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DNA INTERSTRAND CROSSLINK REPAIR IN MAMMALIAN CELLS: STEP BY STEP

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Abstract

Interstrand DNA crosslinks (ICLs) are formed by natural products of metabolism and by chemotherapeutic reagents. Work in *E. coli* identified a two cycle repair scheme involving incisions on one strand on either side of the ICL (unhooking) producing a gapped intermediate with the incised oligonucleotide attached to the intact strand. The gap is filled by recombinational repair or lesion bypass synthesis. The remaining monoadduct is then removed by Nucleotide Excision Repair (NER). Despite considerable effort, our understanding of each step in mammalian cells is still quite limited. In part this reflects the variety of crosslinking compounds, each with distinct structural features, used by different investigators. Also, multiple repair pathways are involved, variably operative during the cell cycle. G₁ phase repair requires functions from NER, although the mechanism of recognition has not been determined. Repair can be initiated by encounters with the transcriptional apparatus, or a replication fork. In the case of the latter, the reconstruction of a replication fork, stalled or broken by collision with an ICL, adds to the complexity of the repair process. The enzymology of unhooking, the identity of the lesion bypass polymerases required to fill the first repair gap, and the functions involved in the second repair cycle are all subjects of active inquiry. Here we will review current understanding of each step in ICL repair in mammalian cells.

Keywords

Chemotherapy; cisplatin; psoralen; unhooking; lesion bypass; replication arrest

INTRODUCTION

ICLs, DNA adducts that link both strands of the duplex, are among the most dangerous DNA lesions. They are obligate blockers of replication and transcription, and, unlike monoadducts, cannot be carried through a proliferative cycle without repair. If not removed they can provoke chromosomal breakage, rearrangements, or cell death (it has been estimated that 20–40 unrepaired ICLs can kill a mammalian cell (Dronkert and Kanaar, 2001; McHugh *et al.*, 2001; Lawley and Phillips, 1996). They have been categorized as “cytotoxic” lesions, and it has been argued that their accumulation over time contributes to genomic instability and aging in tissues and organs (Mitchell *et al.*, 2003). Hypersensitivity to crosslinking agents is a common feature of cells derived from individuals with genome instability disorders such as Fanconi Anemia (FA). Because of their heightened toxicity in proliferating cells, relative to monoadduct forming compounds, crosslinking agents have received extensive application as chemotherapeutic drugs (Vogel *et al.*, 1998; Lawley and Phillips, 1996). Thus, ICLs can be

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seen as both dangerous and useful, each view contributing to the increasing interest in delineating the pathways involved in their repair. Considering the molecular challenge of repairing lesions that engage both strands of the duplex it is understandable that multiple repair pathways are involved, and that repair is more complex than for monoadducts.

Excellent comprehensive reviews on crosslink repair have been published in recent years including those by Dronkert and Kanaar (Dronkert and Kanaar, 2001), Miller and colleagues (Noll *et al.*, 2006; Noll *et al.*, 2004), and Lehoczy, McHugh, and Chovanec (Lehoczy *et al.*, 2007). These authors have described the crosslinking agents employed in most studies, discussed the structure of DNA crosslinked by these compounds, and presented the state of knowledge of the repair pathways in the major experimental organisms: *E. coli*, yeast, and mammalian cells. More focused reviews have also been published on: the chemistry of DNA crosslinking compounds used as antitumor drugs (Rajski and Williams, 1998); the clinical significance of crosslink repair pathways (McHugh *et al.*, 2001)); repair in *E. coli* (Lage *et al.*, 2003); Fanconi Anemia and crosslink repair (Niedernhofer *et al.*, 2005; Andreassen and Ren, 2009; Thompson and Hinz, 2009; Moldovan and D'Andrea, 2009; Patel and Joenje, 2007; de Winter and Joenje, 2009); crosslink damage and aging (Grillari *et al.*, 2007); and crosslink repair and chromosome radial formation (McCabe *et al.*, 2009). In this review we will focus on the repair of ICLs in mammalian cells and discuss some of the major unresolved issues. We apologize to colleagues whose work could not be referenced due to space limitations.

There are several DNA repair pathways that respond to the multiplicity of lesions introduced in DNA by radiation and reactive chemicals. These include Nucleotide Excision Repair (NER) and Transcription Coupled Repair (TCR), Base Excision Repair (BER), MisMatch Repair (MMR), the two Double Strand Break (DSB) Repair pathways-Nonhomologous End Joining (NHEJ), and Homology Directed Repair (HDR), Single Strand Break (SSB) Repair, and Translesion Synthesis (TLS), which may be viewed as an adduct tolerance pathway. We have a relatively detailed knowledge of these pathways, the result of intense investigation over several decades, utilizing technologies based in genetics, biochemistry, biophysics, and immunology. In contrast, despite considerable effort, and steady inquiry during the same several decades, our understanding of crosslink repair is less advanced. This situation is due to several factors.

The structure of crosslinked DNA differs as a function of the chemistry of the crosslinking compound. Thus some ICLs are extremely distorting, others much less so. Furthermore, the extent of distortion can be dependent on sequence context. Since helical distortion is an important determinant of DNA repair, this structural variability can be a confounding variable in comparing results from experiments with different agents. Crosslinking agents do not produce only ICLs, indeed these are usually a minority product. This can muddle the interpretation of experiments which examine the response of cells to agents that produce multiple lesions, most of which are extraneous to the purpose of the experiment. One lesson that has emerged from crosslink repair studies in yeast is that functions from multiple repair pathways are engaged (Saffran *et al.*, 2004; Grossmann *et al.*, 2001; Lehoczy *et al.*, 2007). Indeed, all of the pathways listed above are thought to contribute to the repair of ICLs. Consequently, it can be difficult to distinguish the contribution to ICL repair by components of those pathways, from their response to the lesions (also present) that defined the pathway. In an effort to circumvent some of these problems a number of investigators have developed cell free systems (extracts, purified proteins) in which duplexes with defined ICLs have been introduced. This approach has a long and productive history in the DNA repair field, and has been very powerful in detailing the individual steps in the pathways listed above. However key, perhaps unknown, components may not be present in the mixtures, thus overweighting the role of some factors at the expense of others. Finally, it is clear that crosslink repair can

occur at any time of the cell cycle, but some pathways are operative only in certain phases of the cycle. These distinctions may be lost in experiments performed with cells in **asynchronous** culture, or extracts derived from those cultures.

INTERSTRAND DNA CROSSLINKING AGENTS

In this section we will discuss the most commonly employed experimental crosslinking compounds. As noted above these have been very capably reviewed by others, and we will not attempt to reproduce those efforts. Instead the focus will be on two features of these agents that bear on the interpretation of repair experiments: the multiplicity of adducts following the treatment of cells with these compounds; and the structural consequences for DNA carrying the crosslink. Most of the discussion will be on compounds that can be used to treat cells. However there has been recent work with synthetic substrates in which site specific ICLs have been introduced into duplex oligonucleotides. These are particularly useful for *in vitro* experiments and will also be described (Fig. 1a, b).

Nitrogen mustards

Nitrogen mustards are small bifunctional alkylating compounds that contain N,N-bis-(2-chloroethyl) amine as the defining component. They react with DNA usually at the N7 of guanine (Rajski and Williams, 1998), although reactions with adenine have been described (Balcome *et al.*, 2004). The resultant monoadduct can react with water to terminate the reaction, or there may be a second reaction with guanine (or adenine (Balcome *et al.*, 2004)) on the opposite strand to form an interstrand DNA crosslink. Reaction with cysteines in chromosomal proteins, producing protein DNA crosslinks is also possible (Thomas *et al.*, 1978; Loeber *et al.*, 2009). The DNA monoadduct is the dominant product (90–95%), while of the ICLs (~5%) the N7 to N7 guanine-guanine diadduct is the major component. The reactive guanines are the distal bases in a 5'GNC step (Millard *et al.*, 1990; Ojwang *et al.*, 1989). The minimal distance between the guanines is 8.9 Å, while the length of the diethyleneamine 5 atom tether is only 7.5 Å (Rink and Hopkins, 1995). The helix is necessarily distorted and has a helical axis bend of 12.4–16.8° (Fan and Gold, 1999; Rink and Hopkins, 1995). Structural analysis is compromised by the instability of N7 alkylated guanines which depurinate, eliminating the crosslink. This problem has been addressed in recent clever work from the Schärer group (Angelov *et al.*, 2009). They synthesized duplex oligonucleotides in which the crosslinked guanines were each replaced with 7-deazapurines with an aldehyde at C7. They used a post synthetic reductive double amination reaction linking the two guanines via an alkyl diamine from the aldehydes. The choice of an appropriate diamine linker allowed formation of a crosslink with a longer bridge than with a conventional nitrogen mustard, thus relieving the distortion associated with those adducts. The 7-deazapurines are more resistant to depurination than guanines, and therefore are stable substrates for subsequent experimentation.

Mitomycin C

Mitomycin C has received extensive use in the clinic as an anticancer chemotherapeutic and in the laboratory as a crosslinking agent. The compound requires reduction of its quinone ring (by oxidoreductases (Sartorelli *et al.*, 1994)) to form a quinone methide. This reacts with the N² exocyclic amine of guanine, yielding a monoadduct in the minor groove. Monoadducts form preferentially at 5'CpG sites, while crosslink formation has an absolute requirement for this sequence. This is the result of a specific hydrogen bond formed between the C10 oxygen atom of mitomycin and the 2-NH₂ of the guanine in the strand opposite the guanine engaged in the initial monoadduct reaction (Gargiulo *et al.*, 1995; Weidner *et al.*, 1990). In effect, the mitomycin molecule is positioned for the two reactions required for crosslink formation, before the first, to form the monoadduct, has taken place. This remarkable structural feature notwithstanding, ICLs are only one of six characterized adducts. In addition to the interstrand

crosslink (about 10% of products), there are four monoadducts, as well as the intrastrand crosslink between two adjacent guanines (Palom *et al.*, 2000). Furthermore, DNA reactive oxygen radicals are produced during the reduction (Pritsos and Sartorelli, 1986).

The mitomycin crosslink between guanines in the (C-G)•(G-C) sequence imposes minimal distortion on the duplex, with no bending (Norman *et al.*, 1990; Rink *et al.*, 1996). The G-mit-G linkage does not disrupt or distort the C:G and G:C base pairs involved in the crosslink. Molecular modeling suggests that the crosslinked guanines remain in parallel planes, thus stacking of adjacent bases is not perturbed. In contrast, the GpG intrastrand crosslink induces a 14.6° bend, imposing a kink in the DNA and a distortion of the adducted guanines (Rink *et al.*, 1996).

Cisplatin

Cisplatin (cis-Diamminedichloroplatinum, cis-DDP) (Rosenberg *et al.*, 1965; Rosenberg, 1977), has been widely used in chemotherapy with striking success against testicular cancer. The compound undergoes displacement of the chlorides by water to form the activated form, cis-[Pt(NH₃)₂(H₂O)₂]²⁺, which reacts with a range of cellular targets including RNA, protein, phospholipids, as well as DNA. The major DNA adducts are the 1,2 intrastrand crosslinks at GpG (65%) and ApG (25%) sites. ICLs (up to 8%) form at GpC sites linking the N7 of both guanines (Jamieson and Lippard, 1999; Malinge *et al.*, 1999). In contrast to the nitrogen mustard adducts, the N7-platinum dG adducts are no more likely to depurinate than the unmodified dG (Baik *et al.*, 2002). There is substantial distortion of both the intrastrand and interstrand crosslink (Poklar *et al.*, 1996; Malinge *et al.*, 1994; Malinge *et al.*, 1999). In particular, DNA with a GpC ICL is unwound by 80° with a 45° bend towards the minor groove (Malinge *et al.*, 1994). Important structural features are the flipping of the complementary cytosines into an extrahelical conformation, and the local change from B-DNA to the left handed Z-DNA (Huang *et al.*, 1995). The ICLs are not persistently stable but can convert spontaneously to intrastrand crosslinks with a half life of 120 hrs (Perez *et al.*, 1997).

Psoralens

Psoralens are tricyclic planar molecules with a furan ring fused to a coumarin. They are natural products and have been isolated from plant and fungal sources (see Cimino *et al.*, 1985 for an extensive review). Reaction with DNA requires photoactivation by exposure to 320–400 nm light, and this is the basis of the well known psoralen/UVA (PUVA) therapy for psoriasis, vitiligo and other skin disorders (Stern, 2007; Millington and Levell, 2007). The requirement for photoactivation permits temporal control of the reaction, distinguishing psoralens from the compounds described above.

Psoralens intercalate into DNA with a preference for 5'TA steps (Sinden and Hagerman, 1984) (Saenz-Méndez *et al.*, 2007) and runs of (TA) are hotspots for modification (Boyer *et al.*, 1988). Formation of an ICL proceeds via a two step, two photon reaction. Absorption of the first photon supports cycloaddition between the ethylenic bonds in either the furan or pyrone ring with the C₅–C₆ double bond of thymine to form furan or pyrone monoadducts. While the pyrone monoadduct is unable to react further, the furan monoadduct may absorb another photon and react with the thymine on the other strand forming a crosslink. The frequency of pyrone vs. furan monoadducts is influenced by the peripheral substituents on the psoralen nucleus. For example, 4,5',8 trimethyl psoralen (TMP), with a 4-CH₃, shows a pronounced bias towards furan addition (98%) (Kanne *et al.*, 1984), and thus can be converted in the second step to an ICL. In contrast, 8-methoxy psoralen (8-MOP) forms 20% pyrone side, non convertible, monoadducts. The distinction between the two compounds reflects the presence of the methyl on the C-4 position of psoralen, which imposes a steric constraint on formation of the TMP pyrone monoadduct. Photoactivation with conventional laboratory UVA lamps yields mixtures

of monoadducts and ICLs regardless of the psoralen derivative (Lai *et al.*, 2008). However, with C-4 methyl psoralens it is possible to drive the adduct distribution to almost exclusively ICLs using a high powered laser for photoactivation (Johnston *et al.*, 1977).

The structure of duplex oligonucleotides with various psoralen ICLs has been examined by gel migration, solution NMR, and X ray crystallography (Sinden and Hagerman, 1984; Haran and Crothers, 1988). NMR analysis of a duplex with an 4'-aminomethyl-4,5',8-trimethyl psoralen (AMT) crosslink revealed only minor bending ($<10^\circ$) of the duplex, with distortion of the central 4 base pairs (Hwang *et al.*, 1996). Another study of an oligonucleotide duplex crosslinked by HMT concluded that the helix was unwound by 28° , was not bent, and had a disruption of the DNA structure in the immediate vicinity of the crosslink that returned to normal within 3 base pairs. The authors suggested that the conformational flexibility of the sugar phosphate backbone in the region of the crosslink was a signal for recognition by the DNA repair apparatus (Spielmann *et al.*, 1995b; Spielmann *et al.*, 1995a).

X ray crystallography of HMT crosslinked duplexes demonstrated substantial distortion at the thymine linked to the pyrone ring, although there was no bending, and the structural perturbation was localized to the vicinity of the crosslink (Eichman *et al.*, 2001). In all these analyses the duplex oligonucleotides contained only two T:A base pairs, sufficient for crosslinking. The relatively greater stability of the G:C pairs in the remainder of the duplex could influence the conclusions regarding distortion induced by the crosslink. This point was considered in a structural analysis (by electrophoretic mobility) of psoralen crosslinked duplexes containing A:T and G:C pairs, as well as mismatches. The authors concluded that the degree of local distortion imposed by a psoralen crosslink would reflect the local sequence, with inherently less stable sequences subject to greater deformation (Kumaresan *et al.*, 1992).

In typical biological experiments crosslinking agents react throughout the cell. Thus, ICLs are introduced randomly wherever there is DNA, in both nucleus and mitochondria. However, with psoralens two strategies have been developed that offer greater control of crosslink location. Both technologies exploit the requirement for photoactivation, and the synthetic opportunities for conjugation of psoralens to other moieties, without loss of reactivity.

Targeted psoralen crosslink formation mediated by Triple Helix Forming Oligonucleotides

One approach is based on triple helix forming oligonucleotides (TFOs). The DNA triple helix consists of a third strand of nucleic acid that lies in the major groove of an intact duplex (Felsenfeld *et al.*, 1957). The most stable structures are formed on polypurine:polypyrimidine elements, which are over represented in the human genome (Manor *et al.*, 1988; Schroth and Ho, 1995; Behe, 1995). TFOs can be attached to psoralen via linker arms such that, following triplex formation, a crosslink can be introduced at a specific site by photoactivation (Giovannangeli *et al.*, 1992). This demonstration was followed by a series of publications from the Glazer lab that described mutagenesis and gene conversion induced by triplex targeted psoralen in shuttle vector plasmids (Havre *et al.*, 1993; Wang *et al.*, 1995; Raha *et al.*, 1996; Faruqi *et al.*, 1996) (for reviews see Seidman and Glazer, 2003; Chin *et al.*, 2007; Chin and Glazer, 2009). This plasmid based approach has been exploited in more recent work on the interaction of triplex –psoralen crosslinks with repair factors (Christensen *et al.*, 2008; Lange *et al.*, 2009). Bioactive TFOs that can introduce psoralen ICLs at specific chromosomal target sites in living cells, have also been developed (Majumdar *et al.*, 1998; Puri *et al.*, 2001; Puri *et al.*, 2002; Puri *et al.*, 2004; Seidman *et al.*, 2005; Majumdar *et al.*, 2003; Shahid *et al.*, 2006). These have been used to target mutagenesis and gene conversion in the genome (Majumdar *et al.*, 2003; Majumdar *et al.*, 2008).

Laser localized ICLs

Laser/confocal microscopy has had a major impact on DNA repair studies. Defined subnuclear regions in living cells can be treated with laser light of specific wavelength and intensity to introduce localized DNA damage. The lesions that have received attention are base oxidation products, which can be detected by specific antibodies (Lan *et al.*, 2004), and, of course, DSBs, for which there are surrogate protein markers such as phosphorylated histone H2AX (Rogakou *et al.*, 1999). Psoralens would seem to lend themselves to experiments employing localization by laser photoactivation. However, there have been no reagents available for detection of psoralen ICLs *in situ*. This limitation was overcome by synthesizing psoralen linked to an antigenic tag, digoxigenin. This sterol has received extensive use as an immunotag, and reliable commercial antibodies are available. The equivalent derivative of angelicin, a photoreactive analogue of psoralen, which can form only monoadducts, was also synthesized. Living cells, treated with the tagged compound, were exposed to laser photactivation in a defined region of the nucleus (Thazhathveetil *et al.*, 2007). The location of the ICLs or monoadducts was visualized by immunofluorescence following fixing and staining. Adduct removal could be monitored over time in repair proficient and deficient cells (Fig. 2). In order to distinguish phenomena due to psoralen ICLs, rather than monoadducts or ancillary damage from the laser, parallel experiments with digpsoralen and dig-angelicin were performed.

Synthetic substrates

In the preceding discussion we have considered crosslinking agents that can act directly, or after activation, on DNA *in vivo* and *in vitro*. DNA duplexes crosslinked by a variety of linkages have been constructed by chemical synthesis by a several investigators and have been the subject of biochemical and structural analysis. Incorporated into plasmids, they have been used as probes of repair and mutagenesis in living cells and extracts. Some of these ICLs will be mentioned briefly here.

N²-N² guanine ICLs formed by trimethylene (as a malondialdehyde and acrolein mimic), in either the 5'CpG or 5'GpC orientation, were synthesized by Harris and coworkers (Dooley *et al.*, 2001). The 5'CpG crosslink increased the thermal stability of the duplex, while NMR analyses and molecular modeling indicated that the DNA was minimally distorted (Dooley *et al.*, 2003). In contrast, the 5'GpC crosslink reduced the T_m value, and was distorting, introducing a bend and twist in the helix (Dooley *et al.*, 2003). Duplexes and plasmids with a closely related 5'CpG crosslink were also prepared by Hecht, Moriya and colleagues (Lao and Hecht, 2005; Liu *et al.*, 2006).

Miller and coworkers synthesized a group of ICLs differing in extent of duplex distortion (reviewed in Noll *et al.*, 2004). Three constructs reflect the different disposition of the N⁴C-ethyl-N⁴C linkage: a direct C-C mismatch, or staggered in the 5'CpG or 5'GpC orientation. Of these, the 5'GpC crosslink is the most distorting because the ethyl linker is not long enough to span the distance between the N⁴ amino groups of the cytosine, while in the 5'CpG orientation there is little effect on the helix (Noll *et al.*, 2005). The C-C “mispaired” crosslink causes bending toward the major groove by an angle of ~27°. There is perturbation of stacking of adjacent bases although this is localized and fully accommodated within three bases on either side of the lesion (Webba da *et al.*, 2002). This group also synthesized oligonucleotide duplexes containing an N3T-ethyl-N3T crosslink as a T-T mispair, somewhat analogous to the crosslinked C-C mispair. Structural analysis indicated that the ethyl crosslink can be accommodated in the duplex with little distortion and no hydrogen-bond disruptions (da Silva *et al.*, 2004). Thus the two “mispair” ICLs differed in extent of local distortion. Another important difference is the influence of the modifications on hydrogen bonding with complementary bases. The attachment of the ethyl linker to the N3 on the thymine interferes

with hydrogen bonding, while linkage of the ethyl group to the exocyclic amines of the cytosines does not preclude their participation in hydrogen bonding.

CROSSLINK REPAIR: THE BASIC MODEL

The problem of crosslink repair was addressed many years ago in experiments in *E. coli*. This work resulted in the well known “Cole” model which accounted for the sensitivity to crosslinking agents of strains deficient in NER and HR, and the virtual intolerance of strains with deficiencies in both pathways (Cole, 1973; Cole *et al.*, 1976). This model was further modified by work from other laboratories (Sladek *et al.*, 1989a; Sladek *et al.*, 1989b; Van *et al.*, 1986; Cheng *et al.*, 1991) (see Lage *et al.*, 2003; Vidal *et al.*, 2006 for an appraisal of the role of individual components of the *E. coli* NER apparatus).

In this scheme the NER proteins recognize a crosslink and then incise one strand on either side of the ICL. This produces an “unhooked” substrate with the excised fragment still attached to the non-incised strand by the crosslinking agent. A gap is introduced by the exonuclease activity of DNA pol I in the region adjacent to the 3' side of the crosslink. This is the substrate for a RecA mediated strand exchange with an undamaged homologous chromosome. The still crosslinked oligonucleotide incision product is forced out of the restored duplex, forming a large monoadduct, which can be repaired by conventional NER. In the absence of a functional recombination pathway, the gap may be filled by lesion bypass, by pol II in the case of a nitrogen mustard N7-N7 guanine crosslink (Berardini *et al.*, 1997; Berardini *et al.*, 1999), and pol IV with an acrolein N2-N2 guanine crosslink (Kumari *et al.*, 2008). These are the elements of a major two cycle repair pathway found in all organisms- recognition followed by unhooking, gap formation, then gap repair by recombination or lesion bypass synthesis (Fig. 3). This process converts the crosslink to a monoadduct, after which the second cycle of repair can occur, by, it is presumed, conventional NER. Although this scheme would seem to provide a general solution to crosslink repair, there are quite significant differences and variations in yeast and vertebrate cells.

CROSSLINK REPAIR IN MAMMALIAN CELLS

Although the process in mammalian cells is more complex, as in bacteria, the essential step is the unhooking event. This can be regarded as the central node of a multi entry flow chart. In this discussion we will follow the crosslink through the stages in this schematic (Fig. 4). We will first consider repair in the absence of replication, and then repair triggered by the encounter at a replication fork.

In contrast to the apparent situation in *E. coli* there are three routes for crosslink detection in mammalian cells. Adducts can be recognized in otherwise unperturbed duplex DNA, by factors that recognize DNA damage. Crosslink discovery might also be via encounter with the transcription machinery. Finally, ICLs could block a replication fork, triggering a repair response that would remove the crosslink and restore replication. Brief consideration of these scenarios points to the relevance of cell cycle status, with the latter pathway option available only in S phase. The influence of the cell cycle on crosslink repair has been emphasized in insightful work from McHugh and colleagues (Barber *et al.*, 2005; McHugh and Sarkar, 2006).

Crosslink recognition in the absence of replication

DNA lesions are recognized as distortions in the local structure of the duplex due to destabilization of base stacking, with an attendant increase in helical flexibility (Isaacs and Spielmann, 2004; Maillard *et al.*, 2007a; Maillard *et al.*, 2008; Yang, 2008). This appears to be the common denominator for recognition by the BER, NER, and MMR damage detection

proteins, although there are important structural distinctions that underlie the lesion specificity of each repair system. Thus, while MMR can recognize certain base adducts such as cisplatin and aminofluorene and acetylaminofluorene, NER does not recognize mismatched bases (see Isaacs and Spielmann, 2004; Yang, 2008 for discussion). Binding of damaged DNA by recognition proteins typically accentuates the distortion inherent to the lesion. Crystal structures of glycosylases bound to their substrates reveal nucleotide flipping, discontinuous base stacking, and DNA kinking as characteristic of these complexes (Fuxreiter *et al.*, 2002). DNA containing a single mismatched base pair is bent by about 45° on binding by the human MutSa complex (the heterodimer of Msh2:Msh6).

Entry into the global genome repair pathway of NER begins with adduct recognition by the XPC-HR23B-Centrin complex. Crystallographic analysis of a truncation product of the yeast XPC homolog, Rad4, bound to a duplex oligonucleotide containing a cyclopurine dimer indicates the DNA is highly kinked with a 42° bend. This is consistent with previous reports of bending of damaged DNA by XPC (Janicijevic *et al.*, 2003). The protein binds 11 consecutive base pairs of the adjacent undamaged sequence, making contacts with the phosphodiester and ribose group. At the damage site the protein inserts a β -hairpin through the DNA duplex, forcing both the intrastrand crosslinked bases, and the undamaged complementary bases, out of the helix. The flipped out bases of the undamaged strand are bound by the protein, while the adducted nucleotides are disordered and are not engaged by the protein (Min and Pavletich, 2007). The binding of XPC protein to undamaged single stranded DNA, rather than to the actual lesion is the basis of the well known versatility of damage recognition by the XPC complex (Sugasawa *et al.*, 2002; Sugawara and Hanaoka, 2007). The interaction of XPC with both the adjacent undamaged duplex and the region distorted by the lesion has been emphasized by Naegeli and colleagues, who have developed a bipartite binding model of damage recognition and binding (Maillard *et al.*, 2007b; Maillard *et al.*, 2008; Camenisch *et al.*, 2009).

The other early recognition factor associated with NER, the DDB1-DDB2 complex, binds to UV lesions and a variety of other forms of DNA damage. The current view is that DDB1-DDB2 assists the recruitment of XPC to DNA damage that might otherwise be overlooked by XPC (Fitch *et al.*, 2003; Wang *et al.*, 2004). DDB1-DDB2 exists *in vivo* as a member of a CUL4-E3 ubiquitin ligase. This complex polyubiquitinates XPC, DDB2 and CUL4 (Li *et al.*, 2006) as a key step in what has been termed a lesion “hand off” transfer from DDB1-DDB2 to XPC (Sugasawa *et al.*, 2005). The crystal structures of DDB1-DDB2 bound to a 6–4 UV photoproduct shows that DDB2 makes contacts with the damaged DNA, inserting a three residue hairpin into the duplex from the minor groove. This widens the minor groove, unwinds the DNA in the vicinity of the adduct, flips the adducted bases out of the helix, and kinks the DNA by about 40° (Scrima *et al.*, 2008).

While such substantial distortions of DNA would face a serious energy barrier in undamaged DNA, the damage eases the deformation of the DNA by the recognition proteins, reducing the energetic cost of complex formation (Isaacs and Spielmann, 2004; Fuxreiter *et al.*, 2002; Min and Pavletich, 2007). Thus, recognition by XPC and subsequent repair, of lesions that introduce greater distortion and reduce the T_m value of the adducted duplex, is more efficient than that of lesions that introduce little or no distortion (Geacintov *et al.*, 2002). The sequence context can influence the structural consequences of DNA adducts and modulate the efficiency of recognition and repair (Kropachev *et al.*, 2009) (Cai *et al.*, 2009).

So how are ICLs, in the absence of transcription or replication, recognized? The work on monoadduct recognition emphasizes the importance of helical distortion-local denaturation, disruption of base stacking, etc. However, while ICLs may perturb the duplex (see above), affected bases obviously cannot flip out of the helix. Furthermore, crosslinks, with some

exceptions (Noronha *et al.*, 2002; Dooley *et al.*, 2003), typically stabilize duplexes. Assuming helical distortions are central to the process, it would seem likely that perturbation of the helix adjacent to the crosslinked bases would be the attractant for recognition proteins. This interpretation would merge current thinking about adduct recognition with the special challenge presented by crosslinks. To pursue this reasoning we might then ask: which recognition proteins are involved in the early stages of repair of ICLs, and is there the expected correspondence between efficiency of recognition and extent of ICL induced distortion? As will become apparent, there is very little direct information on this point and what is available is not always in accord with expectation.

The role of damage recognition factors in ICL repair can be considered experimentally in several different ways. A traditional approach is to measure the effect on survival of cells with deficiencies in genes involved in lesion recognition following treatment with crosslinking compounds. Typically these experiments employ unsynchronized cultures and take a few days for an endpoint. Cells vary in the time they spend in each phase of the cell cycle, and this can influence the outcome and confuse comparisons of results with different cells. For example, the popular DT40 chicken cells divide rapidly, and asynchronous populations have only 15% of the cells in G₁ phase, reducing their utility for studies of genes involved in repair in the absence of replication (Nojima *et al.*, 2005). The results of survival assays are a summation of the capacity of the multiple crosslink repair pathways to protect cells from the killing effects of ICLs, as well as the monoadducts also introduced by the compounds. The influence of a defect in a gene involved in one pathway can be masked by other pathways. This point was emphasized in a thoughtful study in yeast from the Doetsch group which showed that multiple repair pathways were involved in resistance to crosslinking agents. They found different contributions of individual pathways to the response to the different compounds, indicating that conclusions based on one compound were not necessarily applicable to another (Beljanski *et al.*, 2004).

An alternative approach is to construct plasmids with a defined crosslink at a specific site, and introduce these into repair proficient or deficient cells. The readout of these assays may require transcription (expression of a reporter gene) or replication (plasmid yield, mutagenesis). Plasmid constructs or duplex oligonucleotides have also been incubated in cell free extracts in which incision can be directly monitored. These experiments report the activity of the functions that survive preparation, and are silent on those that do not. Each assay system has strengths and weaknesses, and contradictory results are not uncommon. In the following section we will discuss the role of damage recognition proteins in the response to crosslinking agents, as reported by these different assays.

XPC

In yeast genetic evidence indicates a requirement for **Rad4 (the XPC equivalent)** during ICL repair (Wu *et al.*, 2004), including G₁ phase repair (Sarkar *et al.*, 2006). In mammalian cells, although it has been known for many years that NER functions are involved in crosslink repair (Kaye *et al.*, 1980; Hoy *et al.*, 1985a), there has been relatively little focus on XPC. XPC deficient cells treated with cisplatin were no more sensitive than wild type cells, while cells deficient in the transcriptional coupled repair factors CSA and CSB, and the post recognition NER factors, XPA, XPD, XPF, and XPG were sensitive (Furuta *et al.*, 2002; McKay *et al.*, 2001). These results implied that the XPC recognition function was not important for cell survival after cisplatin treatment, although this assay cannot identify the toxic adduct(s) (see De Silva *et al.*, 2002 for a discussion of this issue). The response of the NER incision apparatus to a cisplatin crosslinked duplex oligonucleotide was examined in a cell free extract system. While incisions were observed with an intrastrand crosslink, none were detected with the interstrand crosslink (Zamble *et al.*, 1996). Furthermore, the “futile cycle” incisions (below),

seen with psoralen crosslinked substrates (Mu *et al.*, 2000) were not observed. These results, although clearly limited, suggest that the XPC complex does not recognize cisplatin ICLs despite the strong distortion imposed by this adduct. In contrast, RPA binds cisplatin ICLs with greater affinity than the intrastrand crosslink (Patrick *et al.*, 2008), although the biological significance of this observation is unclear.

The involvement of XPC in the repair of psoralen ICLs has been addressed in relatively few experiments. In recent work, a modest sensitivity to TMP/UVA was shown by cells with a deficiency in XPC. The XPC protein was recruited rapidly to sites of laser localized TMP ICLs in wild type G₁ phase cells, while the psoralen ICLs were not removed in XPC deficient G₁ phase cells (Muniandy *et al.*, 2009). Host cell reactivation experiments with a reporter plasmid carrying psoralen ICLs demonstrated a major contribution of XPC to the repair process (Chen *et al.*, 2003). Legerski, Li, and colleagues constructed plasmids containing a single psoralen crosslink placed between a promoter and a reporter gene. The plasmids were introduced into wild type and repair deficient cells and the expression of the reporter gene measured as a reflection of repair competence. They found that NER factors were required, although the influence of the deficiency of XPC was not as pronounced as that of the other NER functions (XPA, ERCC1 etc.). This result was consistent with roles for both transcription coupled as well as global genome repair of the psoralen ICL (Wang *et al.*, 2001). In an analogous experiment the repair of a plasmid carrying a defined mitomycin crosslink was examined in repair proficient and deficient cells. Interestingly, with this construct the decline in repair due to the XPC deficiency was dramatic and equivalent to that in the other NER mutant cells. Repair was also reduced in cells with mutations in the CSA or CSB protein (involved in TCR), although not as strongly as in the XPC deficient cells (Zheng *et al.*, 2003). The authors called attention to the involvement of a TCR pathway (see below). However their data also indicated role for XPC, which is not a component of TCR.

A comparison of these results from different experimental systems suggests that the requirement for XPC was at variance with expectation. There was no apparent involvement in the repair of ICLs formed by cisplatin, the most distorting lesion, while there was a clear requirement with the MMC crosslink, the least distorting. In the instances where XPC is involved in recognition it is not clear what structural features of an ICL are essential for binding. Although the XPC complex can bind a triplex-psoralen crosslink (Thoma *et al.*, 2005), it is not known how the complex would respond to psoralen crosslinks without the conjugation to the triplex oligonucleotide. Nor is it known whether crosslinks formed by other compounds would be bound by the XPC complex, or how these results would correlate to the data from biological experiments.

XPE

XPE deficient cells are not sensitive to psoralen (Muniandy *et al.*, 2009). Binding of DNA with different lesions by the XPE binding factor (as it was then known) was reported by (Payne and Chu, 1994). No binding to 8-MOP ICLs or monoadducts was observed (see also Reardon *et al.*, 1993). Furthermore, XPE deficient cells are proficient in crosslink unhooking activity (Bredberg and Soderhall, 1985; Muniandy *et al.*, 2009). DDB2 was recruited slowly to laser localized psoralen ICLs, in contrast to the rapid accumulation of XPC (Muniandy *et al.*, 2009). It seems that the DDB1-DDB2 complex is not involved in recognition of psoralen ICLs.

MMR

The role of mismatch repair functions in the repair of ICLs has yet to be resolved. Cells deficient in MSH2, the common component of the MutS α (MSH2-MSH6) and MutS β (MSH2-MSH3) mismatch recognition complexes) are sensitive to MMC (Fiumicino *et al.*, 2000) and psoralen (Wu *et al.*, 2005). Recently the Lippard group demonstrated binding of an oligonucleotide

duplex with a cisplatin interstrand crosslink by MutS β , as well as PARP-1, DNA Ligase III, XRCC1, and Ku80, Ku70 (Zhu and Lippard, 2009). The Legerski group showed PCNA stimulated binding of a TMP crosslinked duplex oligonucleotide by the MutS β complex. Incision of the crosslinked substrate in cell free extracts was dependent on MSH2, as well as ERCC1, and XPF (see below), suggesting activity of the MutS β complex in the absence of replication (Zhang *et al.*, 2002). These results contrast with those from experiments in yeast in which a role for mismatch repair factors was assigned to a post unhooking step in S phase (Barber *et al.*, 2005). In the experiments with laser localized psoralen ICLs in G₁ phase cells the removal of the psoralen adducts was unaffected by deficiency in MSH2. Furthermore, recruitment of MSH3 was dependent on functional XPC, suggesting that it entered the repair pathway after recognition by XPC (Muniandy *et al.*, 2009). The linkage between mismatch repair and replication raises the possibility that the involvement of MMR functions in ICL repair might be coupled to repair synthesis, rather than recognition.

Other recognition candidates

Binding of psoralen crosslinked DNA by a complex of human α Spectrin II and the Fanconi Anemia proteins FANCA, FANCC, and FANCG has been shown by the Lambert group (McMahon *et al.*, 2001). The key member of this complex is α Spectrin II which bound the crosslinked DNA with specificity as compared to non crosslinked DNA, but could not distinguish monoadducted DNA and undamaged DNA. Cells treated with siRNA against α Spectrin II showed an increase in chromosome abnormalities, and were modestly more sensitive to MMC than control cultures (McMahon *et al.*, 2009). However, the biological significance of crosslink binding by α Spectrin II has yet to be established.

It should be apparent from this overview of crosslink recognition that there is not a consensus on the role of recognition proteins in crosslink repair. The multiplicity of assay systems coupled with the disparate crosslinking agents has resulted in considerable uncertainty. It is quite possible that the mode of recognition could vary as a function of the structural characteristics of crosslinks formed by different compounds. Systematic experiments to address this possibility have not been performed.

Transcription coupled repair

The experiments showing impaired reactivation of crosslinked reporter plasmids in cells with defects in TCR (Zheng *et al.*, 2003) were in agreement with earlier work from the Hanawalt laboratory, which first demonstrated TCR of psoralen ICLs (Vos and Hanawalt, 1987; Islas *et al.*, 1994). As mentioned above, the genetics of cisplatin ICL repair are consistent with an involvement of TCR (Furuta *et al.*, 2002; McKay *et al.*, 2001). Furthermore, an analysis of mutations induced by MMC or psoralen in the plasmid reporter systems indicated a strong bias for the non transcribed strand, reflecting preferential incision in the transcribed strand (Zheng *et al.*, 2003; Wang *et al.*, 2001). Earlier experiments in yeast also argued for crosslink repair by TCR (Meniel *et al.*, 1995a; Meniel *et al.*, 1995b). However, while the results with plasmids are in accord with TCR of ICLs, there is an inherent bias in the design of the experiments. Typically, the plasmids express a reporter gene and the lesion is placed between the (invariably strong) promoter and translation start site. Transcription, required for TCR, is also required for the endpoint of the assay. Thus, only those molecules that are transcribed can contribute to the readout, and they also become candidates for TCR. Repair by non TCR pathways, of plasmids that do not become transcription templates, is not reported in these assays.

After recognition, in the absence of replication

It is generally assumed that, following recognition, NER functions are involved in further processing, much as with monoadducts. As mentioned above, cells with deficiencies in NER functions are not markedly sensitive to crosslinking agents such as nitrogen mustard (De Silva

et al., 2000), although there may be some variation in sensitivity as a function of the compound (Kaye *et al.*, 1980). A clearer indication of a role for NER activities comes from the experiments with crosslinked reporter plasmids transfected into NER proficient and deficient cells. As discussed in the preceding paragraph the results of experiments with mitomycin or psoralen crosslinked plasmids demonstrated a role for NER and TCR functions in supporting expression of the reporter gene. In both sets of experiments sharp declines in reporter activity were seen with host cells with deficiencies in XPA, XPD, and XPB (Wang *et al.*, 2001; Zheng *et al.*, 2003). An implication of results with cells vs. plasmids is that the crosslinked plasmids are repaired by NER dependent pathways, while genomic ICLs have access to additional, NER independent, pathways.

Post recognition: unhooking

The key event in crosslink repair is the unhooking step, and thus the identification of the critical factors is clearly important. Given the requirement for the NER pathways in bacteria and yeast the roles of the incision nucleases of the mammalian NER pathway, XPG (3' of the adduct) and ERCC1-XPF (5'), are of particular interest. Cells with deficiencies in XPF or ERCC1 are extremely sensitive to crosslinking agents, while those with defective XPG, like cells with defects in the other NER genes, generally show modest sensitivity (but see below) (Hoy *et al.*, 1985b; Hoy *et al.*, 1985a; Collins, 1993; Damia *et al.*, 1996; De Silva *et al.*, 2000; Clingen *et al.*, 2007).

Unhooking in living cells can be monitored by the well known alkaline “comet” assay. The assay provides a measure of the electrophoretic behavior of DNA from single cells embedded in agarose (Collins, 2004). DNA in cells is organized in large supercoiled loops. Strand breaks relax the DNA, which can be unwound in an alkaline environment. The relaxed and unwound DNA will migrate towards the anode in an electrophoretic field. Although breaks can be introduced deliberately by radiation or other treatments, there are sufficient spontaneous breaks such that alkaline electrophoresis of untreated cells reveals a “head” (compact DNA in the vestige of the nucleus) and a “tail” (the more rapidly migrating nicked and relaxed material). This has the appearance of a comet. The pattern from cells that have been exposed to crosslinking agents is markedly different in that there is no tail, as the DNA is bound by the crosslinks. Unhooking of the ICLs restores the tail, and this serves as an assay of cellular competence to perform this step. While the assay does require care and attention to detail to perform reproducibly, it has several major advantages over other whole cell assays. The most important is that monoadducts do not interfere with detection of ICLs (Wu *et al.*, 2009). Consequently it is possible to measure the response of cells to ICLs, regardless of the monoadducts formed by the crosslinking agent. Unhooking in wild type cells may take a few hours (with judicious dosing), and so it is feasible to perform experiments in which synchronized cells are treated and monitored in a particular phase of the cell cycle. Note that if an agent also forms or induces appreciable levels of single or double strand breaks this can compromise the analysis of crosslink repair (De Silva *et al.*, 2000). Thus, if cells become apoptotic as a result of treatment, the interpretation of comet tail recovery can be confounded (Kumaresan *et al.*, 2007).

Unhooking of ICLs formed by nitrogen mustard (Murray and Meyn, 1986) and psoralen (Rothfuss and Grompe, 2004) in G₁ phase synchronized cells has been reported. In the psoralen experiments Rothfuss and Grompe made the interesting observation that the process in synchronized G₁ phase cells was substantially faster than in asynchronous cells. Unhooking in psoralen/UVA treated G₁ phase cells deficient in XPD was greatly reduced relative to wild type cells (Richards *et al.*, 2005) indicating a requirement for NER activities in G₁. However, most studies of the influence of repair gene deficiency on comet tail recovery have used asynchronous cultures. Deficiencies in XPG, and XPB did not alter the kinetics of unhooking

in cells treated with nitrogen mustard relative to wild type cells (De Silva *et al.*, 2000). Similarly, non synchronized human cells deficient in XPG also showed wild type kinetics of unhooking following treatment with psoralen/UVA (Rothfuss and Grompe, 2004). Cells with mutant XPF or ERCC1 genes were unable to unhook nitrogen mustard ICLs (De Silva *et al.*, 2000). Thus unhooking of nitrogen mustard and psoralen ICLs was clearly dependent on XPF/ERCC1. The other NER functions, including XPG, were not required, insofar as asynchronous cultures were concerned. Rather different conclusions were reached in experiments with cisplatin ICLs. XPF/ERCC1 deficient cells were much more sensitive than those with defects in XPB, XPD, and XPG, but all were equally impaired in unhooking (De Silva *et al.*, 2002). The authors suggested that the cisplatin crosslink was not the lesion critical to the survival assay, since inability to unhook was not directly matched by the impact on survival. This conclusion emphasizes the problems of elucidating the dynamics of crosslink repair with compounds that produce ICLs as minor products, while the more abundant single strand adducts can be highly toxic.

Incision of crosslinked model substrates has been examined in cell free extracts and in incubations with purified proteins. In a well known experiment, linear duplexes, or a plasmid, with a psoralen crosslink were incubated in human cell free extracts. The activities in the extract incised the substrates at two sites, both 5' of the crosslink, releasing a 22–28 base long fragment, without removing the ICL. In effect the repair reaction had occurred to one side of the crosslink. The reaction was also performed with purified NER factors, XPA, RPA, TFIIH, XPC, XPG, ERCC1-XPF. These were necessary and sufficient for the dual incisions adjacent to the crosslink (Bessho *et al.*, 1997). In a follow-up study the same group found that the resultant gap was filled in by what was termed “futile repair synthesis” (Mu *et al.*, 2000). Adjacent dual incision products were also recovered in recent work from the Miller laboratory in which duplexes with ICLs of differing degrees of distortion were incubated in cell free extracts (Smeaton *et al.*, 2008). The appearance of the dual incision fragments was a function of the distortion of the ICL and dependent on NER functions. In light of the discussion above, it would seem that the results from the two labs reflect an effort by the repair apparatus to repair the distortion adjacent to the ICL, rather than removing the cause of the distortion. What remains to be determined is whether this is a peculiarity of the *in vitro* conditions, perhaps missing some key factor(s), or an accurate representation of events that occur, at least some of the time, *in vivo*.

A different endpoint was measured in another cell free extract system by Legerski and colleagues. They found that DNA synthesis on both a psoralen crosslinked plasmid and undamaged plasmid in the same extract was dependent on the ICL and ERCC1-XPF, and the recombinational repair genes XRCC2 and XRCC3. Synthesis was not affected by the absence of XPA, XPC, and XPG in the extracts. The author suggested that synthesis was the result of Break Induced Replication (BIR) (Malkova *et al.*, 1996). This would be stimulated by incision of the crosslinked plasmid by the ERCC1-XPF complex followed by strand transfer to the undamaged plasmid and extension by DNA synthesis. The authors used this assay to identify a requirement for MutS β in the recognition and unhooking of psoralen ICLs (Zhang *et al.*, 2002). Neither XPC nor XPG were necessary for incision. RPA was required for both incision and DNA synthesis, while PCNA was essential only for synthesis, although stimulatory for incision (Li *et al.*, 2000; Zhang *et al.*, 2003). An involvement of additional factors was indicated as the result of fractionation of the extracts. A complex containing the Pso4 pre-mRNA splicing factor, and the Werner Syndrome (WS) helicase was found to stimulate ICL induced repair synthesis. This was supported by a reduced reactivation of crosslinked expression reporter plasmids in cells with siRNA knockdown of members of the Pso4 complex (Zhang *et al.*, 2005). Extracts from WS cell line were inactive in the extract repair synthesis assay. As noted by the authors of this report, Pso4 is bound to the nuclear matrix, perhaps reflecting earlier

indications that DNA repair factories are associated with the nuclear matrix (Koehler and Hanawalt, 1996).

Unhooking of psoralen crosslinked linear duplexes has been studied in extracts of chromatin associated proteins (Kumaresan *et al.*, 1995; Kumaresan *et al.*, 2007). The 3' and 5' incisions were 9 nucleotides apart, and were dependent on XPF. These incisions were reduced in extracts from cells deficient in FANCA (Kumaresan and Lambert, 2000), or in extracts in which antibodies against FANCA were added (Kumaresan *et al.*, 2007). Similar results were obtained with extracts from cells with defects in other Fanconi proteins, including FANCB, C, D2, F, and G. Suppression was generally not complete, with the 3' incision more resistant to the defect in Fanconi protein. It should be noted that a role for Fanconi proteins in unhooking is controversial. There are reports indicating partial (Papadopoulou *et al.*, 1987; Averbek *et al.*, 1988) (Matsumoto *et al.*, 1989; Li *et al.*, 1999), or no (Poll *et al.*, 1984; Pichierri *et al.*, 2002; Zhang *et al.*, 2002; Rothfuss and Grompe, 2004) requirement, with some variability depending on complementation group.

Unhooking in cell extracts has also been characterized by Smeaton *et al.* (Smeaton *et al.*, 2008). They prepared duplex oligonucleotides with different ICLs that varied in the degree of distortion imposed on the DNA. As mentioned above they observed the NER dependent dual 5' incisions described in earlier work by Bessho *et al.* (Bessho *et al.*, 1997). However, remarkably, they demonstrated NER independent unhooking of the crosslinked substrates, also stimulated by crosslink induced distortion. This unhooking reaction was unaffected by the absence of ERCC1-XPF.

Although the work of Smeaton *et al.* suggests that there may be an alternative to ERCC1-XPF, most current models assume a fundamental contribution of this complex to unhooking (but see below (Bergstrahl and Sekelsky, 2008)). This view is based in part on the *in vivo* data, but also on a very influential publication by the Wood group, who showed that a complex of the purified proteins could unhook a psoralen crosslinked oligonucleotide in the vicinity of a fork (Kuraoka *et al.*, 2000). The complex was not active on an intact duplex with a crosslink, an interesting contrast to the incision activities in various cell extracts discussed above (Mu *et al.*, 2000; Kumaresan *et al.*, 2002; Smeaton *et al.*, 2008). The dependence on a forked structure prompted speculation that unhooking of ICLs at stalled replication forks could occur in an ERCC1-XPF dependent, but otherwise NER independent, fashion. The incision product was a single strand crosslinked to 4 nucleotides. Similar results were obtained in a later study (Fisher *et al.*, 2008). Recent work shows that ERCC1-XPF does not function alone. Instead it is found associated with the SLX4 protein, which acts as a scaffold for this complex as well as two other structure specific nucleases, MUS81-EME1 and SLX1. The SLX4 association enhances the activity of the nucleases, XPF, MUS81, SLX1, in these complexes (Munoz *et al.*, 2009; Fekairi *et al.*, 2009; Andersen *et al.*, 2009). Cells treated with siRNA to knock down SLX4 expression are sensitive to crosslinking agents, although the sensitivity is not as profound as that seen in the XPF or ERCC1 deficient cells. On the other hand the knock down cells are not sensitive to UV light arguing that SLX4 is involved in the transactions of ERCC1-XPF outside of classical NER. However, ERCC1-XPF is involved in recombination (Sargent *et al.*, 1997; Adair *et al.*, 2000; Niedernhofer *et al.*, 2001), double strand break repair (Ahmad *et al.*, 2008), as well as adduct repair. Thus, whether SLX4 plays a role in unhooking, or other pathways associated with crosslink repair (reconstruction of blocked replication forks, DSB repair, etc.) remains to be determined.

Based on their biochemical studies the Sancar group made the interesting proposal that ERCC1-XPF in combination with RPA could act as a 3'-5' exonuclease on a linear duplex containing a crosslink. The product of this reaction was one strand of the original duplex attached to a single thymine via the psoralen crosslink (Mu *et al.*, 2000). Digestion of one of the strands past

the crosslink would unhook the crosslink and generate a stretch of single stranded DNA. It seems reasonable to consider that this could be done by other nucleases, and could be an alternative to incision based unhooking.

Recognition by replication fork encounter

The demonstration of unhooking by ERCC1-XPF of crosslinked forked substrates by the Wood group solidified the concept of crosslink recognition by replication fork collision. This recognition pathway and its implications have been the subject of much discussion since then. An aspect of this research has been the shift from an experimental concern for repair/removal of ICLs as chemical entities, to a focus on the resolution of the consequences of the encounter-stalled and collapsed replication forks. This reflects the strong general interest in recombinational repair, which is essential for efficient and faithful replication and protects against genomic rearrangements that may have clinical consequences (Thompson and Schild, 2002; Thompson and Hinz, 2009). It is also well established that cells with defects in recombination repair functions are sensitive to crosslinking agents, and mutations in some of these genes are linked to cancer (Liu *et al.*, 1998; Sasaki *et al.*, 2004; De Silva *et al.*, 2000). Double strand breaks induced by crosslinking compounds figure prominently in most of these studies. DSB formation can be monitored by pulsed field electrophoresis, and, of course, by the appearance of γ H2AX foci (Rogakou *et al.*, 1999), which has been widely used as a surrogate marker. However, it appears that there are other inducers of γ H2AX foci, such as single strand DNA at repair foci and chromatin swelling (Clingen *et al.*, 2008; Baure *et al.*, 2009; Marti *et al.*, 2006). Consequently, other measurements of DSB formation are important to avoid misinterpretation of γ H2AX foci.

In the following section we will discuss experiments intended to address crosslink repair in the context of a replication fork. As discussed above, while experiments may be designed with this as the explicit focus, other pathways of discovery and repair are functional throughout the cell cycle (Barber *et al.*, 2005). Thus results of experiments in cells synchronized in S phase may reflect the activity of multiple repair pathways.

Grompe and colleagues showed that synchronized cells treated with psoralen/UVA required passage through S phase to elicit chromosome breakage or cell cycle delay. They proposed that replication fork arrest at a crosslink triggers cellular responses. They further argued that at least the initial steps of crosslink recognition and repair occurred exclusively in S phase (Akkari *et al.*, 2000) (but see below). A similar proposal was made by McHugh, Hartley and colleagues, who called attention to the DSBs generated only during S phase in cells treated with crosslinkers. They developed a model in which double strand break formation at a crosslink stalled fork was followed by unhooking by ERCC1-XPF, and recombinational repair of the resultant gap (De Silva *et al.*, 2000; McHugh *et al.*, 2001). This model accounted for the double strand breaks found in S phase cells treated with crosslinkers and, reminiscent of the Cole model, suggested that recombination was a pathway for gap repair, as well as fork reconstruction. Of course with models that invoke HDR, there is a need for homologous sequences that would span the crosslink site. This could not come from the daughter duplexes at the fork, since neither could replicate through the crosslink. Instead gap repair by recombination might be expected in G₂ phase with one sister chromatid supplying information to repair the other. However, when this issue was considered in synchronized yeast cells it was found that recombinational repair was not a major contributor to crosslink repair in G₂ phase (Barber *et al.*, 2005).

Double strand breaks were provoked by nitrogen mustard and cisplatin in replicating, but not stationary phase, yeast cells (McHugh *et al.*, 2000). Similar observations were made in CHO cells treated with nitrogen mustard (De Silva *et al.*, 2000). Two scenarios could account for this. In one report (Rothfuss and Grompe, 2004) the authors suggested that unhooking could

occur in G₁ phase cells. In the ensuing S phase the encounter of replication forks with unrepaired nicks and gaps generated by unhooking would produce single sided double strand breaks. This process would not require a specific nuclease for production of the DSBs, which would occur well after unhooking. The alternative view, very much in current vogue, is that there is a repair pathway in S phase, initiated by collision of a crosslink with a fork. Support for this comes from experiments with crosslinked replicating plasmids (Liu *et al.*, 2006) and actively dividing cells (Niedernhofer *et al.*, 2004). Cleavage by a nuclease at a fork, of a template strand upstream of the crosslink, would release one arm of the replication fork. This would generate a DSB, and a substrate that would require a second incision to complete unhooking (Kuraoka *et al.*, 2000; De Silva *et al.*, 2000; Bessho, 2003).

The enzymology of DSB formation has been considered in several studies. Although one report described a requirement for ERCC1-XPF for γ H2AX focus formation in response to psoralen/UVA (Mogi and Oh, 2006), other investigators, using electrophoretic techniques, found that ERCC1-XPF was not necessary for nitrogen mustard or MMC induced DSB formation (De Silva *et al.*, 2000; Niedernhofer *et al.*, 2004). Instead it has been proposed that the Mus81-Eme1 nuclease, a structure specific endonuclease with a preference for replication fork like structures, is required. This was shown in experiments with wild type and Mus81 deficient cells treated with for 24–30 hrs with MMC (Hanada *et al.*, 2006). The cells accumulated in S phase and the appearance of double strand breaks was dependent on Mus81-Eme1. This was in accord with proposals that this nuclease is involved in converting replication blocking structures to DSBs, which can then enter a recombinational repair pathway to restore the replication fork (Hanada *et al.*, 2007). It is noteworthy that when cells were treated for 1 hr with MMC, breaks appeared without requirement for Mus81-Eme1 (Dendouga *et al.*, 2005; Hanada *et al.*, 2006). Following exposure to MMC Rad51 foci appeared in both wild type and Mus81 knock out cells. In wild type cells the foci declined over time while they persisted in the mutant cells. This argued that Mus81-Eme1 was involved in resolving a late step in the recombination pathways that repaired crosslink induced DSBs (Dendouga *et al.*, 2005). It was suggested that the discrepancy between the results of experiments with long and short exposure to MMC was reflection of the small fraction of ICLs in comparison to monoadducts, such that the monoadducts were the actual agents of replication fork block and DSB formation in the short exposure experiments (Hanada *et al.*, 2006). The longer exposure would allow greater crosslink accumulation which would then block replication forks, now requiring Mus81-Eme1 for cleavage. This explanation requires a qualitative difference between forks blocked by monoadducts and ICLs, and overlooks the continued production of monoadducts during the longer exposure. The recovery of conflicting results from two versions of an experiment with the same crosslinking agent, which produces several other adducts, is an excellent example of the challenge facing investigators in the field. It would be of interest to address the role of Mus81-Eme1 using psoralen/UVA as the damaging agent, as the production of adducts is immediate to the light exposure.

Mus81-Eme1 recruitment at a stalled fork requires SNM1B, one of three mammalian orthologues of the SNM1 gene in *S. cerevisiae*. SNM1B deficient cells are sensitive to crosslinking agents such as MMC and cisplatin (Demuth *et al.*, 2004), but not UV (Bae *et al.*, 2008). SNM1B interacts with the MRN complex, indirectly with FANCD2, and is required for activation of ATM and Chk2 after MMC treatment. SNM1B binds Mus81 *in vitro*, and is necessary for the production of DSBs in cells treated with MMC (Bae *et al.*, 2008), **and these authors have** proposed that SNM1B is essential for the conversion of a crosslink stalled fork to a broken fork via cleavage by Mus81-Eme1.

Although not required for DSB formation, and thus no longer seen as the exclusive agent of unhooking during fork related repair, ERCC1-XPF is required for the resolution of the breaks as monitored by the decline in γ H2AX foci (Niedernhofer *et al.*, 2004). Results from a plasmid

based assay support this conclusion. A DSB, adjacent to a crosslink, stimulated repair via a homology dependent pathway. Repair was dependent on ERCC1-XPF, Rev3, and components of the FA and MMR pathways (Zhang *et al.*, 2007). Data from these experiments support a model in which the template strand for leading strand synthesis, at a crosslink stalled fork, is cleaved by Mus81-Eme1. The cleavage, in response to a stalled fork rather than the crosslink as such, would be the first of the two incisions required for unhooking. This generates a single sided DSB (the leading side daughter duplex) and a still crosslinked parental duplex with gap adjacent to the crosslink. This would be the substrate for incision on the 5' side of the crosslink by ERCC1-XPF (Niedernhofer *et al.*, 2004). Although not a feature of the original models, presumably this could also be engaged by an exonuclease(s) that could digest one strand of the parental duplex through the crosslink, leaving a single base crosslinked to the other strand (Mu *et al.*, 2000). Extended exonuclease digestion would generate the single strand patches that have been shown to appear following exposure of cells to crosslinking agents such as MMC (Lee *et al.*, 2006).

A contrary role for ERCC1-XPF was advanced by Bergstralh and Sekelsky. They argued that this complex was not involved in unhooking associated with replicative repair. Instead they proposed that unhooking at a blocked replication fork was dependent on Mus81-Eme1 and MutS β /MutL (Bergstralh and Sekelsky, 2008). In their proposal the requirement for ERCC1-XPF would be after the actual repair of the crosslink, during the reconstruction of the fork by recombination. This scenario would remove ERCC1-XPF from a direct involvement in formation of the repair gap, the filling of which by TLS would generate base substitution mutations. However, in experiments with a triplex targeted crosslink it was found that the ERCC1-XPF complex was required for the appearance of point mutations at the crosslink site (Richards *et al.*, 2005). This observation would appear to support the more conventional view of ERCC1-XPF as essential for unhooking.

The Fanconi Anemia pathway

Aside from the question of the enzymology of incision, crosslink discovery by replication has received considerable elaboration in the last few years particularly as events at the blocked fork are concerned. In effect, the models for crosslink discovery and unhooking at replication forks have become embedded in larger schemes that address the cellular response to stalled forks. How blocked forks are broken and resolved has been the subject of recent excellent reviews and primary publications, and is beyond the scope of this article (Collis *et al.*, 2008; Youds *et al.*, 2009; Thompson and Hinz, 2009) (Osman and Whitby, 2007; Budzowska and Kanaar, 2009; Sun *et al.*, 2008). The FA proteins play prominent roles in multiple steps in the process. FANCM and FAAP24 are thought to recognize blocked forks and, via fork reversal, enable access to a cavalcade of enzymes and proteins that repair the damage (Gari *et al.*, 2008a; Gari *et al.*, 2008b). The activity of the core complex ((A-C, E-G, L, FAAP100) is required for the central event of the FA pathway, the monoubiquitination of FANCD2 and FANCI. Core complex proteins are additionally important for lesion bypass synthesis (Mirchandani *et al.*, 2008). FANCD2 and FANCI, in concert with the chromatin remodeler Tip60 (Hejna *et al.*, 2008), are involved in the reconstruction of the broken fork via HDR which also engages FANCD1/BRCA2 and FANCN/PALB2 (see (Thompson and Hinz, 2009; Moldovan and D'Andrea, 2009)).

A linkage between the activity of ERCC1-XPF and the activation and localization of FANCD2 has been suggested by recent work from Moses, Grompe and their colleagues (McCabe *et al.*, 2008). They reported a decrease in monoubiquitination of, and focus formation by, FANCD2 in ERCC1 deficient cells after MMC or hydroxyurea treatment. They proposed that double strand break formation (provoked by both compounds) was the critical event that triggered the FANCD2 response, and that this required the activity of ERCC1-XPF. This

question has also been addressed by McHugh and associates. Although they found that MMC induced monoubiquitination of FANCD2 was not affected by ERCC1 deficiency, they did find reduced focus formation and a reduction in chromatin associated FANCD2 (Bhagwat *et al.*, 2009). They suggested that unhooking by ERCC1-XPF was required for stable chromatin association of FANCD2, and subsequent double strand break repair. It should be noted that these results describe FANCD2 recruitment after the action of ERCC1-XPF, in contrast to models in which FANCD2 accumulation at stalled forks precedes ERCC1-XPF (Thompson and Hinz, 2009).

This focus on double strand breaks, subsequent to a stalled fork, as the inducer of the Fanconi pathway is consistent with work showing activation of FANCD2 in response to agents that introduce breaks independent of replication (Nakanishi *et al.*, 2002; Roques *et al.*, 2009). The possibility that a blocked, but unbroken, fork is also an inducer cannot be considered with most compounds in general use- aphidicolin, hydroxyurea, MMC etc.,- because they induce double strand breaks. However, FANCD2 is monoubiquitinated and appears in foci in cellssynchronized in S phase by double thymidine block, which does not provoke double strand breaks (Bolderson *et al.*, 2004; Taniguchi *et al.*, 2002). This would imply multiple entry points for FANCD2- at stalled forks, as well as the breaks that can form at stalled forks-and could reconcile differences in current models of fork reconstruction.

Role of recombinational repair

There has been a shift in the recent models away from positing recombinational pathways as involved in both repair of the incision gap and the replication fork. The current view is that gap filling is accomplished exclusively by lesion bypass polymerases. Recombinational repair to rebuild the fork proceeds only after both cycles of repair of the crosslink adducts (Niedzwiedz *et al.*, 2004; Patel and Joenje, 2007) (Thompson and Hinz, 2009; Niedernhofer *et al.*, 2005; Mirchandani and D'Andrea, 2006). The models account for the sensitivity of HDR deficient cells to crosslinking agents by placing these functions downstream of the unhooking/gap repair events, and do not invoke them as engaged directly in these processes (Sasaki *et al.*, 2004; Clingen *et al.*, 2008) (although there may be an exception in the instance of unhooking of cisplatin in which XRCC3, a Rad51 paralogue, has been implicated (De Silva *et al.*, 2002)). Thus, for example, in experiments with a replicating crosslinked plasmid, unhooked, but incompletely repaired, intermediates accumulated in cell extracts derived from BRCA2 deficient cells (Cipak *et al.*, 2006). Cell extracts prepared from BRCA2 complemented cells were able to complete the repair process. It should be noted that all the models describe events at a single replication fork as it encounters a crosslink.

The double fork collision model

An alternative view has been proposed by Walter and colleagues who examined replication and repair in a *Xenopus* egg extract of a plasmid with ICLs formed by either the non distorting 7-deaza-guanine nitrogen mustard analogue from the Schärer group (Angelov *et al.*, 2009) or by cisplatin. In this system the NER pathway appears to be inactive. However the plasmid is replicated bi-directionally and both forks collide with the crosslink. They followed the progress of replication and length of the daughter strands by ³²P- α -dATP incorporation. The daughter strands stopped 20 (cisplatin) or 24 (nitrogen mustard) bases from the crosslink. The authors suggested that the DNA around the cisplatin crosslink was more easily unwound, and thus the fork could come somewhat closer. Eventually the leading strands advanced to within 1 nucleotide of the crosslink. This was followed by unhooking by unidentified nucleases, creating a structure in which translesion synthesis occurred via extension of the nascent leading strand past the crosslinked base. The collision of the forks with the crosslink induced Chk1 phosphorylation activating the ATR signaling pathway, and ubiquitination of FANCD2. The authors of this report called attention to two features of their model that distinguished it from

earlier proposals. The first is the collision of two replication forks instead of one. The second is the use of a nascent leading strand as the primer for lesion bypass synthesis, rather than the parental strand, as in the single fork models. In this scenario the unhooking incisions yield the equivalent of a double sided double strand break. This is the classic substrate for repair by NHEJ, and a failure to keep the broken ends sequestered could result in joining with deletion. Furthermore, although the FA pathway was activated, their model does not invoke a fork reversal step, one of the functions proposed for FANCM (Gari *et al.*, 2008a).

After unhooking: processing and gap filling

The intermediate unhooked by incisions is likely to require further processing prior to gap synthesis and the start of the second repair cycle that removes the resultant monoadduct. These events must occur regardless of cell cycle status. Unhooking of the psoralen crosslinked substrate by purified ERCC1-XPF released a 4 base oligonucleotide crosslinked to the unhooked strand (Kuraoka *et al.*, 2000). In the NER independent unhooking reactions described by the Miller group the incised oligonucleotide varied from 4 to 11 bases depending on the chemistry of the crosslink (Smeaton *et al.*, 2008). The structural chemistry of the crosslink (extent of distortion) can reduce the stability of the hybrid of the incised oligonucleotide and the non incised strand, or it can enhance the stability, relative to the non crosslinked hybrid (Noronha *et al.*, 2002). Consequently there may be a processing step required for elaboration of a repair “gap” on the incised strand. This could require a helicase. Unwinding of the unhooked product in G₁ phase cells would presumably rely on the NER helicases. In the context of a blocked or broken replication fork and NER independent repair, one candidate for this putative activity would be the FANCD1 helicase (Peng *et al.*, 2007; Gupta *et al.*, 2007; Wu and Brosh, Jr., 2009; Thompson and Hinz, 2009). FANCD1 deficient cells are sensitive to crosslinking agents, and it has the right polarity, 5'-3', to allow it to load on the uncleaved parental strand and unwind, at least to the crosslinked base. Experiments in *C. elegans* indicated that the FANCD1 equivalent protein was not required for FANCD2 ubiquitination, but did not address a more specific role (Youds *et al.*, 2008).

Digestion of the oligonucleotide to a crosslinked di- or mono-nucleotide is probably necessary to enable repair synthesis by lesion bypass polymerases and PCNA. A candidate nuclease, Pso2 (Snm1) (Li *et al.*, 2005), was identified in experiments in yeast as important in S phase repair, although it has been assigned a role in G₁ phase as well (Barber *et al.*, 2005; McHugh and Sarkar, 2006). The human SNM1 protein is a 5'-exonuclease, specific for single stranded DNA (Hejna *et al.*, 2007). It is a member of a family that includes, among others, SNM1B/Apollo, involved in replication fork collapse ((Bae *et al.*, 2008), see above), and SNM1C/Artemis, involved in NHEJ and V(D)J recombination (Ma *et al.*, 2002). Mouse cells deficient in SNM1 were sensitive to MMC (Dronkert *et al.*, 2000), as were human cells in which SNM1 expression was suppressed by siRNA (Hemphill *et al.*, 2008). Unlike yeast, the mouse SNM1^{-/-} ES cells are not sensitive to other crosslinking agents, such as cisplatin, psoralen, or nitrogen mustard (Dronkert *et al.*, 2000). This may reflect the relatively non distorting nature of MMC ICLs, perhaps suggesting that alternative functions process the other, more distorting adducts, but a definitive explanation has not been established. Curiously, SNM1A has been shown to partially rescue yeast pso2/snm1 mutants exposed to nitrogen mustard and cisplatin (Hazrati *et al.*, 2008). Unfortunately MMC was not employed in those experiments. Digestion of the incised oligonucleotide in the 3'-5' direction would also be expected to enhance lesion bypass synthesis (Minko *et al.*, 2008). This function might be supplied by the ERCC1-XPF complex as discussed above, other nucleases, or it has also been suggested that polδ could provide this activity (Nick McElhinny *et al.*, 2006).

Repair synthesis to fill the gap produced by unhooking and subsequent processing is an essential step that is required to complete the first repair cycle and restore the duplex. Since

synthesis past an adducted base is unavoidable, there is a requirement for recruitment of error prone lesion bypass polymerases. These polymerases, many in the Y family, have been the subject of intense investigation since their discovery, and have been described in recent reviews (Yang and Woodgate, 2007; Budzowska and Kanaar, 2009; Broyde *et al.*, 2008; Lehmann, 2006). Understandably, most work with these polymerases has been with monoadducts, which, if unrepaired, pose a problem for replication fork progression during S phase. The Lehmann group described the switch from replicative polymerases to the translesion polymerases via mono-ubiquitination of the replication processivity factor PCNA (Kannouche and Lehmann, 2004). All Y family TLS polymerases bind the mono-ubiquitinated form of PCNA, and different polymerases are specific for different forms of DNA damage (Lehmann *et al.*, 2007). The signal for this modification is a stalled replication fork (Kannouche *et al.*, 2003). However, crosslink repair in any cell cycle phase requires translesion bypass synthesis, and PCNA monoubiquitination in G₁ phase obviously cannot be triggered by a stalled replication fork. This issue was addressed in experiments in yeast by Sarkar *et al.*, who showed rapid mono-ubiquitination of PCNA in G₁ phase yeast cells treated with crosslinking agents, but not UV (Sarkar *et al.*, 2006). PCNA modification required a functional pol32, a component of the replicative polymerase pol δ , which is also involved in repair. PCNA mono-ubiquitination was not dependent on Rev3, a component of pol ζ . Thus it appears that the encounter by pol δ of the monoadduct product of crosslink unhooking is the signal for PCNA mono-ubiquitination and the recruitment of TLS polymerases. These would then complete synthesis of the repair gap.

While accurate bypass of UV induced thymine dimers can be accomplished by pol η alone, many lesions require the action of two polymerases: one to insert a base across from the site of the lesion, and pol ζ to extend that insertion (Johnson *et al.*, 2000). Recent elegant work with a plasmid model showed that bypass of benzopyrene or cisplatin-GG (intrastrand) adducts required a variable combination of two polymerases. Depending on the adduct, pol κ or pol η were responsible for insertion of correct or mutating bases while pol ζ was required for extension (Shachar *et al.*, 2009). These studies were with classical monoadducts and the equivalent studies have not been performed on monoadducts formed by unhooking of ICLs of different compounds. However some information about the relevant polymerases is available from disparate experimental systems.

DT40 cells deficient in RAD18, which regulates TLS as a ubiquitin E3 ligase for PCNA, and Rev3, the catalytic subunit of pol ζ , were very sensitive to nitrogen mustard, MMC, and cisplatin (Nojima *et al.*, 2005). Rev1, is a deoxycytidyl transferase that interacts with other Y family polymerases and with Rev7, a subunit of pol ζ (Guo *et al.*, 2003; Masuda *et al.*, 2003; Akagi *et al.*, 2009). Cells deficient in Rev1 show increased chromosome breakage and radial formation after treatment with MMC, as compared to controls (Mirchandani *et al.*, 2008). Interestingly, chromosome radials, which are the fusion of broken sister chromatids of disparate chromosomes, are a hallmark of FA cells treated with crosslinking agents. A direct connection between Rev1 and the FA pathway is suggested by the reduction in Rev1 focus formation after DNA damage in FANCG deficient cells (Mirchandani *et al.*, 2008)(see below).

A role for pol η was shown in experiments with both psoralen and MMC ICLs. Early work demonstrated the sensitivity of cells from an XP variant donor (deficient in pol η) to psoralen/UVA (Misra and Vos, 1993). Plasmids carrying a triple helix targeted psoralen crosslink showed an elevated frequency and altered mutation pattern after passage through cells deficient in pol η (Raha *et al.*, 1996). The Legerski group showed a substantial reduction in reactivation of an expression plasmid with a defined mitomycin crosslink after transfection into pol η deficient cells (Zheng *et al.*, 2003). Psoralen/UVA treated Pol η deficient cells show an enhanced production of γ H2AX foci, suggesting that this polymerase contributes to repair of crosslinks and reduces the probability that they will encounter a fork, resulting in a DSB (Mogi *et al.*, 2008). While this observation is consistent with that scenario, as noted above and by the

authors of the report, there are inducers of γ H2AX foci other than DSBs, and they may be activated in the absence of pol η .

Bypass in model systems has been examined with substrates that mimic the product of unhooking. Minko *et al.* found that plasmids carrying an G-G interstrand acrolein crosslink (similar to that formed by MMC) were replicated in mammalian cells with very low levels of mutagenesis, suggesting that most bypass events were error free (Minko *et al.*, 2008). Human pol κ could synthesize past a substrate designed to mimic the unhooked acrolein, incorporating dCTP across from the adducted G. The most efficient bypass was obtained with a substrate with a dinucleotide attached via the crosslink to the template strand. The authors concluded that pol κ could accurately bypass the G-N²-N²-G crosslink. In contrast, bypass by the combination of Rev1 and pol ζ was inefficient. Knockdown of pol κ in cultured human cells followed by treatment with MMC resulted in an enhanced frequency of radial chromosomes, again reminiscent of the phenotype of FA cells.

Replication bypass of model unhooked substrates has been examined from another point of view in the work of Smeaton *et al.* (Smeaton *et al.*, 2009). They prepared oligonucleotide substrates with a base crosslinked to another base. However, the chemistry of the crosslink was such that in some constructs hydrogen bonding was obstructed (N3T-ethyl-N3T), while in others it was not (N4C-ethyl-N4C). Bypass of the non obstructing lesion in mammalian cell extracts was efficient and error free. Bypass was not detected with the obstructing substrates. These data emphasize the relationship between the chemical structure of the crosslink and the efficiency of lesion bypass synthesis. Furthermore, they suggest that, depending on the structure of the crosslink remnant, ICL repair could be blocked even after unhooking.

Analysis of the dynamics of lesion bypass in the context of crosslink repair is a relatively recent development, and not all candidate polymerases have been considered experimentally. For example, *Drosophila* cells deficient in the polymerases encoded by the *mus308* gene are sensitive to crosslinking agents (Harris *et al.*, 1996; Oshige *et al.*, 1999). There are two mammalian homologues, polN and polQ(θ) (Seki *et al.*, 2003; Marini *et al.*, 2003; Takata *et al.*, 2006; Seki and Wood, 2008). While a recent study indicates a role for polN (Zietlow *et al.*, 2009), the participation of polQ(θ) in gapfilling during crosslink repair has not been addressed. Given the different structures of the unhooked crosslinks formed by the compounds discussed here it is likely that there will be some variety in the combinations of bypass polymerases involved in completion of the first repair cycle. The mechanistic role of the FA proteins in bypass is very much an open question. Current models place FA functions at blocked replication forks, and their contribution to lesion bypass and pol ζ dependent mutagenesis as an S phase function. However, bypass must occur during G₁ and G₂ phase repair. Whether there are differences in the functions involved in gap filling in the context of a blocked fork or in the absence of replication remains to be determined.

Another issue that has been raised in recent work is the extent of repair synthesis. In classical NER the repair gap is about 30 nucleotides. However, the Gautier group, working with a plasmid with a single ICL in a *Xenopus* egg extract, observed synthesis corresponding to about 300 nucleotides (Ben-Yehoyada *et al.*, 2009). Whether this reflects fill-in of an extended repair gap (which would be preceded by the exposure of a similarly sized single strand region), or “nick translation” that would replace intact sequence was not determined.

Crosslink mutagenesis

Bypass of an adduct can have mutagenic consequences. It is well established that pol ζ is required for monoadduct mutagenesis, as explained by the two step/two polymerase model of lesion bypass (Johnson *et al.*, 2000; Lawrence *et al.*, 2000). Similarly, it plays a role in the mutagenesis of ICLs. Thus, the frequency of base substitutions at the site of a triplex targeted

crosslink was markedly reduced in cells in which the expression of pol ζ was suppressed (Richards *et al.*, 2005). Furthermore, mutagenesis of crosslinked plasmids declined in Rev1 and Rev3 mutant host cells (Shen *et al.*, 2006). There is a connection between components of the FA pathway and mutagenesis of both monoadducts and ICLs (Niedzwiedz *et al.*, 2004; Thompson *et al.*, 2005). It has been known for some time that FA cells are hypomutagenic in response to psoralen (Papadopoulo *et al.*, 1990a; Papadopoulo *et al.*, 1990b; Hinz *et al.*, 2006). Recent work shows that mutagenesis requires the function of the FA core complex, but not FANCD2 (Mirchandani *et al.*, 2008). What the molecular interaction between the FA proteins and mutagenic bypass is not clear. As noted above the FA core complex makes a contribution to Rev1 focus formation, but interactions between FA core complex members and Rev1 have not been detected (Mirchandani *et al.*, 2008).

Although many details of lesion bypass during crosslink repair remain obscure, the pattern of mutagenesis can be informative. The “diagonal” nature of crosslinking agents such as psoralen makes it possible to deduce, from the mutation spectra, the identity of the incised strand and the strand carrying the crosslinked base during lesion bypass synthesis. One might imagine that if unhooking occurred randomly such that there was an equal probability that either strand could be incised, then bypass synthesis would also occur on either strand at a particular crosslink site averaged over a population of cells. Thus it would be expected that mutations at both of the bases in a crosslink would be recovered. However, in several studies the pattern of mutations induced by psoralen showed a remarkable asymmetry (Sage *et al.*, 1993; Laquerbe *et al.*, 1995; Yang *et al.*, 1994; Chiou and Yang, 1995; Sage *et al.*, 1993). The pattern was consistent with preferential incision of the transcribed strand, such that the unhooked crosslink would be on the non-transcribed strand during bypass synthesis. The identical asymmetry in base substitution was recovered in experiments with a triplex targeted crosslink at a genomic site (Richards *et al.*, 2005). With one exception (Sage *et al.*, 1993) these reports described experiments with HPRT as the mutation marker gene. There is an origin of replication located in the region of the HPRT promoter (Cohen *et al.*, 2002), and so the direction of transcription and the leading strand of replication would be the same. Thus, the mutation pattern is consistent with initiation of repair by the encounter of ICLs with the transcriptional apparatus or a single replication fork. The data are not consistent with the pattern of mutations-at both positions of the crosslink- that would be expected if two forks had collided on opposite sides of the same crosslink (Raschle *et al.*, 2008). However it should be noted that the data set is quite limited, in that mutation patterns in only two genes have been analyzed. It is reasonable to expect that there will be examples of the dual fork model operative at the level of the genome, and that the mutation pattern will be indicative of that pathway.

The second repair cycle

The completion of repair synthesis restores the duplex and forces the crosslinked base, linked to the unhooked strand, out of the helix. This is the substrate for the second cycle of repair. Recent evidence suggests that the unhooked extra helical psoralen crosslink is recognized by the XPE complex. Recruitment of DDB2 to laser localized ICLs occurred much later than XPC protein, and was dependent on XPC and repair synthesis (Muniandy *et al.*, 2009). These data support a scenario in which entry into an NER dependent pathway in G₁ phase cells converts a psoralen ICL, which is not recognized by the XPE complex (Payne and Chu, 1994), to a structure that is. Although the XPE complex is not required for any stage of psoralen crosslink repair, it serves as a marker of the completion of the first repair cycle and the start of the second. At this point it is generally assumed that this is a task for the NER pathway, although there is only limited evidence, from an *in vitro* system, in direct support of this view (Cipak *et al.*, 2006).

An alternative suggestion for the second repair cycle has been proposed in work with the NEIL1 glycosylase. Cells deficient in this activity are sensitive to psoralen/UVA (Couve-Privat *et al.*, 2007). Recently, this group prepared three stranded oligonucleotide structures designed to mimic an unhooked psoralen crosslink attached to a 9-mer oligonucleotide. These constructs were substrates for NEIL1, which was able to cleave the glycosidic bond between the psoralen-thymine adduct and the sugar, producing a thymine base crosslinked to the 9-mer oligonucleotide (Couve *et al.*, 2009). They suggested that the glycosylase, which has a relatively small active site, would bind only the ejected thymine, and not the bulky crosslinked oligonucleotide. Glycosylases other than NEIL1 might also be involved in crosslink repair. Samson and colleagues found that cells derived from knock-out mice in which the alkyladenine glycosylase (also known as methylpurine glycosylase, MPG (Miao *et al.*, 2000)) gene was inactivated were sensitive to psoralen (Allan *et al.*, 1998), and had reduced and delayed formation of γ H2AX foci in comparison to wild type cells. However, the purified protein did not bind a psoralen crosslinked oligonucleotide duplex, arguing against a direct role in crosslink recognition (Maor-Shoshani *et al.*, 2008). If these enzymes do remove the crosslinked monoadduct that appears at the start of the second repair cycle they would provide an alternative to the NER pathway, perhaps explaining the relatively mild sensitivity of NER deficient cells (other than ERCC1-XPF) to crosslinking agents. If there is an involvement of a glycosylase then it might be expected that other members of the BER pathway, AP endonuclease, pol β , etc, would also be engaged. This possibility has not received attention.

Conclusions

It is customary to introduce discussions of crosslink repair by noting our poor understanding of the process. Indeed, in this review we have called attention to inconsistencies, contradictions, and uncertainties in the literature. In large part this is because of the multiplicity of crosslinking agents used by investigators, and the differing properties of those agents and their products. This is unlike the experience in the elucidation of a pathway such as NER in which so much of the inquiry was with UV photoproducts. Conclusions from that work could serve as a reference for studies on monoadducts formed by other agents. This situation obviously does not apply to the crosslink field. However, this state of affairs is not likely to be long lived. The maturation of the monoadduct repair fields and the migration of insight and technology to ICL repair will have a major impact. Advances in the Fanconi Anemia field have had a significant influence on, and expanded the interest in, ICL repair. Additionally, the value of DNA repair analyses for the development of improved chemotherapy regimens has been recognized. The prominence of crosslinking agents in the clinic adds the potential of practical application to the rationale for these studies. In the not too distant future we can expect considerable clarification of what has proven to be a very challenging problem.

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Figure 1. DNA ICL forming agents. (A) Common crosslinking agents used in ICL experiments. (B) DNA-ICL structures including synthetic substrates.

Figure 2.

Laser localized lesions and repair in G₁ mammalian cells. Localized lesions (green stripe) formed by dig-tagged psoralen in WT and XPC deficient cells. Cells are stained with a cell cycle marker (NPAT, red spots) to show cells in G₁ phase. NPAT shows 2 spots in G₁ cells and four spots in S/G₂ cells, and the spots may be present in different focal planes. A color version of this figure is available in the online version of this manuscript.

Figure 3. The 'Cole' model. A schematic of ICL repair in *E. coli*. After the 1st cycle of incision, the gap is either filled by strand exchange or repair synthesis bypassing the adducted base. A color version of this figure is available in the online version of this manuscript.

Figure 4.

Entry into an ICL repair pathway depends on the mode of recognition. (A) In the context of a non-replicating DNA, the distortion of the DNA helical structure caused by the lesion attracts protein(s) involved in the global damage surveillance of DNA. This process has been shown to involve proteins of the NER pathway, with XPC leading the initial recognition. The first incision step on either side of the lesion on one strand of the duplex by XPF-ERCC1 complex (and perhaps XPG) generates a gapped structure which serves as a substrate for bypass polymerases. The now flipped out monoadduct-like structure can be recognized by DDB2 and perhaps also by glycosylases such as MPG or Nei1. Again, the NER pathway will initiate the second cycle of repair and remove the remaining adduct on the opposite strand. (B) Stalling of RNA polymerases at the site of lesion during transcription can also serve as a means of ICL recognition in non-replicating DNA. (C) and (D), a stalled replication fork, either due to a single or dual fork encounter, is attractive to proteins of the Fanconi Anemia (FA) pathway and proteins such as Mus81-EME1/2, Snn1B, and MRN. Initial recognition is thought to be mediated by the FancM-FAAP24 complex, which then becomes part of the FA core complex. The FA core complex is a prerequisite to recruit the FancD2 and FancI proteins which are modified via ubiquitination and phosphorylation. The ICL is incised by XPF-ERCC1 and Mus81-EME1 on the leading strand, generating a DSB at the fork. In the case of converging forks (D), the first incision cycle may occur on either strand. The gapped structure will be filled in by lesion bypass polymerases, including pol ζ , polk, polt, polN, pol η and Rev1. When a single fork is stalled by the ICL polymerase(s) will extend a parental strand to fill the gap. When two forks converge on the ICL a leading daughter strand is extended to bypass the lesion. Upon removal of the remaining single adduct on the opposite strand, in an NER dependent pathway, the broken fork will be reconstructed by recombinational repair. A color version of this figure is available in the online version of this manuscript.