DNA interstrand cross-link repair in *Saccharomyces cerevisiae*

Peter Lehoczky¹, Peter J. McHugh² & Miroslav Chovanec¹

¹Department of Molecular Genetics, Cancer Research Institute, Bratislava, Slovak Republic; and ²Cancer Research UK Laboratories, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, UK

Correspondence: Miroslav Chovanec, Department of Molecular Genetics, Cancer Research Institute, Vlárská 7, 833 91 Bratislava 37, Slovak Republic. Tel.: +421 2 59327332; fax: +421 2 59327350; e-mail: miroslav.chovanec@savba.sk

Received 23 May 2006; revised 20 September 2006; accepted 20 September 2006. First published online 9 November 2006.

DOI:10.1111/j.1574-6976.2006.00046.x

Editor: Martin Kupiec

**Keywords**

anticancer drugs; DNA interstrand cross-links; *Saccharomyces cerevisiae*; nucleotide excision repair; homologous recombination repair; translesion polymerases.

**Abstract**

DNA interstrand cross-links (ICL) present a formidable challenge to the cellular DNA repair apparatus. For *Escherichia coli*, a pathway which combines nucleotide excision repair (NER) and homologous recombination repair (HRR) to eliminate ICL has been characterized in detail, both genetically and biochemically. Mechanisms of ICL repair in eukaryotes have proved more difficult to define, primarily as a result of the fact that several pathways appear to compete for ICL repair intermediates, and also because these competing activities are regulated in the cell cycle. The budding yeast *Saccharomyces cerevisiae* has proven a powerful model for dissection of ICL repair. Important roles for NER, HRR and postreplication/translesion synthesis pathways have all been identified. Here we review, with reference to similarities and differences in higher eukaryotes, what has been discovered to date concerning ICL repair in this simple eukaryote.

**Introduction**

Damage to DNA can be broadly subdivided into two categories according to the number of DNA strands involved. For damage that involves one DNA strand only, the complementary strand in the duplex DNA molecule provides a template to accurately restore the original sequence information. In contrast, lesions involving both DNA strands are potentially far more deleterious, as they are noninformative. This latter class of DNA lesions includes DNA interstrand cross-links (ICL) (Dronkert & Kanaar, 2001), complex DNA lesions that are extremely toxic due to the block to essential DNA transactions, for example transcription and DNA replication, they induce (Ruhland & Brendel, 1979; Magana-Schwencke et al., 1982).

Because of their high cytotoxicity and corresponding potent antitumor activity, ICL-forming agents are widely used in cancer treatment (for a review, see McHugh et al., 2001). As the biological activities of these agents can be significantly affected by enzymatic activation, the kinetics of binding to the relevant biological targets and cellular import and export, all these factors have to be considered to gain a complete insight into the cytotoxic effects of ICL-forming agents. In addition, there is emerging evidence that naturally occurring cross-linking agents might be responsible for some of the DNA damage burden of certain cells. For example, furocoumarins (psoralen-related compounds) are present in medicinal plants (Smith et al., 2004) and in a variety of edible plants such as parsley, celery, carrot and citrus fruits (Manderfeld et al., 1997). However, the levels of psoralens in these plants are far below the lower limit used in clinical treatment, and the blood level of these molecules after consumption of these plants is under the detection limit of high pressure liquid chromatography. Ultraviolet (UV) light has been shown to be capable of inducing a small number of ICL (Love et al., 1986) and, uniquely amongst metals, Cr ions are able to form stable ICL (O’Brien et al., 2003). A few endogenously produced metabolites also form ICL under physiological conditions, e.g. malondialdehyde, a product of lipid peroxidation and prostaglandin biosynthesis (Niedernhofer et al., 2003), and might be a major source of endogenously generated ICL experienced by all cells.

In this review, we focus on ICL repair. The emphasis is mainly on the budding yeast *Saccharomyces cerevisiae*, as this organism has proved an excellent model system for the study of DNA repair pathways (Henriques et al., 1997). In addition, there is good evidence that many basic cellular processes in yeast are conserved in human cells, including DNA damage response and repair pathways. Moreover, yeast strains defective in one or more repair pathways can be...
extremely useful when screening and validating molecules that can damage DNA, as well as in identifying potentially clinically effective drug combinations.

Interstrand cross-link-forming agents

Apart from inducing ICL, all the agents discussed here produce other types of DNA damage, in particular DNA mono-adducts, intrastrand cross-links (IaCL) and DNA-protein cross-links. As different and overlapping cellular outcomes could result from the processing of this variety of lesion, the contribution of ICL to the cytotoxicity of specific agents has been difficult to define clearly in some cases. Nevertheless, there is an expanding list of compelling studies indicating the particularly lethal impact of ICL (Chaney & Sancar, 1996). The effectiveness of DNA repair, inhibition of DNA synthesis and transcription, cell-cycle checkpoint response and induction of cell death by apoptosis and other means, are likely to be the critical steps in determining ICL toxicity. In this section, we discuss the basic biochemistry of ICL-generating agents, the impact of the chemical structure of ICL on DNA repair and the evidence for repair-dependent resistance of human neoplastic cells to these drugs.

The psoralens are furanocoumarins, which despite their mutagenicity (Bernd et al., 1999; Kevekordes et al., 1999) are still used in photo-chemotherapy of inflammatory skin diseases, such as psoriasis, vitiligo and eczema (Engin & Oguz, 2005). The treatment regimen termed psoralen UV-A irradiation is the most widespread procedure practiced in dermatology, although topical psoralen application, mainly in the form of creams and gels, is now preferred to systemic administration.

Historically, many of the studies concerning ICL repair in yeast were carried out using psoralens, mainly 8-methoxypsoralen (8-MOP). It is thought that 8-MOP creates ICL in three steps. First, the asymmetric, tricyclic, planar molecule intercalates between the stacked base pairs and, after photosensitization with UV-A, the furan ring of the molecule binds covalently to the DNA, forming a mono-adduct. A subsequent UV irradiation event promotes a second cyclo-addition of the pyrone ring to the complementary DNA strand, producing a very stable ICL (for a review, see Bethea et al., 1999). The extent of the reaction can be controlled by the dose and wavelength of the UV irradiation. As much as 40% of the 8-MOP-induced mono-adducts can be converted into ICL. It has been estimated that the LD_{57} for 8-MOP corresponds to about 60 mono-adducts and 30 ICL per genome for dividing haploid wild-type yeast cells (Bammann & Brendel, 1989). The full UV-A spectrum (320–400 nm) induces ICL efficiently, whereas longer wavelengths (UV-A over 400 nm) produce mainly mono-adducts (Averbeck et al., 1992; Bethea et al., 1999). Each psoralen derivative discussed in this review can be activated by the appropriate UV dose to form ICL, unless stated otherwise.

Psoralens form adducts with pyrimidines, predominantly with thymine residues, and create ICL principally at d(TpA):d(TpA) sequences (van Houten et al., 1986a; Friedberg et al., 1995). An important tool for the investigation of ICL repair is the availability of monofunctional psoralens, which form only mono-adducts, allowing direct comparison with the effects of ICL-forming psoralens; this enables investigators to determine the degree to which the ICL contribute to the cellular effects of these agents. For example, monofunctional angelicin causes only a negligible decrease in the viability of wild-type yeast cells at the same doses where 8-MOP is highly toxic. This, therefore, is almost certainly due to DNA ICL induction (Grossmann et al., 2001). Another feature of psoralen ICL is that they can be targeted to any required DNA sequence by triplex-forming oligonucleotides (TFO) as shown in both yeast (Barre et al., 1999a, b) and higher eukaryotes (Vásquez et al., 2002; Thoma et al., 2005). TFO are short oligomers that bind with high affinity and specificity, via Hoogsteen or reverse-Hoogsteen hydrogen bonds, to their cognate recognition sequences, fitting into the major groove of double-stranded DNA (dsDNA). Psoralens linked to TFO can efficiently block RNA polymerase II elongation and can introduce DNA damage at specific sequences, thus providing a tool to study the processing of helical distortions and ICL at the subgenome level. It has been shown that psoralen ICL on their own also block transcription (for a review, see Reddy & Vásquez, 2005), and are capable of inhibiting DNA replication (Bessho, 2003), although in mammalian cells the processes of transcription and replication may differentially be affected by DNA alterations caused by the processing of psoralen ICL (Wang et al., 2001b).

The various psoralen derivatives cause different degrees of change in the architecture of the DNA helix and this fact helps explain their variable toxicities. For example, the 4’-(aminomethyl)-4,5’,8-trimethylpsoralen (AMT)-adducted DNA duplex was shown to experience 56° unwinding and 53° bending into the major groove (Tomic et al., 1987), although a more recent study suggests that AMT does not significantly bend the DNA (Hwang et al., 1996). This contradiction might be explained by the fact that the structures of ICL in DNA substantially depend on DNA sequence context (Kumarasen et al., 1992). DNA duplex containing an 8-MOP adduct experiences a similar kink to that containing AMT adduct (Pearlman et al., 1985). In contrast, it has been reported that the DNA helix is locally unwound by 25° for the 4’-(hydroxymethyl)-4,5’,8-trimethylpsoralen (HMT)-induced ICL, and there is no significant bend in the helix axis, because the adenine residues adjacent to the ICL remain coplanar with it (Spielmann et al., 1995).
It should be noted that modification of proteins and lipids by psoralens, although at levels undetectable with current techniques, may also contribute to the cytotoxic and clinical effects. The pyrone ring of 8-MOP is preferentially adducted with proteins and lipids, whereas the furan side reacts with DNA (Bethea et al., 1999). Therefore, protein-DNA cross-links should also be considered when evaluating the overall cellular response.

The major goal of cancer chemotherapy is to commit tumor cells to death by apoptosis or other processes. Although it is not clear how cell death is induced by ICL formation, it is of great clinical interest to understand the underlying mechanisms. A commonly used chemotherapeutic agent that generates ICL, is cis-platinum diammine-dichloride (CDDP, cisplatin). This agent is widely used in the chemotherapy in spite of its systemic toxicity, primarily against head and neck cancers (Haddad et al., 2003), esophageal, gastric, epithelial lung, pancreatic, colon, bladder and particularly testicular and ovarian tumors (reviewed in Boulilas & Vougiouka, 2004). CDDP circulating in the blood or dissolved in physiological solution is neutralized. It must be activated through spontaneous hydrating reactions, which involve the sequential substitution of the cis-chloro ligands with water molecules to be able to react with DNA. Aquation is accomplished in the nucleus, because the concentration of chloride ions is far lower in this organelle compared to the extracellular environment (for a review, see Brabec, 2002). CDDP forms adducts with purines, preferentially with the N7 position of guanines, and the ICL occur principally at d(GpC):d(GpC) sites. Notably, 1,2-IaCL and 1,3-IaCL represent 90% of the total CDDP lesion burden, while ICL account for only 5% (Friedberg et al., 1995; Brabec, 2002). The relative contribution of the different types of damage to the pharmacological properties of CDDP is uncertain. Genetic studies suggest that the IaCL are highly toxic to bacteria (Keller et al., 2001) and recent studies also argue for their toxicity in yeast (McA’Nulty & Lippard, 1996) and mammalian cells (Lawley & Phillips, 1996).

Structurally, CDDP ICL are highly distorting, with the double-helix displaying over 70° unwinding and greater than 40° bending toward the minor groove (Coste et al., 1999). Consequently, the helix is locally (about 5bp) reversed to a left-handed, Z-DNA form (Perez et al., 1997). The ICL exhibit other dramatic features, e.g. the cytosine residues, complementary to the adducted guanines, are extruded from the helix (Malinge et al., 1999), potentially creating substrates similar to those which are easily recognizable for the mismatch repair (MMR) proteins (Barrett et al., 1998). The structure of the ICL is stabilized by intermolecular contacts involving the extra-helical cytosines. The CDDP ICL are not highly stable, with a half-life of 29h. The resulting mono-adducts primarily reorganize into 1aCL, where water molecules function as a driving force in this rearrangement. In addition to the structural parameters of the distorted helix, the network of water molecules surrounding platinum in the minor groove might be relevant for the mechanism of CDDP ICL recognition by proteins (Perez et al., 1997; Coste et al., 1999; Malinge et al., 1999). Interestingly, the transplatin isomer is clinically ineffective, possibly because of the less severe distortion of DNA by ICL that is induced by this isomer. In addition, efficient repair of the large proportion (50%) of mono-adducts formed might be a key factor (Heiger-Bernays et al., 1990). The slow repair of CDDP mono-adducts would also explain the greater accumulation of ICL after long drug exposures (Wilborn & Brendel, 1989).

RNA polymerase II efficiently bypasses transplatin adducts in vivo (Mello et al., 1995). In contrast, CDDP IaCL and ICL block RNA elongation (Corda et al., 1993; Tornagletti et al., 2003) and DNA replication in vitro (Villani et al., 1988; Heiger-Bernays et al., 1990) and in vivo (Sorensen & Eastman, 1988). Inhibition of DNA replication does not involve DNA polymerase β (Hoffmann et al., 1995) and is only transient in vivo, as special mechanisms (see relevant section of this review) can rescue the process. Clinical resistance to platinum drugs (for reviews, see Brabec, 2002; Brabec & Kašpárková, 2002; Siddik, 2003) can arise when cells enhance their ability to replicate DNA past the adduct and then initiate DNA repair (Chaney & Sancar, 1996), or when MMR cannot operate (Jordan & Carmofosneco, 2000).

Many proteins not directly implicated in the damage response can modify the cellular response to CDDP, including transcription factors and the high mobility group (HMG) proteins (for a review, see Torigoe et al., 2005). As it is very unlikely that primitive organisms encountered CDDP-like molecules, cellular proteins presumably recognize CDDP adducts because these lock DNA in a conformation favorable for interaction with these proteins. The HMGB1 protein selectively recognizes and further bends DNA distorted by 1,2-IaCL CDDP adducts (Reeves & Adair, 2005) and the key alteration needed for HMGB1 binding to ICL is an extra DNA unwinding of about 40° (Kašpárková et al., 2003). This recognition might be mediated through intercalation of a hydrophobic residue of HMGB1 into the site originally occupied by the extra-helical cytosines, thus resembling the structure of two G:T mismatches in complex with the MUG glycosylase (Kartalou & Essigmann, 2001). The binding of the HMG domain bound to 1,2-IaCL appears to enhance CDDP cytotoxicity by interfering with DNA repair, mainly by blocking nucleotide excision repair (NER) (Wozniak & Blasiak, 2002), which is an essential pathway for platinum adduct removal. Up-regulation of NER activity coupled to transcription is also associated with resistance to CDDP in tumor cells (Siddik, 2003). Therefore, it has been proposed that the HMGB proteins bound to DNA prevent access of NER factors and this...
‘shielding mechanism’ hinders DNA repair. For instance, the budding yeast transcription repressor, Ixr1, blocks the excision step of DNA repair (McANulty & Lippard, 1996; McANulty et al., 1996), although a lack of the fission yeast Cmb1, a related HMG protein, leads to higher sensitivity to CDDP (Kunz et al., 2003). Mammalian NER factors are not able to excise HMG-shielded IaCL in vitro (Zamble et al., 1996). Importantly, HMG factors do not bind 1,3-IaCL and transplatin adducts and therefore their repair is not blocked (Kartalou & Essigmann, 2001). Some other non-HMG proteins also affect IaCL repair, e.g. linker histone H1 has even greater affinity for CDDP lesions than HMGB (Yaneva et al., 1997). The Escherichia coli MutS MMR factor recognizes the 1,2-d(GpG) IaCL, especially compound IaCL lesions, i.e. IaCL with a mismatched base opposite. However, there is no binding to the 1,3-IaCL and surprisingly no affinity for the ICL (Fourrier et al., 2003). Yeast photolyase recognizes CDDP adducts, but has no affinity for DNA modified with transplatin. Contrary to the situation in E. coli, yeast cells deficient in photolyase are more resistant to CDDP, but not to transplatin, compared with the wild-type cells (Fox et al., 1994), probably because of the missing ‘shielding effect’. There are other proteins with high binding affinity for CDDP adducts, but it is not known whether ICL are their substrates, as the most studies did not determine the type of lesion involved. The level and persistence of DNA adducts induced by CDDP correlates directly with cytotoxicity in higher eukaryotes (Siddik, 2003). Nevertheless, there is no straightforward association between the number of ICL and survival in yeast (Wilborn & Brendel, 1989).

The nitrogen mustard family are used as chemotherapeutic agents against multiple myeloma, lymphoma, leukemia, ovarian carcinoma and various other tumors (Balcome et al., 2004). Similarly to psoralens, nitrogen mustards conjugated to TFO can be used for a targeted gene modification in mammals (Singer et al., 1999). Experimentally, the most commonly used nitrogen mustard is bis(2-chloroethyl)methylamine (HN2, checlothrethamine), a bifunctional alkylating agent. HN2 forms predominantly (90%) N7 guanine mono-adducts in the major DNA groove and a small proportion (1–5%) of ICL joining the two N7 atoms of guanines preferentially at the sequence d(GpNpC):d(GpNpC). The HN2 ICL introduce a demonstrable, but subtle, bending into the DNA helix (Rink & Hopkins, 1995). In addition, the formation of guanine-adenine and adenine-adenine ICL and IaCL after HN2 treatment has been demonstrated (Balcome et al., 2004). HN2 produces different types of mutations, such as base substitutions, intragenic and multilocus deletions, as well as chromosomal rearrangements (Povirk & Shuker, 1994). Further complexity resides in the thermolability of glycosylic bonds of the HN2 ICL, which leads to spontaneous depurination. The aromatic derivative, melphalan, produces more stable ICL (Povirk & Shuker, 1994; Bauer & Povirk, 1997). Evidence suggesting that resistance to nitrogen mustards in mammalian tumor cells can be influenced by increases in homologous recombination repair (HRR) (Wang et al., 2001c) and nonhomologous end-joining (NHEJ) activity has been presented (Panasci et al., 2001). It has been clearly shown that HN2 ICL are the critical cytotoxic lesions in budding and fission yeasts (Chaney & Sancar, 1996; McHugh et al., 1999; Lambert et al., 2003). The yeast photolyase recognizes HN2 adducts and sensitizes cells to this drug similarly as to CDDP, likely because of the ‘shielding effect’ hindering DNA repair (Fox et al., 1994).

Derivatives of nitrosoureas are liposoluble, and penetrate the hematopoietic barrier. Their mechanism of action differs from that of the above agents, and therefore little cross-resistance occurs among them. Bis(2-chloroethyl)nitrosourea (BCNU, carmustine) is an alkylating agent widely used in the therapy of intracranial tumors and malignant melanomas. BCNU decomposes in aqueous solutions to form reactive intermediates, but the precise structure of the alkylating form has not been definitively determined. BCNU creates exocyclic DNA adducts (Hang et al., 2003), the majority of which are linked to the O6 position of guanine. After metabolic activation, the O6-guanine adducts undergo an intermolecular rearrangement, thereby producing a dadduct between the N1 of the guanine and N3 of the complementary cytosine, with no obvious sequence selectivity. This lesion is reminiscent of the ICL generated by HN2 (Fischhaber et al., 1999). Although the ICL constitute only a small fraction (below 8%) of the total adducts, they appear to be the main cytotoxic lesions (Wiencke & Wieland, 1995). The BCNU-induced lesions, probably the ICL, inhibit transcription in vitro (Pieper et al., 1995) and in vivo (Rutman & Avadhan, 1976), although DNA replication does not seem to be affected (Beljanski et al., 2004). Notably, BCNU cytotoxicity can be compromised by development of drug resistance involving the DNA repair protein, O6-alkylguanine-DNA alkyltransferase (ATase), a suicide protein which repairs the mono-adducts in one step, thereby greatly reducing the ICL formation (Farkasová et al., 2000; Drablos et al., 2004). BCNU adducts do not cause G2/M cell cycle arrest in yeast (Beljanski et al., 2004), indicating a difference between yeast and mammals as to cellular response to BCNU treatment. That the resistance of some brain tumor cells is not dependent on increased activity of ATase suggests the involvement of base excision repair (BER) and NER in the elimination of mono-adducts (Baciod et al., 2002). BCNU induces mainly base substitutions in yeast and mammalian cells (Hang et al., 2003) and deletions being observed in the former organism (Kunz & Mis, 1989).

Mitomycin C (MMC), a natural quinine-based antibiotic, is a useful bio-reductive agent in the treatment of gastric, pancreatic and nonsmall cell lung cancers (Verweij &
Interestingly, DNA replication is only slightly reduced in mammalian tumor cells by MMC, whereas RNA synthesis is significantly inhibited (Yajima et al., 1998). MMC itself does not react with DNA, but requires several pathways of metabolic activation leading to DNA adduct formation. ICL are created in two alkylation steps only after enzymatic activation of MMC by cytochrome P450 reductase or other reductases (Volpato et al., 2005). In contrast to other cross-linking chemotherapeutic drugs, MMC cross-links the d(CpG) sequence with relatively high selectivity, and therefore the mammalian genome can only poorly be cross-linked by MMC (Tomasz, 1995). The ICL, which correspond to 13–14% of the total MMC adducts (Warren et al., 1998), join the N2 atoms of guanine residues in the minor groove and distort DNA to only negligible extent (Cummings et al., 1998). RNA synthesis is not affected in mammalian tumor cells by MMC, whereas DNA synthesis is significantly inhibited (Yajima et al., 1990). Interestingly, DNA replication is only slightly reduced in yeast (Beljanski et al., 2004).

**Interstrand cross-link repair**

**Interstrand cross-link repair in *E. coli***

To date, all evidence indicates that there is no single DNA repair pathway capable of eliminating ICL. The first model for ICL repair in *E. coli* was proposed over 30 years ago (Cole, 1973). Subsequent biochemical analysis using psoralen-induced ICL supported the hypothesis that the sequential action of NER and HRR pathways may be sufficient to repair ICL in an error-free manner (for an additional review, see Dronkert & Kanaar, 2001) (Fig. 1a). In the case of psoralen ICL, the Uvr(A)2BC endonuclease first performs two incisions in one DNA strand surrounding the ICL on its furan side (at the 8th phosphodiester bond 5′ and 5th phosphodiester bond 3′ to the cross-linked nucleotide), thereby generating a 13-base oligonucleotide covalently bound to the complementary DNA strand by the ICL (Fig. 1a, step i). The incised strand is resected by the 5′-exonuclease activity of DNA polymerase I (Pol I) which is unable to synthesize on this template because the ICL interferes with its polymerase activity (Fig. 1a, step ii). RecA polymerizes onto this enlarged single-stranded DNA (ssDNA) region in the 5′ → 3′ direction, producing a helical protofilament (Register III & Griffith, 1985) (Fig. 1a, steps iii and iv). The cross-linked oligonucleotide is accommodated within the RecA filament (Fig. 1a, steps v and vi). The homologous pairing of the two DNA molecules gives rise to heteroduplex DNA (hDNA) with a three-stranded region (Sladek et al., 1989b). Strand exchange, facilitated by RecA, starts in the ssDNA gap and proceeds in the direction of the filament formation. Concurrently, RecA monomers dissociate from the tail end of the filament and the cross-over migrates spontaneously, thereby extending the regions of hDNA (Sladek et al., 1989a, b). Surprisingly, RuvAB is capable of mediating branch migration through a psoralen ICL (George et al., 2000). Subsequently, Pol I elongates the 3′ end of the exchanged strand in the donor molecule, and the gap in the recipient molecule is accordingly filled in after the reannealing of this newly synthesized strand. Then Uvr(A)2BC makes two incisions in the other DNA strand on the pyrone side of the ICL (Sladek et al., 1989a, b) (Fig. 1a, step vii). Most likely, the incision activity of Uvr(A)2BC on this side of the ICL is stimulated by a three-stranded DNA structure (Cheng et al., 1988, 1991). The resulting ssDNA gap is progressively filled in by Pol I, thus a cross-linked dsDNA fragment and two intact chromosomes are produced (Fig. 1a, steps viii and ix). In vivo, many other factors take part in ICL repair, some of them greatly enhancing the efficiency (e.g. UvrD helicase) and others regulating the particular steps of the process (Sladek et al., 1989b). Biochemical and genetic experiments using HN2...
revealed an alternative pathway dependent on DNA polymerase II (Pol β), which acts on ICL when a homologous template is not available (Fig. 1b). The data suggest that the reaction is again initiated by incisions made by Uvr(A)2BC (Fig. 1b, step i) and Pol β then synthesizes across the gap spanning the cross-linked oligonucleotide (Fig. 1b, step ii). Next, a second round of incisions by Uvr(A)2BC removes the DNA fragment containing the cross-link (Fig. 1b, step iii). Survival data suggested that both HRR and translesion DNA synthesis are NER-dependent (Berardini et al., 1999). BER does not seem to play an important role in the repair of HN2 ICL in E. coli (Berardini et al., 1997). There is also evidence for at least two pathways of cellular response to CDDP or MMC, both of which are dependent on RecA binding to ssDNA and facilitating translesion DNA synthesis by Pol β (Keller et al., 2001). The RecBCD pathway is necessary for the initiation of HRR by linear DNA and for DNA double-strand break (DSB) repair, while the RecFOR branch aids the binding of RecA during the processing of ssDNA gaps associated with ICL processing (Keller et al., 2001). HRR is crucially important for resistance to CDDP, while MMC stimulates the expression of the SOS regulon to a higher degree, pointing to incomplete ICL repair or persistence of DNA lesions (Keller et al., 2001).

**Interstrand cross-link repair in S. cerevisiae**

ICL repair in eukaryotes is still not well understood. The increased sophistication of the repair processes is essential because of the complex genome structure and highly differentiated phases of the cell cycle. In S. cerevisiae, ICL repair utilizes factors from all of the three major repair groups (RAD3/NER, RAD6/postreplication repair–PRR and RAD52/HRR), originally defined by the epistatic relationships of mutants in response to UV- and ionizing radiation-induced DNA damage (Cox & Game, 1974), as well as genes that have been identified by subsequent studies (BER factors, MMR factors, etc.). It is clear, however, and will be illustrated by examples throughout this review, that the interpretation of the apparent ‘epistatic’ and ‘nonepistatic’ genetic interactions of the various repair pathways involved in ICL repair is very complex. This reflects the fact that multiple pathways compete for intermediates during the various stages of ICL repair, and these can be overlapping, partially overlapping or entirely distinct from one another. Furthermore, they are cell cycle-phase dependent in some cases. The relative contribution of all the pathways known to operate in yeast ICL repair are considered in turn here and we attempt to define their relationship to one another where possible. Where appropriate, well characterized similarities or differences in the mechanism of ICL repair in mammalian cells are briefly discussed.

**Nucleotide excision repair**

NER is a highly versatile mechanism of DNA repair, recognizing and dealing with a wide variety of helix-distorting lesions, such as UV-induced photoproducts, cyclobutane pyrimidine dimers and (6-4) photoproducts (CPD and 6-4 PP, respectively). NER is mainly adapted to remove bulky adducts interfering with normal base pairing and helical geometry (Costa et al., 2003). NER in eukaryotic cells is a multistage process that initially recognizes damaged DNA, unwinds it locally, produces incisions (nicks) upstream and downstream of the lesion, and displaces the excised oligonucleotide. The gap, left after removal of the oligonucleotide, is repaired by DNA polymerases δ and ε, and the resynthesized segment is ligated by DNA ligase I. It has been suggested that four stable NER subcomplexes (NEF1–4) exist in yeast (Sweder & Madura, 2002). DNA incisions, performed by NER, are absolutely required for the early steps of yeast ICL repair. As for E. coli, it is thought that the initial incision reactions release/uncouple the ICL, leaving an excised oligonucleotide connected to the opposite strand by the ICL (van Houten et al., 1986b), although such reactions have yet to be biochemically characterized or reconstituted in yeast. ICL incision after 8-MOP treatment could not be detected in mitochondria, and was depressed in nuclear DNA of stationary phase yeast cells (Magana-Schwencke et al., 1982), compared to exponential phase yeast cells (Miller et al., 1982b). Given that psoralen ICL are incised rapidly in G1 synchronized populations (Meniel et al., 1997), it appears that growth to stationary phase might specifically depress ICL incision. The nicks formed at ICL are more stable than those at CPD (Miller et al., 1982b), indicating that ICL removal is not as rapid a process as UV photoproduction repair, probably because of the complex postincision transactions required to complete ICL repair. The proteins involved in ICL recognition in yeast have not been examined in detail, and therefore we must speculate from genetic data and what is known about the biochemistry of the canonical NER apparatus. We propose that damage recognition proteins of NER, perhaps with additional factors co-opted for this particular pathway, will likely identify the abnormal structure of cross-linked DNA to initiate DNA repair. The NEF2 complex, consisting of Rad4 and Rad23, has high affinity to DNA damaged by UV (Jansen et al., 1998). NEF2 also recruits other repair factors to the site of the lesion. It is believed that turnover of Rad4 – controlled by its ubiquitination – is involved in the overall regulation of NER. Rad23 has dual roles in NER by stabilizing Rad4, possibly regulating the proteolysis of additional factors as well as contributing to damage recognition (Ortolan et al., 2004). Certainly, Rad4 is critical for normal resistance to cross-links induced by HN2, CDDP and MMC (McHugh et al., 1999; Wu et al., 2004b). Further evidence
that NEF2 may have a role in the recognition of psoralen ICL was obtained from a study of the replication and integration of plasmids bearing a HMT ICL, where these events are greatly reduced in a rad4 mutant (Saffran et al., 2004). The NEF4 complex consisting of Rad7 and Rad16 is required for the repair of the nontranscribed regions of DNA (Verhaeg et al., 1994). The NEF4 complex may possess a ubiquitin-ligase activity that regulates NER by modulating the steady-state level of Rad4 (Ramsey et al., 2004). Indeed, the Rad16 protein has a ring finger domain characteristic for E3 ubiquitin-ligases that determine substrate specificity. Rad16 enables the action of NER in heterochromatin and is related to the Snf2/Swi2 family of ATP-dependent chromatin-remodeling DNA helicases. In general, remodeling complexes can alter the position or the structure of a nucleosome to make the DNA more accessible, and the character and location of DNA damage will determine whether these proteins precede or follow the binding of repair factors (Martin & Winston, 2003). The low sensitivity of rad16 strain to CDDP, MMC and DEO indicates either efficient residual excision repair or less frequent lesion induction in the silent regions of chromatin (Saffi et al., 2000; Wu et al., 2004b). In support of the latter possibility, bulk ICL incisions occur normally in rad16 cells (Miller et al., 1982b), but no incision of 8-MOP ICL occurs at the inactive HML locus (Meniel et al., 1995b), signifying the involvement of Rad16 in ICL repair in nonexpressed regions. The rad7 mutant exhibits a defect in the removal of TMP ICL (Miller et al., 1982a,b), but its sensitivity to CDDP or MMC is slight (Wu et al., 2004b).

A second protein potentially contributing to DNA damage recognition during NER is Rad14. It exists in the NEF1 complex with the Rad1-Rad10 endonuclease (Guzder et al., 1996). As rad14 strains are killed by 8-MOP more efficiently than the HRR-compromised strains (Cohen et al., 2002), it appears that for resistance to psoralen, ICL incision is more critical than recombination. Although Rad14 plays a central role in NER, the repair of HN2 mono-adducts (apparently carried out by the 3-methyladenine DNA glycosylase, Mag1) seems to function even in the absence of this protein. Thus, the extreme sensitivity of the respective mutant in both stationary and exponential phases (McHugh et al., 1999) hints at the lethal consequence of the unrepaired ICL. The elevated frequency of mutations observed in rad14 cells following HN2 treatment results from the action of translesion DNA synthesis (TLS) by Pol ζ on HN2 adducts (McHugh et al., 1999).

Many components of the NER apparatus also have roles in other pathways, potentially adding complexity to the interpretation the results obtained from genetic experiments. For example, the Rad1-Rad10 heterodimer, a component of NEF1, is also involved in recombinogenic processes (Kearney et al., 2001) as well as in suppressing gross chromosomal rearrangement events, such as de novo telomere addition or translocations (Hwang et al., 2005). Biochemically, Rad1-Rad10 is a junction-specific endonuclease, incising at the 3'-junction at the transition between dsDNA and ssDNA regions in partially duplex DNA substrates, such as bubble or flap structures, generated during various DNA transactions. Therefore, in NER this nuclease makes the incision 5' to the adduct in the bubble created by the Rad3 and Rad25 helicases. Furthermore, this dimer removes nonhomologous DNA ends during recombination and other DNA repair processes (Davies et al., 1995). Such 3' DNA overhangs can be removed by this heterodimer even if the DNA end is blocked or damaged with covalently bound adduct (Guzder et al., 2004). This endonuclease activity plays a key role in ICL repair, as the absence of Rad1-Rad10 abolishes the incisions of TMP ICL and causes high sensitivity to TMP (Miller et al., 1982b) and CDDP (Abe et al., 1994). Rad1 takes part in the repair of plasmid-born 8-MOP adducts (Magana-Schwencke & Averbeck, 1991) and in the integration of plasmids carrying a single HMT ICL. Although similar transformation frequencies can be observed in rad1 and wild-type cells (Greenberg et al., 2001; Saffran et al., 2004), half of the stable transformants obtained with plasmids cross-linked in vitro by AMT in rad1 cells result from gene conversion events instead of crossovers, and this is not applicable to other NER factors examined, e.g. Rad4 (Saffran et al., 1992). The frequency of crossovers is reduced by RAD1 inactivation even if a nonreplicating plasmid is used in the assay. Moreover, the integration triggered by enzymatically created DSB is suppressed in rad1 cells (Saffran et al., 1994), indicating an alteration in DSB repair fidelity. However, the requirement of these proteins for survival after psoralen addition is comparable to that of other NER factors (Chanet et al., 1985). Therefore, despite these additional roles, Rad1-Rad10 is believed to primarily operate in DNA incision during the repair of HN2 ICL (Barber et al., 2005), suggesting that although the Rad1-Rad10 complex might alter the outcome of HRR reactions downstream of ICL incision, this might not significantly impact on cell survival.

The largest yeast NER complex, NEF3, contains Rad2 (the NER endonuclease producing incisions 3' to the adduct) and TFIIH (an RNA polymerase II-associated transcription factor with two associated helicases, Rad3 and Rad25). Nuclease of the Rad2 family possess both 5' flap endonuclease and the 5'-exonuclease activities required for maturation of Okazaki fragments and mutation avoidance during DNA replication (Sun et al., 2003). Like inactivation of RAD1, deletion of RAD2 greatly increases the sensitivity of yeast cells to CDDP, HN2, MMC and 8-MOP (Wilborn & Brendel, 1989; Wu et al., 2004b). In the corresponding mutants, DNA incision is blocked (Chanet et al., 1985; Meniel et al., 1995a) and the DNA damage-induced
recombination is initiated only after DNA replication in the presence of 8-MOP (Saeki & Machida, 1991), thereby underlining the key function of both NER endonucleases in ICL repair in yeast. All yeast strains deficient in NER demonstrate a lower rate of HRR repair, whereas conversion of a gene upstream from the HMT ICL placed on plasmid DNA is less affected than downstream or bidirectional gene conversion. Altogether, NER is crucial for survival in the presence of bifunctional agents and it partly determines the course of the recombination stage of repair (Saffran et al., 1994). Note that the above-mentioned ICL-forming agents generate predominantly mono-adducts which cause toxicity in NER mutants. Some studies did not affirm the importance of ICL in cell killing, as they did not account for the specific contribution of ICL to the enhanced sensitivity.

A key issue to consider when evaluating the role of the NER apparatus during the incision and processing of ICL in yeast vs. higher eukaryotes is the greatly increased importance of the XPF-ERCC1 (Rad1-Rad10 homolog) structure-specific nuclease in mammalian cells. Rodent cells disrupted for XPF-ERCC1 are far more sensitive to cross-linking agents than isogenic cells lacking other ‘essential’ components of the NER apparatus e.g. XPG (Rad2), XPD (Rad25) and XPF (Rad3) (Hoy et al., 1985a; Andersson et al., 1996; Damia et al., 1996). The reasons for this are still unclear. Given the vital role for XPF-ERCC1 in NER, and also the role of this nuclease in certain homologous recombination processes, two likely scenarios (which are not mutually exclusive) can be envisaged. First, it is possible that XPF-ERCC1 catalyzes a novel type of incision reaction at ICL which does not require other ‘classical’ components of the NER apparatus. How damage recognition would be achieved in this scenario is not clear, but two possibilities that have been suggested include a role for the MUTSβ MMR recognition factor, or perhaps that structures or protein complexes formed when replication forks stall at ICL recruit XPF-ERCC1 and trigger incision, and therefore that any specialized incision reactions XPF-ERCC1 catalyzes might be restricted to S phase. In support of the former model, efficient ICL incision has been shown to require MUTSβ in the presence of RPA in vitro, which are together stimulated by PCNA (Li et al., 1999; Zhang et al., 2002, 2003). In support of the latter proposal, ICL incision reactions requiring only XPF and ERCC1 have been preferentially observed on a substrate that mimics a replication fork (Kuraoka et al., 2000), although it should be noted that other investigators have observed such reactions on normal duplex (Bessho et al., 1997; Mu et al., 2000). Cellular analysis also revealed an ICL incision defect in XPF- and ERCC1-defective CHO cells treated with HN2, although the cells were treated in asynchronous culture, which did not permit cell cycle effects to be evaluated (De Silva et al., 2000). A second possibility is that XPF-ERCC1 influences a recombination reaction that is important in the latter steps of ICL repair. Few data are available to support this assertion directly, although it has been shown that the repair of ICL-associated DSB is reduced in ERCC1 knockout mouse cells (Niedernhofer et al., 2004). Whether this reflects a defect in ICL incision which is a prerequisite for the repair of ICL-associated DSB as in yeast (Barber et al., 2005), or reveals a direct effect of XPF-ERCC1 on the recombination reactions that repair the DSB, is not clear. Therefore, the reasons for the extreme sensitivity of XPF/ERCC1-defective cells remain uncertain, and elucidation of this will significantly aid our understanding of mammalian ICL repair.

**Transcription-coupled and preferential ICL repair**

The close association between NER and transcription has been described in detail elsewhere (for reviews, see Friedberg et al., 1994; Svejstrup, 2002; Reardon & Sancar, 2005). Two nonessential NER factors, Rad26 and Rad28, are members of the nucleosome remodeling Swi2/Snf2 family and couple NER to transcription, such that the transcribed strand of active genes is preferentially repaired compared to nontranscribed strand. It has been confirmed that repair of psoralen ICL is often faster in active genes than in inactive chromatin (Islas et al., 1994) and that mutations induced by MMC (Zheng et al., 2003) or psoralens (Meniel et al., 1995a; Wang et al., 2001b) occur mainly on the nontranscribed strand, suggesting favored ICL repair, i.e. the participation of transcription-coupled repair (TCR) directing the incisions toward the transcribed strand. In yeast, the absence of Rad26 and Rad28 does not reduce resistance to CDDP, HN2 or MMC (Wu et al., 2004b; P. McHugh, unpublished data), thus their involvement in ICL repair is unclear. However, incisions at 8-MOP ICL occur preferentially in the transcriptionally active MATα locus compared to the silent HMLα locus (Meniel et al., 1995b). This is not conditioned by the cell cycle progression and is therefore probably linked to the euchromatin-like structure, as the distribution of ICL is equal at the two loci. However, it was also been reported that at low level of 8-MOP ICL, no preference for expressed strand was observed (Saffran et al., 1994). Thus their involvement in ICL repair is unclear. However, incisions at 8-MOP ICL occur preferentially in the transcriptionally active MATα locus compared to the silent HMLα locus (Meniel et al., 1995b). This is not conditioned by the cell cycle progression and is therefore probably linked to the euchromatin-like structure, as the distribution of ICL is equal at the two loci. However, it was also been reported that at low level of 8-MOP ICL, no preference for expressed strand was observed (Saffran et al., 1994).
the influence of transcription, some incision also occurs on the nontranscribed strand, if it carries the furan, leading to mutations in both DNA strands. Remarkably, the insertions are also biased with respect to transcription, suggesting that PRR, or a putative pathway which precedes PRR, is coupled to transcription (Barre et al., 1999a). TLS opposes the thymine linked to the pyrone results in insertions of A residues and T → A or T → C substitutions. Bypass of the furan-adducted T yields G insertions and T → G substitutions. Therefore, the geometry of the psoralen ICL has an impact on the nucleotide incorporation opposite the lesion (Barre et al., 1999a). In plasmids carrying an HMT ICL, preferential incidence of substitutions in the nontranscribed strand was observed in the wild-type and HRR-compromised cells, whereas there was no strand bias in NER-defective cells, (Safran et al., 2004).

The role of the PSO gene products in ICL repair

Genetic screens aimed at uncovering novel factors influencing the repair of ICL have revealed 10 distinct PSO (‘psoralen sensitive’) genes to date. Whereas products of eight of them (Psol, Ps02, Ps03, Ps04, Ps05, Ps08, Ps09 and Ps10), seem to be involved in the repair or tolerance of DNA damage, Ps06 and Ps07 influence physiological processes unrelated to nucleic acid metabolism. Therefore, the function of them in cellular response to photoactivated psoralens is rather indirect and will not be considered in detail here (reviewed in Brendel et al., 2003).

The first three S. cerevisiae mutants sensitive to photoactivated psoralens were isolated and characterized over two decades ago (Henriques & Moustacchi, 1980a). The psol mutant, apart from its sensitivity to photoactivated psoralens, is cross-sensitive to UV and ionizing radiation (Henriques & Moustacchi, 1980a, b). For 8-MOP photoaddition, the psol mutation was shown to be epistatic to the rad6, rad52, and ps02 mutations, whereas it was synergistic to rad3. As psol further interacted synergistically with rad3 and rad52 and epistatically with rad6 after UV irradiation, and epistatically with rad6 and rad52 after ionizing radiation exposure, the PSO1 gene was proposed to belong to the RAD6/PRR epistasis group (Henriques & Moustacchi, 1981). In support of this proposal, allelism between psol and rev3 was demonstrated (Cassier-Chauvat & Moustacchi, 1988). REV3 encodes a catalytic subunit of DNA polymerase Pol ζ and the reader is referred to the next section for further details on this polymerase and its role in ICL repair.

The ps02 mutant is specifically sensitive to psoralen photoaddition (Henriques & Moustacchi, 1980a) and genetic studies revealed allelism between ps02 and snm1 (Cassier-Chauvat & Moustacchi, 1988), where the latter mutation was isolated on the basis of conferring a specific sensitivity to HN2 (Ruhland et al., 1981a, b). Although genetic studies in S. cerevisiae initially assigned PSO2 to the RAD3/NER epistasis group for sensitivity to cross-linking agents, there are fundamental distinguishing features between the ps02- and NER-defective cells with respect to ICL repair. Density gradient centrifugation experiments, conducted by Magana-Schwenecke and coworkers (Magana-Schwenecke et al., 1982), clearly demonstrated the formation and repair of DNA single-strand breaks (SSB) and DSB in wild-type, exponential phase yeast cells exposed to 8-MOP photoaddition. In the rad3 mutants the ICL incision step was almost completely abrogated, contrasting with ps02 cells that produced both SSB and DSB, but were unable to subsequently reconstitute dsDNA. Hence ps02 mutants are proficient in the incision step of ICL repair, but are defective at some downstream processing event. Nevertheless, investigations into the genetic relationships of PSO2 with other key repair genes have shown a lack of epistasis with the RAD52/HR group (Henriques & Moustacchi, 1981), whereas data regarding the interaction with RAD6/PPR pathway are inconsistent. At the biochemical level, the Ps02 protein is a member of the metal-β-lactamase (MBL) superfamily of enzymes that share a conserved hydrolytic domain that can bind one or two metal ions (Aravind et al., 1999; Callebaut et al., 2002). Ps02 has been reported to possess 5'-nuclease activity on single- and double-strand oligonucleotide substrates (Li et al., 2005), and this activity is dependent upon an active MBL domain, as a point mutation in the predicted active site (D252A mutant) loses most of this biochemical activity and yeast strains harboring PSO2-D252A exhibit a null phenotype with respect to cross-linking drug sensitivity (Li & Moses, 2003; Li et al., 2005). The Ps02 protein also possesses a second domain highly conserved in the MBL-containing family of nucleic acid processing enzymes, namely a β-CASP domain, self-identified by the key members of this family (CPSF, Snml/Ps02 and Artemis) (Callebaut et al., 2002). CPSF is an mRNA processing enzyme (Chanfreau et al., 1996), whereas Artemis (when complexed with DNA-PKcs) is an endonuclease that plays a relatively well-defined role in V(DJ) recombination (Ma et al., 2002), as well as likely playing a nucleolytic role in NHEJ (Rooney et al., 2003). Identified roles of Ps02 in ICL repair that involve interaction to specific repair pathways (e.g. HRR and MMR) will be discussed later, in the context of the relevant sections of this review.

In a similar manner to ps02, the ps03 mutation confers a specific sensitivity to psoralen addition (Henriques & Moustacchi, 1980a). With regard to this sensitivity, ps03 displays epistatic interaction with psol, ps02 and rad3, and synergistic interaction with rad6 and rad52. Therefore, PS03 was initially assigned to the RAD3/NER epistasis group (Benfato et al., 1992). Although allelism between PS03 and RNR4, which encodes a second small subunit of ribonucleotide reductase (Huang & Elledge, 1997; Wang et al., 1997),
was suggested (Brendel et al., 2003), experimental work showing this has never been reported.

As the x9 mutant, originally isolated on the basis of its slight sensitivity to ionizing radiation, was found to be extremely sensitive to 8-MOP photoaddition and to exhibit no allelism with ps01, ps02 or ps03, it was designated ps04 (Henriques et al., 1989). PSO4 was allocated to the RAD52/ HRR epistasis group and suggested to participate in the rejoining step of ICL repair (de Morais et al., 1996). Its allelism with PRP19, an essential gene encoding spliceosome-associated protein (Cheng et al., 1993), was reported (Grey et al., 1996). A putative human homolog of Ps04 has been identified (Mahajan & Mitchell, 2003) and shown to be a component of a larger complex also containing CDC5L, PLRG1 and SPF27 that is required for processing of psoralen ICL in vitro (Zhang et al., 2005). Of note, this complex might also contain the Werner’s syndrome helicase/nuclEase, where the helicase activity was proposed to influence ICL processing. This would be consistent with the observation that cells derived from Werner’s patients are sensitive to ICL-inducing agents (Poot et al., 2001, 2002).

In 1994, three novel ps0 mutants (ps05, ps06 and ps07) were reported. Similarly to ps01, these mutants exhibited cross-sensitivity to UV, radiomimetic mutagens, and to chemicals inducing oxidative stress (Querol et al., 1994). Based on the complementation approach, allelism between ps05 and rad16 was demonstrated (Paesi-Toresan et al., 1995). Rad16 is component of the Nef4 complex that is required for the repair of the nontranscribed regions of DNA, and the reader is referred to the section on NER for further details on the role of this protein in ICL repair. Sequencing of the gene that complemented the sensitivity of the ps06 mutant to the psoralen photoaddition revealed that ERG3, encoding the C-5 sterol desaturase required for ergosterol biosynthesis (Arthington et al., 1991), is allelic to PSO6 (Schmidt et al., 1999). Furthermore, the sensitivity of the ps07 mutant to 4-nitroquinoline-1-oxide was rescued by the DNA fragment containing COX11 (Pungartnik et al., 1999), a gene encoding a protein that is tightly associated with the mitochondrial membrane (Tzagoloff et al., 1990) and is essential for cytochrome c oxidase assembly (Carr et al., 2002). Therefore, cellular response of ps06 and ps07 cells to mutagen treatment may be caused by altered metabolism of these mutagens which leads to changes in membrane lipids and in complex IV of the respiratory chain. Consistent with this, ergosterol and cytochrome c oxidase may be considered important factors in modulation of cellular response to mutagen treatment. Another ps0 mutant, ps08, was shown to act synergistically with rad2, rad3, rad4, rad9 and rad50. Based on this and further findings, PS08 was placed into the RAD6/PRR epistasis group. Indeed, subsequent work displayed allelism between PS08 and RAD6 (Rolla et al., 2002). Rad6 is a E2 ubiquitin-conjugating enzyme, a function of which is described and discussed in detail in next section. Molecular cloning of the PSO9 gene, achieved by complementing UV-C and 8-MOP photoaddition sensitivities of the ps09 mutant, revealed its allelism with MEC3 (Cardone et al., 2006), a product of which is a component of the Rad17-Mec3-Ddc1 complex that is essential to DNA damage checkpoint response in yeast. The DNA damage checkpoint response in yeast has been the subject of several reviews (Weinert et al., 1994; Lydall & Weinert, 1997; Foiani et al., 2000). The PSO10 gene has not yet been cloned. The corresponding mutant exhibits high sensitivity to photoactivated psoralens and UV-C and low induced mutability, suggesting that Pso10 either regulates error-prone repair or is itself an active DNA repair protein/enzyme. In accordance with the phenotype of the mutant, PSO10 was allocated to the RAD6/PRR epistasis group (Brendel et al., 2003).

**DNA damage bypass, translesion synthesis and postreplication repair**

Eukaryotic cells have evolved various surveillance and DNA lesion repair and tolerance machineries to ensure the processing or stabilization of stalled replication forks. Replication is re-established by HRR and/or by shutting the interfering lesions into the pathways of PRR that can lead to error-free or error-prone lesion bypass. The designation of PRR reflects the ability of this system to convert low molecular weight DNA, formed spontaneously or in response to exogenous genotoxic stress in replicating cells, into larger DNA molecules without (or prior to) removal of the original lesion (Torres-Ramos et al., 2002; Barbour & Xiao, 2003).

The Rad6 and Rad18 proteins play a central role in PRR in yeast. Rad6 is a E2 ubiquitin-conjugating enzyme and Rad18 a E3 ubiquitin ligase, guiding substrate specificity. As a complex, Rad6-Rad18 regulates several distinct pathways of PRR and TLS via modifications of PCNA (Pol30) (reviewed by Friedberg et al., 2005; Ulrich et al., 2005). These include TLS by DNA polymerases Pol ζ (Rev3 and Rev7) and/or Pol η (Rad30). A different, error-free, pathway relies on another ubiquitin-conjugating complex, Mms2-Ubc13, as well as on a further (putative) E3 ligase Rad5 and, Rad18. Recently, an alternate avoidance mechanism controlled by Mgs1 has also been suggested (Barbour & Xiao, 2003). Covalent modification of PCNA, by the addition of a single ubiquitin moiety, is necessary for UV-stimulated mutagenesis by Pol ζ, and activates damage-induced TLS by Pol η directly. While conjugation of a single ubiquitin to PCNA at the highly conserved lysine 164 residue (K164) activates TLS, poly-ubiquitination at K164 involving Rad5-Mms2-Ubc13 permits template switch DNA synthesis, perhaps involving the reversal of replication forks. Moreover, HRR-mediated lesion bypass can be suppressed by SUMO
modification of PCNA at K164 (with a minor effect mediated by SUMOylation at K127), which is induced even during an unperturbed S phase. This suppression of HRR is regulated by PCNA-SUMO association with Srs2 (Papouli et al., 2005; Pfander et al., 2005), a helicase capable of disrupting Rad51 ssDNA filaments (Krečić et al., 2003). Moreover, another helicase of the RecQ family, Sgs1, is involved in the dissolution of Rad51-dependent cruciform structures originating from sister chromatid exchange (SCE) events at damaged replication forks (Liberi et al., 2005).

In contrast to mono-adducts, ICL are highly mutagenic. 5-MOP induces base substitutions – created mainly by PRR in combination with NER – and insertions – obtained if the ICL are processed by PRR in the absence of NER (Barre et al., 1999b). Few ICL-induced mutations can be identified in yeast with abolished PRR. Only substitutions can be detected after the processing of HMT ICL by cells defective in NER (Safran et al., 2004). ICL-mediated mutagenesis is almost completely disabled in cells lacking TLS polymerase Pol ζ. Pol ζ has no 3′ → 5′ proofreading activity, exhibits low fidelity and processivity, with half of the molecules dissociating from a DNA template after the addition of only 3–4 nucleotides. Pol ζ is capable of nucleotide insertion, albeit at high mutation rate, opposite certain adducts, e.g. the 5′ thymine of a T-T (6–4) PP, and efficiently extends the DNA end at a misinsertion made by Pol η at the 3′ thymine of the lesion (Lawrence, 2002; Gibbs et al., 2005). Nucleotide incorporation against the uncoupled ICL is important for the survival of cells in G1 as the absence of Rev3, which is the catalytic subunit of Pol ζ, decreases the survival of stationary and G1 phase cells, but not of exponential cells, treated with HN2 (McHugh et al., 2000; Sarkar et al., 2006). This reaction appears to be controlled in a manner reminiscent of damage tolerance at replication forks. The data suggest that, following ICL incision by the NER apparatus, a ‘classical’ gap-filling reaction is attempted (by Pol δ), but thwarted by the presence of the cross-linked moiety. This triggers the Rad6-Rad18-dependent monoubiquitination of PCNA at K164, triggering activation of Rev1 and Pol ζ, leading to TLS past the incised ICL intermediate (Sarkar et al., 2006). This study also revealed that Pso2 is a member of this G1 phase pathway, and acts at the same stage, or later than, Pol ζ. Alternative mechanisms appear to be utilized to cope with the HN2 ICL repair intermediates outside of G0/G1, as rev3 deletion has much smaller effects in other cell cycle phases (Sarkar et al., 2006). Rev3 is also needed to deal with 8-MOP ICL (Cohen et al., 2002). Interestingly, although there is evidence for an S phase, replication-associated bypass of these adducts (Chanet et al., 1983, 1985; Grossmann et al., 2000, 2001) as well as adducts induced by CDDP and HN2 (Grossmann et al., 1999, 2000; Beljanski et al., 2004), this bypass is not blocked in rev3 cells, indicating that Pol ζ is not required to complete bulk DNA replication (Grossmann et al., 2000; Beljanski et al., 2004). Rad52-mediated mechanisms might enable normal S phase, as CDDP and HN2 treatments significantly increase recombination frequency (Beljanski et al., 2004). CDDP strongly reduces the viability of the rev3 mutants passing through the cell cycle, so PRR might be more important for dealing with the IaCL this agent induces than the ICL (Grossmann et al., 2000; Beljanski et al., 2004; Wu et al., 2004b). PRR plays a key role in the elimination of BCNU adducts, because amongst yeast having a defect in DNA repair, only strains with impaired PRR are considerably more sensitive to this agent than the wild-type cells (Simon et al., 2000; Beljanski et al., 2004). In conclusion, Rev3 likely plays a cell cycle phase-specific role in the repair of HN2- and 8-MOP-induced ICL, and is likely to be very important in the tolerance of CDDP IaCL adducts.

Multiple polymerases capable of TLS has been identified in mammalian cells (Goodman & Tippin, 2000; Lehmann, 2005), but to date only two of them, Pol ζ and Pol η, have been reported to directly influence ICL repair (Zheng et al., 2003; Wu et al., 2004a; Richards et al., 2005; Wittschieben et al., 2006). In particular, it has been suggested that TLS mediated by Pol ζ contributes to the development of cellular resistance to CDDP (Wu et al., 2004a). Pol η (encoded by the RAD30 gene), inserts nucleotides with high accuracy against lesions capable of base-pairing, e.g. the polymerase replicates past the T-T cyclobutane dimers before fork collapse can occur. This enzyme, however, does not have a primary role in yeast ICL repair because CDDP, MMC or 8-MOP does not increase the sensitivity or alter the cell cycle of the rad30 mutant cells relative to wild-type cells (Grossmann et al., 2001; Wu et al., 2004b). As yeast rev3 rad30 double mutant shows the same response to HN2 and CDDP as the rev3 single mutant, Rad30 cannot substitute for Rev3 in the repair or tolerance of the ICL or IaCL adducts these drugs induce in this lower eukaryote (Grossmann et al., 2001; Sarkar et al., 2006). Mammalian Pol η is not required for recombination-independent repair of ICL if these are generated by psoralen (Wang et al., 2001b) but, surprisingly, it is essential if they are created by MMC. The absence of Pol η only reduces, but does not abolish, occurrence of MMC-induced mutations (Zheng et al., 2003), and therefore the other lesion bypass polymerase must also take part in the process. In contrast to nonrepliing plasmids that require NER (Wang et al., 2001b), in proliferating mammalian cells the NER functions, other than those dependent on Rad1-Rad10, are dispensable for base substitutions at the site of TFO-targeted HMT ICL (Richards et al., 2005). This discrepancy can be explained by hypothesis that the encounter of a replication fork with an ICL can compensate for defects in NER. On the other hand, the abolition of NER increases deletion formation at the site of the ICL (Richards et al., 2005). Notably, ICL-coupled TFO can impair incisions at
the ICL and totally inhibit the repair DNA synthesis, in accordance with the delayed repair of ICL in the reporter gene and decreased binding of the NER damage recognition factor to them (Guillonneau et al., 2004). Nonetheless, the affinity of binding is strongly influenced by the character of the triplex, chiefly by the extent of helix distortion (Vasquez et al., 2002). The repair of TFO not associated with ICL relies on TCR, requires both the lesion sensing factors of NER, and is partly error-prone (Wang et al., 2001a).

Cells defective in rad6 display a pleiotropic phenotype, indicating the involvement of this factor in many cellular processes. Although Rad6 is not needed for the maintenance of 8-MOP adducted plasmids (Magana-Schwencke & Averbbeck, 1991), the repair of 8-MOP ICL is impaired in a rad6 strain (Chanet et al., 1985). The strain is also sensitive to CDDP, HN2 and MMC, with the sensitivity being higher than that for cells with impaired HRR (Simon et al., 2000). Extreme sensitivity to MMC and CDDP has also been observed in rad5 cells, whereas rad18 and mms2 cells (the latter being impaired only in the ‘error-free’ branch of PRR) show milder, albeit still high, sensitivity to these agents (Wu et al., 2004b), thus confirming the key role of the Rad5-Mms2-Ubc13 arm of PRR in ICL repair. The Rad6-Rad18 complex appears to play a major role in controlling the DNA repair synthesis in the gaps created by the endonucleolytic processing of these ICL, through activation of Pol ζ in G1 cells, and through as yet uncharacterized means in other cell cycle phases. Here the involvement of Rad5-Mms2-Ubc13 arm is very likely implicated, but remains to be explored in detail.

Homologous recombinational repair and nonhomologous end-joining

Numerous reports indicate that diploid cells are more resistant to ICL-forming agents than haploid cells (Henriques & Moustacchi, 1980a; Fedorova & Marfin, 1982; Fedorova & Kozhina, 1987). In haploid yeast, DSB induced by 8-MOP (Magana-Schwencke et al., 1982), CDDP (Frankenberg-Schwager et al., 2005) or HN2 (McHugh et al., 2000), can be repaired only when HRR is functional. Psoralen adducts located on plasmids stimulate multiple integrations of damaged plasmids, and even of those partly homologous cotransformed plasmids that do not contain the lesion, resulting in tandem arrays at the locus of homology or loci of homologies, respectively (Saffran et al., 1991, 1994). 8-MOP stimulates HRR at transcribed genes in diploid yeast and increases the frequency of reciprocal translocations, linked to ectopic gene conversions, in a dose-dependent manner (Dardalhon et al., 1998). Hence, HRR of ICL may possibly be connected to transcription. The expression of RAD51 is required for most, but not all, HRR events and is up-regulated in response to 8-MOP. Presumably, HRR repair of psoralen ICL is augmented by increased Rad51 levels (Cohen et al., 2002). Therefore, collective evidence indicates that HRR is involved in the repair of psoralen ICL in haploid and diploid cells and in fact cooperates with NER analogous to prokaryotes (Cole, 1973) to form a single ICL repair system in yeast (Henriques & Moustacchi, 1980b; Zacharyczyk et al., 1981). The reduced ability of diploid ps02 cells to recombine following treatment with psoralens (Henriques & Moustacchi, 1980a; Saeki et al., 1983) highlights the influence of Pso2 in HRR. Impaired repair of ICL at or before the HRR stage might also explain the similar resistance of diploid and haploid pso2 mutants to psoralens (Henriques & Moustacchi, 1980a). Sporulation and ionizing radiation resistance is normal in the absence of Pso2, and therefore the protein is likely not involved in meiotic recombination or general DSB repair, but controls a step specific in ICL repair (Henriques & Moustacchi, 1980a). In apparent contradiction, the induction of recombinational events by HN2 is enhanced in the diploid pso2/pso2 mutant (Saeki et al., 1983). Nonetheless, when the induction is expressed as a function of survival, it can be seen that cross-over events are less inducible if Pso2 is not present. The rate of spontaneous intra-chromosomal recombination between two inverted repeats in the haploid is not affected by loss of Pso2 (Barber et al., 2005).

In exponential phase, rad51 or rad52 haploid cells are sensitive to ICL-forming agents, including CDDP (Hannan et al., 1984; Abe et al., 1994; Durant et al., 1999; Beljanski et al., 2004), HN2 (McHugh et al., 2000; Beljanski et al., 2004) and 8-MOP (Henriques & Moustacchi, 1980b; de Morais et al., 1996; Grossmann et al., 2001), indicating that the contribution of HRR to ICL repair might be required in cells passing through S phase. This notion is strengthened by the fact that no sensitivity to 8-MOP (Henriques & Moustacchi, 1980b) or HN2 (McHugh et al., 2000) has been observed in these cells if they were treated in stationary phase, suggesting that there is an ICL repair pathway efficiently operating in stationary phase that is independent of HRR (Frankenberg-Schwager et al., 2005). Instead, this pathway likely relies on TLS (McHugh et al., 2000; Sarkar et al., 2006). In mammals, RAD51 is not necessary for the TMP ICL-induced repair DNA synthesis, which can be detected in the plasmid with the ICL and, unexpectedly, in the undamaged donor plasmid, too (Li et al., 1999). These are not nonspecific DNA polymerization reactions triggered by DNA lesions, as psoralen mono-adducts and DSB have no such effects. This indicates the involvement of HRR in the processing of ICL, even though the damage-free plasmid does not have to be homologous to the one bearing the ICL. In this regard, a special HRR mechanism called break-induced replication (BIR, see text below) requires only short regions of homology to commence strand invasion, but the
resulting D-loop is expanded via DNA synthesis. One form of the yeast BIR is independent of Rad51, but requires Rad1 (Malkova et al., 1996). Consistent with the potential involvement of BIR in ICL repair, the mammalian homologs of Rad1 and Rad10, but not the other endonuclease nor the NER recognition factors, are crucial for the TMP-induced repair synthesis (Li et al., 1999). Accordingly, defects in the S‘ NER endonuclease renders cells hypersensitive to CDDP (Cruil et al., 2003), but the other NER mutants show less sensitivity to ICL-forming agents, such as MMC (Hoy et al., 1985b) or the analogs of cyclophosphamide (Andersson et al., 1996). Nonetheless, NER may function in the second round of incisions removing the ICL, but this event is not reflected by survival. However, not all types of ICL require HRR to be repaired, because the absence of Rad52 causes wild-type-like viability in response to BCNU (Beljanski et al., 2004).

Sister chromatid DNA repair in mitotic cells involves participation of Rad54 (Arbel et al., 1999), the absence of which sensitizes cells to 8-MOP and HN2 (Henriques & Moustacchi, 1980b; McHugh et al., 2000). Cells depleted of Rad54 or Mre11 show similar sensitivity to HN2, and comparable defect in the rejoining of chromosome breaks as rad52 cells, arguing that SCE and also the Mre11-Rad50-Xrs2 (MRX) complex play a substantial role in ICL repair. MRX seems to act in an ICL repair pathway distinct from NHEJ, presumably HRR. The increase in sensitivity of exponential rad50 cells to 8-MOP is absent in nongrowing cells (Henriques & Moustacchi, 1980b), in agreement with the possible involvement of MRX in the SCE initiated by the ICL during DNA replication. Rad55 and Rad57 factors form a highly stable complex in yeast and are related to the RecA/Rad51 family of recombinases (reviewed in Dudás & Chovanec, 2004). Inactivation of the Rad55-Rad57 heterodimer severely impairs the repair and the replication of plasmids containing an HMT ICL (Safran et al., 2004) and sensitizes growing cells to 8-MOP (Henriques & Moustacchi, 1980b), CDDP and MMC (Wu et al., 2004b). Curiously, the rad55 mutant remains distinctly sensitive in stationary phase, whereas rad57 cells display wild-type-like response (Henriques & Moustacchi, 1980b), indicating some additional role of Rad55. In mammals, the counterpart of this factor is required for the formation of RAD51 foci upon CDDP treatment (Bishop et al., 1998; Wang et al., 2001b). Rad59, a homolog of Rad52, is necessary for Rad51-independent intra-chromosomal recombination and single-strand annealing (SSA) (Prado et al., 2003) and is also required for survival in the presence of CDDP adducts (Wu et al., 2004b).

HN2 was moderately effective in killing the cells depleted of Rad59, but had no impact on them in terms of DSB repair, suggesting that this factor is probably needed for a subset of HRR events not associated with ICL (McHugh et al., 2000).

Diploid yeast cells lacking various NHEJ components are not sensitive to CDDP or MMC (Wu et al., 2004b). Moreover, NHEJ does not appreciably enhance the cell survival after HN2 exposure, irrespective of the cell cycle phase (McHugh et al., 2000), suggesting that NHEJ may function only as a minor, back-up, system that probably acts on ICL-induced DSB if HRR is disabled. CDDP IaCL adducts block translocation of the mammalian KU proteins (Turchi et al., 2000; Kysela et al., 2003) and hinder the repair of DSB by NHEJ (Diggel et al., 2005). Therefore, the limited role of NHEJ in CDDP-treated cells (Belenkov et al., 2002; Cruil et al., 2003) may be explained by these facts, or it simply reflects the low frequency of DSB induced by this agent. There is some controversy about the involvement of the major components of mammalian NHEJ in processes combating the deleterious effects of HN2 (De Silva et al., 2000; Muller et al., 2000), but strong evidence for HRR influencing the sensitivity of mammalian cells to HN2, MMC and psoralen has been presented (Caldecott & Jeggo, 1991; De Silva et al., 2000; Panasci et al., 2002).

### The origin and repair of ICL-associated DNA double-strand breaks

DSB are not associated with the repair of ICL in bacteria, but have been universally identified in yeast and in higher eukaryotes treated with ICL-forming agents. The generation of DSB appears to be highly dependent on ICL induction, as DSB are clearly present in nongrowing cells (Henriques & Moustacchi, 1980b), in agreement with the possible involvement of MRX in the SCE initiated by the ICL during DNA replication. Rad55 and Rad57 factors form a highly stable complex in yeast and are related to the RecA/Rad51 family of recombinases (reviewed in Dudás & Chovanec, 2004). Inactivation of the Rad55-Rad57 heterodimer severely impairs the repair and the replication of plasmids containing an HMT ICL (Safran et al., 2004) and sensitizes growing cells to 8-MOP (Henriques & Moustacchi, 1980b), CDDP and MMC (Wu et al., 2004b). Curiously, the rad55 mutant remains distinctly sensitive in stationary phase, whereas rad57 cells display wild-type-like response (Henriques & Moustacchi, 1980b), indicating some additional role of Rad55. In mammals, the counterpart of this factor is required for the formation of RAD51 foci upon CDDP treatment (Bishop et al., 1998; Wang et al., 2001b). Rad59, a homolog of Rad52, is necessary for Rad51-independent intra-chromosomal recombination and single-strand annealing (SSA) (Prado et al., 2003) and is also required for survival in the presence of CDDP adducts (Wu et al., 2004b).

HN2 was moderately effective in killing the cells depleted of Rad59, but had no impact on them in terms of DSB repair, suggesting that this factor is probably needed for a subset of HRR events not associated with ICL (McHugh et al., 2000).
also applied to measure the DNA incision activity at ICL (Grossmann et al., 2000, 2001).

The generation of DSB reaches its maximum closely after the ICL treatment in yeast (Magana-Schwencke et al., 1982; McHugh et al., 2000). It is believed that DSB are not obligate intermediates of ICL repair but result when the replication forks are stalled at the unprocessed ICL or meet partially repaired (incised) ICL (Fig. 2a). This assumption is substantiated by the finding that no DSB are created in quiescent yeast after treatment with 8-MOP (Dardalhon & Averbeck, 1995), CDDP (Frankenberg-Schwager et al., 2005) or HN2 (McHugh et al., 2000). Similar observations were made in mammalian cells treated with HN2 or CDDP (De Silva et al., 2000). Mammalian cells also require replication to evoke DSB in the vicinity of HMT ICL (Akkari et al., 2000). ICL-associated DSB apparently differ from those created by ionizing radiation or restriction endonucleases, as they are repaired much more slowly (Niedernhofer et al., 2004). It is possible that SSB produced during ICL incision are transformed into DSB at replication. Alternatively or additionally, the DSB formed after ICL treatment may be a direct result of replication fork collapse, as already suggested. Such disintegration may leave a DNA molecule, in contrast to breaks generated by nucleases or ionizing radiation, with only a single available end (Fig. 2) and a duplex linked by an adduct affecting both strands. This perhaps explains why the pattern of recombination induced by the enzymatically created DSB is not dependent on NER, while it is modulated by NER factors during ICL repair (Saffran et al., 1994), despite the almost identical recombination profiles induced by ICL and DSB in wild-type yeast (Saffran et al., 1994). However, gene conversions downstream from the DNA damage are triggered much more effectively by a DSB than by a psoralen ICL (Saffran et al., 2004).

There is recent direct evidence for DSB generation by the inhibition of replication using plasmid DNA containing a single psoralen ICL in mammalian cell free extracts (Bessho, 2003). Furthermore, incubation of yeast cells in nongrowth medium increases their survival after CDDP treatment, even if HRR is blocked. This increase seems to be due to the

![Diagram of DNA replication-dependent fork breakage and DNA double-strand break](image-url)
processing of the ICL, possibly by NER and TLS, as considerably fewer DSB are created after this liquid holding recovery (Frankenberg-Schwager et al., 2005). No incisions at HMT ICL can be observed in mammalian cells held in G1 by starvation (Akkari et al., 2000). No DSB can be seen in G2/G1 arrested cells regardless of the ICL-forming agent (Niedernhofer et al., 2004; Rothfuss & Grompe, 2004) or in G2 after HMT treatment (Akkari et al., 2000). Most likely, therefore, the majority of DSB are created at replication forks colliding with ICL in vivo and they need not be formed in vitro (Guillonneau et al., 2004).

In a similar manner to yeast (McHugh et al., 2000), HN2-associated DSB are detectable in mammalian cells, in both NER-deficient and proficient cells (De Silva et al., 2000), confirming the NER-independent origin of these breaks. In contrast, there are reports that the generation of DSB after psoralen treatment requires functional NER and occurs within 15 min of the postincubation period (Jachymczyk et al., 1981; Magana-Schwencke et al., 1982; Dardalhon & Averbeck, 1995). Thus, it seems likely that incision reactions as well as impaired replication might be responsible for some psoralen-induced DSB. The 8-MOP ICL are uncoupled producing SSB and DSB in ps2 (Magana-Schwencke et al., 1982), ps4 (de Morais et al., 1996), rad6 (Dardalhon & Averbeck, 1995), rad50 (Dardalhon et al., 1998) and rad51 mutant yeast strains (Averbeck & Averbeck, 1998). The wild-type cells are able to restore most of the broken DNA within 24 h, but rejoining of the chromosomes after 8-MOP treatment is impaired to different extent in the above mutants. Psoralens also cause breakage of the chromosomes in mammalian cells (Rothfuss & Grompe, 2004). In yeast, DSB may be involved in CDDP-mediated cytotoxicity as well, although these are harder to detect, and are only induced by high doses of this agent. In fact, the detection of these breaks is possible only in cells defective in HRR, but not in wild-type cells (Frankenberg-Schwager et al., 2005), suggesting that they perhaps do not represent the preferred processing pathway but are induced when recombination processing is not available. After HN2 treatment, no rejoining occurs in cells lacking NER (Barber et al., 2005), but rad52 cells show residual repair, likely by NHEJ, whereas the Rad51-controlled pathway is responsible only for a subset of resealing events (McHugh et al., 2000). As it seems likely that the incisions preceding DSB formation may arise independently of NER, a search for the candidate endonucleases has been conducted. As cleavage of the structures produced at replication forks blocked by ICL can be responsible for the breaks, candidate factors involved in this process were investigated. Examples of how two structure-specific nucleases Rad1-Rad10 and Mus81-Mms4 might ‘see’ and cleave replication forks, based on the known biochemistry of these nucleases, are illustrated in Fig. 2b. However, the MMR, rad1, rad2, rad27, mus81, srs2, sgs1, top1, top2, top3 and mre11 mutants all exhibit normal DSB formation, so these do not individually seem to be the key factors or endonucleases involved (McHugh et al., 2000; P. McHugh, unpublished data). We consider it highly likely that there is significant redundancy in the apparatus processing forks stalled by severe DNA damage and therefore strains multiply disrupted for several of these activities will need to be analyzed to identify the factors involved.

The distribution of 8-MOP-induced DSB is influenced by local chromatin structure with preferential occurrence in the intergenic segments that contain promoters and terminators. The incidence of DSB in the 5’ section of an active gene is somewhat higher if cells are treated in stationary phase rather than in exponential phase. Modification of this DNA sequence by inverting the gene correlates with the local alteration of chromatin structure and affects the positions and frequency of DSB, which are presumably produced at preexisting sites in inter-nucleosomal DNA (Dardalhon et al., 1998).

**Base excision repair and mismatch repair**

Endogenous DNA lesions caused by base hydrolysis, attack by reactive oxygen species or endogenous metabolites and coenzymes may cause covalent alterations of DNA bases, which may have pro-mutagenic or cytotoxic effects. These lesions are eliminated by BER, commonly initiated by a lesion-specific DNA glycosylase that recognizes the altered base, pushes it into an extrahelical position and catalyzes cleavage of the base-sugar bond, leaving an abasic (AP) site. Subsequently, the AP site is recognized and incised by a separate AP endonuclease (Apn1, Apn2) or in some cases cleaved by an AP lyase activity associated with the glycosylase (e.g. Ntg1, Ntg2, Ogg1), producing SSB with 5’- or 3’-blocked termini, respectively. Apn1 and Apn2 by virtue of their 3’-phosphodiesterase activity are involved in the processing of 3’-blocked ends. Repair is finalized by gap-filling and ligation.

Cells lacking Apn1 and Ogg1 are not sensitive to HN2 (McHugh et al., 1999). Furthermore, these cells are normally resistant to CDDP and MMC, in a similar manner to ung1 (the corresponding gene encodes uracil DNA glycosylase), ntg1, ntg2, apn2 and mag1 cells (Wu et al., 2004b). Surprisingly, the sensitivity of mag1 cells to HN2 is comparable to that of HRR-deficient rad52 cells. On the other hand, mag1 cells are 10 times more resistant to the agent than rad4 cells, indicating that the less toxic mono-adducts can be substrates for BER. As the rad4 mag1 double mutant is as sensitive to HN2 as the rad4 single mutant (McHugh et al., 1999), NER and BER may have overlapping functions in repairing the corresponding lesions. It also appears likely that Mag1 might mitigate the effects of HN2 by removing mono-adducts that could go on to form cross-links from the
DNA, reducing the pool of cross-link precursor lesions. Strikingly, this double mutant, although unable to remove either mono-adducts or cross-links, demonstrates wild-type-like DSB induction (McHugh et al., 2000), indicating that ICL are the main cause of DSB generation. Deletion of the OGG1 gene did not alter the response of yeast cells to 8-MOP (Cohen et al., 2002), CDDP or MMC (Wu et al., 2004b). A strain severely impaired in BER (the nag1 nag2 apn1 triple mutant) shows wild-type-like survival after CDDP or HN2 exposure. Notably, inactivation of these genes does not affect the sensitivities of rad1, rev3 or rad52 strains to the drug, strengthening the notion that BER is not involved in the processing of ICL in yeast (Beljanski et al., 2004). In a similar manner to yeast, XRCC1 is not essential to deal with CDDP in mammalian cells (Crul et al., 2003).

MMR identifies and then removes the unpaired or mispaired bases from newly synthesized DNA. Mismatches primarily arise by flaws in replication or as an outcome of base modifications, or are created during recombination between homologous sequences. Hypothetically, MMR uses 5’ ends of Okazaki fragments and/or PCNA as strand discrimination signals to distinguish between template and nascent DNA. The MMR proteins are evolutionarily conserved and play a fundamental role in avoiding mutations, modulating control over meiotic cross-over and preventing the exchange of divergent DNA sequences. Furthermore, they may participate under certain conditions in DSB repair and in stabilizing stalled replication forks (recently reviewed in Iyer et al., 2006; Jiricny, 2006). The MutSx (Msh2-Msh6 heterodimer) is primarily needed for the repair of single mismatches, whereas MutSβ (Msh2-Msh3 heterodimer) preferentially participates in the repair of small DNA loops, sensing insertions and deletions (Sugawara et al., 2004). The specificity of these complexes is enhanced by MutLx (Mlh1-Pms1), which is essential for MMR, and by MutLβ (Mlh1-Mlh3) with ambiguous assignment. All of the mentioned complexes help to avoid frame-shift mutations (Marti et al., 2002; Bignami et al., 2003; Stojic et al., 2004).

It has been shown that human MUTSβ, but not MUTSα or MLH1, is involved in the recognition and uncoupling of psoralen ICL in mammalian cell extracts (Zhang et al., 2002). The direct binding of human MUTSβ to mismatched DNA (Clark et al., 2000) as well as to TMP ICL is stimulated by PCNA, but the initial processing of the ICL is different from the classic MMR. MMR-compromised yeast cells (msh2, msh6 and pms1 mutants) show no difference in survival after CDDP or HN2 treatment relative to the wild-type cells (Beljanski et al., 2004). Furthermore, these mutations do not strongly modify the sensitivity of rad1 cells, suggesting that MMR does not have a major role in ICL repair (Beljanski et al., 2004). However, the loss of the MSH2, MSH3, MSH6, MLH1 or MLH2 genes has been reported to mildly augment resistance to CDDP and carbo-platin, maybe as a consequence of stalling replication and/or withdrawal of MMR activity that interferes with other DNA repair pathways. The acquisition of CDDP resistance is likely not due to the mutator phenotype of MMR-deficient cells, as disruption of PMS1 causes an elevated mutation frequency but does not influence sensitivity. As inactivation of MSH2, MLH1 or MLH2 has no effect on survival in a rad52 or rad1 background, MMR might interfere with lesion tolerance during DNA replication by regulating the levels of recombinational bypass. Consistent with this, wild-type cells experience elevated resistance to CDDP during stationary phase, but sensitivity of the MMR mutants is not affected by growth phase (Durant et al., 1999). However, increased resistance may also be due to defective cell cycle responses. Moreover, MMR could remove the nucleotides inserted opposite the CDDP lesions by TLS, and the resulting futile cycles of such attempts at their repair may lead to cell death. Loss of lethal signals by corrupting human MUTSα or MUTL complexes, but not MUTSβ functions, also correlates with the increased replicative bypass of, and resistance to, CDDP adducts in mammalian cells (Vaisman et al., 1998). Note that these MMR effects on CDDP resistance might not reflect events at ICL, but could exclusively reflect the effects of IaCL, which form the bulk of the lesions. However, there are certainly reports of mammalian MMR mutants that are less resistant to MMC than the wild-type cells (Fiumicino et al., 2000). Maybe, the balance between DNA repair and apoptosis compensates for the effect of each other, as lack of human MUTSα does not affect sensitivity to BCNU, but represses apoptosis (Hickman & Samson, 1999). Human MUTSβ may also play a dual role in DNA repair and signal transduction (Zhang et al., 2002).

It has been shown that Psq2 has no role in MMR. Nonetheless, Msh2 (MutSx and MutSβ) in cooperation with Exo1 can partly compensate for the absence of Psq2 in the repair of HN2 ICL formed in S phase. MutLx and MutLβ complexes are not essential for this functional redundancy (Barber et al., 2005). Neither Msh2 nor Mlh1 are needed for the induction of extrachromosomal recombination between tandem repeats of a gene by psoralen ICL targeted into one gene copy via TFO. These lesions may possibly be eliminated by forming DSB with the help of NER and/or NER-independent incisions that surround the ICL and thus lead to deletions, while the intramolecular recombination reflects SSA followed by microhomology-dependent end-joining (Faruqi et al., 2000).

**Conclusions - several DNA repair pathways act in concert to eliminate ICL**

The existence of at least three/four systems participating in the repair of CDDP ICL and IaCL (‘Psq2’ pathway/NER, unpublished data) is a major clue towards understanding this complex process. It is also possible that IaCL lesions could be created by excision repair rather than by direct photoreactivation, as the UV sensitivity of Psq2 cells is increased (a similar finding was recently reported by Goksoyr et al., 2007). One of the remaining questions is the role of the oxidative damage repair competence in the repair of CDDP ICL. However, it is well established that there is a cross-talk between NER and MMR pathways and that repair of ICL is modulated by their interaction. Many DNA repair systems have a broader role than simply removing DNA lesions, as they are implicated in genome maintenance and cell cycle control. It is likely that ICL have a role in cell cycle checkpoint control, as supported by the loss of the ability of yeast to deal with CDDP in mammalian cells (Crul et al., 2004). In a similar manner to yeast, XRCC1 is not essential to deal with CDDP in mammalian cells (Crul et al., 2003).
HRR and TLS) is suggested by the epistatic analysis carried out in the respective yeast mutants (Grossmann et al., 2001; Li & Moses, 2003; Beljanski et al., 2004): each pathway is involved in cross-link repair, as a defect in any of them greatly reduces the survival. Supposedly, there are no additional systems acting on ICL, because theoretically one ICL per genome is lethal in the pso2 rad51 rev3 triple mutant, although evidence for the involvement of additional Rad6-Rad18-controlled pathways (e.g., the Rad5-Mms2-Ubc13 branch) is strong, and is absent from the aforementioned experiments. From the data mentioned in this review it is evident that TLS is required in G1, whereas PRR and HRR dominate in S and G2/M cells, for processing of 8-MOP and HN2 ICL. The epistasis of the NER genes and pso2 with rev3 only applies to G1 cells, and is lost if the ICL are created in growing or G2 phase cells (Sarkar et al., 2006). Detailed molecular evidence illustrates how such a pathway could operate in G1 cells, with thwarted postincision gap-filling being required to trigger TLS. As with replication-associated TLS, the covalent modification of PCNA by ubiquitin is absolutely key to these ICL repair transactions. The pathway which dominates outside of G1 and early S is likely, by analogy with E. coli, to take advantage of an undamaged sister chromatid to effect error-free repair. This could either involve postincision HRR, mediated by the Rad52 group of recombination factors, or PRR involving Rad6-Rad18 and the Rad5-Mms2-Ubc13 ‘error-free tolerance’ pathway. Furthermore, it certainly cannot be excluded that these pathways interact under certain circumstances. These cell cycle phase-specific models might not be completely applicable for CDDP-induced DNA damage, where the dominant IaCL adducts add complexity to the interpretation of results. However, the sensitivity profiles of strains blocked in HRR, NER or PRR, assessed in large scale studies using yeast deletion pools, are quite similar for CDDP and HN2 treatments, indicating, overall, a similar response to these DNA repair proteins and evolution of repair systems. Nucleic Acids Res 27: 1223–1242.


Acknowledgements

We apologize to all colleagues whose work has not been cited due to space limitations. Work in the MC laboratory is supported by the VEGA Grant Agency of the Slovak Republic (grants no. 2/3091/24 and 2/6082/26) and project 2003 SP 51 028 08/0028 08 01 from the national program Use of Cancer Genomics to Improve the Human Population Health. PMcI’s laboratory is supported by Cancer Research UK.

References


Fischhaber PL, Gall AS, Duncan JA & Hopkins PB (1999) Direct demonstration in synthetic oligonucleotides that N,N’-bis(2-chloroethyl)-nitosourea cross links N1 of deoxyguanosine to...
N3 of deoxyctydine on opposite strands of duplex DNA. *Cancer Res* **59**: 4363–4368.


Mechanism of DNA interstrand cross-link repair in yeast


Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC & Cheney SG (1998) The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and...


