DNA repair and cell cycle checkpoints in response to DNA interstrand cross-linking agents in *Saccharomyces cerevisiae*

by

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a dissertation

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Abbreviations/Definitions

CDDP - cisplatin

8-MOP - 8-methoxy psoralen

MMS - methyl methane sulfonate

HU - hydroxyurea

ONPG - *o*-nitrophenyl-ß-D-galactopyranoside

UV - ultra violet light

UV-A - long wavelength UV (365 nm)

UV-C - short wavelength UV (254 nm)

DSB - double stranded break

D37 - dose of drug giving 1 lethal hit per cell

NER - nucleotide excision repair

MEC - Mitosis Entry Checkpoint

DUN - Damage Uninducible

CDC - cell division cycle

RAD - Radiation Sensitive

SNM - sensitivity to nitrogen mustard

PSO - psoralen sensitive

REV - defective in reversion mutations

ts - temperature sensitive

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Abstract

DNA interstrand cross-linking agents are used in the treatment of many types of cancer. Despite their wide use in the clinic, the repair of and cell-cycle checkpoint response to DNA cross-links is not well understood. To better describe cross-link repair and cell cycle checkpoint responses to interstrand cross-link damage, baker's yeast (*S. cerevisiae*) was used as a model system. The genetic requirements for survival in the face of cross-link damage were assessed. *snm1*, *rev3* and *rad51* mutants were more sensitive to cisplatin (CDDP) than UV irradiation, suggesting a specific role in cross-link repair. Excision repair genes appear to be required for cross-link repair: *rad1* and *rad14* mutants were sensitive to CDDP as well as UV irradiation. Of the checkpoint mutants tested *mec1, rad53* and *mec3* mutants were the most sensitive to CDDP, while *rad9*, *rad17*, *rad24* and *dun1* mutants displayed only modest sensitivity to CDDP. This suggests that some, but not all, checkpoint functions are essential for cross-link repair.

The cell cycle checkpoint response to CDDP was examined. G2 appears to be the primary checkpoint in wildtype cells following cross-link damage. G1-synchronized cells treated with levels of CDDP causing 40-60% lethality displayed normal DNA replication kinetics as measured by flow cytometry. The lack of S-phase delay suggests that cross-links can be accommodated during replication. Mec1 function was required for G2 arrest. The epistatic relationships between *SNM1*, *REV3* and *RAD51* were assessed with respect to CDDP sensitivity. All double mutants were more sensitive than any of the singles to CDDP; therefore they are in three different epistasis groups. The progression through the cell cycle of *snm1*, *rev3* and *rad51* mutants was examined. Surprisingly, replication occurred normally as measured by FACS analysis in *snm1*, *rev3* and *rad51* mutants even at high levels of lethality (>80% cell death). A prolonged G2 arrest after CDDP treatment in G1 was observed for *snm1*, *rev3* and *rad51* mutants suggesting that these genes are required for the repair of cross-links after replication. High levels of cross-link damage were shown to induce a S-phase delay which was *MEC1*

dependent. The incision of cross-links was shown to be normal in *snm1* and *rev3* mutants. It is possible that Snm1 and Rev3 function downstream of the incision event in the repair of cross-links. Finally, the ability of *dun1* mutants to induce a reporter construct containing a damage response element (DRE) was assessed. The data show that Dun1 is required for induction of DRE containing genes after treatment with CDDP.

Introduction

Cells experience DNA damage from both internal and external sources. To survive, the cells must repair that damage. To remove lesions the cell marshals DNA repair enzymes (for review see Friedberg *et al*, 1995). To make time for repair, the cell slows progression through the cell cycle. In eukaryotic cells this delay in the cell cycle is called a checkpoint. Checkpoints are an active response; that is, removal of checkpoint gene function causes cells to progress through the cell cycle in the presence of unrepaired DNA damage (for review see Elledge, 1996; Weinert, 1998). Cell cycle checkpoints and DNA repair have been well characterized in yeast with respect to many types of damage from various agents. The agents tested include hydroxyurea (HU), methyl methane sulfonate (MMS), ultraviolet (UV) light, and gamma-irradiation to name a few. The DNA repair and cell cycle checkpoint response to agents which cause interstrand cross-links, however, are not well described. The studies in this thesis assess the effects of DNA cross-links on the yeast *S. cerevisiae*. *S. cerevisiae* is used as a model system because it is the best genetically characterized organism with respect to DNA repair and cell cycle checkpoints.

Cross-linkers

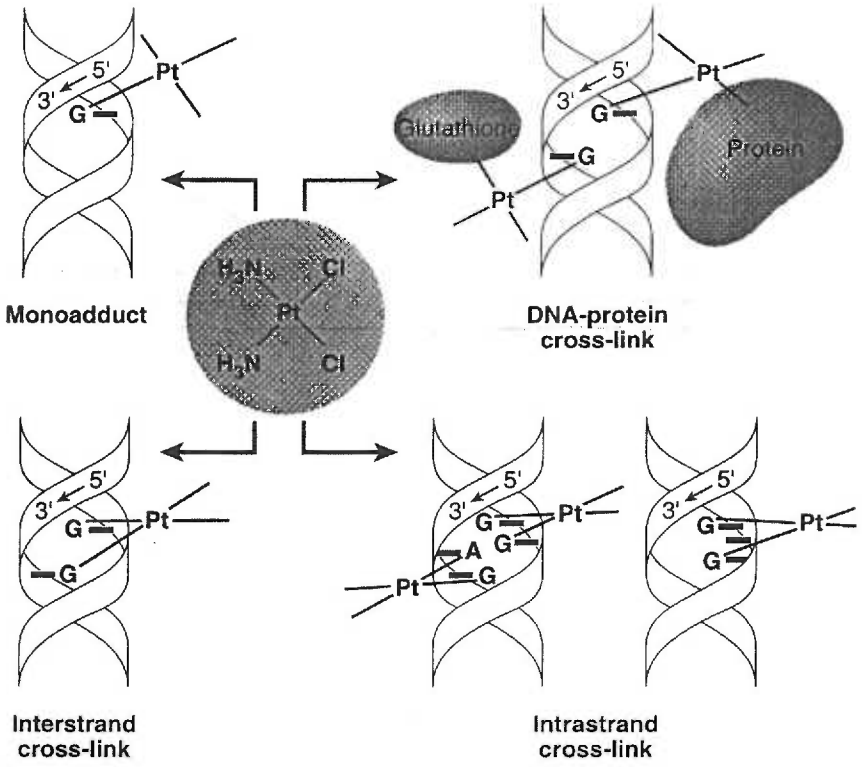
DNA interstrand cross-links are caused by certain anticancer drugs including cisplatin (CDDP) and 8-methoxypsoralen (8-MOP). The biological effects of CDDP were originally discovered serendipitously by Rosenburg and co-workers (1967) who were examining the effects of electric current on the cell division of *E. coli*. They found that an electrolysis product of the platinum electrode was responsible for causing the filamentous growth observed in their experiments. After further description of the effects of CDDP on bacterial cells, Rosenburg et al (1969) began testing the effects of CDDP on solid sarcomas in mice. They found that the drug caused tumor regression within 6 days.

CDDP has now been used successfully in many types of cancer treatment including testicular cancer, ovarian cancer, lung cancer, and bladder cancer (Lippert, 1999 and references therein). CDDP has been particularly effective against testicular cancer. Before the discovery of the anti-tumor capabilities of CDDP, fewer than 5% of the patients diagnosed with testicular cancer survived long term. Today, there is an estimated 90 % cure rate for testicular cancer due, in part, to the efficacy of CDDP (reviewed in Lippert *et al*, 1998). Though not as widely used as CDDP, psoralen does have clinical application in the treatment of psoriasis - a skin disorder characterized by hyperproliferation of epithelial cells (Friedberg *et al*, 1995).

Action of CDDP and 8-MOP

Cisplatin produces three types of DNA lesions: interstrand cross-links; monoadducts; and intrastrand cross-links (reviewed in Lippert, 1999) (Fig. 1). The primary site for adduct formation of the platinum molecule is the N7 of purines. The intrastrand lesions formed are [1,2-d(GpG)]>[1,2-dApG)]>[1,3d(GpNpG)], with interstrand cross-links occurring primarily at d(GpC)•d(GpC) sites. 8-MOP + UVA light produces monoadducts and cross-links at purines, primarily interacting with T residues. Interstrand cross-links form at d(TpA)•d(TpA) sequences (Friedberg *et al*, 1995). 1-5% of the lesions produced by CDDP is an interstrand cross-link (Lippert, 1998) while 8-MOP + UVA treatment produces at best 25% cross-linking (Henriques *et al*, 1997). Though the relative contribution of various lesions to toxicity is not known, interstrand cross-links are thought to be very difficult to repair because as few as 20 interstrand cross-links per genome induced by 8-MOP + UVA can kill a population of wild-type yeast below the D37 (the 37% survival level (D37) represents one lethal hit per cell). It takes about 1,000 fold more intrastrand adducts from UV-C to achieve the same level of killing.

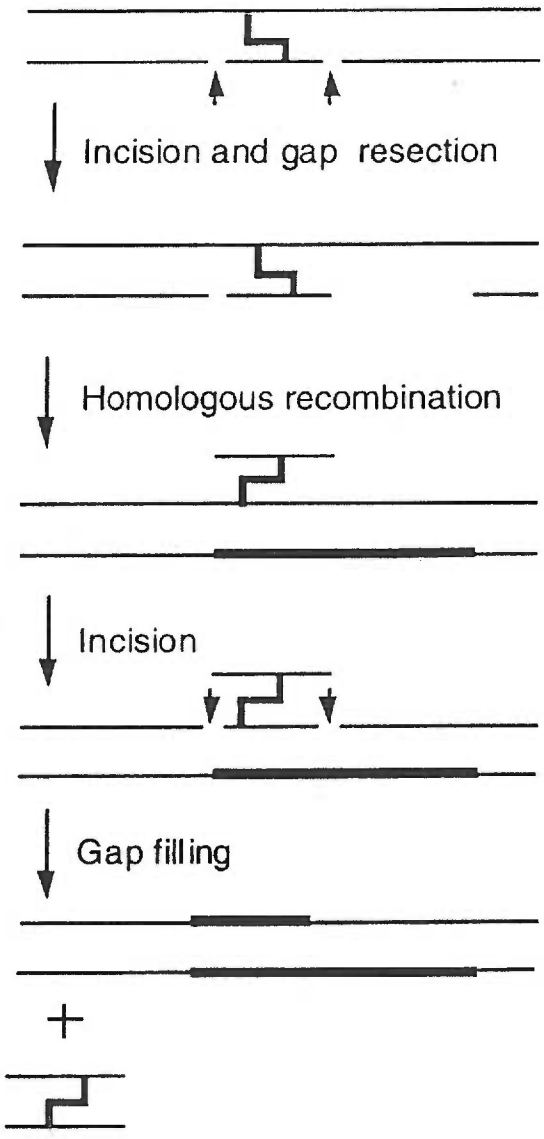
Fig. 1. DNA damage induced by cisplatin. From (Eastman, 1987; Friedberg *et al*, 1995). Interstrand cross-links comprise 1-5% of the total lesions while intrastrand cross-links, monoadducts, and DNA-protein cross-links make up the remainder of the damage.


5'
3'
G
Pt
Monoadduct
Glutathione
Protein
DNA-protein
cross-link
Cl
Interstrand
cross-link
A
Intrastrand
cross-link

Repair of DNA cross-links

DNA interstrand cross-links pose a unique problem for the cell's repair processes. By damaging bases on nearly opposite sides of the helix, the cell does not have complementary information to serve as a template for repair following incision as it does for adducts affecting only one strand. An early model for how nucleotide excision repair (NER) and recombination repair act together to accomplish repair of a cross-link was first proposed by Cole (1973). In this work, Cole treated wildtype and *recA*$^-$ *E. coli* cells with psoralen + UVA and measured the integrity of the DNA over time with alkaline sucrose gradients. He found that in wild-type cells treated with psoralen + UVA, the DNA was cut into discrete pieces. Following a post-treatment incubation, the broken DNA was restored to full length molecules in wild-type strains but not in *recA*$^-$ strains. Cole concluded that RecA must be carrying out recombination to restore the duplex. Strand exchanges were also measured. Cole found that wild-type, but not *recA*$^-$ cells were able to carry out strand exchanges. These findings led Cole to propose the model where a homologous sequence from another chromosome is used to "patch" across from an incised monoadduct (Fig. 2). Later work performed by Cole and his coworkers (1976) demonstrated *in vivo* that the incision of DNA cross-links required uvrA, uvrB, uvrC and polA functions. Additional experiments performed *in vitro* support Cole's model by demonstrating that the ABC exinuclease is required for incision of cross-linked duplex DNA substrates (Van Houten *et al*, 1986) and RecA activity is required for strand exchange following exonucleolytic digest of incisions on either side of a cross-link (Sladek *et al*, 1989). Clearly interstrand cross-link repair in *E. coli* is complex and requires components from at least two repair pathways: nucleotide excision repair and recombination repair.

Fig. 2. Cole's model for DNA repair of interstrand cross-links in *E. coli*. Adapted from Cole (1973) (Li *et al*, 1999). Later work (Van Houten *et al*, 1986) demonstrated that in *E. coli*, incision is first carried out by the UvrABC endonuclease. This is followed by the 5' - 3' exo activity by Pol I. Next, RecA mediates strand exchange and recombination. Once recombintion is complete, Uvr ABC cleaves the remaining monoadduct and repair synthesis seals the gap.


Incision and gap resection
Homologous recombination
Incision
Gap filling

In yeast, studies have shown that members of the RAD3/nucleotide excision repair group, the RAD6/postreplication repair group and RAD52/recombination repair group are required for the repair of DNA interstrand cross-links (Jachymczyk *et al*, 1981; Miller *et al*, 1982 ; Hartwell *et al*, 1997) (Table 1). *In vivo*, the incision step of the interstrand cross-link repair process requires the function of several nucleotide excision repair (NER) genes. Jachymczyk *et al* (1981) first demonstrated that *rad3* mutants, treated with 8-MOP + UVA and allowed time to repair did not experience a reduction in the percentage of dsDNA as measured by density gradient CsCl sedimentation. This suggests that *rad3* mutants cannot incise interstrand cross-links. Additional mutants were tested by Miller *et al* (1982) who demonstrated that *rad1*, *rad2*, *rad3*, *rad4*, *rad10* and *mms19* mutants were unable to nick interstrand cross-linked DNA as measured by alkaline sucrose gradients. They also found that *rad14* mutants were slower at incision and *rad16* mutants had normal incision kinetics after cross-link treatment. Magaña Schwenke *et al* (1982) tested *rad3* and *pso2/snm1* mutants and found that only *rad3* mutants were deficient in incising DNA cross-links as measured by denaturation/renaturation followed by CsCl sedimentation. Using a target specific technique developed by Vos and Hanawalt (1987), Meniel *et al* (1995) demonstrated that *rad1* and *rad2* mutants were deficient in cross-link incision but *rad52* and *snm1/pso2* mutants were proficient in cross-link incision. It has also been shown that the incision step occurs more rapidly on the transcriptionally active MAT-α locus than on the inactive HML-α locus following cross-link damage (Meniel *et al*, 1995; 1997a; 1997b) suggesting that incision of interstrand cross-links may be coupled to transcription. This group also described incision in cells at all different phases of the cell cycle. They accomplished this by synchronizing cells in G1 by separating out nonbudded/G1 cells by elutriation. Cells were then treated with a high dose of 8-MOP + UVA at various times after release and the %ds DNA was measured on a denaturing gel. They found that incisions could occur at any phase of the cell cycle and that incision occurred at a faster rate on the transcriptionally active MAT-α locus than on the transcriptionally inactive HML-α locus (Meniel *et al*, 1997b). Magaña-Schwenke *et al* (1982) found that incision was slower in

G1/stationary-phase cells than in asynchronous cultures.

In addition to single strand incisions, double strand breaks (DSB) also occur following cross-link treatment in yeast. Jachymczyk *et al* (1981) were the first to demonstrate that *rad51* mutants did not repair DSBs after cross-linking while wildtype cells did. They measured the amount of DSBs by neutral sucrose gradients. Magaña-Schwenke *et al* (1982) also describe repair of DSBs after interstrand cross-link damage in wildtype cells and *snm1* mutants (described in detail below) were defective in restoring the DSB following treatment with 8-MOP + UVA. From the studies mentioned above it seems clear that later steps in the repair of DNA cross-links in yeast appear to require recombinational repair enzymes to process the DSB intermediate.

Many of the mutants mentioned above, such as the NER mutants, display similar or greater sensitivity to UV-C light and other agents in comparison to interstrand cross-linking agents. However, yeast mutants specifically sensitive to the interstrand cross-linking agents nitrogen-mustard (Ruhland *et al*, 1981) and 8-MOP (Henriques and Moustacchi, 1980) have been identified (see Henriques *et al*, 1997 for review). These mutants with interstrand cross-link specific sensitivities are of particular importance to the studies in this thesis as they may represent specific defects in interstrand cross-link repair. Two such mutants, *snm1* and *pso2-1* were found to be allelic (Cassier-Chauvat and Moustacchi, 1988). *snm1/pso2* mutants display greater sensitivity to DNA cross-linking agents than to UV-C light (Henriques and Moustacchi, 1980). The sequence of the *SNM1* gene suggests that it encodes a possible Cys2Cys2 Zn-finger domain protein (Richter *et al*, 1992). The promoter has been shown to contain a damage response element (DRE) which has homology to the promoters of previously known damage inducible genes (Wolter *et al*, 1996). Expression of *SNM1* was induced by cross-linking agents (Wolter *et al*, 1996), but not by monofunctional agents such as methyl methane sulfonate (MMS) (Wolter *et al*, 1996).

Table 1.

Epistasis groupings of repair genes. Adapted from Friedberg *et al*, (1995).

RAD3 group	RAD52 group	RAD6 group
RAD1	*RAD50*	***RAD5 (REV2) (SNM2)***
RAD2	***RAD51***	***RAD6***
RAD3	***RAD52***	*RAD8**
RAD4	***RAD53***	*RAD9**
RAD7	*RAD54*	*RAD15**
RAD10	*RAD55*	***RAD18***
RAD14	*RAD56*	*RADH**
SSL1	*RAD57*	*REV1*
SSL2 (RAD25)	*RAD24*	***REV3 (PSO1)***
TFB1	*XRS2*	*CDC9*
RAD16 (PSO5)		*REV5*
RAD23		*REV6**
CDC8		*REV7*
CDC9		*CDC7**
MMS19		*CDC8*
PSO2 (SNM1)		*MMS3**
PSO3		***PSO4****
UVS12		*UMR1-7*

The genes in bold have been shown by the work presented in this thesis and by others to be required for survival after cross-link damage. All genes are assigned to epistasis groups based on UV-C and IR sensitivity in all cases. Genes denoted by an asterisk are assigned on the basis of limited phenotypic characterization. Genes shown in parenthesis are allelic to those primarily listed.

PSO1, another gene found to be required for normal survival after cross-link damage (Henriques and Moustacchi, 1980), is allelic to *REV3* (Cassier-Chauvat and Moustacchi, 1988). Rev3 together with Rev7 forms DNA polymerase ζ which can bypass T=T dimers in an error prone fashion (Nelson *et al*, 1996). The specific role for the Rev3 gene product in the repair of interstrand cross-links has not been established. A third gene, *RAD51*, is also known to be required for survival after interstrand cross-link damage as well as other types of DNA damage (Jachymczyk *et al*, 1981; Friedberg *et al*, 1995). The *SNM1*, *REV3* and *RAD51* genes define three different epistasis groups based on sensitivity of various double mutants to UV-C light although there is still some ambiguity with respect to interstrand cross-linking agents. *SNM1* and *REV3* have been reported to have an epistatic relationship with respect to 8-MOP sensitivity, but are not epistatic with respect to UV-C light sensitivity (reviewed in Henriques *et al*, 1997). This finding suggests that interactions defined by one type of damage may not be true when tested with other types of damage.

Other genes identified in the screen performed by Henriques and Moustacchi (1980) include *pso3*, *pso4*, *pso5*, *pso6* and *pso7* though their specific roles in interstrand cross-link repair are not well described (Henriques *et al*, 1997). The gene mutated in the *pso3-1* mutant has not yet been cloned. *PSO4* has been shown to encode a spliceosome associated protein *PRP19* (Henriques, 1997). *pso4-1* mutants, like *snm1* and *rad51* mutants, can incise 8-MOP cross-links but cannot repair DSBs which occur after 8-MOP + UVA treatment. Unfortunately deletion of *PSO4/PRP19* is lethal in haploid cells (Cheng et al, 1993; Grey et al, 1996). *PSO5*, *PSO6* and *PSO7* may not be specific for cross-link repair as they display greater sensitivity to monofunctional damage from 3-CP + UVA than to interstrand cross-link damage induced by 8-MOP + UVA (Henriques *et al*, 1997).

Cell cycle checkpoints

Cell cycle checkpoints monitor the cell for defects and arrest the cell cycle to make time to remedy any defects. Cell cycle arrest caused by checkpoints enhances survival after several types of DNA damage including cross-links (reviewed in Elledge, 1996; Weinert, 1998). In human cells, defects in cell cycle checkpoint genes such as p53 and ATM lead to genomic instability and cancer (reviewed in Elledge, 1998). Yeast cells have four distinct damage-inducible checkpoints. The G1 checkpoint prevents cells from entering S-phase with unrepaired DNA damage (Siede *et al*, 1993, 1994, 1996; Allen *et al*, 1994, Sidovara *et al*, 1997). The intra-S checkpoint slows replication in the presence of monoadducts made after MMS (methyl methane sulfonate) treatment (Paulovich *et al*, 1995, 1997). The S/M checkpoint arrests cells in S-phase following nucleotide pool depletion by inhibition of ribonucleotide reductase (Rnr3) by HU (Navas *et al*, 1995, 1996; Allen *et al*, 1994; Weinert *et al*, 1994). Finally, the G2/M checkpoint prevents cells from completing mitosis with unrepaired intrastrand adducts and DSBs induced by UV, IR, and other types of damage (Weinert *et al*, 1988, 1994; Allen *et al*, 1994)(Fig. 3A).

Checkpoint models are based upon the following: i) recognition of DNA damage; ii) signal transduction; and iii)signaling targets (Fig. 3A). Several proteins have been proposed to act as sensor molecules by directly recognizing damage and/or processing DNA damage into recognizable intermediates. These include *RAD9, RAD17, RAD24, MEC3* and *DDC1* (Lydall *et al*, 1995; Longhese *et al*, 1997). Weinert and Hartwell (1988) identified *RAD9* as the first checkpoint gene. They found that *rad9* mutants failed to arrest in G2 after γ-irradiation. Later work suggested that Rad9, Rad17, Rad24 and Mec3 may act as a complex to detect DNA damage (Lydall and Weinert, 1995) in checkpoints at all three phases of the cell cycle (reviewed in Weinert, 1998). *Ddc1* is also involved in G1, G2 and S-phase damage inducible checkpoints but is not required for slowing the cell cycle after HU treatment (Longhese *et al*, 1997). Based on epistatic interactions between

DDC1 and *MEC3*, Ddc1 is thought to act in the same pathway with Mec3, Rad17 and Rad24 (Longhese *et al*, 1997).

Some checkpoint proteins have dual functions in replication and checkpoints and are thought to signal arrest when the polymerase stalls at a lesion or is starved for nucleotides; these include Pol2 (Navas *et al*, 1995, 1997), Dbp11 (Araki *et al*, 1995) and *Rfc5* (Sugimoto *et al*, 1996, 1997). Pol2 (Dun2) was first identified to have checkpoint like functions in a screen for strains that were unable to induce damage inducible genes following treatment with HU (Zhou and Elledge, 1993). Later Navas and coworkers (1995) demonstrated that this mutant was specifically defective in intra S-phase delay following HU treatment. They suggested that Pol2 acts in a parallel pathway relative to the Rad9 pathway (described above) to sense damage in S-phase (Navas *et al*, 1996). Dbp11 when overexpressed, suppresses a temperature sensitive (ts) *dpb2-1* (subunit of Pol2) mutant (Araki *et al*, 1995). *dbp11* null mutants are inviable but a thermosensitive *dbp11-1* has been isolated. The *dbp11-1* cells do not replicate DNA at the normal rate and experience premature cell divisions at the restrictive temperature suggesting a role both in replication and checkpoint control (Araki *et al*, 1995). Another replication component, Rfc5 (replication factor c) has also been shown to have a dual role in replication and the S-phase checkpoint. Rfc5 mutants were originally identified as a ts mutant which could be rescued by over expression of Rad53 (this protein is thought to be a signal transduction component of the checkpoint as described later). *rfc5* ts mutants displayed slow S-phase kinetics at the restrictive temperature suggesting a primary role in DNA replication (Sugimoto *et al*, 1996). Later work demonstrated that Rfc5 was essential for regulation of the Rad53 protein kinase and that rfc5-1 mutants were defective in the MMS induced S-phase delay (Sugimoto, 1997). DNA damage sensitive DNA primase mutants (*pri1-M4*) also appear defective in both replication and cell cycle checkpoint delays (Marini *et al*, 1997). The above results clearly show that replication and damage sensing functions are tightly coupled.

Following damage recognition, sensor molecules signal intermediate signaling

molecules (Sanchez *et al*, 1996; Sun *et al*, 1996; Longhese *et al*, 1997). Currently the kinases Mec1, Rad53 (Allen *et al*, 1994; Weinert *et al*, 1994; Sun *et al*, 1996; Snachez *et al*, 1996), and Dun1 (Pati *et al*, 1997) are thought to act downstream of the sensor molecules as signal transducers. *MEC1* was first identified in a screen using a *cdc13-1* ts mutant (Weinert *et al*, 1994). *cdc13* mutants arrest in G2/M following a shift to the restrictive temperature because they accumulate single stranded DNA at their telomeres (Garvik *et al*, 1995). The *mec1* mutants identified in the *cdc13* based screen could not survive the temperature shift due to their failure to arrest. Later, Mec1 was shown to be a lipid kinase with homology to several other checkpoint proteins including mammalian ATM and DNA-PK (reviewed in Zakian, 1995). Mec1 has been shown to control phosphorylation of Rad53 in response to DNA damage (Snachez *et al*, 1996; Sun *et al*, 1996). *RAD53* was also identified in the *cdc13* based screen by Weinert *et al* (1994) as a checkpoint protein. It is thought to act downstream of Mec1 based on phosphorylation studies (Sanchez *et al*, 1996; Sun *et al*, 1996) and on genetic studies (Gardener *et al*, 1999). Dun1, originally isolated in a screen for damage uninducible (DUN) strains (Zhou *et al*, 1993), was thought to be a downstream target of cell cycle checkpoints. A later study demonstrated a defect in G2 arrest (Pati *et al*, 1997). The current model for the G2 checkpoint places Mec1 upstream of two parallel pathways consisting of 1)Rad53 and Dun1, and 2)Pds1 (Fig 3B. Gardner *et al*, 1999)

Once signal transducers have been activated by the sensors, the signal transducers act on downstream targets to assure cell cycle arrest and the induction of repair genes. Targets for the G1 checkpoint arrest mechanism include the phosphorylation of Swi6 which down-regulates transcription of G1 cyclins *CLN1* and *CLN2* (Sidovora *et al*, 1997). The target for the S-phase delay and G2 phase arrest are less well defined. One possible target for the G2 arrest checkpoint is Cdc28 though the reports on its role in the damage induced cell cycle response are conflicting (Li *et al*, 1997; Amon *et al*, 1992; Sorger *et al*, 1992). The reports by Amon *et al* (1992) and Sorger and Murray (1992) demonstrated that $p34^{cdc28}$

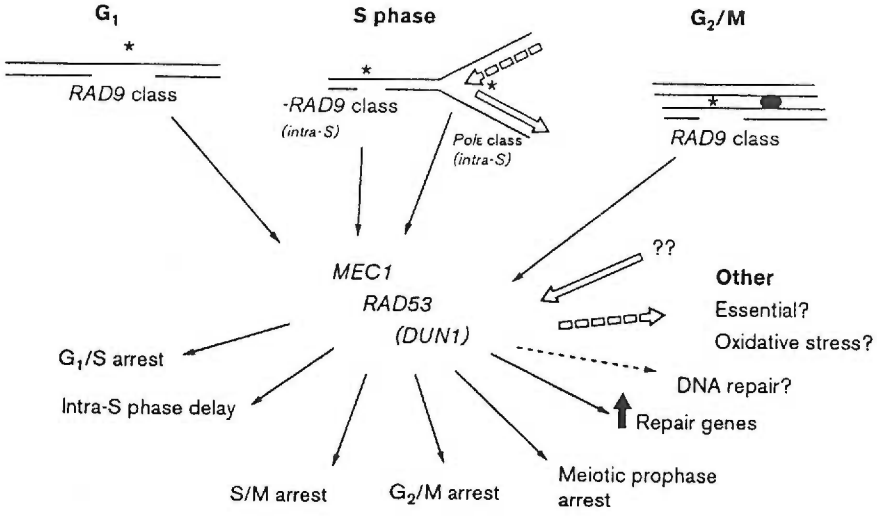
mutants which contained substitutions at a highly conserved tyrosine (residue is found in species from *S. pombe* to mammals) had perfectly normal cell division and arrest characteristics. Later Li and Lai (1997) demonstrated that a mutant allele of *CDC28* (*cdc28-5M*) was defective in damage induced checkpoints and could not survive a shift to the restrictive temperature in a *cdc13-1* background. It seems clear that another component of this protein (other than the conserved tyrosine (Y19)) is important for regulation of the G2/M transition. Pds1 is thought to be a target for Mec1 and acts in late G2 (Fig 3B, Cohen-Fix *et al*, 1997). Recent work has shown that Mec1 and Rad53 act by inhibiting the firing of late origins of replication though the specific targets involved are not known (Longhese *et al*, 1999).

Thesis summary

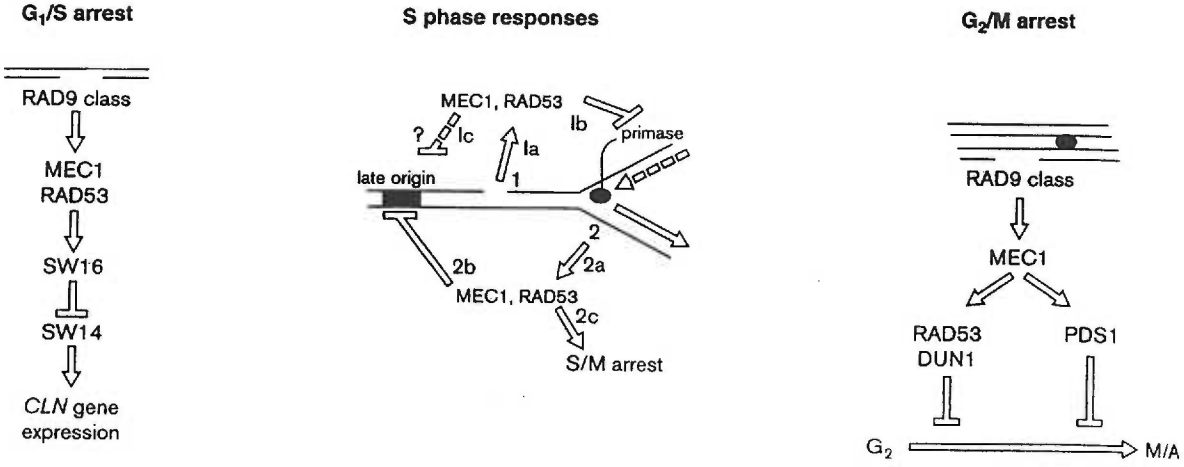
To better understand the cell cycle effects and repair requirements for survival after XL damage, yeast is used as a model system because it is perhaps the best characterized organism with respect to DNA repair and cell cycle checkpoints. The studies reported in this thesis assess the contribution of various repair and checkpoint functions to survival following cross-link damage by measuring the ability of various mutants to survive treatment with cross-linkers. The cell-cycle arrest characteristics of wild-type cells in response to treatment with cisplatin and 8-MOP are described with both synchronous and asynchronous cultures. The epistatic relationships of *SNM1*, *REV3* and *RAD51* with respect to cross-links were also studied. This was accomplished through the construction of isogenic mutant strains with disruptions in the *SNM1*, *REV3* and *RAD51* genes alone and in combination and testing their sensitivity to cross-links. The effects of cisplatin on the cell cycle of *snm1*, *rev3* and *rad51* mutants is assessed. In addition, the incision of interstrand cross-links is examined in *snm1* and *rev3* mutants by following the amount of double stranded DNA over time after cisplatin treatment. The effects of high levels of CDDP damage on S-phase progression are also studied in wild-type and *mec1* checkpoint-deficient cells. Finally, the induction of DRE containing genes following

Fig. 3. Cell cycle checkpoints in yeast. From Weinert (1998). **A** Sensors. In this model DNA damage can occur in any phase of the cell cycle. This damage may be processed to an intermediate such as ssDNA. It is then recognized by sensor molecules which pass a signal via. phosphorylation events to signal transduction molecules (*MEC1*, *RAD53* and *DUN1*). These transducers then signal cell cycle arrest and the induction of repair genes. **B**. Targets of the arrest response. If damage is sensed in G1, the signal is passed to *SWI6* which inhibits the activation of *CLN1* and *CLN2* cyclins by *SWI4*. *CLN1* and *CLN2* drive the G1/S transition in the cell cycle. In S-phase, signals are sent to components of the replication apparatus such as late origins of replication via unknown intermediate molecules to cause a delay (Longhese *et al*, 1999).

A


G1
S phase
G2/M
RAD9 class
-RAD9 class
(intra-S)
Polε class
(intra-S)
RAD9 class
??
Other
Essential?
Oxidative stress?
MEC1
RAD53
(DUN1)
G1/S arrest
Intra-S phase delay
DNA repair?
Repair genes
S/M arrest
G2/M arrest
Meiotic prophase arrest

B


G1/S arrest
RAD9 class
MEC1
RAD53
SW16
SW14
CLN gene expression
S phase responses
MEC1, RAD53
?
Ic
Ia
Ib
primase
late origin
1
2
2b
2a
MEC1, RAD53
2c
S/M arrest
G2/M arrest
RAD9 class
MEC1
RAD53
DUN1
PDS1
G2
M/A

CDDP damage is described for wildtype and *dun1* mutant strains.

When wild-type cells were treated with CDDP in synchronous or asynchronous cultures, the cells arrested primarily with G2 DNA content. The requirement of checkpoint function is reflected by the sensitivity of *mec1*, *mec3* and *rad53* mutants which have defects in several cell-cycle checkpoints. Surprisingly, other checkpoint mutants such as *rad9*, *rad17*, *rad24* and *dun1* showed little or no increased sensitivity to CDDP. *mec1* mutants displayed a complete defect in G2 arrest following CDDP treatment. The results also showed that in isogenic *snm1Δ*, *rev3Δ* and *rad51Δ* strains, which were highly sensitive to cross-links (< 20% survival at doses allowing 80% survival of wild-type strains), S-phase progressed normally after CDDP treatment. This indicates that Snm1, Rev3 or Rad51 are not required for DNA replication after CDDP exposure. CDDP damage caused the mutant strains to arrest permanently in G2, presumably due to inability to complete repair of cross-links adequately. Despite the failure to exit G2, *snm1Δ* and *rev3Δ* mutants incised cisplatin DNA interstrand cross-links normally. The absence of S-phase delay is a saturable phenomenon, as high levels of cross-link damage from CDDP or 8-MOP + UVA in wild-type cells (< 40% survival) elicited a cell cycle delay in S-phase. The S-phase delay seen at high levels of damage is dependent on the function of Mec1. *SNM1*, *REV3* and *RAD51* are in different epistasis groups with respect to cisplatin damage. This suggests that they function in separate pathways to repair DNA cross-links. Finally, the data demonstrate that *dun1* mutants display a defect in induction of DRE containing genes following treatment with CDDP.

Materials and Methods

Strains and plasmids

The strains used in this study are described in Table 2. Strains Y286, Y205, Y207, Y254, Des348, Des377, Des378, D370 and D377, in the CRY1 background, were from S. Elledge, Baylor College of Medicine. Strains AAA18 and AAA83 were from T. Weinert, University of Arizona and are in the A364a background. Strain 8301 was from L. Hartwell, Fred Hutchinson Cancer Research Center, Seattle. pZZ13 is a CEN plasmid containing the *RNR3* promoter fused to a *lacZ* reporter gene (S. Elledge, Baylor College of Medicine). KGY117 contains the pZZ13 plasmid as a reporter construct (Elledge and Davis, 1989). Plasmid pBAD70 (Desany et al., 1998) contains an expression cassette, GAP-RNR1 which suppresses the lethality of the *mec1Δ* mutation in Des378 and the *rad53Δ* mutation in Des 377. KGY114 contains the pYES2 plasmid (Invitrogen).

pSNM1-TV was constructed by first PCR amplifying flanking regions of the *SNM1* ORF from yeast genomic DNA using the following primer pairs. Arm1, primer 1: 5'-ACCGCGGTCACGCAGGTTATCAATACCTTCTCC-3'. Arm1, primer 2: 5'-TTCTAGATGCTGCTTAACATGAGTTTTGACGC-3'. Arm2 primer 1: 5'-TGTCGACTGAGACAATTCAGAAGTGGTTGG. Arm2 primer 2: 5'-TCTCGAGTCCCGACAACATCTTCAATGTC-3'. Following amplification the arms were TA-cloned into pCR2.1 (Invitrogen). These arms were then subcloned into pCK+ (obtained from R.M. Liskay) which is a pBluescript based plasmid with multicloning sites flanking a *hisG::URA3::hisG* marker (Alani *et al*, 1987). This marker contains *hisG* sequences from *S. typhimurium* which surround the *URA3* gene from *S. cerevisiae*. Arm1 and Arm2 were subloned into the multicloning sites of pCK+ via *Sac*II and *Xba*I and *Sal*I and *Kpn*I respectively. Digestion of pSNM1-TV with *Kpn*I and *Xba*I resulted in a *hisG:URA3:hisG* targeting cassette that replaced the endogenous *SNM1* gene between nt -37 and +1066. pRAD51-TV was constructed by Alex Ward by first amplifying arms with the following primers: Arm1,

primer 1: 5'-CGAGCTCGCGAATCCCGCAATAAAGG-3'. Arm1, primer 2: 5'-ACCTAGGCACTGAAGCTGTGACTCTG-3'. Arm2 primer 1: 5'-GGATCCCATTTTTATCTTCATTTCC-3'. Arm2 primer2: 5'-GGTACCCCTTGTGATTTTTCCTTC-3'. Following amplification, the arms were TA-cloned into pCR2.1-TOPO (Invitrogen). They were then subcloned into pCK+ as follows. Arm1 was subcloned using *Sac*I and *Avr*II and Arm 2 was subcloned using *Bam*HI and *Kpn*I. The resulting construct, when cut with *Sac*I and *Kpn*I, added a *hisG:URA3:hisG* cassette that disrupted the endogenous RAD51 gene between nt +29 and nt +79 with respect to the initiation ATG. pYPG-101 was from D. Hinkle. Linearization of this plasmid with *Kpn*I and transformation into a target yeast strain resulted in the disruption of the *REV3* gene sequence with the *hisG:URA3:hisG* marker. Strains S4 and TWY177 were obtained from T. Weinert, University of Arizona, and are in the A364a background. Strains KGY114, KGY120 and JBY115 contain the pYES2 plasmid (Invitrogen) for use as a marker in the DNA cross-link analysis. Cells were transformed with linear targeting cassettes using a lithium acetate based protocol similar to (Gietz *et al*, 1992). Selection was first on SD-URA (BIO-101) to select for integrates. All strains, except *RAD51* deletions, were then selected on 5-FOA (5-fluoro-orotic acid) plates to remove the *URA3* marker, leaving only a single *hisG* sequence inserted (Alani *et al*, 1987). This strategy allowed the construction of multiple disruptions in the same strain background. Genotyping of deletion mutant strains was done by PCR and Southern blot with technical assistance from Jessica Brown and Alex Ward to verify correct insertion of the targeting constructs.

Table 2
Strains used in this study

Strain	Genotype
D370	*MAT***a**, *can1-100, ade2-1, his 3-11, 15, leu2-3,112, trp1-1, ura3-1*
D377	*MAT***a**, *ade2-1, ura3-1, trp1-1, his3-11, leu2-3,112, can1-100, rad51Δ:: LEU2*
Y205	*MAT*α, *ade2-1, his3, leu2-3, 112, lys2, trp1, ura3-Δ100, rnr3::RNR3-URA3-LEU2*
Y207	Y205 + pZZ13 (*HIS3*)
Y254	Y205, *dun1-3*
Y286	*MAT*α, *can1-100, ade2-1, his3-11, 15, leu2-3,112,trp1-1, ura3-1, dun1-Δ100::HIS3*
KGY117	Y254 + pZZ13 (*HIS3*)
Des348	*MAT***a**, *can1-100, ade2-1, his 3-11, 15, leu2-3,112, trp1-1, ura3-1+ pBAD70*
Des377	*MAT***a**, *can1-100, ade2-1, his 3-11, 15, leu2-3,112, trp1-1, ura3-1, rad53Δ::HIS3 + pBAD70*
Des378	*MAT***a**, *can1-100, ade2-1, his 3-11, 15, leu2-3,112, trp1-1, ura3-1, mec1Δ::HIS3 + pBAD70*
AAA18	*MAT*α, *ura3, trp1, leu2, his3*
TWY109	*MAT*α, *ura3, leu2, his3, his7, can1, sap3, rad52-1*
TWY177	*MAT***a**, *leu2, trp1, his3, ura3, mec1-1*
TWY178	*MAT*α, *trp1, ura3, mec2-1*
TWY179	*MAT***a**, *leu2, his7, ura3, mec3-1*
TWY281	*MAT*α, *ura3, trp1, his7, rad17-1*
TWY298	*MAT*α, *ura3, his3, trp1, leu2, rad24-1*
TWY398	*MAT***a**, *his7, ura3, leu2, trp1, rad9::LEU2*
S4	*MAT***a**, *ura3, trp1, leu2, his7*
AAA69	*MAT***a**, *ura3, leu2, trp1, his3, rad1Δ*

Table 2 (continued)

Strain	genotype
AAA83	*MAT***a**, *ura3, his3, leu2, trp1 rad14Δ::HIS3*
8301	*MAT***a** *SCR::URA3 ade2 ade3-130 leu1-1 cyh2 ura3-52 can1 trp1*
	*MAT***a** *ade2 ade3-130 leu1-1 cyh2 ura3-52 can1 TRP1*
	sap3 TYR1
	sap3 tyr1
KGY112	as S4, *snm1::hisG*
KGY114	S4 + pYES2 (*URA3*)
KGY120	as KGY112, + pYES2
KGY216	as S4 *rev3::hisG*
JBY115	as KGY216 + pYES2
AWY114	as S4 *rad51::hisG:URA3:hisG*
AWY112	as S4 *snm1::hisG, rev3::hisG:URA3:hisG*
AWY115	as S4 *snm1::hisG, rad51::hisG:URA3:hisG*
AWY116	as S4 *rev3::hisG, rad51::hisG:URA3:hisG*
AWY117	as S4 *snm1::hisG, rev3::hisG, rad51::hisG:URA3:hisG*
TWY177	*MAT***a**, *leu2, trp1, his3, ura3, mec1-1*

Cell Cycle analysis

Asynchronous cultures were obtained by growing the cells overnight in YPD medium at 30˚C. Cultures were then arrested in G1 by the addition of α-mating factor (SIGMA) to a final concentration of 5 µg/ml and incubated at 30˚C for 2 h. Following arrest, cells were treated with or without CDDP or 8-MOP + UVA at various concentrations (for doses given in specific experiments see legends). After treatment cells were released from the α-factor block by resuspension in YPD containing 0.1 mg/ml pronase. For survival analysis, treated and untreated cultures were plated at various dilutions on YPD plates. FACS preparations were done as described in (Paulovich *et al*, 1995) with minor modifications. Briefly, 0.2 - 2 X 10^7 cells were harvested at various times and fixed in 1 ml of ice-cold 70% ethanol overnight at 4˚C. Following fixation, cells were pelleted and resuspended in 50 mM sodium citrate (pH 7.5). 25 µl of RNaseA (10 mg/ml) was added and the cells were incubated at 50˚C for 1 h. Next, 50 µl of proteinase K (20 mg/ml) was added and the cells were again incubated at 50˚C for 1 h. After treatment with proteinase K, 1 ml of staining solution (16 µg/ml propidium iodide - 50 mM sodium citrate, pH 7.5) was added. The samples were placed at 4˚C in the dark and analyzed on a Becton Dickinson FACScalibur flow cytometer the next day. Each histogram represents about 15,000 cells. For budded analysis, cells were fixed in 3.75% formaldehyde, 0.12 M NaCl. 200 cells were scored for each timepoint.

Measurement of cross-linking

12-15 OD units of cells were harvested by centrifugation, washed once in 5 ml DOB and frozen on liquid nitrogen. DNA was isolated using a MasterPure Yeast DNA Purification Kit (Epicentre Technologies). 2-5 µg of DNA was digested with *Bam*HI. After digestion, a phenol-chloroform extraction was performed, the DNA was precipitated and then resuspended in 8.1 µl TE. Denaturation and Southern

blot analysis was performed as in (Vos *et al*, 1989) with the following specifics. For denaturing, 5.4 µl of 1.0 N NaOH was added. 5.4 µl of TE was added to the undenatured control. The samples to be denatured were then incubated at 56°C for 10 min. and then placed on ice for 2 min. 1.5 µl of 10X DRGE loading buffer (10 mM Tris 100 mM EDTA, 40% sucrose and 0.5% bromophenol blue pH 8) was then added and the DNA was separated by size on a 0.7% agarose gel run overnight at 23 V. 1 X TPE (Sambrook *et al*, 1989) was used as buffer. Following separation, the DNA was transferred to Hybond XL nylon membrane (Amersham Pharmacia Biotech) by alkaline transfer. The membrane was then probed with a random-primed labeled *Bgl*I-*Xho*I fragment from pYES2. The blot was imaged using a Molecular Dynamics Phosphorimager. Band intensities were quantitated using NIH Image v1.57 software by taking a vertical slice of density, printing the histogram, cutting out the peaks and weighing the peaks to estimate density. The % cross-linking was calculated as follows:

$$\%\ \text{ds DNA} = \frac{\text{density of dsDNA}}{\text{density of dsDNA} + \text{density of ssDNA}} \times 100$$

To calculate the number of cross-links per molecule, we first calculated the percent of undamaged molecules by subtracting the %ds DNA from 100. This number was then divided by 100 to give the fraction of undamaged molecules. Next we used the Poisson distribution to calculate lesions per plasmid molecule as follows:

$$\text{Lesions per 6 kb molecule} = -\ln(\text{Fraction of undamaged 6 kb molecules})$$

The number of lesions per 6 kb molecule were then extrapolated for other target sizes such as one replicon (36 kb) and the entire genome (14,000 kb) (Guthrie and Fink, 1991). The lower limit of this assay is 5% ds DNA for a 6 kb target. cross-linking levels reported that are below 5% ds DNA on a 6 kb target are an estimate

from regression analysis.

Calculation of the number of platinum molecules involved in interstrand cross-links relative to the number of platinum molecules bound to DNA in other forms was done as follows. DNA was extracted from CDDP treated cells and the DNA concentration was determined spectrophotometrically at 260 nm and by ethidium bromide staining of agarose gels. The molar concentration of DNA was converted to the molar concentration of genomes in the sample based on the following conversion:

$$\frac{g \times dsDNA}{ml} \times \frac{mol \cdot bp}{640g \times dsDNA} \times \frac{1genome}{14 \times 10^6 bp} = [genomes]M$$

Once the DNA concentration was determined for the sample and converted to genome concentration, the DNA samples were sent to Columbia Analytical Services (CAS) Inc. (Kelso, WA) for analysis by atomic flame mass spectroscopy. Results from CAS were reported as µg/L platinum per sample. From this number, the molar concentration of platinum was determined. The mol fraction of platinum molecules per genome was then determined by comparing the molar concentration of platinum in the sample to the genome concentration. Finally, the number of interstrand cross-links per platinum adduct was obtained by dividing the mol fraction of interstrand cross-links per genome (as calculated from denaturing gel assays as above) by the mol fraction of platinum adducts per genome.

Survival analysis and treatment with DNA damaging agents

For CDDP kill curves, asynchronous cells were treated at a cell density (OD_{600}) of 1. CDDP was dissolved in DOB immediately prior to use at a concentration of 1 to 2 mM. Cells were treated for 2 h in DOB at various concentrations of CDDP. Following treatment the cells were pelleted and resuspended in fresh DOB to a density of 1 OD_{600} unit. Next, the cells were

diluted and plated in duplicate onto YPD plates. Colonies were grown 3-5 days at 30˚C prior to counting. % survival was calculated as follows:

$$\% \text{ survival} = \frac{\text{cfu treated plate}}{\text{cfu untreated plate}} X\ 100$$

8-MOP was prepared in 95% EtOH at a concentration of 1 mg/ml and added to cultures directly for incubation prior to various times of irradiation for 15 min at 10 $Jm^{-2}s^{-1}$ 365 nm UVA light. While they were irradiated with UVA, the cells were constantly mixed on a rotating platform.

Induction of RNR3-lacZ fusion

Cells were grown to mid-log and 2 X 10^7 cells were harvested. The pellets were resuspended in 1 ml DOB medium with various concentrations of CDDP. After 30 min, cells were centrifuged and resuspended in medium lacking histidine and incubated for 4 h at 30˚C. A sample was taken for survival analysis and for analysis of ß-galactosidase activity (Elledge and Davis, 1989). Cells were resuspended in 0.5 ml assay solution containing 0.4 ml Z-buffer (0.1 M sodium phosphate [pH7.0], 1 mM $MgSO_4$), 0.02 ml 0.1% SDS, 0.08 ml 4 mg/ml *o*-nitrophenyl-ß-D-galactopyranoside (ONPG) in 0.1 M potassium phosphate (pH 7.0), and 15 µl chloroform. They were then vortexed, and incubated at 30˚C until a yellow color developed. To stop the reaction, 0.2 ml of 1 M Na_2CO_3 was added. A unit of activity was defined as OD_{420}/h and specific activity was defined as units/OD_{600} of cultures.

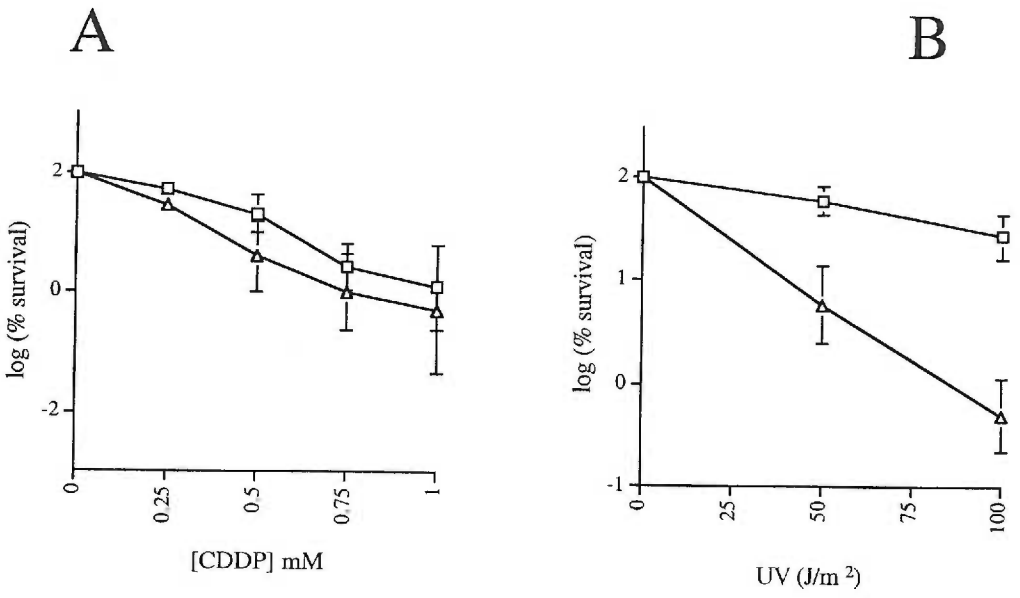
Results

Sensitivity of cell cycle and repair mutants to cisplatin

To evaluate the requirements of various checkpoint and repair specific genes in the repair of cross-links, we tested the sensitivities of each of the mutant strains with respect to an isogenic parent. The series of strains tested (Table 3) included yeast mutants known to be defective in NER but not in checkpoints, such as *rad1* and *rad14*, as well as checkpoint mutants. Typical results of a survival test are shown in Fig. 4. As an index of relative sensitivity, the slope ratio was obtained by dividing the slope of survival vs. dose of CDDP for the mutant strain by the slope of survival vs. dose of CDDP of an isogenic normal strain (McA'Nulty and Lippard, 1996). The sensitivity of each of the strains to UV-C also was determined to define which strains were specifically sensitive to interstrand cross-links and which were sensitive to intrastrand adducts from UV-C. Those strains displaying only sensitivity to cross-links are more likely to be involved directly in specific cross-link repair pathways.

Several conclusions are apparent from the strains tested. (1) Excision repair was needed for optimal survival. Two nucleotide excision repair (NER) mutants, *rad1* and *rad14*, showed increased sensitivity to CDDP compared to an isogenic strain as previously noted (reviewed in Brendel and Ruhland, 1984). It has been reported that excision repair is needed for repair of cross-links *in vitro* (Zamble *et al*, 1996; Bessho *et al*, 1997). However, it cannot be determined from our data what amount of increased cytotoxicity in the *rad1* or *rad14* mutants is due specifically to failure of repair of interstrand cross-links. (2) The major checkpoint functions encoded by the *MEC* genes and *RAD53* are needed for normal survival after exposure to CDDP. However, the sensitivity of *mec* mutants to CDDP is not as great as it is for UV-C, suggesting the possibility of Mec1 and Rad53 independent checkpoint functions for the assessment of interstrand cross-links. (3) *dun1* mutants, have been shown to be defective in inducing genes in response to DNA damage from UV-C or HU (Zhou and Elledge, 1993). Also, Dun1 appears to function in the

Fig. 4. Survival curves of wild-type (AAA18) -o- and rad17-1 (TWY281) -Δ- strains after CDDP (A) and UV-C (B). The data are the average of 4 trials for CDDP and 3 trials from UV. Bars represent the standard deviation for each point.


A
B
log (% survival)
2
0
-2
0
0.25
0.5
0.75
1
[CDDP] mM
log (% survival)
2
1
0
-1
0
25
50
75
100
UV (J/m 2)

G2 checkpoint as *dun1* mutants display a partial defect in G2 arrest after UV-C (Pati *et al,* 1997). Despite having increased sensitivity to HU and MMS, *dun1* mutants tolerated CDDP normally. Normal survival of *dun1* mutants after cross-link damage implies that increased expression of the genes responsive to induction by Dun1, and/or the checkpoint response requiring Dun1 are not essential for repair of cross-links. (4) Certain recombination functions are needed for normal survival after DNA cross-linking. Both *rad52* and *rad51* mutants showed increased sensitivity to CDDP compared to isogenic strains, with *rad51* mutants being more sensitive to CDDP than to UV. The increased sensitivity of *rad51* mutants to CDDP relative to UV-C suggests a more specific requirement for Rad51 in the repair of interstrand cross-links than the repair of intrastrand adducts. Both strains were sensitive to ionizing radiation (slope ratio of 13.9 for *rad51* and 12.9 for *rad52*) indicating that both Rad52 and Rad51 mediated events are required for the repair of double strand breaks (DSB) as has been reported previously (reviewed in Lee *et al* 1999). This result also confirms that the *rad52* mutant strain that was tested, though not a deletion, was disrupted for DSB repair activity. It may be that Rad52 mediated events are not as essential for cross-link repair as those carried out by Rad51. (5) The *rad9*, *rad17* and *rad24* mutants while clearly UV-C sensitive, show less relative sensitivity to CDDP. These gene products are thought to comprise part of the primary response complex for sensing DNA damage (Lydall and Weinert, 1995), and act in the G1, S-phase and G2 checkpoints (reviewed in Weinert, 1998. Lower sensitivity suggests that the functions of Rad9, Rad17 and Rad24 may not be as essential for cross-link surveillance as Mec1 and Rad53. (6) Two strains, *snm1/pso2* and *rev3/pso2* originally identified in screens for interstrand cross-link sensitive mutants (Ruhland *et al*, 1981; Henriques and Moustacchi, 1980) were also tested for UV-C and CDDP sensitivity. The results show that in each strain, CDDP sensitivity was greater than UV-C light sensitivity indicating that these genes are more critical for the repair of cross-links than for the repair of UV-C damage.

Taken together, the results demonstrate that all checkpoint functions may not be required for DNA cross-link surveillance. Genes from the three yeast epistasis groups for DNA repair (the RAD3/excision repair group, the RAD52/recombination repair group and the RAD6/postreplication repair group (Friedberg, 1988; Prakash *et al.* 1993)) are required for interstrand cross-link repair including, at a minimum, *MEC1, MEC3, RAD51, RAD52, RAD53, RAD1, RAD14, SNM1, REV3, RAD6* and *RAD18* (Henriques *et al.* 1997; Friedberg, 1988; Hartwell *et al.* 1997) (Table 1, Table 3).

Table 3

Sensitivities of mutant strains

Strain	UV[a]	cisplatin[a]
rad9Δ	4.09±1.0(2)	1.74±0.5(2)
rad17-1	4.3±1.5(3)	1.4 ±0.3 (4)
rad24-1	5.4±0.4(2)	1.9±0.5(4)
rad53Δ	4.6±1.1(2)	3.08±1.2(3)
mec1Δ	7.0±0.04(2)	4.14±1.8(3)
mec3-1	5.9±0.6(2)	2.38±0.7(4)
dun1-Δ100	2.7±0.8(7)	1.3±0.2(6)
dun1-3	2.2±0.1(2)	1.3±0.04(2)
rad1Δ	21.39±2.7(2)	5.39±0.74(2)
rad14Δ	22.54±1.0(2)	4.31±0.65(2)
rad51Δ	2.2±1.1(2)	16.3±3.4(2)
rad52	2.8±0.3(2)	2.5±0.1(2)
snm1Δ	1.63±0.5(2)	8.08±0.43(3)[c]
rev3Δ	1.79±0.4(2)	6.23±0.56(6)[c]

[a]number represents the slope ratio. The D37 values for CDDP and UV-C for the A364a background are 0.36mM and 39 J/m^2 respectively. D37 values are 0.20 mM CDDP and 34 J/m^2 UV-C light for the CRY1 background. Numbers in parentheses indicate the number of trials.[c]Performed by Alex Ward

CDDP causes primarily an arrest with G2 DNA content

To examine the effects of CDDP on the cell cycle of wild-type yeast, cells were treated with CDDP and followed through the cell cycle. Cell cycle phase was assessed by flow cytometry and by cell morphology. Exposure of wild-type cells to CDDP led to a G2 arrest, but not an apparent G1 or S-phase delay as measured by FACS analysis or budded cell count (Fig. 5). FACS analysis of cell cycle progression with wild-type cells from either asynchronous cultures or cultures synchronized with α-factor gave similar results (Fig. 6). S-phase after CDDP treatment during α-factor arrest was normal for transit time and there was no accumulation in a peak between G1 and G2 DNA content, indicating no S-phase checkpoint induction. A *mec1* mutant showed a lack of G2/M arrest (Fig. 6), consistent with a role for that gene product in activating a response to cross-links, as with other types of DNA damage (Elledge, 1996; Paulovich and Hartwell, 1995; Paulovich *et al.* 1997; Allen *et al*, 1994; Weinert *et al*, 1994). Interestingly, the *rad9* mutant did not show a complete G2/M delay (Fig. 6), similar to *rad17* and *rad24* mutants (not shown). Apparently, the partial delay is sufficient to permit nearly normal levels of repair and survival without Rad9 function. These results indicate that a G2 arrest is induced by CDDP and that loosing the ability to arrest, as is apparent for *mec1* mutants, correlates with decreased survival (Table 3).

Cisplatin does not induce S-phase delay

A lack of G1 or S-phase delay suggested that DNA interstrand cross-links did not cause an inhibition of replication. To evaluate this further, the effects of continuous exposure to MMS compared to cisplatin were determined (Fig. 7). After release from α-factor, MMS caused a significant S-phase delay, as reported (Paulovich and Hartwell, 1995), while no delay was detectable in cells treated with a continuous dose of CDDP. This observation was true in both synchronized (Fig. 7) and

Fig. 5. Effect of CDDP on synchronized S4 yeast cells. Cells were arrested and held 30 min. in α-factor with or without 1mM CDDP, then released as described in methods. FACS profiles and budded cell plots represent untreated (A, C) and CDDP-treated (B, D) cells. 200 cells were scored for morphology and the percent of each form plotted as non-budded -□-, small budded -◊-, and large budded -○-. Survival was 76% for this treatment.

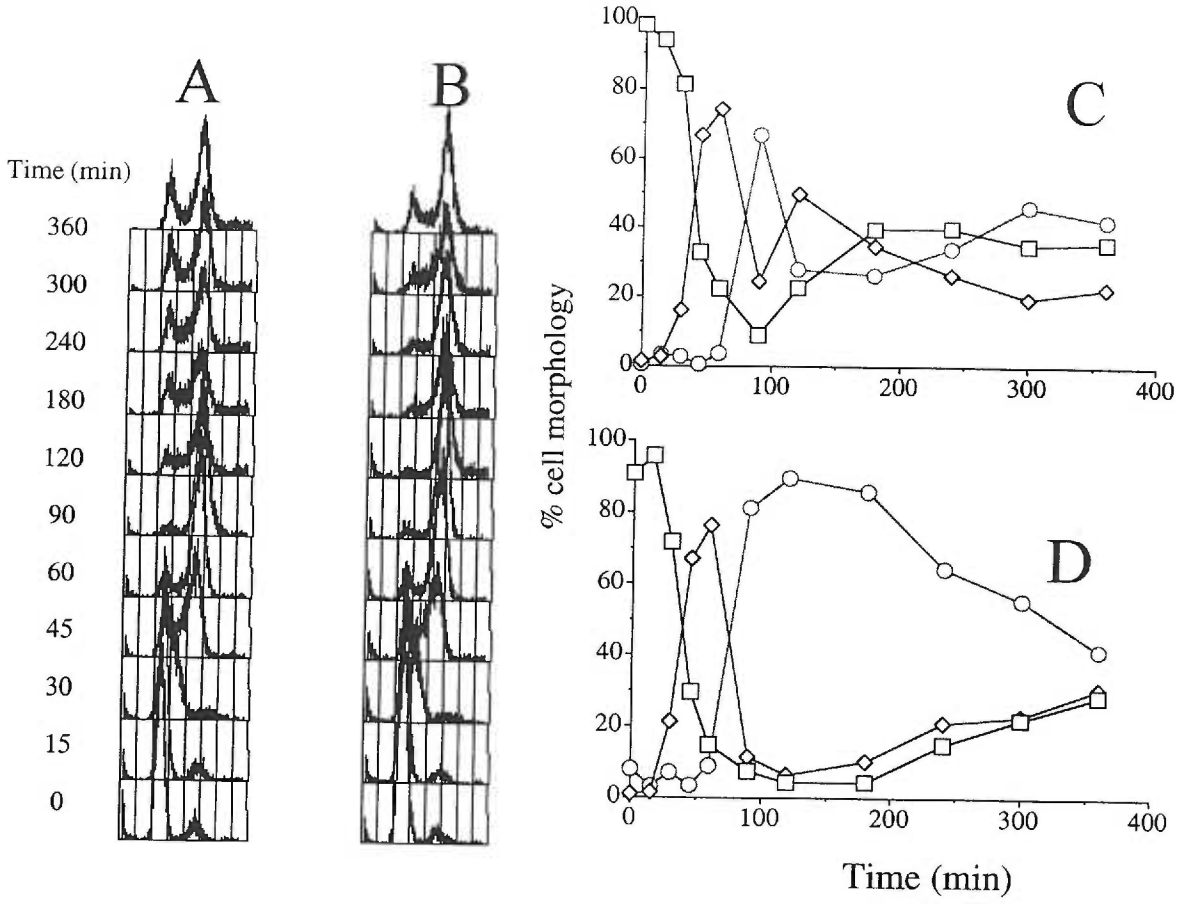

A
B
C
D
Time (min)
360
300
240
180
120
90
60
45
30
15
0
% cell morphology
100
80
60
40
20
0
0
100
200
300
400
Time (min)

Fig. 6. Effects of CDDP exposure on the cell cycle of checkpoint mutants. Asynchronous cultures of wild-type (AAA18), *mec1*-1(TWY177), and *rad9*Δ (TWY398) strains were exposed to 1mM CDDP for 30 min. and time points were taken for FACS analysis as described in Methods. Survival was 88% for AAA18, 73% for TWY177, and 64% for TWY398.

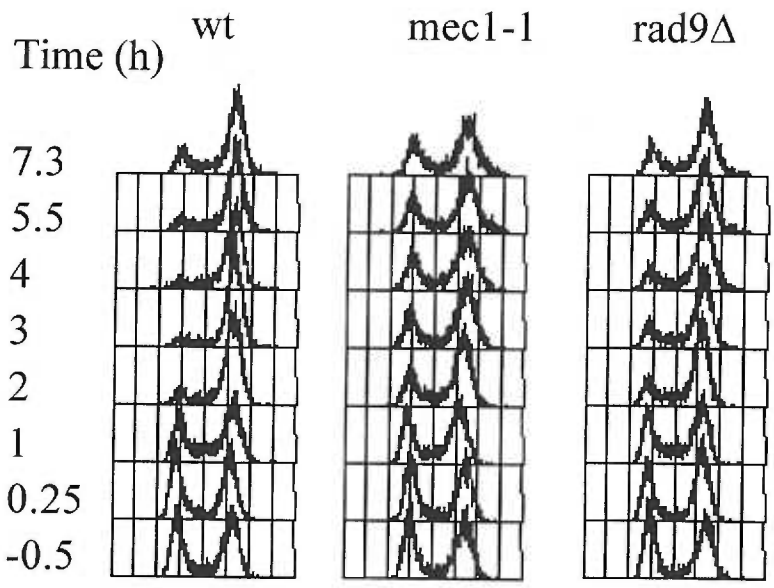

wt
mec1-1
rad9Δ
Time (h)
7.3
5.5
4
3
2
1
0.25
-0.5

Fig. 7. Effects of continuous exposure of CDDP or MMS on a synchronous culture of wild-type (S4) cells as measured by FACS analysis. Cells were arrested with α-factor as described and then released into the presence of MMS or CDDP (A) control, (B) 0.033% MMS, and (C) 250 mM CDDP. Samples taken at 1 hr. after release from α-factor showed survival to be 77% for MMS and 93% for CDDP relative to the control.

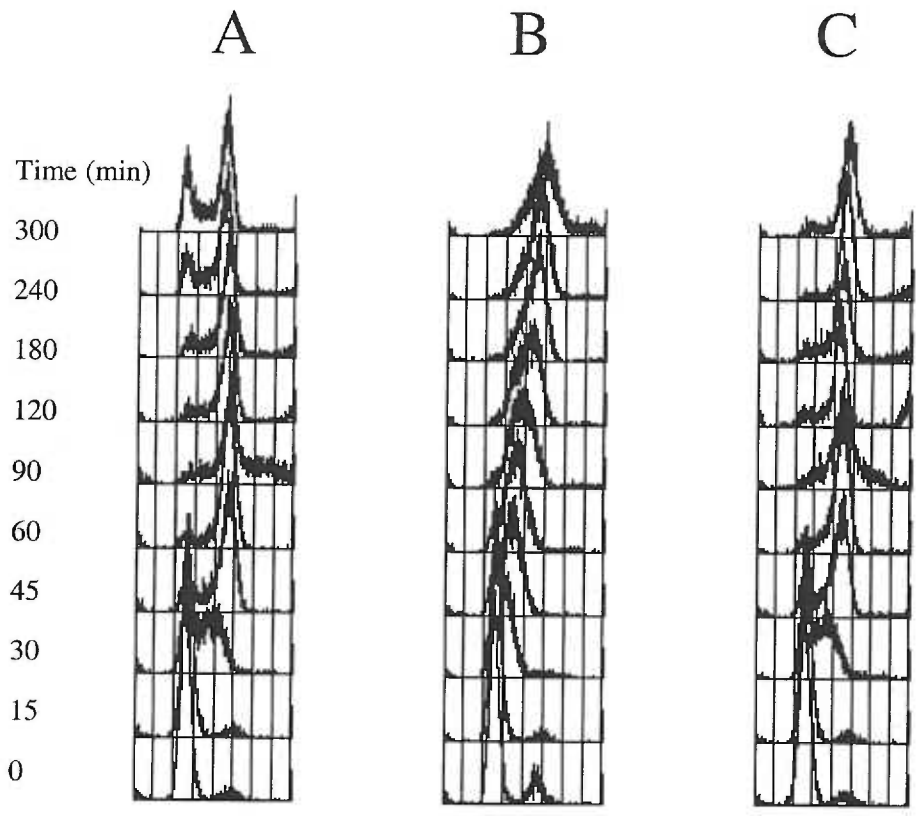
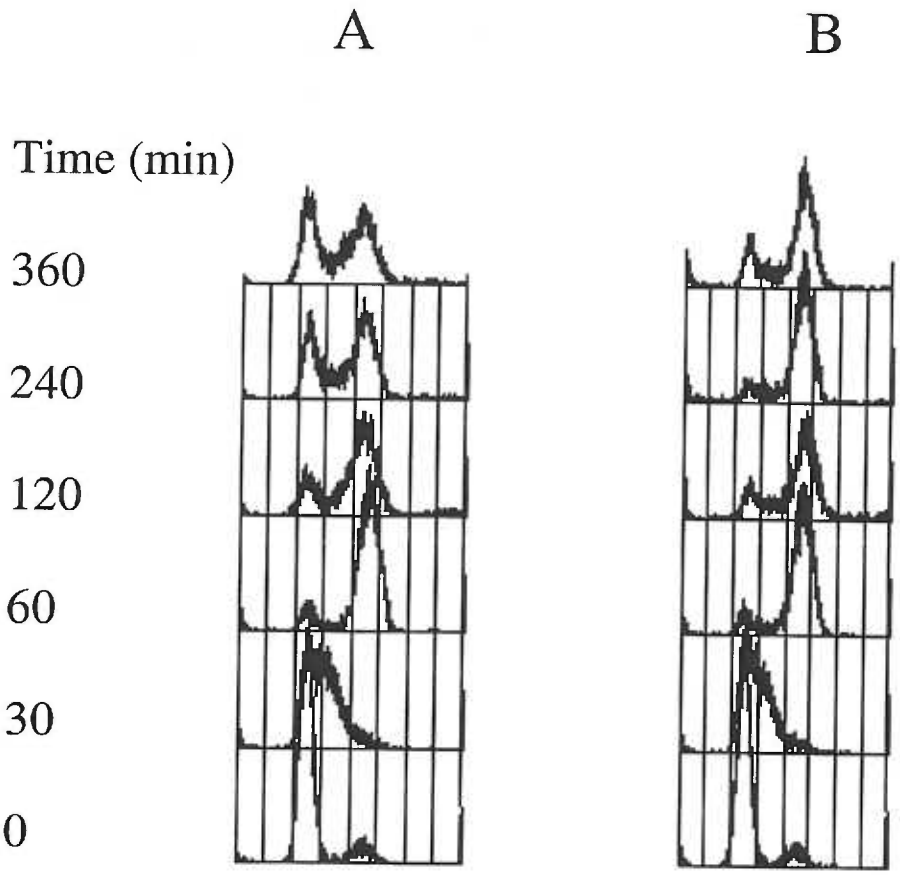

A
B
C
Time (min)
300
240
180
120
90
60
45
30
15
0

Fig. 8. Effect of CDDP on synchronized diploid cells. Strain 8301 untreated (A) or treated (B) with 1 mM CDDP for 30 min. prior to release from α-factor.


A
B
Time (min)
360
240
120
60
30
0

non-synchronized cultures (data not shown).

It may be that repair must proceed to generate the necessary intermediate which could signal a checkpoint dependent arrest. If this were the case, and homologous recombination was the primary repair pathway for interstrand cross-link repair, then a haploid cell would not have homologs until G2/M and might defer repair until after S-phase. Diploid cells, on the other hand, would have a homolog present in G1 phase of the cell cycle and may proceed with repair in G1 or S-phase and thus generate an intermediate which could trigger arrest. To asses the ability of dipoids to transit S-phase with normal kinetics, a diploid strain was arrested in G1 and treated with CDDP. Surprisingly, in synchronized diploid cells damaged in G1, no S-phase delay was noted after release into the cell cycle (Fig. 8). This suggests that repair may not create the necessary intermediate to signal a checkpoint in haploid or diploid cells until G2 DNA content is reached.

Epistasis analysis of SNM1, REV3 and RAD51 with respect to cross-link sensitivity.

As my previous studies indicate, the repair of DNA cross-links in yeast requires the function of genes assigned to three epistasis groups that are primarily assigned by UV-C light (Table 1, Table 3). It may be that cross-links will require different interactions between the pathways as predicted by Cole's model (Fig. 1) which postulates that NER and recombination repair are required sequentially to repair a cross-link. If this were true, genes acting in separate pathways for excision repair may act in the same pathway for recombination repair. One previous study defined such an ambiguity with respect to 8-MOP + UVA sensitivity for *snm1* and *rev3* mutants (Henriques and Moustacchi, 1981). Henriques and Moustacchi (1981) suggest that Snm1 and Rev3 may act in the same pathway to repair interstrand cross-links but not in the same pathway to repair other damage induced by UV. The three genes identified as specifically required for survival after CDDP treatment (*SNM1, REV3* and *RAD51*) (Table 3) were studied to asses whether or not they function in the same pathway to repair interstrand cross-links. This was accomplished

by comparing the phenotype of the double mutants with the single mutants. The double mutants, *snm1Δ rev3Δ, snm1Δ rad51Δ,* and *rev3Δ rad51Δ* were all more sensitive to CDDP than any of the single mutants indicating non-epistasis (Fig. 9, Table 4). *rev3Δ rad51Δ* double mutant strains showed greater than additive sensitivity compared to the other double mutants suggesting that the relationships of these pathways to interstrand cross-link repair may be more complex than simple parallel pathways. It may be that Rev3 and Rad51 compete for a similar substrate as synergistic effects in double mutants are thought to describe competition for a common substrate (Morrison *et al*, 1993). Alternatively, Rev3 and Rad51 may act sequentially, one protein creating substrate for the other (Fig. 10). The triple mutant showed more than additive sensitivity compared to any of the three double mutants confirming that Snm1, Rev3 and Rad51 may be functioning in three different pathways. Additionally, an Snm1 dependent pathway and a Rev3/Rad51 dependent pathway may form separate branches competing for a single intermediate (Fig. 10).

Fig. 9. Epistasis analysis of *SNM1*, *REV3* and *RAD51*. Cells were treated as described in methods.

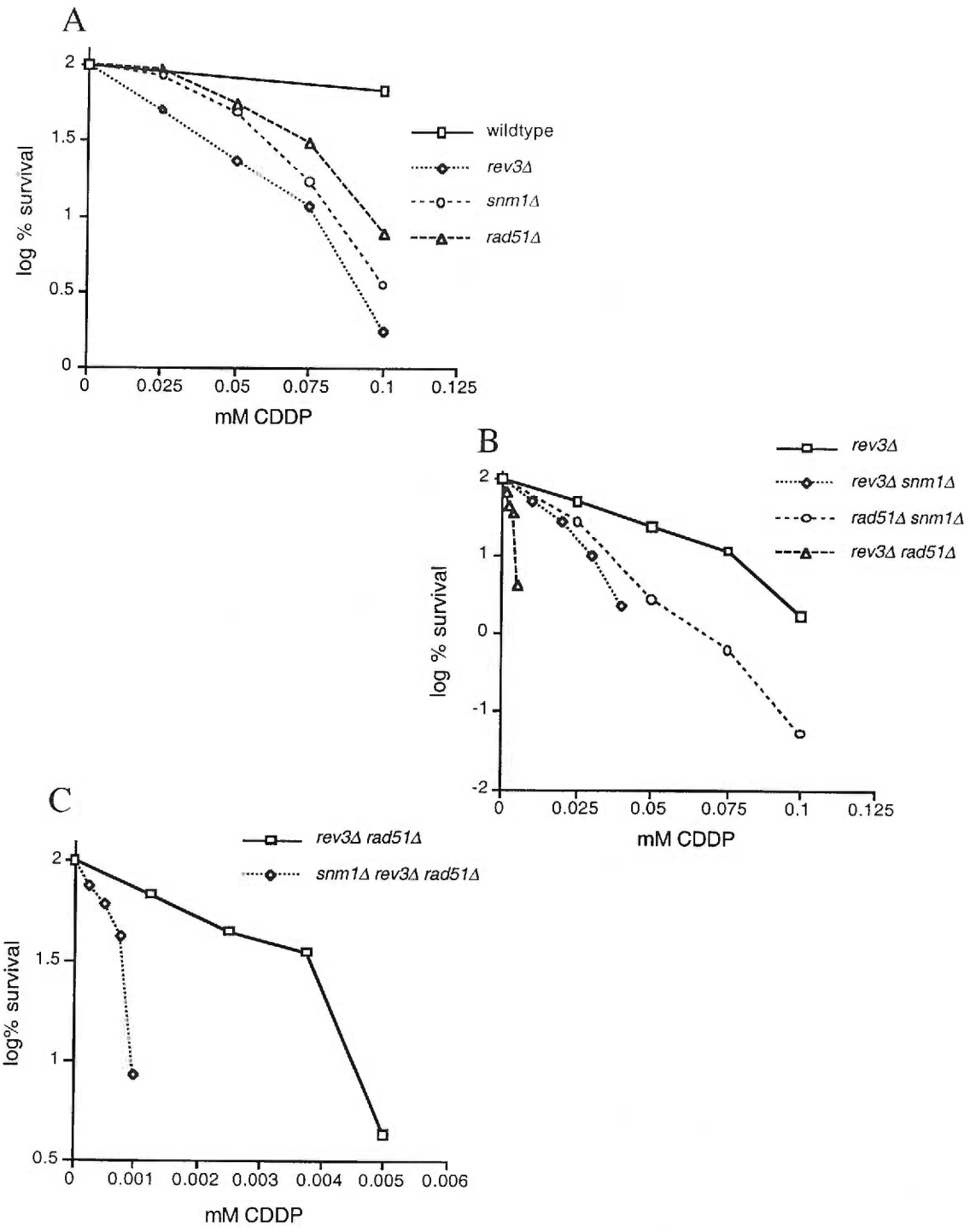
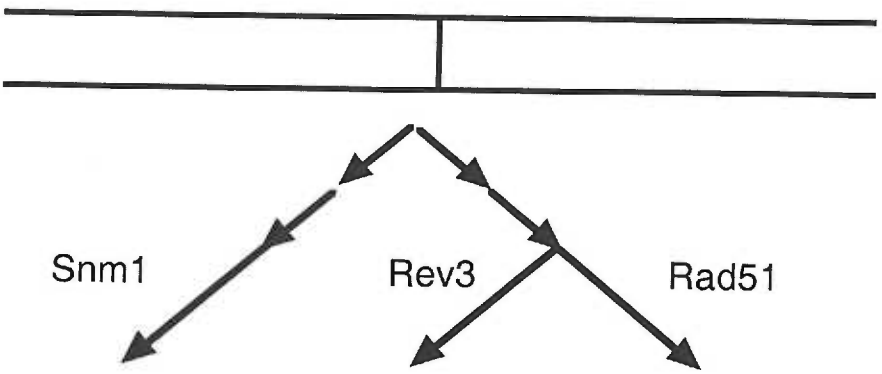

A
log % survival
mM CDDP
wildtype
rev3Δ
snm1Δ
rad51Δ
B
log % survival
mM CDDP
rev3Δ
rev3Δ snm1Δ
rad51Δ snm1Δ
rev3Δ rad51Δ
C
log% survival
mM CDDP
rev3Δ rad51Δ
snm1Δ rev3Δ rad51Δ

Fig. 10. The epistatic interactions suggest that Snm1 Rev3 and Rad51 function in separate pathways. Rev3 and Rad51 may compete for a similar substrate as suggested by the greater than additive sensitivity of the double mutant relative to either single. Snm1 may act in a separate pathway from Rev3 and Rad51 and compete with the Rev3/Rad51 pathway for an upstream intermediate in the repair process. Multiple arrows are drawn leading up to Snm1, Rev3 and Rad51 functions because this pathway may require several steps to generate the intermediate templates required.


Snm1
Rev3
Rad51

Correlation of % survival with interstrand cross-linking levels.

To assess the extent of interstrand cross-links formation at the doses used, cross-link quantitation was done (Fig. 11) (Vos and Hanawalt, 1987) in parallel with survival assays. As expected, interstrand cross-linking increased linearly with dose (Fig. 12). A standard curve based on these data allowed estimation of the number of cross-links present at one lethal hit per cell (D37) in the wild-type and mutant strains (Table 4). While wild-type cells had about 200 cross-links per genome at the D37, approximately 30-50 interstrand cross-links/cell were present at the D37 in the *snm1Δ, rev3Δ* and *rad51Δ* mutants (Table 4). The double mutants tolerated even fewer cross-links (3-14) and the triple tolerated only about one cross-link per genome at the D37 suggesting that the triple mutant represents a complete null phenotype with respect to interstrand cross-link repair.

Table 4

Sensitivities of mutant strains to cisplatin DNA cross-links

Strain	Slope Ratio[a]	D37 (mM CDDP)	# cross-links per genome @D37 ± SD
wildtype	-	0.25	201 ± 122
snm1Δ	6.92	0.052	40 ± 24
rev3Δ	8.03	0.036	28 ± 17
rad51Δ	5.19	0.069	54 ± 32
snm1Δ rev3Δ	19.4	0.016	12 ± 7.3
snm1Δ rad51Δ	15.7	0.019	14 ± 8.7
rev3Δ rad51Δ	118	0.0038	2.9 ± 1.7
snm1Δ rev3Δ rad51Δ	461	0.00078	0.6 ± 0.36

[a]Data for slope ratios obtained by Alex Ward.

Fig. 11. Cross-linking of DNA. Wildtype cells were treated with CDDP and cross-linking of pYES2 was measured as described in methods. Percent crosslinking was as follows: 0.25 mM = 6.25; 0.5 mM = 8.9; 0.75 = 12.7; 1.0 = 19.2.

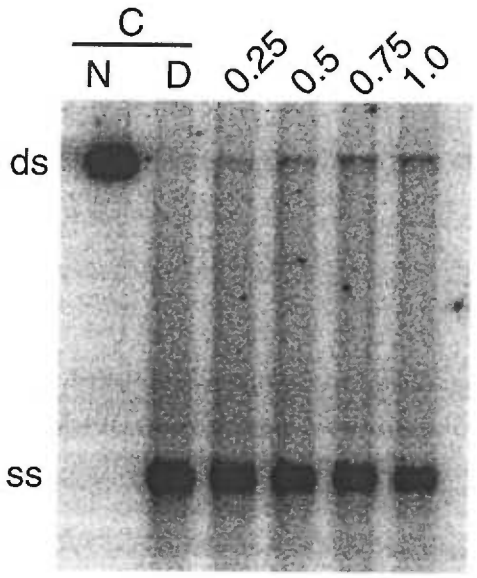
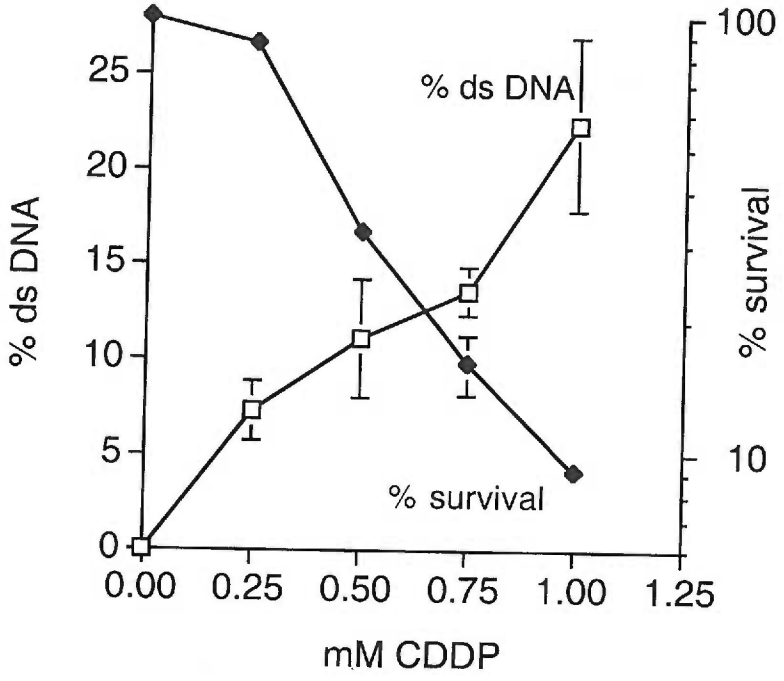

C
N
D
0.25
0.5
0.75
1.0
ds
ss

Fig. 12. Percent survival and cross-linking level as a function of CDDP concentration. Cells were exposed for 2 h to various concentrations of CDDP. Following treatment DNA was isolated for cross-link quantitation and cells were analyzed for survival. The % ds DNA is the percentage of ds DNA of the pYES2 plasmid obtained from a denaturing/renaturing Southern blot. Data represent two independent experiments. Error bars represent standard deviation at each dose.

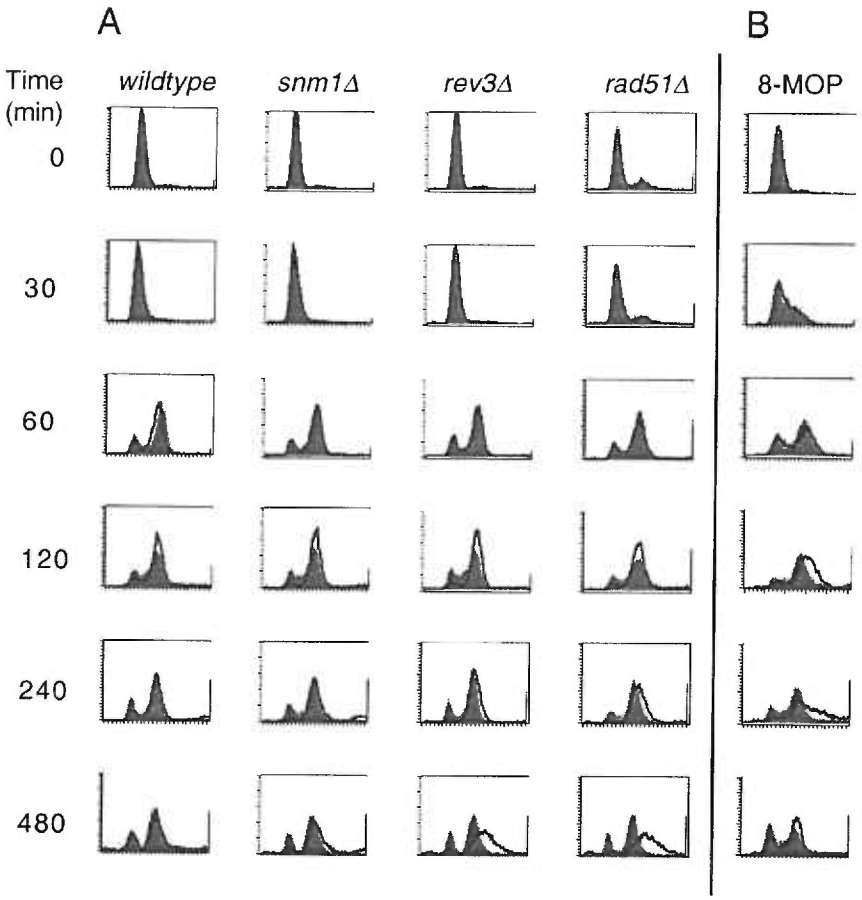

% ds DNA
25
20
15
10
5
0
100
10
% survival
% ds DNA
% survival
0.00
0.25
0.50
0.75
1.00
1.25
mM CDDP

To determine the extent of cross-linking per cisplatin adduct formed, atomic flame mass spectroscopy was done on DNA from CDDP-treated cells. By comparing the total platinum content of the DNA obtained from mass spectroscopy to the number of interstrand cross-links per DNA molecule as determined by denaturing/renaturing southern blots (Vos, 1987), it was found that 1.5 % (±0.4) of the platinum-DNA adducts were interstrand cross-links. Other lesions are likely to be monoadducts and intrastrand cross-links (Fig. 1). This amount of inter-strand cross-linking per platinum molecule is close to values obtained by others (reviewed in Lippert, 1999). Our results demonstrate that, at the D37 of wild-type cells, there are about 100-300 interstrand cross-links (Table 4) and 7,000 to 20,000 other adducts. This number of CDDP interstrand cross-links present at the D37 is significantly higher than the number of 8-MOP interstrand cross-links at the same level of killing as reported by others in different strain backgrounds (Henriques *et al*, 1997). It may be that 8-MOP cross-links are more toxic to cells or that the strain background tested in the earlier studies is more sensitive to cross-link damage.

Wild-type, snm1Δ, rev3Δ and rad51Δ cells demonstrate normal S-phase kinetics after CDDP treatment.

G1-synchronized wild-type cells treated with cisplatin and released into the cell cycle arrest primarily with G2 DNA content (Fig. 5). That there is no detectable S-phase or G1 delay following cross-link damage is surprising - other types of damage (UV-C and MMS) elicit the G1 and S checkpoints at survival levels similar to those reached with CDDP (Siede *et al*, 1993; 1994; 1996; Paulovich *et al*, 1995, 1997). Cells might accommodate cross-link damage during replication and defer completion of repair until the G2 phase of the cell cycle. To test whether a mutant defective in cross-link repair would show a normal S-phase transit after CDDP, we monitored the cell cycle response of a strain with a deleted *SNM1* gene. The cells were arrested in G1 with α-factor and exposed to a dose of cisplatin known to cause a reversible G2 arrest in wild-type cells. At this dose *snm1Δ* cells were less than

Fig. 13. Cell Cycle progression of DNA repair mutants following cisplatin damage. A. Cells were arrested in G1 with α-factor. Following arrest they were treated with 0.5 mM CDDP for 1h. and released into the cell cycle (solid lines). Untreated controls were worked up in parallel (shaded histograms). Fractions were taken for cell cycle analysis at various times. Percent survival was: wild-type > 90%, *snm1Δ* = 20%, *rev3Δ* = 12% and *rad51Δ* = 8%. B. Wild-type cells were arrested as described above and exposed to 0.1 µg/ml 8-MOP for 15 min prior to irradiation with UVA light. Percent survival for this experiment was > 90%. The peak shifting observed in the later time points is the result of mitochondial DNA replication (observed by DAPI staining of cells).


A
B
Time (min)
wildtype
snm1Δ
rev3Δ
rad51Δ
8-MOP
0
30
60
120
240
480

10% viable. Similar to wild-type cells the *snm1Δ* cells exhibited no cell cycle delay until reaching G2 (Fig. 13A). Unlike the wild-type cells, however, the *snm1Δ* cells remained arrested in G2 suggesting that while Snm1 is not necessary for normal progression through S-phase following cross-link damage, it is required for exit from the G2 checkpoint.

Rev3 is known to function as an error-prone DNA polymerase which can bypass DNA damage during replication (Nelson *et al*, 1996). It is possible that such a polymerase would be required for normal replication kinetics in the face of cross-link damage. Consistent with a role in cross-link processing, *rev3(pso1)* mutants were identified in a screen for cross-link sensitive mutants (Henriques and Moustacchi, 1980). A *rev3Δ* mutant isogenic to the *snm1Δ* mutant was constructed to test cell cycle progression in the presence of interstrand cross-links. Cells were arrested in G1, treated with cisplatin and released for cell cycle analysis. The *rev3Δ* strain, like *snm1Δ* proceeded normally through S-phase and arrested in G2 (Fig. 13A). Therefore Rev3 is not required for normal replication kinetics after CDDP exposure.

Recombination is known to be a key process in cross-link repair. It is possible that repair of cross-links may occur before or during S-phase to allow normal replication kinetics. Should recombinational repair be disrupted, S-phase progression may be inhibited. We therefore tested the ability of a recombination repair deficient strain, *rad51Δ*, to transit S-phase after treatment with CDDP. The *rad51Δ* mutant replicated DNA at the normal rate, even though manifesting marked sensitivity to CDDP, and experienced a terminal G2 arrest following CDDP damage in G1 (Fig. 13A). The results indicate that the activities of Snm1, Rev3 and Rad51 are not required for normal progression through S-phase following CDDP damage, and that the mutants arrest in G2 as a result of being unable to repair CDDP damage.

Psoralen interstrand cross-links also do not elicit a G1 or S-phase delay and cause only a G2 checkpoint.

To investigate whether the findings with cisplatin were also true for other DNA interstrand cross-linking agents, the cell cycle response of wild-type cells to 8-MOP + UVA was tested. Cells were arrested in G1 with α-factor, exposed to 8-MOP + UVA while still arrested and monitored using flow cytometry after release into the cell cycle. A dose of 8-MOP + UVA giving approximately 70% survival in wild-type cells induced only a G2 delay; no G1 or S-phase delay was observed (Fig. 13B). This finding is consistent with the observations for CDDP (Fig. 13A, Fig. 5) and enhances the evidence that cross-links are bypassed and elicit a cell cycle checkpoint in G2.

Saturation of cross-link accommodation during S-phase.

The fact that cells can transit the S-phase of the cell cycle in the presence of cross-link damage in both wild-type and repair deficient cells indicates that there exists a mechanism for allowing replication to proceed in the presence of interstrand cross-links. It could be that a polymerase is capable of replicating past a cross-link by switching strands or that at this level of cross-linking, bidirectional replication can proceed up to the cross-link. Whatever the mechanism, the cell can accommodate cross-links during replication. However, this mechanism might become saturated at high levels of interstrand cross-linking. Therefore the ability of cells to pass through the cell cycle normally in the presence of higher levels of interstrand cross-link damage was examined. G1-arrested cells were exposed to varying concentrations of CDDP for 1 h prior to release. Following release into the cell cycle, cells were harvested and analyzed by flow cytometry. At high levels of CDDP damage (< 40% survival), progression from G1 to G2 DNA content was slowed (Fig. 14A). Most of this delay is accounted for by a delay in S-phase, though some delay in entry into S-phase is observed. The threshold of dose at which a significant amount

of S-phase delay is observed is 1.5 mM CDDP for 1 h (Fig. 14A). This dose caused 300-800 cross-links per 14,000 kb genome or 1 - 2 cross-links per 36 kb replicon (Guthrie and Fink, 1991) (Fig. 15). The results suggest that there may be a saturable accommodation mechanism that allows the cell to have normal replication kinetics in the presence of cisplatin DNA damage and levels of lethality approaching 60% cell death. This observation, in conjunction with the normal replication kinetics observed in the mutant cells (Fig. 13A), indicates that the S-phase delay, or lack of it, is based on the degree of cross-linking and not survival or the ability to repair cross-links as the *snm1Δ*, *rev3Δ* and *rad51Δ* mutants had normal replication kinetics despite high lethality.

To assess the effect of an alternate cross-linking agent, G1-arrested cells were exposed to high levels of 8-MOP damage. A similar delay in S-phase progression was observed suggesting that S-phase delay after high levels of cross-linking is a general response for cross-link damage (Fig. 14B) induced by both CDDP and 8-MOP + UVA treatment.

Delay of S-phase is dependent on MEC1 function.

The S-phase delay observed in heavily damaged wild-type cells could either be the physical inhibition of replication by cisplatin lesions and/or the function of a S-phase checkpoint. *mec1-1* mutants are defective in the MMS-induced S-phase checkpoint (Paulovich *et al*, 1995; 1997). To determine whether a checkpoint was contributing to S-phase delay, the S-phase kinetics of *mec1-1* mutants with high levels of CDDP damage were tested. Wild-type and *mec1-1* cells were arrested with α-factor, treated with 2 mM of CDDP for 1 h and released. Whereas both wild-type and *mec1-1* cells showed a delay of entry into S at this dose, the *mec1-1* mutants did not show a delay in S-phase progression (Fig. 16). Replication progressed within 60 min in treated *mec1-1,* cells which is equivalent to replication

Fig. 14. Effect of high CDDP doses on cell cycle progression. Treated cultures are represented by solid lines and untreated controls are represented by shaded histograms. A. wild-type cells were arrested in G1 with α-factor. Following arrest, cells were exposed to varying doses of CDDP for 1 h. They were then released into the cell cycle and fractions were taken for FACS analysis. Percent survival was as follows: 0.5 mM CDDP = 76%; 1.0 mM CDDP = 35%; 1.5 mM CDDP = 19%; and 2.0 mM CDDP = 12%. B. Wild-type cells were arrested as in A. Cells were exposed to 0.4 µg/ml 8-MOP prior to irradiation with UVA light. Percent survival for this experiment was 7.6%. The peak shifting observed in the later time points is the result of mitochondial DNA replication (observed by DAPI staining of cells).

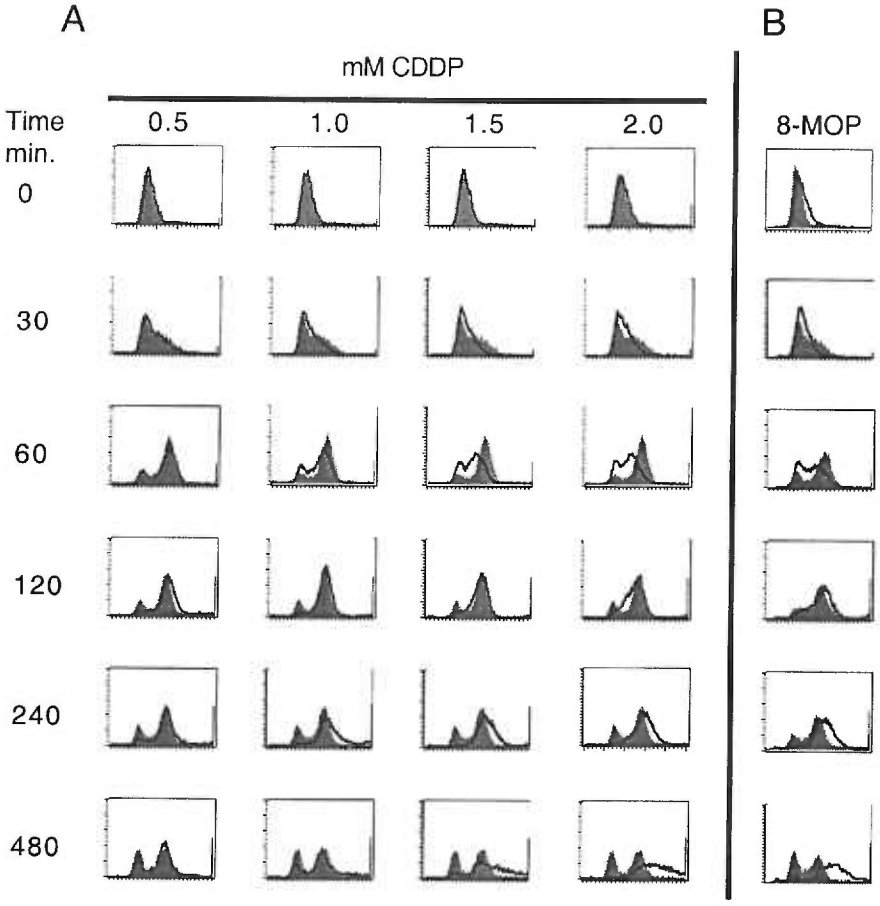

A
B
mM CDDP
Time
min.
0.5
1.0
1.5
2.0
8-MOP
0
30
60
120
240
480

Fig. 15. Quantitation of cross-linking levels in CDDP treated cells. Southern blot of plasmid DNA from treated and untreated cells. Control lanes N and D stand for native and denatured respectively. CDDP treated lanes correspond to same doses as in Fig. 14A.

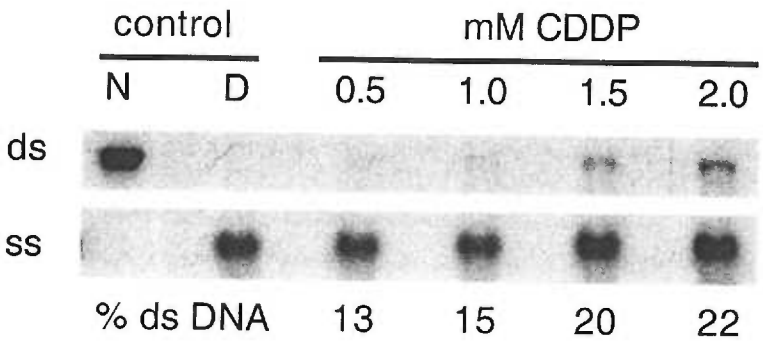
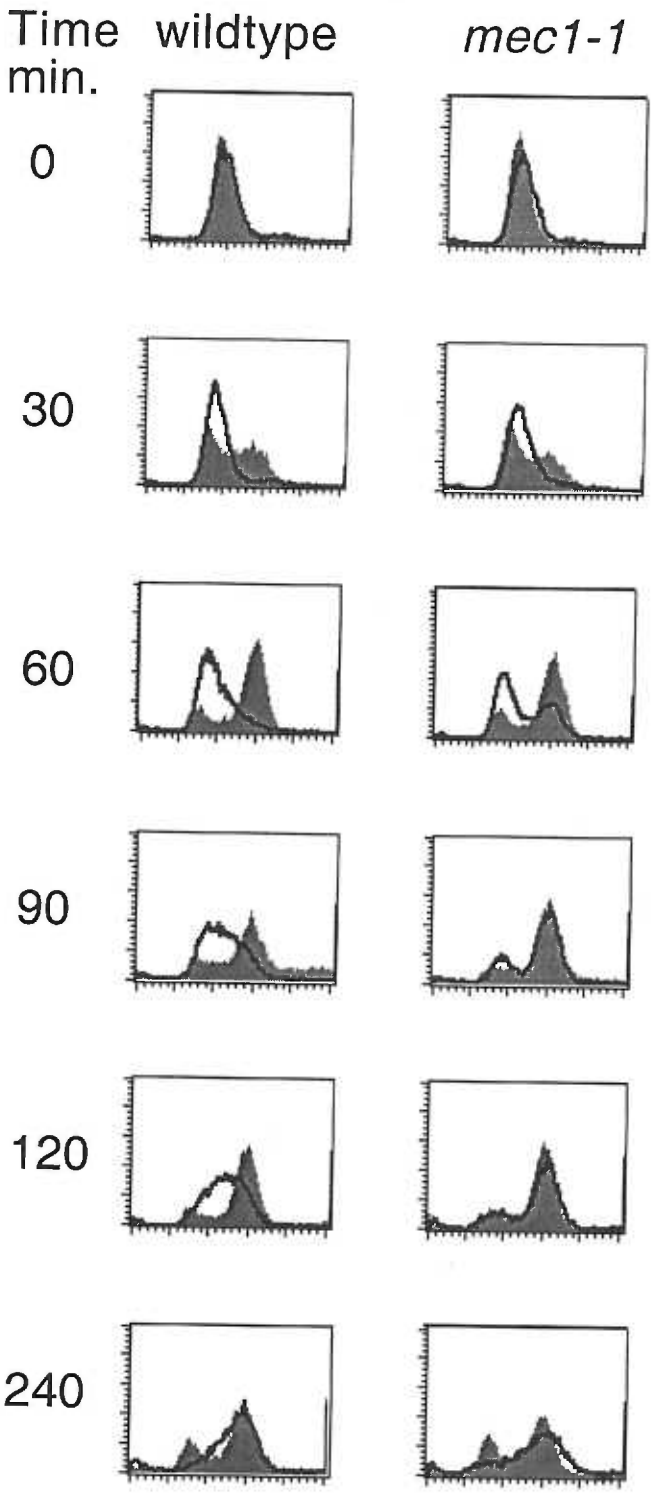

control
mM CDDP
N
D
0.5
1.0
1.5
2.0
ds
ss
% ds DNA
13
15
20
22

Fig. 16. Cell cycle progression in wild-type and *mec1-1* cells following exposure to high levels of CDDP. Cells were first arrested in G1 with mating pheromone. They were then treated with 2 mM CDDP for 1 h prior to release into the cell cycle. Fractions of the culture were taken for FACS analysis at various times. Treated cells are represented by solid lines and untreated cells are represented by shaded histograms. Percent survival for this experiment was: wild-type = 2%, *mec1-1* < 0.15%.


Time
min.
wildtype
mec1-1
0
30
60
90
120
240

times observed in untreated cells. The findings demonstrate that the intra-S-phase delay observed at high cross-linking levels was significantly dependent on a Mec1 checkpoint function.

snm1Δ and rev3Δ mutants incise CDDP DNA cross-links normally.

The incision of cross-links induced by 8-MOP + UVA in yeast has been demonstrated by a variety of techniques (Jachymczyk *et al*, 1981; Miller *et al*, 1982; Magaña-Schwenke *et al*, 1982; Meniel *et al*, 1995a, 1995b, 1997). Here, wild-type, *snm1Δ* and *rev3Δ* strains were tested for ability to incise CDDP DNA interstrand cross-links with a denaturing/renaturing Southern blot technique (Vos and Hanawalt, 1987). This allowed quantitation of the amount of interstrand cross-linked DNA, since only duplexes with interstrand cross-links would remain double stranded after denaturation. Based upon this assay, wild-type, *snm1Δ* and *rev3Δ* cells were able to incise DNA at similar rates as shown by the disappearance of dsDNA over time (Fig. 17). The finding that *snm1Δ* and *rev3Δ* mutants incise CDDP interstrand cross-links, but have increased sensitivity to CDDP, suggests that the functions of Snm1 and Rev3 are not required for initial incision of interstrand cross-links, but are needed for subsequent repair steps.

Cisplatin induces DRE-dependent genes

Transcriptional induction of repair genes is an important aspect of checkpoint function. Surprisingly, our survival data revealed that *dun1* mutants had normal survival after interstrand cross-linking suggesting that transcriptional induction was not necessary for survival. To confirm that there was indeed a defect in transcriptional induction of repair genes in this mutant, the induction of a DRE-element containing reporter construct was measured (Zhou and Elledge, 1993) after treatment with CDDP. The ribonucleotide reductase gene, *RNR3*, has a regulatory region containing a DRE which responds to DNA damage (Elledge and Davis, 1989;

Wolter et al, 1996). CDDP induced expression of the reporter gene in wild-type cells, but the expression was absent in *dun1* cells (Fig. 18) as expected (Wolter *et al.* 1996). Therefore, it appears that the genes up-regulated by the Dun1 protein are not needed at increased levels for normal survival after DNA cross-links.

Fig. 17. Incision of cross-links in repair deficient cells. Asynchronous cells were exposed to 0.75 mM CDDP for 2.5 h. DNA was prepared for Southern blotting as described in methods. Lanes N and D represent untreated wild-type DNA which was either nondenatured or denatured. The % ds DNA was calculated as described in methods. % survival for this experiment was: wild-type = 24%, *snm1Δ* = 0.02% and *rev3Δ* = 0.18%.

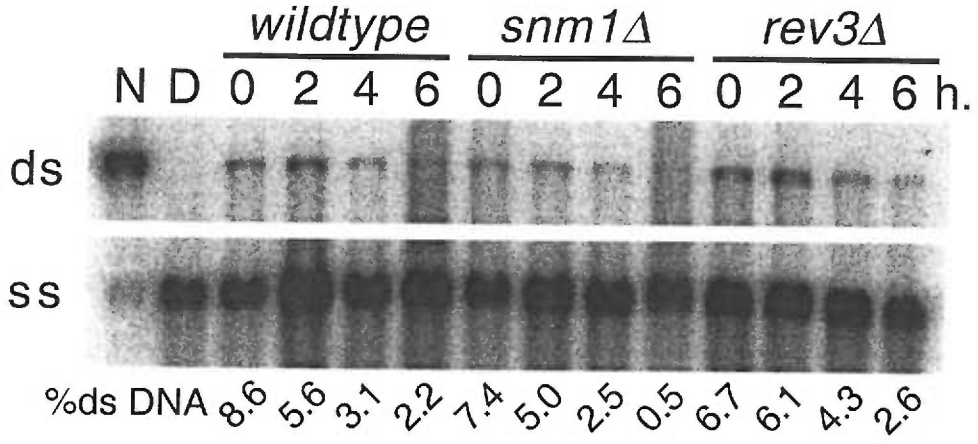
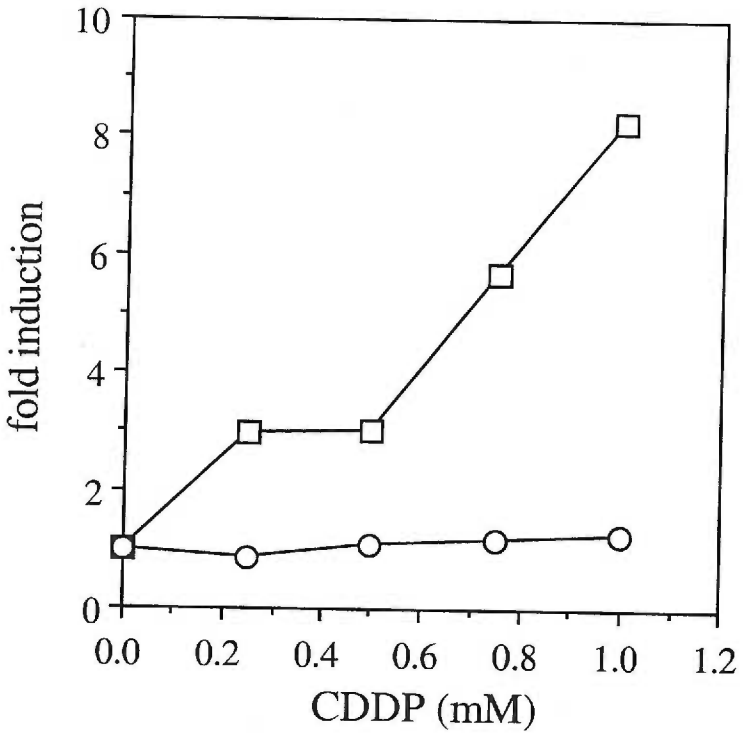

wildtype
snm1Δ
rev3Δ
N D 0 2 4 6 0 2 4 6 0 2 4 6 h.
ds
ss
%ds DNA 8.6 5.6 3.1 2.2 7.4 5.0 2.5 0.5 6.7 6.1 4.3 2.6

Fig. 18. Induction of a DRE in response to CDDP in wild-type (S4) -□- and *dun1* (KGY117) -○- cells. β-galactosidase activity was measured as an index of induction as described in methods.


10
8
6
4
2
0
fold induction
0.0
0.2
0.4
0.6
0.8
1.0
1.2
CDDP (mM)

Discussion

Sensitivity of repair and checkpoint mutants and epistasis analysis with respect to interstrand cross-link damage

The results presented here confirm that components of the RAD3/excision repair group, the RAD6/postreplication repair group, and the RAD52/recombination repair group are required for survival following treatment with cross-linking agents. The genes required for survival include checkpoint genes *MEC1*, *MEC3* and *RAD53*, excision repair group genes *RAD1*, *RAD14* and *SNM1*, recombination repair group genes *RAD51* and *RAD52* and postreplication repair group genes *RAD6*, *RAD18* (Hartwell *et al*, 1997), and *REV3*. It is surprising that the *rad51* mutants are highly sensitive to γ-IR and CDDP while *rad52* mutants are highly sensitive to γ-IR but not CDDP. Two possibilities may explain this finding. First, Rad51 could carry out a function that is more central to cross-link repair than Rad52. One function that is unique to Rad51 is recombination with regions of chromatin that are otherwise inaccessible (Paques and Haber, 1999 and references therein). It may be that this process is important for cross-link repair. Alternatively, disruption of Rad51 may unmask a deleterious cross-link processing pathway. It is possible that disruption of the deleterious pathway would rescue the cross-link sensitive phenotype of *rad51* mutants. A screen for suppressor mutants resistant to DNA cross-links in a *rad51* mutant background may reveal which genes are involved in such a pathway.

Interestingly, some mutants tested (*RAD51*, *REV3* and *SNM1*) showed increased sensitivity to CDDP relative to UV. It is possible that *RAD51*, *REV3* and *SNM1* represent three possible pathways for the repair of cross-links as they fall into separate epistasis groups with respect to cross-link sensitivity. Furthermore, the triple mutant displays greater sensitivity than any of the doubles and tolerates only 1 interstrand cross-link per genome at the D37 suggesting a complete null phenotype for interstrand cross-link repair. It is interesting to note that the double mutant *rad51*

rev3 displayed greater sensitivity than the *snm1 rad51* or the *snm1 rev3* doubles. It may be that Rad51 and Rev3 compete for a similar substrate (Fig. 10). However, the possibility that Rev3 and Rad51 may be acting in a more linear pathway with one protein creating substrate for the other cannot be excluded. The triple mutant also displayed a synergistic interaction. It is possible that Snm1 defines one pathway acting on a similar substrate and a parallel pathway involving Rad51 and Rev3 or in a linear sequential pathway feeding into Rev3 and Rad51 dependent functions (Fig. 10).

Cell cycle checkpoints after cross-link damage

Wild-type cells that are synchronized in the G1 phase of the cell cycle with α-factor and treated with DNA cross-linking agents do not arrest in G1 or S-phase. This result is in contrast to the G1 arrest observed after UV-C treatment of cells in G1 (Siede et al, 1993, 1994, 1996) or the S-phase delay observed after constant MMS treatment (Paulovich *et al*, 1995, 1997) at similar levels of toxicity. *snm1Δ, rev3Δ* and *rad51Δ* mutants also show no delay in G1 or S-phase at similar doses of CDDP, despite a high level of killing. Therefore survival is unrelated to the normal S-phase progression (as determined by DNA content) observed following cross-link damage. Yeast cells apparently possess mechanisms allowing normal replication kinetics, even prior to death, in the presence of irreparable DNA interstrand cross-links.

Apparently *REV3* is not essential for the bulk of S-phase replication in the presence of CDDP-induced DNA damage. If the Rev3/Rev7 DNA polymerase does play a role, it may synthesize only short patches of DNA during the repair of cross-links (Fig. 19) and therefore may not be essential for normal S-phase kinetics. The results reported here are consistent with the results obtained by Paulovich and coworkers (1997) demonstrating that the S-phase delay following MMS treatment of asynchronous cells is not more severe in *rev3Δ* mutants than in wild-type strains following treatment with MMS. If Rev3 were required for the damage-resistant DNA

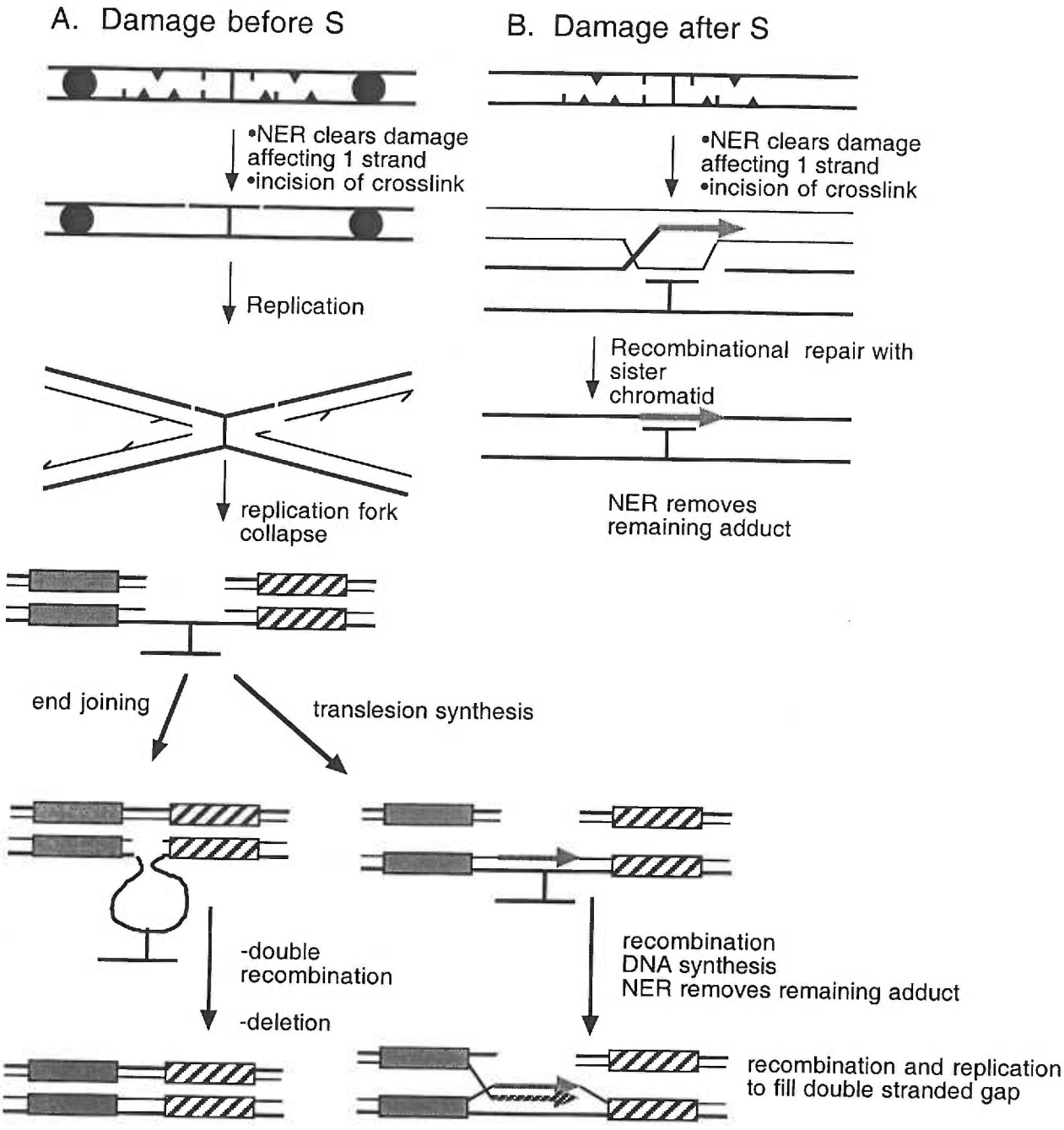
synthesis, then S-phase would be slower in *rev3* mutants.

Rad51 is known to be involved in many recombination pathways (reviewed in Paques and Haber, 1999). Its primary role appears to be the strand invasion step of recombination. It seems clear from my studies that the process of *RAD51* dependent recombination is dispensable for normal replication kinetics in the presence of cross-link damage. The results suggest that Rad51 may act to repair interstrand cross-links after S-phase. Rad51 may be necessary for repairing double strand breaks (DSB) occurring following cross-link damage (Jachmczyk *et al*, 1981) (Fig. 19). *SNM1* also seems to have a role after S-phase, possibly representing an alternate pathway for repairing the DSB intermediate as the incision step in *snm1* mutants is normal (Magaña-Schwencke *et al*, 1995, this thesis). The prolonged arrest in G2 following cross-link damage suggests that the cell is attempting repair or assessing repair in this phase of the cell cycle and cannot complete repair without the functions of Snm1, Rad51 and Rev3.

What triggers the checkpoint after cross-link damage?

The work presented here suggests that interstrand cross-links themselves do not elicit a checkpoint directly, but rather the act of either replicating past or repairing the cross-links generates an intermediate triggering the checkpoint when sufficient numbers accumulate. Possible intermediates that would signal a checkpoint include patches of single stranded DNA (Garvic *et al*, 1995; Lydall and Weinert, 1995) and/or DSBs (Lee *et al*, 1998). Single stranded gaps could be caused by bypass replication events (such as observed for UV-C light (Prakash, 1981)) or by repair on DNA cross-links. DSBs could be generated by replicating up to ss nicks (Kuzminov, 1995) (Fig. 19) or during the process of repairing cross-links (Jachymczyk *et al*, 1981; Magaña-Schwencke *et al*, 1982). Other lesions that CDDP produces such as monoadducts and intrastrand cross-links may readily be repaired by excision repair which is capable of acting in G1 (Siede *et al*, 1994).

Fig. 19. Model for replication in the presence of interstrand DNA cross-links in yeast. Large circles represent replication origins. Small triangles and short lines represent intrastrand adducts and monoadducts respectively. The interstrand cross-link is represented by the vertical line in the center of the replicon. Shaded boxes represent regions of homology. Following CDDP damage in G1, NER (nucleotide excision repair) clears monoadducts and intrastrand cross-links from the template. Incision of the cross-link may also occur. Replication proceeds until a single stranded nick is reached. The replication fork then collapses leaving an unrepaired DSB. The DSB is then repaired by pathways which may require additional recombinational and incisional events. Snm1 may act to restore the DSB while Rev3 may be required to replicate past the monoadduct left after replication fork collapse. Rad 51 may be required for some of the recombination events illustrated.


A. Damage before S
B. Damage after S
•NER clears damage affecting 1 strand
•incision of crosslink
Replication
replication fork collapse
•NER clears damage affecting 1 strand
•incision of crosslink
Recombinational repair with sister chromatid
NER removes remaining adduct
end joining
translesion synthesis
-double recombination
-deletion
recombination
DNA synthesis
NER removes remaining adduct
recombination and replication to fill double stranded gap

Studies with CDDP-treated DNA show that cell extracts lacking various NER proteins are deficient in repair synthesis suggesting that NER acts on some types of CDDP lesions (Wang *et al*, 1993).

Delay of S-phase after high cross-linking levels

In response to high levels of CDDP and 8-MOP damage cells are delayed in S-phase. The S-phase delay is ablated in checkpoint deficient *mec1-1* mutants. This Mec1-dependent component of the S-phase delay may be elicited by the accumulation of other lesions made by CDDP and 8-MOP because only about 1 out of 100 lesions is an interstrand cross-link in CDDP treated cells. NER may be saturated, leaving many interstrand adducts and monoadducts to be repaired later in the cell cycle or tolerated in S-phase (Paulovich *et al*, 1998). Nucleotide excision repair (NER)-deficient *rad14Δ* cells remain in early S following low levels of UV-C damage in G1 (Siede *et al*, 1994; Neecke *et al*, 1999). Low doses of CDDP might elicit the same response in a NER deficient strain. Recently it has been shown that the early S-phase delay in *rad14Δ* cells is dependent on the functions of Mec1 and Rad53 (Neecke *et al*, 1999). Mec1 and Rad53 appear to delay S-phase by signaling the late origins of replication to delay firing (Neecke *et al*, 1999). It is possible that high levels of CDDP also inhibit late origin firing in a Mec1 and Rad53 dependent fashion. This could be tested by measuring the timing of firing for early and late origins (measured by 2-D gels as performed by Neecke *et al*, (1999)) after CDDP treatment in G1-arrested cells.

Accommodation of damage in S-phase - future studies

It is surprising that DNA replication proceeds with normal kinetics at high levels of CDDP damage in *mec1* mutants. This finding underscores the possibility that mechanisms exist for damage resistant DNA replication. To better understand this phenomena, defined substrates with interstrand cross-links could be used in primer

extension assays carried out with yeast cell extracts. If extracts are capable of synthesis in the presence of cross-links, biochemical fractionation of the activity would be one approach to identifying the necessary components. Alternatively, purified candidate proteins could be used to reconstitute activity (Vaisman *et al*, 1999).

Strand switching may be required for synthesis past cross-link lesions. In the haploid yeast system, a cross-linked template would have no homologous sequence to switch to in replication past an interstrand cross-link. It may may be that strand switching can occur by switching to sequences with less or no homology to the damaged sequence. A plasmid system has been developed to assess such synthesis. In fact, RecA protein and other *E. coli* proteins can switch strands and replicate (Morel *et al*, 1997).

The function of SNM1, REV3 and RAD51

Studies on the interstrand cross-link repair process in yeast are limited. Incision of cross-links has been described for specific targets (Meniel *et al*, 1995) and the whole genome (Jachymczyk *et al*, 1981; Magaña-Schwencke *et al*, 1982). Studies have shown that Rad1, Rad2 and Rad3 (Jachymczyk *et al*, 1981; Magaña-Schwenke, 1995; Meniel, 1995) but not Snm1 (Magaña-Schwenke *et al*, 1982, Meniel *et al*, 1995, this study), Rad52 (Meniel *et al*, 1995) or Rev3 (this study) are required for normal incision kinetics of cross-links. Repair of DSBs that accumulate after cross-link damage requires Snm1 (Magaña-Schwencke, 1995) and Rad51 (Jachymczyk *et al*, 1981; Magaña-Schwencke, 1997). It is possible that the DSB is not the result of repair of DNA cross-links but is rather the result of replicating incised DNA cross-links (Fig. 19). As Snm1 and Rad 51 are in different epistasis groups with respect to repair of cross-link damage, they may function in different pathways to repair DSBs that occur following replication fork collapse (Fig. 19). Following S-phase Rev3 may function to replicate past monoadducts left after replication fork collapse. Our results support a model for Snm1, Rev3 and Rad51 functioning in separate pathways and acting on structures that occur following replication (Fig. 19).

Lesion spectrum after CDDP - What does it tell us about cytotoxicity?

The studies in this thesis show that wildtype yeast have 100-300 interstrand cross-links at the D37. This number is somewhat higher than what has been reported for 8-MOP + UVA (20 interstrand cross-links at the D37 (Henriques *et al*, 1997). The higher number of CDDP lesions measured at the D37 may be due to differences in the strain backgrounds tested. Alternatively, 8-MOP interstrand cross-links may be more toxic to the cell than CDDP interstrand cross-links. If 1.5% of the total platinum lesions are involved in an interstrand cross-link then there are about 7,000-20,000 other platinum DNA lesions present at the D37 in wild-type cells. This data cannot confirm that the interstrand cross-link is the primary cytotoxic lesion. To evaluate this further, the relative toxicities of monofunctional platinum compounds and platinum compounds specific for interstrand and intrastrand cross-links must be evaluated. Monofunctional compounds are available but, as of yet, a compound that makes only intrastrand or interstrand cross-links has not been produced.

Repair of DNA cross-links and checkpoint response to cross-links – future directions

Studies which monitor interstrand cross-link repair by measuring only incision or the repair of DSBs are limited by not delineating important intermediates in the repair process. Therefore future studies must utilize plasmid substrates constructed to contain only one interstrand cross-link as in Li *et al* (1999). In addition to defining intermediates, these substrates could be used to address how the Snm1, Rev3 and Rad51 proteins function *in vitro*. To assess the function of Snm1, Rev3 and Rad51, extracts from cells lacking *SNM1*, *REV3* and *RAD51* should be examined for their ability to act on single interstrand cross-link containing substrates. Further studies should work toward the use of purified proteins to repair a cross-linked substrate. This has been accomplished for excision repair and transcription coupled repair (Aboussekhra*et al*, 1995, Svejstup *et al*, 1995, reviewed in Friedberg *et al*, 1995).

Future work on cross-link repair and cell cycle checkpoints in yeast must focus on identifying the physical interactions and signaling interactions between checkpoint proteins and between repair molecules. Identifying binding partners for cross-link repair proteins Snm1, Rev3 and Rad51 and checkpoint proteins Rad9, Rad17, Rad24, Mec3, Mec1, Rad53 and Dun1 with two hybrid assays will be one important approach. Also, to identify interactions during a checkpoint dependent cell-cycle arrest, immunoprecipitations using antibodies for specific checkpoint and repair proteins should be performed on extracts prepared from interstrand cross-link damage arrested cells. Alternatively, epitope tags could be fused to repair and checkpoint gene sequences. This would allow purification of complexes involved in checkpoints or repair by immunoprecipitation or affinity chromatography on extracts from interstrand cross-link arrested cells.

Clinical relevance of cell cycle checkpoints and CDDP

The work presented here demonstrates that loss of G2 checkpoint sensitizes yeast cells to CDDP. Future application of CDDP in chemotherapy will benefit from greater knowledge of the checkpoint phenotype of tumor cells. Those tumors that display a loss of G2 checkpoint function may be more sensitive to CDDP killing. Some work has been done to disrupt the G2 checkpoint in mammalian cells with drugs such as caffeine and UCN-01 (7-hydroxystaurosporin) (Lippert, 1998 and references therein). These drugs, in combination with CDDP, may be useful in treating tumors with p53 defects. $p53^{-/-}$ cells are not more sensitive to CDDP or IR (Reviewed in Lippert, 1998; Weinert, 1997). Indeed, these cells display a loss of G1 checkpoint and have p53 independent G2 and S-phase checkpoints which may compensate. If the G2 checkpoint were abrogated in $p53^{-/-}$ cells by UCN-01, these cells would be hyper-sensitized to CDDP. One potential problem with this

type of therapy would be an increase in damage and mutation to non-tumor cells. Further work in mouse models will be necessary before the use of such a regimen in the clinic is warranted.

Other future directions

In addition to chemotherapy induced cross-links, cross-links from other agents may occur. In fact, that this repair process is conserved in mammalian cells suggests that cross-links occur from sources other than CDDP and 8-MOP which are rarely encountered by mammalian cells. Identifying these sources of damage is one important unexplored area. One possible additional source of external interstrand cross-linking is UV-C light. Some studies suggest that 0.1-1% of UV-C lesions are interstrand cross-links (reviewed in Henriques *et al*, 1997). Additional chemical toxins may also cross-link DNA. Understanding the repair process of these lesions, the intermediates, and errors resulting from repair of cross-link damage are important areas for future study.

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