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Ultraviolet Light-Induced and Spontaneous Recombination in Eukaryotes

Roles of DNA Damage and DNA Repair Proteins

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1. INTRODUCTION

The major biological effects of ultraviolet (UV) radiation are caused by DNA damage (for reviews see Vol. 1, Chapters 15, 17, and 18; Vol. 2, Chapter 15, and refs. *120,181*). UV damage can result in genetic mutations that may promote tumorigenesis (*205*). Indeed, solar UV is the primary cause of skin cancer, the most prevalent form of cancer (*21,94,189*). There is concern about loss of the stratospheric ozone layer, which may result in additional exposure of the earth's surface to UV and consequently an even greater incidence of skin cancer. Therefore, it is important to determine the genetic effects of UV damage. One such effect is recombination, which can result in genetic rearrangements that may contribute to carcinogenesis.

There are three types of UV radiation: UVA (320–400 nm), UVB (280–320 nm), and UVC (200–280 nm). Although UVC is the most damaging to cells, only UVA and UVB penetrate the atmosphere, with most of the deleterious effects attributable to UVB (*181*). UVB and UVC produce more than a dozen photoproducts in DNA. Among these, cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6–4) photoproducts [(6–4)PDs] are the most common, comprising ~75% and ~25% of the total, respectively (*65,119*). UV radiation acts as both initiator and promoter of carcinogenesis, and it is likely that these properties are owing to CPD and (6-4)PD damage (*95,205*). Because the earth is constantly exposed to UV, it is not surprising that cells have evolved several DNA repair mechanisms that protect genetic information from the harmful effects of solar UV radiation (*29,63*).

1.1. Repair of UV-Induced DNA Damage

Most UV lesions in DNA are repaired by nucleotide-excision repair (NER). In eukaryotes this pathway involves incision on either side of a lesion followed by excision of 27–29 nucleotide oligomers. The remaining undamaged DNA strand is used as a template for DNA synthesis to restore the double-stranded DNA (Vol. 1, Chapter 15). In humans, defects in NER cause UV hypersensitivity and have been associated with three

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hereditary diseases, xeroderma pigmentosum (XP), Cockayne's syndrome, and trichothiodystrophy (Vol. 2, Chapter 18). Repair of UV-induced DNA lesions is heterogeneous across the genome, with the actively transcribed strands preferentially repaired over nontranscribed strands of active genes or nontranscribed DNA (*17,116,188*). Such transcription-coupled repair (TCR) is conserved from bacteria to mammals (Vol. 1, Chapter 9 and Vol. 2, Chapters 10 and 18). DNA lesions in nontranscribed sequences or in inactive chromatin are repaired by a general NER pathway termed "global genome repair."

In addition to NER, UV lesions are repaired by several alternative pathways (*203*). Photolyase reverses UV damage by a visible light-dependent reaction called photoreactivation (see Vol. 2, Chapter 2 and ref. *152*). Photolyases specific for CPDs and (6-4)PDs have been identified (*179*). There are also UV damage-specific glycosylases, which produce a nick at one of the glycosyl bonds in a dimer, generating an abasic site that is a substrate for base-excision repair (BER) (*63*). Another mechanism involves a UV-damage endonuclease (UVDE), which introduces an incision 5′ to CPDs and (6-4)PDs and initiates a excision-repair process (*19,201*). Most UV damage is repaired rapidly, although some lesions may persist through several cell divisions before being repaired, producing mutations, or stimulating recombination.

1.2. Cellular Effects of UV-Induced DNA Damage

Cells exposed to UV may undergo cell-cycle arrest, usually at S phase or the G1/S boundary, and then repair or tolerate the damage before resuming the cell cycle, or they may die, often by apoptosis (*153*). Although UV lesions can inhibit DNA synthesis, lesions may persist because of bypass mechanisms (*30,175*). It is well-established that bulky DNA lesions such as CPDs and (6-4)PDs block transcription elongation in vivo and in vitro (*48,187*). Partial and often nonfunctional RNA transcripts are formed from genes containing one or more lesions in transcribed strands. Eukaryotes also respond to UV damage by inducing transcription of a large set of genes. This gene induction facilitates cell-cycle arrest, DNA repair, and adaptation to the insult (*5,44–46,80*). A major consequence of UV irradiation is enhanced recombination. Recombination is a fundamental process in normal metabolism and repair of DNA damage (reviewed in refs. *131,176*). Recombinational repair can restore genetic information but also can produce mutations and chromosome rearrangements that may have deleterious consequences. For example, recombination may contribute to carcinogenesis by causing loss of heterozygosity that inactivates tumor-suppressor genes (e.g., *p53*), or by activating oncogenes by gene duplication, amplification, inversion, deletion, or translocation.

Recombination can occur between homologous or nonhomologous sequences. Homologous recombination involves interactions between DNA sequences sharing significant lengths of homology (>200 bp). Typical homologous recombination substrates are diagrammed in Fig. 1A. Interacting regions may be at allelic positions on homologs (in diploid cells), termed "allelic recombination." Ectopic recombination includes a variety of interactions between nonallelic repeats, such as repeats present on a single chromosome (direct or inverted orientations), at nonallelic positions on homologs, or on nonhomologous chromosomes. Recombination can be conservative, including reciprocal exchanges (crossing-over) and nonreciprocal exchange (gene conversion), or it can be nonconservative, such as half-crossovers or intrachromosomal deletions between direct repeats via single-strand annealing (SSA). In SSA, DNA ends are processed to

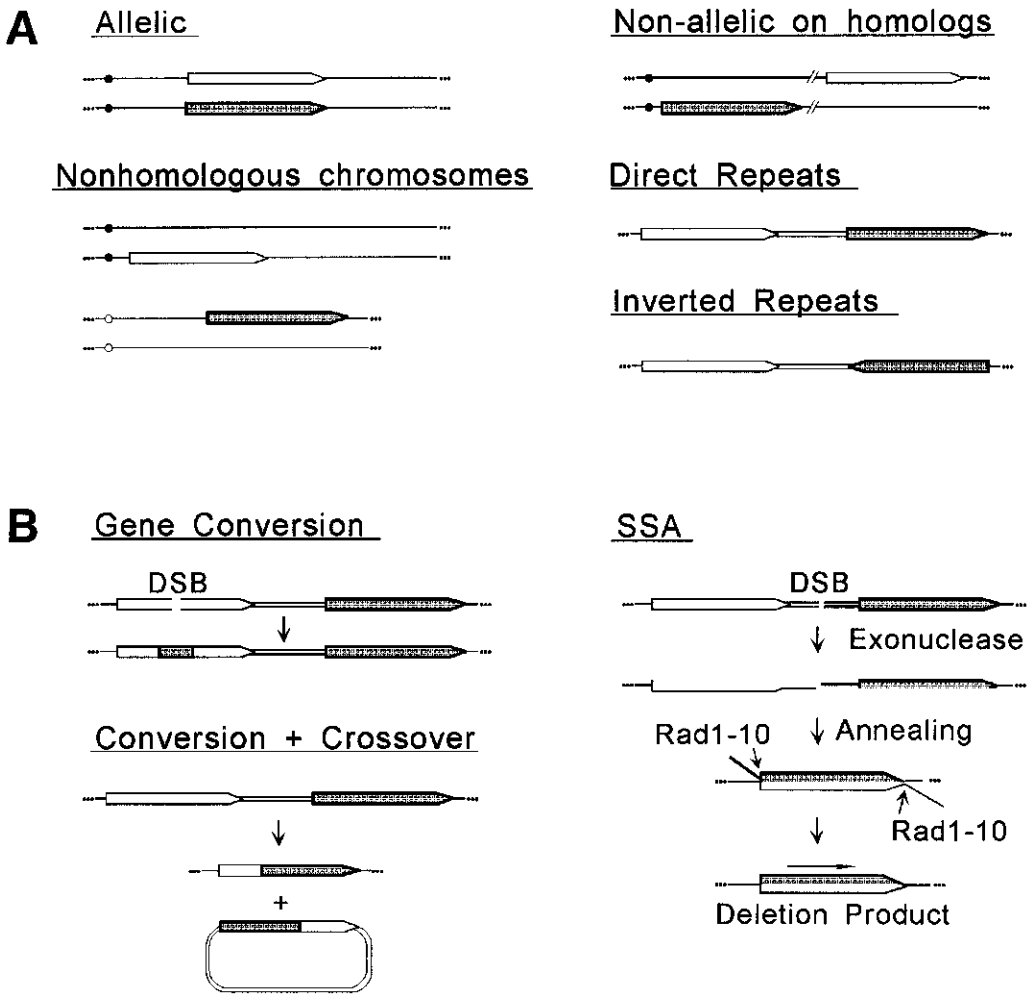

Fig. 1. (A) Types of recombination substrates and events. Repeated regions are shown by boxes, with open and shaded boxes indicating different alleles. Allelic interactions occur between genes present at identical positions in homologous chromosomes. All other interactions are ectopic, including interactions between nonallelic repeats on homologs, repeats on nonhomologous chromosomes, and linked repeats in direct or inverted orientation. **(B)** Types of recombination events. Gene conversion can occur between any repeated regions, shown here for direct repeats. Gene conversion is a nonreciprocal transfer of information from a donor locus (shaded) to a recipient locus (open); typically a damaged allele is the recipient. Gene conversion conserves the gross structure of the recombination substrate. Conversions are often associated with crossovers, which in direct repeats leads to a deletion product plus an excised, circular product that is normally lost. For nonallelic interchromosomal interactions, crossovers result in translocations. Depending on the arrangement of the interacting alleles with respect to centromeres, translocations may be balanced or unbalanced, with unbalanced translocations producing dicentric and acentric fragments (not shown). For inverted repeats, conversion associated with a crossover leads to inversion of DNA between repeats (not shown). SSA occurs between direct repeats and may be initiated by a DSB that is processed by a single-strand exonuclease to expose single-stranded complementary regions. Annealing produces 3′ tails at duplex DNA junctions that are processed by Rad1p/10p endonuclease to produce a deletion product similar to a crossover product. However, unlike crossing over, SSA is nonconservative because no circular product is formed.

expose single-stranded regions that can anneal to form an apparent crossover product with deletion of the DNA between repeats. SSA is common in direct-repeat recombination in yeast (*133*). In mammalian cells, SSA is the predominant mode of extrachromosomal recombination (*42,101,102*), but gene conversion is predominant for chromosomal events (*18,103,130,173*). Sister chromatid recombination (including sister chromatid exchange [SCE] and gene conversion) can be detected at the molecular level only for interactions between linked repeats. In mammalian cells, SCE can be detected in whole chromosomes by microscopic examination. Each type of recombination substrate provides a limited view of the possible recombination events. Recombination substrates are often designed with selectable markers to allow detection of rare events, but selection strongly restricts the types of events detected. Nonhomologous recombination includes nonhomologous end-joining (NHEJ), a mechanism for repairing double-strand breaks (DSBs), and the integration of exogenous DNA into a nonhomologous chromosomal locus (described herein as illegitimate recombination). In this review we focus on the mechanisms and genetic control of UV-induced recombination in eukaryotic cells with emphasis on the yeast *Saccharomyces cerevisiae* and mammalian cells. We also discuss the effects of mutations in UV-repair genes on spontaneous and UV-induced recombination because these provide important insights into recombination mechanisms. For a discussion of recombinational repair in prokaryotes, see the recent review by Cox (*39*).

2. UV-INDUCED RECOMBINATION IN *SACCHAROMYCES CEREVISIAE*

Resistance to the cytotoxic effects of UV in *S. cerevisiae* reflects three types of mechanisms, including NER mediated by genes in the *RAD3* epistasis group (Vol. 1, Chapter 15), damage tolerance mechanisms (so-called error-prone or mutagenic-repair pathways such as translesion synthesis) mediated by genes in the *RAD6* epistasis group (*63*), and recombinational repair of DSBs mediated by genes in the *RAD52* epistasis group (see Vol. 1, Chapter 16 and ref. *133*). DSBs are highly recombinogenic lesions. It is thought that UV or the repair of UV damage does not directly produce double-stranded damage such as DSBs (except perhaps at very high doses), yet UV is highly recombinogenic in yeast and there is considerable indirect evidence suggesting that a significant fraction of UV-induced recombination in yeast involves a DSB intermediate. Yeast is either homozygous or heterozygous at the mating-type locus, *MAT,* and *MAT* genotype strongly influences recombination and DSB repair. *MAT* status influences both UV survival (in UV-sensitive mutants) and UV-induced recombination; these *MAT*-specific topics are discussed in Chapter 5.

2.1. Potential Roles for DSBs and Replication in UV-Induced Recombination

There are several reasonable models for how UV damage might stimulate recombination. (1) UV damage might stimulate recombination by altering DNA structure, perhaps by inducing bending (*81,174*) because bending influences recombination (*147,162*). However, the degree of bending at UV dimers has been questioned (*192,202*) and there is no direct evidence that bending at UV damage stimulates recombination. (2) Recombination might be enhanced as a consequence of UV repair. The principal mechanism of UV repair is NER, which exposes short single-stranded regions that might promote pairing/strand exchange with regions of homology elsewhere in the

genome. However, this view is inconsistent with many studies that indicate that UV-induced recombination usually increases when NER is disabled (Subheadings 3.1. and 5.1.). (3) UV damage might stimulate recombination indirectly, i.e., at undamaged loci, perhaps as a result of gene induction (Subheadings 2.2.). (4) An early model to account for UV-induced recombination in *Escherichia coli* suggested that UV-induced postreplication gaps serve as regions for strand exchange (*149*). (5) Increasing evidence favors the idea that single-strand damage such as UV dimers can be converted to recombinogenic DSBs during replication.

UV lesions may be converted to DSBs when replication forks encounter single-strand breaks (SSBs) or gaps resulting from incomplete processing of UV damage by NER. However, this would predict that recombination would depend on NER and this is not the case (Subheadings 3.1. and 5.1.). Alternatively, DSBs could arise when a replication fork encounters a post-replication gap. Following UV irradiation of bacteria, daughter strands gaps appear at intervals approximating the locations of lesions on the template strands, suggesting that these gaps arise during replication bypass of UV lesions (reviewed in ref. *39*). An arrested replication fork can be processed into a double-stranded end by fork reversal or direct breakage (reviewed in refs. *39,75,93,110,148*). Bacterial studies have shown that replication forks that arrest as a consequence of metabolic processes or DNA damage can restart by a recombinational mechanism, a process termed recombination-dependent DNA replication (*6,93*). DSBs at arrested replication forks have been observed in *E. coli,* in a mechanism dependent on the Holliday junction resolving and branch-migration enzyme complex RuvABC (*118,157*). DSBs have been directly observed following exposure to UV in both yeast and mammalian cells (*61,194*), and there is evidence that a significant fraction of yeast-cell killing by UV results from such DSBs (*91*).

Members of the *RAD52* epistasis group, such as *RAD50, RAD51,* and *RAD54* are important for recombinational repair of DSBs, and *RAD52* is essential for nearly all types of DSB-induced recombination (*133*). A number of studies have shown that UV-induced recombination is dependent on *RAD52* and other *RAD52* group members, consistent with the idea that UV-induced recombination proceeds through a DSB intermediate. For example, UV-induced sister chromatid recombination is eliminated in *rad52* mutants, and reduced by half in *rad50* (end-processing) mutants (*87*). The enhancement of integrative transformation by UV treatment of plasmid DNA is completely abolished in *rad52* mutants (*150*). The UV repair protein Rad3p is a helicase that is essential for viability and NER. Some mutant *rad3* alleles, termed *rem,* display a hyper-recombination phenotype and are synthetically lethal with *rad52 (121).* Song et al. *(163)* argued that the hyper-recombination seen in *rem* strains reflects enhanced conversion of spontaneous damage to DSBs, thus accounting for the *RAD52*-dependence of *rem*-enhanced recombination.

More direct evidence that UV-induced recombination proceeds through a DSB intermediate in yeast comes from studies of chromosomal recombination between partial, nontandem direct repeats *(64).* To stimulate recombination, I-*Sce*I or gpII recognition sequences were located between the repeated genes to allow targeted DSBs and SSBs, respectively, or by exposing cells to γ or UV radiation. Because the recombination substrates were themselves temperature-sensitive mutants of essential genes (*cdc28* and *tub1*), recombination could be studied in arrested cells at the restrictive temperature, or in

dividing cells at the permissive temperature. As expected, DSBs induced by I-*Sce*I nuclease or γ-rays enhanced recombination, both in dividing and nondividing cells. In contrast, SSBs created by gpII showed modestly enhanced recombination in dividing cells, but no enhancement in arrested cells, suggesting that recombination required conversion of SSBs to DSBs during replication. Interestingly, moderate UV doses induced recombination only in dividing cells, but high UV doses enhanced recombination in both dividing and nondividing cells. These results are consistent with the idea that single-strand damage at moderate UV doses is converted to recombinogenic DSBs during replication, whereas at high doses, DSBs may be produced directly by NER processing of closely opposed lesions, obviating the need for lesion conversion during replication. In *rad1* mutants, SCE is replication-dependent, providing additional evidence that replication converts UV damage into recombinogenic lesions *(138),* although the same SCE assay in *RAD1* cells showed a significant component of replication-independent SCE *(86).* The role of Rad1p in UV-induced recombination is considered further in subheading 3.1.

2.2. *Indirect Stimulation of Recombination*

Recombination between chromosomes of unirradiated cells is induced following mating with UV-irradiated cells *(51).* Because the indirect induction of recombination persists for several cell generations *(50),* it seems unlikely that it results from persistent DSBs, although persistence of the primary lesions (which may later be converted to DSBs) remains a possibility. Lesions may persist owing to tolerance mechanisms such as translesion synthesis and lesion bypass (reviewed in ref. *63).* UV lesions may stimulate recombination in undamaged DNA by a triparental mechanism, as with DSBs *(146).*

Alternatively, indirect stimulation of recombination by UV might reflect induction of genes that enhance recombination, an idea supported by several lines of evidence. For example, many genes are induced by UV and other DNA-damaging agents, including several with key roles in recombination such as *RAD51, RAD52,* and *RAD54 (1,38).* The induction of *RAD51* and related genes by UV is conserved through evolution, as UV induces human *RAD51* and *RAD51L1* (also known as *REC2* and *RAD51B*) *(139).* Overexpression of yeast *RAD52* in human cells increases extrachromosomal recombination *(84),* and overexpression of human *hRAD51* increases recombination in human cells *(200).* Also, the UV-sensitivity of yeast *rad51* mutants can be suppressed by overexpression of *RAD54 (36)* (Subheading 3.5.). Although the immediate stimulatory effects of UV on recombination might be at least partially owing to induction of *RAD51, RAD52,* and *RAD54* and/or other genes (reviewed in ref. *62),* the observed indirect stimulation persists much longer than gene induction. Indirect stimulation of recombination may reflect the combined effects of lesion persistence, lesion transfer, triparental recombination, and gene induction.

3. RECOMBINATION IN YEAST MUTANTS WITH DEFECTS IN DNA REPAIR

3.1 *Recombination in RAD3 (NER) Epistasis Group Mutants*

The *RAD3* epistasis group is comprised of a large number of genes that encode proteins involved in the repair of UV damage; those with known effects on recombination are listed in Table 1. Of these, *RAD1* and *RAD10* have been studied most extensively.

Table 1
Partial Listing of *S. cerevisiae* Genes Involved in UV Damage Repair, Tolerance, Checkpoints, and Recombination

Gene	Biochemical activity and function	Reference
***RAD3* epistasis group:**		
RAD1	Complexes with Rad10p, cleaves 3′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD2	Endonuclease cleaves 5′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD3	5′→3′ helicase, DNA-dependent ATPase, unwinds DNA at lesion	(Vol. 1, Chapter 15)
RAD4	Complexes with Rad23p, UV damage recognition	*(73)*
RAD7	UV-inducible, required for repair of nontranscribed DNA	(Vol. 1, Chapter 15)
RAD10	Complexes with Rad1p, cleaves 3′ single-stranded tails at single-strand/duplex DNA junctions	(Vol. 1, Chapter 15)
RAD14	UV damage recognition	(Vol. 1, Chapter 15)
RAD23	UV-inducible, complexes with Rad4p, UV damage recognition	(Vol. 1, Chapter 15 and ref. 73)
DUN1	Protein kinase, controls transcriptional response to DNA damage; partial checkpoint defect	*(8,54)*
SSL1	Essential gene involved in transcription and NER, component of TFIIH	(Vol. 1, Chapter 15)
***RAD6* epistasis group:**		
RAD5	DNA-dependent helicase, zinc finger motif	(Vol. 1, Chapter 15 and ref. *85)*
RAD6	UV-inducible, ubiquitin-conjugating enzyme	(Vol. 1, Chapter 15)
RAD18	UV-inducible, binds ssDNA	(Vol. 1, Chapter 15)
Checkpoint genes:		
RAD9	Controls G1/S, G2/M checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 17)
RAD17	Controls G1/S, G2/M, and meiosis I checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 15)
RAD24	Controls G1/S, G2/M, and meiosis I checkpoints; partial control of S checkpoint	(Vol. 1, Chapter 17)
***RAD52* epistasis group and other genes:**		
RAD51	UV-inducible, strand exchange, and homologous pairing activities; mutant mildly UV-sensitive	(Vol. 1, Chapter 16 and ref. *133)*
RAD52	UV-inducible, DNA end-binding activity, activates Rad51p	(Vol. 1, Chapter 16 and ref. *133)*
RAD54	UV-inducible, helicase (?), activates Rad51p	(Vol. 1, Chapter 16 and ref. *133)*
EXO1	UV-inducible, structure-specific endonuclease, acts in NER-independent UV repair	*(143)*
PSO4	Repair and recombination defects, role in mRNA splicing *(PRP19),* also in *RAD6* epistasis group	*(68)*

For a complete listing of UV repair and checkpoint genes, see Vol. 1, Chapters 15 and 17.

The Rad1p/10p complex is a structure-specific endonuclease that cleaves 3′ single-stranded tails at the junctions of single-stranded and duplex DNA. As such, Rad1p/10p is important for SSA and DSB-induced gene conversion when the broken ends are not fully homologous to donor sequences (Fig. 1B) *(83),* although there is a minor Rad1p-independent pathway for removing nonhomologous single-stranded tails *(37).*

Mutants defective in NER allow one to test the hypothesis that recombination is enhanced by strand breaks and/or single-stranded regions created during NER. In this view, NER-defective mutants would be expected to have lower levels of UV-induced recombination than NER-proficient cells. Although various NER mutants have different effects on UV-induced recombination depending on the type of recombination substrate and cell-cycle phase, most NER mutants display increased levels of UV-induced recombination. Thus, recombination is enhanced by DNA damage, not by repair.

Kadyk and Hartwell *(86)* showed that NER-defective *rad1* mutants display higher frequencies of UV-induced sister chromatid recombination than wild-type. The sister chromatid recombination assay measured both gene conversion and reciprocal exchange. These events in *rad1* cells were dependent on replication: there was no stimulation in cells irradiated in G2 until they passed through the next round of replication. These results are consistent with the replication-dependent conversion of UV lesions to recombinogenic (DSB?) lesions. In contrast, allelic recombination showed a dose-dependent increase in Rad$^+$ cells, but no induction was seen at any dose in *rad1* mutants. There appear to be at least two distinct mechanisms of UV-induced sister chromatid recombination because *rad1* mutants gave rise exclusively to gene conversions, whereas *RAD1* cells gave rise to 75% gene conversion and 25% reciprocal exchange *(86).*

Integrative transformation of UV-damaged (nonreplicative) plasmid DNA showed a clear dose-dependent increase in Rad$^+$ cells. A much sharper increase at a low dose was seen in *rad1* and *rad3* mutants, but there was no further increase at higher doses *(150),* suggesting a saturated process. In Rad$^+$ cells a significant fraction of transformants had multiple copies of the integrated plasmid and the number of copies increased with UV dose. Multiple copies likely arise via SSA that can produce concatemers that subsequently integrate into the target chromosome. Because Rad1p/10p endonuclease is required to process SSA intermediates *(83),* it is not surprising that *rad1* mutants yielded more gene conversions and fewer transformants with multiple integrated copies; in this system gene conversion does not require Rad1p/10p. In *rad3* and *rad4* mutants, conversions were as rare as in Rad$^+$ *(150),* indicating that the shift from integration toward gene conversion in *rad1* is independent of the *rad1* NER defect.

A comprehensive study of NER mutants revealed a variety of effects on spontaneous recombination, including Ty and non-Ty direct repeat recombination, and ectopic Ty and non-Ty gene conversion *(99).* In a *rad1* mutant, direct-repeat recombination decreased slightly at Ty and decreased by ~threefold at the non-Ty substrate. In contrast, conversion increased by ~twofold at Ty and non-Ty loci. Interestingly, *rad10* had similar effects on both types of direct-repeat recombination, but no effect on conversion, suggesting that Rad1p and/or Rad10p have roles that are independent of their endonucleolytic roles in the Rad1p/10p complex. Because *rad1* had stronger effects, it was not surprising that the recombination phenotype of the *rad1 rad10* double mutant was essentially the same as *rad1.* Mutations in *rad2, rad4, rad7, rad14,* and *rad23* had little or no effect on any of these recombination endpoints. The decrease in direct-repeat

recombination in the *rad1* and *rad10* single mutants, and the double mutant, can be understood in terms of the important role that Rad1p/10p endonuclease plays in SSA (Fig. 1B). It was suggested that the increase in conversion in *rad1* reflects channeling of DNA damage from direct-repeat recombination toward conversion, although this does not adequately explain the lack of effect in other NER-defective mutants (*rad2,4,7,10,14,* and *23*). Together these data suggest a role for *rad1* in gene conversion that is independent of its role in NER, perhaps through interactions with the mismatch repair (MMR) system (Subheadings 3.2. and 3.4.).

Deletions in direct-repeat recombination substrates are not solely a consequence of SSA. A study of spontaneous deletions in short direct repeats in wild-type cells and *rad1* mutants revealed that *rad1* reduced deletions by 10-fold with 415 bp repeats, indicative of an SSA defect, but only fivefold with 223 bp repeats, and only threefold with 103 bp repeats (A. Bailis, personal communication). Because Rad1p/10p is required to remove nonhomologous termini >60 bp in length during SSA *(59),* it appears that a significant fraction of these events proceed by a mechanism distinct from SSA, such as single-end invasion. These results also indicate that the relative efficiency of the alternative mechanism is inversely proportional to repeat length.

Rad3p has DNA-DNA and DNA-RNA helicase activities. Rad3p is an essential component of the TFIIH transcription initiation factor and it is important for NER. Maines et al. *(109)* isolated an allele of *rad3* called *rad3-G595R* that confers temperature-sensitive growth, a mild reduction in transcription, and increased spontaneous deletions specifically in short direct repeats (<200 bp). *rad3-G595R* also enhances integrative transformation of plasmid DNA sharing limited homology to a chromosomal target. *rad3-G595R* is not UV-sensitive, indicating that NER is functional. Short-repeat recombination can cause significant genome instability even in organisms with relatively little repetitive DNA, such as yeast. The hyper-recombination phenotype of *rad3-G595R* allele is distinct from the *rem* alleles of *RAD3* (Subheading 2.1.) because *rem* only enhances recombination between long regions of homology, such as allelic gene conversion. *rad3-G595R* mutants also display reduced processing at DSB ends. These data were explained by a model in which a helicase defect in *rad3-G595R* reduces end-processing, leading to longer-lived single-stranded tails in short repeats and thereby promoting SSA (or integrative transformation), whereas rapid processing of DSB ends in *RAD3* strains quickly eliminates short repeats and they are unavailable for recombination. Ss11p is another component of TFIIH known to interact with Rad3p. A mutant allele of *SSL1 (ssl1-T242I)* was isolated as a suppressor of *rad3-G595R* temperature sensitivity. Interestingly, *ssl1-T242I* alone mimicked several of the *rad3-G595R* phenotypes, including increased short-repeat recombination, whereas the double mutant had wild-type (low) levels of short-repeat recombination and normal end-processing *(109).* These findings confirm a close association of Rad3p and Ss11p and they provide an interesting example of compensatory mutations in a pair of interacting, multifunctional proteins.

3.2. Recombination in RAD6 (Damage Tolerance) Epistasis Group Mutants

Ty and non-Ty direct repeat recombination and Ty and non-Ty ectopic gene conversion were examined in mutants in the *RAD6* epistasis group *(99). rad5* and *rad18* single mutants had increased levels (3- to 20-fold) of all four types of recombination, and sim-

ilar increases were seen in the *rad5 rad18* double mutant, indicating that these genes are epistatic with respect to spontaneous recombination. Interestingly, these genes also have roles in mutagenesis but they do not show the same epistasis in mutagenesis assays. The high levels of recombination seen in *rad5* and *rad18* are reduced to the (low) *rad1* levels in *rad1 rad5* and *rad1 rad18* double mutants. That a *rad1* mutation can eliminate the increase in gene conversion seen in *rad5* and *rad18* mutants provides additional support for the idea that Rad1p has a direct role in gene conversion distinct from its role (with Rad10p) in processing SSA intermediates.

Rad5p has been implicated in channeling repair of transformed plasmids with a double-strand gap (with noncohesive termini) from NHEJ to a gene-conversion pathway. In *RAD5* cells, 99% of repair led to gene conversion with chromosomal sequences donating information (1% had point mutations). In contrast, only 25% of transformants in *rad5* mutants resulted from gap repair, and 75% resulted from NHEJ, yielding up to 8 bp deletions. These results implicate Rad5p in DNA end-protection or in a more direct (positive) role in homologous recombination *(3).* This transformation system was recently used to test *rad18* and *rad5 rad18* double mutants. *rad18* mutants display essentially 100% gene conversion (much higher than the 25% with *rad5*), although 12% had point mutations outside the gap-repair tract, likely reflecting *rad18*-enhanced mutagenesis. The *rad5 rad18* double mutant displayed an interesting mixture of single mutant phenotypes: gene conversion was intermediate (90%), suggesting that *rad18* suppresses to a large extent the *rad5* defect in gene conversion, and there were few point mutations suggesting that *rad5* suppresses the *rad18* mutagenesis phenotype. As with *rad5,* nonconverted products in the *rad5 rad18* double mutant arose by NHEJ, but deletions were much larger, up to several hundred bp in length (F. Eckardt-Schupp, personal communication). These results suggest that Rad5p and Rad18p may operate together in various DNA repair processes, perhaps in one or more complexes.

Although spontaneous Ty recombination is increased in UV-repair mutants, Ty recombination is minimally enhanced by UV *(96).* It is curious that Ty recombination is refractory to induction by UV because Ty recombination is strongly enhanced by targeted DSBs *(137).*

3.3. Recombination in Checkpoint Mutants

Another class of mutants that are sensitive to UV light are those with defective checkpoints (Vol. 1, Chapter 17), including *dun1, mec1, mec3, rad9, rad17,* and *rad24.* Checkpoint systems are thought to delay cell-cycle progression in response to DNA damage, presumably to allow time for repair before replication or mitosis. If replication of damaged DNA creates recombinogenic lesions (i.e., DSBs), checkpoint mutants would be expected to display hyperrecombination phenotypes, and this is generally true.

Dun1p is a member of a family of protein kinases that includes the checkpoint proteins Rad53p, Mec1p (a relative of mammalian ATM), and Hrr25p. *dun1* mutants display several phenotypes including inability to induce *RNR3* (encoding ribonucleotide reductase) and *MAG1* (encoding a methyladenine DNA glycosylase), a partial G2 checkpoint defect, and increased sensitivity to UV and MMS (Vol. 1, Chapter 18). In addition, *dun1* mutants display increased spontaneous and UV-induced recombination, including gene conversion and SCE *(54);* increased SCE requires replication of DNA containing UV lesions because *dun1* does not increase SCE in G2-arrested cells. It is

possible that the increased spontaneous recombination in *dun1* reflects increased replication of DNA containing endogenous lesions. However, because *dun1* has pleiotropic effects, *dun1* recombination phenotypes might reflect other factors, such as nucleotide imbalance or defective phosphorylation of repair proteins *(54).*

Rad9p controls the G1/S and G2/M checkpoints and it partially controls the S checkpoint. As with *dun1, rad9* mutants display increased spontaneous and UV-induced recombination, measured by reciprocal translocation. However, *rad9* mutation had little or no effect on SCE. It was proposed that Rad9p channels UV and spontaneous damage toward SCE, thus reducing translocations *(53).* Rad24p controls the G1/S, G2/M, and meiosis I checkpoints, and it partially controls the S checkpoint (Vol. 1, Chapter 17). Paulovich et al. *(138)* showed that SCE has different dependencies on Rad9p and Rad24p depending on the status of *RAD1.* Thus, SCE in *rad1* mutants depends in part on Rad9p and Rad24p, but SCE is independent of these genes in *RAD1* cells. A model accounting for these data suggests that *RAD1* cells have two pathways for stimulating SCE: replication past excision-repair tracts forming recombinogenic daughter-strand gaps and replication past unrepaired lesions (perhaps forming DSBs). In *rad1* mutants, UV lesions are not excised, so only the latter pathway is available. Apparently Rad9p and Rad24p play roles in the pathway that processes unrepaired lesions into SCE events, but their precise functions remain unclear.

Mutant *mec1* (also called *esr1*) strains are sensitive to UV and MMS *(88). MEC1* is related to other known checkpoint genes such as *Schizosaccharomyces pombe* rad3$^+$. Interestingly, *mec1* mutation increases mitotic recombination but strongly reduces meiotic recombination; the meiotic phenotype likely reflects, at least in part, the fact that *MEC1* is induced 20-fold during meiosis. These complex phenotypes, as well as the observation that *mec1* null mutants are inviable, argues for numerous roles for Mec1p in both mitosis and meiosis.

3.4. Relationships Among MMR, UV Repair, and UV-Induced Recombination

The MMR system is intricately linked to recombination, and there is increasing evidence for functional overlap between MMR and UV repair. In *E. coli,* UV-induced recombination in UV repair-defective *(uvrA)* mutants is strongly dependent on MMR, and it has been suggested that excision repair and MMR might act in a coordinated manner to form recombinogenic substrates *(55,56).* MMR and NER both recognize several different types of non-B form DNA arising from DNA damage and errors in replication. Mismatches are formed in recombination intermediates during strand invasion, branch migration, and strand annealing ("pairing"), and MMR strongly influences the outcome of recombination events. For example, DSB-induced gene conversion is largely a consequence of MMR of heteroduplex DNA *(140,198).* Spontaneous recombination frequencies are also controlled by the MMR system: levels are substantially reduced by sequence divergence ("homeologous recombination"), but levels are increased to those seen with homologous substrates in MMR-defective strains, such as *msh2* and *msh3* mutants *(129,* and refs. therein).

In mammals and *E. coli,* MMR-defective mutants display modest UV sensitivity. These results are consistent with the findings that TCR of UV damage is absent in *E. coli mutS* and *mutL* mutants, and in human cells with mutations in either *hMLH1* or *hMSH2 (114,115).* Although yeast MMR-defective mutants are not more sensitive to

UV killing than wild-type cells, and there is no dependence of TCR on the MMR system *(171),* UV-sensitivity of yeast NER mutants is increased by an *msh2* mutation *(13).* Furthermore, Msh2p interacts with many NER proteins, including Rad1p, Rad2p, Rad3p, Rad10p, Rad14p, and Rad25p, supporting the idea that MMR and NER proteins exist in complex(es) that may function together in some forms of DNA repair *(13).*

As discussed in Subheading 3.1., Rad1p/10p is important for processing SSA recombination intermediates. Interestingly, Rad1p/10p-dependent processing during SSA also involves the mismatch-recognition proteins Msh2p and Msh3p *(154).* This finding prompted an examination of the effects of UV-repair proteins on spontaneous homeologous recombination between inverted repeats with four heterologies *(129).* In this system, *msh3* and *msh2* mutations increased recombination 8- to 22-fold, respectively, whereas *rad1* and *rad10* mutations increased recombination 6- to 8-fold. The *rad1* and *rad10* effects likely reflect changes in structure-specific processing of recombination intermediates, rather than effects of defective NER, because neither *rad2* nor *rad14* increased homeologous recombination *(129).* These results indicate that Rad1p and Rad10p function with Msh2p and Msh3p in reducing homeologous recombination.

Exo1p is a member of a family of structure-specific nucleases that includes Rad2p (XPG homolog) and Rad27p (flap endonuclease; FEN1); the Exo1p substrate specificity more closely aligns it with Rad27p. *EXO1* is also related to *DIN7* and both are induced by UV. An *exo1 rad2* double mutant is more UV-sensitive than either single mutant, and similar results were obtained when *exo1* was combined with *rad51, rad52,* or *msh2,* suggesting that Exo1p confers resistance to UV independently of NER, recombination, and MMR *(143).* However, Exo1p does have a role in MMR in *S. cerevisiae,* as does exo1$^+$ in *S. pombe (172,182),* and Exo1p interacts with Msh2p *(178).* In *S. cerevisiae, exo1* mutants show reduced spontaneous deletion (SSA) events in direct repeats. When *exo1* was combined with either *rad1* or *rad52,* SSA was not reduced below the levels seen in the single *rad* mutants, indicating that Exo1p acts in the Rad52p- and Rad1p-dependent pathway, at least for deletion in direct repeats *(57).* Exo1p homologs have been identified in *S. cerevisiae, S. pombe,* Drosophila, and humans *(47,57,172,177),* and it is functionally conserved: expression of human *EXO1* complements several yeast *exo1* phenotypes, including UV sensitivity, reduced spontaneous recombination, and increased mutation *(144).* The precise role of Exo1p in recombination is unclear, but it seems likely that it processes DNA ends.

3.5. Recombination Promotes UV Resistance: Roles of PSO4, RAD51, and RPA/RFA1

As discussed in Subheading 2.1., several lines of evidence implicate recombination as a key factor promoting cell survival of UV damage. Additional support for this idea comes from analysis of *PSO4, RAD51,* and replication protein A (RPA). The phenotypes of *pso4* mutants parallel those of *E. coli* recA mutants: both are sensitive to mutagenic chemicals and radiation, and both show defects in induced mutagenesis and recombination. *PSO4* has therefore been assigned to both *RAD6* and *RAD52* epistasis groups. *pso4* mutants display decreased spontaneous allelic and direct-repeat recombination *(4). pso4* mutants are almost completely blocked for UV-induced direct-repeat recombination, with both gene conversion and reciprocal exchange affected *(112).* Together with the UV-sensitivity of *pso4* mutants, these data are consistent with the

idea that UV-resistance is dependent in part on recombinational repair. This idea gains support from several other findings: expression of RecA in yeast increases resistance to UV *(25);* expression of RecA increases UV-induced recombination *(190);* the UV-sensitivity of *rad51* mutants can be suppressed by overexpression of another recombinational repair protein, Rad54p *(36).*

RPA has three evolutionarily conserved subunits of 70, 36, and 14 kDa; the 70 kDa subunit is encoded by *RFA1* and has been subjected to considerable mutant analysis. RPA is involved in DNA replication *(24)* and is an essential component of NER *(72).* RPA has roles in replication initiation and elongation, stabilizing single-stranded DNA (ssDNA) generated at replication forks, and it stimulates DNA polymerase α. *S. cerevisiae RFA1* is an essential gene, consistent with its key role in DNA replication *(24,79).* Many *rfa1* mutants are temperature-sensitive and they display a variety of other phenotypes, including slow growth, UV sensitivity, checkpoint defects, and altered recombination *(26,58,106,107,159,184).* RPA (or the Rfa1p subunit) stimulates strand exchange by Sep1p, enhances homologous pairing and strand exchange by Rad51p, and mediates Rad51p-Rad52p interactions *(79,128,168–170).*

Although many UV-sensitive mutants display increased levels of recombination, *rfa1* mutants have reduced levels of spontaneous allelic and DSB-induced plasmid × chromosome recombination *(58,107).* A comprehensive screen identified 24 *rfa1* mutants, many of which were temperature- and UV-sensitive. Although UV sensitivity might reflect defects in NER, many of these *rfa1* mutants were also sensitive to MMS and HO nuclease, and they had defects in HO nuclease-induced recombination. On the basis of these results it was proposed that the UV sensitivity of these mutants may reflect a defect in DSB repair *(184).* Another *rfa1* allele *(rfa1-D228Y)* was also UV sensitive, but this mutation increased spontaneous direct-repeat recombination. As with other *rfa1* mutants, *rfa1-D228Y* mutants are deficient in spontaneous allelic recombination, and direct-repeat recombination yields principally deletions by a Rad52p-independent mechanism. Thus, *rfa1-D228Y* appears to channel recombination intermediates from a conversion to a nonconversion (Rad52p-independent) pathway *(159).* In this view, the UV sensitivity of *rfa1-D228Y* is consistent with a defect in DSB repair.

4. UV-INDUCED RECOMBINATION IN MAMMALIAN CELLS: *GENETIC CONSEQUENCES*

4.1. UV-Induced Extrachromosomal Recombination

Several strategies have been employed to monitor UV-induced recombination in extrachromosomal DNA substrates. The cells, exogenous DNA, or both can be irradiated and the frequency that stable transfectants are recovered is taken as a measure of illegitimate recombination. By using two inactive copies of a selectable marker, either on separate plasmids or as repeats on a single plasmid, one can monitor extrachromosomal homologous recombination. However, in these assays both homologous and illegitimate recombination must occur to generate a stable transfectant, and it is sometimes difficult to distinguish the effects of UV on these distinct events. Treatment of plasmid DNA with UV prior to transfection enhances transfection efficiency and/or extrachromosomal homologous recombination *(67,78).* UV-enhanced extrachromosomal homologous recombination does not require plasmid replication *(27).* Irradiation of cells

usually enhances transfection, although no effects were seen with some cell types (reviewed in ref. *78).* Thus, UV generally enhances extrachromosomal recombination.

4.2. UV-Induced Recombination Between Chromosomal Repeats

Spontaneously derived duplications in the hypoxanthine phosphoribosyl transferase *(HPRT)* gene were used in several studies to investigate UV-induced homologous recombination in chromosomal repeats. Because *HPRT* is present on the X chromosome, it is hemizygous in male cells and functionally hemizygous in female cells. These *hprt* substrates can form a functional *HPRT* gene upon deletion of the duplicated sequence. Two studies demonstrated that UV doses of 8 to 20 J/m^2 enhanced recombination two- to sixfold above spontaneous levels in an *hprt* gene containing a 13.7 kbp intragenic duplication of exons 2 and 3 *(7,97).* A similar duplication of *HPRT* exon 2 and its flanking regions in a Chinese hamster ovary (CHO) V79 derivative showed a fivefold increase in deletions following a UV dose of 10 J/m^2 *(204).* Another V79-derived cell line with a different *hprt* duplication showed similar levels of UV-induced recombination, although in this case functional *HRPT* genes were thought to arise by illegitimate rather than homologous recombination *(77).* The modest increases in UV-induced recombination seen in these *hprt* studies may reflect, at least in part, high spontaneous reversion frequencies.

More detailed product analysis was possible with a substrate carrying directly repeated copies of the herpes *TK* gene inactivated by *Xho*I linker insertions at different positions and integrated into thymidine kinase-deficient mouse L cells. In these cells, a UV dose of 12 J/m^2 increased recombination 30-fold *(196).* Molecular analysis showed that 85–90% of UV-induced TK$^+$ recombinants arose by gene conversion, and similar results were obtained for spontaneous events *(103).* The remainder were deletions, reflecting intrachromosomal crossovers or unequal SCEs. Gene conversions were also predominant for UV-induced and spontaneous recombination with the same *TK* substrate in a human fibroblast cell line *(14),* with hygromycin direct repeats in another human fibroblast cell line *(183),* and with *neo* direct repeats in a CHO cell line *(43).* In contrast, spontaneous and UV-induced recombination between *neo* inverted repeats yielded relatively few simple gene conversions, with most products displaying complex structures consistent with multiple rearrangements *(43).*

A preliminary analysis with *neo* direct-repeat substrates suggests the conversion: deletion ratio for UV-induced events may be influenced by the number of heterozygosities and by transcription (our unpublished results). In these substrates, the *neo* repeats were 1.4 kbp in length, one *neo* was driven by the dexamethasone-inducible MMTV promoter, and the second lacked a promoter. In a substrate with 13 heterozygosities in *neo* and with low-level transcription of MMTV*neo* (dexamethasone absent), UV-induced predominantly gene conversions. In contrast, with high-level transcription (dexamethasone present), most UV-induced recombinants had deletions (reflecting crossovers, SSA, or unequal SCE). Thus for UV-induced events, transcription levels influence the relative frequencies of gene conversions and deletions. This effect may be specific for multiply heterozygous substrates, because UV induced predominantly gene conversion in a related substrate with a single heterozygosity, regardless of transcription levels *(43).* Because these substrates were integrated at different chromosomal loci, this difference may reflect a position effect; targeted substrates will be required to distin-

guish these possibilities. If multiple heterozygosities promote a deletion mechanism (or inhibit gene conversion) for UV-induced events, this would have important implications with regard to stability of mammalian genomes, which have large amounts of diverged, repetitive sequences.

5. MECHANISM OF UV-INDUCED RECOMBINATION IN MAMMALIAN CELLS

5.1. Stimulation of Recombination by DNA Damage or by Repair?

As discussed in Subheading 3.1., recombination might be stimulated by UV damage per se, or as a consequence of repair processing. This question has been addressed in mammalian cells with defects in NER, and by modulating TCR in recombination substrates with regulated promoters. There is a linear relationship between the extent of UV damage to plasmids and illegitimate recombination as measured by transfection frequency *(165).* However, transfection of NER-deficient XP cells with mutagen-damaged plasmids led to increased transfection compared with repair-proficient cells *(165,186),* indicating that damage-induced illegitimate recombination is not enhanced by NER. A similar conclusion was reached for homologous recombination between chromosomal direct repeats as NER-deficient XP cells required lower doses of UV than NER-proficient cells to reach a particular recombination level *(14,15,183).* SCEs observed cytogenetically arise by homologous recombination *(164).* As with direct-repeat recombination, UV-induced SCE was more frequent in NER-deficient XP cells than NER-proficient cells *(2,40).* Because UV repair can be enhanced by increasing transcription levels, an alternative approach to determine whether recombination is enhanced by UV damage or repair is to modulate repair levels by modulating transcription. The advantage of this approach is that recombination is monitored at a single locus, avoiding problems associated with chromosomal position effects *(18).* The effects of transcription on UV-induced *neo* direct-repeat recombination were monitored in CHO cells, with one *neo* driven by the MMTV promoter. Although transcription and UV separately stimulated recombination, increasing transcription levels enhanced TCR and this reduced UV-induced recombination. Together these studies indicate that DNA damage, not repair by NER/TCR, stimulates illegitimate and homologous recombination. There are at least three other repair pathways (photolyase, glycosylases, UVDE) (reviewed in ref. *203).* However, only a small fraction of UV lesions are processed by these alternative repair pathways, and it is unlikely that they significantly impact UV-induced recombination.

A corollary to the idea that recombination is stimulated by UV damage and not repair is that poorly repaired regions may be recombination hotspots. Repair of dimers can vary between genes, bulk chromatin or even between neighboring base positions *(66,180).* For example, along the *p53* gene, sites of skin-cancer mutation hotspots are almost always sites where DNA repair processing is particularly slow *(180).* These sites may have a higher probability for initiating recombination events.

As discussed in Subheading 3.4., there is significant overlap between the NER and MMR systems. Mutations in the human MMR genes *hMSH2* and *hPMS2* render cells slightly more sensitive to UV *(114,115).* Active MMR was not required for spontaneous or UV-induced SCE in a human cell line, indicating that SCE can occur independently

of lesion recognition/processing by MMR *(132).* This is consistent with the report that purified hMutSα (MSH2-MSH6) mismatch recognition complex does not recognize UV-induced DNA photoproducts *(125).* However, hMutSα does recognize compound UV/mismatch lesions *(193),* which are thought to arise during error-prone translesion synthesis *(63).* Compound lesions are removed more efficiently by excision repair and recognized less efficiently by the MMR system *(125).* Compound lesions are highly mutagenic but they do not appear to be sites of recombination *(193).* Thus, neither NER nor MMR is required for UV-induced recombination in mammalian cells.

5.2. Does Transcription Stalling at UV Lesions or Gene Induction Enhance Recombination?

It is well-established that bulky DNA lesions such as CPDs block transcription both in vivo and in vitro *(48,155).* The mRNA levels of genes required for recombination might be reduced if UV lesions blocked transcription in these genes and this could presumably reduce the level of recombination. However, such effects would be expected only at very high doses where a significant fraction of cells suffered damage to genes required for recombination, and only if recombination proteins had short half-lives relative to the rate of repair.

UV induces many genes in mammalian cells *(5,44,46,80,108),* including two in humans with important roles in homologous recombination, *hRAD51* and *hRAD51B (139).* Overexpression of *hRAD51* in human cells increases spontaneous recombination *(200).* Thus, a fraction of UV-induced recombination in mammalian cells may occur at undamaged loci owing to enhanced expression of *RAD51* and other recombination genes. Although there is clear evidence for indirect stimulation of recombination by UV in yeast (Subheading 2.2.), analogous cell-fusion experiments have not been performed in mammalian cells.

5.3. Roles of Replication, PCNA, BRCA1, BRCA2, RAD51, and RPA in UV-Induced Recombination

There is no direct evidence linking replication-fork arrest at UV lesions with recombination in mammalian cells, but indirect evidence suggests a probable connection. Because replication-fork pausing is conserved from bacteria to humans, it seems likely that the mechanisms for processing stalled or arrested forks are also conserved and tightly regulated. Although UV lesions cause significant helix distortion *(92,181),* DNA replication inhibition and cell-cycle arrest does not reflect a direct block by these helix-distorting lesions, but an indirect effect mediated by increased levels of $p21^{Cip1/WAF1}$ *(31,49,195),* an inhibitor of cyclin-dependent kinases *(141).* Thus, in mammalian cells as in yeast, replication of damaged DNA may produce DSBs, and in fact DSBs have been observed following UV exposure of mammalian cells *(194).*

Several proteins have been implicated in UV-induced recombination in mammalian cells, including proliferating cell nuclear antigen (PCNA), BRCA1, BRCA2, RPA, RAD51, and RAD54. PCNA is a replication processivity factor that is essential for DNA replication and cell-cycle arrest, BER, and MMR *(70,111,160,185).* PCNA appears to localize at sites of replication during S phase *(22,23).* Although the interaction between p21 and PCNA inhibits PCNA function in S-phase replication, it does not affect PCNA function in DNA synthesis during repair *(60,98,191).* Germline mutations

in the breast-cancer susceptibility genes, *BRCA1* and *BRCA2,* predispose women to early onset of familial breast and ovarian cancers *(11,35,197).* Both genes are now implicated in recombination (Chapter 10); in particular, *BRCA1* is important for DSB-induced homologous recombination *(123).* In yeast and higher eukaryotes, RAD51 forms nucleoprotein filaments on ssDNA, mediating homologous pairing and strand-exchange reactions *(9,10,71,168).* In human cells, RAD51 nuclear foci increase after UV irradiation; these foci probably represent sites of recombinational repair *(74).* Upon UV irradiation of S-phase MCF7 cells, BRCA1 was found to be phosphorylated and co-localized with PCNA and RAD51 *(156).* A subsequent study showed that BRCA2 is also co-localized with PCNA *(32).* The interactions between BRCA1, BRCA2, and RAD51 and their recruitment to replication forks after UV exposure *(32–34,156)* strongly implicate these proteins in UV-induced recombination.

As in yeast, mammalian RPA is involved in DNA replication *(90),* NER *(124),* and recombinational repair. RPA has a role in both initiation and elongation of replication, stabilizing ssDNA generated at replication forks and stimulating DNA polymerase α activity *(24,89).* In human cells, UV-induced inhibition of replication can be reversed by the addition of RPA, suggesting a role for RPA in this regulatory event *(31).* RPA interacts with RAD52 and this interaction appears to be required for homologous recombination in mammalian cells *(136).* DNA damage causes RAD51 and RPA to form specific foci at ssDNA sites, suggesting that these are sites of recombinational DNA repair *(145).* The limited data in mammalian cells is consistent with the view developed from yeast studies that UV-induced recombination involves an interplay among replication functions (PCNA, RPA) and recombination functions (RAD51, RAD52, BRCA1, BRCA2).

The 70 kDa subunit of RPA interacts with the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) *(158),* and DNA-PKcs is involved in UV-induced replication arrest through modulation of RPA activity *(135).* DNA-PK is a nuclear serine/threonine protein kinase consisting of DNA-PKcs and the Ku heterodimer (Ku70 and Ku80) and this complex has an integral role in NHEJ (Vol. 2, Chapter 16). Thus, the interaction between RPA and DNA-PK might be important for UV-induced illegitimate recombination.

5.4. Negative Regulators of UV-Induced Recombination: p53 and XRCC9

Certain biological effects of UV irradiation are expected to reduce levels of recombination. p53 is induced by UV light, and it has roles in DNA repair, checkpoint control, and apoptosis *(76,161).* Cells lacking p53 or with mutated p53 have higher rates of homologous recombination than wild-type cells *(12,113,167,199).* Therefore UV-induction of p53 might be expected to downregulate recombination. Extrachromosomal recombination induced by site-directed psoralen adducts was similar in cells with wild-type p53 and cells with p53 inactivated by E6 protein *(52).* To date, a direct comparison of UV-induced recombination levels in wild-type and p53 mutant cells has not been made. The ATM protein is thought to function in the same pathway as p53 in G1/S checkpoint control (Vol. 2, Chapter 19), and *atm* mutants also display increased levels of spontaneous homologous recombination *(16,117).* It is possible that the increased spontaneous recombination in *p53* and *atm* mutants is a consequence of the G1/S checkpoint defect, which increases replication of spontaneous damage, as hypothesized for yeast checkpoint mutants (Subheading 3.3.).

The CHO mutant UV40 is hypersensitive to UV but NER-proficient, and UV40 cells are defective in expression of *XRCC9 (105).* UV40 cells show high levels of SCE, elevated chromosomal instability, and increased sensitivity to UV inhibition of replication *(28). XRCC9* was subsequently shown to be homologous to the gene defective in Fanconi anemia group G patients, *FANCG (41).* As with UV40, *FANCG* mutant cells show chromosome instability. Thus, XRCC9, like p53, appears to negatively regulate recombinational repair processes. Observed levels of UV-induced recombination likely reflect a balance between recombination suppression, which preserves gross genomic integrity, and recombination enhancement, which promotes DNA repair and/or recombinational restart of collapsed replication forks.

6. DNA-REPAIR PATHWAYS, DELAYED GENOMIC INSTABILITY, AND CANCER

It is becoming clear that there is considerable overlap among various DNA-repair pathways. The similar effect of *msh2, msh3, rad1,* and *rad10* on homeologous recombination (Subheading 3.4.) provides a striking example of overlap between MMR and UV-repair pathways (reviewed in ref. *151).* Cellular responses to DNA-damaging agents reflect complex networks that operate from the nucleotide to the chromosome and whole-cell levels, including repair systems such as NER, and tolerance systems such as translesion synthesis, lesion bypass via recombination, and checkpoints. In mammalian cells, these networks produce a balance between repair of DNA damage and cell death by apoptosis. It is now well-established that defects in NER increase spontaneous and UV-induced mutagenesis and recombination. Cancer predisposition associated with NER defects may result both from enhanced mutagenic and recombinogenic effects of spontaneous and UV-induced DNA damage.

Genomic instability is a hallmark of cancer. Instability is expressed in many ways, including chromosome rearrangement, aneuploidy, gene amplification, increased mutation rate, and increased instability of short repeated sequences such as micro- and minisatellites *(20,69,100,122,126,134,142).* Cells displaying one type of instability may or may not display other types. Thus, instability may result from dysregulation of any of a number of "stability functions." It has long been known that DNA damage caused by radiation can be converted rapidly to mutations and chromosome aberrations. It recent years it has become apparent that radiation also has delayed effects, inducing genomic instability and/or mutations many generations after exposure. Although most research on delayed instability has focused on the effects of ionizing radiation *(104,122,126),* an early report indicated that UV can induce delayed chromosome aberrations *(82)* and there is evidence that UV induces a delayed mutator phenotype *(166).* At least for ionizing radiation, delayed effects appear to reflect epigenetic changes, rather than direct genetic effects *(127).* It is possible that epigenetic changes are responsible for the indirect stimulation of recombination by UV seen in *S. cerevisiae* (Subheading 2.2.). UV is a complete carcinogen and it is reasonable to suspect that this is at least part owing to its direct mutagenic effects. With our better understanding of the broader effects of UV, a central question is whether indirect effects, such as induced mutator and chromosome instability phenotypes, also contribute to UV carcinogenesis.

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14

Telomeres, DNA Repair Proteins, and Making Ends Meet

Susan M. Bailey, Julianne Meyne, and Edwin H. Goodwin

1. INTRODUCTION

Telomeres are unique structures at the physical ends of linear eukaryotic chromosomes. They were first described over 60 years ago by Hermann Muller in his classic studies of the fruit fly *Drosophilia melanogaster (78).* He coined the name 'telomere' from the Greek—*telos* meaning end and *meros* meaning part—based on their chromosome end protection function. Shortly thereafter, Barbara McClintock's cytogenetic observations in maize demonstrated that broken chromosomes could fuse with one another to form dicentric chromosomes *(72,73).* These early studies brought to light the fact that natural chromosome ends are distinguished from random breaks and protected from illegitimate end-joining reactions. How the cell accomplishes this critical discrimination is still under investigation.

Telomeres continue to hold scientist's fascination today, particularly as new cytogenetic and molecular biology technologies have opened additional doors of understanding into their structure and function. Amazingly, telomeres have been found to be involved not only in chromosome stability, but also in chromosome replication, nuclear architecture, gene expression, human tumor formation, aging, and senescence *(118).* Most recently, and perhaps most surprisingly, DNA repair proteins have been discovered to play an essential role in the normal end-capping function of chromosomal termini.

2. TELOMERE BIOLOGY

Telomeric DNA consists of tandem arrays of short, repetitive G-rich sequences that are oriented 5′-to-3′ towards the end of the chromosome *(7,9),* forming a 3′ single-stranded G-rich overhang *(66,113).* Together with an ever-increasing number of known telomeric binding proteins, a dynamic terminal structure is created that "caps" both ends of linear chromosomal DNA molecules and provides protection from exonucleolytic attack and degradation, as well as preserves genomic stability by preventing undesired end-joining reactions *(10,88,119,120).* Because there are no genes contained in repetitive DNA, it has been thought of as "junk" DNA. However, as can easily be

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seen from the vital functions that telomeres perform, this way of thinking about these special regions of the genome requires reexamination.

2.1. Telomere Replication

The importance of telomeres to the cell is evidenced by the fact that functional telomeres are essential for continuous cellular proliferation, an observation that has profound implications in our understanding of aging and cancer *(48,49,77).* Although telomeres are vital for chromosomal stability and cell survival, telomere maintenance presents several unique problems to the cell. First, telomeres are faced with an end-replication problem. Because conventional DNA polymerases replicate only in the 5′-to-3′ direction, can only extend existing DNA or RNA chains, and need a complementary strand to provide a template, they are unable to replicate to the very end of a linear duplex *(83,111).* During semiconservative DNA replication, short RNA primers (8–12 bp) made by RNA primase are required to initiate lagging-strand replication, which are then extended by DNA polymerase to form Okazaki fragments. As these RNA primers are removed and fragments ligated together, there are no means to synthesize the lagging-strand sequence complementary to the short region at the very end of a chromosome, so a gap results. It has also been proposed that the end replication problem is a result of the inability of leading-strand synthesis to produce a 3′ overhang *(65).* In this scenario, a 5′-to-3′ exonuclease is required to recreate the 3′ overhang after replication, causing telomere strands duplicated by leading-strand synthesis to be shorter than the parental telomere that served as a template during replication *(Fig. 1).* According to a revised model of telomere replication *(66,112),* DNA is lost from both ends of the chromosomes, due to degradation of the 5′ C-rich strand by an S-phase-specific exonuclease activity, resulting in long 3′ overhangs at both ends. Others demonstrate that telomeres generated by leading- versus lagging-strand DNA synthesis differ and suggest that each chromosome has one telomere with a long G-rich overhang (200 ± 75-nucleotide) and one that is either blunt-ended or has a short G-rich overhang (≤12 nucleotides) *(116).* By whichever mechanism, without some type of compensatory mechanism, it is certain that with each cell division telomeric sequence is lost. The average rate of loss in mammalian cells has been estimated to be between 50 and 75 bp/telomere/cell cycle *(89,116).* It also appears that the rate of loss is not constant, but rather fluctuates greatly from cell cycle to cell cycle *(79).*

In germ line cells (and a majority of tumors), the specialized ribonucleoprotein telomerase compensates for telomeric repeat loss *(37).* Telomerase contains an internal RNA template for the 5′-to-3′ addition of TTAGGG repeats to extend the single-stranded G-rich strand of the chromosome end *(38).* However, in most adult human somatic cells telomerase is inactive or present at very low levels *(55).* Hayflick recognized in the 1960s that normal cells undergo a finite number of doublings in cell culture *(50).* It has since been suggested that the progressive erosion of telomeres may be responsible for placing an upper limit on the proliferative capacity of somatic cells. According to this hypothesis, once a critically shortened length is reached, the cell no longer recognizes the telomere as a natural chromosome end. Instead, critically shortened telomeres are misidentified by DNA surveillance enzymes as ends created by double-strand breaks, an event that triggers cell-cycle arrest. Tumor cells are thought to overcome this barrier by reactivating telomerase, thereby maintaining their telomeres

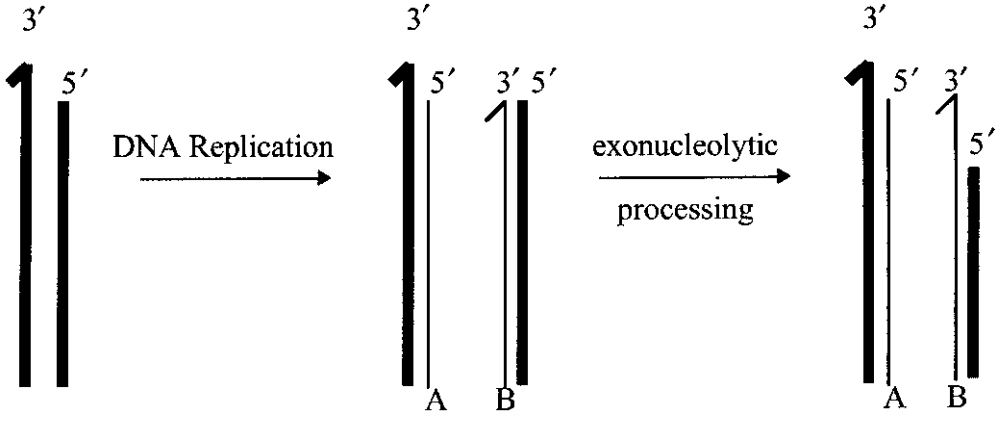

Fig. 1. Mechanism of telomere shortening. The parental 3′ and 5′ telomere DNA strands, shown here as dark lines, are replicated by lagging and leading strand synthesis respectively (newly synthesized DNA is shown by lighter lines). The telomere replicated by lagging strand synthesis *(A)* has a 3′ overhang because this mode of replication can not proceed to the very end of linear DNA. However, this may not necessarily indicate that this telomere has shortened because the parental telomere also had a 3′ overhang. The telomere replicated by leading strand synthesis, *(B)* is initially blunt-ended and requires exonucleolytic processing of the 5′ strand to recreate a 3′ overhang. This processed 5′ strand will then serve as a shorter template for replication in the following cell cycle. Therefore, in this hypothetical mechanism, exonucleolytic processing of the telomere replicated by leading strand synthesis is the source of telomere shortening.

and avoiding senescence *(1,20).* In addition, telomerase-independent pathways, or alternative lengthening of telomeres (ALT), have been proposed as a means to maintain telomere length *(8,18).* These presumably recombination-based mechanisms use telomeric DNA on other chromosomes as a template for extending telomeres. Two such possible mechanisms are illustrated in Fig. 2.

2.2. Telomere-Associated Proteins

Two human telomere-specific DNA binding proteins, TRF1 (TTAGGG repeat binding factor 1) and TRF2 have been identified *(15).* TRF1 is a negative regulator of telomerase activity at chromosomal termini and thus of telomere length, i.e., overexpression of TRF1 leads to progressive telomere shortening, while inhibition increases telomere length *(108).* TRF2 has also been shown to be a negative regulator of telomere length *(101),* as well as protecting chromosome ends from end-to-end fusion events *(109).* TRF2 is the first telomere-associated protein implicated in the maintenance of the correct terminal DNA structure necessary for proper telomere function. A clue as to what that physical structure might be was uncovered by the discovery that TRF2 can remodel mammalian telomeric DNA into large duplex loops, termed t loops *(39).* A t loop is created when a telomere end loops back and the single-stranded G-rich tail invades an interior segment of duplex telomeric DNA. TRF1, which can induce bending, looping, and pairing of duplex DNA *(5,6),* and TRF2, which can induce invasion of the 3′ single-stranded tail into duplex telomeric DNA *(39),* bind duplex telomeric DNA in vivo and both appear to be involved in the formation of t loops *(101).* By sequestering telomeric

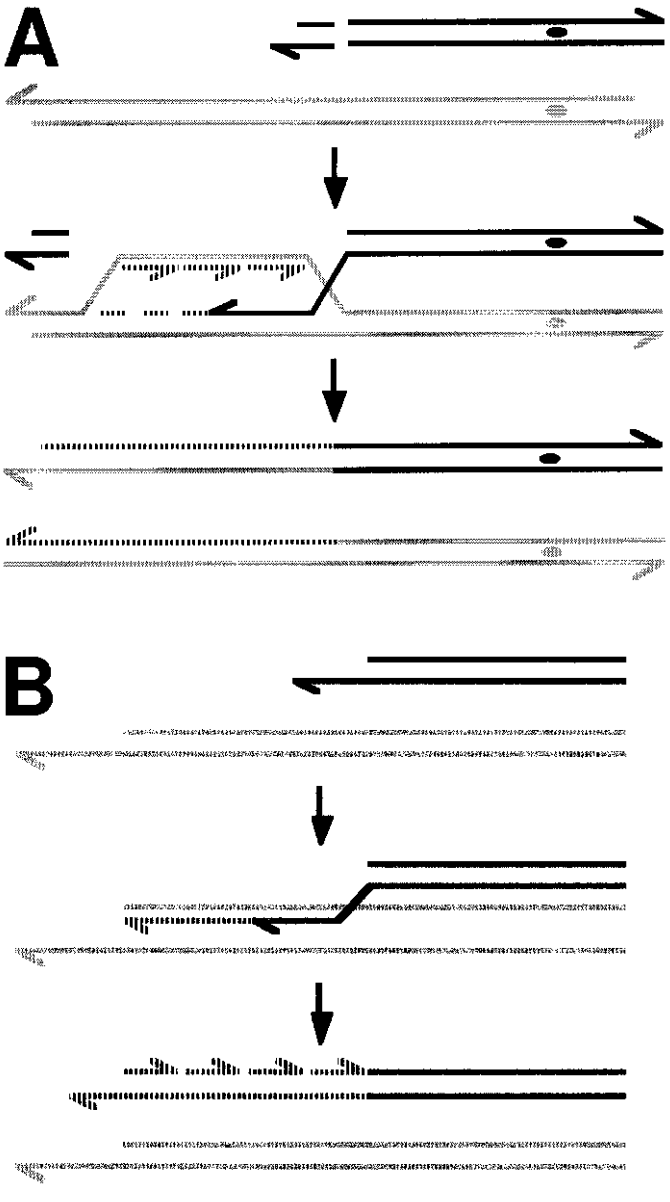

Fig. 2. (A) Telomere elongation by semiconservative DNA synthesis *(43).* Strand invasion by the 3′ terminus of one strand from the centromeric side of a DSB in the dark chromosome initiates leading-strand DNA synthesis in the telomere of the lighter chromosome. Lagging-strand synthesis occurs in the displaced strand (indicated by dashed lines). Following completion of DNA synthesis, the intermediate structure is resolved. The distal end of the DSB is either degraded or engages in unproductive recombination with another target. **(B)** Telomere elongation by conservative DNA synthesis *(118).* Strand invasion of a template telomere initiates leading-strand DNA synthesis. The intermediate structure is resolved and the complementary strand is synthesized by either lagging-strand synthesis or is primed by a terminal hairpin created by the G-rich strand folding back on itself.

termini, t loops may effectively conceal natural chromosome ends from the cell's DNA repair machinery.

TIN2, a TRF1-interacting protein that co-localizes with TRF1 on human metaphase chromosome termini, has also been identified *(56).* Expression of mutant TIN2 causes elongation of telomeres in a telomerase-dependent manner, suggesting that TIN2 mediates TRF1 function and thereby negatively regulates telomere length. Tankyrase, another TRF1-interacting protein, is discussed in Subheading 3.4. Additional telomere-associated proteins with roles in DNA repair are discussed in Subheading 3.

3. DNA REPAIR PROTEINS AND TELOMERE FUNCTION

DNA double-strand breaks (DSBs) represent a major threat to the integrity of the genome because of their potential for causing lethality, mutagenesis, and carcinogenesis if they are left unrepaired or are misrepaired. Many exogenous agents, including ionizing radiation and a number of anticancer drugs (e.g., bleomycin) cause DSBs, as do endogenous free radicals—the natural by-products of oxidative metabolism. DSBs also occur normally as intermediates in site-specific V(D)J recombination, the process that helps to generate the wide variety of antigen-binding sites necessary for antibody and T-cell receptor proteins during lymphocyte development. Consequently, all cell types possess multiple, as well as very effective, mechanisms for the repair of DSBs *(52,121).*

3.1. Modes of DSB Repair

Eukaryotic cells accomplish DNA DSB-repair via at least two pathways, homologous recombination (HR) and nonhomologous (or illegitimate) end joining (NHEJ) *(54,43).* In yeast, the predominant DSB-repair pathway is HR, which involves the exchange of genetic information between a damaged chromosome and an undamaged homolog, sister chromatid, or other region of shared homology. Repair of a DNA DSB by HR occurs by means of replication, using the homologous strand as a template, so typically there is no loss of genetic information. Although the significance of HR in mammalian cells is emerging *(64,105),* currently the majority of DSBs in mammalian cells appear to be repaired by NHEJ, a process which requires little or no homology between the two recombining molecules *(104),* but usually alters the DNA sequence at the point of joining (small deletions or insertions). NHEJ proteins have been shown to be critical for maintaining mammalian genomic stability *(31).* Yeast also possess a NHEJ pathway, which is especially important in haploid G1 phase cells since no homolog is present *(96).* Crucial components of the NHEJ DNA repair pathway appear to be conserved between yeast and mammalian systems *(23).* For example, *Saccharomyces cerevisiae* homologues of both human Ku70 (yKu70 or Hdf1) and Ku80 (yKu80 or Hdf2) have been identified and found to play important roles in NHEJ *(13,14,29,30,53).* One notable exception, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), appears to be restricted to higher eukaryotes, as a yeast homologue has not been identified.

Human Ku was originally identified as an autoimmune antigen in patients with polymyositis-scleroderma overlap syndrome *(76).* Ku is the most abundant DNA end-binding protein in both mammalian and yeast cells, and it recognizes many DNA structures in a DNA-sequence-independent manner. Ku is a heterodimer composed of 70 and 86 kDa subunits, which are encoded by the *XRCC6* and *XRCC5* genes, respectively *(28).*

Ku binds with high affinity to double-stranded DNA ends, whether blunt, overhanging or hairpin in structure *(27).* Mutant cells lacking Ku are deficient in the repair of DSBs, as well as in recombination of the immunoglobulin V(D)J region, and are hypersensitive to radiation *(41),* yet the precise role of Ku in DNA double-strand break repair remains elusive. Ku may function as a DNA damage sensor, rapidly identifying and binding to broken ends, then recruiting, or targeting, other proteins to the site. Once bound, Ku could signal the presence of DNA damage through its association with the ~465 kDa catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which together with the Ku heterodimer comprise DNA-PK, a nuclear serine/threonine kinase *(17,99).* The kinase activity of DNA-PKcs is activated when complexed with DNA-bound Ku *(34).* DNA-PKcs has also been shown to bind DNA itself (i.e., without Ku) but have limited protein kinase activity *(45).* Enlightening structural analysis of DNA-PKcs suggests that activation of the kinase requires interactions with both double- and single-stranded DNA *(46,62).* It becomes interesting and perhaps most important to recognize that only very specific types of DNA structure mediate DNA-PKcs activation. Ku therefore, may serve to stabilize the interaction between the free DNA ends and the catalytic subunit, provide protection from degradation and/or contribute to, or control the chromatin structure necessary for the assembly of a functional Ku/DNA-PKcs/XRCC4/Ligase IV NHEJ repair complex *(90).*

XRCC4 (X-ray repair cross-complementing gene 4) *(63)* encodes a small nuclear phosphoprotein of 334 amino acids, is an effective substrate for DNA-PK *in vitro,* and has been shown to be tightly associated with DNA Ligase IV *(22),* a component necessary for the final step of rejoining broken chromosomal ends *(115).* DNA Ligase IV is also a specific *in vitro* substrate of DNA-PK *(57).* An important role for *XRCC4* in normal development and suppression of tumorigenesis has been demonstrated *(32).*

The Mre11/Rad50/Xrs2 (yeast) complex also functions in DSB repair by both HR and NHEJ *(42),* but its precise role is unclear. It has been demonstrated that the Mre11/Rad50 complex possesses both endonuclease and 3′-to-5′ exonuclease activities *(106),* and so it may process, or prepare, the broken DNA ends for ligation, or it may play more of a structural role *(82).* Well-conserved human homolog of RAD50 and MRE11 have been identified and appear to be physically associated *(26,85).* A complex consisting of human RAD50, MRE11, and another protein of about 95 kDa (p95), has been purified *(106).* The p95 protein, termed Nibrin *(110),* was found to be mutated in Nijmegen Breakage Syndrome (NBS). Nibrin deficiency leads to the chromosomal instability, radiation sensitivity, and cancer predisposition seen in patients with this rare autosomal recessive disorder. Nibrin, encoded by the *NBS1* gene, may be a functional homolog of yeast Xrs2. The function of Nibrin is currently unclear, but p53 upregulation in response to irradiation is abrogated in NBS cells, suggesting a role in DNA-damage signaling and cell cycle checkpoint control *(16).* It is interesting to note that human MRE11 and RAD50 proteins form discrete nuclear foci at sites of damage in response to DSB-inducing agents. These foci do not form in NBS cells, suggesting that Nibrin is required for the localization and/or stabilization of this complex at DSBs *(71).*

Nanoelectrospray tandem mass spectrometry, protein blotting, and indirect immunofluorescence studies have revealed a cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 at human telomeres *(122).* RAD50 and MRE11 co-localized with TRF1 and TRF2 and were present at most interphase telomeres. NBS1 was associated with TRF2 and telomeres only in S-phase and only in a minority of the cells. The

presence of the RAD50/MRE11/NBS1 complex at human telomeres and its association with TRF2 supports a role in mammalian telomere function, perhaps in t loop formation. For additional details about the MRE11/RAD50/NBS1 complex, *see* Chapter 7.

3.2. DSB Repair and Chromosome Instability

Structural chromosomal aberrations are a conspicuous visual manifestation of the misrepair or nonrepair of DSBs *(19).* They can be separated into two distinct classes, chromosome-type aberrations that involve both chromatids of a mitotic chromosome and chromatid-type aberrations, which involve only one chromatid. Aberrations can be classified further based on the unique structures that are formed. Examples include dicentric chromosomes that by definition have two centromeres, acentric fragments having no centromere, ring chromosomes, inversions where a chromosomal segment has become detached and then reintegrated into the same chromosome in an inverted position, translocations created by the exchange of pieces of broken chromosomes, and terminal deletions that are created by unrejoined DSB. Robertsonian translocations are a particular type of exchange that deserve special mention both because they are widespread in mammals and because they will become important in the discussion that follows. Robertsonian translocations are created by the joining of two acrocentric (centromere at one end) chromosomes at their centromeres to form a metacentric (centromere at the middle) chromosome *(91).* In mouse, it has been shown that the p-arm (short-arm) telomeres are deleted when acrocentric chromosomes enter into Robertsonian rearrangements *(33,80,97).*

The consequence for the cell varies depending on the type of chromosomal aberration present. For example, the two centromeres of a dicentric chromosome an asymmetrical exchange, may be pulled towards opposite poles during anaphase resulting in mechanical difficulty in the separation of daughter cells, an event that is often lethal. Acentric fragments also do not segregate properly during mitosis since they do not have a centromere. Some daughter cells consequently suffer the loss of genetic material that may unmask recessive lethal mutations. In contrast, symmetrical exchanges like inversions and translocations are rarely lethal, but may cause mutation of genes located at the breakpoints involved in the rearrangement. Cancer cells frequently contain very specific inversions or translocations, having oncogenes or tumor suppressor genes located at the breakpoints *(102).* Observations such as these illustrate nicely the importance of effective DSB repair in maintaining a stable genetic inheritance.

As the physical ends of linear DNA duplexes, telomeres run the risk of being misidentified as double-strand breaks in need of repair and ligation. The cell must be able to distinguish between natural chromosome ends (telomeres) and broken double strands, as the consequences of illegitimate telomeric end joining, e.g., dicentric chromosomes and Robertsonian translocations, would be disastrous to the cell. With this in mind, recent evidence (primarily in *S. cerevisiae)* that surprisingly locates several proteins of the DNA repair arsenal at telomeres, presents an intriguing paradox. Why are DNA repair proteins, whose function is to bind and join double-stranded ends, present at the telomere where fusion is undoubtedly to be avoided, and what role(s) do they play in normal telomere function?

3.3. DNA Repair Proteins in Yeast Telomere Biology

In addition to functioning in the vital processes of DNA repair, NHEJ proteins have also been found to be required for normal telomere structure and function, maintenance,

and telomere-associated transcriptional silencing *(12).* In vivo crosslinking experiments have demonstrated that the Ku heterodimer binds yeast telomeres *(36).* The absence of the Ku heterodimer affects the perinuclear clustering of telomeres normally seen in wild-type yeast cells, indicating that Ku is required to establish the normal structural arrangement of telomeres in interphase nuclei *(59).* It has also been proposed that Ku protects the telomere from nucleolytic and recombinational activities *(86).* Furthermore, yeast strains defective in Ku70 or Ku80 lose the majority, but not all, of their terminal telomere repeats *(13,87).* Disruption of the *RAD50, MRE11,* or *XRS2* genes also leads to telomere shortening *(3,81).* The roles these proteins play in telomere maintenance and function is currently unclear. The idea that DNA repair proteins may contribute to a terminal end-binding complex that in turn recruits still other proteins necessary for normal telomere function is supported by the finding that yKu70p interacts with Sir4p *(107),* which in turn interacts with Rap1p *(67),* a key regulator of telomere length.

Sir2p, Sir3p, and Sir4p also interact with histones H3 and H4 and function in transcriptional silencing at telomeres by inducing a condensed, inaccessible heterochromatic state in the vicinity of the telomere. As a result, genes at or near telomeres are subject to transcriptional repression, a phenomenon termed telomere position effect (TPE) *(35).* Disruption of Ku debilitates this telomere-associated silencing *(44).* Mutation of *Sir* genes leads to decreased Ku-dependent NHEJ, indicating that in yeast these silencing proteins may also play a role in NHEJ *(60).* In addition, redistribution of yeast telomeric Ku and Sir proteins in response to DNA strand breaks has been demonstrated *(70,75).* These results suggest that Ku recruits the Sir protein complex to sites of DNA damage, inducing a heterochromatin-like state around the broken ends, perhaps through an interaction with nucleosomal DNA *(40).* Nucleases and recombination enzymes may then be excluded from the broken ends, so that degradation and undesirable recombination, such as joining reactions with other DNA ends, are avoided. A model of telomeric silencing might also involve rapid binding of Ku to telomeric DNA and interaction with the Sir protein complex through Sir4p. The resulting chromatin condensation then serves to both repress transcription and protect chromosome ends from degradation and recombination reactions *(107).*

3.4. DNA Repair Proteins in Mammalian Telomere Biology

Telomeres in both yeast and mammalian cells are tandem arrays of G-rich repetitive DNA, but it is important to recognize that differences also exist between the two. Yeast telomeres each consist of ~250–350 base pairs (bp) of the variable TG_{1-3} sequence *(95),* while each mammalian telomere consists of ~5–10 kilo base pairs (kb) in human *(68,69)* to ~50–100 kb in mouse *(58,123)* of the TTAGGG repeat sequence. Mammalian telomeres end in long (130–270 bp) single-stranded G-rich overhangs *(66,116),* whereas long single-stranded overhangs occur at yeast telomeres only briefly in late S-phase *(25,114),* otherwise, yeast telomeres have only an ~10 nucleotide G-rich overhang *(120).* Therefore, although there are similarities, it is not unreasonable to expect differences in the requirements for telomere end-binding activities and protection to exist between the two organisms.

Also in contrast to yeast, the study of DNA repair proteins in mammalian telomere biology has focused primarily on illegitimate recombination events that are observable as telomeric associations and chromosomal end fusions. Chromosomal end-to-end

fusion has been attributed to a loss of telomere function caused by telomere shortening *(21).* In agreement with this hypothesis, the numbers of end-to-end associations have been observed to increase as telomere length decreased over several generations in mouse cells lacking the telomerase RNA subunit (mTR) *(11).* However, analysis of two human cell lines with severely shortened telomeres revealed that they were not detectably compromised in end-capping function *(93).* These results suggest that telomeric length is not the only factor that determines the fusigenic behavior of chromosome ends. In addition, no clear link between short telomeres and fusigenic potential could be demonstrated in murine severe combined immunodeficiency *(scid)* and Chinese hamster ovary (CHO) immortalized cell lines, leading to the speculation that chromosomal fusion may be caused by abnormalities in the structure of telomeric chromatin (98). Interestingly, measurement of telomere lengths in four different *scid* mice by Southern blot analysis of terminal restriction fragments (TRF) resolved on pulsed-field gels, as well as by quantitative fluorescence *in situ* hybridization (Q-FISH) analysis, revealed that *scid* mice consistently had longer telomeres than isogenic wild-type control animals *(47).* Measurements of telomere length in wild-type and Ku80 deficient mice have also revealed that, in contrast to yeast, Ku deficiency does not result in telomere shortening, and in fact telomeres are moderately elongated in Ku mutant cell lines *(94).*

Chromosomal end-to-end associations have been observed in tumor cells and in several human diseases including ataxia telangiectasia (AT). AT is an inherited autosomal recessive disorder featuring chromosomal instability and a high incidence of cancer (*see* Vol. II, Chapter 19). The ataxia telangiectasia mutated (ATM) gene product is defective in AT and is a member of the phosphatidylinositol 3-kinase superfamily, as is DNA-PK and the yeast Tel1 protein. It has been reported that telomeres in normal diploid and Epstein-Barr virus-transformed AT fibroblasts are shorter than in repair-proficient human fibroblasts *(84,117)* and that telomeres shorten at an accelerated rate in AT patients *(74).* However, a separate and more extensive study concluded that SV-40 transformed AT cells did not have a defect in telomere maintenance *(103).*

In mammalian cells, the effectiveness of telomeric end capping can be evaluated with fluorescent *in situ* hybridization (FISH). FISH has proven to be a powerful cytogenetic tool that reveals the chromosomal location of DNA target sequences with homology to fluorescently labeled nucleic acid probes. Spontaneously arising chromosome aberrations can be inspected for telomere signal at the point of fusion between the two chromosomes, the presence of which would give an unambiguous indication that telomeric end capping has failed. Such a strategy was recently employed to assess the ability of normal and DNA repair-deficient mouse cell lines to cap and protect chromosome ends *(2).* Telomeric fusions were not observed in any of the repair-proficient control cell lines examined. However, spontaneously arising fusions in which telomere sequence was retained at the points of joining were observed in mutant cell lines deficient in either Ku70, Ku80, or DNA-PKcs, clearly indicating that telomeric end-capping had failed in these mutants *(Fig. 3).* A mutant cell line deficient in p53 was also examined, but telomere-positive fusions were not observed at levels significantly above wild type controls. DNA-PKcs-deficient *scid* cells were analyzed in both low passage primary cultures and again after spontaneous immortalization. One type of fusion product, telomere-positive Robertsonian translocations, became especially prevalent after immortalization and their numbers continued to increase with further

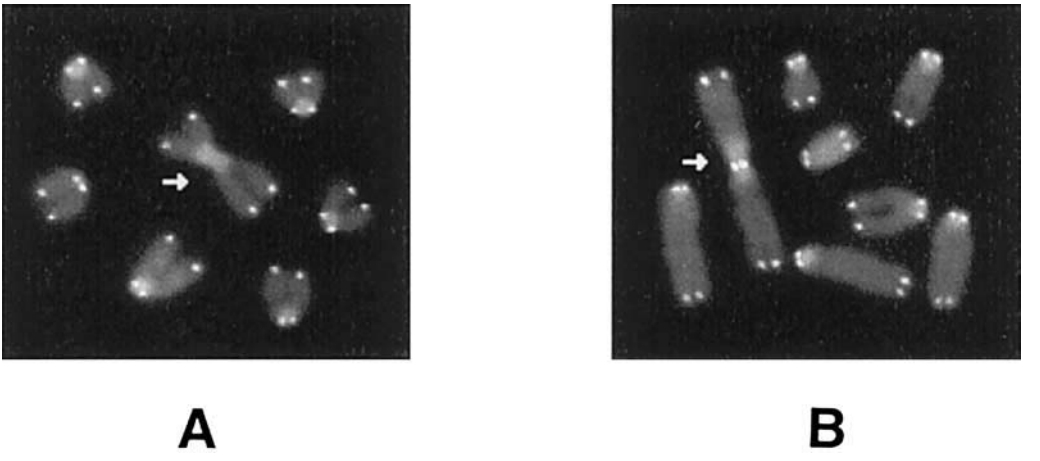

Fig. 3. Partial metaphase spreads illustrating typical mouse acrocentric chromosomes and Robertsonian translocations *(arrows).* **(A)** Repair-proficient control *without* telomere sequences at the point of fusion. **(B)** Repair-deficient mutant *with* telomere sequences present at the point of fusion.

passaging. Interestingly, these aberrations, which must be dicentrics, were not lethal to the cells possessing them, and in fact appeared to confer a growth advantage since they appeared in all immortalized cells. In contrast, SV-40 virally transformed *scid* cells restored to repair proficiency through transfection with a functional cDNA copy of the human DNA-PKcs gene, exhibited declining numbers of telomeric fusions with each successive passage until the culture became essentially free of these aberrations. Presumably, those telomeric fusions that existed in the population at the time of transfection were unstable, or cells containing them were selected against. These results demonstrate that restoration of DNA-PKcs function to *scid* cells reestablishes efficient telomeric end-capping.

The ability of Ku to bind mammalian telomeric DNA in vitro has been demonstrated *(4).* In addition, Ku has recently been localized to mammalian telomeric repeats using an in vivo crosslinking method *(51),* providing further support for a direct role of Ku as a mammalian telomeric protein. The impaired end-capping phenotype associated with DSB repair deficiency is strikingly similar to that caused by the inducible expression of a dominant negative allele of TRF2 *(109),* although it is less severe. Collectively, these results suggest that there may be at least two mechanisms mediating chromosomal end fusion. The first is a telomere length-dependent mechanism in which shortening of telomeric sequence beyond a critical value leads to loss of telomere function. The second mechanism is telomere length-independent, with telomeric fusion resulting from an inability to maintain a special protective structure at the very terminus of the chromosome and involving DNA-PK. Both mechanisms promote an unstable genetic inheritance that may contribute to the process of carcinogenesis.

Using a yeast two-hybrid screen with TRF1, a negative telomere length regulator (*see* Subheading 2.2.), an additional human telomere-associated protein, termed tankyrase, was isolated *(100).* Tankyrase has a COOH-terminal region with homology to the catalytic domain of poly(ADP-ribose)polymerase (PARP). PARP is an abundant

and highly conserved nuclear enzyme found in most eukaryotic cells that rapidly binds single- and double-stranded DNA, is activated by DNA damage (strand breaks), and catalyzes the formation of poly(ADP-ribose) onto a protein acceptor using nicotinomide adenine dinucleotide (NAD^+) as a substrate *(61).* Tankyrase has PARP-like activity with at least two specific substrates, TRF1 and tankyrase itself, and is strongly inhibited by the PARP inhibitor 3-aminobenzamide (3-AB). Modification of TRF1 and tankyrase by poly(ADP-ribosyl)ation decreases their binding affinity to telomeric DNA, and so may increase access to the telomere by telomerase and/or other end-binding proteins. Indirect immunofluorescence studies of metaphase chromosomes, probing with both anti-tankyrase and anti-TRF1 antibodies, revealed colocalization of the two signals at or near the physical ends of the chromosomes *(100),* further implicating tankyrase as a component of the human telomeric complex. In light of recent evidence that various DNA repair proteins are involved in normal telomere function, and assuming tankyrase plays a similar role to PARP's known role as a negative regulator of recombination, the presence of tankyrase at telomeres makes it a plausible candidate for involvement in suppression of inappropriate recombination activities in telomeric DNA, however a direct role has yet to be demonstrated. Chromosomal instability and telomere shortening have been reported in mice lacking PARP *(24).* In addition, it was observed that the absence of PARP did not affect the presence of telomeric single-stranded overhangs. It is also of interest to note that poly(ADP-ribosyl)ation by PARP has been shown to stimulate the protein kinase activity of DNA-PK *(92).*

What emerges from these yeast and mammalian studies is an as yet incomplete and sometimes contradictory picture that surprisingly places many DNA repair proteins at chromosomal termini where they play an unexpected yet crucial role in normal telomere maintenance and function. Further elucidation of the relationship between telomeres, DNA repair proteins, and "making ends meet" in illegitimate rejoining events will provide valuable new insight into this intriguing paradox. For example, through their contribution to efficient telomeric end capping, NHEJ repair genes help to preserve the fidelity of genetic inheritance. Whether or not other repair genes are similarly required, the molecular mechanisms through which they act, and the in vivo ramifications of ineffective end capping remain topics for future investigation. It is clear that a complete understanding of DNA repair-deficient phenotypes, and in particular how these phenotypes relate to cancer predisposition, will need to include the new role of DNA repair genes in telomere function.

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Conservation of Eukaryotic DNA Repair Mechanisms

Alan R. Lehmann and Elaine M. Taylor

1. INTRODUCTION

The fundamental importance of DNA repair for all organisms has become widely acknowledged in recent years. Evidence for the crucial role of DNA repair for the survival of all organisms comes from (1) the diversity of different repair processes; (2) the remarkable finding that about 2% of the *Escherichia coli* chromosome encodes proteins involved in DNA repair processes; (3) the extraordinary degree of evolutionary conservation of DNA repair mechanisms and proteins in all organisms. DNA repair pathways have been largely conserved from bacteria to mammals. In the vast majority of cases, the proteins that carry out these repair processes are conserved in structure and function in eukaryotes, and in some cases in bacteria as well. This conservation has been of great value in assisting our understanding of the mechanisms of DNA repair. The different strengths of working with yeasts on the one hand, which are very amenable to genetic analysis, and human cells on the other hand, which are often more amenable to biochemistry and provide relationships to human diseases, has greatly accelerated work in this area. Conclusions derived from the genetic and biochemical analysis of repair pathways in yeast can be extrapolated to human systems, and vice versa.

In this chapter we discuss the conservation of DNA repair proteins. It would be impossible to provide a comprehensive review, including all the information from the various genome projects that is now available in the sequence databases. We therefore concentrate mainly on published data on proteins from mammalian cells and the budding yeast, *Saccharomvces cerevisiae.* Where appropriate we also consider the bacterium, *E. coli,* and lessons can also be learned from the fission yeast, *Schizosaccharomyces pombe.* The latter is very distantly related to *S. cerevisiae,* so that conservation between these two yeasts is likely to extend throughout eukaryotes.

2. DNA DAMAGE REVERSAL: *PHOTOLYASES AND METHYLGUANINE METHYLTRANSFERASES*

Two DNA repair processes are unusual in that they require only a single protein that is able to reverse a specific form of damage in the DNA *in situ.* Photoreactivation, the first DNA repair process to be discovered *(57),* is brought about by the enzyme DNA

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photolyase. Photolyase binds to cyclobutane pyrimidine dimers (CPDs) in the dark and reforms the monopyrimidines on exposure to long wavelength UV or visible light. In an extensive study of photolyases from different organisms, Yasui and coworkers *(196)* were able to delineate two distinct classes of photolyases, with little sequence similarity between the two classes. Class I encompasses the enzymes from eubacteria, halobacteria and lower eukaryotes (including *S. cerevisiae*), whereas class II occur in methanobacteria and higher eukaryotes (including birds, fish, and nonplacental mammals). Interestingly, no photolyase has been identified in *S. pombe,* nor is there convincing evidence for photolyase in placental mammals. This interesting and unusual evolutionary development is quite different from that found in other repair pathways discussed in the following sections.

Within the last few years a gene encoding a photolyase that reverses the 6-4 photoproduct rather than the CPD has been identified in *Drosophila melanogaster (86,176).* This photolyase has 20–22% identity with the class I CPD photolyases, and 22–24% identity with blue-light photoreceptors, which appear also to belong to the same superfamily. Two human homologs of this class of genes have been isolated recently *(175,181),* but they appear to encode blue-light receptors rather than active photolyases. This family of genes and their activities has been reviewed by Yasui and Eker in Volume 2, Chapter 2, of this series.

The second damage reversal process is the demethylation of the highly mutagenic methylated base O^6 methylguanine by O^6 methylguanine DNA-methyltransferase (MGMT). The mechanism of action is the same in all organisms, namely the removal of the methyl group onto a cysteine residue in the active site of the protein, the resulting S-methylcysteine causing irreversible inactivation of the protein activity. In *E. coli* there are two proteins with MGMT activity. The Ada protein is highly inducible by methylating agents and has two methyltransferase activities in separate domains of the protein. The N-terminal domain has an activity which removes methyl groups from methylphosphotriesters, whereas the C-terminal domain contains the MGMT activity. The second (constitutive) *E. coli* protein Ogt, and eukaryotic homologs, contain only the latter activity. Both the yeast and human homologs were cloned by their abilities to correct the sensitivity to methylating agents of *E. coli* MGMT mutants *(72,194).* Human MGMT is about 28% identical to both Ogt and the C-terminal domain of Ada, with much higher conservation in the extreme C-terminus, which contains the active site cysteine residue (e.g. refs. *145,170*). Likewise the yeast Mgt1p is 43% identical to the *E. coli* proteins and 34% identical to the human protein over the C-terminal 88 amino acids *(194).*

3. BASE EXCISION REPAIR

DNA base damage can occur spontaneously (e.g., by hydrolytic deamination of cytosine and 5-methylcytosine), as an undesirable byproduct of cellular oxidative processes, or as a result of reactions with simple alkylating agents or ionizing radiation. This type of damage is corrected by the base excision repair (BER) pathway (*see* ref. *92* for a recent review). BER is initiated by a DNA glycosylase, which cleaves the glycosylic bond between the modified base and the sugar-phosphate backbone. DNA glycosylase action thereby excises the damaged moiety as a free base, and generates an abasic site within the DNA. Some DNA glycosylases are specific for a particular substrate (e.g.,

Table 1
Sequence Homology of Proteins Involved in BER

Protein	Activity	% Identity *S. cerevisiae*/man	% Identity *E. coli*/man	Functional complementation man/*E. coli*	Reference
hUDG1	Uracil-DNA glycosylase	40	—	Yes	*(92,124)*
hTDG	Thymine-DNA glycosylase	—	29	No report in literature	
hOGG1	8-oxoguanine-DNA glycosylase	38	—	Yes	*(140,144)*
hMYH	Adenine DNA-glycosylase	(31% *S. pombe*/man)	41	No report in literature	*(102,163)*
MPG	3-methyladenine-DNA glycosylase	—	—	Yes	*(31,121, 148)*
hNTH1	Endonuclease III	50% (over about half the protein)	22.5	*nth* mutants have no phenotype	*(8,43)*
HAP1 (APE1)	AP endonuclease	21	28	yes	*(14,38,79, 142)*

uracil), whereas others are able to recognise several different types of damaged DNA base *(92).* The resulting abasic site is converted to a single nucleotide gap by the action of an AP endonuclease, which cleaves the phosphodiester backbone, and a deoxyribosephosphodiesterase (dRpase), which removes the deoxyribose phosphate from the 5′ end of the cleaved strand. Short-patch gap-filling by a DNA polymerase and DNA ligase completes the repair process.

In recent years many of the genes required for BER have been identified in *E. coli,* yeast, and mammalian cells, revealing a high degree of evolutionary conservation (Table 1). Several of the eukaryotic genes were cloned by complementation of *E. coli* BER mutants. For example, the *S. cerevisiae OGG1* gene, which encodes an 8-oxoguanine DNA glycosylase, was cloned by functional complementation of an *E. coli mutM* mutant *(180).* In this case, Ogg1p can functionally substitute for the MutM glycosylase despite sharing little sequence similarity. A human homolog of *OGG1, hOGG1* (38% identical to the yeast protein at the amino acid level), is able to complement the mutator phenotype of both the yeast *ogg1* and *E. coli mutM* mutants *(140,144).*

The MutY protein of *E. coli* encodes an adenine glycosylase that removes adenines from A/G or A/8-oxoguanine mispairs. Although homologs have been isolated both from human sources with 41% identity, and from *S. pombe* with 28% identity to MutY, no homolog has been found in the *S. cerevisiae* genome *(102,163).* Eukaryotic homologs of the *E. coli* uracil DNA glycosylases *(ung and mug) (62,162),* 3-methyladenine DNA glycosylase *(alkA) (31,121,148)* and endonuclease III *(nth) (8,43)* have also been cloned and characterized (see Table 1). The human uracil glycosylase is able to complement the phenotype of *E. coli ung* mutants, and human MPG protein (AlkA homolog) complements the alkylation sensitivity of *E. coli alkA tag* mutants. (Complementation of *mutY* mutants by the corresponding human genes has not been reported,

and *nth* mutants of *E. coli* do not have a phenotype that can be analyzed in a complementation assay).

In *E. coli* there are two AP endonucleases. The major activity is associated with exonuclease III (Xth), the minor activity being endonuclease IV (Nfo). In human cells, the major AP endonuclease activity is encoded by the *HAP1/APE1* gene. HAP1 endonuclease shares 28% identity with *E. coli* Xth, and is able to complement some of the mutant phenotypes of the *E. coli xth* mutant *(38,142).* A structural homolog, designated *APN2* or *ETH1,* was also identified recently in yeast, but the major AP endonuclease in this organism is the product of the *APN1* gene. Apn1p shares 41% identity with the minor *E. coli* AP endonuclease, Nfo *(139).* Thus the major AP endonuclease in yeast is the homolog of the minor AP endonuclease in *E. coli* and vice versa.

Although the general strategy of BER is highly conserved from bacteria to humans, the later stages of this repair process seem to differ in higher eukaryotes. For example in mammals, in contrast to *E. coli,* a single enzyme, DNA polymerase β, performs both the dRpase and gap filling functions *(105,164).* Moreover, no yeast counterparts have been identified for either DNA ligase III or XRCC1, which are together thought to provide the major DNA joining activity required for the completion of BER in mammals *(21,174).*

4. MISMATCH REPAIR (MMR)

DNA mismatches result from errors during DNA replication. In order to ensure that such mismatches are repaired in the correct orientation, the cell must have a mechanism for distinguishing between parental and daughter strands. In *E. coli* this is effected by methylation at the 6-position of adenines in GATC sequences by the Dam methylase, a process that occurs subsequent to synthesis of the daughter strands (*see* Vol. 1, Chapter 11). The initial steps of MMR are the recognition of the mismatch by the MutS protein, which then recruits the MutL followed by the MutH protein. The latter cuts the DNA opposite the nearest hemi-methylated GATC sequence, and this incision on the daughter strand initiates a long patch excision repair process. Methylation of the parental strands is one of the few repair-related mechanisms that is not conserved in eukaryotes. Thus there are no known eukaryotic homologs of either Dam or MutH. In *S. cerevisiae* there are, however, six MutS and four MutL homologs. Three of the former, Msh2p, Msh3p, and Msh6p, and three of the latter, Mlh1p, Mlh3p and Pms1p have been implicated in MMR. The role of MutS in *E. coli* is taken over in yeast by two separate heterodimers, namely Msh2p/Msh3p and Msh2p/Msh6p, which have specificities for different types of mismatches, the former having preference for single-base mispairs, the latter for small insertion/deletion mismatches. Similarly the function of MutL is for the most part substituted with heterodimers of Mlh1p/Pms1p. Recent work has suggested however, that a proportion of the repair of specific insertion/deletion mispairs by the Msh3p-dependent MMR pathway uses a heterodimeric Mlh1p/Mlh3p complex in place of the Mlh1p/Pms1p *(54).*

All the yeast MMR proteins proteins have human homologs, whose importance has been demonstrated by their association with familial colon cancer. Many patients with hereditary nonpolyposis colon carcinoma (HNPCC) are associated with defects in hMSH2, hMLH1, and hPMS2 *(51,134)* and there is evidence in a few cases for association with hPMS1 *(119),* and hMSH6 *(2).* The phylogenetic relationships between the

Table 2
Sequence Homology of Proteins Involved in MMR

Protein	Activity	% Identity *S. cerevisiae*/man	Reference
hMSH2	Mismatch recognition	40	*(51)*
hMSH3	Mismatch recognition	30	*(59,118)*
hMSH6	Mismatch recognition	29	*(129)*
hMLH1	Binding to Msh2 complex	34	*(130)*
hPMS1	Binding to Msh2 complex	20 (Mlh3p)	*(54)*
hPMS2	Binding to Msh2 complex	32 (Pms1p)	*(119)*

different homologs are discussed in detail by Crouse in Vol. 1, Chapter 19 and by Fishel and Wilson *(52).* The sequence identities between the *E. coli,* yeast, and human proteins that have been implicated in MMR are presented in Table 2. (Note the confusing nomenclature: hPMS2 appears to be the homolog of yeast Pms1p, and hPMS1 appears to be the homolog of yeast Mlh3p.)

5. NUCLEOTIDE EXCISION REPAIR (NER)

Nucleotide excision repair (NER) is a versatile DNA-repair pathway that can act on a wide variety of DNA alterations. It is particularly effective on bulky, helix-distorting lesions, such as UV-induced DNA photoproducts. Relative to BER, NER is a complex process, requiring the concerted action of many proteins, in order to recognize the lesion, open up the DNA structure around the site of the damage and excise the damage-containing strand (*see* ref. *192* for a review). In humans, defects in NER are associated with the inherited syndromes xeroderma pigmentosum (XP), trichothiodystrophy (TTD) and Cockayne Syndrome (CS).

Most of the genes required for NER have been cloned from yeast and from humans, and biochemical studies on their encoded products have identified the functional activities associated with many of these proteins (*see* Table 3 and Volume 2, Chapter 18). Most were cloned by complementation of radiation sensitive mutants but some (e.g., XPF, ref. *161*) have been cloned on the basis of sequence homology with yeast genes. Conversely, when the human *XPB/ERCC3* gene was cloned by complementation of a UV-sensitive rodent mutant, no yeast homolog was known. The homologous *RAD25* was subsequently isolated by hybridization with *XPB* cDNA *(131).* A striking feature of all the NER genes cloned and sequenced to date is the high degree of structural similarity which exists between proteins from such evolutionarily distant organisms. Despite this structural homology, interspecies complementation of NER defects is rare, reflecting the multiplicity of protein-protein interactions required for this complex repair process. In BER, where a number of enzymes act sequentially at the site of damage and little requirement for protein-protein contact might be anticipated, only those parts of the BER enzyme important for catalytic activity need to be conserved in order for cross-species complementation to be effective. In contrast, NER demands the concerted action of a large number of proteins, each of which interacts with other members of the repair complex. Functional complementation of NER defects therefore requires that protein structure must be conserved, not only in those regions important for catalytic function, but also in areas required for protein-protein interactions.

Table 3
Sequence Homology of Proteins Involved in NER

Human Protein	Protein activity	% Identity *S. cerevisiae*/man[a]	% Identity *S. pombe/S. cerevisiae*[b]	Reference
XPA	Damage-specific DNA-binding protein	27 (Rad14p)		*(9)*
XPC	Damage-specific DNA-binding protein	23(Rad4p)		*(93)*
HR23B	Associated with XPC	32 (Rad23p)		*(104)*
XPG (ERCC5)	Damage-specific 3′ endonuclease	24 (Rad2p)	33 (Rad13p)	*(25,154)*
XPF (ERCC4)	Damage-specific 5′ nuclease subunit	26 (Rad1p)	30 (Rad16p)	*(24,161)*
ERCC1	Damage-specific 5′ nuclease subunit	35 (C-terminal half) (Rad10p)	39 (C-terminal half) (Swi10p)	*(143,182)*
XPD (ERCC2)	5′-3′ helicase; subunit of TFIIH	52 (Rad3p)	65 (Rad15p)	*(116,187)*
XPB (ERCC3)	3′-5′ helicase; subunit of TFIIH	55 (Ss12p/Rad25p)		*(131)*
p62	TFIIH subunit	26(Tfb1p)		*(48)*
p52	TFIIH subunit	40 (Tfb2p)		
p44	TFIIH subunit	42 (Ss11p)		
p34	TFIIH subunit	33 (Tfb4p)		

[a] *S. cerevisiae* protein in parenthesis.
[b] *S. pombe* protein in parentheses.

A summary of the functional activities of eukaryotic NER genes cloned to date is presented in Table 3. The XPC protein shows some homology to *S. cerevisiae* Rad4p *(93),* and both interact with a second protein (HR23B in humans and Rad23p in yeast), which is evolutionarily conserved *(68,104).* The XPC-HR23B complex has been recently shown to act at the first step in NER, namely damage recognition (165). This complex is then displaced by XPA. The human XPA protein and its *S. cerevisiae* homolog Rad14p are DNA binding proteins, both of which contain a zinc finger motif *(7,69).* TFIIH is a multiprotein complex that functions in initiation of RNA polymerase II-mediated transcription, and is absolutely required for NER in both yeast and mammalian cells *(169).* Genes for all nine subunits of TFIIH have now been described for both humans and yeast, revealing an extraordinary degree of evolutionary conservation *(48).* In yeast five of these nine components (Rad3p, Rad25p/Ss12p, Ss11p, Tfb1p, and Tfb2p) have thus far been shown definitively to play a role in NER *(48,186),* and it is likely that the whole TFIIH complex operates in both processes, albeit in different forms *(169).* Perhaps the best characterized of the TFIIH subunits are the products of the *RAD25* and *RAD3* genes. Rad25p and its human counterpart XPB function as 3′–5′ DNA helicases, whereas Rad3p and its homologs (XPD in humans and Rad15p in *S. pombe*) are 5′-3′ DNA helicases *(77).* The ATP-dependent helicase activities of XPB and XPD are required for the limited opening of the DNA duplex around the site of a DNA lesion *(47).* The formation of this open DNA intermediate is a prerequisite for dual incision by the NER nucleases *(46).*

A further interesting aspect reflecting evolutionary conservation is the frequency of mutations in *XPD/RAD3* compared to *XPB/RAD25.* In mutant screens for UV-sensitive mutants of *S. cerevisiae,* many *rad3* alleles were isolated. Likewise there are a substantial number of XP and TTD patients mutated at different sites in *XPD (172).* In contrast mutations in *RAD25* were never detected in mutant screens and there are only three known families with mutations in *XPB.* This shows that in both organisms Rad3p/XPD is tolerant of small alterations, which still permit TFIIH to function in transcription. In contrast Rad25p/XPB can only tolerate very few alterations without destroying TFIIH transcription function with resulting fatal consequences.

Two structure-specific nuclease activities are associated with eukaryotic NER, one of which cleaves the DNA on the 3′-side of the damage, the other on the 5′ side. The 3′-endonuclease is encoded by *RAD2* in *S. cerevisiae* and *XPG* in humans *(10,122).* XPG, Rad2p, and *S. pombe* Rad13p all share a high level of sequence identity over two functionally important regions close to the N- and C-termini, respectively *(25,54).* Moreover, in a rare instance of interspecies functional complementation, the *S. cerevisiae RAD2* gene is partially able to rescue the UV sensitivity of a *S. pombe rad13* mutant *(107).* The endonuclease activity which cleaves on the 5′-side of a DNA lesion is associated with a heterodimeric complex, comprised of the Rad1p and Rad10p proteins in *S. cerevisiae* and ERCC1 and XPF from humans *(11,161).* The homologous gene products in *S. pombe,* Rad16p and Swi10p, have also been shown to interact and presumably fulfil the same role in fission yeast *(24).* Curiously, the domains involved in the interaction of ERCC1 and XPF appear to be different from those that modulate Rad1p/Rad10p interactions *(37).*

6. REPAIR OF DSBs

Although we still have relatively little understanding of the enzymatic mechanisms of repair of DSBs, over the last five years there have been tremendous advances in our

understanding of the molecular genetics of this process. Two major pathways have been delineated. The first, involving homologous recombination (HR), appears to be the sole mechanism for repairing DSBs in *E. coli.* In yeast, it is the major pathway, but it plays a less important role in mammalian cells. Conversely, the second pathway, nonhomologous end-joining (NHEJ) has not been found in *E. coli,* plays a minor role in yeast, but is a major pathway for repairing γ-ray induced DSBs in mammals.

6.1. Recombination Repair

The key protein mediating homologous recombination in bacteria is RecA, which forms nucleoprotein filaments and promotes pairing of homologous DNA sequences and strand transfer *(58).* In yeast, repair of DSBs by HR requires the products of the *RAD51, 52, 54, 55* and *57* genes, and the ability to repair DSBs is severely compromised in mutants of these genes. There is evidence that the products of these genes interact to form a multiprotein complex *(33,73,109,166,167).* Rad51p is homologous to RecA. Although the sequence homology is not particularly dramatic (30% over the C-terminal half of the protein, ref. *159*), Rad51p forms nucleoprotein filaments with DNA that are structurally almost indistinguishable from RecA filaments *(123).* The sequence conservation of this group of proteins in eukaryotes is very high (Table 4) and the genes from higher organisms that operate in this pathway have been cloned using techniques based on sequence homology. Homologs of Rad51p have been isolated from fission yeast, chicken and mammalian species, and the degree of sequence (and presumably structural) identity is very striking *(158).* The human protein, like RecA, promotes homologous pairing and strand transfer, but the polarity of the transfer is opposite to that mediated by RecA *(12).* Two other yeast proteins, Rad55p and Rad57p, which promote strand exchange mediated by Rad51p *(167),* are in the same protein family as Rad51 *(82,101).* At least five additional mammalian members of this family have also been recently identified (*see* Table 5). Two of these, *XRCC2* and *XRCC3* were isolated by their ability to correct the mitomycin C (MMC) sensitivity of mutants that are sensitive to ionizing radiation and cross-linking agents *(27,97).* These are therefore implicated in recombination repair. The other family members were identified by screening of the expressed-sequence-tag (EST) databases. Their functions have yet to be elucidated.

Rad54p, which interacts with Rad51p *(33),* also shows a high degree of sequence conservation. Curiously, however, Rad52p, another key protein in HR in yeast, which stimulates pairing mediated by Rad51p *(166),* is much less well conserved than Rad51p or Rad54p *(112,125)* (*see* Table 4). Cells from a recently constructed *rad52* knockout mouse are not sensitive to ionizing radiation at all *(141).* The reason for this is not yet clear, and does not rule out a role for Rad52p in repair of DSBs in mammalian cells. There may be more than one homologous protein with overlapping functions, and it has been shown that overexpression of human *RAD52* confers enhanced radioresistance to cultured monkey cells *(132).* Two human homologues of Rad54p have been identified *(75,81,114)*

Two genes associated with familial breast cancer, *BRCA1* and *BRCA2* have been implicated in repair of ionizing radiation damage. Evidence has been produced that both BRCA1 and BRCA2 interact with hRAD51 (e.g., *see* refs. *156,157*), and that *BRCA2*-defective cells are deficient in their ability to repair DSBs *(1,35).* These results implicate BRCA1 and BRCA2 in HR, but further work is needed to define their precise roles. No homologs of these genes have been identified in lower eukaryotes, implying a

Table 4
Sequence Homology of Key Proteins Involved in HR

Human protein	Activity	% Identity *S. cerevisiae*/ man	% Identity *S. pombe*/ *S. cerevisiae*	Reference
hRAD51	Strand exchange	69	67	*(113,158)*
hRAD52	Cofactor for RAD51	30	27	*(112,125)*
hRAD54		48	51	*(81,114)*
hRAD50	Interacts with hMre11	28	35	*(40)*(E Hartsuiker, personal communication)
hMRE11	3′–5′ Exonuclease	34	50 (over 500 aa)	*(137,171)*
NBS1 (Nibrin)	Interacts with hMre11	28 (over 115 aa) with Xrs2p		*(23,183)*

Table 5
RecA/Rad51 Homologs in Humans

Rad51p homolog	Isolation/function	% Identity to *S. cerevisiae* gene	Reference
HsRAD51	Hybridization	67% to Rad51p	*(158)*
XRCC2	Correction of irs1 mutant	20–25% to Rad51p, 55p, 57p	*(27,97)*
XRCC3	Correction of irs1SF mutant	28–31% to Rad51p, 57p	*(97)*
HsRAD51B/ HREC2/ RAD51H2	EST database	30–31% to Rad51p, 57p	*(3,26)*
HsRAD51H3/ RAD51D	EST database	25–27% to Rad51p, 55p, 57p	*(26,138)*
RAD51C	EST database	31% to Rad51p	*(41)*

specific role for the proteins in multicellular organisms. Despite the absence of obvious homologs of BRCA1 and BRCA2 in simpler organisms, BRCA1 contains domains designated BRCT domains, which have been found in many proteins involved in DNA repair and DNA damage responses *(17,22).* This motif is of unknown function and comprises about 100 amino acids in five conserved blocks.

Three other yeast proteins, which are known to form a stable complex and to be involved in repair of radiation damage, are the products of the *RAD50, MRE11,* and *XRS2* genes *(80).* They are involved both in the formation and rejoining of breaks during meiosis, and there is evidence that they are involved in both HR and NHEJ in *S. cerevisiae (20,108,177).* Human homologs of *RAD50* and *MRE11* have been isolated *(40,137).* The protein complex containing human MRE11 and RAD50 was isolated recently and found to contain other subunits. One of these had limited sequence identity to Xrs2p at the N-terminus, and is likely to be its functional homolog. Excitingly this protein was found to be the product of the gene defective in the Nijmegen Breakage Syndrome *(23,183).* This disorder has clinical and cellular characteristics related to

Table 6
Sequence Homology of Proteins Implicated in NHEJ

Human protein	Protein activity	% Identity *S. cerevisiae*/man	Reference
Ku70	DNA end-binding	22 (over about 50% of the protein)	*(49)*
Ku86	DNA end-binding	20	*(18,108)*
DNA-PK_{CS}	Protein kinase	—	*(71)*
Ligase IV	Break joining	24	*(153,173, 191)*
XRCC4	Activator of ligase IV	22	*(74,94)*

ataxia-telangiectasia (A-T) (*see* Section 7.). At the time of writing, there is no evidence for the involvement of these human homologs in NHEJ in humans.

Although HR plays a role in the response of mammalian cells to radiation, as shown by the radiation-sensitivity of cells derived from the *RAD54* knockout mice *(45),* this sensitivity is not very drastic. This suggests that there are alternative routes for repairing DSBs in mammalian cells.

6.2. Nonhomologous End-Joining

A major pathway for repair of DSBs in mammals appears to be NHEJ, as shown by the very marked radiosensitivity of mutant cells defective in this pathway (reviewed in ref. *78*). Several of the proteins involved in NHEJ have recently been identified and characterized. DNA-dependent protein kinase is absolutely required for this process. It is composed of the 70 and 86 kDa subunits of the DNA-end binding Ku protein, and the 460 kDa catalytic subunit (DNA-PK_{CS}), which is recruited when Ku binds to DNA ends (reviewed in ref. *78*). Homologs of the two Ku subunits have been identified in yeast (*see* Table 6). The degree of sequence identity to the mammalian proteins is quite low. Mutants in yeast *KU70* or *KU86* are barely sensitive to ionizing radiation, because of the major role played by HR *(160).* However, in a *rad52* background in which HR can no longer take place, deletion of the *KU* genes results in a substantial further sensitization to radiation *(160).* Evidence for the involvement of Ku in NHEJ in yeast has come from the use of plasmid systems, in which plasmids cut with restriction enzymes are transformed into yeast cells and are substrates for NHEJ. Rejoining of these breaks requires intact yeast *KU* genes *(18,19,108,178).* Despite both the sequence and the functional similarity between yeast and mammalian Ku proteins, there is no evidence either biochemically or from the complete sequence of the yeast genome, that a homolog of DNA-PK_{CS} exists in yeast. In mammals, defects in Ku subunits or in DNA-PK_{CS} result in similar radiation sensitivities, suggesting that the whole complex participates in NHEJ in mammals. This is therefore a curious divergence between yeast and humans, which awaits a satisfactory explanation.

Strong evidence has been produced to show that the joining step of NHEJ is mediated by DNA ligase IV, which forms a tight complex with the XRCC4 protein *(36,63).* The latter had been shown by genetic studies to be involved in NHEJ. This work led to the identification of a homolog of DNA ligase IV in the yeast genome and the demonstration of its involvement in NHEJ in yeast *(153,173,191).* An XRCC4 homolog, designated Lif1p, which interacts strongly with yeast DNA ligase IV has also been identified recently *(74).*

Thus in the NHEJ pathway, we have the interesting finding that the process is conserved from yeast to man and many of the proteins are structurally conserved, but DNA-PK_{CS}, a key protein essential for NHEJ in mammalian cells, does not have an identified structural homolog in yeast.

7. DNA DAMAGE CHECKPOINTS

In addition to the DNA repair processes discussed earlier, eukaryotic cells also possess regulatory mechanisms known as checkpoints, which function to arrest cell-cycle progression in response to DNA damage. These cell cycle delays provide time for the completion of DNA repair before replication or mitotic segregation take place. Failure to respond to DNA damage in this way can lead to an increase in genomic instability, and checkpoint defects in humans are often associated with carcinogenesis.

Much of our current knowledge regarding DNA damage checkpoint control has been derived from genetic studies using the yeasts *S. cerevisiae* and *S. pombe* (reviewed in refs. *29,99,120,188*). Evidence from these organisms suggests that specific protein complexes detect DNA damage and activate a signal transduction pathway to transmit inhibitory signals to the cell cycle machinery. Many of the genes required for DNA damage checkpoint functions have been identified in both budding and fission yeast, and more recently a number of their human homologs have been cloned on the basis of sequence similarity (*see* Table 7). The degree of structural similarity that is evident between checkpoint proteins from different species suggests that DNA-damage checkpoint mechanisms have, like DNA-repair processes, been highly conserved throughout evolution.

In *S. pombe* the products of six genes *(rad1, rad3, rad9, rad17, rad26 and hus1)* required for checkpoint function *(4)* form three complexes, which comprise the upstream components of the checkpoint response. The *S. pombe* Rad3p/Rad26p complex *(42)* is required for all checkpoint functions and is probably involved in the recognition step of the checkpoint response. Homologs of Rad3p have been identified in several organisms, and in *S. cerevisiae* a homolog of Rad26p, designated Ddc2p, has been found with very similar function to Rad26p, but relatively poorly conserved at the sequence level *(126).* As yet no homolog has been identified in the mammalian EST databases. Rad3p and its homologs in *S. cerevisae* (Mec1p) and humans (ATR) are members of a conserved superfamily of phosphatidylinositol 3-kinase-related proteins (PIK-like kinases) *(15,32,84,189).* Despite their structural similarity to lipid kinases, no associated lipid kinase activity has been found for any of these proteins. Instead, protein kinase activity has been demonstrated for both Rad3p and ATR *(15,85).* This kinase activity is essential for their function in checkpoint control *(15,193).* DNA damage-induced phosphorylation of many of the proteins that participate in the DNA-damage checkpoint is dependent on Rad3p/Mec1p function *(96,99,185).* Another closely related member of the PIK-like kinase family is encoded by *ATM,* the gene that is mutated in the inherited, cancer-prone disorder ataxia-telangiectasia (A-T) *(152).* Cells derived from A-T patients are highly sensitive to ionizing radiation and are defective in G1/S, intra-S, and G2/M checkpoints following ionizing radiation *(13,83,128).* ATM shares significant sequence similarity with Rad3p and Mec1p (21% and 19% identity to Rad3 and Mec1 respectively), but it is actually more closely related to the products of the Tel1p proteins in *S. cerevisiae* and *S. pombe* (*see* Table 7) *(65,110,117).* Surprisingly, *S. cerevisiae tel1* mutants do not exhibit a checkpoint defect, although there does seem to

Table 7
Sequence Homology of DNA Damage Checkpoint Proteins

S. pombe protein	*S. cerevisiae* protein	Human protein	% Identity *S. pombe/ S. cerevisiae*	% Identity *S. pombe*/man	Protein properties	Reference
Rad1p	Rad17p	hRAD1	23	27	Potential nuclease Limited homology to PCNA	*(56,103,168,179)*
Rad3p	Mec1p	ATR	23	26	PI3-kinase related	*(15,32,84,189)*
Rad9p	Ddc1p	hRAD9	21	25	Limited homology to PCNA	*(95,100,115)*
Rad17p	Rad24p	hRAD17	20	49	Limited homology to RF-C	*(66,133)*
Rad26p	Ddc2p		14		Interacts with Rad3p	*(4,126)*
Hus1p	Mec3p	hHUS1	16	30	Limited homology to PCNA	*(89,90)*
Chk1p	Chk1p	hCHK1	32	29	Protein kinase	*(53,150)*
Rad24p and Rad25p	Bmh1p and Bmh2p	14-3-3 proteins	71	55	14-3-3 proteins	*(55)*
Crb2p/Rhp9p	Rad9p		26		BRCT domain protein	*(146,155,190)*
Cds1p	Rad53p	hCHK2	35	30	Protein kinase	*(5,16,106,111)*
Rad4p/Cut5p	Dpb11p	database	27			*(6,50,147)*
Cut2p	Pds1p		20		Anaphase inhibitor	*(60,195)*
Te11p	Te11p	ATM	22	23	PI3-kinase related	*(65,110,117,152)*
		p53			Transcription factor	*(197)*
		p21			Cdk inhibitor	*(67,70)*

be a degree of functional overlap between Mec1p and Tel1p, because Tel1p overexpression can partially complement some of the radiation sensitivity associated with a *mec1* mutant *(110).*

The *S. pombe* proteins Rad1p, Rad9p, and Hus1p all have some sequence similarity to PCNA *(30).* There is direct evidence for physical interactions between these three proteins *(30,89),* their *S. cerevisiae* homologs (Rad17p, Ddc1p, and Mec3p respectively) *(88,127),* and their human homologs *(184).* Furthermore, both hRAD1 and hRAD9 can partially rescue the checkpoint defects of the respective *S. pombe* mutants, implying functional as well as structural conservation in the DNA-damage checkpoint *(56,95).* It has been suggested that Rad1p, Rad9p, and Hus1p might form a heterotrimeric ring with a similar structure to the homotrimer formed by PCNA.

Both *S. pombe* Rad17p and its homolog in *S. cerevisiae,* Rad24p, show some sequence similarity to the components of replication factor C *(66).* A physical interaction between Rad24p and the four smaller components of replication factor C has been demonstrated in *S. cerevisiae (64).*

Although the DNA damage-sensing aspects of checkpoint control seem to be highly conserved throughout eukaryotes, the downstream signal amplification and cell-cycle responses differ somewhat between organisms. For example, in *S. cerevisiae* a major target of the DNA-damage checkpoint is the anaphase inhibitor Pds1p, which becomes phosphorylated after DNA-damage in a Mec1p-dependent manner *(34).* Pds1p is absolutely required to block cell-cycle progression in mitosis (at the metaphase-anaphase transition), following DNA damage *(195).* The fission yeast homolog of Pds1p, Cut2p, is also required to prevent the premature separation of sister chromatids during the normal cell cycle, but is apparently not a major target of the DNA-damage checkpoint *(60).* Instead, in fission yeast and in human cells, DNA damage leads to cell cycle arrest prior to the onset of mitosis, at G2/M. In *S. pombe* this arrest operates through a Chk1p protein kinase-dependent mechanism, which serves to maintain an inhibitory tyrosine phosphorylation on the cell-cycle kinase Cdc2p *(61).* Mammalian homologs of Chk1p have been identified and characterized, and they appear to operate in a similar manner to *S. pombe* Chk1p *(98,136,150).*

The *S. cerevisiae* protein kinase Rad53p is a major downstream target of Mec1p in the checkpoint response to DNA damage *(149),* and is required for G2 arrest. Rad53p is also required for the intra-S checkpoint in *S. cerevisiae* by arresting DNA replication in response to damage and preventing the firing of late origins of replication, as well as for the transcriptional induction of a number of DNA repair genes *(5,135,151).* The fission yeast homolog of Rad53p, Cds1p, functions to inhibit DNA replication when DNA damage is incurred, but does not appear to play a major role in inhibiting mitosis *(96).* Thus Rad53p and Cds1p are clearly structural and functional homologs and both require Mec1p/Rad3p for activation, but Rad53p is an essential component of all DNA damage checkpoints, whereas Cds1p is not. A human homolog of Rad53p/Cds1p has been identified and designated hChk2. Its activity is dependent on the ATM protein kinase *(16,106).* hChk2 is required to maintain the G2 checkpoint after ionizing radiation *(76).*

The apparent conservation of G2/M checkpoint proteins means that the genetic analysis of DNA-damage checkpoints in yeast is likely to be very informative with regard to higher eukaryotic-checkpoint control. To date, much of the work regarding

DNA-damage checkpoints in mammalian cells has focused on the G1/S cell-cycle transition and its regulation by the p53 tumor-suppressor protein. DNA damage results in the stabilization of p53. This upregulation of p53 is dependent on ATM, ATR, and Chk2 *(28).* Depending on the cell type and the nature of the damaging agent, p53 can have either of two downstream effects. In some cell types, p53 upregulates the cyclin-dependent kinase (CDK) inhibitor p21 *(44).* p21 binds, in multiple copies, to several cyclin-CDK complexes (e.g., cyclin D1-Cdk4, cyclin E-Cdk2, cyclin A-Cdk2, cyclin A-Cdc2) and in so doing prevents cells from exiting G1 *(67,70).* In cells such as human fibroblasts this G1 arrest is permanent *(39).* In other cell types upregulation of p53 results in cell death by apoptosis (reviewed in ref. *87*). It is thus likely that the principal function of p53 is not to assist in cell survival by allowing time for repair of DNA damage during G1 arrest. Its function is rather to remove from the cycling population those cells that have received substantial amounts of genetic damage, thereby protecting the whole organism. This protective function is only likely to be relevant to multicellular organisms, consistent with the fact that no p53 homologs have been identified in single cell organisms.

8. CONCLUSIONS

The high degree of evolutionary conservation of proteins involved in different DNA repair processes attests to their crucial importance in all organisms. The differences in the nature and conservation of these repair processes highlight different mechanisms and constraints. Of the seven processes discussed above, four (methyltransferases, BER, MMR, and HR) are conserved both structurally and functionally from *E. coli* to humans, although in MMR and HR, there is an increasing degree of complexity as we go up the evolutionary ladder. This is exemplified by the six eukaryotic homologs of the MMR protein, MutS, and in HR, the three RecA homologs in yeast and at least six in humans. With BER, although sequence conservation is not particularly striking, in many cases the genes from one organism can complement corresponding mutants in other organisms. This is consistent with BER being a sequential process of different enzymes acting on DNA substrates. With NER, functional complementation is rare, presumably because of the involvement of multiprotein complexes. It is surprising therefore, that several instances of partial complementation of yeast cell-cycle checkpoint mutants by homologous human genes have been reported. Because checkpoint proteins also appear to act in multiprotein complexes, functional complementation would not have been anticipated.

In NER, MMR, and HR every component has a sequence homolog conserved between yeast and humans, whereas in NHEJ a crucial protein in mammals does not have a sequence homolog in yeast. It should be noted, however, that there are increasing numbers of examples of proteins that have no discernible sequence similarity, yet when 3D stuctures have been solved, the structures of the proteins are almost superimposable. This is exemplified by the sliding-clamp proteins that are required to maintain the processivity of DNA polymerases in pro- and eukaryotes. In the latter case, this function is performed by PCNA, which forms a trimeric ring around the DNA, clamping DNA polymerase δ to the DNA, whereas in the former it is the β subunit of DNA polymerase III that performs this function as a dimer. The structures of the two rings are strikingly similar even though there is no significant sequence similarity *(91).* Thus, in instances

where there are no obvious sequence homologs between two organisms, the possibility of structural and functional homology is not excluded.

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