). While homologous recombination is considered to be the main DSB repair pathway in lower eukaryotic cells, DSBs in higher eukaryotes, including mammals, appear to be repaired mainly via the NHEJ pathway [1]. Defects in end-joining generally result in increased sensitivity to ionizing radiation, as well as immunodeficiency, caused by failure to complete V(D)J recombination [2, 3].

In contrast to the homologous recombination repair pathway, NHEJ does not require a DNA template. DNA ends are directly ligated, although processing of the DNA ends prior to ligation is required for a subset of DSBs. For instance, missing 5' phosphates necessitate addition of a novel phosphate group by polynucleotide kinase [4]. Furthermore, the Artemis protein, in complex with the DNA-dependent protein kinase (DNA-PK), is probably required for processing of non-matching DNA ends and the opening of DNA hairpin structures which are formed during V(D)J recombination [5].

The ligase IV/XRCC4 complex and the DNA-dependent protein kinase (DNA-PK) are the core components of the NHEJ pathway [2], although the involvement of additional factors has been shown by both genetic and biochemical studies [6, 7]. DNA-PK consists of the Ku70/80 heterodimer and a 470 kD catalytic subunit (DNA-PK_{CS}), which functions as a serine/threonine protein kinase.

Ku70/80 binds with high affinity to DNA ends, which suggests that it has a role in damage recognition [8]. Its recently elucidated three dimensional structure shows that the heterodimer forms a hollow ring around the DNA helix [9], which allows

migration along the axis of the DNA molecule to internal positions and even transfer between DNA ends [10, 11]. In addition, the involvement of Ku 70/80 containing protein complexes in end-to-end association of linear DNA molecules and formation of DNA loops has been demonstrated by scanning force microscopy [12-14]. Although these structures were observed to be a minority of the protein-DNA complexes, they suggest a role for Ku 70/80 or the complete DNA-PK holo-enzyme in mediating the formation of DNA synapsis. In addition, the Ku heterodimer also attracts DNA-PK_{cs} towards the DNA ends and stimulates its kinase activity [8], which is required for efficient DSB rejoining [15].

Several studies have demonstrated a role for DNA-PK in recruitment of the ligase IV/XRCC4 complex towards DNA ends [16-19], which is required for ligation of the DSB by ligase IV. The finding that XRCC4 is a target for the DNA-PK_{cs} protein kinase [20], further supports a model in which both the DNA-PK holo-enzyme and ligase IV/XRCC4 are present at the DNA ends and have a cooperative role in rejoining of the break.

In addition to a function in attracting ligase IV/XRCC4 towards the DNA break, DNA-PK has recently been found to mediate association of two DNA molecules. This synapsis activated the DNA-PK_{cs} protein kinase [21]. Many proteins have been found to be *in vitro* targets for the DNA-PK_{cs} kinase, although the biological relevance is not clear in most cases. Some interesting candidate target proteins are the tumor suppressor protein p53 and the recently identified Artemis protein. The regulation of Artemis nuclease activity may be a good explanation for the observation that DNA-PK_{cs} deficiency leads to accumulation of DNA hairpins during V(D)J recombination [22].

Interestingly, DNA-PK_{cs} can also phosphorylate itself [23, 24], which can lead to dissociation of the catalytic subunit from the DNA and inactivation of its kinase activity. DNA-PK_{cs} autophosphorylation takes place *in vivo* and mutants of DNA-PK that miss one or two of the main autophosphorylation sites are less well able to complement the radiosensitive phenotype of a DNA-PK_{cs} deficient cell line than the wild type protein [25-28].

In this paper, we present evidence that DNA-PK_{cs} requires synapsis of two DNA ends for autophosphorylation. Interestingly, remodeling of DNA-PK complexes bound to DNA ends and subsequent ligation require conditions that allow for DNA-PK_{cs} autophosphorylation, suggesting that DNA-PK regulates end-joining by restricting accessibility to DNA ends until they are synapsed.

Materials and Methods

Protein purification

DNA-PK for general use in all assays was purified from human placenta, according to a protocol adapted from Chan et al. [29]. After homogenization, ammonium sulphate

precipitation, low-pressure purification on DEAE sepharose, S sepharose and a second DEAE sepharose column according to Chan et al., the extract was loaded directly onto a 1 ml Resource Q FPLC column (Amersham Pharmacia). No double-stranded DNA cellulose column was used, in order to obtain a preparation that contained both the DNA-PK catalytic subunit and the Ku 70/80 heterodimer. DNA-PK was bound to Resource Q in buffer B (20 mM Tris pH 7,5, 100 mM KCl, 0.02% Tween-20, 2 mM DTT) and was eluted with a linear gradient from 100 mM to 1 M KCl. Active fractions were eluted at approximately 400 mM KCl in the gradient and contained both the DNA-PK catalytic subunit and the Ku 70/80 heterodimer, as confirmed by Coomassie staining and Western blot analysis. The final preparation was dialysed to 25 mM Hepes pH 7.5, 50 mM KCl, 5% glycerol and 2 mM DTT.

The preparation was estimated to be approximately 70% pure, containing 100 ng/μl DNA-PK_{cs} and Ku 70/80 in a molar ratio of 1:3. Specific activity of the preparation was determined to be 1400 pmol ATP/min/μg with the SignaTECT DNA-dependent Protein Kinase Assay System (Promega), according to the manufacturers protocol. Total yield of DNA-PK_{cs} from 450 g of placenta was 100 μg.

Highly pure preparations of DNA-PK_{cs} and Ku70/80 (>90% pure), for use in the ligation assay, were obtained from Hela cells according to Chan et al. [29].

Also for use in the ligation assay, next to the above mentioned preparation, a second DNA-PK preparation from human placenta was made by replacing the final resource Q colomn by a mono S purification step, using a similar gradient from 100 mM to 1M KCl.

Hmg 1 protein was expressed as a fusion protein with Glutathion-S-transferase (GST) in *E. coli*, using an expression plasmid containing HMG boxes A and B (amino acid residues 1-123). *E. coli* containing the expression vector was grown at 37°C to OD_{600} = 0.5 and expression was induced by addition of 0.5 mM IPTG. After three hours, cells were harvested by centrifugation, lysed in PBS containing 0.1 mg/ml lysozyme and sonicated. The lysate was cleared by centrifugation for 30 minutes at 12,000 x *g*. Subsequently, the supernatant was loaded onto a Glutathione Sepharose 4B (Sigma-Aldrich) column, washed with 5 column volumes of PBS and eluted with 15 mM Glutathione in 50 mM Tris.HCl, pH 8. GST-HMG containing fractions were pooled and dialyzed against 25 mM Tris.HCl, pH 8, 150 mM KCl, 2 mM DTT, 10% (v/v) glycerol.

DNA-PK_{cs} autophosphorylation

Different concentrations of DNA-PK were incubated with 0.5 nM DNA substrate (either a 250 or a 1000 bp fragment) in the presence of radiolabeled γ-^{32P}ATP (Amersham Pharmacia, final activity 0.4 μCi/μl), 25 mM Hepes.KOH pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 0.1 mM ATP and 0.1 mg/ml BSA. Reaction mixtures were incubated for 30 minutes at 37°C and the reaction was terminated by addition of SDS-PAGE loading buffer. Reaction mixtures were separated by 6% SDS-PAGE gel electrophoresis.

Incorporation of radiolabeled phosphate into the DNA-PK_{cs} band was determined by phospho-imaging (Image Quant phosphorimager).

The autophosphorylation assay has been performed at least four times, yielding reproducible results.

In vitro ligation reactions

Ligation reactions were performed in the presence of a 965 bp overall radiolabeled DNA substrate, made by PCR reaction on pDVG137 using primers DAR5 and NEB1224 (sequence details available upon request), yielding a 1.9 kb fragment. This fragment was gel purified and cohesive ends were introduced by Sph I digestion, followed by gel purification of the 965 bp fragment. The reaction system contained DNA-PK at a concentration of approximately 10 ng/µl. Ligation reactions were initiated by addition of 3 units T4 DNA ligase (Promega). After incubation at 37°C for 30 minutes, reaction mixtures were treated overnight with 0.5 µg/µl proteinase K in the presence of 0.1% SDS at 50°C. Subsequently, proteins were removed by phenol/chloroform extraction, after which the ligation products were separated by gel electrophoresis in a 1.3% agarose gel in TBE buffer, containing 0.5 µg/ml ethidiumbromide. Reaction products were visualized by phospho-imaging.

When using *E. coli* DNA ligase, DNA-PK and the DNA substrate were incubated for 30 minutes at 37°C under the reaction conditions above described, after which ligation buffer (New England Biolabs) and 5 Units *E. coli* DNA ligase (New England Biolabs) were added. The ligation reaction took place at 16°C for 90 minutes.

When wortmannin was included in the ligation reaction mixtures, it was added as a stock solution in DMSO to a final concentration of 10 µM wortmannin and 5% DMSO in the reaction mixtures.

Exonuclease digestions

Exonuclease digestion reactions were performed under identical buffer conditions as were used for the ligation reactions. DNA-PK (10 ng/µl) was incubated with 0.5 nM radioactively labeled DNA substrate for 30 minutes at 37°C before exonuclease was added. Nucleases were added at concentrations that were just sufficient for complete digestion of an unprotected DNA substrate.

For Bal31 digestion, $CaCl_2$ was added to a final concentration of 12.5 mM and digestion was performed by incubation for 5 minutes at room temperature. Each reaction mixture contained 5×10^{-3} units of Bal31 (Life Technologies). The reactions were stopped by addition of 10 mM EGTA, 0.1% SDS and 0.5 ng/µl proteinase K. After incubation at 50°C for more than two hours, DNA was purified by phenol/ chloroform extraction and ethanol precipitation. The DNA ends were separated from the DNA substrate by restriction endonuclease digestion with either Eco47III or XhoI, which both digest close to either DNA end. Digestion products were separated by

polyacrylamide gel electrophoresis on a 12% denaturing gel in TBE buffer and were visualized by phospho-imaging.

Exonuclease V (Amersham Bioscience) was added to the reaction mixtures to a final concentration of 5 x 10^{-3} units and incubation was performed for 10 minutes at 37°C. The reactions were stopped by addition of 0.1% SDS. Digestion products were separated by electrophoresis on a 1% agarose gel in TBE buffer and were visualized by phospho-imaging. DNA substrate was a 5' labeled 1.2 kb fragment with 5' protruding ends.

Footprinting experiments

Protein/DNA complexes were assembled under the same reaction conditions as were used in the ligation assays. DNA substrate was a 5' radioactively labeled 874 bp fragment, made by PCR on pECFP-C1 (Clontech), using DG159 and 5' radioactively labeled DG156 as primers (sequence details available upon request), followed by gel purification of the 874 bp fragment. After incubation of this DNA substrate with DNA-PK, DNAse I was added to a final concentration of 250 ng/ml and digestion was allowed to proceed for 15 minutes at room temperature. The digestion reactions were terminated by addition of 10 mM EDTA and 0.1% SDS, and DNA was purified by proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. One volume of loading buffer (95% deionised formamide, 20 mM EDTA) was added and incubation took place for 10 minutes at 95°C. Electrophoresis was performed on a 12% denaturing polyacrylamide gel in TBE buffer, followed by phospho-imaging.

Results

DNA-PK forms protein-DNA complexes at the DNA ends

We studied the behavior of DNA-PK on DNA ends, especially in situations where these DNA ends are brought in close proximity. We first confirmed the presence of protein-DNA complexes at the DNA termini by testing the susceptibility of a 5' radioactively labeled DNA substrate for exonuclease digestion in the presence or absence of purified DNA-PK (figure 1A). As expected, DNA-PK rendered the DNA substrate unsusceptible to digestion by exonuclease V, whereas the naked substrate was completely degraded (compare lanes 2 and 4), indicating that DNA associated proteins block access of the exonuclease to both ends of the DNA. Similar results were obtained by using Bal31 exonuclease (data not shown), suggesting that DNA associated proteins reside at the DNA termini, sterically inhibiting exonuclease.

Protein - DNA complexes were studied in more detail by DNAse footprinting (figure 1B). This clearly revealed the presence of protein complexes at the DNA termini, which span a specific region of a few dozen nucleotides. By comparing with a 27 nt fragment, obtained by NheI digestion of the DNA substrate, we determined that DNA-PK covers 31-33 nucleotides of the DNA terminus, which is similar to what

has been reported before [30]. In addition to this area of DNAse protection, a clear difference in DNAse sensitivity can also be observed at approximately 38 nt and 43 nt from the end (arrowheads in figure 1B), suggesting that this part of the DNA may also be in contact with DNA-PK.

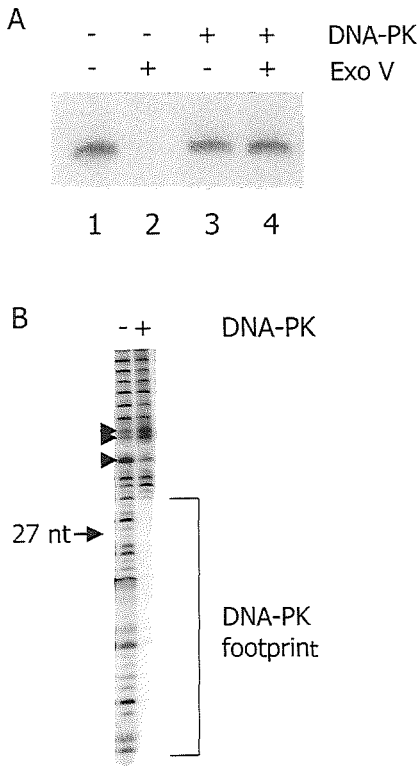

Figure 1 DNA-PK associates with DNA termini. (A) Analysis by nuclease protection assay. A 5' radioactively labeled 1.2 kb DNA substrate was first incubated with (lanes 3 and 4) or without (lanes 1 and 2) DNA-PK. Subsequently, susceptibility of the DNA-protein complex for degradation by exonuclease V was tested (lanes 2 and 4). **(B)** DNAse I footprint of DNA-PK, associated with the terminus of a DNA substrate. An 874 bp DNA substrate with one 5' radioactively labeled strand was first incubated with (lane 2) or without (lane 1) DNA-PK. Subsequently, the reaction mixtures were incubated with DNAse I. The position of a 27 nt marker fragment has been indicated on the left.

Effect of ATP on DNA-PK complexes

Conditions that allow for DNA-PK phosphorylation have been reported to alter the composition of DNA end-bound complexes [23]. In order to study the effect of ATP addition on DNA end accessibility, we tested susceptibility of the DNA termini for Bal31 exonuclease in both the presence and the absence of ATP (fig 2A). An overall ^{32}P labeled DNA substrate was incubated with purified DNA-PK, followed by a short Bal31 digestion. Subsequently, the DNA ends were separated from the substrate by digestion with restriction endonuclease, which allowed visualization of Bal31 digestion of the DNA termini.

Upon binding of DNA-PK to DNA in the absence of ATP, both strands of the DNA end were protected against digestion by Bal31 (lane 3). However, in the presence of ATP, the DNA ends became more susceptible to exonuclease digestion, as indicated by a 3-fold reduction in the amount of undigested DNA ends (lane 4). Substitution of

ATP by AMP-PNP, an ATP analog that cannot be cleaved at the γ-phosphate, did not result in an increased susceptibility of DNA ends for Bal31 degradation (lane 5). We conclude that ATP hydrolysis between the β- and γ-phosphate is required for remodeling of the DNA end bound protein complexes.

DNA-PK_{CS} autophosphorylation has been shown to disrupt the DNA-PK complex [23]. We therefore analyzed the end-bound DNA-PK complexes in more detail. However, footprint analysis did not reveal dissociation of the bulk of end bound proteins upon addition of ATP (figure 2B). However, the footprint appears to be somewhat weakened after ATP addition, indicating that dissociation of a fraction of bound DNA-PK may occur (see also discussion).

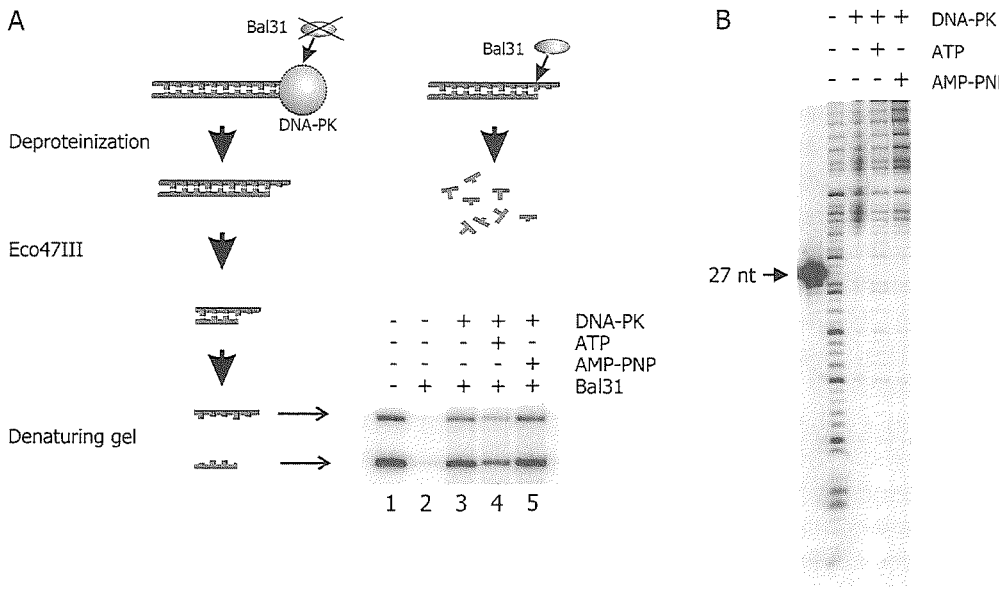

Figure 2 ATP renders the DNA termini with complexed DNA-PK more susceptible to nucleolytic degradation. (A) Bal31 exonuclease protection assay. An overall radioactively labeled DNA substrate with 3' protruding ends was incubated either in the presence (lanes 3, 4 and 5) or in the absence of DNA-PK (lanes 1 and 2). During this incubation, ATP (lane 4) or AMP-PNP (lane 5) were present as indicated. After a period known to be sufficient for binding and autophosphorylation of DNA-PK, Bal31 was added. Subsequently, the DNA termini were separated from the DNA substrate by digestion with Eco47III. Denaturing gel electrophoresis separated both strands of the terminal fragment, which are visible as two products with different mobility. The difference in intensity between these bands, can be explained by a lower content of radioactive adenosine in the upper band. **(B)** DNAse footprint analysis. An 874 bp DNA substrate with one 5' radioactively labeled strand was incubated either in the absence (lane 1) or in the presence (lanes 2, 3 and 4) of DNA-PK. ATP (lane 3) and AMP-PNP (lane 4) were present as indicated.

Autophosphorylation of DNA-PKCS requires synapsis of DNA ends

The previous experiments suggested that the kinase activity of DNA-PKCS, which catalyses hydrolysis of the phosphodiester bond between the β- and the γ-phosphate of ATP, might be involved in remodeling or disruption of DNA-PK complexes at DNA termini. In order to get a better understanding of the events leading to the alterations in

these complexes, we investigated DNA-PKCS autophosphorylation under conditions that favor or disfavor synapsis of DNA ends.

First, we quantified the autophosphorylation rate after incubation of increasing amounts of DNA-PK with a fixed amount of linear DNA. This type of assay yielded a linear dose response curve (figure 3A). Subsequently, we set out to determine whether synapsis of DNA ends is necessary for efficient DNA-PK_{cs} autophosphorylation. If this would be the case, one would expect that this reaction takes place at a rate that depends on the ability of the DNA termini to reach each other. We therefore set up an assay to measure the efficiency of DNA-PK_{cs} autophosphorylation using differently sized DNA substrates (figure 3B). When a short (250 bp) DNA substrate is used, which is too short to circularize efficiently, synapsis will mainly depend on intermolecular end to end interactions, whereas the use of a longer (1000 bp) substrate allows efficient circularization, which supports synapsis upon both inter- and intramolecular interactions. Since intramolecular DNA end interactions will predominate at low DNA concentrations, autophosphorylation rates should be significantly higher when using the 1000 bp substrate, provided that DNA-PK is present in quantities that allow occupation of both ends of the same DNA molecule. On the other hand, if synapsis is not important, autophosphorylation should only depend on the number of DNA ends present in the reaction mixture, irrespective of the possibility to circularize the activating DNA molecule.

The level of autophosphorylation was determined by measuring the incorporation of radiolabeled phosphate into DNA-PK_{cs} in reaction mixtures containing a short (250 bp) and a long (1000 bp) DNA substrate in the presence of radiolabeled ATP (figure 3B). A much higher level of DNA-PK_{cs} autophosphorylation was measured when the 1000 bp DNA substrate was used, compared to the levels that were determined upon incubation with the shorter 250 bp substrate (figure 3C). This finding supports the proposed model that DNA-PK_{cs} autophosphorylation takes place upon synapsis of DNA ends.

To verify that the observed difference in autophosphorylation level is indeed caused by the predicted difference in circularization efficiency of both DNA substrates, high mobility group 1 (Hmg1) protein was added. This protein facilitates DNA bending and thus increases the efficiency of intramolecular DNA end synapsis for the 250 bp substrate. Indeed, the autophosphorylation level in reactions containing the 250 bp substrate in the presence of Hmg1 is similar to the activity observed in reactions containing the 1000 bp substrate (figure 3C), whereas Hmg1 addition to the 1000 bp substrate did not significantly change the autophosphorylation level.

In addition, we verified that autophosphorylation requires DNA end-to-end synapsis and not any other event in which DNA is bent back upon itself. To this end, the autophosphorylation assay was performed with increasing concentrations of DNA (1000 bp fragment) and a constant amount of DNA-PK (figure 3D). DNA : DNA-PK_{cs} molar ratios ranged from 1:10 to 3:1. We observed that such a titration curve has a clear optimum, which finding is consistent with a model in which both DNA

ends with bound DNA-PK have to be brought together before autophosphorylation can proceed. Any other event in which DNA bends back upon itself, would result in autophosphorylation levels that increase with DNA concentration up to a maximum when all DNA-PK is bound to a DNA end, which should remain constant upon further increase of DNA concentration. We observed that the maximum level of DNA-PK_{cs} autophosphorylation was reached when the ratio of DNA-PK_{cs} : DNA was estimated to be 2:1 (figure 3D), suggesting that both ends of the same DNA molecule should be occupied by DNA-PK for optimal activity. Therefore, we conclude that synapsis of two DNA-PK bound DNA ends is required for autophosphorylation.

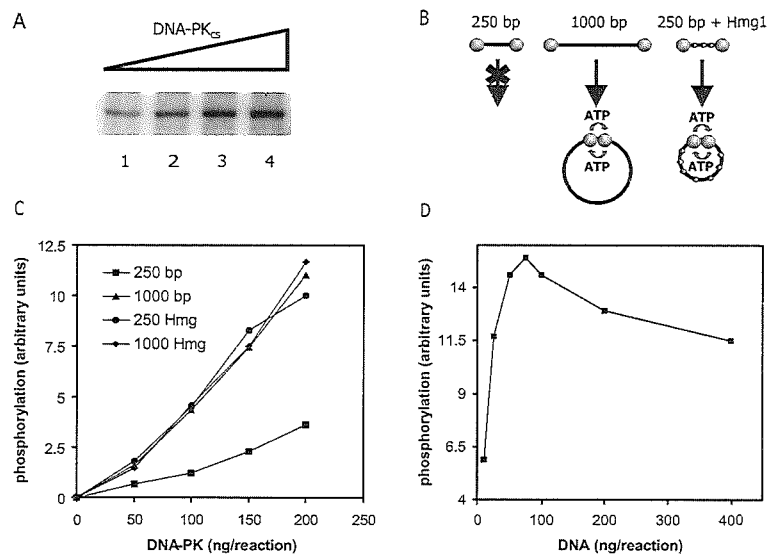

Figure 3 Autophosphorylation of DNA-PK_{cs} takes place at DNA synapses. (A) The incorporation of radiolabeled phosphate into DNA-PK_{cs} under conditions that allow for DNA-PK autophosphorylation, increases in a linear fasion with the amount of added DNA-PK. DNA-PK was added at amounts ranging from 50 ng (lane 1) to 200 ng (lane 4). These amounts were also used in the assay described in figures B and C. **(B and C)** Two differently sized DNA substrates, a 250 bp and a 1000 bp fragment, were incubated at low concentrations with DNA-PK in the presence of radioactively labeled ATP. Reaction mixtures were separated by denaturing polyacrylamide gel electrophoresis and the efficiency of DNA-PK_{cs} autophosphorylation was determined by measuring the incorporation of radioactive phosphate into DNA-PK_{cs}. The y-axis of the figure represents phosphate incorporation in arbitrary units, the x-axis represents the total amount of DNA-PK_{cs} in the reaction mixtures. **(D)** Autophosphorylation of DNA-PK_{cs} as a function of DNA concentration. DNA-PK_{cs} concentration was kept constant at 100 ng/reaction. A 1000 bp DNA fragment was added in amounts ranging from 10 to 400 ng/reaction (DNA : DNA-PK_{cs} molar ratio ranging from 1:10 to 3:1). The autophosphorylation level reaches a maximum, estimated at a DNA-PK_{cs} : DNA molar ratio of 2:1 (= 70 ng DNA per reaction).

The kinase activity of DNA-PK_{cs} is required for ligation of DNA ends

The results presented above demonstrate that autophosphorylation of DNA-PK_{cs} takes place at the synapsis of two DNA ends. Furthermore, footprinting and exonuclease digestion assays showed that the protein kinase activity of DNA-PK_{cs} is required

to modify protein-DNA complexes. We further investigated how this ATP dependent change in the end-bound DNA-PK complex influences accessibility of DNA ends to DNA ligases under various reaction conditions.

Ligation of DNA ends was studied *in vitro*, using a 965 bp radiolabeled DNA substrate with 3' protruding ends, under the same reaction conditions that were used to demonstrate DNA-PK autophosphorylation. Ligation reactions were performed at low DNA concentrations, strongly favoring intramolecular ligation. Using either T4 DNA ligase (figure 4A, lanes 2 and 3) or *E. coli* DNA ligase (figure 4B, lanes 2, 3 and 4), intramolecular ligation of the substrate was achieved.

Subsequently, ligation was studied in reaction mixtures that contained the DNA substrate and DNA-PK, either in the presence or the absence of ATP. When incubation took place without ATP, we used several strategies to ensure that ligation could still proceed at the same rate as would be observed in the presence of ATP. First, we used AMP-PNP, an ATP analog that supports activity of T4 DNA ligase, but cannot function as a cofactor for DNA-PK_{cs} (figure 4A). In the absence of DNA-PK, the substrate was clearly ligated intramolecularly (lane 3). Strikingly, a dramatically reduced level of ligation (five-fold) was observed in reaction mixtures to which DNA-PK was added (lane 5). A similar result was obtained when *E. coli* DNA ligase was used. This ligase requires NAD^+ as a cofactor instead of ATP, which allows omission of ATP in these reactions, without loss of ligation activity. Consistent with the previous experiment, addition of DNA-PK inhibited ligation of the DNA substrate in the absence of ATP (figure 4B, lane 5). In conclusion, these data indicate that the presence of DNA-PK under conditions that do not allow for phosphorylation, renders the DNA ends unavailable for ligation.

In contrast, efficient ligation of the substrate was restored in reaction mixtures containing DNA-PK if ATP was present (figure 4A, lane 4 and figure 4B, lane 6), indicating that ATP is required for ligation to proceed, even when the ligase itself does not need ATP for its activity.

Interestingly, ligation products were different from the intramolecular joining products that were formed in reactions without DNA-PK. These products had a lower mobility, suggesting that they were formed via an intermolecular joining route, even though the intramolecular event should be highly favored at the low DNA concentrations used in this assay. Addition of the Ku 70/80 heterodimer to the reaction mixture did not result in a shift in the nature of ligation products, indicating that this activity requires DNA-PK_{cs} (data not shown). The DNA-PK dependent shift from intramolecular to intermolecular ligation was also observed when *E. coli* DNA ligase was used instead of T4 DNA ligase (figure 4B, lane 6). We confirmed that the lower mobility DNA species were linear multimers of the substrate by exonuclease V digestion; intramolecular ligation products could not be degraded by an exonuclease, whereas non-circular intermolecular products were exonuclease sensitive (data not shown).

The need for ATP hydrolysis suggested that DNA-PK_{cs} protein kinase activity

was required for ligation. We therefore included the DNA-PK_{cs} inhibitor wortmannin in ligation reactions with DNA-PK and ATP and observed that inhibition of DNA-PK_{cs} kinase activity indeed reduced ligation efficiency of the DNA substrate two-fold (figure 4C). As expected, wortmannin did not influence T4 DNA ligase activity (data not shown). Wortmannin was included in the reactions at a final concentration of 10 μM, which effectively inhibits autophosphorylation of DNA-PK_{cs} (figure 4D).

Both the increased accessibility of DNA ends upon ATP addition and the shift from intra- to intermolecular joining products in the presence of DNA-PK have been observed with two differently purified DNA-PK preparations, obtained from human placenta (see materials and methods section for details of the purification procedure). In addition, similar results were obtained by using a commercially available DNA-PK preparation from Hela cells (Promega) and a highly pure DNA-PK preparation from Hela cells. In all cases we observed an identical shift of ligation products and a similar requirement for the presence of ATP for efficient ligation to proceed. These data are reproducible and strongly argue against a possible contaminating factor that might contribute to the observed effects.

In conclusion, our data show that DNA-PK renders the DNA termini unavailable for ligation, until an ATP dependent event takes place that requires the kinase activity of DNA-PK_{cs}, most likely DNA-PK_{cs} autophosphorylation.

Discussion

Autophosphorylation of DNA-PK_{cs} requires synapsis of DNA ends

The discovery that DNA-PK is involved in NHEJ was a major breakthrough in this area of research [8]. However, the role of this protein kinase in bringing together DNA ends and stimulating the subsequent ligation step is not yet understood in much detail. In this study we have investigated the role of DNA-PK in the early steps of NHEJ. As reported before [30], we found that DNA-PK forms specific complexes at DNA termini, covering approximately three turns of the helix. Upon addition of ATP, DNA-PK_{cs} can undergo autophosphorylation. We demonstrated that this reaction only occurs efficiently when both DNA termini of the same substrate molecule can reach each other and are occupied by DNA-PK, showing that synapsis of two DNA-PK bound termini is required for efficient autophosphorylation. Our data are consistent with either DNA-PK_{cs} autophosphorylation in *trans* or in *cis* and with the observation that DNA-PK activity on a peptide substrate was most efficient under conditions that allowed for synapsis [21].

DNA-PK_{cs} kinase activity is required for accessibility of DNA ends

As seen before [31], the DNA-PK complexes blocked access to the DNA ends: both exonucleases and ligases were not able to exert their activity in the presence

of DNA-PK. However, the presence of ATP caused a change: the ends became more accessible for Bal31 exonuclease and ligase. AMP-PNP could not substitute for ATP, showing that all these changes required ATP hydrolysis, probably of the γ-phosphodiester bond.

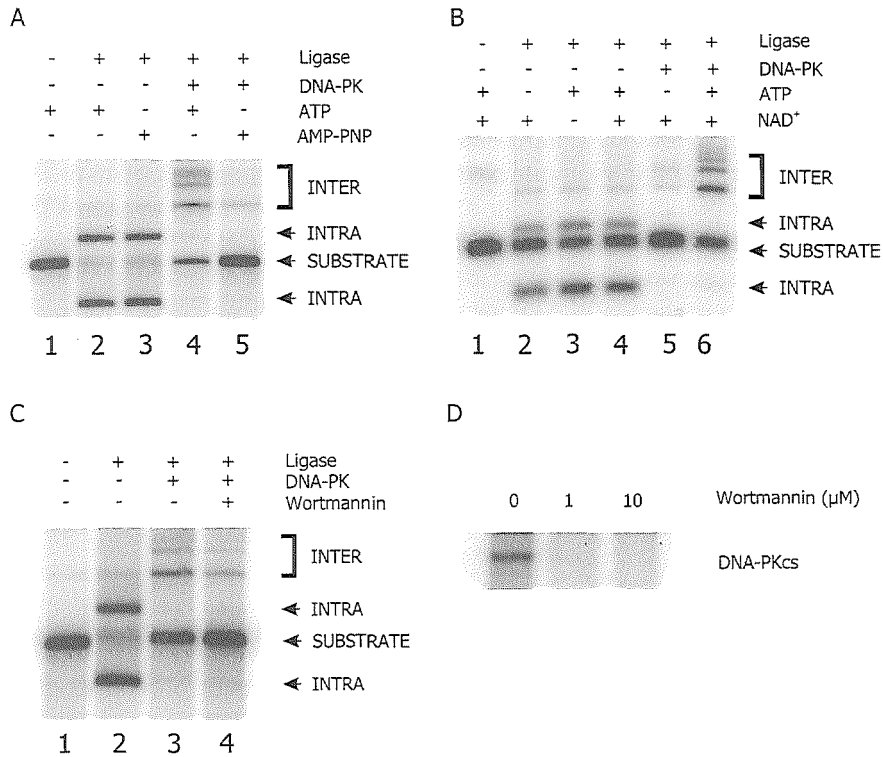

Figure 4 Ligation in the presence of DNA-PK requires ATP hydrolysis and an active DNA-PK$_{cs}$ kinase. (A) An overall labeled DNA substrate with cohesive ends was incubated with T4 DNA ligase, either in the absence (lanes 1, 2 and 3) or the presence (lane 4 and 5) of DNA-PK. ATP or AMP-PNP was present as indicated. Ligation products were separated by agarose gel electrophoresis. The nature of the ligation products, identified as intra- or intermolecular ligation products, was confirmed by exonuclease V digestion. Note that intramolecular ligation products can be either ligated on one strand (open circular form) or on both strands (covalently closed circular form). **(B)** An overall labeled DNA substrate with cohesive ends was incubated with *E. coli* DNA ligase, either in the absence (lanes 1, 2, 3 and 4) or the presence (lane 5 and 6) of DNA-PK. ATP and/or NAD$^+$ were present as indicated. **(C)** An overall labeled DNA substrate with cohesive ends was incubated with T4 DNA ligase, either in the absence (lanes 1 and 2) or the presence (lane 3 and 4) of DNA-PK. All reactions mixtures contained ATP. The DNA-PK$_{cs}$ kinase inhibitor wortmannin was added in lane 4. Total levels of ligation products in all lanes were decreased in comparison to figure 4A, due to the presence of DMSO in the reaction mixtures. **(D)** Wortmannin inhibits autophosphorylation of DNA-PK$_{cs}$. Incorporation of radiolabeled phosphate into DNA-PK$_{cs}$ was determined in the absence and presence of 1 μM or 10 μM wortmannin. Even 1 μM wortmannin completely inhibits DNA-PK$_{cs}$ autophosphorylation.

The most obvious activity that could be involved in these changes is the protein kinase activity of DNA-PK, which hydrolyzes this phosphodiester bond. Indeed, we

found that the PI-3 kinase inhibitor wortmannin reduced the efficiency of DNA end ligation in the presence of DNA-PK, suggesting that DNA-PK autophosphorylation may be required for the remodeling of end-bound protein complexes. We found that four different DNA-PK preparations (two from human placenta and two from Hela cells), including a highly pure preparation (>90%), had a similar effect in the ligation assay, rendering a contribution of incidental contaminants unlikely.

Although both DNA-PK_{cs} and Ku can be autophosphorylated, only the protein kinase activity of the catalytic subunit has been shown to be regulated by phosphorylation [23]. Therefore, we consider the most obvious explanation for our results that the regulation of DNA end accessibility is executed, at least in part, through DNA-PK_{cs} autophosphorylation. This would provide a biochemical explanation for the observation that DNA-PK_{cs} autophosphorylation is required for efficient DSB rejoining *in vivo* [25, 26, 28].

The increased accessibility of the DNA ends to Bal31 exonuclease and ligase shows that a change in the protein-DNA complex takes place upon ATP addition. This provides further evidence for the suggestion of Ding et al. [28], that DNA-PK_{cs} autophosphorylation mediates a conformational change that is required for end processing. In agreement with their findings, our footprint analysis did not provide evidence for complete dissociation of the protein complex. This observation is also consistent with the results of Yoo and Dynan [32], but contradicts several other studies [23, 24] that presented evidence for decreased affinity of DNA-PK_{cs} for the DNA-Ku complex after autophosphorylation, resulting in disassembly of DNA-PK_{cs} from the DNA and loss of DNA-PK activity. The apparent discrepancy between these findings and our observations can possibly be explained by incomplete phosphorylation of our DNA-PK_{cs}. However, our findings may also be consistent with the observation that DNA-PK_{cs} dissociates from DNA upon autophosphorylation. The molar excess of DNA-PK used in our autophosphorylation assay may allow dissociation of phosphorylated DNA-PK_{cs} and subsequent reloading of a still unphosphorylated DNA-PK_{cs} molecule. This would be consistent with our observation of a slightly less pronounced footprint upon ATP addition. The window of time between dissociation of the phosphorylated DNA-PK_{cs} molecule and reassociation of a new DNA-PK_{cs} molecule would allow binding of ligases or nucleases to the DNA termini.

The nature of the synaptic complexes

As discussed above, the specific DNase footprint indicates the presence of a specific protein-DNA complex under our assay conditions. Interestingly, the presence of DNA-PK severely affected the nature of the joining products after ligation. Intermolecular ligation events were favored over intramolecular ones in the presence of DNA-PK, whereas intramolecular joining products were mainly detected in the absence of DNA-PK. Similar observations have been made previously in ligation reactions in crude nuclear extracts or with purified components. Addition of crude fractions containing

DNA-PK to DNA substrates resulted in formation of oligomeric ligation products [31, 33]. This reaction could be inhibited by addition of wortmannin, suggesting that the kinase activity of DNA-PK_{cs} was involved. Multimeric ligation products were also observed in reactions containing purified DNA-PK and the XRCC4/ligase IV complex, but not in reactions containing only XRCC4/ligase IV [18]. We found that the presence of DNA-PK stimulated intermolecular ligation by the two tested ligases: T4 DNA ligase and *E. coli* ligase. Therefore, the reported shift from an intramolecular to an intermolecular ligation pathway is likely to be independent of the ligase and can be attributed exclusively to the action of DNA-PK.

Chen et al. [18] proposed that DNA-PK might stimulate association of DNA molecules, which would increase the likelihood of intermolecular joining. However, this cannot explain some of our results: we found that autophosphorylation depended on synapsis of the two ends of the same DNA molecule under the conditions used in the ligation reaction, indicating that association with the other end of the same DNA molecule predominates at these low DNA concentrations. We assume that the two synapsed DNA termini do not dissociate after DNA-PK_{cs} autophosphorylation. However, we found that the two ends of the same DNA molecule are not joined efficiently. These two findings may be reconciled by the following model (see figure 5). Upon binding of DNA-PK to DNA termini, the DNA ends can form a synapse, held together by protein-protein contacts. Synapsis activates autophosphorylation activity, which results in remodeling of the complex, allowing access to processing enzymes as well as DNA ligase. However, the ends are held in such a way that ligation of these ends is sterically highly unfavorable. The only way to perform a ligation reaction under these conditions is intermolecular ligation.

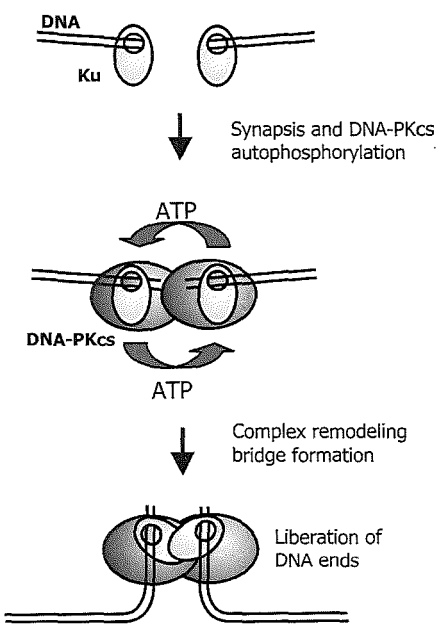

Figure 5 Model for DNA-PK_{cs} autophosphorylation and its possible significance for *in vivo* end-joining. After the occurrence of a DSB, DNA-PK binds to the termini of both DNA fragments, thereby sterically protecting the termini against degradation by nucleases or (premature) ligation. Upon synapsis, DNA-PK_{cs} autophosphorylation induces a (subtle) remodeling of the protein – DNA complex. This alteration of the end bound complex makes the extreme ends accessible for ligases (or processing enzymes). Upon DNA-PK_{cs} autophosphorylation, the termini are held together by the protein complexes, which function as a molecular bridge between the broken DNA fragments. Such a bridge would ensure stable association of both ends without blocking access to the extreme termini.

Implications for end-joining in vivo

The model of figure 5 can explain the results from our *in vitro* assays, and has several implications for end-joining *in vivo*. It explains how DNA ends are protected from degradation until they encounter the other end. DNA-PK_{cs} autophosphorylation would enable end processing while the ends are held together. This configuration ensures synapsis of the ends in a tight complex without blocking the extreme termini. This is especially important for the more complex types of breaks generated by ionizing radiation, which may require extensive processing before ligation can take place.

One major problem has not been solved by our experiments: why are the synapsed ends not ligated to each other? If a similar block would exist *in vivo*, it would cause a very high level of chromosomal aberrations. Obviously, there should be factors that modulate the structure of this complex *in vivo* in such a way that the ends that are brought together can be ligated. As the intermolecular ligation does not depend on the DNA ligase, we assume that other factor(s) may be required for this switch. We are currently investigating whether DNA bending proteins such as Hmg1 or Hmg2 might be involved in the modulation of this process.

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Chapter 5

DNA-dependent protein kinase autophosphorylation regulates the ligation step of non-homologous end-joining

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Abstract

DNA-dependent protein kinase (DNA-PK) is one of the central enzymes that function in the non-homologous end-joining (NHEJ) pathway of DNA double-strand break repair. The enzyme consists of the Ku70/80 heterodimer and a catalytic subunit (DNA-PK_{CS}) that displays serine/threonine protein kinase activity. DNA-PK_{CS} needs to undergo autophosphorylation in order to promote efficient end-joining. We show that DNA-PK autophosphorylation is required to make DNA ends available for joining by the NHEJ enzyme complex ligase IV/XRCC4. Complexes containing DNA and DNA-PK could be isolated after DNA-PK autophosphorylation and such complexes supported end-joining after addition of ligase IV/XRCC4, even when DNA-PK_{CS} kinase activity was disabled during the joining step. DNA end accessibility could be reversed by protein dephosphorylation, showing that DNA-PK phosphorylation is indeed required to create and maintain an accessible configuration. DNA-PK_{CS} alone was sufficient to support ligase IV/XRCC4 mediated joining, while the Ku70/80 heterodimer stabilized the protein-DNA complex.

Introduction

DNA double-strand breaks (DSB's) are especially genotoxic DNA lesions, since they can lead to chromosomal breakage, fragmentation and translocation, if unrepaired. DSB's can be caused by exogeneous agents, such as ionizing radiation or mutagenic chemicals, but also by radicals that emerge during normal cellular metabolism. In addition, DSB's are generated during V(D)J recombination, which is an essential process in the development of functional B- and T-lymphocytes. It is therefore of vital importance that each cell is equipped with enzymatic machineries that mediate DSB repair.

At least two distinct DSB repair pathways have evolved: homologous recombination (HR) and non-homologous end-joining (NHEJ) [1-4]. NHEJ is considered to be the prevailing pathway during the G0 and G1 phases of the cell cycle in mammalian cells, since this repair pathway does not require the presence of an intact DNA template, in contrast to HR. NHEJ involves juxtaposition of DNA ends by an enzymatic machinery and subsequent ligation. When DNA termini are incompatible or damaged, processing is necessary before ligation can proceed. It is generally accepted, that the direct joining of two DNA termini would require at least four steps, namely the detection of a DSB, the formation of a molecular bridge that holds the DNA ends together, processing activities that yield ligatable ends and, finally, a ligation step [5].

The enzymatic core of NHEJ consists of at least two protein complexes: the DNA-dependent protein kinase (DNA-PK) holo-enzyme and the DNA ligase IV/XRCC4 complex [6, 7]. The DNA-PK holo-enzyme consists of the Ku70/80 heterodimer and a 470 kDa catalytic subunit (DNA-PK_{CS}) with serine/threonine protein kinase activity.

Ku70/80 displays high affinity for DNA ends, a characteristic that suggests a role for this protein in recognition of DNA damage [8]. The three-dimensional structure of Ku70/80 reveals that the dimer forms a hollow ring around the DNA helix [9], that can slide along the DNA axis. This ATP independent migration of Ku70/80 facilitates relocation of the heterodimer to internal positions on the DNA molecule and even transfer between DNA ends [10, 11].

The formation of a kinase competent DNA-PK complex by Ku70/80 and DNA-PK_{CS} requires simultaneous binding of these enzymes to a DNA terminus [8]. Since Ku70/80 has much higher affinity for DNA ends than DNA-PK_{CS}, this heterodimer most likely binds to DNA termini first and subsequently attracts DNA-PK_{CS} towards the DSB site.

Multiple lines of evidence suggest that DNA-PK stimulates DNA end association. Scanning force microscopy showed that either Ku70/80 or the complete DNA-PK holo-enzyme can mediate DNA synapsis [12-14]. In addition, an *in vitro* experiment demonstated DNA-PK mediated association of DNA molecules [15].

Stimulation of DNA end synapsis by DNA-PK can also be deduced from the observation that DNA-PK alters the pathway via which DNA molecules are ligated in *in vitro* end-joining assays. Several authors have reported the formation of oligomeric, intermolecular DNA joining products in a DNA-PK dependent manner, even under conditions that would favor intramolecular ligation [16-19]. This feature has been explained in two ways that are not mutually exclusive: DNA-PK may stimulate intermolecular end associations and/or suppress intramolecular ligation [16].

Several targets of the DNA-PK_{CS} kinase have been identified *in vitro*, including XRCC4, p53 and Ku70/80. The finding that DNA-PK both mediates XRCC4 phosphorylation [20] and attracts ligase IV/XRCC4 towards the DNA ends [19, 21-23], suggests a cooperative role for both DNA-PK and ligase IV/XRCC4 in rejoining of the break. The relevant *in vivo* function of the DNA-PK_{CS} serine/threonine kinase is not yet fully understood, but there is convincing evidence that the kinase is involved in regulation of the Artemis nuclease activity, which is required for successful opening of DNA hairpins during V(D)J recombination [24].

The DNA-PK_{CS} kinase not only mediates phosphorylation of these heterologous substrates, but can also phosphorylate itself [25, 26]. This autophosphorylation takes place *in vivo* and mutations within the autophosphorylation sites of DNA-PK_{CS} result in a radiosensitive phenotype [27-29], evidencing the involvement of DNA-PK_{CS} autophosphorylation in efficient DSB rejoining. *In vitro* experiments showed that DNA termini with bound DNA-PK require DNA-PK autophosphorylation to enhance accessibility for both nucleases and ligases, suggesting that DNA-PK_{CS} autophosphorylation regulates processing or ligation activities at DNA termini [16]. This hypothesis is further supported by the finding that mutations in DNA-PK_{CS} autophosphorylation sites reduced nucleolytic processing of DNA termini *in vivo* [29].

Here, we provide evidence that DNA-PK phosphorylation induces a stable

conformational change in the DNA-bound DNA-PK complex. We show that DNA-PK phosphorylation status can stably influence the accessibility of DNA termini for ligase IV/XRCC4 and that this accessibility can be reversed by the action of protein phosphatase.

Materials and methods

Purification of DNA-PK

Highly pure preparations of both DNA-PK_{CS} and Ku70/80 (>90% pure on Coommassie stained gel) were obtained from HeLa cells according to a modified version of the conventional protocol by Chan *et al.* [30]. Immunoblot analysis did not reveal detectable contamination of Ku70/80 in the DNA-PK_{CS} preparation.
DNA-PK for general use in all assays was purified from human placenta, according to a previously described method [16]. The final preparation was dialysed to 25 mM Hepes pH 7.5, 50 mM KCl, 5% glycerol and 2 mM DTT. The preparation was estimated to be approximately 70% pure, containing 100 ng/µl DNA-PK_{CS} and Ku70/80 in a molar ratio of 1:3.

Purification of ligase IV/XRCC4

Preparations of ligase IV/XRCC4 were obtained by overexpression in *E. coli*. The host bacterial strain Rosetta pLysS (Novagen) was therefore transformed with the construct pMJ4052, containing C-terminally histidine tagged hDNA ligase IV and untagged hXRCC4 [31]. Cells were grown at 37°C, until OD_{600} reached 0.6. Dual expression of ligase IV and XRCC4 was than induced by addition of 1 mM IPTG, followed by overnight incubation at 15°C. Cells were harvested by centrifugation, resuspended in PBS and frozen at –80°C.

The ligase IV/XRCC4 complex was purified, using the histidine tag of ligase IV. Cells were resuspended in lysis buffer (500 mM NaCl, 20 mM Hepes pH 8, 10% glycerol, 2 mM β-mercapto-ethanol, 2 mM imidazole, 0.1 % NP-40), homogenized by sonication and the mixture was clarified by 45 minutes centrifugation at 20.000 rpm. For batch purification, a 10 ml Ni-NTA sepharose resin column was used. Proteins were bound to the column in lysis buffer. Unbound proteins were removed by washing with 3 x 40 ml lysis buffer and bound proteins were eluted with 40ml lysis buffer + 200 mM imidazole. The Ni-NTA eluate was diluted 3X with zero salt buffer Z (20 mM Hepes pH 8, 10% glycerol, 2 mM DTT, 1mM EDTA, 0.02% NP-40) and loaded on a 15 ml Q sepharose column. The column was washed with > 5 column volumes of buffer Z + 100 mM KCl and subsequently eluted by applying a gradient from 100 mM to 500 mM KCl in 200 ml buffer Z. Ligase IV/XRCC4 eluted at approximately 300 mM KCl. The peak fractions were pooled and dialyzed overnight against 5L buffer Z + 50 mM KCl. Uncomplexed XRCC4 was removed by chromatography on a 15 ml S sepharose column. Free XRCC4 did not bind to this column. After washing with > 5 column

volumes of buffer Z + 50 mM KCl, a 200 ml gradient elution from 50 mM to 500 mM KCl in buffer Z was applied. The ligase IV/XRCC4 complex eluted at approximately 250 mM KCl. The obtained preparation reached >95% purity on Coommassie stained gel. The yield was approximately 2 mg/l bacterial culture.

Deadenylation of ligase IV/XRCC4

Ligase IV/XRCC4 was deadenylated by incubation in a reaction mixture containing buffer Z, 250 mM KCl, 5 mM Na_2 pyrophosphate and 10 mM $MgCl_2$, for 2 hours at ambient temperature. The incubation mixture was subsequently diluted with zero salt buffer Z and deadenylated ligase IV/XRCC4 was isolated by repurification on an S sepharose column as described above.

In vitro ligation reactions

The standard ligation reaction was performed in the presence of a 965 bp overall radiolabeled DNA substrate with 3' protruding cohesive ends. This substrate was obtained by radioactive PCR on pDvG137, using primers DAR5 and NEB1224 (sequence details available upon request). This PCR reaction yielded a 1.9 kb fragment, which was subsequently gel purified. Cohesive ends were introduced by SphI digestion, followed by gel purification of the formed 965 bp fragment.

A DNA substrate with 5' protruding, complementary ends was made by radioactive PCR on pDvG172, using primers DAR5 and FM30 (sequence details available upon request). This PCR reaction yielded a 1 kb fragment, which was subsequently gel purified. Cohesive ends were introduced by EagI digestion, followed by gel purification of the substrate.

Ligation mixtures (10 µl) contained 25 mM HEPES-KOH pH 7.5, 0.1 mg/ml BSA, 10 mM $MgCl_2$, 10 mM KCl, 1 mM ATP, 1 mM DTT, 10 ng/µl DNA-PK_{CS},1 ng/µl DNA substrate and either 0.3 U/µl T4 DNA ligase (Promega) or 20 ng/µl ligase IV/XRCC4. The use of ligase IV/XRCC4 added 20 mM KCl extra to the reaction mixtures. After incubation at 37°C for 30 minutes, all reactions were stopped by addition of 0.1% SDS. Proteins were removed by treatment with 0.5 µg/µl proteinase K for several hours at 50°C, followed by phenol/chloroform extraction. Ligation products were separated by gel electrophoresis in a 1.5% agarose gel in TBE buffer, containing 0.5 µg/ml ethidium bromide. Reaction products were visualized by phosphor-imaging (Molecular Dynamics instrument, Image Quant software).

Exonuclease V digestion

Exonuclease V digestion of ligation products was carried out as follows. First, the DNA substrate and DNA-PK were incubated under standard ligation conditions, as described above. Reactions were stopped by addition of 0.1% SDS and proteins were removed by proteinase K treatment and phenol/chloroform extraction. DNA ligation products were subsequently retrieved by ethanol precipitation in the presence of

dextran. Exonuclease V digestion of DNA took place at 37°C for 15 minutes in a buffer containing 66 mM glycine.NaOH pH 9.4, 30 mM $MgCl_2$, 0.6 mM ATP and 1 U/µl exonuclease V (Amersham Bioscience).

Isolation of phosphorylated DNA - DNA-PK complexes

DNA-PK autophosphorylation was allowed to proceed by incubating a DNA substrate with 3' protruding, complementary termini with DNA-PK and ATP for 30 minutes at 37°C in a reaction volume of 100 µl, under the standard ligation conditions described above (but without ligase). Subsequently, protein-DNA complexes were separated from ATP by gravitational passage over a 1 ml Sephadex G50 column (Amersham Pharmacia), equilibrated in reaction buffer without ATP. Protein-DNA species were detected by measuring radioactivity of eluate fractions. Radioactive fractions were pooled and divided in aliquots. Aliquots were supplemented with either 1 mM ATP or 1 mM AMP-PNP as indicated. Ligation was initiated by addition of approximately 20 ng/µl deadenylated ligase IV/XRCC4.

$DNA\text{-}PK_{CS}$ autophosphorylation assay

Reactions were performed in standard ligation buffer, containing [γ-^{32}P]ATP (Amersham Pharmacia, final activity approximately 0.4 µCi/µl), 10 ng/µl $DNA\text{-}PK_{CS}$, 10 ng/µl Ku70/80 and 1 ng/µl of a 1000 bp DNA substrate. Autophosphorylation levels were determined as previously described [16].

PP1 phosphatase effects in ligation assays

Protein phosphatase 1 (PP1) and its specific inhibitor, protein phosphatase inhibitor 2 (I-2) were both obtained from New England Biolabs. When included into ligation or autophosphorylation assays, final concentrations amounted to 0.12 Units/µl PP1 and 50 ng/µl I-2. PP1 activity required the presence of 1 mM $MnCl_2$ in the reaction mixtures.

Results

DNA-PK autophosphorylation and intermolecular DNA ligation

Previously, we showed that inhibition of ligation by binding of DNA-PK to DNA termini can be reversed by an ATP dependent remodeling of DNA-protein complexes that requires the $DNA\text{-}PK_{CS}$ kinase activity, most likely DNA-PK autophosphorylation [16]. Interestingly, DNA-PK induced a change in the nature of DNA products that were formed upon joining, shifting them almost exclusively towards intermolecular species. Here, we studied the effect of the structure of the DNA terminus on both joining efficiency and the type of junctions that were formed upon ligation.

We first examined T4 DNA ligase mediated joining of DNA ends, using radiolabeled DNA substrates with either 3' or 5' protruding, complementary ends

(figure 1A and B). Reactions either contained ATP, which supports both ligation and DNA-PK_{cs} kinase activity, or AMP-PNP, which supports ligation but not DNA-PK_{cs} kinase activity.

Ligation of both 3' and 5' protruding termini proceeded mostly via an intramolecular ligation route in the absence of DNA-PK (lanes 2 of figures 1A and B) but was shifted towards an intermolecular route when DNA-PK was added to the reaction mixture under conditions that allowed for autophosphorylation (lanes 4 of figures 1A and B). However, the use of AMP-PNP in reactions containing DNA-PK, clearly diminished ligation efficiency (5-fold in the case of a 3' protruding end and 2-fold in the case of a 5' protruding end), showing the involvement of the DNA-PK_{cs} kinase in a process that renders the DNA termini more accessible for T4 DNA ligase. Although both DNA substrates showed a qualitatively comparable shift in ligation route and ATP dependence, the ATP dependence appeared to be somewhat more pronounced for 3' protruding ends. For this reason, we used the 3' DNA substrate for all subsequent assays presented in this paper.

In order to examine whether all ligation products from reactions that contained DNA-PK were linear, we treated them with exonuclease V. Exonuclease V readily digested the linear substrate, but circular DNA products were not degraded (figure 1C, compare lanes 1 and 2). Oligomeric ligation products, retrieved from reaction mixtures containing DNA-PK, were completely digested by exonuclease V (figure 1C, compare lane 3 and 4), showing that all intermolecular joining products are linear and that no circular oligomers were formed. Resolution allowed to identify at least five oligomeric DNA species, corresponding to molecules with a length ranging from 1 kb to 5 kb, with 1 kb intervals.

These T4 ligase mediated *in vitro* ligation studies have the advantage that the effects of DNA-PK on ligation efficiency and joining route can be attributed to DNA-PK_{cs} kinase mediated DNA-PK autophosphorylation, rather than to phosphorylation of the ligase, since a direct functional interaction between mammalian DNA-PK and bacteriophage T4 ligase is unlikely to occur. However, the joining step of NHEJ is carried out by the ligase IV/XRCC4 complex in mammalian cells. We therefore also used this ligase to examine the effect of DNA-PK its ligation characteristics (figure 2A).

In the absence of DNA-PK, ligase IV/XRCC4 ligated the DNA substrate in an almost exclusively intramolecular fashion (figure 2A, lane 2). Both ATP and AMP-PNP were able to support this reaction (figure 2A, lanes 2 and 3). When DNA-PK was added, the joining route shifted from intramolecular to intermolecular, as evidenced by the appearance of linear multimers and the disappearence of circular ligation products (lane 4). Replacing ATP by AMP-PNP virtually abolished ligation (figure 2A, lane 5, ligation efficiency was reduced 8-fold). These effects are even slightly more pronounced than those observed with T4 DNA ligase. However, the shift to intermolecular ligation and the ATP dependence are similar for both ligases and can therefore be attributed solely to the action of DNA-PK.

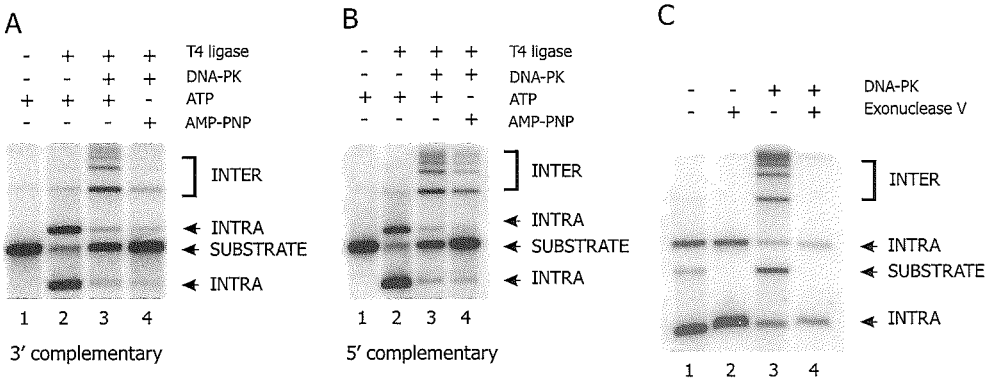

Figure 1 Ligation of DNA ends with associated DNA-PK is enhanced by an ATP dependent process and yields exclusively linear oligomeric joining products. (A) DNA substrate with 3' protruding, complementary termini was incubated with T4 DNA ligase, either in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of DNA-PK. ATP dependence of joining efficiency was tested by adding either ATP or AMP-PNP. Ligation products were separated by agarose gel electrophoresis. Note that intramolecular ligation products are either ligated on one strand (open circular) or two strands (covalently closed). **(B)** A similar experiment as described in A, but performed with a DNA substrate with 5' protruding, complementary termini. **(C)** All intermolecular ligation products that are formed in the presence of DNA-PK are linear oligomers. After completion of ligation in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of DNA-PK, DNA species were isolated and treated with (lanes 2 and 4) or without (lanes 1 and 3) exonuclease V. Intramolecular ligation products are not degraded by exonuclease V activity, whereas linear DNA species are completely degraded.

DNA-PK autophosphorylation stably changes accessibility of DNA ends

It is clear that a DNA-PK mediated phosphorylation event introduces a change in the DNA end-bound protein complexes, resulting in increased accessibility of these DNA ends. In order to examine whether the protein-DNA structures that are formed during such a remodeling event are stable upon isolation, we first incubated the DNA substrate together with ATP and DNA-PK under the standard reaction conditions used in our *in vitro* ligation system. Upon completion (defined as maximum DNA-PK_{CS} autophosphorylation after 30 minutes, data not shown), ATP was removed by gel filtration. When deadenylated ligase IV/XRCC4 was added to the eluate without addition of ATP, virtually no ligation was observed (figure 2B, lane 2), showing that ATP from the initial reaction was effectively removed from the protein-DNA species by gel filtration.

However, in the presence of deadenylated ligase IV/XRCC4 and either ATP or AMP-PNP, ligation proceeded efficiently in both reactions (figure 2B, lanes 3 and 4), showing that complexes of DNA-PK and DNA are stable and that the phosphorylation status of DNA-PK can stably change DNA end accessibility. The slightly higher level of ligation in the presence of ATP (compare lanes 3 and 4, ligation is reduced to 70% when AMP-PNP is used instead of ATP), can probably be attributed either to continuing DNA-PK autophosphorylation in the reaction mixture containing ATP, or to

DNA-PK mediated ligase IV/XRCC4 phosphorylation. However, this is a minor effect compared to the ATP dependence of reactions that did not contain prephosphorylated DNA-PK complexes.

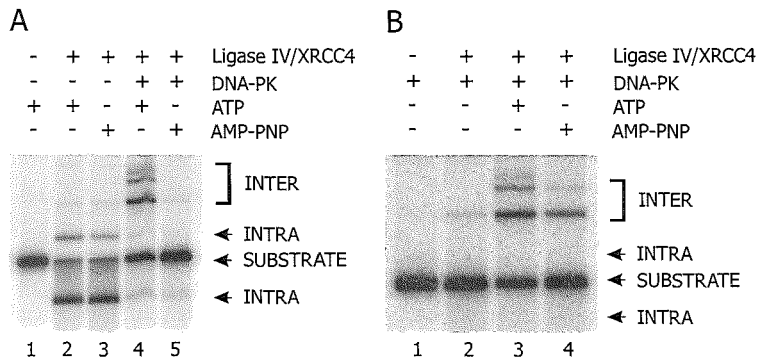

Figure 2 Ligase IV/XRCC4 mediated ligation of DNA termini with bound DNA-PK requires DNA-PK autophosphorylation, resulting in protein-DNA complexes with stably altered DNA end accessibility. (A) A DNA substrate with 3' protruding, complementary termini was incubated with ligase IV/XRCC4, either in the presence (lanes 4 and 5) or absence (lanes 1 - 3) of DNA-PK. ATP dependence of joining efficiency was tested by adding either ATP or AMP-PNP. Ligation products were separated by agarose gel electrophoresis. **(B)** A DNA substrate with 3' protruding, complementary termini was incubated with DNA-PK and ATP for 30 minutes, allowing for autophosphorylation of DNA-PK_{cs}. Upon removal of ATP by gelfiltration, isolated protein-DNA complexes were incubated with deadenylated ligase IV/XRCC4, either in the absence of cofactors (lane 2) or in the presence of ATP (lane 3) or AMP-PNP (lane 4).

Dephosphorylation of end bound DNA-PK complexes reduces ligation

Subsequently, we asked whether the increased accessibility of DNA termini with phosphorylated DNA-PK, could be reversed by dephosphorylating the DNA-PK complex. Therefore, we studied the effect of protein phosphatase 1 (PP1) on both ligation route and ligation efficiency.

First, we tested whether PP1 was able to reverse DNA-PK_{cs} autophosphorylation. As expected, DNA-PK_{cs} autophosphorylation level was strongly reduced by PP1 (figure 3A, compare lanes 1 and 2). Simultaneous addition of PP1 and a specific inhibitor of PP1 activity, I-2, did not result in reduction of autophosphorylation levels (figure 3A, lane 3), proving that I-2 successfully blocks PP1 activity.

The influence of PP1 on ligation was examined by incubating the DNA substrate and ligase IV/XRCC4 with or without DNA-PK under standard ligation assay conditions in the presence of ATP. When applicable, PP1 alone or PP1 along with its inhibitor I-2 was added to the reaction (figure 3B). Next, formation of ligation products was examined.

As expected, intramolecular ligation in reactions without DNA-PK proceeded effectively in the presence of either I-2, PP1 or PP1 + I-2 (figure 3B, lanes 2-5). When

DNA-PK was present, a different picture emerged. Ligation efficiency is reduced 5-fold by PP1 (figure 3B, compare lanes 6 and 8 and figure 3C). Simultaneous addition of both PP1 and its inhibitor I-2 restored the level of ligation to approximately 70% of the untreated value (figure 3B, compare lanes 6 and 9 and figure 3C). These results indicate that DNA-PK phosphorylation status needs to be maintained to allow ligase IV/XRCC4 mediated ligation.

The commercially obtained PP1 preparation showed a low level of nuclease activity, which reduced overall ligation efficiency 1.6-fold (calculated by dividing the average level of unmodified substrate in lanes 6 and 7 by the average level in lanes 8 and 9). To correctly calculate levels of ligation products in reactions containing PP1, measured levels were multiplied with this factor 1.6.

A similar requirement for phosphorylated DNA-PK was observed when using T4 DNA ligase instead of ligase IV/XRCC4 in the ligation assay (figure 3D). Again, intermolecular ligation efficiency was reduced by PP1 (compare lanes 2 and 3), showing that effects on DNA end accessibility are ligase independent.

Effects of ku70/80 and DNA-PK$_{CS}$ on ligation

Up to this point, both DNA-PK$_{CS}$ and ku70/80 were present in all ligation assays. In order to determine the role of each component individually, we used highly pure preparations of either component in the ligation assay.

Addition of Ku70/80 to either T4 DNA ligase or ligase IV/XRCC4 mediated ligation reactions, did not lead to a clear shift in ligation products (figure 4A, lane 2, compare lane 2 with lane 1, which contains the complete DNA-PK holo-enzyme and figure 4B, compare lanes 1 and 2). Upon titrating increasing amounts of Ku70/80 into the ligation reaction, levels of unmodified substrate increased, whereas levels of circular joining products were reduced. This effect was observed when Ku70/80 is in (more than 10X) molar excess of DNA. The decrease in ligation products is directly proportional to the amount of Ku70/80 present, as shown in figure 4C. We also performed ligation reactions which contained higher concentrations of DNA (40 ng/µl instead of 1 ng/µl). In these assays, Ku70/80:DNA molar ratios ranged from approximately 1:1 to 10:1. Neither an increase nor a decrease in joining efficiency was observed when using T4 DNA ligase or ligase IV/XRCC4 (data not shown).

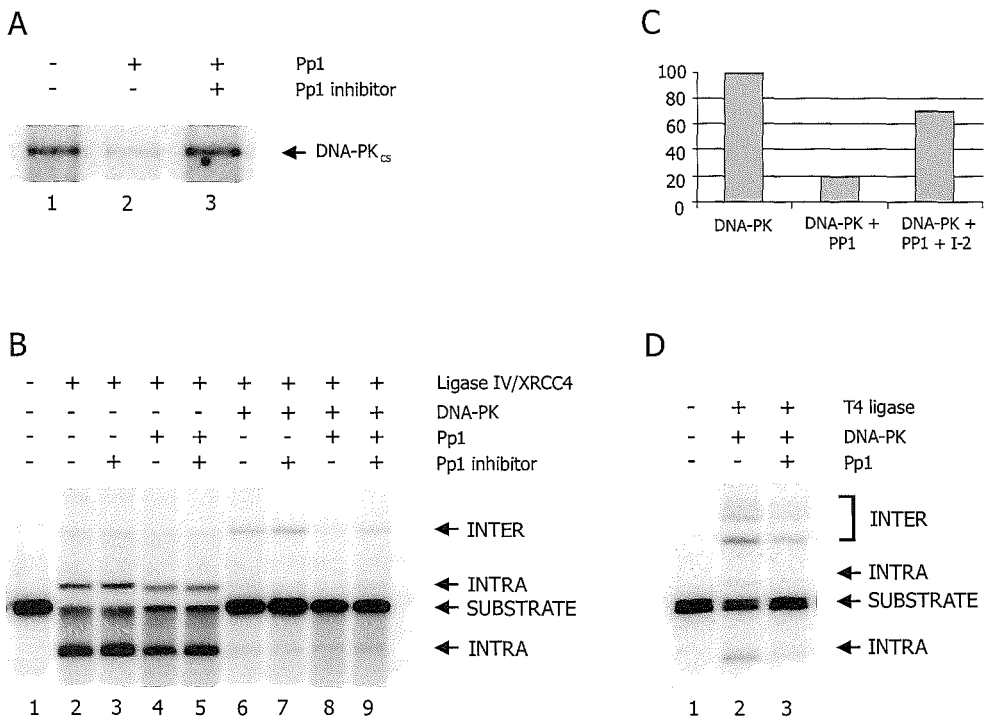

Figure 3 Dephosphorylation of end bound DNA-PK complexes by protein phosphatase 1 reduces ligation efficiency of DNA termini. (A) DNA-PK_{CS} autophosphorylation was measured by the incorporation of radioactively labeled phosphate, in the absence of PP1 (lane 1), in the presence of PP1 (lane 2) or in the presence of both PP1 and its specific inhibitor I-2 (lane 3). **(B)** Ligase IV/XRCC4 mediated ligation of a DNA substrate with 3' protruding, complementary termini was examined in the absence (lanes 1 – 5) or presence (lanes 6 – 9) of DNA-PK, upon addition of either I-2 (lanes 3 and 7), PP1 (lanes 4 and 8) and both PP1 and I-2 (lanes 5 and 9). Note an overall decrease of ligation efficiency, due to the presence of the PP1 cofactor $MnCl_2$ in the reactions. Also note a slight decrease in ligation efficiency in all lanes where PP1 is present, probably due to a weak, contaminating nuclease activity, present in the commercially obtained PP1 preparation. **(C)** Quantification of ligation efficiency of DNA-PK associated DNA substrate in the absence of PP1 (left), the presence of PP1 (middle) or the presence of both PP1 and I-2 (right). Ligation efficiency was determined by measuring the levels of intermolecular ligation products, formed in DNA-PK containing reactions (figure 1B, lanes 6, 8 and 9). Levels were measured by phosphoimager software and corrected for a general decrease of ligation efficiency in the presence of PP1. **(D)** PP1 decreases the efficiency of T4 ligase mediated joining of DNA termini with associated DNA-PK. A DNA substrate with 3' protruding, complementary DNA termini was incubated with T4 ligase and DNA-PK in either the absence (lane 2) or presence (lane 3) of PP1.

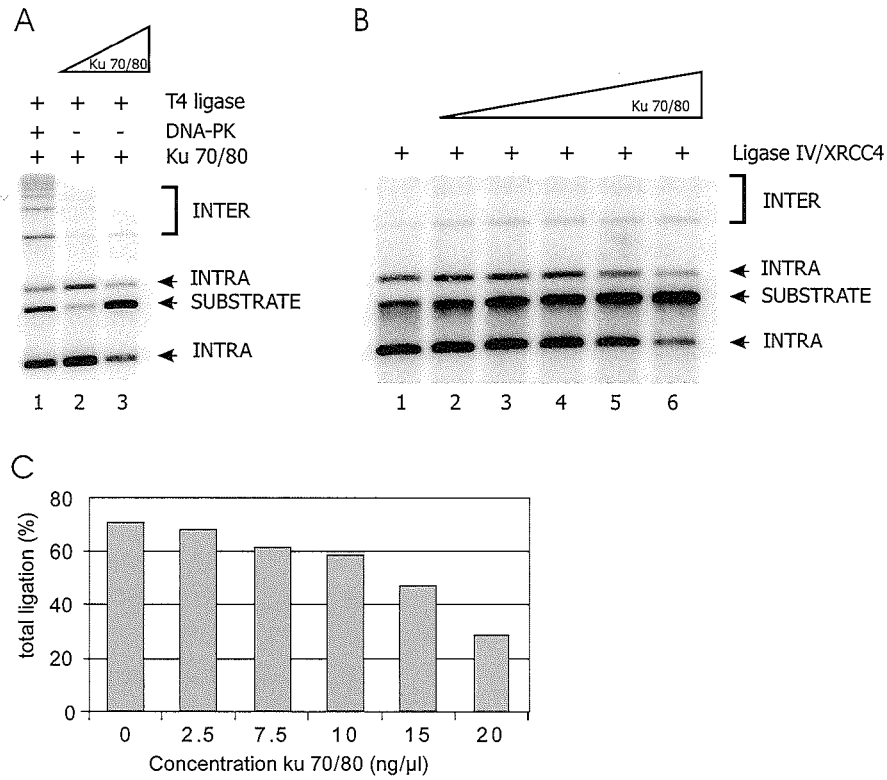

Figure 4 Ku70/80 inhibits both ligase IV/XRCC4 and T4 DNA ligase mediated ligation. (A) A DNA substrate with 3' protruding, complementary ends was incubated with T4 DNA ligase and DNA-PK (lane 1) or two concentrations of purified Ku70/80 (2.5 ng/µl in lane 2 and 7.5 ng/µl in lane 3). **(B)** A similar experiment as in A, with ligase IV/XRCC4 instead of T4 DNA ligase. Increasing Ku70/80 concentrations were added (2.5, 7.5, 10, 15 and 20 ng/µl in lanes 2, 3, 4, 5 and 6, respectively). **(C)** Quantification of the Ku70/80 dependent decrease in ligation efficiency. Total ligation efficiency was determined by measuring the total level of intramolecular ligation products in each lane of figure 4B, and expressing those levels in percentage of total DNA present in the reaction.

Addition of DNA-PK_{cs} alone to a reaction mixture containing T4 DNA ligase, did not result in a complete alteration of ligation route (figure 5A, lane 4). Although some stimulation of intermolecular joining is observed (figure 5A, compare lanes 2 and 4), approximately 85% of total joining products consists of circular monomers. Addition of Ku was necessary for a complete shift in ligation products (figure 5A, lanes 5, 6 and 7). Concentrations of 2.5 ng/µl Ku70/80 and 10 ng/µl DNA-PK_{cs} (lane 5) reduced the level of intramolecular products and increased intermolecular products, compared to the situation where only DNA-PK_{cs} was present (figure 5A, compare lanes 4 and 5). Increasing the Ku70/80 concentration to 5 ng/µl sufficed to almost completely suppress intramolecular joining (figure 5A, lane 6). In this reaction, the molar ratios of DNA:Ku70/80:DNA-PK_{cs} were approximately 1:20:15.

When ligase IV/XRCC4 replaced T4 ligase, a quite different effect of DNA-PK_{cs} emerges (figure 5B). In this case, addition of DNA-PK_{cs} alone shifts 80% of total ligation products towards intermolecular species (figure 5B, lane 3). Furthermore, DNA multimers are larger than observed in systems where the complete DNA-PK holo-enzyme is present (compare figure 5B, lane 3 with figure 5A lane 3 or figure 5B lane 4). We therefore conclude that DNA-PK_{cs} stimulates ligase IV/XRCC4 mediated intermolecular ligation much more efficiently than T4 DNA ligase mediated joining.

Surprisingly, addition of Ku70/80 decreased the total ligation efficiency dramatically (compare figure 5B, lanes 3 and 4 - 8). We did not observe a change in ligation efficiency throughout the Ku70/80 titration range (ranging from 2.5 ng/µl to 20 ng/µl, figure 5B, lanes 4 – 8), showing that the level of intermolecular joining products is independent of the Ku70/80 concentration.

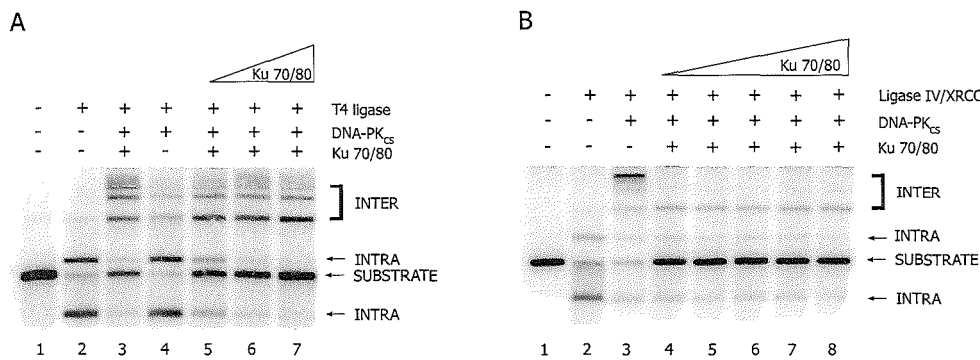

Figure 5 DNA-PK_{cs} stimulates ligase IV/XRCC4 mediated joining, but not T4 DNA ligase mediated joining. (A) T4 DNA ligase mediated ligation in the presence of DNA-PK_{cs} alone (lane 4), or in the presence of a constant amount of DNA-PK_{cs} with increasing Ku70/80 added (lanes 5 – 7). In lanes 5 –7, DNA-PK_{cs} was kept constant at 10 ng/µl, and Ku70/80 was added at a concentration of 2.5 ng/µl (lane 5), 5.0 ng/µl (lane 6) or 7.5 ng/µl (lane 7). **(B)** Ligase IV/XRCC4 mediated ligation in the presence of DNA-PK_{cs} alone (lane 3), or in the presence of a constant amount of DNA-PK_{cs} with increasing Ku70/80 (lanes 4 – 8). In lanes 4 –8, DNA-PK_{cs} was kept constant at 10 ng/µl, and Ku70/80 was added at increasing concentrations (2.5, 7.5, 10, 15 and 20 ng/µl in lanes 4, 5, 6, 7 and 8, respectively).

Ku70/80 stabilizes complexes of DNA with DNA-PK_{cs} and ligase IV/XRCC4

The observation that DNA end-joining by ligase IV/XRCC4 is stimulated by DNA-PK_{cs} alone, but reduced when both DNA-PK_{cs} and Ku70/80 are present, raises the question why Ku70/80 is required for NHEJ *in vivo*. Therefore, we set out to examine the stability of DNA-PK_{cs} – DNA and DNA-PK – DNA complexes under more physiological conditions: at higher salt concentrations. We first established that ligase IV/XRCC4 could still mediate ligation at increasing salt concentrations (figure 6A). Even at 100 mM KCl, the ligation reaction did proceed, although the joining efficiency dropped to approximately 50% upon increasing KCl concentration from 30 mM to 100 mM (as quantified in figure 6C).

Subsequently, we studied end-joining in the standard reaction, containing either DNA-PK_{cs} alone or both DNA-PK_{cs} and Ku70/80, at increasing salt concentrations (ranging from 30 mM to 100 mM KCl, figure 5B). Upon increasing the KCl concentration to 50 mM, intermolecular joining in DNA-PK_{cs} containing reactions dropped 17-fold, compared to 30 mM KCl reactions (figure 6C and figure 6B, compare lanes 1 and 2). Since ligase IV/XRCC4 activity is still 92% at this KCl concentration (compared to 30 mM KCl), this dramatic decrease in ligation products is most probably due to inherent instability of complexes of DNA with DNA-PK_{cs} and ligase IV/XRCC4.

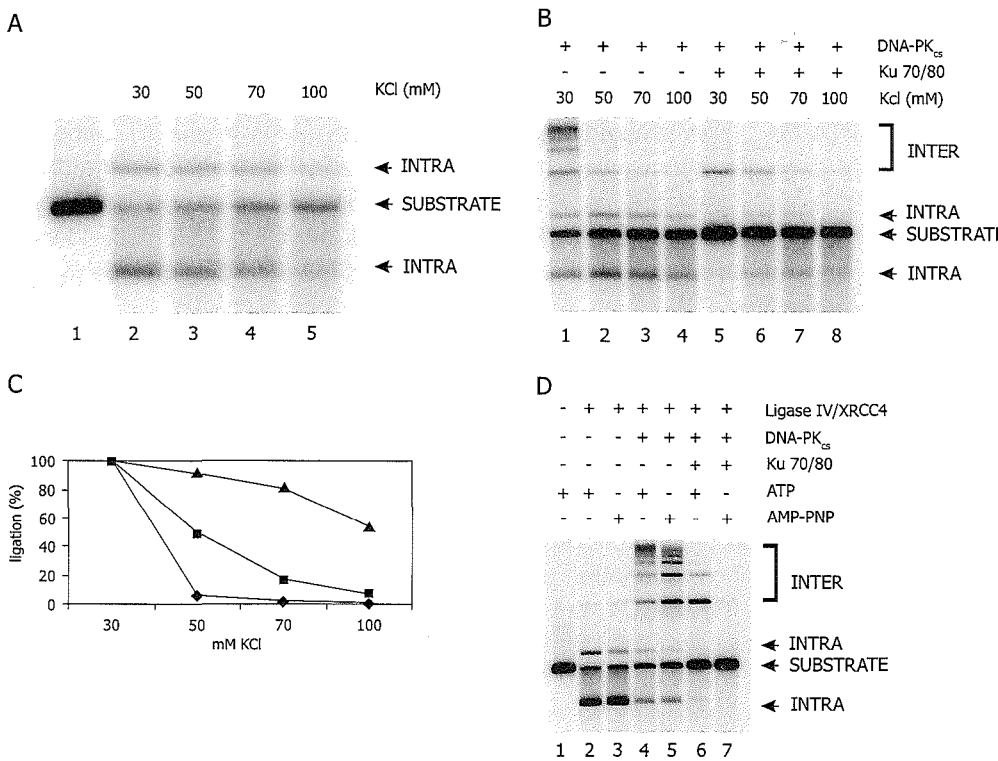

Figure 6 The effects of DNA-PK components on ligase IV/XRCC4 mediated joining at various salt concentrations. (A) Efficiency of ligase IV/XRCC4 mediated joining at different salt concentrations. Ligation reactions were performed at 30, 50, 70 and 100 mM KCl. The levels of total ligation products in each reaction were measured and depicted graphically in C. The level of total ligation products obtained at 30 mM KCl was arbitrarily set at 100%. **(B)** Ligase IV/XRCC4 mediated joining in the presence of DNA-PK_{cs} alone (lanes 1 – 4) or the complete DNA-PK holo-enzyme (lanes 5 – 8) at different salt concentrations. Ligation reactions were performed at 30, 50, 70 and 100 mM KCl. The levels of total intermolecular ligation products in each reaction were measured and depicted graphically in C. The level of total ligation products obtained at 30 mM KCl was arbitrarily set at 100%. **(C)** Quantification of ligation efficiencies. Triangles: ligase IV/XRCC4 mediated ligation in the absence of DNA-PK. Squares: ligase IV/XRCC4 mediated ligation in the presence of the complete DNA-PK holo-enzyme. Diamonds: ligase IV/XRCC4 mediated ligation in the presence of DNA-PK_{cs} alone. **(D)** ATP dependence of ligase IV/XRCC4 mediated ligation in the absence of DNA-PK or in the presence of either DNA-PK_{cs} alone (lanes 4 and 5) or the complete DNA-PK holo-enzyme (lanes 6 and 7). ATP dependence was examined by adding either ATP (lanes 2, 4 and 6) or AMP-PNP (lanes 3, 5 and 7).

When Ku70/80 was present, the ligation reaction proved to be more resistant to higher salt concentrations (figure 6C, figure 6B, compare lanes 5 and 6). Intermolecular ligation efficiency was still 50% at 50 mM KCl when both DNA-PK_{cs} and Ku70/80 were added to the reaction. We therefore conclude that although DNA-PK_{cs} alone greatly stimulates ligase IV/XRCC4 at low salt concentrations, Ku70/80 must be present to stabilize protein – DNA complexes under more physiological conditions.

If Ku70/80 really enhances stability of protein - DNA complexes, one would expect that DNA-PK autophosphorylation may be less important to increase DNA end accessibility in situations where only DNA-PK_{cs} is present at DNA termini, than in situations where the complete DNA-PK holo-enzyme is present. This was indeed the case: replacing ATP with AMP-PNP did not significantly inhibit intermolecular joining when only DNA-PK_{cs} was present (figure 6D, compare lanes 4 and 5), although intermolecular joining products were mostly shorter and the level of unmodified substrate was slightly elevated, indicating that ligation efficiency was somewhat decreased. In contrast, addition of Ku70/80 introduced the clear ATP dependence that we always observed when examining the complete DNA-PK holo-enzyme (figure 6D, compare lanes 6 and 7).

Discussion

DNA-PK autophosphorylation stably changes DNA end accessibility

We here show that ligation of DNA termini with associated DNA-PK requires an ATP driven, DNA-PK dependent process in order to proceed effectively. This must be due to kinase activity, since protein dephosphorylation in turn prevents efficient ligation of DNA termini. This phosphorylation event did not only lead to increased DNA end accessibility for ligase IV/XRCC4, but also for T4 DNA ligase or *E. coli* ligase, as presented in a previous paper [16]. Since no biologically relevant interaction between DNA-PK and T4 DNA ligase or *E. coli* ligase is known or expected, the target of the DNA-PK mediated phosphorylation event that enhances DNA end accessibility, must be the DNA-PK holo-enzyme itself. This conclusion is consistent with our finding that DNA-PK mediated ligase IV/XRCC4 phosphorylation is not required for efficient ligation (figure 2B). We therefore infer that DNA-PK autophosphorylation facilitates DNA end accessibility.

Our experiments demonstrate that the protein – DNA complexes that are formed upon DNA-PK autophosphorylation are stable and can be readily isolated. When the DNA-PK – DNA complex is pre-phosphorylated and subsequently separated from ATP, ligation further proceeds in an ATP independent manner, suggesting that DNA-PK autophosphorylation is the sole action that needs to be performed in order to facilitate DNA end accessibility. Furthermore, we conclude that the change in ligation route does not depend on phosphorylation of ligase IV/XRCC4 by DNA-PK, since ligation of prephosphorylated complexes containing DNA and DNA-PK proceeds in an

intermolecular fashion, even in the presence of AMP-PNP (see figure 2B). Moreover, Ku70/80 alone hardly affects the ligation route, indicating that the catalytic subunit mediates the shift from intramolecular to intermolecular ligation.

An important issue that is still a matter of debate in the NHEJ field, is the fate of DNA-PK_{cs} after autophosphorylation. Complete dissociation of DNA-PK_{cs} after autophosphorylation has been reported [25, 26], but is contradicted by several other studies that suggest a more moderate conformational alteration of protein - DNA complexes [16, 29, 32]. In this paper, we have presented evidence that DNA-PK_{cs} must be present for a distinct shifting of ligation products towards intermolecular species. The finding that ligation of isolated complexes containing prephosphorylated DNA-PK and DNA proceeds in an exclusively intermolecular fashion, can most easily be explained when we assume that DNA-PK_{cs} is still associated with the DNA – Ku70/80 complex after DNA-PK_{cs} autophosphorylation. These results argue for a conformational alteration of protein-DNA complexes, rather than for complete dissociation of DNA-PK_{cs}. Another possibility would be, that DNA-PK_{cs} stably alters the Ku-DNA complex in an ATP dependent manner and subsequently dissociates from the DNA. However, footprinting experiments that were conducted under conditions that allow for DNA-PK autophosphorylation, did not indicate such a dissociation [16].

Taken together, our data plea for a very central role of DNA-PK_{cs} in regulating end accessibility during NHEJ. This conclusion introduces a discrepancy between *in vitro* and *in vivo* observations, since the phenotype of DNA-PK_{cs} deficiency is relatively mild. DNA-PK_{cs} deficient mice are viable, in contrast to ligase IV or XRCC4 deficient mice. This discrepancy may be explained by redundancy between DNA-PK_{cs} and another, related PI-3 like kinase such as ATM. The fact that simultaneous deletion of both DNA-PK_{cs} and ATM is not viable in mice, supports this hypothesis [33, 34]. Furthermore, end-joining reactions *in vitro* may be different from *in vivo* NHEJ, since cellular DNA substrates exist in a chromatin context, which may put other constraints on the NHEJ reaction than the naked DNA in our assays. Moreover, the *in vivo* situation may be more complex due to interaction of the core components with additional NHEJ factors that have been either shown or predicted to exist [35, 36].

Interactions between DNA-PK subunits and ligase IV/XRCC4

We here show that DNA-PK_{cs} is able to cause a partial shift in joining products from intra- to intermolecular species in the presence of T4 DNA ligase, but that it requires the presence of ligase IV/XRCC4 to facilitate this shifting effectively. This difference between both ligases can most easily be explained by a direct interaction between DNA-PK_{cs} and ligase IV/XRCC4. Such interaction could possibly stabilize complexes containing DNA-PK_{cs} and DNA and has been reported before [19, 21].

The amount of multimeric DNA joining products that is formed during ligation in the presence of DNA-PK_{cs} alone, suggests a role for DNA-PK_{cs} not only in

inhibition of intramolecular ligation, but also in active stimulation of intermolecular ligation. This stimulatory effect of DNA-PK_{cs} on ligase IV/XRCC4 mediated ligation was only observed at low salt concentrations, indicating that either the interaction between DNA-PK_{cs} and ligase IV/XRCC4 or between ligase IV/XRCC4 and DNA is not very stable at physiological salt conditions. Addition of Ku70/80 decreases the total ligation efficiency, but alleviates the salt sensitivity of the ligation system. This effect of Ku70/80 can be due either to a stabilizing effect of the heterodimer on complexes containing DNA-PK_{cs} and DNA or to a direct interaction between Ku and ligase IV/XRCC4.

Several authors observed interactions between Ku and ligase IV/XRCC4, suggesting either a physical recruitment of ligase IV/XRCC4 by Ku or a stimulation of ligation activity in a Ku dependent manner [21, 22, 37]. Contrasting, other investigators found an inhibitory effect of Ku on ligase IV/XRCC4 mediated joining [19], or either a stimulatory or inhibiting effect, depending on the ratio of Ku:DNA [38]. In our reaction system, using a molar excess of Ku protein versus DNA termini (as is the physiological situation), Ku70/80 clearly inhibits ligase IV/XRCC4 mediated intramolecular (but not intermolecular) ligation. When Ku70/80:DNA ratios were more or less equimolar, neither a decrease nor an increase in joining efficiency could be observed.

Our findings indicate a trade-off between ligation efficiency and stability. The presence of Ku70/80 at DNA termini makes them less available for ligation, but stabilizes the total protein – DNA complex. Interestingly, we found that it is the Ku70/80 component of the DNA-PK holo-enzyme that introduces the need for DNA-PK autophosphorylation in order to facilitate efficient ligation, suggesting that complexes containing DNA-PK and DNA are stabilized by the presence of Ku to such an extent that an energy consuming process is required to make DNA ends accessible again. This mechanism would possess regulatory capability, allowing DNA-PK to stably occupy DNA ends, preventing premature processing or degradation, until DNA-PK autophosphorylation enables the next step in the NHEJ process.

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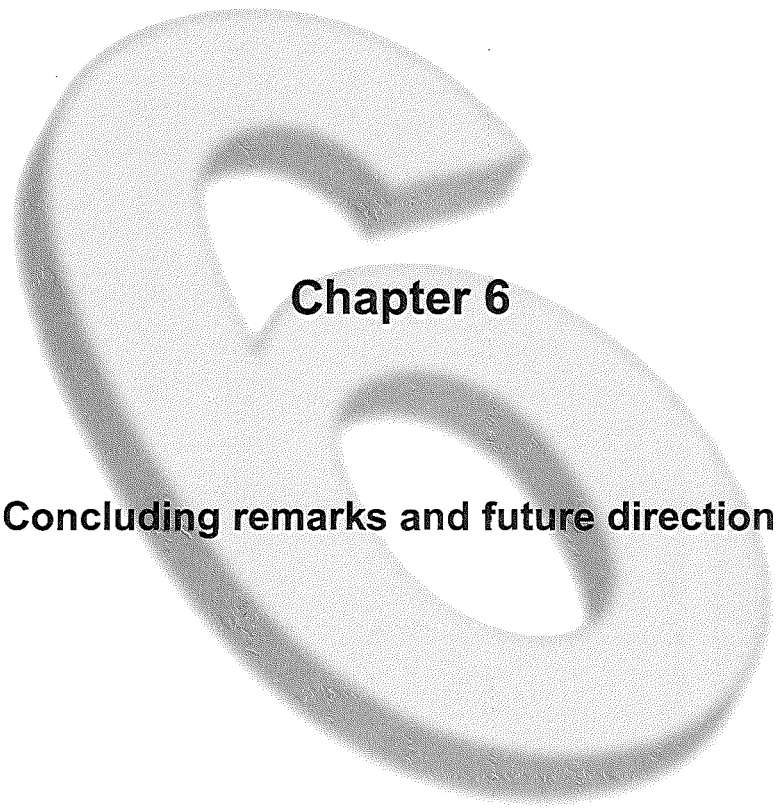

Chapter 6

Concluding remarks and future directions

The contribution of this thesis to NHEJ research

The subject of non-homologous end-joining (NHEJ) is a relatively new area of research. Detailed insights into the molecular mechanism of NHEJ were obtained not earlier than the mid 1990's, when the cloning of NHEJ genes made biochemical studies with purified enzymes possible. In a relatively short period, it became clear that the DNA-PK holo-enzyme not only binds to DNA ends, but that it has the potential to tether DNA termini and to interact with the ligase IV/XRCC4 complex (see chapter 2 for an overview). These data suggested that DNA-PK functions as a kind of molecular anchor during NHEJ, mediating the formation of a multi-protein complex that bridges the two ends of a broken DNA molecule and recruiting enzymes that are required to rejoin the DNA ends. Although this model is attractive and may very well reflect part of the truth, it is certainly simplistic and does not account for all observations.

A relevant and timely question that is not answered by the 'bridging-model', concerns the relevance of the DNA-PK_{cs} kinase activity, which is most likely not necessary for binding and tethering of DNA. Since DNA-PK_{cs} is a member of the PI-3 like kinase family, like ATM, it may function in the signal transduction cascade upstream of the NHEJ process. On the other hand, DNA-PK_{cs} may exert its main function by activation of factors that play directly in the NHEJ process. In vitro studies have shown that DNA-PK_{cs} is able to phosphorylate p53, XRCC4, Ku70/80 and DNA-PK_{cs} itself, observations that are consistent with both hypotheses and do not unequivocally answer the question of the biologically relevant target of DNA-PK_{cs} kinase activity [1]. The situation has become even more complicated after the finding that DNA-PK_{cs} forms a functional complex with the Artemis protein, which may mediate processing of DNA termini [2].

The research efforts that are presented in this thesis, provide novel insights into the biochemistry of DNA-PK at DNA termini and may contribute to a better understanding of its function during NHEJ. The findings discussed in chapters 4 and 5 highlight the importance of DNA-PK_{cs} autophosphorylation in regulating access of enzymes, either nucleases or ligases, to DNA termini that are occupied by DNA-PK. This autophosphorylation takes place, either in *cis* or in *trans*, in a synaptic complex of DNA-PK and two DNA termini. These data suggest a model in which unphosphorylated DNA-PK associates with DNA ends and protects them from premature degradation until both DNA termini are brought together in a protein – DNA complex. Within this complex, DNA-PK functions in a 'switch-like' fashion, regulating access of ligase IV/ XRCC4 or a processing enzyme to the DNA termini by means of its phosphorylation status.

This model predicts that deficiencies in DNA-PK_{cs} autophosphorylation would strongly reduce cellular DSB processing or repair via the NHEJ pathway. Interestingly, several studies have been published recently, in which the influence of mutations in known DNA-PK_{cs} autophosphorylation sites were examined *in vivo* [3-5] These reports unequivocally show that cellular ionizing radiation resistance and

normal DSB rejoining requires DNA-PK_{cs} autophosphorylation. One of these studies shows a strong reduction in processing of rare coding junctions that are formed during V(D)J recombination, upon mutation of DNA-PK_{cs} autophosphorylation sites [3]. In conclusion, all available *in vivo* data on DNA-PK_{cs} autophosphorylation suggest a more or less central role for DNA-PK in regulation of DSB rejoining efficiency and possibly in end processing, very consistent with the 'switch' function model presented in this thesis.

The 'switch' model of DNA-PK autophosphorylation is also consistent with biochemical and atomic force microscopy studies that indicate the presence of DNA-PK at synapses of DNA ends and explains why a protein kinase is required at these places [6-8]. It certainly does not exclude the possibility that DNA-PK_{cs} kinase activity is also necessary for the activation of other NHEJ factors. On the contrary, it may very well be that the Artemis protein (or another processing enzyme) is specifically recruited to the DNA-PK – DNA complex and regulated in a very organized fashion by the DNA-PK_{cs} kinase. This regulation may take place on two levels: 1. the nucleolytic activity of Artemis is regulated by DNA-PK_{cs} mediated phosphorylation [2] and 2. the access of activated Artemis to the DNA termini is regulated by DNA-PK_{cs} autophosphorylation (as suggested in chapters 4 and 5).

In addition, the 'switch' model leaves room for a possible interaction between DNA-PK and ligase IV/XRCC4 at the DNA end synapses [9, 10]. Indeed, several experiments described in chapter 5 suggest an interaction between DNA-PK_{cs} and the ligase IV/XRCC4 complex (at least under low ionic strength conditions). DNA-PK_{cs} either recruits ligase IV/XRCC4 to the DNA termini or stimulates the joining activity of ligase IV. However, the *in vivo* relevance of such an interaction still requires experimental confirmation. In any case, end-joining by ligase IV/XRCC4 still proceeds after complete autophosphorylation (and thus inactivation) of DNA-PK_{cs}, indicating that the DNA-PK_{cs} kinase activity may not be necessary for ligase IV/XRCC4 recruitment (chapter 5).

Future directions

Research on DNA-PK

The 'switch'-model described above, in which DNA-PK_{cs} autophosphorylation regulates processing and joining of DNA termini, is a very powerful tool in explaining the observed *in vivo* requirement for DNA-PK_{cs} autophosphorylation during normal DSB rejoining. The use of purified mutant DNA-PK_{cs} protein in *in vitro* end-joining assays would be very beneficial to further strengthen this hypothesis. In particular, DNA-PK_{cs} protein with mutations in known autophosphorylation sites would be useful. However, expression of (mutant) DNA-PK_{cs} in a bacterial or baculovirus system and subsequent purification is twarthed by either recombination events that disrupt the reading frame of the overexpression construct, or by unacceptably low expression levels.

This technical problem has, up to the present, placed constraints on both the purification of wild type DNA-PK_{cs} (as explained in chapter 3) and on the availability of mutant DNA-PK_{cs} protein. Clearly, research on NHEJ would greatly benefit from improvement in the technique of recombinant overexpression of DNA-PK_{cs}.

The research presented in chapters 4 and 5 does not completely explain the role of Ku70/80 in the DNA-PK_{cs} autophosphorylation mediated regulation of DNA end accessibility. The presence of Ku70/80 will certainly be needed to stimulate DNA-PK_{cs} kinase activity, necessary for DNA-PK_{cs} autophosphorylation. In addition, Ku70/80 most likely stabilizes the DNA-PK_{cs} – DNA complex, as suggested in chapter 5. Obviously, upon DNA-PK_{cs} autophosphorylation a conformational change takes place in the end-bound protein complexes, which liberates DNA termini or increases their accessibility. However, it is still unclear whether Ku70/80 plays an active role in this remodeling process, or that it solely depends on DNA-PK_{cs}. Ku70/80 phosphorylation by the DNA-PK_{cs} kinase may be required in order to enable the remodeling process. *In vitro* study on the effect of Ku70/80 protein with mutations in known phosphorylation sites, is an efficient way to address these questions. The availability of Ku80 protein with C-terminal deletions would also be beneficial, since activation of DNA-PK_{cs} by Ku is reported to be mediated by the Ku80 C-terminal domain [11].

Another interesting problem in the NHEJ field that still awaits further explanation, is the alteration of ligation route by DNA-PK. This phenomenon has been observed by many researchers, but has never been explained [9, 12-14]. In chapter 4, a working model is presented that may explain why DNA molecules are exclusively ligated in an intermolecular fashion in the presence of (autophosphorylated) DNA-PK, even though an intramolecular route is distinctly preferred when DNA-PK is absent. This model infers that the two ends of the DNA substrate molecule are physically tethered by the end-bound DNA-PK complex, which is perfectly consistent with a role of DNA-PK in the formation of a molecular bridge between DNA termini. However, NHEJ *in vivo* would be disabeled when the tethered DNA termini are not able to ligate. The relief of this block may *in vivo* very well require an additional factor, which is possibly needed to remove proteins that are strongly associated with the DNA termini. The fact that such a factor has not been found yet, suggests that it may be an enzyme with an activity that is masked by redundancy. Identification of this hypothetical missing factor may be commenced by addition of fractionated cellular extracts to *in vitro* end-joining reactions and subsequent downstream processing of fractions that are able to reverse DNA-PK induced shifting of ligation route.

General NHEJ research

Research on NHEJ is in general complicated by the fact that not all enzymes involved are known. Several authors have reported the existence of factors that contribute to the NHEJ pathway, but their mechanisms of action and their place in the NHEJ process remain unresolved [15, 16]. Identification of processing enzymes is difficult

since their activities may be redundant and not NHEJ specific, as explained in chapter 2. The molecular mechanism of NHEJ will, however, never be completely understood before all factors involved are known and their possible interactions with the core components, DNA-PK and ligase IV/XRCC4, are mapped. Isolation of missing factors will require the use of biochemical as well as genetic methods. *In vitro* end-joining assays, as described in this thesis, can surely contribute to studies on the effects of fractionated cellular extracts on ligation efficiency. In the past, such studies have already proven to be fruitful. They are, however, not yet used to the full extent of their capacity.

The value of biochemical end-joining assays is significant, but limited by the fact that DNA substrates *in vivo* exist in a chromatin context. Information on the composition and structure of such substrates is very limited and therefore almost impossible to mimic *in vitro*. Chromatin structure may place specific demands on the NHEJ mechanism, introducing the need for chromatin modifying enzymes, like helicases. Such enzymes will most likely not be recognized when cellular extracts are used in end-joining assays with naked DNA substrates. Hence, another approach will have to be contemplated for the isolation and characterization of factors that mediate regulation of DSB repair in a chromatin context.

Two major techniques that will contribute to a better understanding of the NHEJ mechanism are atomic force microscopy (AFM) and fluorescence microscopy. AFM has already proven its value in elucidating the role of Ku and DNA-PK_{cs} in binding and tethering DNA ends [7, 8, 17]. When mutant variants of core enzymes become available, AFM may be very useful to examine association of these mutant proteins to either DNA or other proteins. *In vivo* verification of the data thus obtained can be most conveniently done by means of microscopic colocalization studies on fluorescently labeled NHEJ (mutant) proteins. In addition, fluorescence microscopy studies will possibly yield data on *in vivo* recruitment kinetics of mutant and wild-type enzymes to DSB sites, although the ratio of free protein to available DSB sites may be unfavorable for these kinds of measurements.

X-ray crystallographic imaging of the NHEJ enzymes will undoubtedly lead to a better understanding of protein–protein or protein–DNA interactions. The resolution of the Ku70/80 three-dimensional structure has been an important step forward in the NHEJ field and a very good example of protein structure-function relation [18]. Especially data on the structure of DNA-PK_{cs} are needed, since they may provide insight into both the DNA binding (or tethering) abilities of this protein and the mechanism of its phosphorylation induced remodeling. Low resolution images of DNA-PK_{cs} have already been made available, but crystalization of the enzyme has not been reported yet [19, 20]. It is to be expected that crystalization of smaller parts or domains of DNA-PK_{cs} will prove to be fruitful, so interesting data are likely to emerge in the coming years.

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Nederlandse samenvatting

De cel ligt aan de basis van ieder leven en mag letterlijk beschouwd worden als de allerkleinste bouwsteen daarvan. Het is een microscopisch klein, met vocht gevuld, driedimensionaal compartiment met een min of meer autonome stofwisseling. Binnen de grenzen van de cel bevindt zich een nog kleiner compartiment, de celkern, waarin het DNA is gelegen. DNA is een chemische verbinding die tijdens de celdeling van generatie op generatie wordt doorgegeven. Men kan zich de allerkleinste eenheid van die verbinding, het DNA molecuul, het beste voorstellen als een heel lange, dunne draad. Wanneer we de lange draad van het DNA molecuul in detail bekijken, dan blijkt het een ketting te zijn die is opgebouwd uit vele miljoenen schakels. Iedere schakel noemen we een 'base'. De volgorde waarin de basen aan elkaar geschakeld zijn, vormt een soort van morsecode: de genetische code. Die code fungeert als een blauwdruk voor de vervaardiging van duizenden verschillende eiwitten, elk met een eigen functie. Eiwitten kunnen dienen als signaalstoffen die cellulaire processen reguleren. Dergelijke signalen bepalen bijvoorbeeld tot wat voor celtype een cel zich gaat ontwikkelen, wanneer een cel zich gaat delen, wat voor stoffen deze uitscheidt, enzovoorts. Eiwitten kunnen ook fungeren als enzymen, een soort van moleculaire machines die bepaalde chemische reacties uitvoeren.

Als de precieze volgorde van de DNA basen verandert, bijvoorbeeld ten gevolge van een beschadiging, dan is het heel goed mogelijk dat bepaalde eiwitten niet meer correct worden vervaardigd. Daardoor kunnen belangrijke processen in de cel volledig ontsporen. Wanneer de schade aan het DNA erg groot is, sterft de cel meestal af. Maar wanneer een cel de DNA verandering overleeft, is er grote kans dat dit veranderde DNA door middel van celdeling aan de volgende generatie cellen wordt doorgegeven. Soms kan DNA schade de functie van een cel en het delingsproces zo ontregelen, dat een wildgroei van de cel optreedt. Men spreekt dan van kanker.

Er zijn tal van zaken die het DNA kunnen beschadigen, doordat ze in staat zijn een chemische reactie met het DNA molecuul aan te gaan. Ultraviolette en radioactieve straling zijn bekende oorzaken van DNA schade. Soms introduceert de cel zelf fouten in het DNA. Gelukkig hebben alle bekende cellen enzymen ter beschikking, die beschadigingen aan het DNA weer kunnen herstellen. Voor iedere soort beschadiging is in de loop der evolutie een set reparatie-enzymen ontwikkeld. In **hoofdstuk 1** is een overzicht gegeven van de soorten DNA beschadigingen die kunnen optreden en van de verschillende reparatieprocessen die door de enzymen worden uitgevoerd.

Dit proefschrift concentreert zich op één bepaalde soort DNA schade, namelijk de zogenaamde dubbel-strengs breuken. Dit zijn breuken in het DNA molecuul, die er de oorzaak van zijn dat stukjes van chromosomen (een soort opgerolde DNA moleculen die zich in de celkern bevinden) letterlijk afbreken. Deze stukjes gaan verloren of worden aan andere chromosomen vastgemaakt, waardoor een volledige chaos in de celkern ontstaat. Het is dus van groot belang dat dergelijke breuken gerepareerd kunnen worden.

Eén van de twee reparatieprocessen die dubbel-strengs breuken kunnen

aanpakken, wordt aangeduid met de term 'non-homologous end-joining' (NHEJ). De enzymen die een rol spelen bij dit proces, zijn door gecoordineerde samenwerking in staat om een 'brug' te slaan tussen de beide fragmenten die ontstaan na het breken van het DNA molecuul. Deze brug brengt de uiteinden bij elkaar en maakt ze weer aan elkaar vast, een proces dat 'ligatie' wordt genoemd. We weten dat er minimaal vier enzymen betrokken zijn bij NHEJ, hoewel er goede aanwijzingen bestaan dat er nog meer factoren een rol spelen. De bekende spelers hebben de volgende namen: XRCC4, ligase IV, Ku70/80 en DNA-afhankelijk proteïne kinase (DNA-PK_{cs}). **Hoofdstuk 2** is een gedetailleerde samenvatting van de meest relevante gegevens over het NHEJ proces, de functies van de bekende NHEJ enzymen en de onderlinge samenwerking van die enzymen.

Hoewel de centrale NHEJ enzymen bekend zijn, is het nog geenszins volledig duidelijk wat ze precies doen tijdens het herstel van DNA breuken. In dit proefschrift worden proeven beschreven, die tot doel hebben het NHEJ proces in een reageerbuis (*in vitro*) na te bootsen. De nadruk ligt daarbij op bestudering van de eigenschappen van het enzym DNA-PK_{cs}.

Om zulke proefnemingen te kunnen verrichten, moesten de vier bovengenoemde NHEJ enzymen eerst in zuivere vorm geïsoleerd worden. Daarna werd bestudeerd op welke wijze ze een interactie aangaan met stukjes DNA en met elkaar. In **hoofdstuk 3** is beschreven hoe het enzym DNA-PK_{cs} via een gecompliceerde zuiveringsprocedure gewonnen kan worden uit menselijke nageboorten (placenta) of gekweekte menselijke cellen. Om een indruk te geven van de omvang van een dergelijke zuivering: een placenta van een halve kilogram levert ongeveer 50 microgram DNA-PK_{cs} op: een onzichtbare hoeveelheid.

In **hoofdstukken 4, 5 en 6** worden de *in vitro* proefnemingen aan DNA-PK_{cs} en de andere HNEJ enzymen beschreven en uitgelegd. Deze proeven tonen aan dat DNA-PK_{cs} en Ku70/80 samen aan de uiteinden van DNA moleculen binden en daar een eiwit-DNA complex vormen. Dit complex beschermt de uiteinden van het DNA molecuul tegen cellulaire enzymen die DNA kunnen modificeren. Het zorgt er tevens voor dat ligase IV, het enzym dat tijdens NHEJ de ligatie verricht, DNA fragmenten niet aan elkaar kan koppelen.

Het DNA-PK_{cs} enzym blijkt een actieve rol te spelen bij het bijeen brengen van twee DNA fragmenten. De eiwitcomplexen die zich op de DNA uiteinden bevinden, bestaande uit DNA-PK_{cs} en Ku70/80, vormen een soort van moleculaire 'brug' tussen twee stukken DNA. In deze brug bevinden zich waarschijnlijk meerdere DNA-PK_{cs} moleculen. Een aantal proeven dat in hoofdstuk 4 uiteen is gezet, laat zien dat een DNA-PK_{cs} molecuul in zo'n brug in staat is om een fosfaatgroep aan een ander DNA-PK_{cs} molecuul te koppelen. Deze reactie wordt autofosforylatie genoemd. DNA-PK_{cs} autofosforylatie blijkt een verandering teweeg te brengen in het eiwit-DNA complex, waardoor de uiteinden ineens wél beschikbaar worden voor ligatie.

Deze bevindingen doen sterk vermoeden dat DNA-PK_{cs} autofosforylatie de ligatiestap in het NHEJ proces reguleert, simpelweg door ligase IV toegang te

verlenen tot de DNA uiteinden. Die verhoogde toegankelijkheid van de DNA uiteinden wordt dan precies op het juiste moment bewerkstelligd, namelijk op het ogenblik dat de DNA fragmenten in een DNA-eiwit complex bij elkaar worden gebracht. De *in vitro* proeven suggereren overigens wel, dat de situatie in de cel niet zo heel simpel is. Waarschijnlijk zijn er nog onbekende factoren nodig om de DNA uiteinden die in zo'n complex gevangen zitten, netjes aan elkaar te ligeren.

Enkele experimenten die in hoofdstuk 5 beschreven zijn, maken aannemelijk dat DNA-PK_{cs} een directe interactie aangaat met ligase IV. Deze interactie stimuleert het ligase, maar zij treedt alleen op onder bepaalde laboratoriumcondities die niet geheel overeenkomen met de cellulaire situatie. Waarschijnlijk is de aanwezigheid van Ku70/80 nodig om de interactie tussen ligase IV en DNA-PK_{cs} te versterken.

Als we nu alle gegevens over DNA-PK_{cs} bij elkaar nemen, dan blijkt dit enzym een heel centrale rol te spelen in het NHEJ proces. DNA-PK_{cs} is betrokken bij het bij elkaar brengen van de DNA uiteinden die ontstaan na het breken van een DNA molecuul. Zolang die uiteinden elkaar nog niet gevonden hebben, beschermt DNA-PK_{cs} ze tegen afbraak, voortijdige modificatie of ligatie. Zodra de DNA uiteinden bijeen zijn gebracht in een eiwit-DNA complex, reguleert DNA-PK_{cs} de toegankelijkheid van de uiteinden door middel van autofosforylatie. DNA-PK_{cs} verzorgt daarna waarschijnlijk ook nog de recrutering van ligase IV naar de vrijgemaakte DNA uiteinden. Deze gegevens voorzien ons van nieuwe inzichten in de wijze waarop coordinatie van modificatie en ligatie plaatsvindt tijdens het NHEJ gestuurde herstel van dubbelstrengs DNA breuken.

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Naschrift

Een sotternij

Iedere overeenkomst met bestaande personen of gebeurtenissen is geheel toevallig. Dit in tegenstelling tot de diverse anachronismen: deze zijn bewust aangebracht.

Setting is een alchemistisch laboratorium, waarin een grote oven door zwetende dagloners roodgloeiend wordt gehouden met behulp van een blaasbalg. Temidden van sulfurische dampen betreedt een magere magister het toneel, zenuwachtig heen en weer hippend op zijn hielen en tenen, de schaarse haren wolkend in een aureool van geleerdheid. Op zijn zwarte gewaad prijkt de orde van het gulden hoornvlies, een blijk van verdienste die uitsluitend wordt toegekend aan magisters die verder kunnen zien dan anderen.

Magister
Verzamelt u allen en opent uw oren,
de tijd die ik vraag is voor u niet verloren:
'k wil u vertellen hoe in beelden en dromen,
de waarheid met vleugels tot mij is gekomen.
Een gloednieuw idee is in mijn geest gerijpt,
verstandig is hij, die 't volledig begrijpt.
Nog één experiment moeten wij ondernemen,
en dan zal de wereld spoedig vernemen,
hoe zonder veel moeite hier plots'ling verrijzen,
de heilige graal en het steentje der wijzen.
Leerling! Nu snel op je benen gerept,
en vindt de bewijzen voor mijn groots concept!
Hier zijn de plannen, in kannen en kruiken,
pasklaar, gereed en direct te gebruiken:
resultaat en conclusie, 't staat allemaal hier,
netjes beschreven op dit stuk papier.
Wat nog ontbreekt is de proef op de som,
wie die niet kan lev'ren is echt veel te dom.
Want als nu het proefje mijn doelen niet haalt,
dan is het de leerling wiens kunde hier faalt,
maar mocht hij toch slagen naar mijn mooie plan,
dan ben ik als meester een heel begaafd man!

Leerling
Maar meester, rijst hier alweer een nieuw plan?
Ik weet niet of ik dit nog wel bijhouden kan.
Tientallen proeven en honderden gralen,
zijn door mijn hoofd heen en weer aan het dwalen.
Iedere dag verandert de missie!
Mag ik vermelden, mét uw permissie:
'k waardeer dat ik mij aan uw kennis mag laven,
maar gist'ren lag de graal in het westen begraven,
vandaag moet ik hem in het oosten gaan zoeken,
en morgen, misschien, doet u mij uit de doeken,
dat we de graal in het zuiden gaan vinden;
ons plan de campagne slaat om als de winden.
Wat ons ontbreekt is één duid'lijk doel,
niet gebaseerd op hints of gevoel,
maar bereid van een deeg dat goed is doorkneden,
zodat ik mijn tijd efficient kan besteden,
aan 't grondig bewerken van één goed project,
want slechts dán is het moog'lijk dat men data verwekt,
die naadloos zullen passen in een groter geheel,
dán valt ons zeker een paper ten deel.
Maar blijven we toch onze aandacht verspreiden,
dán is de voortgang niet méér dan bescheiden.

Magister
Waarom blijf je me hiermee vermoeien?
Je mening kan mij bij benad'ring niet boeien.
Het is gewoon luiheid die je hier camoufleert,
Ik denk dat je meer slaap dan een paper begeert.
Als je wilt dat je werk ons ooit nog zal baten,
zul je 't één moeten doen en 't ander niet laten.
Maar een simpele proef blijkt voor jou al te veel,
ik bak echt geen brood van dit heel slechte meel.
Ik doe de proef zelf wel, die moeite is klein,
zo'n erg grote klus kan dat nauwelijks zijn.

Een geweldige ontploffing doet het toneel op zijn grondvesten schudden. Met een wolk van stof als nasleep treedt de Magister Magistralis binnen, de witte haren strak achterover gekamd. Trots draagt deze indrukwekkende figuur de orde van de gelaarsde arend, een onderscheiding voor mensen die zouden kunnen vliegen op vleugels van roem, maar uit bescheidenheid liever blijven lopen.

Magister Magistralis
Ach, beste jongen, dit spijt mij nu zeer,
de verstrooidheid, 't is zeker, die kwelt me hier weer!
Het valt ook niet mee om professor te zijn,
die baan zit echt vol venijn en chagrijn!

Koor der professoren
Ja dat is waar, onze zorgen zijn groot,
aan vele gevaren staat het onderzoek bloot.
Japanners, Chinezen en Amerikanen,
offeren daag'lijks hun bloed, zweet en tranen.
Ongelijk is de strijd, het doet ons veel leed,
maar soms komt een paper gewoon niet gereed.
Ons treft geen blaam, onze leiding is goed,
maar wanneer Den Haag nauw'lijks bijdrage doet,
ontbreekt 't aan geschut in een bloedig gevecht.
Daarnáást moet nog nodig iets anders gezegd:
personeel, tegenwoordig, is hopeloos slecht,
met zó weinig inzet komt er nooit iets terecht!
Win eens een oorlog met zwangere vrouwen!
En leerlingen, denkend aan kind'ren en trouwen!
Verlof en vakantie is al wat ze boeit,
en daardoor wordt altijd de planning verknoeid.

Jan Peter
U bent zo ondankbaar, geleerden met baarden!
Den Haag geeft u gratis haar normen en waarden!
Mijn normen zijn daag'lijks te zien op de buis,
maar de waarden die blijven bij mij in de kluis.
Tot hoger donatie ben ik niet bereid,
besteedt maar de poet met een beter beleid!

Koor van de gevestigde orde
(in canon met Jan Peter)
Nóg harder werken dan wij nu al doen?
En dat voor een schijntje en heel weinig poen?
Laat leerling-magister maar prutsen en knoeien,
het eindresultaat scheidt de slechten en goeien.
Het geeft ze de kans om talent te bewijzen,
en mocht er toevallig geen vinding verrijzen,
dan kunnen wij zeggen: óns treft geen blaam,
leerling en baas zijn zo onbekwaam.

Magister
(na tien minuten naar de Graal te hebben gezocht)
Wat een gedoe, dat gepruts in het lab,
die buffer is op, da's geen leuke grap,
naar een pot of een pan zoek ik soms drie kwartier,
maak maar eens tempo op deze manier!
Op korte termijn behaal ik geen eer,
terwijl het juist snel moet, ik wil zo veel meer!
Alles gaat langzaam en echt veel te stroef,
laat zitten, vergeet, abondeer deze proef!

Dankwoord

Alvorens het doek neerdaalt over ons blijspel, wil de auteur nog een woord richten tot het verzameld publiek. Niet in de laatste plaats om alle onzekerheid omtrent het lot van de onfortuinlijke leerling-magister weg te nemen. Wees gerust: het gaat hem voorspoedig. Hij schreef een mooie magisters-thesis, zonder ooit de Steen der

Wijzen te hebben gevonden. Maar dit laatste is zoals het zijn moet. Want zolang de Steen zich verbergt, zólang wordt de waarde van een ontdekking nog bepaald door de bezieling die aan die verovering ten grondslag lag. In de woorden van Bernardo Trevisano: 'om goud te maken, moet men van goud uitgaan.'

Wat deze stelling betekent voor de waarde van mijn eigen proefschrift, laat ik ter beoordeling aan een eminenter gezelschap. Liever gebruik ik de resterende regels van dit naschrift voor een hoger doeleinde, namelijk het neerschrijven van een laudatio op de mensen die me terzijde stonden bij de incarnatie van dit boekje. Menig lezer van onderhavig naschrift heeft waarschijnlijk al nerveus heen en weer gebladerd, zoekend naar de sectie waarin dan eindelijk het langverwachte dankwoord wordt uitgesproken. En terecht, dierbare lezer! Want waarlijk, al dat geschrijf heeft lang genoeg geduurd. De sluitende strofe komt met hoge snelheid dichterbij en slechts weinig regels scheiden ons nog van het stuk karton dat de grens van mijn werkstuk in ruimte en tijd vastlegt. Laat ik dus niet langer talmen met het vernoemen van de mensen wiens bijdrage aan dit boekje een vermelding rechtvaardigt.

Het is, geloof ik, geen usage om het nawoord te beginnen met een dankbetuiging aan je ouders. Maar ik ben nooit iemand geweest die zich veel gelegen laat aan protocollen, zeker niet wanneer de rechtvaardigheid gediend is met een afwijking daarvan. Want in waarheid heb ik aan niemand meer te danken dan aan twee mensen die nooit twijfelden of ze me moesten stimuleren bij het najagen van dromen. Toen ik op mijn veertiende of vijftiende aangaf dat ik graag een eigen laboratorium wilde bouwen, nauwelijks het verschil tussen zoutzuur en zwavelzuur wetende, is de vraag nooit geweest *of* dat lab wel gebouwd mocht worden, maar alleen *hoe* zoiets dan zou moeten gebeuren. Ik ben de dag niet vergeten dat ik thuiskwam met een zwaar dementerende fotometer die wat betreft zijn voorkomen het midden hield tussen een bescheiden kamerharmonium en zo'n toestel waar captain James T. Kirk mee door de tijd reist. Mijn vader zal zich het ding herinneren! Beste pa, in de manier waarop je ernaar keek zat je analyse van het project al besloten. Niettemin heb je het gevaarte twee trappen omhoog gesleept, alleen om me de deceptie van een realistisch scenario te besparen. Vele jaren later heb je de tijdmachine weer over diezelfde trappen naar beneden gesleurd, toen reparatie inmiddels onmogelijk was gebleken.

Laat ik vervolgen met de naam van Dik van Gent. Beste Dik, ik geloof dat ik in eerste instantie mijn bewondering moet uitspreken voor het feit dat je het vijf jaar hebt uitgehouden met een grillige dromer. Onder je begeleiding ben ik gegroeid van een zelfingenomen student vol warrige idealen, tot een wetenschapper die een aardige indruk heeft van wat er in ons vakgebied omgaat. Je hebt me geleerd dat professor Sickbock een creatie van Marten Toonder is, die in het echte leven geen bestaansrecht heeft. Dergelijke transmutatie vereist soms het verlies van pluimen en veren en is in essentie geen pijnloos proces. Graag bedank ik je op deze plaats voor de wijze waarop je me in dergelijke momenten van groeipijn toch richting hebt gegeven, al was het maar door me gewoon alle denkbare richtingen op te sturen.

Vanaf het moment dat onze samenwerking een aanvang nam, begreep ik dat je een groot adept bent van de edele kunst der speculatie. Ik geloof dat de gemiddelde halfwaardetijd van jouw speculaties significant korter is dan de levensduur van een stuk speculaas. Daarom noem ik ze in gedachten vaak 'speculaasies'. Een van de grootste inzichten die ik de afgelopen jaren heb verworven, is het besef dat het succes van een beginnend wetenschapper afhangt van zijn talent om een veelbelovend plan te onderscheiden van een 'speculaasie'.

Een andere belangrijke verworvenheid die ik aan je te danken heb, is het inzicht dat wetenschap en schermsport veel overeenkomsten vertonen: succes hangt af van een delicate balans tussen snelheid en precisie, twee diametraal geplaatste grootheden die slechts na veel oefening enigszins met elkaar verenigbaar zijn. Rest me nog om te memoreren aan je bijna encyclopedische kennis van het vakgebied en je onmiskenbare talent om theorie en praktijk tot één geheel te smeden.

Jan Hoeijmakers, beste Jan, ik ben je zeer dankbaar voor het onvoorwaardelijk vertrouwen dat je in me gesteld hebt. Vooral op de momenten dat mijn onderzoek gestrand leek en ik veel liever een betrekking als hoefsmid had aanvaard, bleek jouw vertrouwen op een goede afloop van solide hardhout. Ik denk dat dit optimisme kenmerkend is voor je, zowel voor de wetenschapper als voor de mens. Ik ben er ook van overtuigd dat datzelfde optimisme de grondslag is van je succes, van je bewonderenswaardige inzet voor de wetenschap en van je doorzettingsvermogen in moeilijke tijden.

Bogdan 'Bobby' Florea, als kersverse doctor ben je de aangewezen persoon gebleken om mijn promotie-perikelen te relativeren. In die door het lot aangewezen hoedanigheid als 'mental coach', heb je veel verbeterd aan mijn visie op het doen van onderzoek: ik denk dat je de perfecte balans hebt gevonden tussen het nastreven van persoonlijk geluk en het dienen van de wetenschap. Je ijver zal vroeg of laat resulteren in een mooie ontdekking, zoveel is zeker. Je aanwezigheid is zeer gunstig gebleken voor de sfeer op het lab en de denkbeelden die je zowel frequent als ontijdig lanceerde als onaantastbare waarheden, hebben vaak voor hilarische momenten gezorgd. Ik ben blij in jou een medestander gevonden te hebben die zo af en toe kon helpen om het Mannelijk Front tegen het Vrouwelijk Blok te verdedigen. Dit laatste zelfs ondanks het feit dat je masculine temperament vaak minder beschaafde manifestaties zoekt. Soms, als jij je dissonante adagio van duistere barbarij door het lab liet galmen, was ik toch zo gelukkig dat ik niet leef in jouw wereld van door hormoonbalans en electro-impuls geregeerde primaten. Niettemin heb ik je met plezier zien komen en met spijt zien gaan. Bedankt dat je mijn paranimf wilt zijn.

Linda Brugmans, ik ken, geloof ik, niemand met een zonniger en gelijkmatiger karakter. Het was een genoegen met je samen te werken, temeer daar je prettige humeur virulent genoeg is gebleken om het mijne bij tijd en wijle met een vleugje hartelijkheid te infecteren. Bedankt dat je mijn paranimf wilt zijn.

Als ik op deze wijze doorga, zal ik het aanhechten van een extra katern 'dankwoord' in overweging moeten nemen. Laten we daarom het tempo wat

verhogen. Koos Jaspers, graag wil ik je hier bedanken voor de vele gesprekken die we hebben gehad over alles-behalve-moleculaire-biologie. Vaak heb ik versteld gestaan van je zeer grote eruditie en van de verscheidenheid aan onderwerpen waarover je op zinvolle wijze kan uitwijden. Nicole Verkaik, bedankt voor je bijdrage aan het belangrijkste gedeelte van mijn proefschrift (hoofdstuk 4). Guido Keijzers zij hier vermeld voor technische ondersteuning. Hanny Odijk ben ik erkentelijk voor alle mogelijke vormen van goede raad en advies. Anja Raams en Esther Appeldoorn (het duo Knabbel en Babbel), uiteraard verdienen jullie als oudgedienden ook een plaatsje in dit naschrift. Tom de Vries Lentsch bedank ik hartelijk voor de layout van dit boekje. Iedereen die mijn artistieke capaciteiten kent, weet dat deze thesis nooit in de huidige vorm gegoten zou zijn zonder professionele hulp. Jorie Versmissen, hartelijk dank voor je aangename gezelschap en uiteraard voor het aanleveren van de prachtige tekst van Bertrand Russell. Claire Wyman, ik spreek je maar in het Nederlands aan, wetende dat je die taal beter machtig bent dan je echtgenoot, zeer bedankt voor de inleiding in het mystieke vakgebied van de 'atomic force microscopy'.

De laatste stofe van dit dankwoord wil ik graag gebruiken om vergeving te vragen voor al mijn falen, mijn dromerijen, mijn driftig en soms onverdraaglijk karakter. Behalve een zeker cynisme, voorwaar mijn onbetwiste handelsmerk, heb ik tijdens mijn verblijf in Rotterdam een belangrijke waarheid gevonden. Namelijk het besef dat het beschrijven van een ding niet hetzelfde is als het begrijpen van haar essentie. En dat beschouwing, zelfs van de ogenschijnlijk simpelste dingen, ver verwijderd is van doorgronding. De strijd die een denkend wezen voert om grip te krijgen op de essenties van zijn bestaan, vindt immers plaats in een rationeel luchtledige dat nog steeds beter toegankelijk is voor artiesten dan voor geleerden. Het is soms een hard gelag, te beseffen dat de noeste vorsersarbeid ons niet hoger voert dan boekhouding van de schepping en dat wij, wetenschappers, wel geblakerd zijn door het vuur der bezieling, maar niet gelouterd en niet gekomen tot het ware inzicht. Onbegrepen blijft de aard van die wonderlijke zelfstandigheid waaruit we bestaan. Men diene in mijn woorden geen geringschatting van wetenschappelijk werk te ontdekken. Dat vijfhonderd jaar nauwgezet onderzoek zijn vruchten afwerpt, is een makkelijk aan te tonen feit. Liever word ik geopereerd door een eigentijdse chirurg, bijgestaan door eeuwen van kennis, dan dat ik een keisnijding onderga in een middeleeuws lazaret. Mij past dankbaarheid dat ik een bijdrage, hoe gering ook, heb mogen leveren aan die opvlucht.

Eric Weterings, 28 november 2004