Non-homologous end joining in class switch recombination: the beginning of the end

Ashwin Kotnis, Likun Du, Chonghai Liu, Sergey W. Popov and Qiang Pan-Hammarström*

Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden

Immunoglobulin class switch recombination (CSR) is initiated by a B-cell-specific factor, activation-induced deaminase, probably through deamination of deoxycytidine residues within the switch (S) regions. The initial lesions in the S regions are subsequently processed, resulting in the production of DNA double-strand breaks (DSBs). These breaks will then be recognized, edited and repaired, finally leading to the recombination of the two S regions. Two major repair pathways have been implicated in CSR, the predominant non-homologous end joining (NHEJ) and the alternative end-joining (A-EJ) pathways. The former requires not only components of the 'classical' NHEJ machinery, i.e. Ku70/Ku80, DNA-dependent protein kinase catalytic subunit, DNA ligase IV and XRCC4, but also a number of DNA-damage sensors or adaptors, such as ataxia–telangiectasia mutated, γH2AX, 53BP1, MDC1, the Mre11–Rad50–NBS1 complex and the ataxia telangiectasia and Rad3-related protein (ATR). The latter pathway is not well characterized yet and probably requires microhomologies. In this review, we will focus on the current knowledge of the predominant NHEJ pathway in CSR and will also give a perspective on the A-EJ pathway.

Keywords: class switch recombination; non-homologous end joining; alternative NHEJ; microhomology

1. INTRODUCTION

Immunoglobulin M (IgM) is the primordial antibody class and has been supplemented during evolution by antibody classes (IgG, IgA and IgE) with improved, more specialized, effector functions. The change in antibody class is effectuated by a looping out–deletion–recombination process called class switch recombination (CSR), where the constant region gene of the heavy chain (Cm) is replaced by a downstream constant region gene (Cγ, Ca or Cδ; Stavnezer et al. 2008). The process requires germ line (GL) transcription of the unarranged C region genes (Stavnezer 1996; Chaudhuri & Alt 2004) and is initiated by a B-cell-specific factor, activation-induced cytidine deaminase (AID; Muramatsu et al. 2000; Revy et al. 2000). The modes of action of AID are discussed elsewhere in this issue.

CSR involves DNA regions, called ‘switch (S) regions’, that are located in the introns upstream of each C region gene, except Cδ. S regions are composed of tandemly repeated sequences that contain common pentameric sequences (GAGCT and GGGCT), but differ in the overall length of the repetitive region, the actual sequence of the repeats and the number of polymorphic alleles (Pan-Hammarstrom et al. 2007). In both humans and mice, Sμ, Sζ and Sε are closely related and characterized by a dense clustering of pentameric repeats, with or without a higher ordered structure. The Sγ regions, however, share very little homology with the respective Sμ regions (Pan-Hammarstrom et al. 2007).

The initial lesions introduced by AID in the S regions are subsequently processed, leading to the production of DNA double-strand breaks (DSBs; discussed elsewhere in this issue). There are two major mechanisms for the repair of DSBs, homologous recombination (HR) and non-homologous end joining (NHEJ). The former is dependent on sequence homology and is the most active in the late S/G2 phase. The latter uses little or no sequence homology, which is sometimes imprecise and functions throughout the cell cycle. NHEJ is therefore considered to be the principal mechanism used in CSR, as AID-dependent DSBs are introduced and repaired mainly in the G1 phase of the cell cycle (Schrader et al. 2007) and the nature of S region sequences (lack of long stretches of perfect homology between the different S regions) would theoretically not support HR.

The ‘classical’ NHEJ machinery requires a number of factors, including Ku70/Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase IV, XRCC4, Artemis and XLF (Cernunnos; Lieber 2008). During the past decade, evidence has accumulated that many of these factors are actually required for the CSR process. When the classical...
Table 1. CSR phenotype in cells deficient for various DNA repair factors that might be involved in the NHEJ pathway during CSR.

<table>
<thead>
<tr>
<th>protein</th>
<th>model</th>
<th>CSR efficiency</th>
<th>proliferation defect</th>
<th>GL transcription type of junctions</th>
<th>significant shift towards use of microhomology</th>
<th>frequency of junctional mutations*</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATM</td>
<td>human</td>
<td>reduced (IgA)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Su–Sz</td>
<td>yes (7.2 versus 1.8 bp)</td>
<td>reduced</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgA, IgG1, IgG2a, IgG3, IgE)</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>yes (2.5 versus 1.2 bp)</td>
<td>reduced</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG1, IgG2b, IgG3)</td>
<td>7–50% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>yes (2.6 versus 1.2 bp)</td>
</tr>
<tr>
<td>H2AX</td>
<td>mouse</td>
<td>reduced (IgG1)</td>
<td>24–50% of controls</td>
<td>no</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG3)</td>
<td>30% of controls</td>
<td>no</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG1)</td>
<td>30–75% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (1.8 versus 1.7 bp)</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG1)</td>
<td>7% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (0.9 versus 1.1 bp)</td>
</tr>
<tr>
<td>53BP1</td>
<td>mouse</td>
<td>reduced (IgG1)</td>
<td>2–10% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (2.9 versus 1.8 bp)</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (all Ig classes)</td>
<td>2–10% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (2.5 versus 2.0 bp)</td>
</tr>
<tr>
<td>NBS</td>
<td>human</td>
<td>reduced (IgA and IgG)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Su–Sx</td>
<td>yes (3.6 versus 1.8 bp)</td>
<td>normal</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG1 and IgG3)</td>
<td>approx. 50% of controls</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy</td>
<td>yes (2.3 versus 1.2 bp)</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgG1)</td>
<td>approx. 50% of controls</td>
<td>yes</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (1.4 versus 0.8 bp)</td>
</tr>
<tr>
<td>Mre11</td>
<td>human</td>
<td>reduced (IgA and IgG)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Su–Sz</td>
<td>no (2.6 versus 1.8 bp)</td>
<td>reduced C to T</td>
</tr>
<tr>
<td>ATR</td>
<td>human</td>
<td>normal (IgA and IgG)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Su–Sy</td>
<td>no (1.8 versus 1.2 bp)</td>
<td>reduced C to T</td>
</tr>
<tr>
<td>Ku70</td>
<td>mouse</td>
<td>impaired (IgG1, IgG2b, IgG3 and IgE)</td>
<td>not detectable</td>
<td>yes</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>yes (1.8 versus 1.2 bp)</td>
</tr>
<tr>
<td>Ku80</td>
<td>mouse</td>
<td>impaired (IgG1 and IgG3)</td>
<td>not detectable</td>
<td>yes</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>yes (1.2 versus 1.8 bp)</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>mouse</td>
<td>reduced (IgE)</td>
<td>100– to 250-fold less</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (0.9 versus 1.2 bp)</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>impaired (all the isotypes except IgG1)</td>
<td>not detectable</td>
<td>no</td>
<td>normal</td>
<td>Su–Sy1</td>
<td>no (1.8 versus 1.2 bp)</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>reduced (IgA, IgG1, IgG3 and IgG2b)</td>
<td>30–50% of controls</td>
<td>no</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*Continued.*
The predominant end-joining pathway used in normal cells during CSR requires not only components of the classical NHEJ machinery, but also a number of factors that are considered being DNA-damage sensors, transducers or adaptors. Table 1 summarizes the CSR phenotype in mouse knockout and human disease models. A reduced efficiency of CSR and/or an increased usage of microhomology at recombinational junctions are common features when the predominant NHEJ pathway is impaired (table 1).

(a) Ataxia-telangiectasia mutated signalling

(i) ATM

ATM is a phosphoinositol 3-kinase-like kinase (PIKK) that plays a central role in orchestrating a network of cellular responses to DSBs, including cell cycle control, DNA repair and apoptosis (Shiloh 2003; Lavin & Kozlov 2007). ATM deficiency in humans results in a rare, multi-system disorder, ataxia–telangiectasia (A–T), characterized by cerebellar degeneration with ataxia, telangiectasia, chromosomal instability, radiosensitivity and cancer predisposition (Chun & Gatti 2004).

A–T is also recognized as a primary immunodeficiency disorder, with both the cellular and humoral immune systems being affected, leading to recurrent and severe infections (Lavin & Shiloh 1999). IgA deficiency (IgAD) has been observed in 60–80% of patients, and a subgroup suffers from concomitant IgG subclass deficiency (Lavin & Shiloh 1999). During investigations aimed at elucidating the underlying cause of antibody deficiency in A–T patients, we observed that the S_ins-Sigma junctions in patient B cells were characterized by a strong dependence on short, perfectly matched sequence homologies (microhomologies) and devoid of normally occurring mutations around the breakpoints (Pan et al. 2002). More than 60 per cent of the junctions exhibited a microhomology of 4 bp or more with the longest being 21 bp, as illustrated in figure 1 in the very first set of breakpoints we obtained. This is far beyond what one would expect as a consequence of NHEJ, which should theoretically result in no, or a 1–3 bp, microhomology. The serendipitous finding of an aberrant pattern of S_ins–Sigma junctions from A–T patients led us to hypothesize that ATM is involved in the predominantly used NHEJ pathway in CSR. In A–T patients, where ATM function is impaired, the S regions are joined by an A-EJ pathway involving microhomology (Pan et al. 2002). This concept was not fully accepted at that time, as ATM was not considered to be a part of the DSB repair machinery, in particular not with NHEJ. Furthermore, definitive
evidence showing that NHEJ is involved in CSR was still missing.

ATM-deficient mice have been generated in several laboratories and these mice are characterized by growth retardation, infertility, radiosensitivity and development of thymic lymphomas (Barlow et al. 1996; Elson et al. 1996; Xu et al. 1996). The serum level of immunoglobulins is, however, largely normal (Xu et al. 1996) although reduced serum titres of antigen-specific IgG and IgA antibodies have been detected after immunization (Lumsden et al. 2004). Two studies have subsequently shown that B cells from ATM-deficient mice cannot switch to IgG and IgA as efficiently as do wild-type B cells, suggesting an intrinsic B-cell defect during CSR (Lumsden et al. 2004; Reina-San-Martin et al. 2004). Furthermore, the defect is not due to alterations in cell proliferation, GL transcription or 53BP1 focus formation and has thus been suggested to be located either at an early step referred to as synapsis, i.e. holding the DNA ends

Figure 1. The sequences of the first five Sm–Sa junctions derived from A–T patients (Pan et al. 2002). The Sm and Sa1 or Sa2 sequences are aligned above and below the recombined switch junctional sequences. Microhomology was determined by identifying the longest region at the S junction of perfect uninterrupted donor/acceptor identity (solid-line boxes). Imperfect repeats were determined by identifying the longest overlap region at the S junction by allowing one mismatch on either side of the breakpoint (the extra nucleotide beyond the perfect matched sequence identity is indicated by dotted-line boxes). The Sm and Sb breakpoints for each S fragment are indicated by a downward arrowhead and an upward arrowhead, respectively, and their positions in the germ-line sequences (X54713, L19121 and AF030305; Mills et al. 1990; Islam et al. 1994; Pan et al. 2001) are indicated above or below the arrowhead. (a) 21 bp microhomology (28/29 bp imperfect repeat), (b) 12 bp microhomology (19/20 bp imperfect repeat), (c) 6 bp microhomology (9/10 bp imperfect repeat), (d) 7 bp microhomology (10/11 bp imperfect repeat), (e) 7 bp microhomology (11/12 bp imperfect repeat).
in proximity to facilitate subsequent repairs (Reina-San-Martin et al. 2004), or at the break repair step (Lumsden et al. 2004).

One of the two studies (Lumsden et al. 2004) has also shown a significant increase in the length of microhomology at the Sµ–Sy1 junctions (2.6 bp versus 1.2 bp in wild-type cells), not as dramatic as the Sµ–Sz junctions derived from A–T patients (7.2 bp versus 1.8 bp in controls), but very similar to the Sµ–Sy junctions derived from A–T patients (7.2 bp versus 1.2 bp in wild-type cells), not as dramatic as the Sµ–Sz junctions previously (Pan-Hammarstrom et al. 2007), and will present in more detail later, Sµ–Sz and Sµ–Sy junctions are resolved differently both under normal circumstances and in patients with various defects in their DNA repair systems. Owing to the higher degree of homology between Sµ and Sz when compared with Sµ and Sy, the microhomology-based pathway would be a more attractive alternative for Sµ/Sz recombination when the classical NHEJ pathway is impaired. Thus, we would probably observe a more dramatic shift in the use of longer microhomologies in ATM-deficient mouse B cells if the Sµ–Sz junctions were analysed. It is currently unclear though, whether a ‘normal’ appearance of Sµ–Sy junctions, i.e. a normal length of microhomology, would simply indicate a normal end joining or whether it may actually result from yet another A-EJ mechanism that does not require a long microhomology.

The exact function of ATM in the predominant NHEJ pathway in CSR remains unclear. ATM phosphorylates a number of factors that are known to be involved in CSR, including NBS1, Mre11, γH2AX, 53BP1 and MDC1. Thus, one possible role of ATM is to recruit and/or activate other DNA-damage response factors, such as γH2AX, 53BP1, MDC1 and possibly the Mre11 complex, configuring the DNA termini for subsequent repair steps, and/or slowing down cell cycle progression until the repair is complete (Lieber 1999; Lieber et al. 2003; figure 2). As we will discuss below, however, the CSR phenotype resulting from deficiency of any of these ATM substrates is clearly not identical to a deficiency of ATM (table 1). A second possibility is that ATM may have a more direct role in the end-processing step through phosphorylation of a proposed nuclease that participates in NHEJ, i.e. Mre11 or Artemis (figure 2). Artemis would be an interesting candidate, as it is a downstream component in the ATM signalling pathway required for the repair of a subset of radiation-induced DSBs, but dispensable for ATM-dependent cell-cycle checkpoint arrest (Riballo et al. 2004). Artemis, however, appears to be dispensable for efficient CSR (Rooney et al. 2005), although a recent study has provided evidence that Artemis is required for repair of at least part of the CSR-related chromosomal breaks at the Ig locus (Franco et al. 2008). Finally, a recent, large-scale proteomic analysis has identified an impressive list of ATM and ATR substrates (n=700), including some additional factors that have previously been implicated in CSR, such as the mismatch repair factors MSH2, MSH6, MLH1, PMS2, EXO1 and RPA1 (Matsuoka et al. 2007). An increased length of microhomology at Sµ–Sy junctions has indeed been observed in MLH1 and PMS2 knockout mice (Ehrenstein et al. 2001; Schrader et al. 2002), supporting a potential link between ATM/ATR and the mismatch repair factors.

(ii) γH2AX (phosphorylated H2AX)

In response to DNA DSBs by ionizing radiation, H2AX is rapidly phosphorylated by ATM or DNA-PKcs (Burma et al. 2001; Hickson et al. 2004), resulting in the formation of γH2AX foci that spread throughout a megabase-long region flanking the break (Rogakou et al. 1999). In cells undergoing CSR, NBS1 and γH2AX foci are formed and co-localized at the CH region during the G1 phase of the cell cycle, suggesting that these factors might also have a role in response to CSR-induced DSBs (Petersen et al. 2001).

H2AX-deficient mice are growth retarded, immunodeficient, radiosensitive and show male-specific infertility (Celeste et al. 2002). Serum levels of IgG1, IgG3 and IgA are significantly reduced in these mice and in vitro CSR to IgG1 and IgG3 is markedly reduced (approx. 25–30% of wild-type levels; Petersen et al. 2001; Celeste et al. 2002). The impaired CSR in H2AX-deficient cells is not due to an alteration in induction of GL transcription and short-range intra-switch region recombination proceeds normally (Reina-San-Martin et al. 2003). The pattern of Sµ–Sy1 junctions is also largely normal, with a normal length of microhomology at the junctions and only a slightly reduced frequency of mutations around the junctions (Reina-San-Martin et al. 2003). The role of γH2AX in CSR has therefore been proposed to facilitate long-range synopsis of two different S regions (Reina-San-Martin et al. 2003). A recent study has further shown that AID-dependent IgH locus chromosome breaks occur at a high frequency in H2AX-deficient B cells undergoing CSR, suggesting that γH2AX may promote end joining during CSR (Franco et al. 2006). Interestingly, activated B cells deficient in ATM, 53BP1 or MDC1 show a similar phenotype, with an increased number of IgH locus breaks and translocations (Franco et al. 2006), implying that they may have a common function in the predominant NHEJ pathway in CSR. However, the largely normal pattern of Sµ–Sy junctions in H2AX-deficient cells argues against the idea but, as we have discussed above, analysis of Sµ–Sz junctions in these cells will be required before it is defined as normal. Alternatively, ATM and γH2AX, or 53BP1 (as discussed below), play additional roles in CSR, which are independent of each other.

(iii) MDC1

MDC1, yet another ATM substrate (Goldberg et al. 2003; Lou et al. 2003; Stewart et al. 2003), is a mediator or adaptor that mediates γH2AX-dependent chromatin retention of DNA-damage response factors (Stucki & Jackson 2006). MDC1-deficient mice show similar phenotypes as H2AX-deficient mice, including growth retardation, male infertility, immunodeficiency, chromosomal instability and radiosensitivity (Lou et al. 2006). Serum levels of immunoglobulins are normal in MDC1-deficient mice, and a reduction in CSR to approximately 50–75% of wild-type levels has been observed in cultured MDC1-deficient B cells

Phil. Trans. R. Soc. B (2009)
(iv) **53BP1**

53BP1 was first identified as a p53-binding protein (Iwabuchi et al. 1994) and later shown to participate at an early stage in DNA DSB signalling (Schultz et al. 2000; DiTullio et al. 2002; Fernandez-Capetillo et al. 2002; Wang et al. 2002). 53BP1 is phosphorylated by ATM but may also be an activator of ATM in response to DNA DSBs (for review see Mochan et al. 2004). 53BP1-deficient mice are growth retarded, radiosensitive, immunodeficient and predisposed to cancer (Ward et al. 2003), a phenotype that resembles ATM or H2AX knockout mice. Studies on 53BP1-deficient mice have further demonstrated that 53BP1 is dispensable for HR, variable diversity joining (V(D)J) recombination and somatic hypermutation (SHM) but is clearly required for CSR (Manis et al. 2004; Ward et al. 2004). Serum levels of IgG and IgA are substantially reduced (Manis et al. 2004) and the ability of 53BP1-deficient B cells to switch in *vivo* to all the isotypes, is severely impaired (reduced to 2–10% of wild-type levels; Manis et al. 2004; Ward et al. 2004).

The CSR defect is not due to a proliferative block or an alteration of GL transcription (Manis et al. 2004; Ward et al. 2004) and the Sμ–Sy1 junctions reveal no significant differences in the length of microhomology or mutation frequency (Manis et al. 2004; Reina-San-Martin et al. 2007). However, unusual long insertions, not seen in 240 S junctions from wild-type cells, have been observed in two Sμ–Sy1 53BP1−/− junctions, and intra-switch recombination is enhanced in 53BP1-deficient cells (Reina-San-Martin et al. 2007), suggesting a potential role of 53BP1 in synopsis or protecting the DNA ends of the two S regions.

There is thus far no human disease that has been linked to mutations in the gene encoding 53BP1. A recent study has, however, described a patient who suffered from the RIDDLE syndrome (radiosensitivity, immunodeficiency, dysmorphic features and learning difficulties; Stewart et al. 2007). Cells from this patient lack an ability to recruit 53BP1 to the sites of DNA DSBs. Mutations were neither found in the gene encoding 53BP1 itself nor in the genes encoding ATM, MDC1, H2AX and PR-Set7, factors that are known to be involved in regulating the localization of 53BP1 to the breaks (Stewart et al. 2007). It is possible that an as yet unknown protein, acting upstream of 53BP1 during the cellular response to DNA DSBs, is defective in this patient. It is interesting to note that significantly increased microhomology is observed at the Sμ–Sζ junctions amplified from this patient, with 94 per cent of junctions exhibiting a microhomology of 4 bp or more (Stewart et al. 2007). A reduced frequency of insertions and mutations around the Sμ–Sζ breakpoints is also observed. The Sμ–Sy3 junctions from this patient have also been analysed, albeit unfortunately not shown in the paper.
Nevertheless, the altered pattern of Sµ–Sz junctions in this patient suggests that there could be a shift from the dominant NHEJ to the A-EJ pathway when the formation of 53BP1 foci is defective.

The precise role of 53BP1 in CSR remains elusive. The CSR defect observed in 53BP1-deficient mice is more severe than that in ATM- or H2AX-deficient mice, and the pattern in the Sµ–Sy junctions is different from that of ATM-deficient cells (although Sµ–Sz junctions have not been analysed in 53BP1-deficient mice). It is thus likely that in addition to being part of the ATM signalling pathway, 53BP1 also has a role in CSR, which is independent of ATM or γH2AX. A recent study has shown that MDC1 functions primarily in HR/sister chromatic recombination, in a manner that is strictly dependent upon its ability to interact with γH2AX, whereas 53BP1 functions in an XRCC4-dependent NHEJ pathway, through interaction with the dimethylated lysine 20 of histone H4, a process that is independent of γH2AX (Xie et al. 2007). If this is also true for CSR, it would probably explain why CSR is affected in the order of 53BP1−/− more than H2AX−/− more than MDC1−/−.

(v) The Mre11-Rad50-NBS1 complex

The Mre11 complex is a multisubunit nuclease composed of Mre11, Rad50 and NBS1. This complex is required for telomere maintenance, cell-cycle checkpoint signalling, DNA replication, meiotic recombination and efficient repair of DNA DSBs by HR and/or NHEJ (D’Amours & Jackson 2002; Lavin 2007). The Mre11 complex acts as a sensor of DNA HR and/or NHEJ (D’Amours & Jackson 2002; Lavin 2007). This will lead to autophosphorylation and activation of ATM, which in turn will phosphorylate many substrates, including members of the Mre11 complex. The Mre11 complex thus acts both upstream and downstream of ATM. In humans, no mutations in Rad50 have been reported to date. Mutations in genes encoding Mre11 and NBS1 result in two chromosomal instability syndromes, ataxia–telangiectasia-like disorder (ATLD; Stewart et al. 1998) and Nijmegen breakage syndrome (NBS; Varon et al. 1998), where both exhibit features that are characteristic for A–T.

NBS patients are characterized by immunodeficiency, microcephaly, chromosomal instability and a high incidence of lymphoid malignancies (Digweed & Sperling 2004). Deficiency of IgG and/or IgA is observed in 80–90% of the patients (Gregorek et al. 2002), which could be due to a lack of T-cell help and/or an intrinsic B-cell defect. By analysing the in vivo-switched B cells, we have previously shown a reduced level of CSR to IgA in NBS patients (Pan et al. 2002). The recovered Sµ–Sz and Sµ–Sy junctions also show a significantly increased length of microhomology, although not as dramatic as those derived from A–T patients (Pan et al. 2002; table 1). In contrast to A–T and NBS patients, normal immunoglobulin levels are observed in most ATLD patients described to date (Stewart et al. 1999; Delia et al. 2004; Fernet et al. 2005). Significantly reduced numbers of Sµ–Sz and Sµ–Sy clones have, however, also been shown in ATLD patients (Lahdesmaki et al. 2004). There is a trend (not significant) in the use of longer microhomologies at the Sµ–Sz junctions but not in the Sµ–Sy junctions. The dependence of microhomology at the Sµ–Sz or Sµ–Sy junctions is thus A–T>NBS>ATLD>control.

The Sµ–Sz junctions obtained from NBS and ATLD patients are, however, clearly different from those in A–T patients, in at least two respects: first, the blunt end direct joining mechanism (requiring no microhomology) is severely impaired in A–T patients but not in NBS or ATLD patients. Second, the precise joining of complementary DNA ends with 1–3 bp sequence homology, probably executed through a simple religation mechanism (Haber 1999), is normal in A–T patients, but defective in NBS and, to a lesser degree, ATLD patients. Mutations or insertions often occur at junctions with 1–3 bp microhomology in NBS and ATLD patients (Lahdesmaki et al. 2004). Thus, as in cells from A–T patients, the balance between the predominantly used NHEJ and the A-EJ pathways is altered. ATM and the Mre11 complex may, however, act on the predominant end-joining pathway in a different manner or at a different step (Pan et al. 2002; Lahdesmaki et al. 2004). NBS1 and Mre11 may also have roles in CSR, which are independent of each other, as the patterns of mutations and insertions around the Sµ–Sz junctions are clearly different. In NBS patients, all base substitutions were located at G/C nucleotides, and in ATLD patients, the substitutions that occurred most often in controls, C to T transitions, were never observed (Lahdesmaki et al. 2004). These peculiar patterns of base substitutions are, however, specific for the CSR reactions, as they are quite different from the SHM patterns we observed in the VH regions from these patients (Du et al. 2008).

In mice, disruption of any subunit of the Mre11 complex results in embryonic lethality (Xiao & Weaver 1997; Luo et al. 1999; Zhu et al. 2001). A humanized mouse model for NBS has been generated by the introduction of the human 5 bp deletion hypomorphic allele into NBS-deficient mice (DiFiLippantonio et al. 2002). No CSR defect (to IgG1 and IgG3) has, however, been observed in this mouse model. Using a conditional knockout strategy, two studies have shown that CSR to various IgG subclasses is reduced in NBS1-deficient B cells (to approx. 50% of wild-type levels; Kracker et al. 2005; Reina-San-Martin et al. 2005). The CSR defect is not dependent on GL transcription and appears to be due to inefficient recombination at the DNA level (Reina-San-Martin et al. 2005). In both studies, a normal length of microhomology and a normal rate of mutations were found at the Sµ–Sy junctions. It is noteworthy that in one of the studies, residual levels of wild-type NBS (25%) remained in the cells, and thus the effect of NBS1 on CSR may well have been underestimated (Kracker et al. 2005) as some CSR junctions might be derived from NBS-proficient cells. The level of IgA switching and the quality of Sµ–Sz junctions have not been analysed to date in these mice.

(vi) ATR

As discussed earlier, the CSR defect in 53BP1-deficient cells is even more severe than that in ATM-deficient cells. Another possibility is that additional upstream
PIKs, which activate 53BP1, might be involved in CSR. The A–T and Rad3-related protein (ATR) could be one such candidate, as it is closely related to ATM and targets an overlapping set of substrates, including 53BP1 and H2AX (Abraham 2001; Cimprich & Cortez 2008). Loss of ATR in mice results in embryonic lethality (Brown & Baltimore 2000) whereas hypomorphic mutations in the ATR gene have been identified in a few patients with the Seckel syndrome (O’Driscoll et al. 2003). These patients are characterized by intrauterine growth retardation, dwarfism, microcephaly, ‘bird-like’ facial features and mental retardation but no obvious immunodeficiency. Studies on B cells from these patients have shown that the proportion that has switched to IgA and IgG in the peripheral blood is normal (Pan-Hammarstrom et al. 2006). An analysis of the Sμ–Sζ junctions showed a normal ‘blunt end-joining’ and a normal ‘simple religation’ process (joining 1–3 bp complementary ends), but impaired end joining with partially complementary (1–3 bp) DNA ends. There is also a significant increase in the length of microhomology at the Sμ–Sζ junctions (3.0 bp versus 1.8 bp in controls), but only up to 9 bp. Thus, ATR is likely to have a moderate role in the predominant end-joining process during CSR. Whether this is due to its interaction with 53BP1 or H2AX is, however, unknown. An additional speculation is that ATR may also be required in the A-EJ pathway where a longer microhomology (10 bp or more) is required (figure 2).

(b) The classical non-homologous end joining machinery

(i) Ku70/Ku80
Ku is a heterodimer of Ku70 and Ku80. They are probably among the first proteins that bind to the DNA ends at a DSB and the Ku–DNA complex recruits and activates DNA-PKcs (Lieber et al. 2003; Lieber 2008). Ku and DNA-PKcs are proposed to act in the synapsis process (Lieber et al. 2003; Lieber 2008). Ku70 or Ku80 knockout mice are growth retarded, radiosensitive and are severely immunodeficient (Nussenzweig et al. 1996; Gu et al. 1997). B-cell development is arrested at an early stage due to a profound deficiency in V(D)J recombination (Nussenzweig et al. 1996; Gu et al. 1997). To assess the role of Ku in CSR, pre-rearranged V(D)J heavy chain and V J light chain genes were introduced into a Ku70- or Ku80-deficient background. In both cases, CSR to IgG and/or IgE is severely impaired, suggesting that the Ku heterodimer is required for CSR (Casellas et al. 1998; Manis et al. 1998). However, the severe proliferation defect of Ku-deficient B cells has made it difficult to determine whether Ku, and thus NHEJ, has a direct role in end joining during CSR. No CSR junctions have been analysed in Ku70- or Ku80-deficient mice. However, the majority of the recovered coding or RS joints from Ku70-deficient cells exhibited 1–5 bp short homologies (Gu et al. 1997).

(ii) DNA-PKcs
The first piece of evidence that DNA-PKcs might be involved in CSR came from a study using pre-B cell lines derived from severe combined immunodeficiency (Scid) mice, where the ability of these lines’ switch to IgE was impaired (Rolink et al. 1996). These mice carry a spontaneous point mutation in the DNA-PKcs gene, which results in a truncated protein lacking DNA-PKcs kinase activity (Blunt et al. 1996; Danska et al. 1996; Beamish et al. 2000). In a second study, Ig heavy and light chain knock-in DNA-PKcs-null mice were analysed and, despite a normal proliferation, GL transcription and AID induction, CSR to all the Ig classes was severely impaired, with the exception of IgG1, providing convincing evidence that DNA-PKcs is required for CSR (Manis et al. 2002). The importance of DNA-PKcs in CSR has, however, been challenged by studies where Ig heavy and light chain knock-in Scid (Bosma et al. 2002; Cook et al. 2003) or DNA-PKCs C-terminal deleted mice (Kiefer et al. 2007) were analysed. CSR efficiency was either close to normal (Kiefer et al. 2007) or reduced approximately two- to threefold (Bosma et al. 2002; Cook et al. 2003). The difference between the DNA-PKcs-null and Scid mice may suggest that the kinase activity of DNA-PKcs is not essential, or at least replaceable, during CSR, and DNA-PKcs may have a non-catalytic role in CSR, for instance by mediating synapse complex formation (DeFazio et al. 2002).

Based on the Scid studies, one can imagine a DNA-PKcs kinase-independent end-joining process in CSR. It would be of interest to know whether this is still part of NHEJ or it is the A-EJ used in the absence of ATM. In DNA-PKcs-null cells, the Sμ–SγJ junctions appear to be indistinguishable from controls (Manis et al. 2002). In one of the studies on Scid mice, CSR junctions have been analysed and a small increase in microhomology usage has been observed (3.4 bp versus 2.3 bp; Cook et al. 2003). However, this was based on a mixture of junctions (Sμ–SγJ, Sγ–Sζ and Sμ–Sζ). As Sμ–Sζ junctions, similar to Sμ–Sα, tend to show longer microhomologies when NHEJ is impaired (Pan-Hammarstrom et al. 2005; Yan et al. 2007), this analysis is not conclusive. A separate analysis of different types of CSR junctions would be preferable. The pattern of mutations around these CSR junctions is, however, altered, with a significantly reduced number of C mutations (resembling those from ATLD patients), suggesting that the kinase activity of DNA-PKcs may have a role in the later steps of the end-joining process during CSR. This notion is also supported by a recent study showing that DNA-PKcs is required for end joining of a subset of AID-induced CSR breaks (Franco et al. 2008).

(iii) DNA ligase IV and XRCC4
The XRCC4–DNA ligase IV complex is responsible for catalysing the ligation step of NHEJ (Lieber et al. 2003; Lieber 2008). No human disease has yet been shown to be associated with a mutation in the XRCC4 gene, whereas a few patients with defective DNA ligase IV activity have been described. These patients are characterized by microcephaly, growth retardation, radiosensitivity and mild to severe immunodeficiency (O’Driscoll et al. 2001; Buck et al. 2006; Enders et al. 2006; van der Burg et al. 2006). CSR junctions have been characterized in some of these patients and a marked shift to the usage of microhomology at the Sμ–Sζ junctions has been observed (9.8 bp versus...
1.8 bp in controls; Pan-Hammarstrom et al. 2005). This test has been most dramatic among all the patient groups that we have tested to date (DNA ligase IV > ATM > NBS > ATR > ATLD). Almost all junctions (97%) displayed at least 1 bp microhomology, the remaining being 1 bp insertions, thus the ‘blunt end joining’ or ‘direct end joining’ is completely abolished in the DNA ligase IV-deficient cells. Forty-seven per cent of the junctions exhibited a microhomology of 10 bp or more with the longest being 29 bp, which is by far the longest that has been described in human cells. A reduction of mutations or insertions at, or around, the S\textsubscript{µ}–S\textsubscript{z} junctions was also observed.

Despite the dramatic increase of microhomology at the S\textsubscript{µ}–S\textsubscript{z} junctions, the S\textsubscript{µ}–S\textsubscript{γ} junctions only show an increased frequency of insertions, but no significant increase in the length of junctional homology or rate of mutations (Pan-Hammarstrom et al. 2005). This difference cannot solely be due to differences in the homology between the S\textsubscript{µ} and the S\textsubscript{z} or S\textsubscript{γ} regions, as a small, albeit significant, increased length of microhomology has been observed in the S\textsubscript{µ}–S\textsubscript{γ} junctions from ATM-, ATR- and NBS-deficient cells. Furthermore, no significant increase in the rate of insertions has been observed at the S\textsubscript{µ}–S\textsubscript{γ} junctions from these patients. It is possible that yet another alternative pathway is used for joining the S\textsubscript{µ}–S\textsubscript{γ} regions in the DNA ligase IV-deficient patients, where 1 bp insertions are frequently introduced. It is also possible that the residual level of DNA ligase IV activity in the patient cells results in different modes of CSR junction resolution.

Disruption of LIG4 or XRCC4 in mouse results in embryonic lethality. Models that are based on XRCC4-deficient-mouse B cells have therefore been generated by two independent groups using different strategies (Soulas-Spraul et al. 2007; Yan et al. 2007). CSR to IgG or IgE is reduced to approximately 20–50% of control levels in XRCC4-deficient cells (Soulas-Spraul et al. 2007; Yan et al. 2007). In one of the studies, Yan et al. (2007) analysed S\textsubscript{µ}–S\textsubscript{γ} and S\textsubscript{µ}–S\textsubscript{z} junctions and demonstrated that there is a shift towards microhomology-based, alternative joining in the absence of XRCC4. They also found that ‘blunt’ or ‘direct’ end joining is totally abolished in XRCC4-deficient cells. In their preliminary studies, they have also observed a similar lack of direct joining and a bias towards a microhomology-based joining in DNA ligase IV-deficient mouse B cells. These results, taken together with the data obtained from DNA ligase IV-deficient patients, clearly suggest that the classical NHEJ machinery is required for CSR and, in its absence, an A-EJ mechanism is operating.

(c) Additional factors

Increased microhomology at the CSR junctions has also been observed in mice deficient in PMS2, MLH1, MSH4 or MSH5 and in IgA and common variable immunodeficiency (CVID) patients carrying mutations in the MSH5 gene (Ehrenstein et al. 2001; Schrader et al. 2002; Sekine et al. 2007; Pan-Hammarstrom & Hammarstrom 2008). How the different mismatch repair pathways are connected to the NHEJ machinery is currently unclear. In addition to the patient who suffered from the RIDDLE syndrome, a marked shift towards microhomology at the S\textsubscript{µ}–S\textsubscript{z} junctions has also been observed in several patients suffering from another rare form of immunodeficiency (hyper IgM syndrome) with an unknown genetic basis (Peron et al. 2007). The predominant NHEJ pathway in CSR is thus likely to be regulated by additional DNA-damage response/repair factors that remain to be characterized.

3. THE ALTERNATIVE END-JOINING PATHWAY DURING CLASS SWITCH RECOMBINATION

The A-EJ mechanism used in CSR and V(D)J recombination (Corneo et al. 2007) also contributes to the chromosomal translocations that give rise to lymphoid malignancies (Nussenzweig & Nussenzweig 2007; Yan et al. 2007). Compared with the predominant NHEJ pathway, however, we know rather little about the mechanism underlying A-EJ. Deletions, insertions and microhomologies are normally associated with the A-EJ pathway; however, we do not know the kinetics of the repair (fast or slow), during which cell cycle it is operative (G1 or others), how long a microhomology that is required (more than 1 bp, or 4 bp or even longer), whether there is only one or multiple pathways or the identity of the factors actually involved in the A-EJ mechanism. The identification of the factors in the A-EJ pathway may be complicated by the possibility that a short microhomology could be the result of either the NHEJ or A-EJ pathways, whereas lack of microhomology may not necessarily exclude the involvement of A-EJ. Furthermore, some factors regulating the classical NHEJ pathway may also be involved in the A-EJ process. A number of potential factors of the latter will be briefly discussed below.

(a) The Mre11–Rad50–NBS1 complex

The 3′–5′ exonuclease activity of Mre11 has previously been suggested to be involved in microhomology-based end joining, where it degrades the mismatched DNA ends until sequence identity is revealed (Paul & Gellert 2000). Based on the CSR junctions from ATLD and NBS patients, we have previously hypothesized that the Mre11 complex may be required for repairing DNA ends with a short (1–3 bp) homology, which is likely to be part of the NHEJ reaction. The Mre11 complex is, however, unlikely to be involved in A-EJ, if this pathway requires microhomology of 4 bp or longer, as the proportion of junctions with these longer microhomologies is slightly increased, rather than decreased, in both patient groups (Lahdesmaki et al. 2004).

(b) Poly (ADP-ribose) polymerase 1, XRCC1 and DNA ligase III

Poly (ADP-ribose) polymerase 1 (PARP-1) is an abundant nuclear protein that binds to both single-strand breaks (SSBs) and DSBs. It is involved in many cellular responses including base excision repair (BER) and, possibly, HR (Hellday et al. 2005). Together with XRCC1 and DNA ligase III, it has also been proposed as a candidate factor for the alternative, or back-up, pathway of NHEJ (Audebert et al. 2004; Wang et al. 2005, 2006). PARP-1 binds to DNA ends and, under normal conditions, it is in direct competition with Ku and possibly also DNA-PKcs (Wang et al. 2006).
Inhibitors of PARP-1 have been found to increase IgA switching in a B cell line and enhance IgG1 switching in activated splenic B cells (Shockett & Stavnezer 1993). This may imply that PARP-1 would normally inhibit or compete with other factors that participate in the predominant NHEJ pathway during CSR, which fits well with the hypothesis that it may be a component of the A-EJ pathway. Inhibitors of PARP-1 can in fact lead to the activation of ATM in other DNA repair process such as HR (Bryant & Helleday 2006). It would therefore be of considerable interest to test the effect of PARP-1 in CSR on an NHEJ-deficient background. Both ligases I and III seem to be required for microhomology-mediated end joining (Liang et al. 2008) but which of the two ligases is involved in A-EJ during CSR remains to be clarified.

(c) Werner protein

Werner protein (WRN) belongs to the RecQ helicase family and is mutated in the premature ageing disease, Werner’s syndrome (Yu et al. 1996). WRN has been implicated in HR but has also been linked to NHEJ, as its exonuclease activity is stimulated by Ku70/Ku80 (Li & Comai 2000). WRN, together with ligase III, has recently been implicated in A-EJ activity in chronic myeloid leukaemia cells, where knockdown of these factors leads to a significant reduction of microhomology at the repair sites (Sallmyr et al. 2008). However, WRN physically interacts with one of the key WRN factors, the XRCC4–DNA ligase IV complex, which stimulates WRN's exonuclease activity (Kusumoto et al. 2008). Thus, it is unclear which pathway WRN is actually involved in during CSR (if it has a role at all).

4. CONCLUDING REMARKS

Over the past decade, we have begun to understand the complex molecular mechanisms underlying DNA editing, repair and recombination during CSR. Two major repair pathways have been proposed, the predominant NHEJ and the A-EJ involving micro-homologies (figure 2). A more complicated picture will probably emerge when the NHEJ pathway is further dissected and the A-EJ pathway is delineated. The ultimate goal will be to have a three-dimensional dynamic model for CSR, where we would know the molecular basis of the process and the interaction between the DNA repair factors at the switch regions.

This work was supported by the Swedish Research Council, the Swedish Cancer Foundation, the Swedish Society for Medicine and the ‘KID’ programme from the Karolinska Institutet. The authors have no conflicting financial interests.

REFERENCES


DeFazio, L. G., Stansel, R. M., Griffith, J. D. & Chu, G. 2002
Synapsis of DNA ends by DNA-dependent protein kinase.

Delia, D., Pane, M., Buscemi, G., Savio, C., Palmeri, S.,
MRE11 mutations and impaired ATM-dependent responses
in an Italian family with ataxia–telangiectasia–like disorder.
Hum. Mol. Genet. 13, 2155–2163. (doi:10.1038/hmg/ddb221)

Difilippantonio, M. J., Petersen, S., Chen, H. T., Johnson, R.,
Delia, D., Piane, M., Buscemi, G., Savio, C., Palmeri, S.,
et al
Enders, A.
Elson, A., Wang, Y., Daugherty, C. J., Morton, C. C., Zhou,
Ehrenstein, M. R., Rada, C., Jones, A. M., Milstein, C. &
Franco, S., Murphy, M. M., Li, G., Borjeson, T., Boboila, C.
et al
Gregorek, H., Chrzanowska, K. H., Michalkiewicz, J.,
Syczewska, M. & Madalinski, K. 2002 Heterogeneity of
humoral immune abnormalities in children with Nijmegen
breakage syndrome: an 8-year follow-up study in a single

Gu, Y. et al. 1997 Growth retardation and leaky SCID
phenotype of Ku70-deficient mice. Immunity 7, 653–665.
(doi:10.1016/S1074-7613(00)80386-6)

Haber, J. E. 1999 Sir-Ku-ious routes to make ends

Helleday, T., Bryant, H. E. & Schultz, N. 2005 Poly(ADP-
ribose) polymerase (PARP-1) in homologous recombina-
tion and as a target for cancer therapy. Cell Cycle 4,
1176–1178.

Hickson, I. et al. 2004 Identification and characterization of a
novel and specific inhibitor of the ataxia–telangiectasia
mutated kinase ATM. Cancer Res. 64, 9152–9159.
(doi:10.1158/0008-5472.CAN-04-2727)

Islam, K. B., Baskin, B., Nilsson, L., Hammarström, L.,
Sideras, P. & Smith, C. I. 1994 Molecular analysis of IgA
deficiency. Evidence for impaired switching to IgA.

Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R. & Fields,
S. 1994 Two cellular proteins that bind to wild-type but not
(doi:10.1073/pnas.91.13.6098)

Kiefer, K., Oshinsky, J., Kim, J., Nakajima, P. B., Bosma,
G. C. & Bosma, M. J. 2007 The catalytic subunit of
DNA-protein kinase (DNA-PKcs) is not required for Ig
class-switch recombination. Proc. Natl Acad. Sci. USA
104, 2834–2848. (doi:10.1073/pnas.0611359104)

Kracker, S. et al. 2005 Nibrin functions in Ig class-switch
recombination. Proc. Natl Acad. Sci. USA 102,
1584–1589. (doi:10.1073/pnas.0409191102)

Kusumoto, R., Dawut, L., Marchetti, C., Wan Lee, J.,
Vindigni, A., Ramsden, D. & Bohr, V. A. 2008 Werner
protein cooperates with the XRCC4-DNA ligase IV
complex in end-processing. Biochemistry 47, 7548–7556.
(doi:10.1021/bi7023256)

Lahdesmaki, A., Taylor, A. M., Chrzanowska, K. H. & Pan-
Hammarstrom, Q. 2004 Delineation of the role of the
Mre11 complex in class switch recombination. J. Biol. Chem.
279, 16 479–16 487. (doi:10.1074/jbc.M312796200)

Lavin, M. F. 2007 ATM and the Mre11 complex combine to
recognize and signal DNA double-strand breaks. Oncogene
26, 7749–7758. (doi:10.1038/sj.onc.1210880)

Lavin, M. F. & Kozlov, S. 2007 ATM activation and DNA

Primary immunodeficiency diseases (eds H. D. Ochs,
NY: Oxford University Press.

Li, B. & Comai, L. 2000 Functional interaction between Ku
and the Werner syndrome protein in DNA ligase IV
processing. J. Biol. Chem. 275, 39 800.

Liang, L., Deng, L., Nguyen, S. C., Zhao, X., Maulion,
C. D., Shao, C. & Tischfield, J. A. 2008 Human DNA
ligases I and III, but not ligase IV, are required for
microhomology-mediated end joining of DNA double-
(doi:10.1093/nar/gkn184)

Lieber, M. R. 1999 The biochemistry and biological
significance of nonhomologous DNA end joining: an
essential repair process in multicellular eukaryotes. Genes

Lieber, M. R. 2008 The mechanism of human nonhomolog-
(doi:10.1074/jbc.R700039200)

Mechanism and regulation of human non-homologous
(doi:10.1038/nrm1202)

Phytopl. Trans. R. Soc. B (2009)


Petersen, S. et al. 2001 AID is required to initiate Nbs1-gamma-H2AX focus formation and mutations at sites of class switching. Nature 414, 660–665. (doi:10.1038/414660a)


Phil. Trans. R. Soc. B (2009)


